

## **P2X<sub>4</sub> receptor antagonist ameliorates the neurotoxicity induced by local anaesthetic.**

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### **Abstract**

Local anaesthetics are known to produce neurotoxicity at normal doses. Present investigation check the neuroprotective effect of P2X<sub>4</sub> receptor antagonist in local anaesthetic induced neurotoxicity. In this study SH-SY5Y neuronal cell was treated with local anaesthetic (bupivacaine) and P2X<sub>4</sub> receptor antagonist (Sumarin) at different concentrations. Neuroprotective effect of sumarin was evaluated by estimating cell viability, apoptosis, Reactive Oxygen Species (ROS) accumulation, Caspase 3 and 9 activity and morphological changes. Result of study suggested that treatment with sumarin significantly increases ( $p < 0.01$ ) the cell viability and reverse the effect of bupivacaine. It was observed that treatment with sumarin (100  $\mu\text{mol/l}$ ) effectively attenuates the apoptosis, ROS accumulation Caspase 3 and 9 activity and morphological changes induced by bupivacaine (1  $\text{mmol/l}$ ). Thus present study concludes that neuroprotective effect of P2X<sub>4</sub> receptor antagonist in bupivacaine induced neurotoxicity of cell by decreasing the ROS and Caspase activity and thereby decreases the apoptosis of neuronal cell.

**Keywords:** P2X<sub>4</sub> receptor, Neurotoxicity, Local anaesthetic, Apoptosis, Reactive oxygen species.

*Accepted on July 30, 2016*

### **Introduction**

Local anaesthetics are clinically used for the production of anaesthesia, relieving pain associated with post operation and biopsy study [1]. Literature suggested that these drugs are possess cytotoxic property, on the basis of which it produces neurotoxicity when applied for spinal anaesthesia clinically [2,3]. It was observed in the reported study that administration of local anaesthetic through cauda equina, intrathecal route induces apoptosis in neuronal cells by inducing atrophy, degeneration of axon, oedema [4]. Local anaesthetics having higher lipophilicity carry neurotoxic property and evidences links the neurotoxicity associated with bupivacaine administration [5]. There are various mechanisms on the basis of which local anaesthetics induces cytotoxicity/neurotoxicity like apoptosis induced through activation of Caspase pathway, increase in ROS production, mitochondria injury, tyrosin kinase enzyme inhibition, blockage of Na<sup>+</sup> ion channel, increase in release of calcium from rough endoplasmic reticulum and activation of p38 Mitogen-Activated Protein Kinases (MAPK) etc. [6-8]. Even hyperexcitability of the neuron is one of the factors responsible for neurotoxicity induced by local anaesthetic [9].

P2X<sub>4</sub> receptor is related to neuropathic pain and induces injury by up regulation of MAPK and thereby activates the release of brain derived neurotrophic factor in neuronal cells [10]. Thus P2X<sub>4</sub> receptor activation produces hyper excitability of neuron and neuronal injury [11]. Previous studies reported that P2X<sub>4</sub> receptor antagonists reduces the ROS production and cytokine

level, thus decreases the oxidative stress [12]. It also inhibits the apoptosis of neuronal cell by activating the Caspase pathway and decreasing the activation of MAPK [13]. P2X<sub>4</sub> receptor antagonists have also maintained the neurochemical level in the brain tissues and thereby used in the management of epilepsy [14]. Anticonvulsant drugs reported to have neuroprotective in local anaesthetic induced neurotoxicity [15]. On the basis of these facts it is worthy to assess the neuroprotective effect of P2X<sub>4</sub> receptor antagonists in local anaesthetics induced neurotoxicity.

### **Material and Methods**

#### ***Cell line and culture***

Neuronal (SH-SY5Y) cell line was procured from Shanghai Institutes for Biological Sciences, Shanghai, China. In a 5% CO<sub>2</sub> humidified incubator at 37°C cells lines were incubated. DMEM medium was used with (10% v/v) foetal bovine serum, penicillin (100 U/ml) and streptomycin (100  $\mu\text{g/ml}$ ) as a culture medium for the present study.

