Kafkas Univ Vet Fak Derg
25 (1): 1-8, 2019
DOI: 10.9775/kvfd.2018.19940

Kafkas Universitesi Veteriner Fakultesi Dergisi
ISSN: 1300-6045 e-ISSN: 1309-2251
Journal Home-Page: http://vetdergikafkas.org
Online Submission: http://submit.vetdergikafkas.org

Research Article

The Neuroprotective Effect of Pioglitazone on NB2a Mouse Neuroblastoma Cell Culture

Kamil VURAL [1,a] Okan SEYREK [1,b]

[1] Department of Pharmacology of Manisa Celal Bayar University, School of Medicine, TR-45030 Manisa - TURKEY
[a] ORCID: 0000-0002-7975-1266; [b] ORCID: 0000-0003-2253-7987

Article ID: KVFD-2018-19940 Received: 04.05.2018 Accepted: 27.10.2018 Published Online: 06.11.2018

How to Cite This Article

Vural K, Seyrek O: The neuroprotective effect of pioglitazone on NB2a mouse neuroblastoma cell culture. *Kafkas Univ Vet Fak Derg,* 25 (1): 1-8, 2019. DOI: 10.9775/kvfd.2018.19940

Abstract

Pioglitazone (PGT) is a PPAR-γ activator that has neuroprotective properties via different mechanisms. It is thought to be neuroprotective in both acute and chronic use. Chlorpyrifos (CPS) is an Organophosphate insecticide that leads to attention deficit and cognitive problems in children and its neurotoxic effects are well known. This study aims to investigate the neuroprotective effects of PGT on CPS neurotoxicity in NB2a cell in the culture medium. We investigated the cell viability and proliferation using MTT assay and the percentage of neurite inhibition was analysed by measuring neurite outgrowth. Apoptosis was evaluated using the apoptotic index in TUNEL staining. Cell proliferation was found to be significantly reduced by CPS (25 µM), and this concentration-based reduction was prevented by PGT. Neurite outgrowth was inhibited by CPS (25 µM), whereas PGT significantly reversed neurite inhibition at and above 10 µM concentrations. The apoptotic index, which was increased using CPS (25 µM), was observed to reduce using PGT, depending on the concentration. Organophosphate is harmful to human health, and to our knowledge, there is no treatment. In individuals exposed to chlorpyrifos toxicity, acute toxic effects on neurons may be prevented or treated by PGT.

Keywords: *NB2a, Neurotoxicity, Pioglitazone, Neuroprotective, Neurite outgrowth, Apoptosis, Chlorpyrifos*

Pioglitazonun NB2a Fare Nöroblastoma Hücre Kültürüdeki Nöroprotektif Etkisi

Öz

Pioglitazon (PGT), farklı mekanizmalar yoluyla nöroprotektif özelliklere sahip bir PPAR-γ aktivatörüdür. PGT akut ve kronik kullanımının nöroprotektif olduğu düşünülmektedir. Klorpirifos (CPS) çocuklarda dikkat eksikliği ve bilişsel problemlere yol açan bir organofosfat insektisit olup nörotoksik etkileri iyi bilinmektedir. Bu çalışmada, kültür ortamında fare kaynaklı NB2a kanser dizin hücrelerinde CPS nörotoksisitesi üzerine PGT'nin nöroprotektif etkileri olup olmadığının araştırdık. MTT analizini kullanarak hücre proliferasyonu üzerine etkilerini ve ılımlı nörotoksik etkinin bir göstergesi olan Nörotoksisite Tarama Testi ile % nörit inhibisyonu incelendi. Apopitoz, TUNEL boyamada apopitotik indeks kullanılarak değerlendirildi. CPS (25 µM) konsantrasyonda uygulandığında NB2a hücre proliferasyonu azalttı. PGT ise konsantrasyon bağlı olarak klorpirosa bağlı azalmayı önlendi. Klorpirifon 25 µM konsantrasyonda tama yakın Nörit uzamasını inhibe ederken, PGT 10 µM ve üzerindeki konsantrasyonlarda nörit inhibisyonunu önemli ölçüde engelledi. CFS (25 µM) ile artan apopitotik hücre sayısını, PGT 10 uM konsantarasyondan itibaren anlamlı düzeyde azalttığı görüldü. Organofosfatlar insan sağlığına zararlıdır ve toksik etkilerini önlemek için bildiğimiz bir tedavisi yoktur. Klorpirifos toksisitesine maruz kalan bireylerde, nöronlar üzerindeki akut toksik etkileri PGT ile önlenebilir veya tedavi edilebilir.

