Original Research Paper

Pinacidil and levamisole prevent glutamate-induced death of hippocampal neuronal cells through reducing ROS production

Mustafa Shukry[1], Tarek Kamal[2], Radi Ali[3], Foad Farrag[4], Essam Almadaly[5], Ayman A. Saleh[6], Mohammed Abu El-Magd[4]

[1]Department of Physiology, Faculty of Veterinary Medicine, Kafrelsheikh University, Egypt, [2]Department of Biochemistry, Faculty of Veterinary Medicine, Kafrelsheikh University, Egypt, [3]Department of Hygiene and Preventive Medicine, Faculty of Veterinary Medicine, Kafrelsheikh University, Egypt, [4]Department of Anatomy and Embryology, Faculty of Veterinary Medicine, Kafrelsheikh University, Egypt, [5]Department of Theriogenology, Faculty of Veterinary Medicine, Kafrelsheikh University, Egypt, [6]Department of Animal Wealth Development, Genetics & Genetic Engineering, Faculty of Veterinary Medicine, Zagazig University, Egypt

Activators of both adenosine 5′-triphosphate (ATP)-sensitive K^+ (K_{ATP}) channel and cystic fibrosis transmembrane conductance regulator (CFTR) Cl^- channel have significant *in vivo* and *in vitro* neuroprotection against glutamate-induced death of some neuronal cells. Here, the effect of the K_{ATP} channel activator, pinacidil, and the CFTR Cl^- channel opener, levamisole, against glutamate-induced oxidative stress were investigated in mouse hippocampal cells, HT22. The results from cell viability assay (WST-1) showed that pinacidil and levamisole weakly protected cells against glutamate-induced toxicity at 10 μM and their effect increased in a dose-dependent manner till reach maximum protection at 300 μM. Pretreatment with pinacidil or levamisole significantly suppressed the elevation of reactive oxygen species (ROS) triggered by glutamate through stabilising mitochondrial membrane potential and subsequently protected HT22 cells against glutamate-induced death. HT22 cells viability was maintained by pinacidil and levamisole in presence of glutathione inhibitor, BSO. Also, pinacidil and levamisole pretreatment did not induce recovery of glutathione levels decreased by glutamate. Expectedly, this protection was abolished by the K_{ATP} and CFTR Cl^- channels blocker, glibenclamide. Thus, both pinacidil and levamisole protect HT22 cells against glutamate-induced cell death through stabilising mitochondrial membrane potential and subsequently decreasing ROS production.

Keywords: K_{ATP}, CFTR Cl^-, Pinacidil, Levamisole, Glutathione, ROS, HT22

Introduction

Adenosine 5′-triphosphate (ATP)-sensitive potassium (K^+), K_{ATP}, channels are ligand-gated K^+ channels that couple membrane excitability to the metabolic state of the cell. They are the main targets of K^+ channel openers (KCOs). Several different compounds, including diazoxide, cromakalim and pinacidil, have the ability to activate K_{ATP} channels.[1,2] K_{ATP} channels are present in a wide variety of cells such as myocytes, pancreatic beta cells, and neurons.[3] K_{ATP} channels are involved in the relaxation of smooth muscle induced by H_2S.[4] K^+ channel openers protect cardiac myocytes against ischaemic injury[5] and serve as metabolic sensors in the cascade linking insulin secretion to hyperglycaemias[6]

In nervous tissues, K_{ATP} channels regulate the release of neurotransmitters and are involved in protection against glutamate excitotoxicity *in vivo*[7] and in cultured hippocampal neurons.[8] In addition, K_{ATP} channels play an important role in enhancing the resistance of the retina against ischaemic insult[9] and are involved in an endogenous protective mechanism against ischaemia-reperfusion injury in the brain.[10] Subsequently, some KCOs such as diazoxide and iptakalim show neuroprotective effects in brain ischaemia[11] This protective effect mainly functions by the opening of K_{ATP} channels, which hyperpolarises the plasma membrane and reduces the influx of calcium ions. This protective activity is suppressed by K_{ATP}-channel blockers such as glibenclamide.[12]

Correspondence to: Mustafa Shukry, Department of Physiology, Faculty of Veterinary Medicine, Kafrelsheikh University, Egypt. Email: Mostafa_shukry2002@yahoo.com, Mohammed Abu El-Magd, Department of Anatomy and Embryology, Faculty of Veterinary Medicine, Kafrelsheikh University, Egypt, Email: mohrizk73@yahoo.com

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Cystic fibrosis transmembrane conductance regulator (CFTR) is an ABC transporter-class ion channel that transports chloride and thiocyanate ions across epithelial cell membranes.[13] CFTR Cl^- channels and sulphonylurea receptors, a component of K_{ATP} channels, belong to the ATP-binding cassette superfamily, and are widely expressed in various organs including the brain.[14] Levamisole, an opener of CFTR Cl^- channels, protects neuronal cells against death in a dose-dependent manner.[15,16] Both 5-nitro-2-(3-phenylpropylamino) benzoic acid (NPPB) and indyanyl oxyacetic acid (IAA-94) are specific CFTR Cl^- channel blockers.[17,18] This study was conducted to check the possible neuroprotective effect of levamisole and pinacidil and how they can exert this potential action.

