Bulletin of Faculty of Pharmacy, Cairo University 55 (2017) 95–100

Contents lists available at ScienceDirect

Bulletin of Faculty of Pharmacy, Cairo University

journal homepage: www.sciencedirect.com

Original Article

# Neuroprotective effects of potassium channel openers on cerebral ischemia–reperfusion injury in diabetic rats

Anand B. Pithadia [a], Shital S. Panchal [b,*], Denvanshu J. Patel [c]

[a] Department of Pharmacology, Institute of Pharmacy, Nirma University, Ahmedabad, India
[b] Department of Pharmacology, Parul Institute of Pharmacy, Vadodara 391760, Gujarat, India
[c] Department of Pharmacology, Parul Institute of Medical Sciences and Research, Vadodara 391760, Gujarat, India

ARTICLE INFO

*Article history:*
Received 12 March 2016
Received in revised form 16 August 2016
Accepted 21 September 2016
Available online 21 October 2016

*Keywords:*
Stroke
Potassium channel openers
Type I diabetes
Neuroprotection

ABSTRACT

*Objectives:* This study was done to estimate the potential neuroprotective role of potassium channel openers in cerebral ischemia–reperfusion (IR) injury in streptozotocin (STZ) induced type-I diabetic rats (T1DR).

*Methods:* Potassium channel openers – cromakalim, cinnarizine and nicorandil; potassium channel blocker –glibenclamide, insulin (as an antidiabetic standard), telmisartan (as an anti-hypertensive standard agent) and vitamin E (as an antioxidant and antiapoptotic standard agent) were given for 3 days in streptozotocin (45 mg/kg i.v.) induced type I diabetic rats along with middle cerebral artery occlusion. After 24 h of surgery, plasma glucose, neurobehavioral score, cerebral infarct volume, blood pressure and caspase-3 levels were measured to evaluate the mechanism of potassium channel openers (KCOs) for neuroprotection.

*Results:* Following STZ administration and ischemia–reperfusion, blood sugar, neurobehavioral score, cerebral infarct volume and caspase-3 levels were significantly high in diabetic-IR groups. Treatment with cromakalim, cinnarizine, nicorandil, insulin and vitamin E significantly reduce neurobehavioral score while nicorandil and vitamin E significantly reduced cerebral infarct volume. Caspase-3 levels were significantly reduced by cromakalim and nicorandil treated animals. Except insulin and glibenclamide, none of the agents significantly reduce plasma glucose levels.

*Conclusion:* Treatment of ischemic stroke with potassium channel openers in T1DR is neuroprotective. Inhibition of apoptosis may contribute to their neuroprotective effects after stroke in T1DR.



## 1. Introduction

Cerebral ischemic stroke is caused due to obstruction of blood supplied to the brain. It is classified either as ischemic stroke (IS) or hemorrhagic stroke. There are around 83% cases of strokes with IS while the remaining have hemorrhagic brain stroke which results in leakage of blood into the brain. Important etiological factors for pathogenesis include hypercholesterolemia, hypertension and hyperglycemia. It is reported that diabetes mellitus (DM) increases the risk of brain stroke 2 to 3 times more. DM increases the risk of macrovascular and microvascular complications [1,2]. Current treatments options for brain stroke include the use of anti-platelet agents and tissue plasminogen activators (tPA) for their thrombolytic effects. Anti-oxidants such as vitamin C, E and growth factors are found to be neuroprotective in IS. Furthermore, anti-hypertensives, anti-hyperlipidemics as well as oral hypoglycemic agents are beneficial for prevention of IS [3].

However, tPA treatment of stroke after 3 h in patients with DM increases the risk of death and intracerebral hemorrhage [4,5]. Even, reports have found that tPA treatment within 2 h after stroke in type-I diabetic rats significantly increases brain hemorrhage, and increases neurobehavioral score after stroke [6,7]. Thus, there is a need to identify new treatment agents with neuroprotective action in IS and its related disorders like diabetes.

$K^+$ ion channels of CNS (central nervous system) play an important role for providing neuroprotection in animal models of ischemic brain stroke [8]. ATP sensitive potassium channel openers such as nicorandil and cromakalim showed free radical scavenging

Peer review under responsibility of Faculty of Pharmacy, Cairo University.

* Corresponding author at: Department of Pharmacology, Institute of Pharmacy, Nirma University, Sarkhej-Gandhinagar Highway, Chandlodia, Gota, Ahmedabad 382481, Gujarat, India.

*E-mail addresses:* abpithadia@hotmail.com (A.B. Pithadia), shital.panchal@nirmauni.ac.in (S.S. Panchal), devanshu_1984@yahoo.co.in (D.J. Patel).

http://dx.doi.org/10.1016/j.bfopcu.2016.09.002
1110-0931/

effect and anti-apoptotic effect in streptozotocin-induced diabetic rats and in cultured myocytes [9–11]. They produce neuroprotective action IR injury through anti-apoptotic, anti-oxidant and anti-inflammatory actions in various experimental animal models [12]. Thus they are newer therapeutic treatment targets for neuroprotection in ischemic brain stroke. However, their mechanism of neuroprotection in stroke and its related disorders like DM remains unknown. Present acute type of study assumed that potassium channel openers might treat IR injury in STZ induced type-I diabetes mellitus by inhibiting apoptosis pathways. Animal model of IR was induced by cerebral artery occlusion in STZ induced type - 1 diabetes mellitus (T1DM). The neuroprotective actions of potassium channel openers cromakalim, nicorandil and cinnarizine were determined to find out the mechanism of action in diabetic rats.

## 2. Materials and method

### 2.1. Drugs and chemicals

All the drugs and chemicals for study were of laboratory grade. Except vitamin E, all other drug solutions were freshly prepared in distilled water and given intraperitoneally (i.p.) in appropriate doses as written in the experimental design section. STZ solution was dissolved in phosphate buffer and given 45 mg/kg intravenously (i.v.). Vitamin E suspension was prepared by dissolving it in 4% tween 80 and given orally. Here, cromakalim, cinnarizine and nicorandil were taken as potassium channel opener agents. Glibenclamide was taken as potassium channel blocker. Insulin, telmisartan and vitamin E were taken as antidiabetic, antihypertensive and antioxidant-antiapoptotic standard agents respectively.

### 2.2. Animals

Adult male Wistar Albino rats weighing between 180 and 210 g were procured from the Animal House of Parul Institute of Pharmacy, Vadodara. The animal experimental protocols, including all use, care, and operative procedures, were approved by the Institutional Animal Ethics Committee (IAEC). Every effort was made to minimize the number of animals used and their suffering. Animals were maintained at 18 ± 2 °C in polypropylene cages with food and water *ad libitum*. Animals were divided into sixteen groups.

### 2.3. Experimental design

Group 1 (normal control, $n = 6$) animals were administered with tween 80 (4%). Group 2 (Diabetic control, $n = 6$) animals were administered with STZ (45 mg/kg i.v) on 1st day. On day 3, diabetic glucosuria was confirmed using Diachex urine strip. Group 3 (Diabetic Sham surgery operated, $n = 6$) animals were given with tween 80 (4%) for 3 days along with STZ on 1st day. Sham surgery was done on 2nd day. Group 4 (IR control, $n = 12$) animals received tween 80 (4%) for 3 days. IR was done on 2nd day from initiation of experiment. Group 5 ($n = 12$), 6 ($n = 12$), 7($n = 12$) and 8 ($n = 12$) were induced with IR and treated with cromakalim (10 mg/kg i.p.) [13], cinnarizine (5 mg/kg ip) [14], nicorandil (5 mg/kg ip) [15] and vitamin E (150 mg/kg orally) [16] respectively for 3 days. Group 9 (Diabetic-IR control, $n = 12$) animals were administered with tween 80 (4%) for 3 days after STZ (45 mg/kg i.v.) induced diabetes. Cerebral IR was done on 2nd day. Group 10 animals ($n = 12$) were administered with cromakalim (10 mg/kg i.p. for 3 days) along with STZ (45 mg/kg i.v. on 1st day) along with IR injury on 2nd day. Similarly group 11 ($n = 12$), 12 ($n = 12$), 13 ($n = 12$), 14 ($n = 12$), 15 ($n = 12$), and 16 ($n = 12$), were treated with cinnarizine (5 mg/kg ip), nicorandil (5 mg/kg ip), glibenclamide (5 mg/kg i.p), [17] insulin (5 IU/day) [18], telmisartan (10 mg/kg i.p) [19] and vitamin E (150 mg/kg orally for 3 days) respectively.

### 2.4. Induction of cerebral ischemia–reperfusion injury

Cerebral IR was induced as per transient middle cerebral artery occlusion (tMCAO) method of Wang et al. [13]. Rats were anesthetized with an i.p. injection of 100 mg/kg ketamine. A 2–3 cm incision was made in the middle of the neck line, separating the left carotid artery, the superior thyroid artery, and the occipital artery, as well as the internal and external carotid communicating arteries. The occipital artery branches of external carotid artery (ECA) were isolated and tied with a cotton thread. Cotton thread was tied loosely around the ECA stump near the bifurcation. Then internal carotid artery (ICA) and common carotid artery (CCA) were temporarily occluded by a fine vessel clip. Through a small incision to the ECA stump, blunt Poly-L-lysine coated 4-0 monofilament was inserted from the left external carotid artery into the left internal carotid artery to a depth of 18.0 mm, vessel clip from ICA removed. After a variable length of suture had been inserted into the ECA stump, resistance was felt and slight curving of suture was observed, indicating that the suture had passed the middle cerebral artery (MCA) origin and reached to proximal segment of anterior cerebral artery (ACA-it has small diameter). Hence the suture had blocked all sources of blood from ICA, ACA and posterior cerebral artery. Finally the vessel clip from CCA was removed to restore the blood flow. The midline incision was closed, leaving the suture protruding so it could be withdrawn to allow reperfusion. The thread was maintained for 2 h and subsequently removed to restore blood flow to the common carotid and internal carotid arteries. Here 18 mm of suture was pulled back until resistance was felt, indicating that the tip cleared the ACA-ICA lumen and was in the ECA stump, then trimmed. The animals were transferred to a fresh cage with free access to food and water.

### 2.5. Tissue homogenate preparation

Brain samples were washed with isotonic saline and homogenized using ice-cold 10% w/v 0.1 M phosphate buffer of pH 7.4. Supernatant was obtained by centrifuging the homogenate at 12000 rpm (20 min). This supernatant was used to estimate caspase-3 [20].

### 2.6. Tissue total protein level

It was estimated as per the method of Lowry et al. [21] using bovine serum albumin, alkaline copper reagent Solution A (2% sodium carbonate in 0.1 N NaOH solution in distilled water), solution B (0.5% copper sulfate in 1.0% sodium potassium tartarate) and Folin's phenol reagent.

### 2.7. Plasma glucose levels

Glucometer (One Touch Ultra 2, Lifescan Inc, USA) was used to estimate glucose levels.

### 2.8. Neurobehavioral score

Neurobehavioral score was obtained for the group after 24 h of IR injury [22]. This score was monitored for group 4 to 16. Score 0: no behavioral deficit; score 1: forelimb flexion and positive tail suspension test; Score 2: Reduced hold of the forelimb when tail pulled; Score 3: Spontaneous circling or contralateral circling movement when tail pulled; Score 4: Spontaneous circling; Score 5: Death.

### 2.9. Cerebral infarct volume

Coronal brain sections (2 mm thickness) were made from the forebrain region and immersed with 2% 2, 3, 5 triphenyltetrazolium chloride solution at 37 °C for 30 min. These slices were kept into 10% paraformaldehyde solution for fixing purpose. Total infarct volume was calculated by summing up the unstained infarct areas in each section and multiplying it by slice thickness of 2 mm [23].

### 2.10. Caspase-3 activity assay

Caspase 3 levels of brain tissue homogenate were measured by the use of an ELISA-based assay kit, produced by Shanghai Crystal day Biotech Co., Ltd., China.

### 2.11. Blood pressure

It was measured by the invasive method before sacrificing the animals [24].

### 2.12. Statistical methods

All the data were expressed as mean ± SEM and evaluated using a one-way ANOVA and Tukey's post hoc test to identify two group differences at $p < 0.05$.

## 3. Results

### 3.1. Plasma glucose levels

STZ (45 mg/kg i.v.) significantly increase plasma glucose levels in diabetic animals (565.33 ± 9.61), diabetic sham operated (530.16 ± 25.83) and diabetic IR animals (479.16 ± 31.56) animals in comparison with normal animals (138.5 ± 13.3). Treatment with glibenclamide and insulin significantly decreases blood glucose levels to 147 ± 13.61, 137.66 ± 16.27 respectively in comparison with diabetic IR animals (Fig. 1).

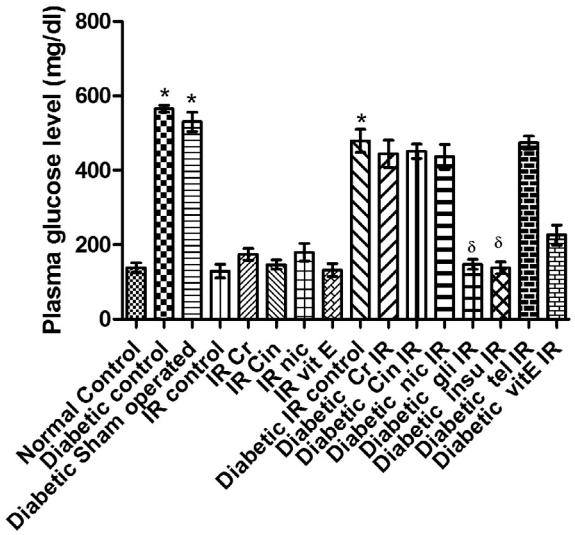



**Fig. 1.** Effect of cromakalim, cinnarizine, nicorandil and vitamin E on plasma glucose levels. Data presented as mean ± SEM, asteriks (*) indicate significant difference from the normal control and (δ) indicate significant difference from diabetic IR control rats at $p < 0.05$.

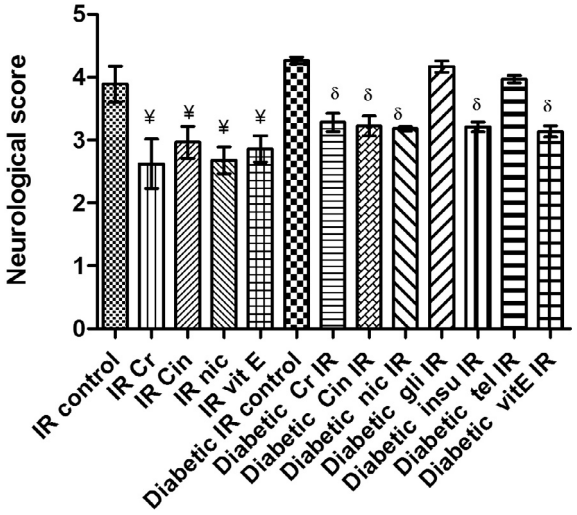



**Fig. 2.** Effect of cromakalim, cinnarizine, nicorandil and vitamin E on neurological score. Data presented as mean ± SEM, asteriks (¥) indicate significant difference from the IR control rats, (δ) indicate significant difference from diabetic IR control rats at $p < 0.05$.

### 3.2. Neurobehavioral score

Treatment of cromakalim, cinnarizine, nicorandil and vitamin E significantly reduced brain neurobehavioral score (2.62 ± 0.39, 2.96 ± 0.25, 2.88 ± 0.21, 2.86 ± 0.21 respectively) when compared with IR animals (3.89 ± 0.28). Treatment with cromakalim, cinnarizine, nicorandil, insulin and vitamin E significantly reduced neurobehavioral score to 3.14 ± 0.14, 3.22 ± 0.15, 3.18 ± 0.05, 3.22 ± 0.07 and 3.56 ± 0.208 when compared with diabetic IR (4.26 ± 0.25) animals (Fig. 2).

