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Research Report

The role of ASIC1a in neuroprotection elicited by quercetin in focal cerebral ischemia

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ABSTRACT

One of the major instigators to neuronal cell death and brain damage following cerebral ischemia is calcium dysregulation. The intracellular calcium overload resulting from glutamate excitotoxicity is considered a major determinant for neuronal loss during cerebral ischemia. Moreover, ASIC1a activation due to acidosis also promotes intracellular calcium overload during ischemic insult. Interestingly, ASIC1a was found to be inhibited by some flavonoids which carry an anti-inflammatory property particularly quercetin, which could be exploited in hypoxic conditions like cerebral ischemia. This encourages us to investigate the neuroprotective effect of quercetin besides its possible downstream signaling mechanism in focal cerebral ischemia. The treatment of quercetin 30 min before ischemia and 4 h after reperfusion shows significant protection from ischemic injury as noticed by reduction in cerebral infarct volume and neurobehavioral deficit. In addition to earlier calcium dependent rise in the levels of nitrite and MDA exhibited marked reduction ($P<0.01$) in their levels when given quercetin pretreatment in ischemic brain regions. The quercetin treatment also reduced the spectrin break down products (SBDP) caused by ischemic activation of calcium dependent protease calpain. In ex-vivo study, it was also observed that quercetin inhibited the acid mediated intracellular calcium levels in rat brain synaptoneurosomes. These studies suggest the neuroprotective role of quercetin in focal cerebral ischemia by regulation of ASIC1a.

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1. Introduction

Acid sensing ion channels (ASICs) have been shown to promote glutamate independent intraneuronal calcium overload during cerebral ischemia. These ASICs belong to the degenerin–epithelial sodium channel superfamily which are widely distributed in neuronal system and play important role in a number of physiological processes (Garcia-Anoveros et al., 2001; Wemmie et al., 2004). Primarily, all ASICs are Na^+ selective channel which on activation at normal resting potential produce exclusively inward currents resulting in membrane depolarization and the excitation of neurons (Baron et al., 2002;

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Abbreviations: ROS, reactive oxygen species; nNOS, Neuronal nitric oxide synthase; SBDPs, spectrin breakdown products; ASIC, acid-sensing ion channels; COX, cycloxygenase; cGMP, 3′5′-cyclic guanosine monophosphate; TTC, triphenyltetrazolium chloride; PBS, phosphate buffer saline

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Lilley et al., 2004). Nevertheless homomeric ASIC1a, a common ASIC subtype in central nervous system upon acidic activation also induces direct Ca^{2+} entry through these channels (Waldmann et al., 1997; Chu et al., 2002; Yermolaieva et al., 2004). The ASIC mediated membrane depolarization is also believed to facilitate the activation of voltage gated Ca^{2+} channels and NMDA receptor-gated channels which further promotes neuronal excitation and Ca^{2+} accumulation (Wemmie et al., 2002).

As acidosis is central and prominent metabolic feature of ischemic brain, the inhibition of ASIC1a channels offers a new neuroprotective approach in cerebral stroke management. Moreover, inhibition of ASIC1a with flavonoids remains unknown against neuroprotection in animal models of cerebral ischemia. Flavonoids are believed to act as health-promoting substances and some of them have antioxidant and anti-inflammatory properties (Middleton et al., 2000, Havsteen, 2002). Quercetin has been reported to display both anti-inflammatory and antiischemic effects and can pass the blood-brain barrier (Youdim et al., 2004; Rogerio et al., 2007). Additionally, quercetin has protective effects on cell function in vitro and in vivo (Zhu et al., 2007; Cho et al., 2006).

Thus, apart from reducing inflammation with selective ASIC1a, inhibitory activity of quercetin could also prove a beneficial effect in reducing the effect of cerebral ischemia. Therefore, the target of the present study was to determine the neuroprotective efficacy of quercetin in rat model of focal cerebral ischemia/reperfusion (I/R) injury and efforts were also use to analyze its regulatory effect on ASIC1a channels mediated downstream survival/damage mechanisms.

2. Results

2.1. Effect of quercetin pretreatment on neurological deficit and cerebral infarct volume

We determined the minimum effective neuroprotective dose of quercetin on the basis of reduction in neurological deficit