Materials and Methods

Chemical and reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise indicated.

Cell viability assay (WST-1 Assay)

Cell viability was determined with 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1, 3- benzene disulphonate (WST-1) using a Cell Counting Kit (Dojindo Laboratories, Kumamoto, Japan). HT22 cells were grown in Dulbecco's modified Eagle's medium (DMEM) (low glucose, Wako Pure Chemicals, Osaka, Japan) supplemented with 10% foetal bovine serum and 1% penicillin-streptomycin (FBS, Equitech-Bio, Inc., Kerrville, TX, USA) at 37°C in 5% CO2. After the treatment of the cells under various experimental conditions, WST-1 was added to the culture at a final concentration of 500 nM, and the plates were re-incubated for 2–4 hours. The absorbance was photometrically measured at 450 nm using a reference wavelength of 690 nm.

Cell death assay (LDH Assay)

The HT22 cells were plated at 10^4 cells/ml in 96 well micro titre plates. After overnight incubation, the plates were incubated in low serum (DMEM supplemented with 0.5% FBS) medium for 48 hours before treatment. Since FBS contains LDH, which increases background absorbance, 16 hours after the treatment the microlitre plate was centrifuged at 250 x g for 10 minutes, and the cell supernatant was used for the LDH assay (Roche Diagnostics Japan, Tokyo, Japan). Cell supernatant (100 µl) was mixed with reaction mixture (100 µl) containing NAD+, iodotetrazolium chloride and sodium lactate, and incubated in dark for 30 minutes in shaker. The amount of formazan salt formed by LDH was measured at 490 nm. Percentage of cell death was calculated according to the instruction manual. Three controls were used: background control (DMEM supplemented with 0.5% FBS only), high control (cell lysed with X-100 Triton, final 8% as triton destroyed the cell which further releases the maximum LDH content to the surrounding medium), and low control (untreated HT22 cells used to determine the spontaneous LDH release).

GSH measurement

HT22 cells were grown on 6-well plates. Cells were collected with ice-cold phosphate-buffered saline (PBS). The cell pellet was resuspended in 120 µl of 0.1M sodium phosphate buffer (pH 8.0) containing 5 mM EDTA, and deproteinized by adding 4 volumes of 25% (*w*/*v*) metaphosphoric acid. The sample was centrifuged for 10 minutes at 14 000 rpm. The resultant supernatant was used for GSH assay using commercial kit OxiSelect™ Total Glutathione (GSSG/GSH) Assay Kit following the instruction of the manufacturer. Briefly, the supernatant (5 µl), 185 µl of 0.1M sodium phosphate buffer (pH 8.0) containing 5 mM EDTA and 10 µl of o-phthaldi aldehyde solution (1 mg/ml in methanol) were added to a 96-well black microplate and incubated at room temperature for 15 minutes. Fluorescence intensity was measured at 420 nm with excitation at 350 nm using a microplate reader (Vaioskan Flash, Thermo Fisher Scientific, Waltham, MA, USA). The resultant pellet was solubilised in 75 µl 0.2M NaOH and used for protein assay. GSH was normalised to cellular protein measured by the DC Protein Assay (Bio-Rad Laboratories, Hercules, CA, USA) using gamma-globulins as a standard.

Measurement of ROS production

HT22 cells were cultured on at a density of 2×10^5 cells/well in poly-D-lysine coated glass bottom 6-well plates (Sigma) with serum-free DMEM without phenol red containing 5 µM 5-(and-6)- chloromethyl-2′, 7′-dichlorodihydrofluorescein diacetate, acetyl ester (CM- H2DCFDA) (Molecular Probes, Eugene, OR, USA) for 20 min in a CO_2 incubator. The medium was replaced by serum-free DMEM without phenol red. The fluorescence of DCF was observed with fluorescence digital microscopy (VB-7000, Keyence, and Osaka, Japan). The intensity of fluorescence was quantified using Keyence image measurement and analysing software (VH-H1A5).