### 3.3. Cerebral infarct volume

Treatment of cromakalim, cinnarizine, nicorandil and vitamin E significantly reduced cerebral infarct volume (48.66 ± 4.58, 54.55 ± 4.49, 41.83 ± 3.02, 47.41 ± 3.45 respectively) when compared with IR control animals (81.45 ± 2.40). Treatment with nicorandil and vitamin E significantly reduced cerebral infarct volume to 43.02 ± 2.49, 45 ± 0.95 when compared with diabetic IR (61.80 ± 3.38) animals (Fig. 3).

### 3.4. Caspase-3 levels

Caspase-3 levels were significantly high in diabetic animals (226.57 ± 65.29), diabetic sham operated (106.94 ± 31.90) and diabetic IR (167.67 ± 49.15) animals when compared with normal animals (89.67 ± 26.35). Treatment of cromakalim, cinnarizine, nicorandil and vitamin E significantly reduced brain caspase-3 levels 110.83 ± 32.60 when compared with IR animals (180.84 ± 52.44). Treatment with cromakalim and nicorandil significantly reduced caspase-3 levels to 92.64 ± 26.66 and 92.99 ± 27.74 when compared with diabetic IR (167.67 ± 49.15) animals (Fig. 4).

### 3.5. Blood pressure

For the results of blood pressure, there was no significance among various groups (Fig. 5).

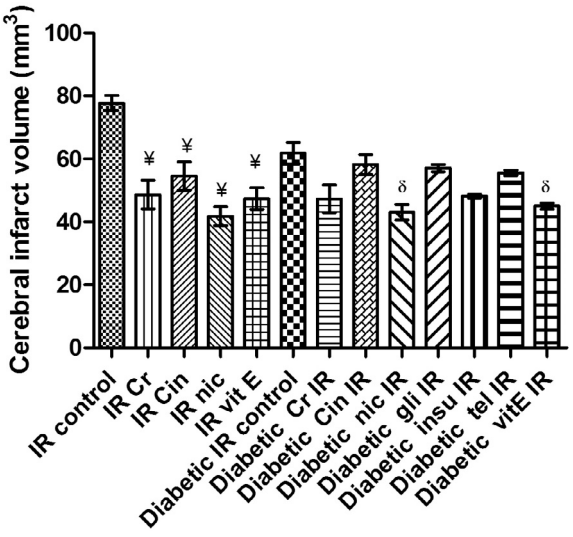



**Fig. 3.** Effect of cromakalim, cinnarizine, nicorandil and vitamin E on cerebral infarct volume. Data presented as mean ± SEM, asteriks (¥) indicate significant difference from the IR control rats, (δ) indicate significant difference from diabetic IR control rats at $p < 0.05$.

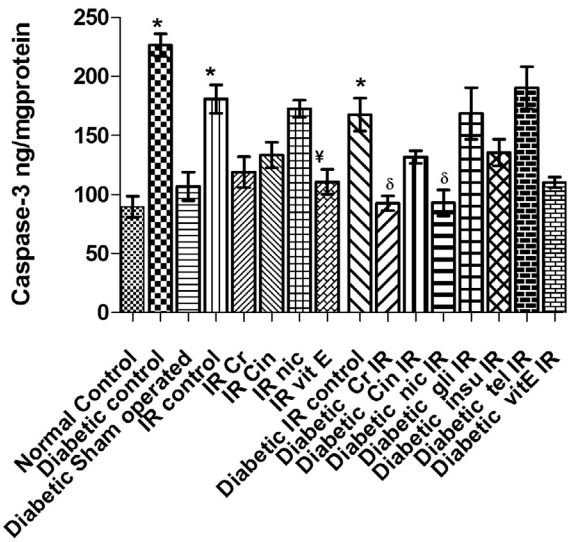



**Fig. 4.** Effect of cromakalim, cinnarizine, nicorandil and vitamin E on caspase-3 levels. Data presented as mean ± SEM, asteriks (∗) indicate significant difference from the normal control rats, (¥) indicate significant difference from diabetic IR control rats and (δ) indicate significant difference from diabetic IR control rats at $p < 0.05$.

## 4. Discussion

In the present study we have investigated the effect of potassium channel openers against IR model of brain injury along with STZ induced diabetes. Resulted symptoms resemble that observed in clinical status. We demonstrated that KCOs improve functional outcome by significantly reducing cerebral infarct volume and caspase enzyme levels. Inhibition of apoptosis may contribute to KCOS induced neuroprotective effect after ischemic stroke of T1DR.

Numerous studies reported high prevalence of stroke associated with diabetes [24]. Experimental studies on rats were done to rule out correlation between ischemic brain stroke and diabetes. Evidence showed that high blood sugar by STZ decreases blood flow to the brain by 37% and it is associated with raised cerebral infract diameter in the penumbral brain region. Oxidative stress, apoptosis and inflammatory changes are thought to be cellular mechanisms for neuronal injury with diabetes [25,26]. STZ diabetic animals with stroke have significantly raised apoptosis when compared to non-diabetic stroke animals [27]. In present study, we had used three KCOs namely cromakalim, nicorandil and cinnarizine. All three agents produced significant neuroprotection in terms of neurobehavioral score in comparison with IR animals and diabetic IR control animals. These effects are consistent with vitamin E and insulin. Further, all three KCOs and vitamin E showed a significant reduction in cerebral infarct volume when compared with IR control animals. However, only nicorandil and vitamin E significantly reduce infarct volume when compared with diabetic IR animals.

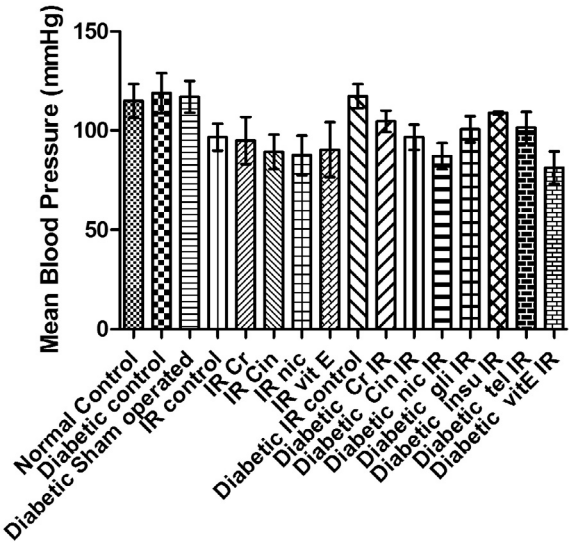



**Fig. 5.** Effect of cromakalim, cinnarizine, nicorandil and vitamin E on mean blood pressure. Data presented as mean ± SEM and indicate non-significant difference among various groups.

Apoptosis is a programed cell death that occurs in chronic neurodegenerative disorders such as stroke, Alzheimer's and Parkinson's diseases. It comprises two pathways intrinsic and extrinsic. Both pathways involve events with a series of caspase enzymes [28,29]. Apoptosis of neuronal cells is an important factor to neurological deficiencies with diabetes. Ischemic stroke exaggerates these neuronal deficiencies. Caspase-3 is one of the most abundant caspases among other members in the rat brain [30]. Recent animal experimental evidence indicates involvement of apoptosis in neurodegeneration after ischemic brain injury. Intracerebroventricular injection of caspases peptide inhibitor reduced caspase end products, cerebral IL-1β levels, reduced tissue damage and significantly improved behavioral deficits in ischemic mouse brain. Furthermore the same pathways and mechanisms are involved in pancreatic β cell destructions that lead to diabetes mellitus and high blood sugar level is associated with pathogenesis of stroke [31,32]. Intracerebral injection of a caspase-3 inhibitor protected against α-amino-3-hydroxy-5-methyl-4-isoxazole propionate mediated excitotoxicity and brain damage. Caspase inhibitors provide neuroprotection to ischemic animals [33]. After hypoxia and ischemia neurons apoptosis mediated via caspase-3 [34].

Studies have shown that caspase-3 mediated apoptosis pathways are activated during acute cerebral ischemia and play a crucial role in ischemic nerve injury at cellular and molecular levels [35,36]. Influx of $K^+$ current and hyperpolarization of cell prevent apoptosis and blocking of $K^+$ ion channels was suggested to induce apoptosis [37]. Data of our study revealed that caspase-3 levels were significantly high in diabetic IR animals. Cromakalim and nicorandil significantly reduced its levels. Therefore, a reduction

in caspase-3 by nicorandil and cromakalim treatment may produce hyperpolarization of cells, attenuate apoptosis and reducing infarct volume after stroke in diabetic animals. These data are comparable with standard ant-oxidant and anti-apoptotic vitamin E treated animals. Vitamin E produced anti-apoptotic and neuroprotective effects by inhibiting caspase-3 enzyme against hypoxia and reperfusion injury to rats [38].

It is known that potassium channel openers produce vasodilation and reduce blood pressure [39]. High blood pressure is one of the causes of hemorrhagic brain stroke [1]. Hence to rule out involvement of such a mechanism of action by KCOs, we had treated animals with antihypertensive telmisartan and more over the blood pressure of each group of animal was noted down. However, telmisartan treated animals didn't show any neuroprotective action and blood pressure of each group of animals remains unaffected. Secondly we had treated animals with two anti-diabetic drugs namely ATP sensitive potassium channel blocker glibenclamide and insulin. Except lowering of blood sugar level, glibenclamide didn't show any significant neuroprotective activity. Insulin treated animals showed a significant lowering of neurobehavioral score in diabetic IR animals. This insulin (antidiabetic standard) mediated action is supported by previous findings that insulin showed neuroprotective action by reducing cerebral infarct volume and apoptosis in diabetic rats with MCAO (middle cerebral artery occlusion) [40]. Thus neuroprotective action by KCOs is independent of blood pressure and glucose lowering activity. It may be due to opening of potassium ion channels and prevention of apoptosis through inhibition of caspase enzyme.

In this study, we for the first time demonstrated cromakalim and nicorandil treatment promotes neuroprotection after stroke in type-I diabetic rats. However, mechanisms by which KCOs produce neuroprotective effects need further study and investigation.

## 5. Conclusions

We found that cromakalim and nicorandil treatment promotes functional outcome after ischemic stroke in type-I diabetic rats. Anti-apoptotic effects may contribute to neuroprotective effects by KCOs after ischemic stroke in type-I diabetic rats.

## Conflict of interest

We declare that we have no conflict of interest.

## Acknowledgement

This study was funded by Gujarat Council on Science and Technology (GUJCOST), Gandhinagar, Gujarat, India/Grant No. GUJCOST/MRP/2014-15/389 dated 30/06/2014.

## References

[1] V.L. Roger, A.S. Go, D.M. Lloyd-Jones, E.J. Benjamin, J.D. Berry, W.B. Borden, D. M. Bravata, et al., Heart disease and stroke statistics.—2012 update: a report from the American Heart Association, Circulation 125 (2012) 2–220.
[2] H. Mast, J.L. Thompson, S.H. Lee, J.P. Mohr, R.L. Sacco, Hypertension and diabetes mellitus as determinants of multiple lacunar infarcts, Stroke 26 (1995) 30–33.
[3] K. Kikuchi, H. Uchikado, M. Morioka, Y. Murai, E. Tanaka, Clinical neuroprotective drugs for treatment and prevention of stroke, Int J Mol Sci 13 (2012) 7739–7761.
[4] J. Alvarez-Sabin, C.A. Molina, J. Montaner, J.F. Arenillas, R. Huertas, M. Ribo, et al., Effects of admission hyperglycemia on stroke outcome in reperfused tissue plasminogen activator–treated patients, Stroke 34 (2003) 1235–1241.
[5] A.Y. Poppe, S.R. Majumdar, T. Jeerakathil, W. Ghali, A.M. Buchan, M.D. Hill, Canadian alteplase for stroke effectiveness Study I. Admission hyperglycemia predicts a worse outcome in stroke patients treated with intravenous thrombolysis, Diabetes Care 32 (2009) 617–622.
[6] R. Ning, M. Chopp, T. Yan, A. Zacharek, C. Zhang, C. Roberts, et al., Tissue plasminogen activator treatment of stroke in type-1 diabetes rats, Neuroscience 222 (2012) 326–332.
[7] X. Fan, J. Qiu, Z. Yu, H. Dai, A.B. Singhal, E.H. Lo, et al., A rat model of studying tissue-type plasminogen activator thrombolysis in ischemic stroke with diabetes, Stroke 43 (2012) 567–570.
[8] K. Kasono, T. Yasu, A. Kakehashi, N. Kinoshita, H. Tamemoto, K. Namai, et al., Nicorandil improves diabetes and rat islet b-cell damage induced by streptozotocin in vivo and in vitro, Eur J Endocrinol 151 (2004) 277–285.
[9] G.C. Alan, E.B. Susan, A.M. John, Therapeutic potential for potassium channel modulator for CNS disorders, Expert Opin Ther Pat 13 (2003) 23–32.
[10] A. Masaharu, T. Yasushi, M. Eduardo, Antiapoptotic effect of nicorandil mediated by mitochondrial atp-sensitive potassium channels in cultured cardiac myocytes, J Am Coll Cardiol 40 (2002) 803–810.
[11] C. Heurteaux, V. Bertaina, C. Widmann, M. Lazdunski, K+ channel openers prevent global ischemia-induced expression of c-fos, c-jun, heat shock protein, and amyloid 3-protein precursor genes and neuronal death in rat hippocampus, Neurobiology 90 (1993) 9431–9435.
[12] H. Zhang, L.C. Song, C.H. Jia, Y.L. Lu, Effects of ATP sensitive potassium channel opener on the mRNA and protein expressions of caspase-12 after cerebral ischemia reperfusion in rats, Neurosci Bull 24 (2008) 7–12.
[13] S. Wang, J. Liu, Q. Chang, Y. Li, Y. Jiang, S. Wang, Neuroprotective effects of cromakalim on cerebral ischemia-reperfusion injury in rats, Neural Regen Res 5 (2010) 678–682.
[14] M.E. Omar, Modulation of visceral nociception, inflammation and gastric mucosal injury by cinnarizine, Drug Target Insights 2 (2007) 29–38.
[15] K. Kasono, T. Yasu, A. Kakehashi, A. Kinoshita, H. Tamemoto, K. Namai, et al., Nicorandil improves diabetes and rat islet b-cell damage induced by streptozotocin in vivo and in vitro, Eur J Endocrinol 151 (2004) 277–285.
[16] O.A. Ebuehi, R.A. Ogedegbe, O.M. Ebuehi, Oral administration of Vitamin C and Vitamin E ameliorates lead-induced hepatotoxicity and oxidative stress in the rat brain, Nig Q J Hosp Med 22 (2) (2014) 85–90.
[17] B. Ömer, L. István, Gy.P. Julius, Effect of the combination of glibenclamide, an ATP-dependent potassium channel blocker, and metoprolol, a cardioselective β-adrenoceptor blocker, during myocardial infarction in conscious rats, Turk J Med Sci 30 (2000) 517–522.
[18] A. Lacruz, T. Baptista, S. de Mendoza, J.M. Mendoza-Guillén, L. Hernández, Antipsychotic drug-induced obesity in rats: correlation between leptin, insulin and body weight during sulpiride treatment, Mol Psychiatry 5 (1) (2000) 70–76.
[19] M. Iqbal, K. Dubey, T. Anwer, A. Ashish, K.K. Pillai, Protective effects of telmisartan against acute doxorubicin-induced cardiotoxicity in rats, Pharmacol Rep 60 (3) (2008) 382–390.
[20] S. Yousuf, F. Atif, M. Ahmad, T. Ishrat, B. Khan, F. Islam, Neuroprotection offered by Majun Khadar, a traditional Unani medicine, during cerebral ischemic damage in rats, Evidence-Based Complementary Alter Med (2011) 1–9.
[21] O.H. Lowry, W.J. Rosenbrough, A.I. Farren, R.J. Randel, Protein measurement with folin phenol reagent, J Biol Reagent 93 (1951) 265–275.
[22] S.A. Menzies, J.T. Hoff, A.L. Betz, Middle cerebral artery occlusion in rats: a neurological and pathological evaluation of reproducible model, Neurosurgery 31 (2009) 100–106.
[23] W. Shilei, L. Junchao, C. Qingxian, L. Yu, J. Yan, W. Shiduan, Neuroprotective effects of cromakalim on cerebral ischemia-reperfusion injury in rats. Correlation with hippocampal metabotropic glutamate receptor 1 alpha and glutamate transporter 1, Neural Regen Res 5 (2010) 678–682.
[24] P. Subramani, R. Ramasamy, Measurement of invasive blood pressure in rats, J Pcol Pcot (2012) 172–177.
[25] M.J. Quast, J. Wei, N.C. Huang, D.G. Brunder, S.L. Sell, J.M. Gonzalez, et al., Perfusion deficit parallels exacerbation of cerebral ischemia/reperfusion injury in hyperglycemic rats, J Cereb Blood Flow Metab 17 (1997) 927–938.
[26] N.N. Rizk, J. Rafols, J.C. Dunbar, Cerebral ischemia induced apoptosis and necrosis in normal and diabetic rats, Brain Res 1053 (2005) 1–9.
[27] J.B. Schulz, M. Weller, M.A. Moskowitz, Caspases as treatment targets in stroke and neurodegenerative diseases, Ann Neurol 45 (1999) 421–429.
[28] M. Endres, S. Namura, M. Shimizu-Sasamata, C. Waeber, L. Zhang, T. Gomez-Isla, et al., Attenuation of delayed neuronal death after mild focal ischemia in mice by inhibition of the caspase family, J Cereb Blood Flow Metab 18 (1998) 238–247.
[29] S. Namura, J. Zhu, K. Fink, M. Endres, A. Srinivasan, K.J. Tomaselli, et al., Activation and cleavage of caspase-3 in apoptosis induced by experimental cerebral ischemia, J Neurosci 18 (1998) 3659–3668.
[30] G.S. Christopher, R.S. Brad, C.R. David, Apoptotic mechanisms after cerebral ischemia, Stroke 40 (2009) 331–339.
[31] A. Paul, J. Krijnen, W.M. Hans, S.S. Niessen, Apoptosis in diabetes, Apoptosis 14 (2009) 1387–1388.
[32] M. Rabuffetti, C. Sciorati, G. Tarozzo, E. Clementi, A.A. Manfredi, M. Beltramo, Inhibition of caspase-1-like activity by Ac-Tyr-Val-Ala-Asp-chloromethyl ketone induces long-lasting neuroprotection in cerebral ischemia through apoptosis reduction and decrease of proinflammatory cytokines, J Neurosci 20 (2000) 4398-04.
[33] C. Manabat, B.H. Han, M. Wendland, N. Derugin, C.K. Fox, J. Choi, et al., Reperfusion differentially induces caspase-3 activation in ischemic core and penumbra after stroke in immature brain, Stroke 34 (2003) 207–213.
[34] S.R. Martini, T.A. Kent, Hyperglycemia in acute ischemic stroke: A vascular perspective, J Cereb Blood Flow Metab 27 (2007) 435–451.