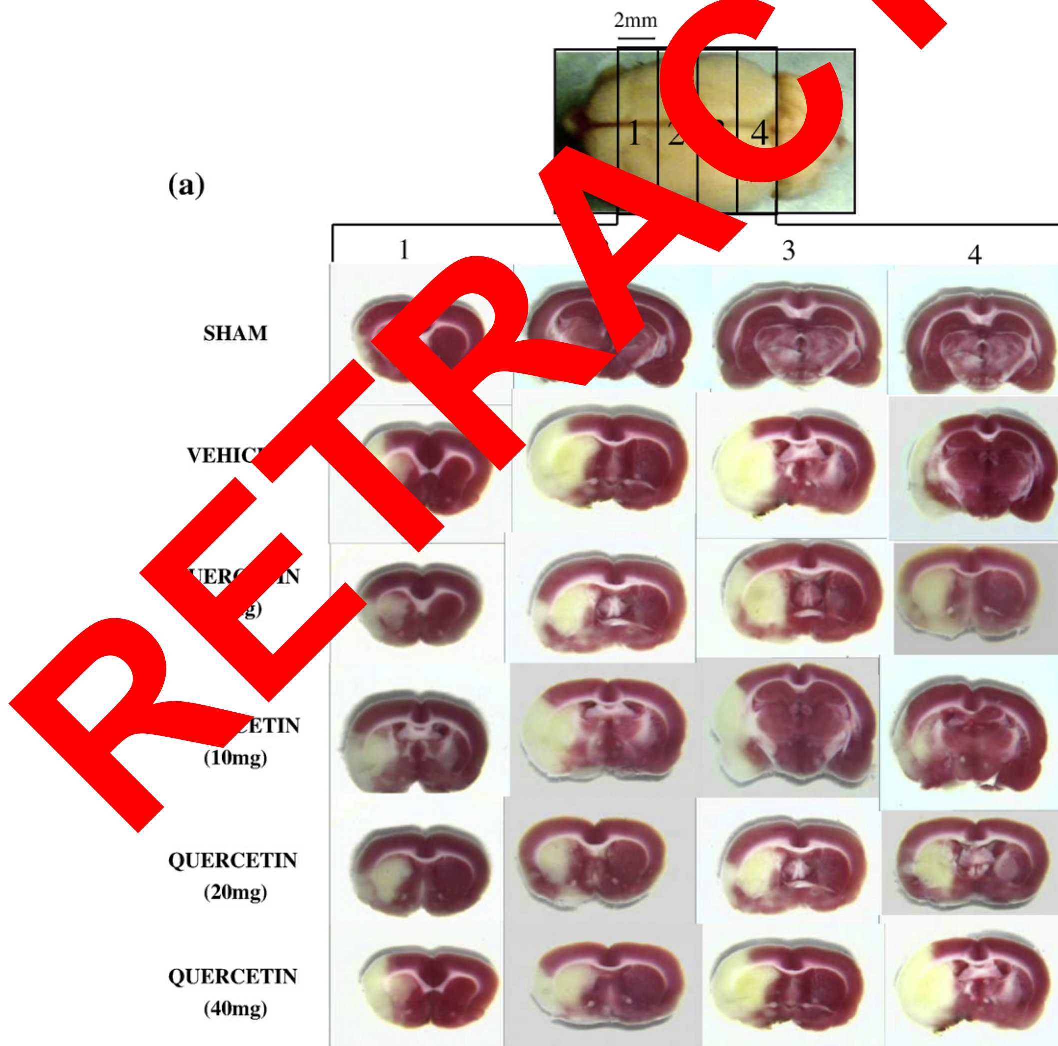

Fig. 1 – Effect of different doses of quercetin pretreatment on (a) representative TTC stained coronal brain sections of sham, vehicle and quercetin treated rats post 1/24 h I/R injury. (b) Cerebral infarct volume of rats following 1/24 h I/R injury. (c) Neurological deficit score. * versus sham and ** versus vehicle.

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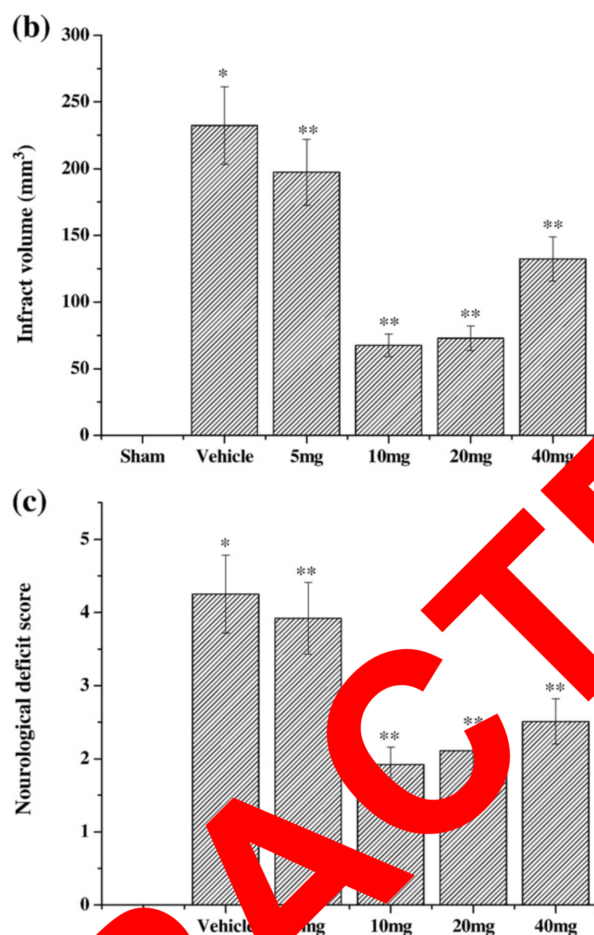


Fig. 1 [illegible]ntinued).

and cerebral infarct volume of [illegible] subjecte[illegible] 1/24 h of I/R injury. In our studies, 1/24 h i[illegible]ry consiste[illegible] produced marked infarcts in both c[illegible]ical a[illegible] subcortical ipsilateral regions of rat brain as e[illegible]nced in T[illegible]ained coronal brain sections (Fig. 1a). N[illegible]ological deficit w[illegible] analyzed on the basis of neurolog[illegible] score[illegible]tained post 1/24 h of I/R injury in all experiment[illegible] The ve[illegible]icle treated group of rats shows signi[illegible]ntly h[illegible]r neuro[illegible]ical deficit as compared to sham g[illegible] [illegible]imals [illegible]ignificant ($P<0.01$) improvement [illegible] neuro[illegible]ical def[illegible] score was found in quercetin pret[illegible]d ra[illegible]s except lowest dose as compared to vehicle [illegible]d group (Fig. 1c). The mean of infarct volume was found to b[illegible]2.43±21.3 mm^3 in vehicle treated control rats whereas pret[illegible]ment with quercetin at 10, 20, 40 mg/kg i.p. doses produced marked reduction in infarct volume, ranging from 67.63±29.3, 72.87±27.2, 132.37±30.4 mm^3 respectively (Fig. 1b). Thus 10 mg/kg quercetin i.p. showed significant ($P<0.01$) improvement in neurological deficit and infarct size, therefore 10 mg/kg quercetin was selected as optimum dose for further studies.

2.2. *Effect of quercetin post-treatment on cerebral infarct volume and neurological deficit*

The minimal neuroprotective dose 10 mg/kg i.p. of quercetin was also significantly effective 2 h post I/R as reflected by improvement in the neurological outcome as well as nearly 48% reduction in the infarct volume as compared to vehicle. Further, 4 h post treatment also significantly ($P<0.05$) improved the neurological deficit and also reduced infarct volume by almost 25% (Fig. 2a, b and c). Thus, present study suggested that quercetin elicits neuroprotection even 4 h post reperfusion with slight decrease in efficacy as compared to pretreatment.

2.3. *Effect of quercetin on brain nitrite levels*

As we know that calcium dependent activation of neuronal nitric oxide synthase (nNOS) has always been associated with early rise in post ischemic nitrite levels in rat brain. Therefore, nitrite levels were measured at 20 min post ischemia in ipsilateral cortex and striatal regions of rat brain. In both brain loci the nitrite levels were found to be significantly higher ($P<0.05$) in vehicle treated as compared to sham group of animal while pretreatment with quercetin (10 mg/kg i.p.) significantly ($P<0.05$) attenuated the increase in nitrite levels in both striatal and cortical regions of ischemic rat brain (Fig. 3a).