Measurement of mitochondrial membrane potential

This was assessed using a live cell assay with the fluorescent lipophilic cationic dye TMRE (tetramethylrhodamine ethylester) (Molecular Probes, Eugene, OR, USA). This dye is positively charged, which accumulates in active mitochondria.[19] After different treatment with pinacidil (300 µM), levamisole (300 µM) and/or glutamate(10 mM) for 24 hours, HT22 cells

were stained with 200 nM TMRE for 30 minutes at 37°C, then washed for three times in medium and re-suspended in PBS. Some samples were incubated with carbonyl cyanide m-chlorophenylhydrazone (CCCP, 10 μM) an uncoupler of electron transport and oxidative phosphorylation was used as a positive control for depolarised mitochondria, for 10 minutes prior to staining with TMRE. The TMRE fluorescence intensity was then measured with excitation at 549 nm and emission at 574 nm using a fluorometer (Tecan, Genios, Maennedort, Switzerland).

Statistical analysis

Data were statistically analysed with GraphPad Prism 5 (GraphPad Software, Inc., LaJolla, CA, USA). The significance of differences between experimental groups was determined by Tukey's multiple comparison tests following ANOVA.

Result

Pinacidil and levamisole prevent glutamate-induced death

To determine the optimal concentration of K_{ATP} channel opener pinacidil and CFTR Cl^- channel opener levamisole required to prevent glutamate-induced cell death, we incubated HT22 cells with glutamate 10 mM alone (control positive) or in combination with either pinacidil or levamisole at concentrations of 10, 30, 60, 120, 300 μM. HT22 cells without any treatment were used as a negative control. WST-1 assay results showed that HT22 cells subjected to 10 mM glutamate (alone) for 24 hours die, while when used in combination with pinacidil or levamisole the majority of the cells remain alive depending on the concentration of pinacidil and levamisole (Fig. 1A-F). Pinacidil and levamisole weakly protected cells against glutamate-induced toxicity at 10 μM and their effect increased in a dose-dependent manner till reach maximum protection at 300 μM with only 5 and 8% of HT22 cells underwent cell death, respectively (Fig. 1E, F). This means that pinacidil and levamisole at 300 μM protects HT22 cells against death induced by 10 mM glutamate. Because 300 μM pinacidil and levamisole showed greater protection than 120 μM, 300 μM was used in the further experiments in this study.

The results of LDH assay support that obtained by WST-1 assay and showed that pinacidil and levamisole at a concentration of 300 μM protect HT22 cells from cell death induced by glutamate (Fig. 2A).

On the other hand, application of 50 μM of the K_{ATP} channels blocker glibenclamide either alone or in combination with 300 μM pinacidil, significantly increased the death rate of HT22 cells (Fig. 3). Similar results were obtained after application of 15 μM of the CFTR Cl^- channels blocker IAA-94 (indyanyl oxyacetic acid-94) either alone or combined with 300 μM levamisole (Fig. 3). This supports the neuroprotective effect of pinacidil and levamisole and indicates that this protection involves K_{ATP} and CFTR Cl^- channels.

Pinacidil and levamisole prevent glutamate-induced death through reduction of ROS production

The exposure of HT22 cells to glutamate induces depletion of glutathione and increases the ROS production, however the majority of the ROS is not simply a by-product of GSH depletion.[20] There are many possible sources of ROS, including mitochondria and a wide array of enzymes such as the monoamine oxidases, tyrosine hydroxylase, l-amino oxidase, the lipoxygenases, cyclo-oxygenase and xanthine oxidase.[21] To check whether the neuroprotective mechanism of pinacidil and levamisole involved change in production of glutathione and/or ROS, glutathione level was measured following addition of either 10 mM glutamate alone or in combination with 300 μM pinacidil or levamisole. Glutathione levels decreased by 10 mM glutamate. However, this glutamate-induced glutathione depletion was not improved after addition of pinacidil or levamisole (Fig. 2B). This indicates that the neuroprotective effect of pinacidil and levamisole is not associated with glutathione recovery. In contrast, 300 μM pinacidil and levamisole co-treated with glutamate group suppressed glutamate-induced ROS production in HT22 cells (Fig. 4A–E).