[35] M.F. Ahmed, S.A. Azza, M.E. Mona, A.K. Sanaa, E.E. Mostafa, Beneficial effects of thymoquinone and omega-3 on intestinal ischemia/reperfusion-induced renal dysfunction in rats, Bull Faculty Pharm Cairo Uni 52 (2014) 171–177.
[36] M. Tagami, K. Yamagata, K. Ikeda, Y. Nara, H. Fujino, A. Kubota, et al., Vitamin E prevents apoptosis in cortical neurons during hypoxia and oxygen reperfusion, Lab Invest 78 (1998) 1415–1429.
[37] C.C. Shieh, M. Coghlan, J.P. Sullivan, M. Gopalakrishnan, Potassium channels: molecular defects, diseases, and therapeutic opportunities, Pharmacol Rev 52 (2000) 557–594.
[38] D.C. Camerino, D. Tricarico, J.F. Desaphy, Ion channel pharmacology, Neurotherapeutics 4 (2007) 184–198.
[39] R. Yu-hua, H. Wang, Iptakalim, an ATP-sensitive potassium channel opener, confers neuroprotection against cerebral ischemia/reperfusion injury in rats by protecting neurovascular unit cells, J Zhejiang Univ Sci B 12 (2011) 835–845.
[40] H. Zhang, Y.Y. Liu, Y. Ma, Y.L. Lu, Protective effect of ATP sensitive potassium channel opener on cerebral ischemia/reperfusion injury and its signal transduction mechanism, Zhongguo Wei Zhong Bing Ji Jiu Yi Xue 19 (2007) 221–224.

*Pithadia AB et al / Journal of Pharmacy Research 2017,11(5),485-489*

**Research Article**
**ISSN: 0974-6943**

**Available online through**
**http://jprsolutions.info**

# Neuroprotective effect of potassium channel openers against hydrogen peroxide ($H_2O_2$) induced neuronal stress: *IN-VITRO* study

**Pithadia AB[1], Soni AK[1], Suhagia BN[1], Soni TG[2], Panchal SS[*3], Navale A[4]**

[1] Department of Pharmacology & Toxicology, Faculty of Pharmacy, Dharmsinh Desai University, College Road, Nadiad-387 001,Gujarat, India
[2]Soni TG Department of Pharmaceutics, Faculty of Pharmacy, Dharmsinh Desai University, College Road, Nadiad-387 001, Gujarat, India
[3]Department of Pharmacology, Institute of Pharmacy, Nirma University,
Sarkhej-Gandhinagar Highway, Chandlodia, Gota, Ahmedabad-382481,Gujarat, India
[4]Department of Pharmacology, Faculty of Pharmacy, Parul University At & P.O.Limda,Tal:Vaghodia,Vadodara-391760, Gujarat, India

**Received on:19-03-2017; Revised on: 30-04-2017; Accepted on: 20-05-2017**

## ABSTRACT

**Aim**: This study was design to assess and establish *in-vitro* neuroprotective role of potassium channel openers (KCOs) against hydrogen peroxide ($H_2O_2$) induced neuronal stress. **Material and methods**: Cell viability assay was done using MTT (3-(4, 5 dimethyl thiazole-2-yl)-2, 5-diphenyl tetrazolium bromide) method to determine $IC_{50}$ value of $H_2O_2$. Based on 50 % Inhibitory Concentration ($IC_{50}$) results of $H_2O_2$, neuroprotective effect of KCOs cromakalim, nicorandil , cinnarizine, and standard vitamin E was determined using U87 cells (human primary glioblastoma cell line). **Results:** Cromakalim, nicorandil and vitamin E significantly produce neuroprotective effects against $H_2O_2$ induced neuronal damage while cinnarizine did not produce significant activity. **Conclusion:** Results of our study demonstrated the neuroprotective role of potassium channel openers against $H_2O_2$ induced oxidative damage to neuronal cells.

**KEY WORDS:** Potassium Channel Openers; *In-vitro*; Neuroprotection

## 1. INTRODUCTION

Potassium channels have been identified by molecular cloning and genetic expression techniques in CNS and they are novel targets for CNS disorders such as Alzheimer's disease, Parkinson's disease, Huntington's disease and stroke. These channels play important role in neuronal physiology of CNS. Their main role is to maintain membrane potential and neuronal excitability [1]. KCOs have been reported to possess anti-apoptotic and antioxidant activity through activating $K^+$ channels [2]. It has been reported that there is 20 % decrease in $H_2O_2$ production in isolated brain mitochondria by KCOs and this effect is abolished by their blockers. Similar cell protective effects were obtained also for neuronal and nephron cells. These cells were exposed to toxic insult of glutamate and $H_2O_2$ [3,4]. Potassium channels are present in plasma membrane as well as in inner mitochondrial membrane of cells. Hence both sites are targeted by KCOs.

Brain is highly susceptible to oxidative damage due to continuous high amount of oxygen consumption. Numerous experimental studies have demonstrated that oxidative stress cause alterations in structure and function of neuronal cells including astrocytes and glial cells [5]. In present study, glial cells were selected as model of neuronal damage by oxidative toxicant $H_2O_2$ which has other potential consequences. Glial cells not only provide nutrients and growth factors for neurons but actively participate in immune reaction and mechanisms. Glial cells also contains other antioxidant enzymes such as superoxide dismutase, glutathione peroxidase which are require for metabolism of xenobiotics and hence they protect brain from oxidative damage [6].

The aim of our study was to evaluate the ability of KCOs to protect culture of human primary glioblastoma cells against $H_2O_2$ induced oxidative cellular damage through *in-vitro* experiments. We investigated the role and mechanism of action of KCOs in neuroprotection against oxidative stress.

***Corresponding author**
***Panchal SS,***
***Department of Pharmacology,***
***Institute of Pharmacy,***
***Nirma University, Sarkhej-Gandhinagar Highway,***
***Chandlodia, Gota, Ahmedabad-382481 Gujarat, India.***

## 2. MATERIALS AND METHODS

Fetal bovine serum (FBS), Phosphate buffered saline (PBS), Dulbecco's Modified Eagle's Medium (Ham's F12) and trypsin were obtained from Sigma Aldrich Co, St Louis, USA. EDTA (Ethylenediaminetetraacetic acid), glucose and antibiotics were

obtained from Hi-Media Laboratories Ltd., Mumbai. Dimethyl sulfoxide (DMSO) , propanol and MTT were procured from E. Merck Ltd., Mumbai, India.

### 2.1 Cell lines and culture medium

U87 (human primary glioblastoma cell line) cell line was procured from National Centre for Cell Sciences, Pune, India. Stock cells was cultured in Ham's F12 supplemented with 10% inactivated FBS, penicillin (100 IU/ml), streptomycin (100 µg/ml) and amphotericin B (5 µg/ml) in an humidified atmosphere of 5% $CO_2$ at 37°C until confluent. The cells were dissociated with cell scrapper. The stock cultures were grown in 25 $cm^2$ culture flasks and all experiments was carried out in 96 microtitre plates (Tarsons India Pvt. Ltd., Kolkata, India).

### 2.2 Preparation of Test Solutions

For studies, each test drug and $H_2O_2$ was weighed and mixed to obtain the desired concentration using DMSO. Volume was made up with Ham's F12 supplemented with 2% inactivated FBS to obtain a stock solution of 1 mg/ml concentration and sterilized by filtration. Serial two fold dilutions were prepared from this for carrying out studies. In Experiment 1, cells were incubated with different concentrations of $H_2O_2$ (2, 5, 50, 100, and 200 nM) for 24 h, and MTT assay was performed to detect $IC_{50}$ value of $H_2O_2$. In Experiment 2, cells were pretreated with different concentrations of each KCOs and vitamin E (2, 5, 10, 20, and 40 µg) for 4 h and then incubated with $H_2O_2$ (effective dose) for 24 h. MTT assay was done to evaluate neuroprotective effect of all agents.

### 2.3 Determination of cell viability by MTT Assay

The ability of the cells to survive in a toxic insult has been the basis of most cytotoxicity assays. This assay is based on the assumption that dead cells or their products do not reduce tetrazolium. The assay depends both on the number of cells present and on the mitochondrial activity per cell. The principle involved is the cleavage of MTT into a blue colored product formazan by mitochondrial enzyme succinate dehydrogenase. The number of cells was found to be proportional to the extent of formazan production by the cells used. The monolayer cell culture was trypsinized and the cell count was adjusted to 1.0 x $10^5$ cells/ml using Ham's F12 medium containing 10% FBS. To each well of the 96 well microtitre plate, 0.1 ml of the diluted cell suspension (approximately 10,000 cells) was added. After 24 h, when a partial monolayer was formed, the supernatant was flicked off, and monolayer was washed with medium and 100 µl of different concentrations of $H_2O_2$ were added on to the partial monolayer in micro titer plates. The plates were then incubated at 37º C for 24 hours in 5% $CO_2$ atmosphere. After 24 hours, the solutions in the wells were discarded and 50 µl of MTT in PBS was added to each well. The plates were gently shaken and incubated for 3 h at 37º C in 5% $CO_2$ atmosphere. The supernatant was removed, 100 µl of propanol was added and the plates were gently shaken to solubilize the formed formazan. The absorbance was measured using a microplate reader at a wavelength of 540 nm. Inhibitory concentration ($IC_{50}$) values were calculated and that dose (effective dose) of $H_2O_2$ was considered for the determination of neuroprotective effect of KCOs and vitamin E[7].

### 2.4 Determination of Neuroprotective activity

The experiment was performed as per the standard protocol. Briefly, exponentially growing U87 cells was trypsinized from the culture flask and $1.5\times10^5$ cells/ml were seeded in 96 microtitre plate. After attaining confluency, culture mediums from the wells were discarded and cells were pre-incubated with different concentrations of test samples and standard vitamin E (2, 5, 10, 20, and 40 µg) for 4 h in culture medium and then incubated with $H_2O_2$ (effective dose) for 24 h at 37ºC with atmosphere of 5% $CO_2$ except control wells. After incubation MTT assay was performed to determine the cell viability. From the absorbance values of test and control groups, percentage cell viability offered by test samples against $H_2O_2$ induced toxicity was calculated with following formulae.

$$\% \text{ cell viability} = (A_s - A_b / A_c - A_b) \times 100$$

Where

$A_s$ = Absorbance of sample (Samples with all reagents and cells)

$A_b$ = Absorbance of blank well

$A_c$ = Absorbance of control (All the reagents with cells except sample)

## 3. STATISTICAL ANALYSIS

Data were expressed as mean ± standard error of mean (SEM). Statistical analysis was analyzed by one way ANOVA followed by Tukey's test for multiple comparisons using GraphPad Prism 5 software. $P < 0.05$ was considered to be statistically significant.

## 4. RESULTS AND DISCUSSION

The dose dependent effect of $H_2O_2$ (2, 5, 50, 100, and 200 nM) on cell proliferation after 24 h. Values are presented as mean ± SEM of three experiments in each group. 50% inhibition concentration value ($IC_{50}$) was found to be 100 nM **(Figure 1)**. **Figure 2 to 5** showed dose-dependent effect of cromakalim, nicorandil, cinnarizine and vitamin E (2, 5, 10, 20, and 40 µg) alone and against $H_2O_2$ -induced changes on cell proliferation. Values are presented as mean ± SEM of three experiments in each group. Treatment with alone KCOs and vitamin

E (2, 5, 10, 20, and 40 µg) did not affect cell proliferation. However their pretreatment dose dependently enhanced cell proliferation against $H_2O_2$ toxicity. These dose dependent protection was non-significant in cinnarizine treated cells, while cromakalim (10, 20, and 40 µg), nicorandil ( 10, 20, and 40 5ØßgÍ) and vitamin E (5, 10, 20, and 40 µg) showed significant protection against $H_2O_2$ toxicity when compared with non-treated cells (*, $p < 0.05$).