2.4. *Effect of quercetin on brain MDA levels (lipid peroxidation)*

MDA is a marker of lipid peroxidation, therefore the MDA levels were measured post 1 h of ischemia in cortex and

striatal regions of rat brain. MDA levels in brain were significantly ($P<0.01$) increased in vehicle treated rats compared to sham rats. The MDA levels in vehicle treated rats were found to be 5.62±0.49 & 6.3±0.63 nmol/mg proteins whereas in quercetin10mg/kg i.p. treated rats were 3.42±0.42 & 3.75±0.75 nmol/mg protein, in cortical and striatal regions, respectively (Fig. 3b). Thus quercetin pretreatment resulted in significant ($P<0.01$) reduction in post ischemic brain MDA levels.

2.5. *Effect of quercetin on spectrin breakdown products (SBDPs)*

Calpain is a calcium dependent protease which cleaves cytoskeletal protein spectrin that results spectrin break down products in ischemic rat brain. These fragments were evaluated by western blot and appeared as ~150 kDa band along with complete spectrin protein (280 kDa). A significant increase ($P<0.01$) in SBDPs was found in post 1/12 h of I/R injury in cortical and striatal brain regions of vehicle treated group. SBDPs level significantly ($P<0.01$) reduced by pretreatment of quercetin (10 mg/kg i.p) approximately 49% in both regions of ischemic rat brain (Fig. 4).

2.6. *Effect of acidosis on $[Ca^{2+}]$i in synaptoneurosomes and its regulation by quercetin*

Preparation of synaptoneurosomes is considered a unique "physiologically active synapse" which imitates in vivo conditions more closely as compared to cultured neurons. Our studies on stimuli induced $[Ca^{2+}]$i influx were performed on synaptoneurosomal preparation which was initially validated by depolarization with KCl (30 mM) that led to a rapid increase in synaptoneurosomal $[Ca^{2+}]$i. The basal level of $[Ca^{2+}]$i observed in synaptoneuro[illegible] 180±18 nM which virtually doubled to 370±2[illegible]M on stim[illegible]tion with KCl. Further stimulation of the syn[illegible]neurosom[illegible] preparation with NMDA (100 μM) [illegible]or se[illegible]ized w[illegible] glycine (100 μM), significantly in[illegible]sed basal [illegible]73±13 nM. While acidification of [illegible]tracell[illegible] medi[illegible] to pH 6.0 by addition of 0.1 N HCl inc[illegible]e[illegible]napton[illegible]urosomal $[Ca^{2+}]$i up to 246.3±8.8 nM [illegible]h slo[illegible]etics [illegible]ompared to NMDA (100 μM) indu[illegible]ise (Fig. 5). [illegible]ingly prior addition of quercetin [illegible]m[illegible]mM) inhib[illegible]d the acid mediated increase in synapto[illegible]osomal $[Ca^{2+}]$i in a dose dependent man[illegible] was witho[illegible]y effect on NMDA mediated $[Ca^{2+}]$i i[illegible]ease. The inhibition was more than 90% as observed w[illegible]h 1 mM of [illegible]uercetin ($P<0.001$). This underlies the

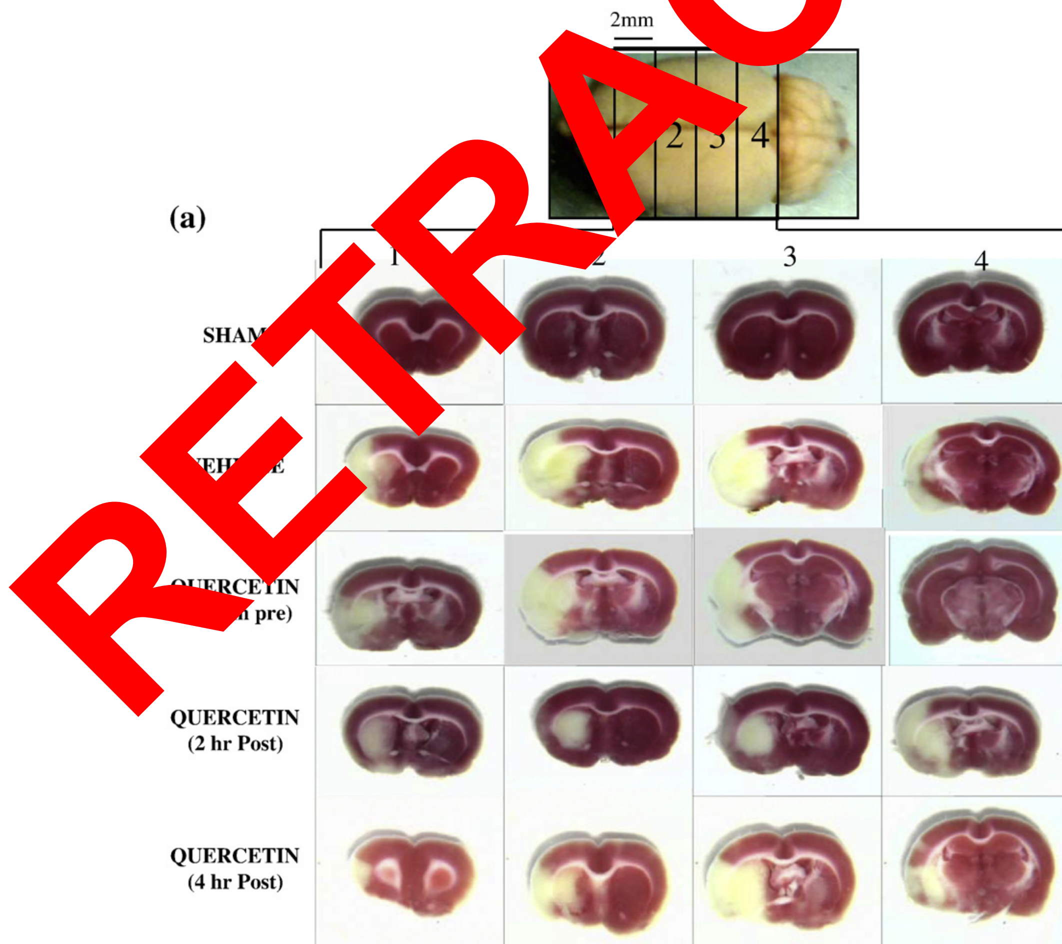
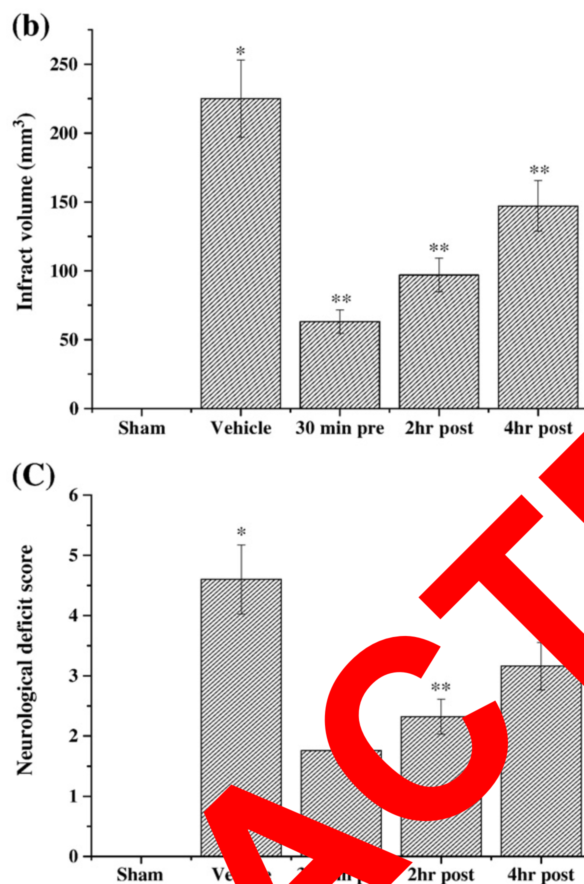