Role of GSH in levamisole and pinacidil protection

Levamisole and pinacidil protect the HT22 cells from glutamate-induced oxytosis, for further investigation of the GSH role in this protection, HT22 cells were treated with L-buthionine sulphoximine (BSO 1 mM), which inhibits gamma glutamyl cysteine synthetase and consequently inhibits GSH synthesis. HT22 cells were treated with BSO 1 mM in presence or absence of levamisole and pinacidil and subsequently cell viability and GSH level were measured. Pinacidil and levamisole can maintain the viability of HT22 cells in presence of BSO but addition of pinacidil or levamisole did not improve BSO-induced glutathione depletion (Fig. 5).

Effects of pinacidil and levamisole on mitochondrial membrane potential

Mitochondrial membrane potential measured by TMRM significantly decreased in glutamate-treated HT22 cells as compared to that of control cells (Fig. 6). Otherwise, HT22 cells pre-incubated with pinacidil and levamisole prevented the depolarisation

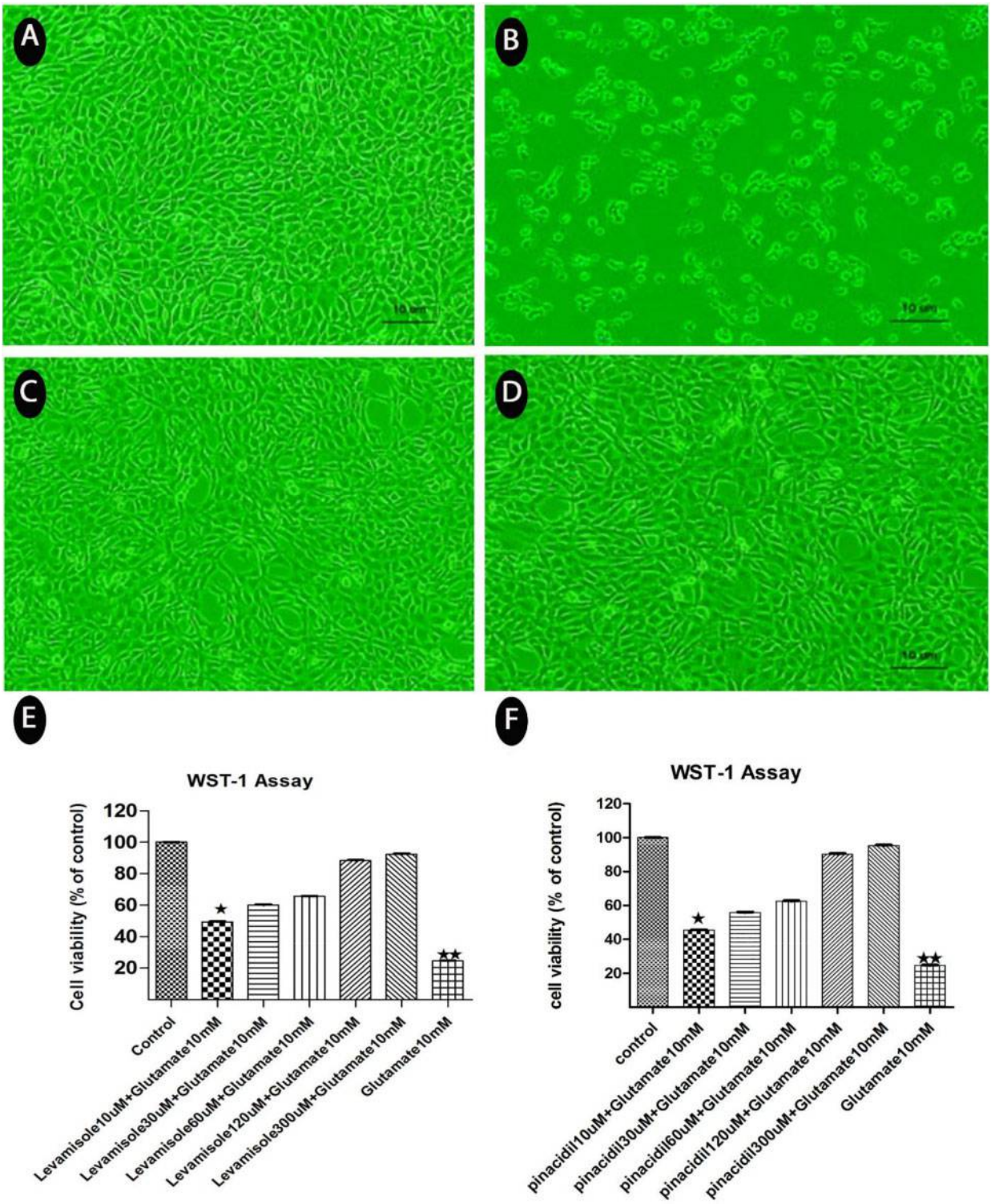

Figure 1 Effects of pinacidil and levamisole on cell viability and glutamate-induced cell death in HT22 cells. Phase-contrast and fluorescence microphotographs of (A) HT22 cells without any treatment were used as a negative control. (B) HT22 cells treated with glutamate10 μM. (C) HT22 cells treated with pinacidil at a concentration of 300 μM with glutamate10 μM. (D) HT22 cells treated with levamisole at a concentration of 300 μM with glutamate10 μM. (E) Levamisole inhibits glutamate-induced cell death. HT22 cells were cultured with various concentrations of