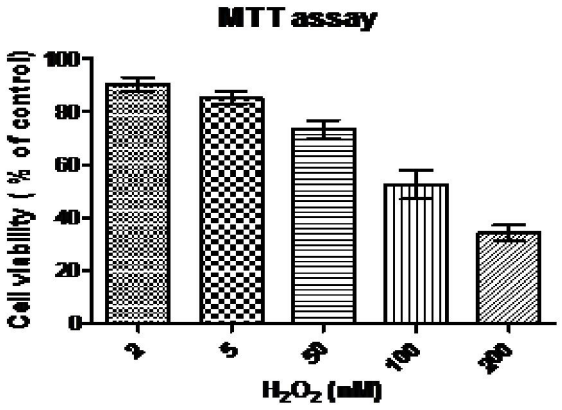



**Figure 1: The dose dependent effect of $H_2O_2$ (2, 5, 50, 100, and 200 nM) on cell proliferation after 24 h.**

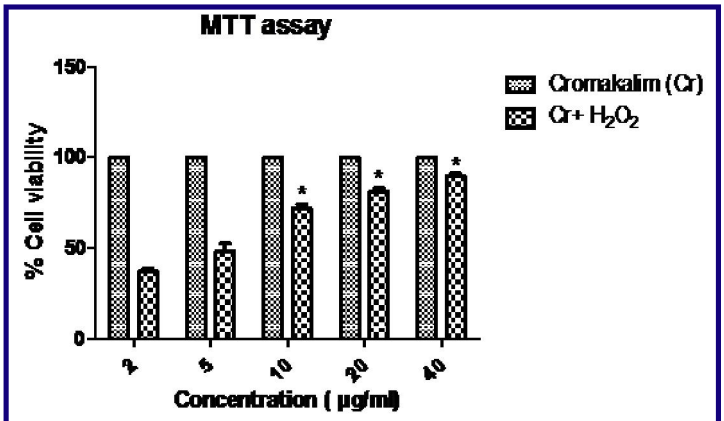



**Figure 2: The dose-dependent effect of cromakalim (2, 5, 10, 20, and 40 µg) alone and against $H_2O_2$ induced changes on cell proliferation.**

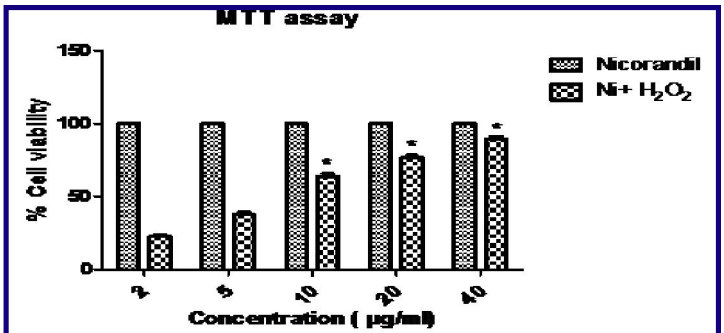



**Figure 3: The dose-dependent effect of nicorandil (2, 5, 10, 20, and 40 µg) alone and against $H_2O_2$ induced changes on cell proliferation.**

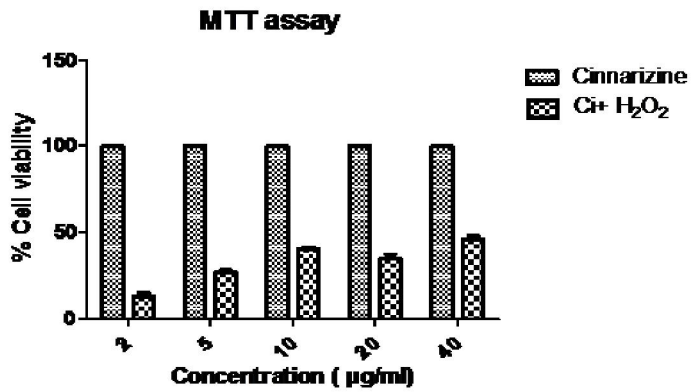



**Figure 4: The dose-dependent effect of cinnarizine (2, 5, 10, 20, and 40 µg) alone and against $H_2O_2$ induced changes on cell proliferation.**

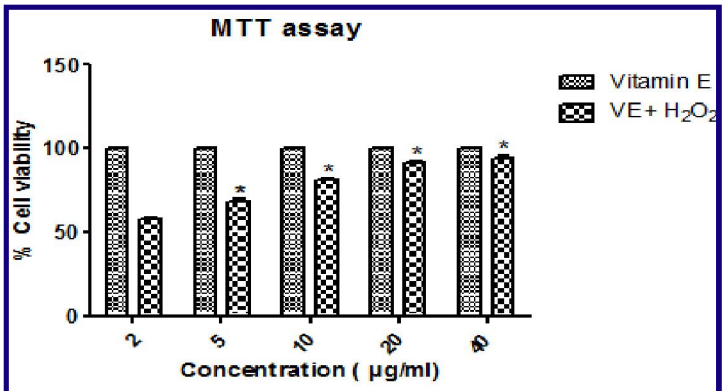



**Figure 5: The dose-dependent effect of vitamin E (2, 5, 10, 20, and 40 µg) alone and against $H_2O_2$ induced changes on cell proliferation.**

Hence we have demonstrated that KCOs protected against $H_2O_2$ induced cell death in cultured neurons. This is consistent with previous findings that cromakalim, nicorandil produced neuroprotective and anti-oxidant effect in ischemic brain stroke model of rodents and prevent neuronal excitotoxicity [8]. Thus present results support the hypothesis that oxidative stress mediated neuronal damage is reversed by cromakalim and nicorandil as well as standard anti-oxidant vitamin E. It is well reported that pretreatment of tissues with KCOs produce cytoprotective effect against various insults and glipizide, a specific blocker of ATP-sensitive $K^+$ channels increase ischemia-induced expression of various genes and this effect was blocked by same channel openers to produce neuroprotection [9, 10]. Even cromakalim prevents glutamate mediated cell death, cell viability, necrosis and oxidative injury in cultures hippocampal neuronal cells [11] and produces neuroprotective effects in diabetic ischemic rats as well as aluminium chloride treated ischemic rats [12, 13]. Hence $K^+$ channel openers seem to be potential drugs for neuronal diseases. Cinnarizine is a blocker of $Ca^{2+}$ channel and used in treatment of vertigo and motion sickness. However it also opens potassium channel to elicit anti-inflammatory, anti-nociceptive, anti-oxidant and gastro- protective properties [14]. In our study, cinnarizine did not

show any significant activity in our study. $H_2O_2$ induced neuronal damage and it is prevented by KCOs as well as standard anti-oxidant vitamin E. During oxidative stress enormous amount of free radicals are generated. Free radicals are unstable chemical species with a single unpaired electron in an outer orbital and readily attack nucleic acids as well as other membrane molecules. In addition, free radicals set up autolytic reactions.

$H_2O_2$ is considered as a toxic intermediate species generated during free radical induced redox reaction [15]. $H_2O_2$ mediated reaction along with hydroxyl radical reacts with unsaturated fatty acids and generates lipid peroxyl radical which finally cause protein denaturation, DNA damage and cell death. Oxidative stress results into ATP depletion, intracellular acidosis, enhanced release of excitatory neurotransmitters and increased $Ca^{2+}$ intracellular and $K^+$ extracellular. KCOs allow entry of $K^+$ ion by opening ion channels and activate them to produce anti-oxidant and neuroprotective action [16, 17].

Structurally cromakalim is the benzopyran derivative and nicorandil is the nicotinamide derivative type of potassium channel opener that showed antioxidant function against lipid peroxidation. Both of these agents have unsaturated double bond. Additionally cromkalim contains hydroxyl group in its structure. These all structural feathers provide H donating ability to stop lipid peroxidation chain reaction [18, 19]. During the antioxidant reaction, vitamin E is converted to a α-tocopherol radical by the donation of labile hydrogen to a lipid peroxyl radical. Vitamin E performs a unique function by interfering free radical chain reactions via catching the free radical. The free hydroxyl group on the aromatic ring is responsible for the antioxidant properties [20].

Recently it is reported that antioxidants delay the development of neurodegenerative diseases in various animal models [21]. Thus, our results for neuroprotection by KCOs against $H_2O_2$ induced cytotoxicity is also consistent with several previously reported neuroprotective activities of KCOs in both *in-vivo* and *in-vitro* models. Based on the present findings, it may be suggested that potassium channel openers may provide neuroprotective effect to rescue the neuronal cells against $H_2O_2$ induced neuronal damage by suppression of oxidative stress response.

## 4. CONCLUSION

In view of our results, we hypothesize that KCOs are able to reverse the effect of $H_2O_2$ induced neuronal damage. However detailed cellular studies and clinical studies are required to establish the mechanisms underlying the beneficial effects of KCOs and to explore their pharmacotherapeutic role in the treatment of neurodegenerative diseases.

## Acknowledgments

This study was funded by Gujarat Council on Science and Technology (GUJCOST), Gandhinagar, Gujarat, India/Grant No. GUJCOST/MRP/ 2014-15/389 dated 30/ 06/2014.

## REFERENCES

1. Alan GC, Susan EB, John AM ,Therapeutic potential for potassium channel modulator for CNS disorders, Expert Opin Ther Pat 2003, 13(1),23-32.
2. Hosseini-Tabatabaei A, Abdollahi M, Potassium channel openers and improvement of toxic stress: Do they have role in the management of inflammatory bowel disease? Inflam Allergy Drug Targets 2008, 7, 129-135.
3. Heinen A, Camara AK, Aldakkak M, Rhodes SS, Riess ML, Stowe DF. Mitochondrial $Ca^{2+}$-induced $K^+$ influx increases respiration and enhances ROS production while maintaining membrane potential, Am J Physiol Cell Physiol 2007, 292, C148–56.
4. Kulawiak B, Kudin AP, Szewczyk A, Wolfram SK: BK channel openers inhibit ROS production of isolated rat brain mitochondria, Exp Neurol 2008, 212,543-547.
5. Goodman Y, and Mattson MP: $K^+$ channel openers protect hippocampal neurons against oxidative injury and amyloid beta-peptide toxicity, Brain Res 1996, 728, 328-332.
6. Kurosinski P, Gotz J: Glial cells under physiologic and pathologic conditions, Archives Neurol 2002, 59(10),1524–1528.
7. Zhe G, Rui-Yuan P, Xiao-Yan Q. Potential Protection of Coeloglossum viride var. Bracteatum Extract against Oxidative Stress in Rat Cortical Neurons, J Anal Meth In Chem, 2013,1-7.
8. Wang SL, Wang P, Chang QX, Cromakalim pretreatment affects mitochondrial structure and function in a rat model of ischemia/reperfusion injury, Neural Regen Res 2008, 3(9), 933-938.
9. Dominika M, Sandra R, Mirandola b, Wolfram S, Kunz B, Mitochondrial potassium channels and reactive oxygen species, FEBS Lett, 2010,1-5.
10. Catherine H, Valtrie B, Catherine W, Michel L, $K^+$ channel openers prevent global ischemia-induced expression of c-fos, c-jun, heat shock protein, and amyloid (3-protein precursor genes and neuronal death in rat hippocampus, Neurobiol, 1993, 90, 9431-9435.
11. Lauritzen I, Weihe J, Lazdunski M, The potassium channel opener levcromakalim prevents glutamate-induced cell death in hippocampal neurons, J Neurochem, 1997, 69, 1570-1579.

**12.** Pithadia AB, Rajesh KS, Geetika MP, Panchal SS, Neuroprotective effects of cromakalim on cerebral ischemia-reperfusion (IR) injury in streptozotocin (STZ) induced type-I diabetic rats, J Pharm Res 2014, 8,773-778.

**13.** Pithadia AB, Panchal S, Neuroprotective effects of cromakalim on cerebral ischemia-reperfusion (IR) injury and aluminum induced toxicity in rat brain, Int J Pharm Pharm Sci, 2014, 6, 392-396.

**14.** Omar ME, Abdel S, Modulation of visceral nociception, inflammation and gastric mucosal injury by Cinnarizine, Drug Target Insights, 2007, 2, 29–38.

**15.** Ademiluyi OA, Oboh G, Aragbaiye FP, Oyeleye SI, Ogunsuyi OB , Antioxidant properties and in vitro a-amylase and a-glucosidase inhibitory properties of phenolics constituents from different varieties of Corchorus spp. J Taibah Uni Med Sci , 2015, 10(3), 278-287.

16. Liu X, Wu JY, Zhou F, The regulation of rotenone-induced inflammatory factor production by ATP-sensitive potassium channel expressed in BV-2 cells, Neurosci Lett, 2006, 394, 131-135.

**17.** Zhu HL, Luo WQ, Wang H , Iptakalim protects against hypoxic brain injury through multiple pathways associated with ATP-sensitive potassium channels, Neurosci, 2008, 157, 884-894.

**18.** Mannhold R. $K_{ATP}$ channel openers: structure–activity relationships and therapeutic potential, Med Res Rev, 2004, 24, 213–266.

**19.** Jahangir A, Shen WK, Terzic A, Potassium channel openers: therapeutic potential in cardiology and medicine, Expert Opin Pharmacother, 2001, 2, 1995–2010.

**20.** Burton GW, Ingold KU, Vitamin E as an in vitro and in vivo antioxidant, Ann NY Acad Sci 1989, 570:7–22.

**21.** Uddin MS, Mamun AA, Hossain MS, Akter F, Iqbal MA, Asaduzzaman M, Exploring the Effect of Phyllanthus emblica L. on Cognitive Performance, Brain Antioxidant Markers and Acetylcholinesterase Activity in Rats: Promising Natural Gift for the Mitigation of Alzheimer's Disease, Annals Neurosci 2016, 23, 218-229.


**Source of support:** Nil , **Conflict of interest: None Declared**

*AB Pithadia et al. / Journal of Pharmacy Research 2014,8(6),773-778*

**Research Article**
**ISSN: 0974-6943**

**Available online through**
**www.jpronline.info**

# Neuroprotective Effects of Cromakalim on Cerebral Ischemia-Reperfusion (IR) Injury In Streptozotocin (STZ) Induced Type-I Diabetic Rats

**AB Pithadia[*1], Rajesh KS[1], Geetika MP[2], SJ Panchal[3]**

*[*1]Department of Pharmacology, Parul Institute of Pharmacy, Vadodara-391760, Gujarat, India*
*[2]Parul Institute of Neurosciences, Parul Sevashram Hospital, Vadodara,Gujarat, India*
*[3]Department of Pharmacology, Institute of Pharmacy, Nirma University, Ahmedabad, India.*

**Received on:21-04-2014; Revised on: 08-05-2014; Accepted on:06-06-2014**

## ABSTRACT

**Objective:**To evaluate neuroprotective effects of cromakalim in diabetic rats. **Methods:** Wistar rats were randomly assigned to 6 groups (n = 36): Normal control, diabetic (STZ) control, diabetic Sham operated, ischemia reperfusion (IR) control, IR-diabetic control and IR-diabetic with cromakalim. Cromakalim 10 mg/kg intraperitonially (i.p.) was administered daily for 28 days in middle cerebral artery occluded rats treated with STZ. At 24 hours post-surgery, neurological score, brain hemisphere weight difference, lipid peroxidation, $Na^+/K^+$ ATPase pump activity and super oxide dismutase level were measured. **Results:** Following cerebral ischemia-reperfusion injury along with STZ, neurological score, brain hemisphere weight difference, malondialdehyde (MDA) levels were significantly high while $Na^+/K^+$ ATPase pump activity and super oxide dismutase(SOD) levels were significantly lower in diseased animals ($P < 0.05$). Treatment with cromakalim significantly reduced neurological score, brain hemisphere weight difference, malondialdehyde levels while raised $Na^+/K^+$ ATPase pump activity and super oxide dismutase levels ($P < 0.05$). **Conclusion:** Hence of results our study demonstrated the neuroprotective potential of cromakalim in cerebral IR injury in type I diabetic rats.