Fig. 2 – Effect of quercetin (10 mg/kg,i.p.) pre and post treatment on (a) representative TTC stained coronal brain sections of sham, vehicle, quercetin pre and post treated rats. (b) Cerebral infarct volume of rat following 1/24 h I/R injury. (c) Neurological deficit score. * versus sham and ** versus vehicle.



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specificity of quercetin in reducing the [Ca^{2+}]i increase induced by acidotoxicity(Fig. 6).

3. Discussion

Acid-sensing ion channels (ASIC) are nonselective ion channels, which are activated in response to decreased extracellular pH. These trimeric channels consist of one or more of six different subunits (ASIC1a, ASIC1b, ASIC2a, ASIC2b, ASIC3, and ASIC4), which vary in their response to pH levels. In the event of cerebral ischemia, the extracellular pH decrease triggers ASIC channel gating, allowing calcium to enter neurons (Xiong et al., 2006; Simon and Xiong, 2006, Pignataro et al., 2007). Although both ASIC1a and ASIC2a subunits are found abundantly in the brain, ASIC1a containing channels are considered to play a major role in calcium-mediated ischemic brain injury (Xiong et al., 2004). Intraneuronal Ca^{2+} overload due to the activation of ASIC, NMDA and other cation channels is the major cause of acute injury in cerebral ischemia which is followed by oxidative stress, inflammation and apoptosis subsequently contributing to the late phase of ischemic injury. The myriad of calcium dependent enzymes like neuronal nitric oxide synthase (nNOS), calpain, phospholipase, xanthine oxidase, ligases and DNases are activated due to elevation in the level of intraneuronal Ca^{2+}. These enzymes are involved in the synthesis of free radicals and catabolism of proteins, phospholipids and nucleic acids (Mehta et al., 2007).The post ischemic reperfusion further exacerbates cerebral injury by generating free radicals (Chan, 1996). These pathological changes cause membrane alterations and breakdown of cytoskeletal structure culminating in severe cerebral damage. The mechanism of action of quercetin Thus, keeping in view above, an effort was made to analyze whether quercetin could also provide neuroprotection by inhibiting ASIC1a mediated acidotoxicity apart from its beneficial anti-inflammatory activity. The anti-inflammatory property of quercetin and other polyphenols, is due to inhibition of cycloxygenase (COX) mediated prostaglandins synthesis, particularly prostaglandin E2 which plays major role in inflammation (Peng et al., 2006). Later these effects were found to be mediated by NFkB and AP-1 activation (Wattel et al., 2004). Thus, our results suggest that quercetin elicit neuroprotection in acute ischemic stroke owing to its ASIC1a inhibitory effect and partly due to anti-inflammatory potential. Hence studies were undertaken to analyze the neuroprotective role of quercetin in ischemic rat brain and its ability to regulate ASIC1a, both in vivo and ex vivo. The studies involved the use of focal cerebral ischemia rat model and analysis of downstream calcium dependent survival and death mechanisms in striatal and cortical regions of ischemic brain. The reason for choosing these regions lie in the observation that following MCA occlusion, cells in the striatum die immediately constituting core region surrounded by a penumbral region represented by cortex where cell death progresses with time (Popp et al., 2009; Verma et al., 2010). It is expected that an ideal neuroprotective should prevent

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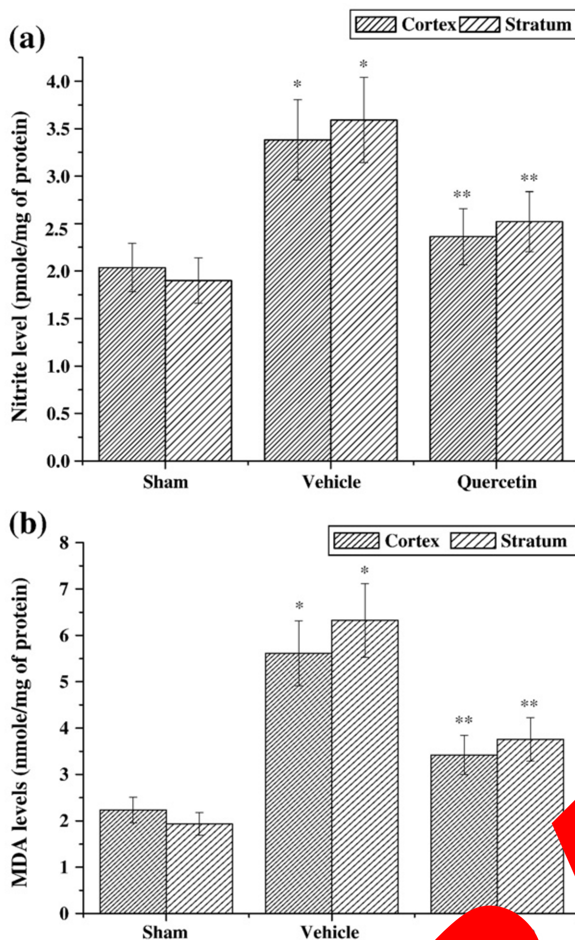