levamisole (10, 30, 60, 120, 300 μM) for 24 hours. (F) Pinacidil prevents glutamate-induced cell death. HT22 cells were cultured with various concentrations of pinacidil (10, 30, 60, 120, 300 μM) for 24 hours. Cell viability was determined by WST-1 assay. Data are means ± SD (n = 8). *P< 0.05, **P< 0.01 for difference from control.

of mitochondrial membrane potential caused by glutamate. This confirms that pinacidil and levamisole prevent glutamate-induced death of HT22 cells through reducing ROS production.

Discussion

Oxytosis, programmed cell death due to oxidative stress, has been studied in mouse hippocampal cell line, HT22[20,22] and primary cultures of cortical neurons.[23]

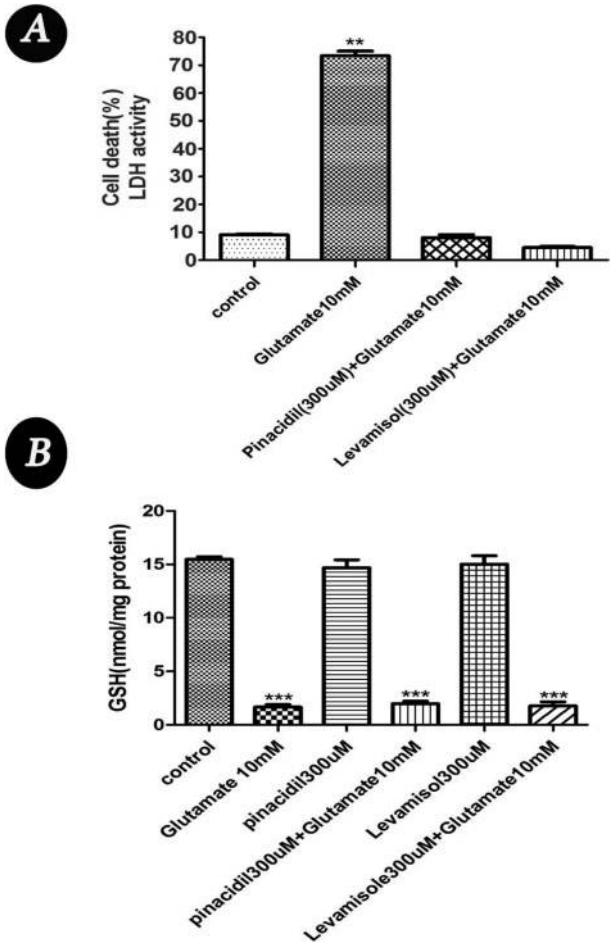

Figure 2 (A) Analysis of LDH activity confirms the inhibitory effect of pinacidil and levamisole on glutamate-induced cell death. Data are means ± SD (n = 8). **P< 0.01 for difference from glutamate-alone treated group. (B) Effects of pinacidil and levamisole on glutathione levels in glutamate-treated HT22 cells. Addition of pinacidil or levamisole did not improve glutamate-induced glutathione depletion. Data are means ± SD (n = 6). ***P< 0.001 for difference from control.

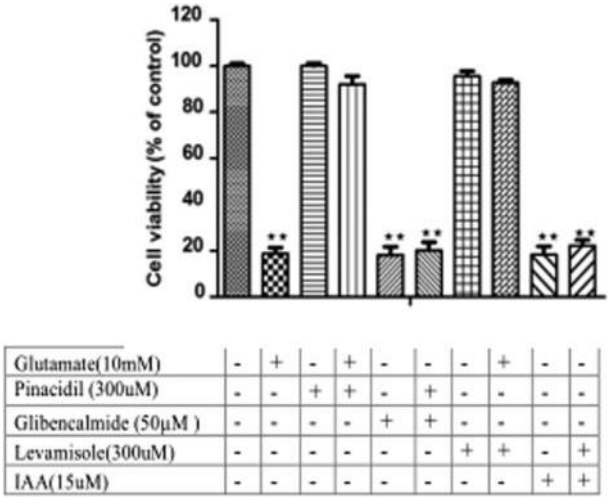

Figure 3 The effect of the K_{ATP} channels blocker glibencalmide (50 μM) and the CFTR Cl^- channels blocker IAA-94 (15 μM), either alone or in combination with 300 μM pinacidil (alone or with glutamate) or levamisole (alone or with glutamate), on cell viability of HT22 (as determined by WST-1 assay). Data are means ± SD (n = 8). **P< 0.01 for difference from control.