**KEY WORDS** cerebral ischemia-reperfusion. cromakalim. STZ. Type I diabetes. oxidative stress.

## 1. INTRODUCTION

Many studies have reported worsening of cerebral ischemia condition in diabetes and diabetes is considered as modifiable risk factor for brain stroke. Preclinical studies have demonstrated that potassium channel modulators show a neuroprotective effect on cerebral ischemia-reperfusion (IR) injury in experimental rats. However, their exact mechanism of action in brain stroke associated with diabetes remains poorly understood. Hence present study was done to investigate the prophylactic use of the cromakalim on neurological function in rats with cerebral IR injury and STZ induced type-I diabetes.

A stroke is an interruption of blood supply to a particular part of the brain. It can be either ischemic stroke(IS) due blockage of blood vessel within the brain and that constitute about 83% of strokes or haemorrhagic stroke due to rupturing of blood vessel and, causing blood to leak into the brain. It is documented that diabetes mellitus increases the risk of stroke by 2 to 3 times by increasing susceptibility to atherosclerosis and proatherogenic risk factors such as hypertension and abnormal blood lipids[1].Northern Manhattan study showed 2.7 fold increased risk of stroke with high fasting glucose. Control of blood sugar has been shown to reduce microvascular complications such as nephropathy, retinopathy and macrovascular complications such as stroke [2].

Hence diabetes is considered as a high risk factor for brain stroke. Current treatment of brain stroke includes tissue plasminogen activators, antiplatelet agents and anticoagulants for their antithrombotic effects. Free radical scavengers, minocycline and growth factors have shown neuroprotective effects in the treatment of stroke, while antihypertensive drugs, lipid-lowering drugs and oral antidiabetic drugs have shown beneficial effects for the prevention of stroke [3]. These agents are associated with number of side effects and hence there is a need to identify new targets and new molecules in central nervous system (CNS) that provide neuroprotection in stroke related disorders such as diabetes mellitus.

Ion channels for $Na^+$, $K^+$, $Ca^{2+}$ and $Cl^-$ are widely expressed in CNS and play a crucial role in controlling a very wide spectrum of physiological processes such as neuronal action potential. It is well reported that their dysfunction can lead to various neurological diseases. Hence new generation of CNS drugs in brain stroke are expected to result from targeting these ion channels. $K^+$ channels are

***Corresponding author.**
***AB Pithadia***
***Department of Pharmacology,***
***Parul Institute of Pharmacy,***
***Vadodara-391760, Gujarat, India***

widely expressed in CNS and their beneficial role has been already established as neuroprotective .Their dysfunction alter neuronal physiological processes and results in pathophysiology of various diseases including stroke, epilepsy, pain and cognition [4] .Hence $K^+$ channels are considered as potential therapeutic targets in the treatment of neurological disorders.

Cromakalim, adenosine triphosphate-sensitive potassium channel ($K_{ATP}$) openers provide neuroprotection against cerebral ischemia-reperfusion injury by anti-inflammatory and anti-oxidant action in rats [5].However its neuroprotective mechanism in stroke related disorder such as diabetes has not been studied. Hence present study hypothesized that $K_{ATP}$ openers could treat symptoms of stroke in type-I diabetes by providing neuroprotection. Rat model of cerebral ischemia-reperfusion was established through middle cerebral artery occlusion using the suture method (5) and this was preceded by STZ to induce type-I diabetes in same animals. The effects of prophylactic use of the $K_{ATP}$ channel opener, cromakalim, on neurological function, brain infract volume and lipid peroxidation parameters were determined to explore the mechanisms of action of cromakalim for neuroprotection in diabetes.

## 2. MATERIALS AND METHOD

### 2.1 Drugs and Chemicals

Cromakalim was gifted from Dr. Anjali Tarai, Assistant Professor, Department of Pharmacology, VSS Medical College, Cuttack, Orissa, India. Other chemicals used in experiment were of laboratory grade. Cromakalim solution was prepared freshly by dissolving it in sterile distilled water and injected i.p. with dose 10 mg/kg. STZ solution was made freshly in phosphate buffer at the beginning of each experiment and given orally at dose of 45 mg/kg i.v.

### 2.2 Animals

Healthy adult female Wistar rats (250 gm) aged 8 months were selected for the study. Animals were maintained at 18± 2 °C and kept in well ventilated animal house in polypropylene cages with free access to food and water. All studies was done with prior permission from IAEC of Parul Institute of Pharmacy (921/PO/AC/05/ CPCSEA) and Study was approved as Protocol No PIPH 21/13. Animals were divided into six groups.

Group 1 served as normal control (n=6) and were treated 4 % tween 80 as vehicle. Group 2, Diabetic control (n=6) animals and were treated with STZ at dose 45 mg/kg i.v (intravenous) injection on day 1. On day 3 Conformation of diabetes was done using Diachex strip. Group 3 served Diabetic Sham operated (n=6) and treated with 4 % tween 80 as vehicle for 28 days after STZ induced diabetes. Sham operation was performed after 3 weeks. Group 4 served as ischemia reperfusion (IR) control (n=6) and received 4 % tween 80 as vehicle for 28 days. Cerebral ischemia –reperfusion was performed on 28$^{th}$ day from initiation of experiment. Group 5 served as diabetic-IR control (n=6) and animals were treated with 4 % tween 80 for 4 weeks after induction of diabetes with STZ at dose 45 mg/kg i.v. Cerebral ischemia –reperfusion was performed on 28$^{th}$ day from initiation of experiment. Group 6 treated with STZ and cromakalim (n=6) along with IR injury. These animals were treated with cromakalim daily for 28 days at dose 10 mg/kg i.p. Cerebral IR was performed on 28$^{th}$ day from initiation of experiment.

Cerebral IR was induced as per transient middle cerebral artery occlusion (tMCAO) method as per Wang et al., 2010. Rats were anesthetized with an i.p. injection of 100 mg/kg ketamine. A 2–3 cm incision was made in the middle of the neck line, separating the left carotid artery, the superior thyroid artery, and the occipital artery, as well as the internal and external carotid communicating arteries. The occipital artery branches of ECA (external carotid artery) were isolated and tied with a cotton thread. Cotton thread was tied loosely around the ECA stump near the bifurcation. Then ICA (internal carotid artery) and CCA (common carotid artery) were temporarily occluded by fine vessel clip. Through a small incision to the ECA stump, blunt Poly-L-lysine coated 4-0 monofilament was inserted from the left external carotid artery into the left internal carotid artery to a depth of 18.0mm, vessel clip from ICA removed. After a variable length of suture had been inserted into ECA stump, resistance was felt and slight curving of suture was observed, indicating that suture had passed MCA (Middle cerebral artery) origin and reached to proximal segment of anterior cerebral artery (ACA-it has small diameter). Hence suture had blocked all sources of blood from ICA, ACA and posterior cerebral artery. Finally vessel clip from CCA was removed to restore blood flow. The midline incision was closed, leaving suture protruding so it could be withdrawn to allow reperfusion. The thread was maintained for 2 hours and subsequently removed to restore blood flow to the common carotid and internal carotid arteries. Here 18 mm of suture was pulled back until resistance was felt, indicating that tip cleared the ACA-ICA lumen and was in ECA stump, then trimmed. The animals were transfer to a fresh cage with free access to food and water.

### 2.3 Tissue homogenate preparation

At the end of the study, animals were sacrificed, brain were dissected out and washed with 0.9% NaCl and stored at –40 °C for further processing. Brain was homogenized in 10% (w/v) ice-cold 0.1 M PBS (pH 7.4). The lipid peroxidation (LPO) was estimated with a part of crude homogenate and the rest homogenate was centrifuged at 12,000 rpm for 20 min to obtain the supernatant (S) that was used for enzymatic estimations (6).

### 2.4 Tissue total protein level

It was measured using bovine serum albumin as standard, alkaline copper reagent Solution A (2% sodium carbonate in 0.1 N NaOH

solution in distilled water) and solution B(0.5% copper sulphate in 1.0% sodium potassium tartarate).50.0 ml of solution A was mixed with 1.0ml of solution B just before use. Folin's phenol reagent was obtained commercially. One volume of Folin's phenol reagent was diluted with 1 volume of distilled water just before use. 20.00 mg standard BSA was dissolved in 100 ml distilled water. Few drops of NaOH were added to aid complete dissolution of BSA(Bovine serum albumin) and to avoid frothing. 10ml of the stock was diluted to 100ml to get a working standard (200 µg/ml). Procedure 0.1ml crude homogenate was used for protein extraction. The protein was extracted by mixing with 5% cold trichloroacetic acid and centrifuged. The pellet was solubilized with 0.5 N sodium hydroxide and stored over-night at room temperature. After neutralization with 0.5 N HCl, 0.2 ml of diluted solution and different concentrations of standard were taken. The volume was made up to 1.0 ml with distilled water. Blank contained 1.0 ml distilled water. To all the tubes 5.0 ml alkaline copper reagent was added and left at room temperature for 10 Min. 0.5 ml of folin's phenol reagent was added and the blue colour developed was read after 20 minutes at 660nm against reagent blank in a spectrophotometer. Protein concentration is expressed as mg/gm of wet brain tissue [7].

**2.5 Blood glucose level**

Serum samples were analyzed for glucose levels using glucometer (One Touch Ultra 2, Lifescan Inc, USA).

**2.6 Malondialdehyde (MDA)**

It is a marker of lipid peroxidation. 1 ml of homogenate was incubated at 37 °C for 10 min. 1 ml of 10% trichloroacetic acid (TCA) chilled (w/v) was added to it and centrifuged at 2500 rpm for 15 min at room temperature. 1 ml of 0.67% TBA was added to 1 ml of supernatant and kept in a boiling water bath for 10–15 min. The tubes were cooled under tap water. After cooling 1 ml of distilled water was added to it and absorbance was taken at 530 nm. The results were expressed as micromoles/mg of protein[8].

**2.7 Superoxide dismutase (SOD)**

An aliquot of 0.25 ml ice-cold chloroform was added to 0.1 ml of supernatant followed by addition of 0.15 ml ice-cold ethanol. The mixture was centrifuged at 3000 rpm for 10 min at 4 °C. 0.2 ml the supernatant was taken and 1.3 ml buffer, 0.5 ml EDTA, and 0.8 ml water were added. Reaction was started by adding 0.2 ml epinephrine. Change in absorbance ΔOD (optical density)/min at 480 nm was read for 3 min. The results were expressed in terms of munits/mg protein. One unit of enzyme activity is defined as the concentration required for the inhibition of the chromogen production by 50 % in one minute under the defined assay conditions[9].

**2.8 $Na^+/K^+$ ATPase pump activity**

The incubation mixture containing 0.1 ml of each i.e tris HCL, 50 mM magnesim sulphate, 650 mM NaCl, 50 mM KCl, 1mM EDTA, 40 mM ATP and brain homogenate. Mixture was incubated at 36 °C for 15 minute. The reaction was arrested by addition of 1ml 10 % TCA and resultant mixture was centrifuged for 5 min. 0.5 ml supernatant was taken,2.5 ml ,0.5 ml of 2.5 % ammonium molybdate and 0.25 ml ANSA (1-amino 2-napthol 4-sulphonic acid)was added. The mixture was incubated at 37 °C for 10 minutes. The intensity of blue color developed against blank at 620 nm. Results were expressed as nmoles of inorganic phosphorus liberated/min/mg protein. Standard calibration curve was prepared using potassium dihydrogen phosphate (2-15 µg/ml) [10].

**2.9 Brain hemisphere weight difference**

The MCA occluded animals were anaesthetised with high dose of pentobarbitone. The skull was opened and whole brain was removed immediately, rinsed with ice cold distilled water followed by 20 % sucrose and dried using bloating paper. The ipsilateral and contralateral parts of brain are isolated and weighed on digital balance. Weight difference was calculated between ipsilateral and contralateral brain region of each animal[11].

**2.10 Neurobehavioral function scores**

Neurological score was observed after 24 hrs of IR [12].This score was observed for group 4, 5 and 6.

Score 0: No apparent deficit

Score 1: Contralateral forelimb flexion when suspended by tail

Score 2: Decreased grip of the contralateral forelimb while tail pulled

Score 3: Spontaneous movement in all direction or contralateral circling only if pulled by tail.

Score 4: Spontaneous contralateral circling

Score 5: Death after recovery from anaesthesia.

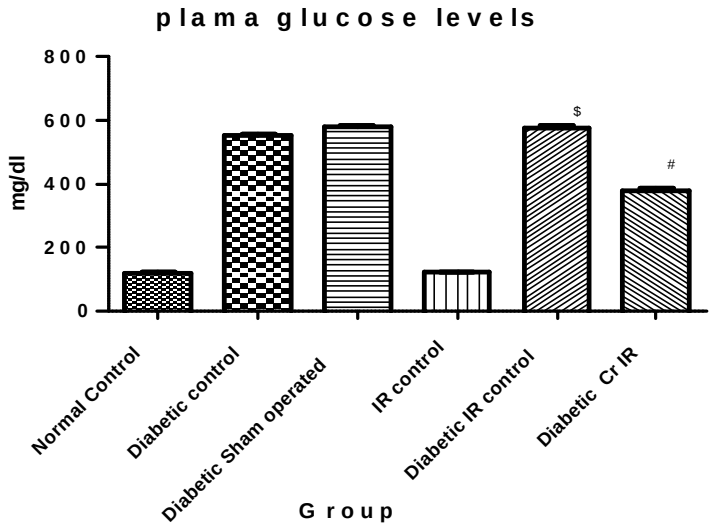
**3. STATISTICAL METHODS**

Data are expressed as mean values ± S.E.M and analyzed using one-way ANOVA followed by Post-hoc Tukey's tests to identify two group differences using Prism software at 5% significance level.

**4. RESULTS**

**4.1 Blood glucose levels**

STZ at dose of 45 mg/kg i.v. significantly raise blood glucose level in diabetic control animals when compared with normal animals (120.6 ± 0.71).An elevated blood glucose level is responsible for higher ischemic injury in diabetic IR animals. Effect of drug on plasma glucose level was measured. Treatment with cromakalim with dose of 10 mg/kg i.p. significantly decreases in blood glucose level (378.67 ± 7.92) when compared with diabetic IR group (575.5 ± 8.6) as well as diabetic control group (553.3 ± 4.28) (Figure 1).

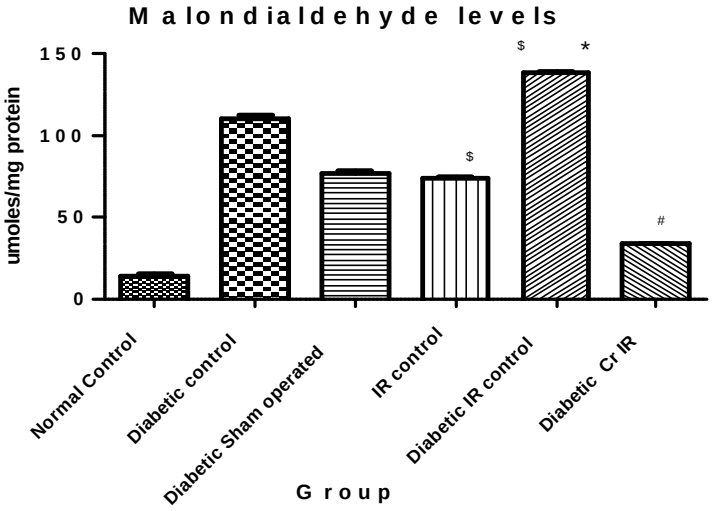



*$ Significant different than normal control (p>0.05)*
*# Significant different than diabetic IR control (p>0.05)*

**Figure 1. Plasma glucose levels**

### 4.2 Brain MDA levels (n=6)

Administration STZ significantly raised MDA level in IR control animals (74.06 ± 1.69) and STZ induced diabetic IR animals (137.88 ± 1.46) when compared with normal animals (14.20 ± 1.86) and when compared between IR control animals and STZ induced diabetic IR animals. Treatment of cromakalim significantly reduced brain MDA level (34.02 ± 0.36) when compared with STZ induced diabetic IR animals (Figure 2).