Fig. 3 – Effect of quercetin (10 mg/kg, i.p[illegible]etreat[illegible]nt on (a) Nitrite and (b) MDA levels in cortic[illegible]l an[illegible] rat brain following post 20 min of [illegible]hemia. [illegible]sus sham, ** versus vehicle.

cell death to avoid inc[illegible]ration of p[illegible]mbral region into necrotic core region[illegible]wever, pretreatm[illegible] with quercetin offered significant [illegible]roprot[illegible]on and a dose of 10 mg/kg of quercetin was foun[illegible]be[illegible]mal in [illegible]roviding neuroprotection as exhibited b[illegible]lative [illegible]ction in [illegible]urological deficit score and cerebral i[illegible]ume. [illegible]rological deficit arises from damag[illegible] the te[illegible]ory of M[illegible] occlusion i.e. caudate putamen and c[illegible] th[illegible] motor function and could be well correlate[illegible] cerebral damage mapped out as infarct. An improveme[illegible] neurological function very well correlated to the decreased [illegible] of infarction in territory of MCA. Moreover, quercetin post treatment at the same dose was also effective in providing neuroprotection when administered even 4 h post reperfusion, though with less efficacy. Further, the inhibitory effect of quercetin on calcium mediated rise in NO levels (Fig. 3a) also supports the neuroprotective effect following ischemic insult. An increase in intraneuronal Ca^{2+} serves as a signal for activation of Ca^{2+} calmodulin dependent and protein kinase C regulated neuronal nitric oxide synthase (nNOS). Activation of nNOS generates nitric oxide (NO) which is a physiological mediator of vasodilation by elevating level of 3′5′-cyclic guanosine monophosphate (cGMP) in vascular smooth muscles. Nevertheless at high concentrations as evidenced during ischemia, NO causes damage to neuronal tissues by combining with the superoxide anion to form peroxynitrite anion which then decomposes to form highly toxic hydroxyl and nitrogen dioxide radicals (Beckman et al., 1990; Radi et al., 1991).

In our studies, NO production was quantified by measuring nitrite, a stable end product of NO (Green et al., 1982). An increase in nitrite level was found in ischemic regions of brain after 20 min of ischemia suggesting an increase in NO synthesis by calcium dependent neuronal nNOS (Fig. 3a). This is reported that nitrite levels were fo[illegible] to increase post 10 min of cerebral ischemia (Kader et[illegible] Özben et al., 2005), we have estimated the nit[illegible] levels af[illegible]20 min of ischemia and found two fold d[illegible]ed nitrit[illegible]evels in quercetin treated group as c[illegible]pared [illegible]e vehi[illegible] (Fig. 3a). Decrease in NO levels a[illegible] quercetin [illegible]t could be associated with decr[illegible]d cal[illegible]n influ[illegible]esulting from ASIC1a inhibition thus [illegible] neuro[illegible]rotection. We also observed signific[illegible]ncrea[illegible] lipid p[illegible]idation (Fig. 3b) as it is evident fro[illegible]creased M[illegible] in ischemic rat brain immediate[illegible]os[illegible]erfusion (S[illegible]ser et al., 2002). MDA is a marker for lipid pero[illegible]tion and its quantification reveals the exte[illegible]euronal me[illegible]ne damage which is high in lipid co[illegible]nt. It is most likely that lipid peroxidation pathway in a p[illegible]ischemic br[illegible]n hemisphere is a result of combined action o[illegible] and free r[illegible]cals generated from activation of nNOS and ph[illegible]olipase [illegible]2 (Radi et al., 1991; Holscher, 1998). The querce[illegible]ted decrease in MDA levels due to inhibition of [illegible]IC1a mediated calcium influx involved in activation of nNOS [illegible]spholipase A2 as well. Intracellular calcium overload [illegible]lso results in activation of calpains which are calcium dependent cystein proteases involved in physiological and pathological processes. Calpain activity is required for the induction of long term potentiation (Oliver et al., 1989;Vanderklish et al., 1996) and is also involved in necrotic cell death through the degradation of several substrates essential for cell survival including enzymes, transcription factors, receptors, transporters, channels and proteins of the cytoskeleton such as spectrin (Wang, 2000). It had been shown that NMDA receptors dependent excitotoxicity in part is also mediated by calcium dependent calpain activation (Siman and Noszek, 1988; Araujo et al., 2004). Influence of quercetin on calpain activity following cerebral ischemia was studied by western blot using antibody against α-spectrin which is substrate for calpain. It is known that following focal or global ischemia, rapid and sustained activation of calpain occurs which produces spectrin break down products (SBDPs) of 145/150 kDa (Jiang et al., 2005; Del Rio et al., 2008).