In addition, K_{ATP} channels are highly expressed in neurons of hippocampus,[24,25] and expression of CFTR Cl^- is greater in hippocampus than in cortex.[14] HT22 cells also lack functional ionotropic glutamate receptors, therefore, serve as an excellent model of glutamate-induced oxidative neurotoxicity. We therefore used HT22 cells in this study to investigate the neuroprotective role of the K_{ATP} channel opener pinacidil and CFTR Cl^- channel opener levamisole against oxidative stress.

Previous studies have investigated that the K_{ATP} channel openers protect neuron against death and reduce abnormal excitatory synaptic activity.[8] Here, we found that the activation of K_{ATP} channels mediates pinacidil-induced neuroprotection for HT22 cells. This conclusion is based on the findings that pinacidil suppresses glutamate toxicity and the K_{ATP} blocker glibenclamide suppresses the pinacidil-induced neuroprotection. Moreover, CFTR Cl^- channels are abundant in the cerebral cortex, hippocampus and HT22 cells.[14,16,26] These transmembrane channels are necessary to maintain normal cell survival in hippocampal neurons.[27] CFTR Cl^- channel opener levamisole supports HT22 cells against oxytosis induced by glutamate.[16] In accordance, we also found a similar neuroprotective effect for levamisole. In supportive, the CFTR Cl^- channel blocker IAA-94 suppresses protection by levamisole (this study) and H2S.[16] This confirms that CFTR Cl^- channels are also involved in protection by levamisole and H2S against oxytosis.

Glutamate induces HT22 mouse hippocampal cell death exclusively through the oxytotic pathway as these cells do not express functional ionotropic receptors.[28] Glutamate-induced cell death is produced by the blockade of the cystine/glutamate uptake, which causes the progressive depletion of glutathione.[29] Glutathione is the major antioxidant within neurons which protects cells against damage initiated by oxidative stress. High levels of extracellular glutamate blocks cystine entrance into neuronal cells which leads to loss of intracellular cysteine.[21,30] Subsequently, the intracellular glutathione becomes depleted and cells die as a result of severe oxidative stress. However, changes in glutathione alone are insufficient for complete neuronal protection. Previous studies have shown that in both primary cultures of cortical neurons[23] and HT22 cells,[16] H2S protects cells from oxytosis mainly by increasing glutathione levels inside the cells. In cortical neurons, H2S enhances the activity of γ-glutamylcysteine synthetase and cystine transporter activity to increase intracellular cysteine and