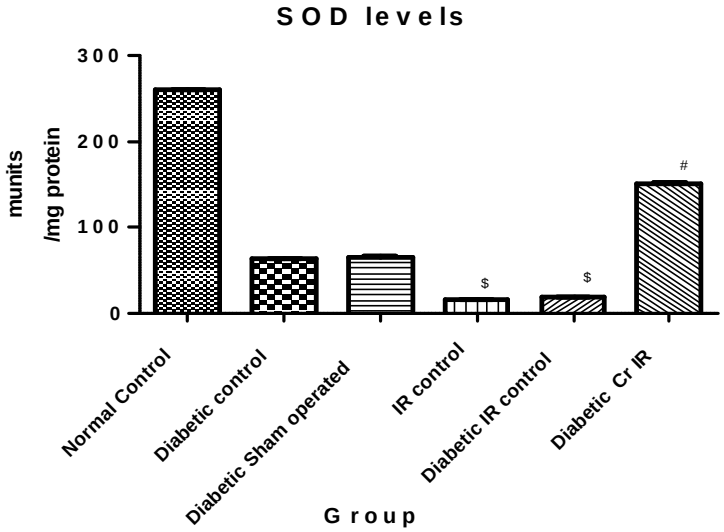



*$ Significant different than normal control (p>0.05)*
** Significant different than IR control group (p>0.05*
*# Significant different than diabetic IR control (p>0.05)*

**Figure 2: Brain malondialdehyde (MDA) levels**

### 4.3. Brain SOD levels (n=6)

Administration STZ significantly reduced SOD level in IR control animals (17.01 ± 0.27) and STZ induced diabetic IR animals (19.83 ± 0.37) when compared with normal animals (259.76 ± 0.80).This is non-significant when compared between IR control animals and STZ induced diabetic IR animals. Treatment of cromakalim significantly raised brain SOD level (150.25 ± 2.10) when compared with STZ induced diabetic IR animals (Figure 3).

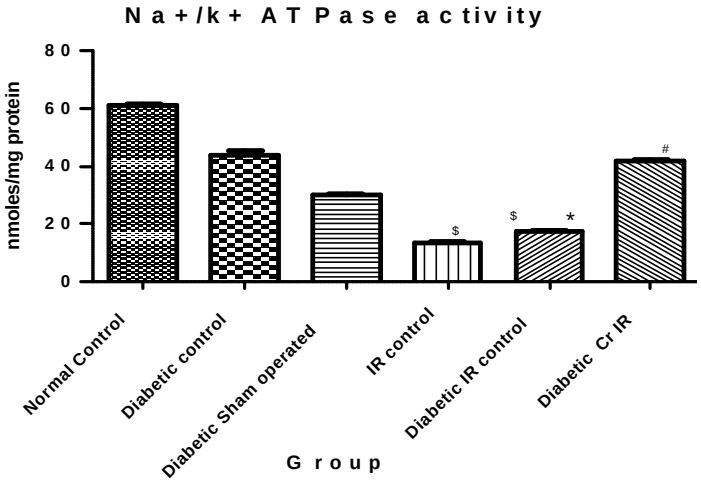



*$ Significant different than normal control (p>0.05)*
*# Significant different than diabetic IR control (p>0.05)*
** Significant different than STZ IR control (p>0.05)*

**Figure 3: Brain SOD levels**

### 4.4. $Na^+/K^+$ ATPase activity (nm/mg protein) (n=6)

Administration STZ significantly reduced $Na^+/K^+$ ATPase activity in IR control animals (13.69 ± 0.32) and STZ induced diabetic IR animals (17.27 ± 0.52) when compared with normal animals (259.76 ± 0.80).This is significant when compared between IR control animals and STZ induced diabetic IR animals. Treatment of cromakalim significantly raised brain SOD level (41.76 ± 0.50) when compared with STZ induced diabetic IR animals (Figure 4).

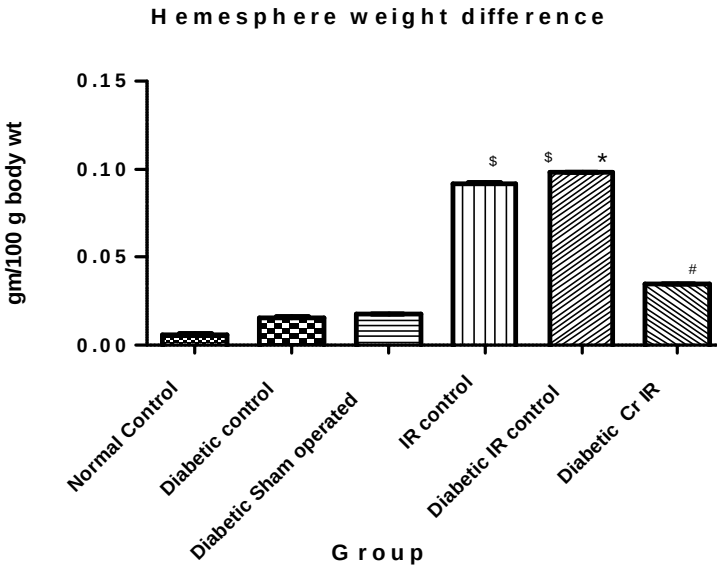



*$ Significant different than normal control (p>0.05)*
** Significant different than IR control group (p>0.05*
*# Significant different than diabetic IR control (p>0.05)*

**Figure 4: $Na^+/K^+$ ATPase activity (nm/mg protein)**

### 4.5 Hemisphere weight difference (n=6)

Administration STZ significantly raised brain hemisphere weight difference in IR control animals (0.091. ± 0.0003) and STZ induced diabetic IR animals (137.88 ± 1.46) when compared with normal animals (0.098 ± 0.0004) and when compared between IR control animals and STZ induced diabetic IR animals. Treatment of cromakalim significantly reduced brain hemisphere weight difference (0.034 ± 0.0003) when compared with STZ induced diabetic IR animals (Figure 5).

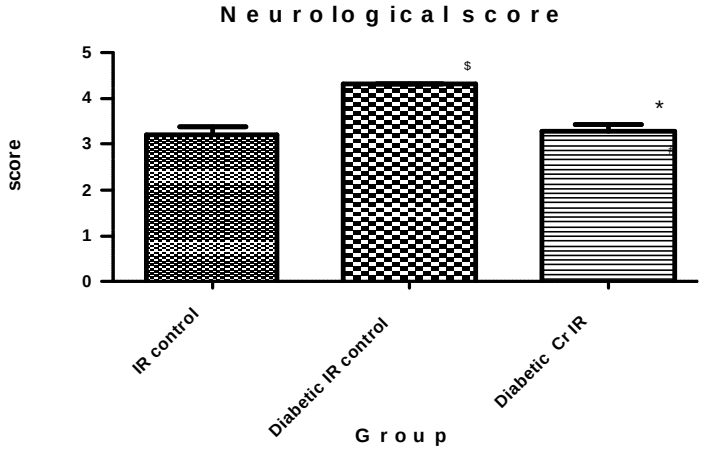



*$ Significant different than normal control (p>0.05)*
** Significant different than IR control group (p>0.05*
*# Significant different than diabetic IR control (p>0.05)*

**Figure 5: Hemisphere weight difference**

### 4.6 Neurological score(n=6)

Treatment of cromakalim significantly reduced brain neurological damage (3.28 ± 0.14) when compared with animals treated with IR along with STZ (4.31 ± 0.006) as well as IR control animals (3.20 ± 0.16) (figure 6). This is significant when compared between IR control animals and STZ induced diabetic IR animals (figure 6).




*Each group contains 6 animals,$ Significant different than IR control group (p>0.05) * Significant different than diabetic IR control (p>0.05)*

**Figure 6: Neurological score**

## 5. DISCUSSION

In present study we have made an attempt to evaluate efficacy of $K_{ATP}$ channel opener cromakalim against IR type of brain stroke model in STZ treated diabetic rats. The symptoms produced by IR along with STZ resembles to a great extent with that observed in clinical status. It is well documented that diabetes significantly increased risk of stroke and mortality following stroke. The Greater Cincinnati–Northern Kentucky Stroke Study (GCNKSS) showed that the risk for ischemic stroke in white diabetic patients is higher at every age-group compared with nondiabetic patients, with highest RR (Relative risk) of 5.3 found in the 45- to 54-year age-group. Diabetic patients are more likely to present with cerebral infarct, indicating that ischemia in diabetic patients is less likely to be reversible [13]. This presents a unique problem for preventing stroke in this population. Numerous studies report the higher incidence of ischemic stroke in association with diabetes. In support of such clinical findings, many preclinical experimental studies were performed to find out correlation between diabetes and occurrence of brain stroke. It is reported that STZ induced diabetes reduced cerebral blood flows up to 37 % and increase infract size in pnumbral region of brain [14]. Mechanism for such injury to brain along with diabetes involves free radical generation and inflammatory changes [15]. Hence to produce such clinical condition, we treated rats with STZ to induce diabetes and in same animals ischemic stroke was induced on 28$^{th}$ day from initiation of experiment. Our objective of study was to investigate neuroprotective effect of cromakalim (potassium channel opener) on cerebral ischemic stroke in STZ induced diabetes in rats. Cromakalim is ATP sensitive potassium channel opener and it improves neurological function and reduced infarction volume in rats [6]. Studies have shown that oxidative stress is high during acute cerebral ischemia and it plays a key role in ischemic nerve injury. It is well reported that during IR injury along with high blood glucose level, anti-oxidant mechanisms are compromised and hence it raise lipid peroxidation (malondialdehyde-MDA level) and reduced super oxide dismutase (SOD) levels in diseased control animals [16]. In present study, cromakalim significantly lowers value of MDA and increased level of SOD in diabetic-IR rats. Thus prevent lipid peroxidation and oxidative stress. This suggest anti-oxidant role of cromakalim to produce neuroprotection in diabetic animals.

Hence, chronic administration of cromakalim was found to reduce reduced oxidative damage induced by ischemia –reperfusion injury along with diabetes. Cromakalim treatment prevents neuronal damage in diabetic-IR rats. This is supported by significant lower value of neurological damage score in cromakalim treated animals when compared with IR control animals and STZ-IR animals.

In further support, cromakalim significantly raised $Na^+/K^+$ ATPase pump activity and reduced brain hemisphere weight difference in its treated group. As it is well understood that neuronal cell damage by

ischemic injury and by STZ induced diabetes interrupt activity of $Na^+/K^+$ ATPase pumps which maintain cellular water level. Due to inhibition of this pump, extracellular potassium accumulates and at the same time that sodium and water are sequestered intracellular and leading to cell swelling, its lysis and raised brain hemisphere weight difference [17]. Hence our report show role of cromakalim in prevention of this cell injury.

In nutshell, our results show that cromakalim significantly improves neurological functions at 24 hours post-surgery, and provide neuroprotection as observed by reduced blood glucose level, malondialdehyde level, the neurological score, brain hemisphere weight difference and by raising SOD level along with $Na^+/K^+$ pump activity when compared with disease control groups. These results suggest that prophylactic use of cromakalim could improve neurological functional and protect neurons as well as stroke related disorders in a rat model of cerebral ischemia-reperfusion injury with STZ induced diabetes.

## 6. CONCLUSION

Results suggest that pre-treatment with cromakalim (10mg/kg i.p.) produce neuroprotective effect in diabetic-IR animals as indicted by reduction in plasma glucose, neurological deficit, malondialdehyde, and raised anti-oxidant enzyme defence. This protective effect might be due to free radical scavenging effect, enhancing anti-oxidant enzyme and reducing blood glucose level.

## 7. ACKNOWLEDGEMENT

The authors are thankful to Dr. Anjali Tarai, Assistant Professor, Department of Pharmacology, VSS Medical College, Cuttack, Orissa India providing gift sample of cromakalim. We wish to express our deep gratitude to Dr J.K.Patel and Dr Devanshu Patel for providing all necessary laboratory facilities at Parul Institute of Pharmacy.

## 8. REFERENCES

1. Goldstein LB, Adams R, Alberts MJ, *et al*. Primary prevention of ischemic stroke: a guideline from the American Heart Association/ American Stroke Association Stroke Council. Circulation. 2006; 113(24):e873-923.
2. Boden-Albala B, Cammack S, Chong J, Wang C, Wright C. Diabetes, fasting glucose levels and risk of ischemic stroke and vascular events: finding from the Northern Manhattan Study (NOMAS).Diabetes Care. 2008; 31:6 1132-37.
3. Kikuchi K, Uchikado H, Morioka M, Murai Y, Tanaka E.Clinical Neuroprotective Drugs for Treatment and Prevention of Stroke. Int J Mol Sci. 2012; 13: 7739-61.
4. Alan GC, Susan EB, John AM. Therapeutic potential for potassium channel modulator for CNS disorders. Expert Opin Ther Pat. 2003; 13(1):23-32.
5. Wang S, Liu J, Chang Q, Li Y, Jiang Y, Wang S. Neuroprotective effects of cromakalim on cerebral ischemia-reperfusion injury in rats. Neural Regen Res. 2010; 5(9):678-82.
6. Sharma P and Singh R. Dichlorvos and lindane induced oxidative stress in rat brain: Protective effects of ginger. Pharmacog Res. 2012; 4(1): 27–32.
7. Lowry OH, Rosenbrough WJ, Farren AI, Randel RJ. Protein measurement with folin phenol reagent. J Biol Reagent. 1951; 93: 265-75.
8. Ohkawa H, Ohishi N, Yagi K. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal Biochem. 1979; 95:351–58.
9. Misra HP, Fridovich I. The role of superoxide anion in the autoxidation of epinephrine and a simple assay for superoxide dismutase. J Biol Chem. 1972; 247:3170–75.
10. Sovoboda P and Mossinger B. Catecholamaines and brain microsomal Na+ -K+ ATPase protection against lipoperoxidative damage.Biochem Pharmacol. 1981; 30: 427-32.
11. Ahmad S. Angiotensin receptor antagonist delays NO deficient stroke in stroke prone rats. Eur J Pharmacol. 1997; 333: 39-45.
12. Menzies SA, Hoff JT, Betz AL.Middle cerebral artery occlusion in rats: A neurological and pathological evaluation of reproducible model. Neurosurg. 2009; 31: 100-6.
13. Ellen L, Brett M. Diabetes, the Metabolic Syndrome, and Ischemic Stroke: Epidemiology and possible mechanisms. Diabetes Care. 2007; 30 (12): 3131-40.
14. Quast MJ, Wei J, Huang NC.Perfusion desficit parallels exacerbation of cerebral ischemia/reperfusion injury in hyperglycemic rats. J Cereb Blood Flow Metab. 1997; 17: 927-38.
15. Martini SR and Kent TA. Vascular effect of hyperglycemia in stroke. J Cereb Blood Flow Metab. 2007; 27: 435-51.
16. Trigunayat A. Effect of ehanolic extract of H.perforatum on oxidative stress induced by cerebral ischemia-reperfusion in rats. Annal Neurosci. 2009; 16(1):6-9.
17. Ramnathan N, Babu C, Justin A, Santhakumari S. Elucidation of neuroprotective role of endogenous GABA and energy metabolites in middle cerebral artery occluded model in rats. Ind J Exp Biol. 2012; 50:391-97.