Quercetin significantly reduces the elevated levels of SBDPs following cerebral ischemia indicating its ability to affect calpain activation. This further confirms the quercetin inhibitory effect on ASIC1a dependent calcium influx which modulates calpain activation. We also analyzed the effect of quercetin on acidosis mediated $[Ca^{2+}]i$ influx on synaptoneurosomes prepared from rat brain. The synaptoneurosomes are brain subcellular preparation enriched in presynaptic and postsynaptic membrane fragments resealed in a vesicular fashion. These are physiologically active if used in a limited time frame for receptor mediated responses like adenylate cyclase activity (Hollingsworth et al., 1985), phosphoinositide hydrolysis (Gusovsky and Daly, 1988), Cl^- uptake (Imamura and Prasad, 2003) and $[Ca^{2+}]i$ influx studies (Benavides et al., 1988; Salinska et al., 2000). In order to validate

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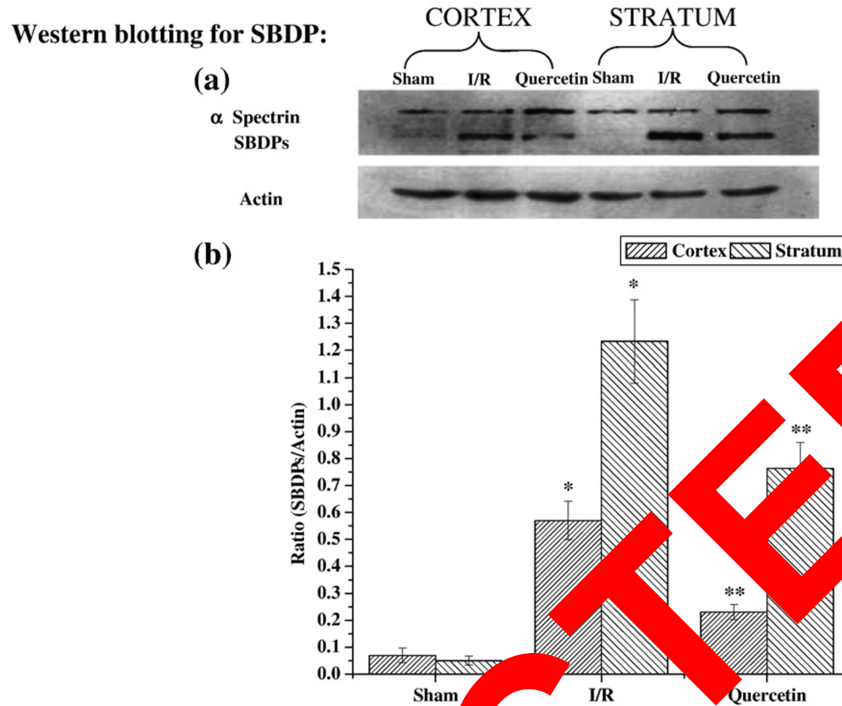

Fig. 4 – (a) Representative western blot showing effect of querceti[illegible]0 mg/kg, i[illegible] pretreatment on calpain mediated spectrin break down products (SBDPs) following 1/12 h I/R injury. (b) Graph s[illegible] the de[illegible]tometric analysis of the ratio of the densities of SBDPs/Actin band. * versus sham and ** versus I/R.

synaptoneurosomal activity, the use of KCl resulted in marked [illegible] in synaptoneurosomal [Ca^{2+}] by opening of vol[illegible]itive C[illegible] channels. Further decrease in extracellula[illegible] fro[illegible].4 to 6[illegible] caused rapid increase in [Ca^{2+}]i level[illegible]us su[illegible]ting the concept of acidosis mediated calci[illegible]m i[illegible] following ischemic injury. The in[illegible]ation w[illegible]ercetin dose dependently inhibited the rise [illegible]$^{2+}$]i, media[illegible]y acidosis which is in conformity with [illegible]viou[illegible]orts.

4. Conclusion

The blood brain barrier permeability, anti-inflammatory and antioxidant property of quercetin was exploited against focal cerebral ischemia and role of ASIC was established in the mechanism of action of quercetin. On the basis of the observation reported in the manuscript it can be

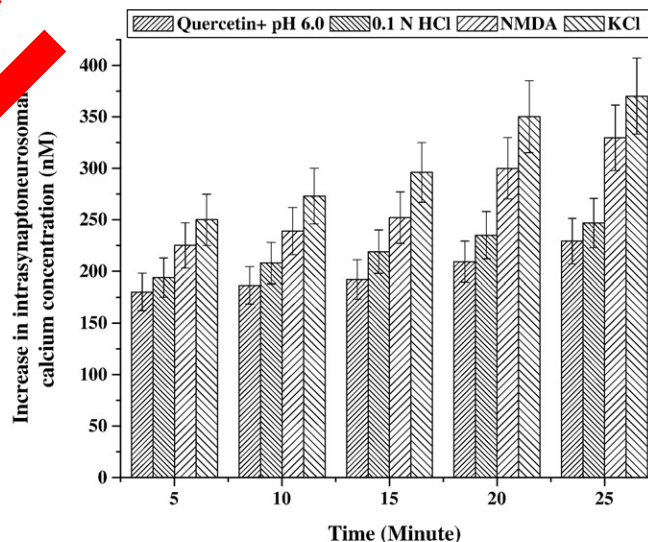

Fig. 5 – Effect of KCl (30 mM), NMDA(100 μM) and pH 6.0 on intrasynaptoneurosomal calcium levels and effect of quercetin (1 mM) on pH 6.0 induced calcium levels.

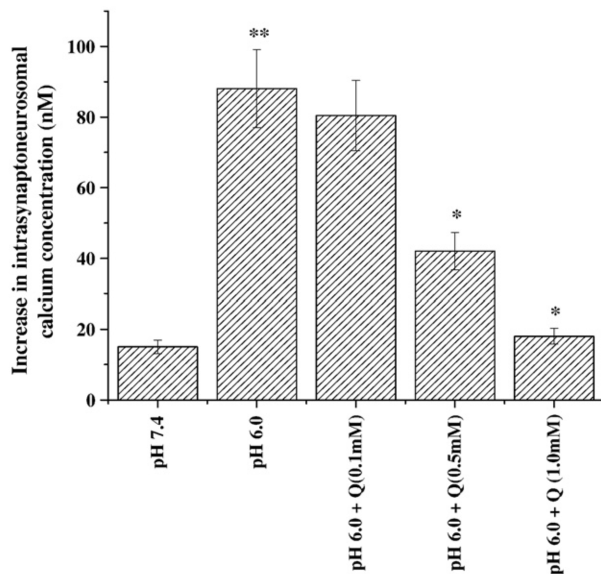

Fig. 6 – Effect of various concentrations of quercetin (0.1–1 mM) on pH 6.0 evoked intrasynaptoneurosomal calcium levels (* significant versus pH 6.0, **significant increase versus pH 7.4).

summarized that the decrease in calpain level and [Ca^{2+}] permeable ASIC shows inhibitory effect of quercetin in f[illegible]al cerebral ischemia. Results of the present study pr[illegible] significant evidence that quercetin inhibits ASIC1a me[illegible]ed acidotoxicty and confers neuroprotection in MCAo [illegible] model of focal cerebral ischemia. Post and [illegible]schem[illegible] neuroprotection improves the applicatio[illegible] qu[illegible]etin in dietary use as well as clinical against t[illegible]schem[illegible]troke.