#### ***MTT assay***

MTT assay was used to assess cellular viability. In which cell ( $5 \times 10^3$  cells/well) were placed at 96 well plates and treated with bupivacaine for the period of the duration of 3 days. MTT assay was performed as described by Xiao et al. MTT (50  $\mu\text{l}$ ) was added to the well plate and keep it for incubation for 4 hr. at 37°C. Thereafter from the reaction mixture supernatant was

washed out and subsequently dimethylsulfoxide (200  $\mu$ l) was added to all the well plate at room temperature. The absorbance was estimated at 570 nm wavelength [16].

### CCK-8 assay

Neuronal cells were placed ( $5 \times 10^3$  cells/well) in 96-well plates. Cell plates were treated with suramin 25, 50 and 100  $\mu$ mol/l for the duration of 6 hr, thereafter media was treated with 1 mmol/l bupivacaine for the duration of 1 day. CCK8 was poured in to each well plate for the period of 3 hr and optical density of the plate was read at a wavelength of 450 nm by spectrophotometric analysis [17].

### Apoptosis assay

Apoptosis of cell was assessed by apoptosis assay in which SH-SY5Y cell lines ( $1 \times 10^6$  cell/well) placed for 1 day in 6 well plates. Cell plates were treated with suramin 25, 50 and 100  $\mu$ mol/l and bupivacaine for the period of the duration of 2 days. Apoptosis of cells were estimated as per the instruction given by manufacturer (BD biosciences) by using apoptosis detection kit. In all the well plate Annexin V-FITC of 5  $\mu$ l was added and kept it for 10 min at 37°C for incubation and thereafter Propidium Iodide (PI) at a volume of 5  $\mu$ l was added to it. Flow cytometer was used for the estimation of cell apoptosis [18].

### Estimation of reactive oxygen species

SH-SY5Y cell lines ( $1 \times 10^6$  cell/well) were placed for 1 day in 6 well plates and separated into four different treatment groups such as control, bupivacaine (1 mmol/l) treated group, suramin (100  $\mu$ mol/l) and suramin (100  $\mu$ mol/l) for 6 hr subsequently bupivacaine (1 mmol/l) for 1 day. ROS accumulation was estimated by DCFH DA fluorescent dye. Cells of all the groups were incubated with DCFH DA for the duration of 20 min at 37°C and then cells were washed with PBS for 3 times. After washing all the cells were resuspended with PBS and flow cytometer used for the estimation of ROS accumulation [17].

### Caspase-3 and Caspase-9 activation assay

SH-SY5Y cell lines ( $1 \times 10^6$  cell/well) were placed for 1 day in 6 well plates and separated into four different treatment groups such as control, bupivacaine (1 mmol/l) treated group, suramin (100  $\mu$ mol/l) and suramin (100  $\mu$ mol/l) for 6 hr subsequently bupivacaine (1mmol/l) for 1 day. Activity of Caspase 3 and 9 was estimated by as per the instructions of Caspase 3 and 9 kit (Tiangen Biotech). All the cells were incubated for 30 min by keeping it with cell lysis buffer. BCA protein reagent was used for the estimation of concentration of protein. Estimation of Caspase 3 and 9 was achieved using micro plate spectrophotometer (Bio Rad Lab., CA, USA) at a wavelength of 405 nm [16].

### Transmission electron microscopy (TEM)

Neuronal cells were treated in the same way as described in ROS measurement and after washing with PBS and fixed by

using 2.5% glutaraldehyde for the duration of 1 hr at 4C and lead uranium was used for the staining of it.

Transmission electron microscope was used for the observation of ultrastructure of cell [17].