**Source of support: Nil, Conflict of interest: None Declared**

Innovare Academic Sciences

International Journal of Pharmacy and Pharmaceutical Sciences

ISSN- 0975-1491 Vol 6, Issue 6, 2014

Original Article

# NEUROPROTECTIVE EFFECTS OF CROMAKALIM ON CEREBRAL ISCHEMIA-REPERFUSION (IR) INJURY AND ALUMINIUM INDUCED TOXICITY IN RAT BRAIN

**[*1]PITHADIA ANAND BHARAT KUMAR, [2]SHITAL PANCHAL**

**[*1]Department of Pharmacology, Parul Institute of Pharmacy, Vadodara,[2] Department of Pharmacology, Institute of Pharmacy, Nirma University, Ahmedabad, Gujarat, India.**
**Email: abpithadia@hotmail.com**

*Received: 29 Apr 2014 Revised and Accepted: 28 May 2014*

**ABSTRACT**

**Objective:** Preclinical studies have demonstrated that potassium channel openers show a neuroprotective effect on cerebral ischemia-reperfusion (IR) injury in rats. However, their mechanism of action and effects in brain stroke related disorders such as Alzheimer's disease (AD) remain poorly understood. Hence present study was done to investigate the prophylactic use of the adenosine triphosphate-sensitive (ATP) potassium channel opener cromakalim on neurological function in rats with cerebral IR injury and its related disorders AD.

**Methods:** Male Wistar rats were randomly assigned to 6 groups (n = 36): Normal control, aluminium chloride($AlCl_3$) control, $AlCl_3$ Sham operated, ischemia reperfusion (IR) control, $AlCl_3$-IR control and $AlCl_3$-IR with cromakalim treatment. Cromakalim10 mg/kg intra peritonially (i.p.) was administered daily for 42 days in middle cerebral artery occluded rats treated with $AlCl_3$. At 24 hours post-surgery, neurological score, brain hemisphere weight difference, brain acetylcholinestarase level, lipid peroxidation, $Na^+/K^+$ ATPase pump activity and super oxide dismutase level were measured.

**Results:** Following cerebral ischemia-reperfusion injury along with aluminium chloride, neurological score,brain hemisphere weight difference, brain acetylcholinestarase level, malondialdehyde levels were significantly high while $Na^+/K^+$ ATPase pump activity and super oxide dismutase(SOD) levels were significantly lower in diseased animals ($P < 0.05$). Treatment with cromakalim significantly reduced neurological score, brain hemisphere weight difference, brain acetyl cholinestarase level, malondialdehyde levels while raised $Na^+/K^+$ ATPase pump activity and super oxide dismutase levels ($P < 0.05$).

**Conclusion:** Hence results of our study demonstrated the neuroprotective potential of cromakalimin cerebral IR injury and its related disorders AD.

**Keywords:** Cerebral ischemia-reperfusion, Cromakalim. Aluminium, Alzheimer's disease, Oxidative stress, Neuroprotection.

## INTRODUCTION

Stroke is an interruption in the supply of blood to a particular part of the brain. There are two main causes of stroke. Either a blood vessel within the brain becomes clogged or blocked (ischemic stroke-IS, about 83% of strokes) or it ruptures, causing blood to leak into the brain (haemorrhagic stroke) [1].It is well reported that stroke more than doubles the risk of developing dementia and AD and around one in three people who have a stroke develop dementia [2].Clinical diagnosis of ADis associated with considerably increased risk of stroke development and patients with AD had a higher risk of IS (ischemic stroke) and ICH (intracerebral hemorrhage) [3]. Further epidemiological studies show that risk factors for AD have a vascular basis and practically all drugs reported to slow the development of AD, improve or increase cerebral perfusion. Evidence that cerebral hypoperfusion appears to precede the cognitive, and degenerative pathology that is present in AD. Current treatment of brain strokeincludes tissue plasminogen activators, antiplatelet agents and anticoagulants for their antithrombotic effects. Reports showed that free radical scavengers, minocycline and growth factors have shown neuroprotective effects in the treatment of stroke, while antihypertensive drugs, lipid-lowering drugs and oral antidiabetic drugs have shown beneficial effects for the prevention of stroke [4]. However, these agents are associated with number of side effects. Therefore, there is a need to identify new targets and molecules in central nervous system (CNS) that provide neuroprotection in stroke related disorders. One of them is the ion channel for $Na^+$, $K^+$, $Ca^{2+}$ and $Cl^-$ . They play a crucial role in controlling a very wide spectrum of physiological processes. Their dysfunction can produce various CNS diseases. Hence new generation of therapeutic agents are expected to result from targeting this ion channels.

Further, it is well established that $K^+$ channels are widely expressed in CNS and are gaining attention for their beneficial roles in neuroprotection. Dysfunction of potassium channel leads to change in neuronal physiological processes and results in pathophysiology of various diseases including stroke, epilepsy, pain and cognition [5].Hence $K^+$ channels are recognized as potential therapeutic targets in the treatment of CNS disorders. Adenosine triphosphate-sensitive potassium channel ($K_{ATP}$) openers such as cromakalim provide neuroprotection against cerebral ischemia-reperfusion injury by reducing inflammation and free radical mediated oxidative stress. Neuroprotective effects of cromakalim have been already established on cerebralischemia-reperfusion injury in rats [6].Hence present study hypothesized that $K_{ATP}$ openers couldreduce strokeas well as stroke related disorderssuch as AD by providing neuroprotection. Rat model of cerebral ischemia-reperfusion was established through middle cerebral artery occlusion using the suture method [6] and this was preceded by $AlCl_3$ to induce cholinergic nerve damage as that of Alzheimer's disease in same animals [7]. The effects of prophylactic useof the $K_{ATP}$ channel opener, cromakalim, on neurologicalfunction, brain acetylcholinestares level and lipid peroxidation parameters were determined to explore the mechanisms of action of cromakalimfor neuroprotection.

## MATERIALS AND METHOD

### Drugs and Chemicals

Cromakalim was gifted from Dr. Anjali Tarai, Assistant Professor, Department of Pharmacology, VSS Medical College, Cuttack, Orissa, India. Other chemicals used in experiment were of laboratory grade. Cromakalim solution was prepared freshly by dissolving it in sterile distilled water and injected i.p. with dose 10 mg/kg. $AlCl_3$ solution was made freshly in sterile distilled water at the beginning of each experiment and given orally at dose of 100 mg/kg.

### Animals

Healthy adult male Wistar rats (250 gm) aged 8 months were selected for the study. Animals were maintained at 18± 2°C and kept in well ventilated animal house in polypropylene cages with free

access to food and water. All studies was done with prior permission from IAEC of Parul Institute of Pharmacy (921/PO/AC/05/ CPCSEA) and Study was approved as Protocol No PIPH 21/13.Animals were divided into six groups. Group 1 served as normal control (n=6) and received distilled water. Group 2 serve as $AlCl_3$ control and received $AlCl_3$at dose 100 mg/kg per oral for a period of 42 days (6 weeks).Group 3 served as $AlCl_3$ Sham operated (n=12) and treated with $AlCl_3$ at dose 100 mg/kg per oral for a period of 42 days (6 weeks).Sham operation was performed on 41th day. Group 4 served as ischemia reperfusion (IR) control (n=6) and received distilled water for 42 days. Cerebral ischemia –reperfusion was performed on 41th day from initiation of experiment. Group 5 served as $AlCl_3$-IR control (n=6) and animals were treated with $AlCl_3$ at dose 100 mg/kg per oral for a period of 42 days (6 weeks). Cerebral ischemia –reperfusion was performed on 41th day from initiation of experiment. Group 6 treated with$AlCl_3$and cromakalim (n=6) along with IR injury. These animals were treated with $AlCl_3$ at dose 100 mg/kg per oral and cromakalim at dose 10 mg/kg (i.p.) for a period of 42 days (6 weeks). Cerebral IR was performed on 41th day from initiation of experiment. Cerebral IR was induced as per MCAO (transient middle cerebral artery occlusion) method [6]. Rats were anesthetized with an i.p. injection of 100 mg/kg ketamine. A 2–3 cm incision was made in the middle of the neck line, separating the left carotid artery, the superior thyroid artery, and the occipital artery, as well as the internal and external carotid communicating arteries. The occipital artery branches of external carotid artery (ECA) were isolated and tied with a cotton thread.Cotton thread was tied loosely around the ECA stump near the bifurcation. Then internal carotid artery (ICA) and common carotid artery (CCA) were temporarily occluded by fine vessel clip.

Through a small incision to the ECA stump, blunt Poly-L-lysine coated 4-0 monofilament was inserted from the left external carotid artery into the left internal carotid artery to a depth of 18.0mm, vessel clip from ICA removed. After a variable length of suture had been inserted into ECA stump, resistance was felt and slight curving of suture was observed, indicating that suture had passed middle cerebral artery (MCA) origin and reached to proximal segment of anterior cerebral artery (ACA-it has small diameter).Hence suture had blocked all sources of blood from ICA, ACA and posterior cerebral artery. Finally vessel clip from CCA was removed to restore blood flow. The midline incision was closed, leaving suture protruding so it could be withdrawn to allow reperfusion.The thread was maintained for 2 hours and subsequently removed to restore blood flow to the common carotid and internal carotid arteries.Here 18 mm of suture was pulled back until resistance was felt, indicating that tip cleared the ACA-ICA lumen and was in ECA stump, then trimmed. The animals were transfer to a fresh cage with free access to food and water.

**Tissue homogenate preparation**

At the end of the study, animals were sacrificed, brain were dissected out and washed with 0.9% NaCl and stored at –40 °C for further processing. Brain was homogenized in 10% (w/v) ice-cold 0.1 M phosphate buffer solution (pH 7.4). The lipid peroxidation (LPO) was estimated with a part of crude homogenate and the rest homogenate was centrifuged at 12,000 rpm for 20 min to obtain the supernatant (S) that was used for enzymatic estimations [7].

**Tissue total protein level**

It was measured using bovine serum albumin as standard, alkaline copper reagent Solution A (2% sodium carbonate in 0.1 N NaOH solution in distilled water) and solution B(0.5% copper sulphate in 1.0% sodium potassium tartarate).50.0 ml of solution A was mixed with 1.0ml of solution B just before use. Folin's phenol reagent was obtained commercially. One volume of Folin's phenol reagent was diluted with 1 volume of distilled water just before use. 20.00 mg standard bovine serum albumin (BSA) was dissolved in 100 ml distilled water. Few drops of NaOH were added to aid complete dissolution of BSA and to avoid frothing. 10ml of the stock was diluted to 100ml to get a working standard (200 µg/ml). Procedure 0.1ml crude homogenate was used for protein extraction. The protein was extracted by mixing with 5% cold trichloroacetic acid and centrifuged. The pellet was solubilized with 0.5 N sodium hydroxide and stored over-night at room temperature. After neutralization with 0.5 N HCl, 0.2 ml of diluted solution and different concentrations of standard were taken. The volume was made up to 1.0 ml with distilled water. Blank contained 1.0 ml distilled water.

To all the tubes 5.0 ml alkaline copper reagent was added and left at room temperature for 10 Min. 0.5 ml of folin's phenol reagent was added and the blue colour developed was read after 20 minutes at 660nm against reagent blank in a spectrophotometer. Protein concentration is expressed as mg/gm of wet brain tissue [8].

**Brain acetylcholinestarase level**

It is a marker of the loss of cholinergic neurons in the forebrain. The assay mixture contained 0.05 ml of supernatant, 3 ml of sodium phosphate buffer (pH 8), 0.1 ml of acetylthiocholine iodide and 0.1 ml of DTNB (Ellman reagent). The change in absorbance was measured for 2 min at 30 s intervals at 412 nm. Results were expressed as micromoles of acetylthiocholine iodide hydrolyzed/ min/mg of protein [9].

**Malondialdehyde (MDA)**

It is a marker of lipid peroxidation. 1 ml of homogenate was incubated at 37 °C for 10 min. 1 ml of 10% trichloroacetic acid (TCA) chilled (w/v) was added to it and centrifuged at 2500 rpm for 15 min at room temperature. 1 ml of 0.67% TBA was added to 1 ml of supernatant and kept in a boiling water bath for 10–15 min. The tubes were cooled under tap water. After cooling 1 ml of distilled water was added to it and absorbance was taken at 530 nm. The results were expressed as micromoles/mg of protein [10].

**Superoxide dismutase (SOD)**

An aliquot of 0.25 ml ice-cold chloroform was added to 0.1 ml of supernatant followed by addition of 0.15 ml ice-cold ethanol. The mixture was centrifuged at 3000 rpm for 10 min at 4 °C. 0.2 ml the supernatant was taken and 1.3 ml buffer, 0.5 ml EDTA, and 0.8 ml water were added. Reaction was started by adding 0.2 ml epinephrine. Change in absorbance ΔOD (optical density)/min at 480 nm was read for 3 min. The results were expressed in terms of munits/mg protein.One unit of enzyme activity is defined as the concentration required for the inhibition of the chromogen production by 50 % in one minute under the defined assay conditions [11].

**$Na^+$/ $K^+$ ATPase pump activity**

The incubation mixture containing 0.1 ml of each i.e tris HCL , 50 mM magnesim sulphate , 650 mM NaCl , 50 mM KCl , 1mM EDTA , 40 mM ATP and brain homogenate . Mixture was incubated at 36 ˚C for 15 minute. The reaction was arrested by addition of 1ml 10 % TCA and resultant mixture was centrifuged for 5 min.0.5 ml supernatant was taken, 2.5 ml,0.5 ml of 2.5 % ammonium molybdate and 0.25 ml ANSA (1-amino 2-napthol 4-sulphonic acid)was added.The mixture was incubated at 37 ˚C for 10 minutes. The intensity of blue color developed against blank at 620 nm. Results were expressed as nmoles of inorganic phosphorus liberated/min/mg protein. Standard calibration curve was prepared using potassium dihydrogen phosphate (2-15 µg/ml) [12].

**Brain hemisphere weight difference**

The MCA occluded animals were anaesthetised with high dose of pentobarbitone. The skull was opened and whole brain was removed immediately, rinsed with ice cold distilled water followed by 20 % sucrose and dried using bloating paper. The ipsilateral and contralateral parts of brain are isolated and weighed on digital balance. Weight difference was calculated between ipsilateral and contralateral brain region of each animal [13].

**Neurobehavioral function scores**

Neurological score was observed after 24 hours of IR[14].This score was observed for group 4, 5 and 6.

**Score 0**: No apparent deficit

**Score 1**: Contralateral forelimb flexion when suspended by tail

**Score 2**: Decreased grip of the contralateral forelimb while tail pulled

Score 3: Spontaneous movement in all direction or contralateral circling only if pulled by tail.

Score 4: Spontaneous contralateral circling

Score 5: Death after recovery from anaesthesia.

**Statistical methods**

Data are expressed as mean values ± S.E.M and analyzed using one-way ANOVA followed by Post-hoc Tukey's tests to identify two group differences using Prism software at 5% significance level.

**RESULTS**

**Brain acetylcholinesterase levels**

Administration aluminium chloride for 42 days significantly raised cholinesterase level in $AlCl_3$ treated control animals (73.07 ± 2.05) and $AlCl_3$ Sham operated animals (64.10 ± 0.84) when compared with normal animals(19.49 ± 0.55).Ischemia reperfusion(IR) injury along with $AlCl_3$also significantly raised (96.86 ± 1.07) cholinesterase level when compared with IR control (24.01 ± 0.54).