5. Experimental pro[illegible]s

5.1. *Reagents*

Quercetin, Fura 2 A[illegible]TTC a[illegible]ther chemicals were obtained from Sigma unless [illegible] specif[illegible]d.

5.2. *A[illegible]*

Male [illegible]gue [illegible] (6 weeks, 250±10 g) in-bred at the Animal [illegible] of the [illegible]tute of Nuclear Medicine and Allied Sciences, De[illegible]ere used for the experiments. Animals were kept under standard [illegible]ratory conditions maintained with the highest standards of animal care and housing. They were allowed free access to food and water and maintained at 12 h day/night cycle. The approved standard procedures and the institutional animal ethical committee guidelines were followed throughout the experiments.

5.3. *Focal cerebral ischemia*

Focal cerebral ischemia was induced by occlusion of the middle cerebral artery (MCA) using a modification of the intraluminal technique (Longa et al., 1989). Rats were anesthetized with kitamine (50 mg/kg i.p.) and maintained a constant body temperature of 37±0.5 °C. The left common carotid artery (CCA) was exposed through a midline incision in the neck region. The neck muscles were separated further to expose external carotid artery (ECA) and internal carotid artery (ICA). A 4.0 cm length 3–0 monofilament nylon suture (Ethicon) was introduced into the ECA lumen through a small nick and gently advanced from the ICA lumen to block the origin of MCA. The approximate length of filament inserted near the bifurcation point to the MCA blockade site was about 18–22 mm. The ECA stump was tightened by thread around th[illegible]raluminal nylon suture to prevent bleeding. Reperfusio[illegible]d by gently removing the monofilament after [illegible] of ischem[illegible] In sham-operated animals, all the procedures [illegible]pt for the [illegible]ertion of the nylon filament were carr[illegible]out. A[illegible]ls were [illegible]llowed to recover from anesthesia a[illegible]on regainin[illegible]ting reflex, were transferred to cag[illegible] the a[illegible]l room, [illegible]n temperature maintained at 26±2.5 °C. [illegible]ere pr[illegible]ided food and water ad-libitum.

5.4. *Dr[illegible]nt and exp[illegible]ental protocol*

Quer[illegible]as dissolv[illegible] normal saline and administered in[illegible]eritonially 30 min p[illegible]or to MCA occlusion and 2 and 4 h a[illegible] ischemic st[illegible]ke at 5 mg, 10 mg, 20 mg, and 40 mg/kg doses to [illegible]termine th[illegible]inimum effective neuroprotective dose pre an[illegible]t ische[illegible] injury. A total of 52 animals were divided in six gro[illegible]sting of sham (n=6), control (n=6) and quercetin [illegible]ated (n=10) for each dose group. The effective dose thus [illegible]ed was administered 2 and 4 h post ischemia in two more groups (n=10 each) to assess the efficacy of quercetin in post ischemic injury. Further, biochemical and molecular studies were undertaken with the effective dose in three more groups viz sham, vehicle and treated (n=6 in each group).

5.5. *Neurobehavioral assessment*

The neurobehavioral assessment was done 24 h post reperfusion (Longa et al., 1989). Briefly, the neurobehavioral deficits was monitored on five point scale with 10 grading scores: a score of 0 indicated no neurologic deficit, a score of 1 means failure to extend opposite forepaw fully, a score of 2 was assigned when contralateral circling was seen. While the rat which was not able to grip the wire mesh and fell on the contralateral side of brain damage, a score of 3 was assigned. Further when the rats were unable to walk spontaneously and had a depressed level of consciousness was given a score of 4. The neurobehavioral scores obtained after testing on each scale were averaged to denote the degree of neurological deficit.

5.6. *Evaluation of cerebral infarct volume*

After neurological examination, rat brain was perfused with normal saline by transcardiac perfusion and isolated in chilled conditions. The cerebellum was removed and the rest of the brain was immediately transferred at −20 °C. Frozen brain was vertically sliced into uniform coronal sections of 2 mm thickness. The brain slices obtained were incubated in TTC (0.5% in 0.1 M PBS) at 37 °C for 30 min (Bederson et al., 1986). TTC stains, viable brain tissue to brick red whereas

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unstained brain tissue i.e. infarcted portion of tissue remains unstained and appears as white. The infarct area thus obtained was measured and quantified by image analysis software (image J). Further, infarct volume was calculated by linear integration of the infarct area of each slice multiplied by the average thickness of brain section and expressed in mm^3.

5.7. *Biochemical analysis*

Biochemical analysis included measurement of nitrite and malondialdehyde 20 min and 60 min post ischemia respectively in cortex and striatal regions of ipsilateral rat brain. The above brain parts were quickly removed by decapitation under ether anesthesia and homogenized (5:1 v/w) in ice cold 0.1 M phosphate buffer, pH 7.4. The tissue homogenate thus obtained was used for estimation as per protocol given below.

5.7.1. *Measurement of nitrite*

Nitrite and nitrate estimations in biological material are commonly used as a marker for nitric oxide (NO) production. The nitrite levels were estimated in affected brain regions using Griess reaction (Guevara et al., 1998). Briefly, the tissue homogenate was centrifuged at 1580 *g* for 15 min at 4 °C and the supernatant thus obtained was deproteinized by mixing with an equal amount of 4% sulfosalicylic acid. Further 350 µl of this reaction mixture was made to react with 350 µl of [illegible] reagent (1:1 mixture of 1% sulfanilamide in 5% phosphor[illegible] and 0.1% N-(1-naphtyl)ethylenediamine in distilled water) [illegible]d incubated for 10 min in the dark at room temperature. [illegible] absorbance of the assay samples was meas[illegible]40 nm [illegible] spectrophotometer (Perkin Elmer, Germ[illegible]). Nitr[illegible] concentrations were calculated using a calibrati[illegible]urve [illegible] from sodium nitrite and expressed as p[illegible]/mg [illegible]tein in tissue homogenate. Total protein conce[illegible]tion of tis[illegible] homogenate was estimated by the Lowry [illegible] (Lowry et al., [illegible]1).