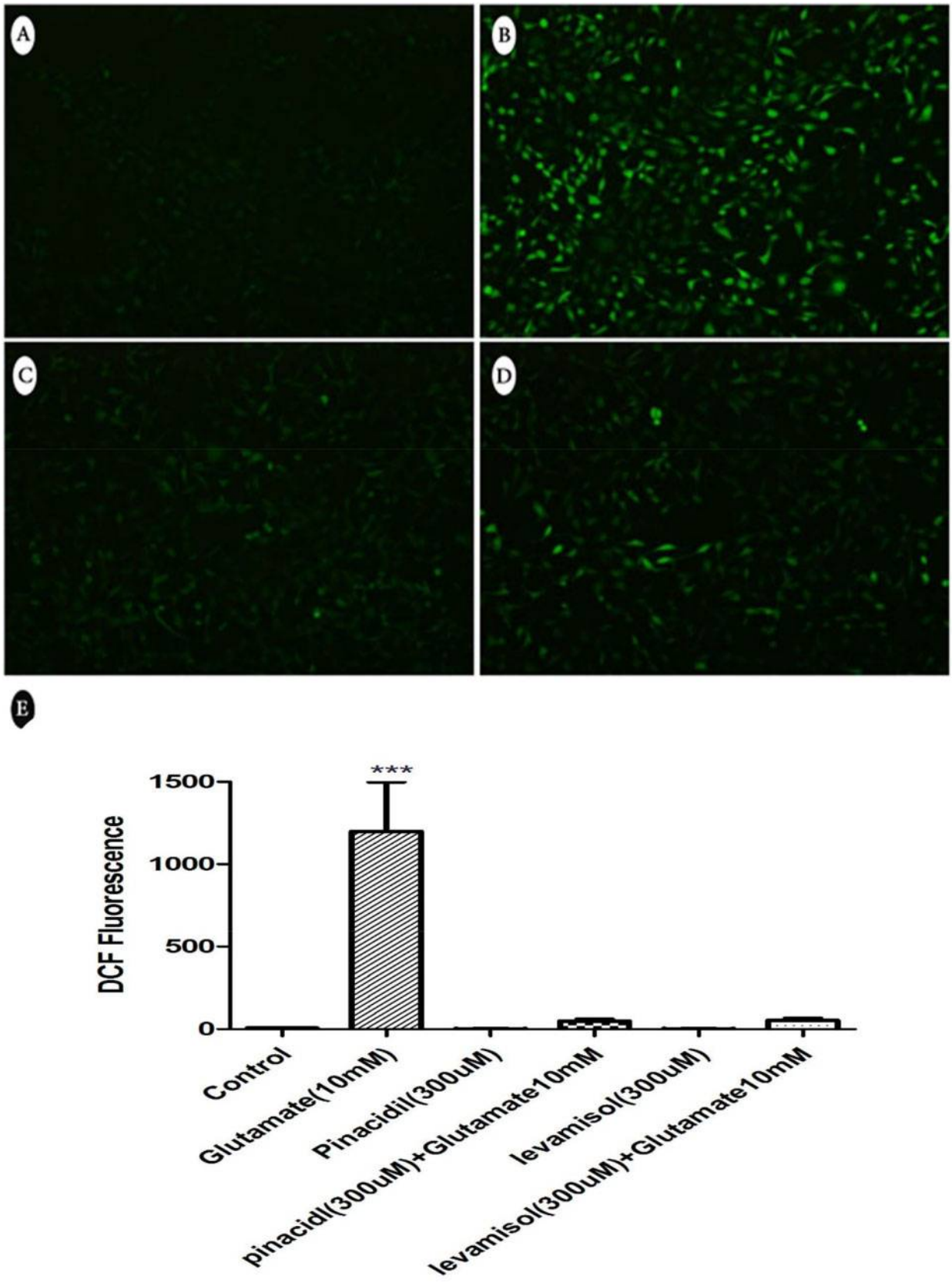

Figure 4 Effects of pinacidil and levamisole on ROS levels in glutamate-treated HT22 cells. Phase-contrast and fluorescence microphotographs of glutamate and/or pinacidil and levamisole-treated HT22 cells. (A, control) (B, HT22 cells treated with glutamate10 μM), (C and D) Addition of pinacidil or levamisole, respectively, led to significant reduction in ROS levels in glutamate-treated HT22 cells. (E) Levels both cytosolic and mitochondrial ROS were measured using CM-H2DCFDA. Fluorescence intensity was quantified using Keyence image measurement and analysing software (VH-H1A5). Data are means ± SD ($n = 6$). *** $P < 0.001$ for difference from control.

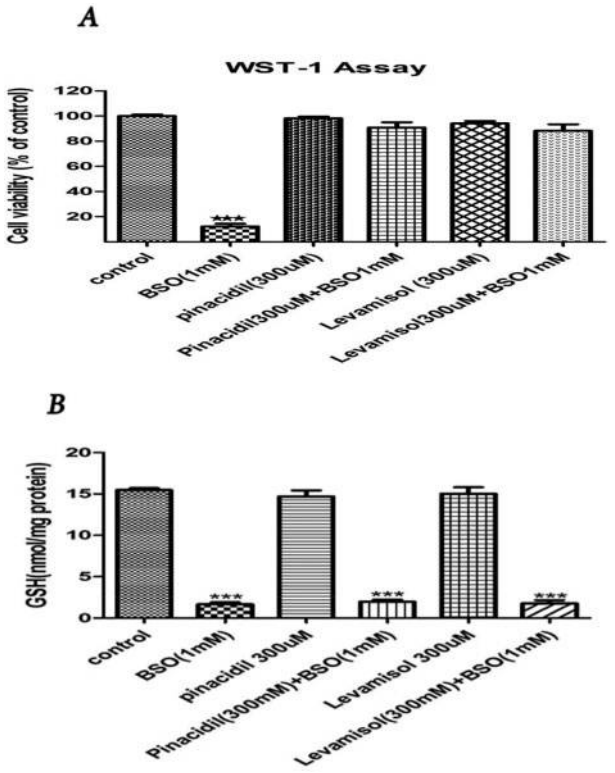

Figure 5 The effect of the buthionine sulphoximine (BSO, 1 mM), either alone or in combination with 300 μM pinacidil or levamisole for 24 hours on cell viability of HT22 (as determined by WST-1 assay). (A) Addition of pinacidil or levamisole can maintain the viability of HT22 cells in presence of BSO. (B) Effects of pinacidil and levamisole on glutathione in BSO-treated HT22 cells. Addition of pinacidil or levamisole did not improve BSO-induced glutathione depletion. Data are means ± SD (n = 6). ***P< 0.001 for difference from control.