### Statistical analysis

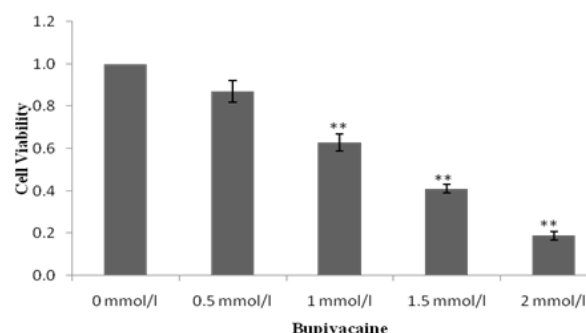
All the values of these experiments were articulated as mean  $\pm$  SD and the data was statistically analysed by one-way ANOVA and thereafter applied to Dunnett post hoc test.  $p < 0.05$  was considered significant statistically.

## Result

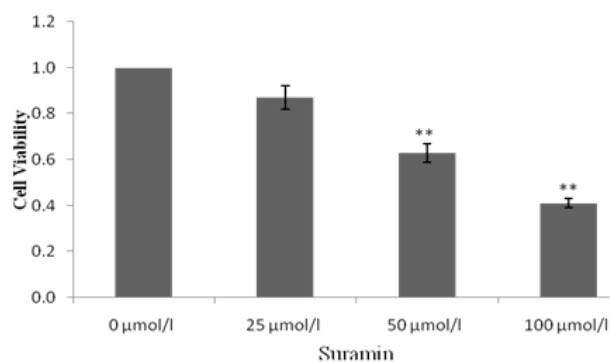
### Estimation of toxic effect of bupivacaine on SH-SY5Y neuronal cell line

MTT assay was used for the estimation of toxic effect of bupivacaine on SH-SY5Y neuronal cell line as shown in Figure 1. It was observed that bupivacaine at dosage 1, 1.5 and 2 mmol/l significantly decreases the viable cell compared to control (0  $\mu$ mol/l).

LD<sub>50</sub> of bupivacaine was found to be 1 mmol/l same as that of previous study and therefore it was at this dose used for the induction of cell death.



**Figure 1.** Cytotoxic effect of bupivacaine on SH-SY5Y neuronal cell line by MTT assay. Values are mean  $\pm$  SD ( $n=6$ ), \*\* $p < 0.01$  vs. control group.



**Figure 2.** Effect of suramin on cellular toxicity induced by bupivacaine by CCK8 assay on SH-SY5Y neuronal cell line. Values are mean  $\pm$  SD ( $n=6$ ), \*\* $p < 0.01$  vs. control group.

**Estimation of effect of suramin on cellular toxicity induced by bupivacaine on SH-SY5Y neuronal cell line**

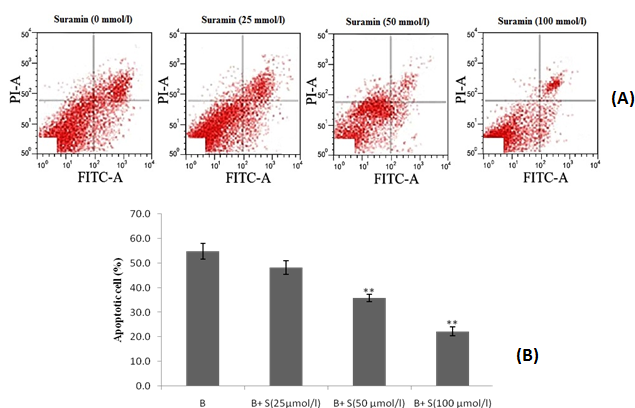
Figure 2 shows the effect of suramin on cellular toxicity induced by bupivacaine by using CCK 8 assay. Treatment with suramin at 50 and 100 µmol/l concentration significantly ( $p < 0.01$ ) attenuates the cytotoxicity induced by local anaesthetic (bupivacaine).

It was observed that suramin shows the cytoprotective activity in a concentration dependent manner.

**Estimation of effect of suramin on apoptosis induced by bupivacaine on SH-SY5Y neuronal cell line**

Effect of suramin on bupivacaine induced apoptosis by using Annexin V-FITC detection kit. There was decrease in bupivacaine induced apoptosis of neuronal cell by suramin treatment as compared to control group as shown in Figure 3A.

Statistical representation also suggested that treatment with suramin significantly ( $p < 0.01$ ) decreases the percentage of apoptosis cell induced by bupivacaine compared to control group and these are in a dose dependent manner as shown in Figure 3B.