5.7.2. *Estimation of m[illegible]ndialdehyde [illegible]DA)*

The MDA is a by[illegible]duct of lipid pe[illegible]idation and an important bioma[illegible] of m[illegible]brane damage. It was determined based on [illegible] with thio barbituric acid (TBA). Briefly, the tissue h[illegible]genate [illegible]0 µl), 30% trichloroacetic acid (300 [illegible], [illegible]HCl (1[illegible]) [illegible] 2% (w/v) TBA in 0.5 M NaOH (300 [illegible] were th[illegible]oughly m[illegible]ed up. After each addition, the tota[illegible] [illegible]ume [illegible] 3 ml with distilled water. The above mixtur[illegible] heated in a water bath at 80 °C for 20 min and centrifuge[illegible] 4000 *g* for 10 min. The resultant pink chromogen formed w[illegible] measured at 532 nm in spectrophotometer. MDA concentration in brain was determined using standard curve and values expressed as nmol/mg tissue protein in homogenate. Total protein concentration of tissue homogenate was determined by Lowry method (Lowry et al., 1951).

5.8. *Western blotting for spectrin break down products (SBDPs)*

The brain tissue was dissected to remove cortex and striatum of ipsilateral side and homogenized in ten volumes of ice cold HEPES buffer (pH 7.4) containing HEPES 1 mM, $MgCl_2$ 1 mM, Sucrose 0.32 M, EDTA 1 M, $NaHCO_3$ 1 M, PMSF 0.1 M and protease inhibitors cocktail. The tissue homogenate was then centrifuged at 800 *g* for 10 min to remove nuclei. The supernatant thus obtained was again centrifuged at 10,000 *g* for 20 min to sediment crude membranes. The resultant crude membrane pellet was suspended in homogenizing buffer, the protein concentration of which was determined using Lowry's method (Lowry et al., 1951). Equal amount of proteins was resolved on SDS-PAGE and transferred on to nitrocellulose membranes. The primary antibodies used for immunoblotting were mouse monoclonal antispectrinα [illegible] rabbit polyclonal anti-actin (Santa Cruz Biotechnolog[illegible]) [illegible]he secondary antibodies used were HRP conjug[illegible] anti-IgG. [illegible] immunoreactive bands were visualized b[illegible]hanced ch[illegible]iluminiscence (ECL) detection (GE H[illegible]ncare[illegible]. The b[illegible] intensity was measured using s[illegible] densitomet[illegible]s by Alpha Imager TM 2200 sof[illegible]re an[illegible]eported [illegible] (SBDPs/Actin) intensity ratio.

5.9. *Mea[illegible]ment of [Ca[illegible] [illegible] brain synaptone[illegible]so[illegible]*

5.9.1. *[illegible]eparation o[illegible] brain synaptoneurosomes*

S[illegible]ptoneurosomes we[illegible] prepared from the rat brain accord[illegible] to the meth[illegible]d described by Hollingsworth et al. (1985) with [illegible]t modific[illegible]n. Briefly, rat brains were rapidly removed a[illegible] cereb[illegible] cortices were dissected and homogenized in 7 volu[illegible]xygenated (95% O_2/5% CO_2) ice-cold Krebs-[illegible]icarbonate buffer (118 mM NaCl, 4.7 mM KC1, 1.0 mM $MgCl_2$, [illegible] $CaCl_2$, 24.9 mM $NaHCO_3$, 1.2 mM KH_2PO_4 and 5 mM glucose, pH 7.4) with a Teflon glass homogenizer (5 strokes). The homogenate was diluted with 30 ml of the same buffer and filtered gently through three layers of a nylon mesh (100 µm). The resulting suspension was again filtered through a 10 µm millipore filter using positive pressure. The filtrate thus obtained was centrifuged at 1000 *g* for 15 min. The resulting pellets, synaptoneurosomes were suspended in Krebs bicarbonate buffer so as to get final protein concentration of 3 mg/ml. This suspension was then incubated at 37 °C for 10 min with Fura2 AM (5 µM).The suspension was centrifuged to remove extra-syanptoneurosomal Fura2 AM and the resulting pellets were again resuspended in Krebs–HEPES buffer ($NaHCO_3$ was substituted with 20 mM HEPES) to get protein concentration of 0.2 mg/ml which was used for intracellular calcium measurement within 90 min.

5.9.2. *Measurement of $[Ca^{2+}]i$*

Measurement of $[Ca^{2+}]i$ was performed at 37 °C under continuous stirring using Varian Fluorescence Spectrophotometer quipped with a water jacketed cuvette holder and magnetic stirring system. Dual wavelength excitation at 340 and 380 nM (the 340/380 ratio) were used and the resting fluorescence was measured at 510 nM. Changes in fluorescence intensity were monitored following the addition of glycine (100 µM), NMDA (100 µM), glutamate (100 µM), KCl (20 mM) and HCl to make buffer solution pH drop to 6.0. The quercetin effect on acidosis mediated $[Ca^{2+}]i$ increase was analyzed by incubating synaptoneurosome preparation with concentrations ranging from 0.1 mM to 1 mM of quercetin prior to addition of HCl. At the end of experiment the maximal fluorescence (F_{max}) and minimal fluorescence (F_{min}) was obtained by adding 10 µl of

10% triton-X and 30 μl of 0.5 mM EGTA respectively. $[Ca^{2+}]i$ was calculated using the equation

$$[Ca^{2+}]i = Kd(R\text{–}Rmin) / (Rmax\text{–}R)(Sf2 / Sb2)$$

where Kd is the dissociation constant value amounted to 225 nM (Grynkiewicz et al., 1985) while R is the fluorescence intensity ratio F1/F2 in which F1 at 340 nm and F2 at 380 nm are the fluorescence for the ion-bound and ion-free indicator respectively. Rmin is the ratio for the ion-free indicator; Rmax the ratio for the ion-saturated indicator; Sf2 and Sb2, fluorescence for the free and the calcium-bound dye at 380 nm respectively.

5.10. *Statistical analysis*

Data are represented as mean ± standard error of mean (SEM). Statistical significant was analyzed using one-way ANOVA followed by Tukey's post hoc test. A P value of <0.05, <0.01 and <0.001 was considered significant.

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