glutathione.[23] In contrast, in the present study the basal levels of glutathione were not changed in the presence or absence of pinacidil and levamisole. In addition, these channel openers only retrieved the cell viability but not the levels of glutathione decreased by glutamate. The activation of K_{ATP} and CFTR Cl^- channels by pinacidil and levamisole may therefore be independent of the increase in the levels of

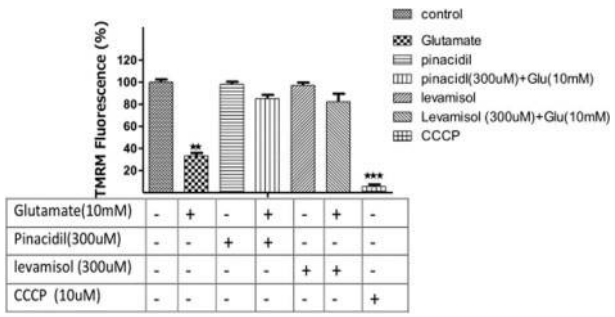

Figure 6 Effects of pinacidil and levamisole on mitochondrial membrane potential. Mitochondrial membrane potential was measured using fluorescent dye TMRE and carbonyl cyanide m-chlorophenylhydrazone (CCCP, 10 μM) was used as positive control for loss of mitochondrial membrane potential. Data are means ± SD (n = 6). **P< 0.01, ***P< 0.001 for difference from control.

glutathione and cysteine or the activation of cystine transport. This notion was supported by the results obtained after application of GSH inhibitor, BSO, which indicated that pinacidil and levamisole can maintain the viability of HT22 cells in presence of BSO. Thus, addition of pinacidil or levamisole did not improve BSO-induced glutathione depletion.

Glutamate-induced oxytosis is also characterised by exponential burst of ROS through an unknown mechanism.[31,32] ROS can be generated following cell lysis, oxidative burst, or the presence of an excess of free radicals such as H2O2.[33–35] Mitochondria are the major ROS-generating sites in mammalian cells. Blockade of complexes in the electron transport chain (ETC) increases the leakage of single electrons to O2 and therefore increases ROS levels.[36] This prompts us to assess whether the neuroprotection effect of both pinacidil and levamisole is dependent on ROS reduction. We found that glutamate-treated cells caused depolarisation of mitochondrial membrane potential. This depolarisation was inhibited following preincubation of cells with pinacidil and levamisole. These findings suggest that stabilising mitochondrial membrane potential contribute to the protective effect of both pinacidil and levamisole and that these two compounds suppress glutamate-induced ROS production in HT22 cells. Therefore, pinacidil and levamisole might directly scavenge free radicals production. Because the activity of K_{ATP} and CFTR Cl^- channels can be blocked by oxidation of thiol groups,[37,38] we suggest that pinacidil and levamisole work by reducing ROS production which in turn reduces oxidised thiol groups and decreases the production of oxidants.

It is therefore possible (after experimental verification) to use the orally administered antihypertensive drug (pinacidil) and anti-helminthic/immunomodulation drug (levamisole) as neuroprotective therapy in neurodegenerative diseases associated with oxidative injury mediated by ROS, including Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis and conditions such as ischaemia and excitotoxicity.[39] The protective actions of the K_{ATP} channels may be accompanied by prevention/attenuation of the changes in Ca homoeostasis and trigger some opioid receptors (need to be experimentally proved) and this may explain the intracellular neuroprotection pathway of this channel. We are currently investigating this hypothesis.

Conclusion

The present study reveals that pinacidil and levamisole protect HT22 cells from oxidative stress by two mechanisms; a) activation of both K_{ATP} and CFTR Cl^- channels, respectively, and b) decrease ROS overproduction induced by glutamate oxytosis through

stabilising mitochondrial membrane potential. Further investigations are needed to elucidate other possible intracellular pathways contributing in the neuroprotective mechanism of pinacidil and levamisole.

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Conflicts of interest No conflict-of-interest.

Ethics approval This work was done according to the ethical principles of Kafrelsheikh university.

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