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Original Article

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

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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 effect and anti-apoptotic effect in streptozotocin-induced diabetic

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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.

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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).

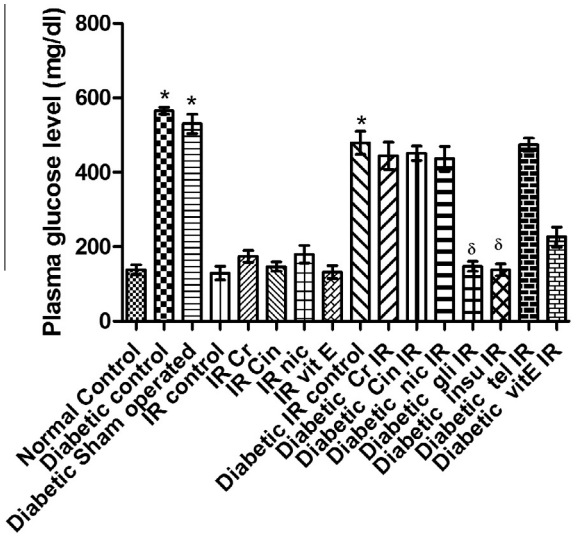

Fig. 1. Effect of cromakalim, cinnarizine, nicorandil and vitamin E on plasma glucose levels. Data presented as mean ± SEM, asteriks (∗) indicate significant difference from the normal control and (δ) indicate significant difference from diabetic IR control rats at $p < 0.05$.

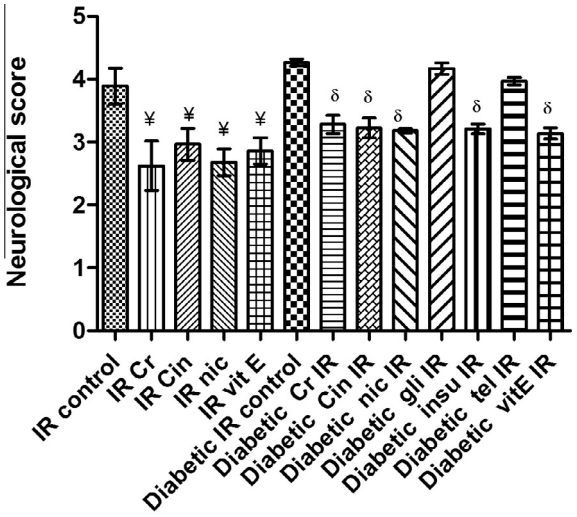

Fig. 2. Effect of cromakalim, cinnarizine, nicorandil and vitamin E on neurological score. Data presented as mean ± SEM, asteriks (¥) indicate significant difference from the IR control rats, (δ) indicate significant difference from diabetic IR control rats at $p < 0.05$.

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).

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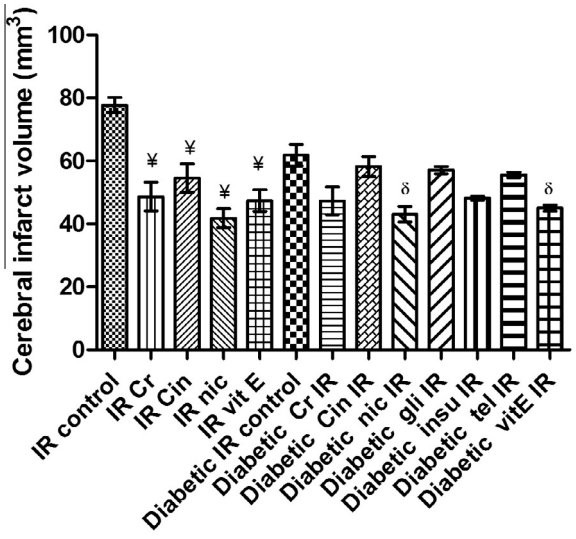

Fig. 3. Effect of cromakalim, cinnarizine, nicorandil and vitamin E on cerebral infarct volume. Data presented as mean ± SEM, asteriks (¥) indicate significant difference from the IR control rats, (δ) indicate significant difference from diabetic IR control rats at $p < 0.05$.

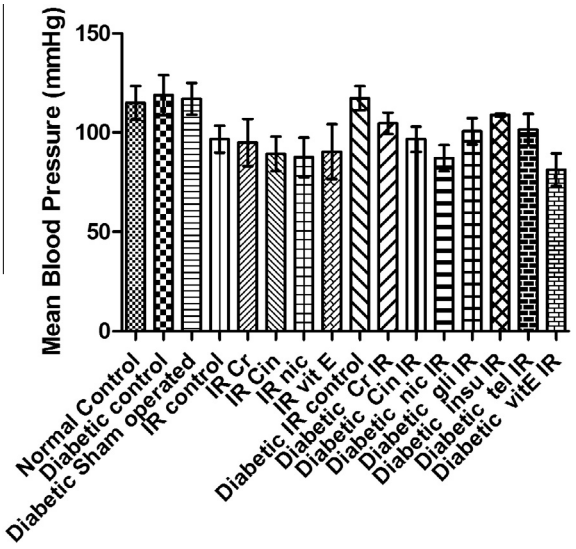

Fig. 5. Effect of cromakalim, cinnarizine, nicorandil and vitamin E on mean blood pressure. Data presented as mean ± SEM and indicate non-significant difference among various groups.

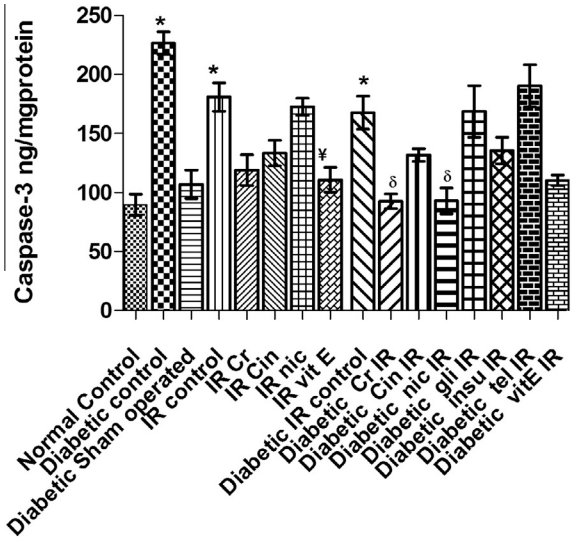

Fig. 4. Effect of cromakalim, cinnarizine, nicorandil and vitamin E on caspase-3 levels. Data presented as mean ± SEM, asteriks (*) indicate significant difference from the normal control rats, (¥) indicate significant difference from diabetic IR control rats and (δ) indicate significant difference from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

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

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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.

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