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

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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.

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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 1.5×10^5 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 (*, $p < 0.05$).

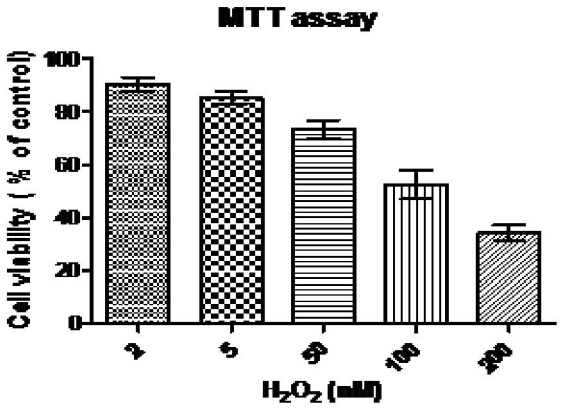

Figure 1: The dose dependent effect of H_2O_2 (2, 5, 50, 100, and 200 nM) on cell proliferation after 24 h.

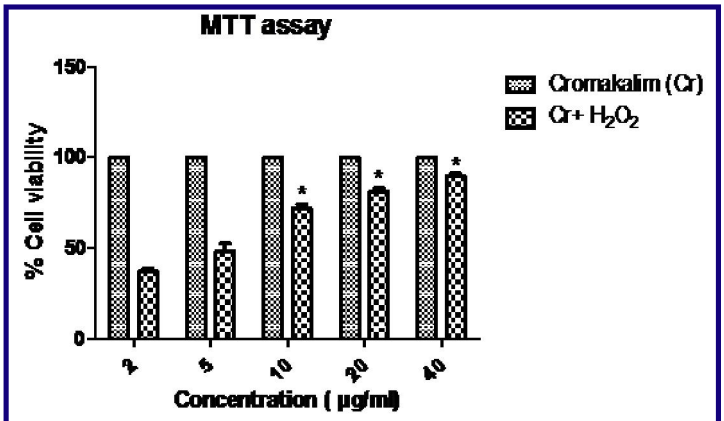

Figure 2: The dose-dependent effect of cromakalim (2, 5, 10, 20, and 40 µg) alone and against H_2O_2 induced changes on cell proliferation.

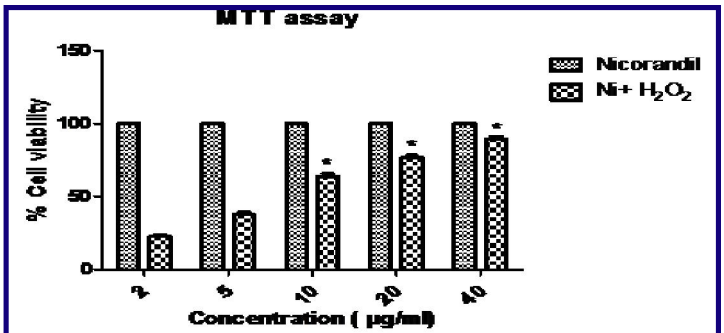

Figure 3: The dose-dependent effect of nicorandil (2, 5, 10, 20, and 40 µg) alone and against H_2O_2 induced changes on cell proliferation.

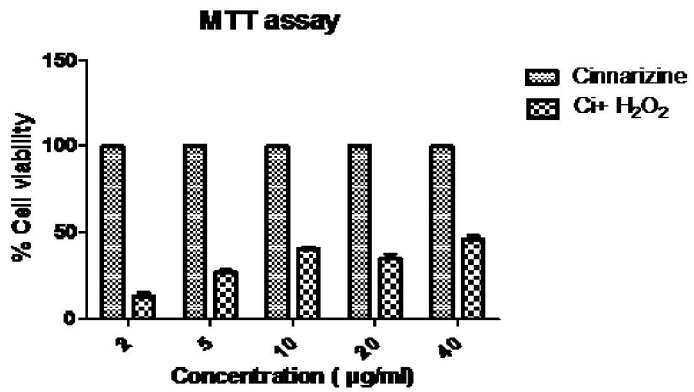

Figure 4: The dose-dependent effect of cinnarizine (2, 5, 10, 20, and 40 µg) alone and against H_2O_2 induced changes on cell proliferation.

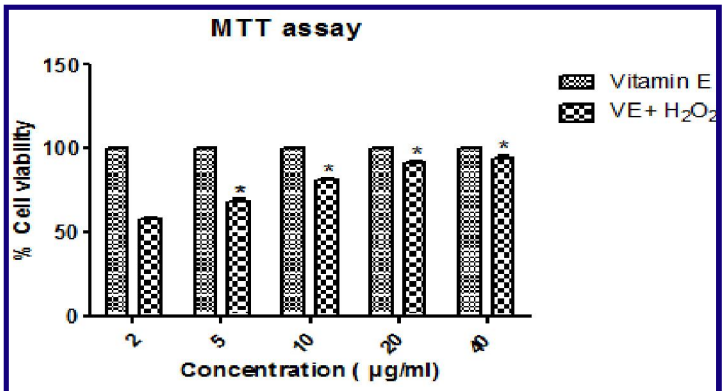

Figure 5: The dose-dependent effect of vitamin E (2, 5, 10, 20, and 40 µg) alone and against H_2O_2 induced changes on cell proliferation.

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

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