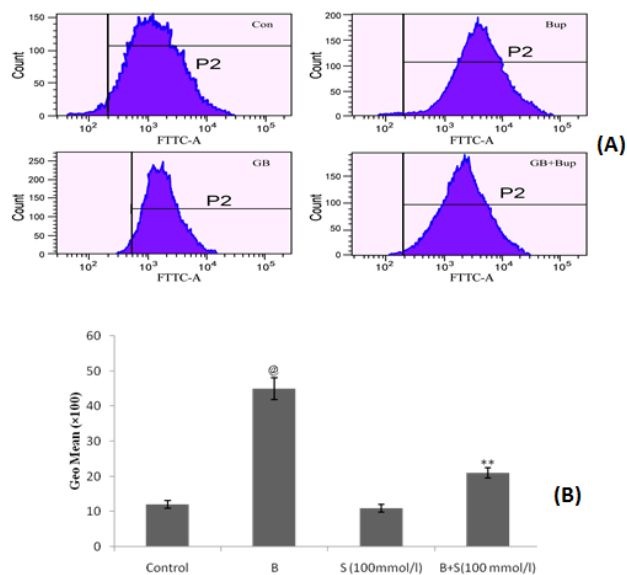


**Figure 3.** Effect of Suramin on Apoptosis Induced by Bupivacaine on SH-SY5Y neuronal cell line. (A). Annexin V-FITC detection. (B). Summarized data. Values are mean ± SD (n=6), \*\* $p < 0.01$  vs. control group.

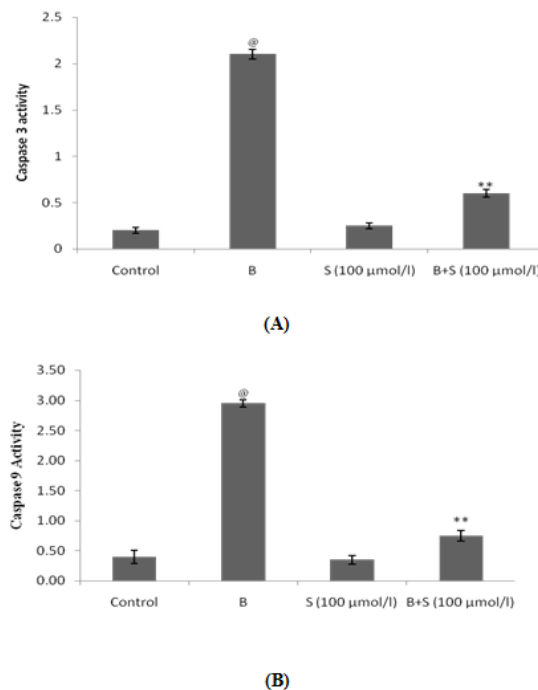
**Estimation of effect of suramin on ROS Production in SH-SY5Y neuronal cell line**

Figure 4 shows the effect of suramin in ROS production in SH-SY5Y neuronal cells by flow cytometer. It was observed that treatment with suramin significantly decreases the accumulation of ROS which was increased by bupivacaine treatment. Accumulation of ROS was estimated by DCFH DA fluorescent signals as shown in Figure 4A.

These results demonstrated that suramin protects the mitochondrial function by reducing the ROS production and increasing the antioxidant capacity.



**Figure 4.** Effect of suramin on ROS Production in SH-SY5Y neuronal cell line. (A). DCFH DA fluorescent signals. (B). Summarized data. Values are mean ± SD (n=6), @ $p < 0.01$  vs. control, \*\* $p < 0.01$  vs. B (Bupivacaine treated).



**Figure 5.** Effect of suramin on activity of caspase 3 and 9 in SH-SY5Y neuronal cell line. (A). Activity of Caspase 3 and (B). Activity of Caspase 9. Values are mean ± SD (n=6), \*\* $p < 0.01$  vs. control group.