Treatment of cromakalim significantly reduced brain actetylcholinesterase level (26.33 ± 0.37) when compared with AlCl3 IR control animals (table 1).

**Brain malondialdehyde(MDA) levels(n=6)**

Administration aluminium chloride for 42 days significantly raised MDA level in $AlCl_3$ treated control animals (128.92 ± 3.46) and $AlCl_3$ Sham operated animals (78.72 ± 1.46) when compared with normal animals(14.20 ± 1.86).Ischemia reperfusion(IR) injury along with $AlCl_3$ also significantly raised (121.143 ± 1.67) MDA level when compared with IR control (77.23 ± 1.94). Treatment of cromakalim significantly reduced brain MDA level (39.90 ± 0.45) when compared with $AlCl_3$ IR control animals (table 2).

**Brain SOD levels (n=6)**

Administration aluminium chloride for 42 days significantly reduced SOD level in $AlCl_3$ treated control animals (84.54 ± 0.83) and $AlCl_3$ Sham operated animals (73.59 ± 0.85) when compared with normal animals(259.76 ± 0.80).Ischemia reperfusion(IR) injury along with $AlCl_3$ also significantly reduced (16.33 ± 1.93) SOD level when compared with IR control (22.78 ± 0.48).

Treatment ofcromakalim significantly raised brain SODlevel (143.49 ± 0.68) when compared with $AlCl_3$ IR control animals (table 3).

**$Na^+/K^+$ ATPase activity (nm/mg protein) (n=6)**

Administration aluminium chloride for 42 days significantly reduced $Na^+/K^+$ ATPase activity level in $AlCl_3$ treated control animals (37.24 ± 0.60) and $AlCl_3$ Sham operated animals (22.12 ± 0.39) when compared with normal animals(61.27 ± 0.47).

Ischemia reperfusion (IR) injury along with $AlCl_3$ also significantly reduced (22.95 ± 0.43) $Na^+/K^+$ ATPase activity when compared with IR control (10.12 ± 0.38). Treatment of cromakalim significantly raised brain $Na^+/K^+$ ATP ase activity (47.96 ± 0.29) when compared with $AlCl_3$ IR control animals (table 4).

**Table 1: Brain cholinesterase activity µ mol of acetyl thiocholine iodide hydrolyzed/ minute (min)/milligram (mg) of protein)(n=6)**

| Groups | Brain cholinesterase levels |
|---|---|
| Normal Control | 19.49 ± 0.55 |
| $AlCl_3$ control | 73.07 ± 2.05* |
| $AlCl_3$ Sham operated | 64.10 ± 0.84* |
| IR control | 24.01 ± 0.54* |
| $AlCl_3$ IR control | 96.86 ± 1.07** |
| $AlCl_3$cromakalim IR | 26.33 ± 0.37*** |

* Significant different than normal control ($p<0.05$) , **Significant different than IR control group($p<0.05$) , *** Significant different than $AlCl_3$ IR control ($p<0.05$)

**Table 2: Brain malondialdehyde (MDA) levels**

| Groups | MDA(µmoles/mg protein) |
|---|---|
| Normal Control | 14.20 ± 1.86 |
| $AlCl_3$ control | 128.92 ± 3.46* |
| $AlCl_3$ Sham operated | 78.72 ± 1.46* |
| IR control | 77.23 ± 1.94* |
| $AlCl_3$ IR control | 121.143 ± 1.67** |
| $AlCl_3$cromakalim IR | 39.90 ± 0.45*** |

* Significant different than normal control ($p<0.05$) , **Significant different than IR control group ($p<0.05$) , ***Significant different than $AlCl_3$ IR control ($p<0.05$

**Table 3: Brain SOD levels**

| Groups | SOD(munits/mg protein) |
|---|---|
| Normal Control | 259.76 ± 0.80 |
| $AlCl_3$ control | 84.54 ± 0.83* |
| $AlCl_3$ Sham operated | 73.59 ± 0.85* |
| IR control | 22.78 ± 0.48* |
| $AlCl_3$ IR control | 16.33 ± 1.93** |
| $AlCl_3$cromakalim IR | 143.49 ± 0.68*** |

* Significant different than normal control ($p<0.05$) , ** Significant different than IR control group ($p<0.05$) , *** Significant different than $AlCl_3$ IR control ($p<0.05$)

**Hemisphere weight difference (n=6)**

Administration aluminium chloride for 42 days significantly raised brain hemisphere weight difference in $AlCl_3$ treated control animals (0.016 ± 0.002) and $AlCl_3$ Sham operated animals (0.022 ± 0.003) when compared with normal animals(0.0062 ± 0.0003).Ischemia reperfusion(IR) injury along with $AlCl_3$ also significantly increased (0.077 ± 0.003) brain hemisphere weight differencewhen compared with normal control. However, this is nonsignificant when compared with IR control (0.071. ± 0.003).

Treatment of cromakalim significantly reducedbrain hemisphere weight difference (0.039 ± 0.0038) when compared with $AlCl_3$ IR control animals (table 5).

**Neurological score (n=6)**

Treatment ofcromakalim significantly reduced brain neurological damage (1.2 ± 0.12) when compared with animals treated with IR along with $AlCl_3$ (4.22 ± 0.15) as well as IR control animals (3.20 ± 0.16) (table 5).

**Table 4: $Na^+/K^+$ ATPase activity (nm/mg protein)**

| Groups | (nm/mg protein) |
|---|---|
| Normal Control | 61.27 ± 0.47 |
| $AlCl_3$ control | 37.24 ± 0.60* |
| $AlCl_3$ Sham operated | 22.12 ± 0.39* |
| IR control | 10.12 ± 0.38* |
| $AlCl_3$ IR control | 22.95 ± 0.43** |
| $AlCl_3$ cromakalim IR | 47.96 ± 0.29*** |

* Significant different than normal control ($p<0.05$) , ** Significant different than IR control group ($p<0.05$) , *** Significant different than $AlCl_3$ IR control ($p<0.05$)

**Table 5: Hemisphere weight difference**

| Groups | Gm/100 gm body weight |
|---|---|
| Normal Control | 0.0062 ± 0.0003 |
| $AlCl_3$ control | 0.016 ± 0.002* |
| $AlCl_3$ Sham operated | 0.022 ± 0.003* |
| IR control | 0.071 ± 0.003* |
| $AlCl_3$ IR control | 0.077 ± 0.003 (NS) |
| $AlCl_3$ cromakalim IR | 0.039 ± 0.0038*** |

* Significant different than normal control ($p<0.05$) , *** Significant different than $AlCl_3$ IR control ($p<0.05$) , NS is Non-significant when compare with IR control

**Table 6: Neurological score**

| Groups | Gm/100 gm body weight |
|---|---|
| IR control | 3.20 ± 0.16 |
| $AlCl_3$ IR control | 4.22 ± 0.15 |
| $AlCl_3$cromakalim IR | 1.2 ± 0.12* |

* Significant different than $AlCl_3$ IR control ($p<0.05$)

**DISCUSSION**

In present study we have made an attempt to evaluate efficacy of $K_{ATP}$ channel opener cromakalim against ischemic-reperfusion type of brain stroke model in $AlCl_3$treated rats. The symptoms produced by IR along with $AlCl_3$ resembles to a great extent with that observed in clinical status. Research studies have demonstrated that three months after ischemic stroke, about 30-40 % of people show signs of dementia, including impairments in learning, attention as well as ability to remember. This vascular dementia mainly results from the interruption of blood supply to brain during stroke. Hence to produce such clinical condition, we treated rats with $AlCl_3$ for 42 days. It has been suggested that aluminium is a contributing factor in the pathogenesis of Alzheimer's disease. Hence to induce neurotoxicity as observed in AD, aluminium chloride was used [7] and cerebral ischemia –reperfusion was performed on 41$^{th}$ day from initiation of experiment in same animals.Our objective of study was to investigate neuroprotective effect of cromakalim (potassium channel opener) on cerebral ischemic stroke in aluminum chloride induced neuronal toxicity in rats. Cromakalim is ATP sensitive potassium channel opener and it improves neurological function and reduced cerebral infarction volume in rats [6].

Studies have shown that oxidative stress is increased during acute cerebral ischemia and plays a key role in ischemic nerve injury. It is well reported that during IR injury along with aluminium anti-oxidant mechanisms are compromised and hence it raise lipid peroxidation (malondialdehyde-MDA level) and reduced super oxide dismutase (SOD) levels in diseased control animal [15].In present study, cromakalim significantly lowers value of MDA and increased level of SOD in IR-aluminium treated rats. Thus prevent lipid peroxidation and oxidative stress.This suggest anti-oxidant role of cromakalim to produce neuroprotection. Aluminium crosses the blood brain barrier and induces inflammatory responses and inhibits long-term potentiation, and causes synaptic structural abnormalities, thereby resulting in profound memory loss [16]. Aluminium raises brain acetylcholinesterase level, cause neurodegeneration and neuroinflammation.It itself results into oxidative damage to brain and cognitive dysfunction.

Aluminium has a biphasic effect on acetylcholinesterase activity, withan initial increase in the activity of this enzyme during the first 2 weeks of exposure followed by a markeddecrease. These results into slow accumulation of aluminium in the brain [17, 18, 19] and thiswould contribute to the increase in acetylcholinesterase activity as observed in the aluminium chloride treated rats.Further, it is well established that more level of same enzyme is responsible for Alzheimer'sdisease to damage cholinergic neurones. Chronic administration of cromakalim was found to reduce brain acetylcholinestarse level and also reduced oxidative damage induced by ischemia –reperfusion injury along with chronic aluminium administration. Hence from data of our study, cromakalim

treatment prevents cholinotoxic effect of aluminium on neurones in IR-aluminium treated rats. This is further supported by significant lower value of neurological damage score in cromakalim treated animals when compared with IR control animals and $AlCl_3$-IR animals.Cromakalim significantly raised $Na^+/K^+$ ATPase pump activity and reduced brain hemisphere weight difference. As it is well understood that neuronal cell damage by ischemic injury and by chronic aluminium administration interrupt activity of $Na^+/K^+$ ATPase pumps which maintain cellular water level. Due to inhibition of this pump, extracellular potassium accumulates and at the same time that sodium and water are sequestered intracellular and leading to cell swelling, its lysis and raised brain hemisphere weight difference [20].Hence our report show role of cromakalim in prevention of this cell injury.

Hence our results show that cromakalim significantly improves neurological functions at 24 hours post-surgery, and provide neuroprotection as observed by reduced acetylcholinestarase level, malondialdehyde level, the neurological score, brain hemisphere weight difference and by raising SOD level along with $Na^+/K^+$ pump activity when compared with disease control groups. These results suggest that prophylactic use of cromakalim could improve neurological functional and protect neurons as well as stroke related disorders in a rat model of cerebral ischemia-reperfusion injury with $AlCl_3$ induced neurotoxicity.

## CONCLUSION

In present study, results suggests that pre-treatment with cromakalim ( 10mg/kg i.p.) produce neuroprotective effect in $AlCl_3$-IR animals as indicted by reduction in, neurological deficit, malondialdehyde, cholinesterase enzyme and raised anti-oxidant enzyme defence. This protective effect might be due to free radical scavenging effect, enhancing anti-oxidant enzyme and reducing cholinesterase activity.

## ACKNOWLEDGEMENTS

The authors are thankful to Dr. Anjali Tarai, Assistant Professor, Department of Pharmacology, VSS Medical College, Cuttack, Orissa, India for providing gift sample of cromakalim and Dr. Rajesh KS, Principal, Parul Institute of Pharmacy for providing necessary infrastructure for experimental work at Parul Institute.

## CONFLICT OF INTEREST

We declare that we have no conflict of interest.

## REFERENCES

1. Goldstein LB, Adams R, Alberts MJ, Appel LJ, Brass LM, Bushnell CD, et al. Primary prevention of ischemic stroke: a guideline from the American Heart Association/American Stroke Association Stroke Council: cosponsored by the Atherosclerotic Peripheral Vascular Disease Interdisciplinary Working Group; Cardiovascular Nursing Council; Clinical Cardiology Council; Nutrition, Physical Activity, and Metabolism Council; and the Quality of Care and Outcomes Research Interdisciplinary Working Group. Circulation 2006;113(24):e873-923.
2. Torre JC. Alzheimer Disease as Vascular Disorder-Nosological Evidence. Stroke 2002;33:1152-62.
3. Chi N-F, Chien L-N, Ku H-L, Hu C-J, Chiou H-Y. Alzheimer disease and risk of stroke: a population-based cohort study. Neurology 2013;80(8):705-11.
4. Kikuchi K, Uchikado H, Morioka M, Murai Y, Tanaka E. Clinical neuroprotective drugs for treatment and prevention of stroke. International journal of molecular sciences 2012;13(6):7739-61.
5. Alan GC, Susan EB, John AM. Therapeutic potential for potassium channel modulator for CNS disorders. Expert Opin Ther Pat 2003;13(1):23-32.
6. Wang S, Liu J, Chang Q, Li Y, Jiang Y. Neuroprotective effects of cromakalim on cerebral ischemia-reperfusion injury in rats. Neural Regen Res 2010;5(9):678-82.
7. Kumar A, Prakash A, Dogra S. Neuroprotective effect of carvedilol against aluminium induced toxicity: possible behavioral and biochemical alterations in rats. Pharmacological reports : PR 2011;63(4):915-23.
8. Lowry OH, Rosenbrough WJ, Farren AI, Randel RJ, J. Protein measurement with folin phenol reagent. Reagent 1951;93:265-75.
9. Ellman GL, Courtney KD, Andres V, Featherstone RM. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem Pharmacol 1961;7:88-95.
10. Ohkawa H, Ohishi N, Yagi K. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal Biochem 1979;95:351–358
11. Misra HP, Fridovich I. The role of superoxide anion in the autoxidation of epinephrine and a simple assay for superoxide dismutase. The Journal of biological chemistry 1972;247(10):3170-5.
12. Sovoboda P, Mossinger B. Catecholamaines and brain microsomal Na+ -K+ ATPase protection against lipoper oxidative damage. Biochem Pharmacol 1981;30:427-32.
13. Ahmad S. Angiotensin receptor antagonists delay nitric oxide-deficient stroke in stroke-prone rats. European journal of pharmacology 1997;333(1):39-45.
14. Menzies SA, Hoff JT, Betz AL. Middle cerebral artery occlusion in rats: A neurological and pathological evaluation of reproducible model. Neurosurg 2009;31:100-6.
15. Trigunayat A. Effect of ehanolic extract of H. perforatum on oxidative stress induced by cerebral ischemiareperfusion in rats Annal Neurosci 2009;16(1):6-9.
16. Roskams AJ, Connor JR. Aluminum access to the brain: a role for transferrin and its receptor. Proceedings of the National Academy of Sciences of the United States of America 1990;87(22):9024-7.
17. Kumar S. Biphasic effect of aluminium on cholinergic enzyme of rat brain. Neuroscience letters 1998;248(2):121-3.
18. Sharma P, Singh R. Dichlorvos and lindane induced oxidative stress in rat brain: Protective effects of ginger. Pharmacognosy research 2012;4(1):27-32.
19. Kawahara M, Muramoto K, Kobayashi K, Mori H, Kuroda Y. Aluminum promotes the aggregation of Alzheimer's amyloid beta-protein in vitro. Biochemical and biophysical research communications 1994;198(2):531-5.
20. Ramnathan N, Babu C, Justin A, Santhakumari S, J. Elucidation of neuroprotective role of endogenous GABA and energy metabolites in middle cerebral artery occluded model in rats. Ind Biol 2012;50:391-7.