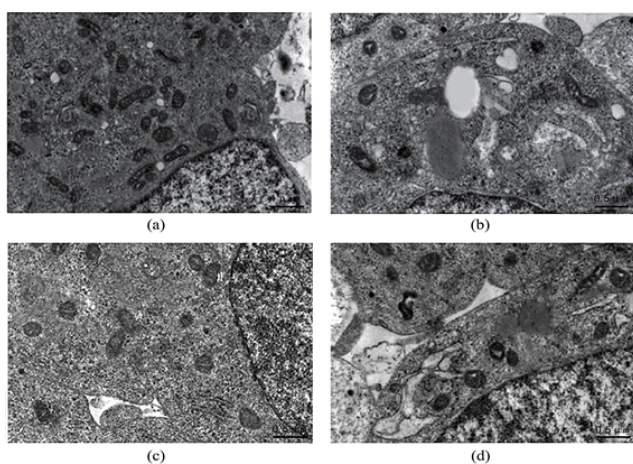
**Estimation of effect of suramin on activity of Caspase 3 and 9 in SH-SY5Y neuronal cell line**

Treatment with bupivacaine significantly ( $p < 0.01$ ) increases the activity of Caspase 3 and 9 protein compared to control group. Whereas, treatment with suramin significantly decreases the activity of Caspase 3 and 9 in SH-SY5Y neuronal cell line

compared to bupivacaine alone treated group as shown in Figure 5.

### **Estimation of structural changes in SH-SY5Y neuronal cell line by TEM**

Normal cellular structure of SHSY5Y cells were normal regular and endoplasmic reticulum and mitochondrial shape as in TEM image of Figure 6a. Whereas bupivacaine treated group shows swollen endoplasmic reticulum and mitochondria as shown in Figure 6b. Treatment with sumarin appears no changes in the normal structure of cell and its organelles as shown in Figure 6c. Moreover, sumarin attenuates the morphological changes induced by bupivacaine treatment as only slightly swollen in the endoplasmic reticulum as appears in Figure 6d.



**Figure 6.** Effect of sumarin on morphological changes. (a). Control group. (b). Bupivacaine (1 mmol/l) treated group. (c) Sumarin (100  $\mu$ mol/l) treated group. (d). Bupivacaine (1 mmol/l) + Sumarin (100  $\mu$ mol/l) treated group.

### **Discussion**

In this study P2X<sub>4</sub> receptor antagonist i.e. sumarin attenuates the neuronal injury induced by local anaesthetic (bupivacaine). Sumarin protects the neuronal cell by decreasing the apoptosis, ROS accumulation, Caspase 3 and 9 activity and morphological changes induced by bupivacaine in SHSY5Y neuronal cells.

Local anaesthetic drugs show neurotoxicity by inducing apoptosis. Apoptosis of cell was activated by extrinsic and intrinsic pathway and bupivacaine induces apoptosis of neuronal cell by activating both the pathway [19]. Excessive ROS production and accumulation increases the oxidative stress and thereby activates apoptosis [20]. Bupivacaine increases the ROS production in the neuronal cell line and treatment with sumarin significantly ( $p < 0.01$ ) decreases the ROS accumulation. Thus sumarin effectively decreases the apoptosis of neuronal cell induced by bupivacaine.

Literature also suggested that local anaesthetic activates Caspase and thereby stimulate the cell death through activation of apoptosis [4]. Drugs decreases the Caspase activity

attenuates the process of apoptosis effectively [21]. This study suggests that sumarin effectively decreases the activity of Caspase 3 and 9 in SHSY5Y neuronal cells. Even TEM study also demonstrated the protective effect of sumarin on morphological characters of mitochondria and endoplasmic reticulum of bupivacaine induced neurotoxicity in cell.

### **Conclusion**

Present investigation concludes the neuroprotective effect of P2X<sub>4</sub> receptor antagonist in local anaesthetic induced neurotoxicity. It also postulates the possible mechanism of action of sumarin as it decreases the apoptosis of neuronal cells by reducing ROS accumulation, decreasing Caspase 3 and 9 activity and protecting the morphological changes.

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