Anahtar sözcükler: *NB2a, Nörotoksisite, Pioglitazone, Nöroprotektif etki, Nörotoksisite tarama testi, Apoptioz, Klorpirifos*

INTRODUCTION

People may be exposed to toxic effects of many chemicals throughout their lives. Some of these chemicals have a neurotoxic effect. Although neurons are highly developed cells, they cannot protect themselves and regenerate in case of damage [1].

Some of them have a neurotoxic effect. Although neurons are highly developed cells, they cannot protect themselves and to be regenerated in case of damage [1].

Chlorpyrifos (CPS: chlorpyrifos, O, O-diethyl O-3,5,6-trichloropyridin-2-yl phosphorothionate, O, O-diethyl O-3,5,6-trichloro-2-pyridyl phosphorothionate, chlor-

İletişim (Correspondence)
+90 533 3427112
kvural@cbu.edu.tr

pyrifos-ethyl) is one of the most extensively used organophosphate (OP) insecticides. CPSwas introduced into the market in the 1960s and is still used widely in agriculture and the home. Following its entry into the body, CPS is metabolically converted into its oxygen or oxon analogue, in which theSulphur of its P=S group is replaced by oxygen. This biotransformation reaction is carried out mainly in the liver by the cytochrome P450 (CYP)-dependent monooxygenase system. CPS activation to CPS-oxon is the main cause of moderate acute toxicity in mammals.CPS, have neurotoxic effects in susceptible species and cause neurodegenerative changes in central and peripheral nerves [2]. Therefore, we decided to use this compound to produce moderate acute toxicity.

Thiazolidinedione group drugs used in the treatment of diabetes act by increasing insulin response in the target cell. Cellular effects occur via the activation of peroxisome proliferator-activated receptors (PPARs) [3]. Activation of PPARγ is known to regulate inflammatory responses.and reduce the expression of many proinflammatory genes (COX-2, iNOS and cytokines), and it is also associated with inflammation in neurodegeneration [4]. Pioglitazone (PGT), a member of this group, is thought to have neuron-protective property through several mechanisms by increasing the gene transcription with the agonistic effect of PPARγ. However, it is not known whether it has a protective effect against neuronal damage in acute and long-term use [5]. PGT is known to have antidiabetic and adverse effects on the heart [6,7]. One of the findings of PGT use is that the medication may have a neuroprotective effect [3].

The differentiation of neurons in culture (evidenced by neurite outgrowth) is a physiological process that is a general indicator of cellular well-being. Its measurement, therefore, provides a useful in vitro model for the assessment of neurotoxicity and has been successfully used to demonstrate the neurotoxic potential of a wide range of agents, including excitatory amino acids. Neurite outgrowth is a specific structural end-point unique to the nervous system and depends upon a number of critical cellular processes, such as axonal transport. The inhibition of neurite outgrowth is only one marker of neurotoxicity that involves differentiating cells; thus, it may be of more relevant to exposure of the developing nervous system, rather than the mature nervous system. The mouse NB2a neuroblastoma cell has been determined to be a sensitive predictor of neurotoxicity, and its relative ease of culture and reproducibility suggested it was suitable for further use and development of experiments involves the assessment of interactions [8]. NB2a is a cell line and is frequently used in the examination of neurotoxic effects on the neuronal cells. The neurotoxic effect of the drugs is evaluated by % inhibition of neurite. If the damage is moderate, the nerve cell withdraws the neurite outgrowth [9].

The aim of this study is to show neuroprotective property of PGT on the OP toxicity. In the damage caused by CPS, the neuroprotective effect created by PGT is an important condition, whichcould be used clinically. The findings will also contribute to understanding the behavioural mechanisms of the nerve cell in pathological conditions.

MATERIAL and METHODS

Ethical Approval

Ethical Approval was given by the Medical Ethics Committee of Manisa Celal Bayar University (No: 10.05.2018 - 20.478.480).

Materials

Mouse NB2a neuroblastoma cells were provided from. European Collection of Cell Cultures (ECACC) (cell line: 89121404). All the chemicals used in the experiment were obtained from Sigma (St. Louis, MO, USA). Tissue culture flasks and culture plates were supplied from Falcon/ Fred Baker (Runcorn, Cheshire, UK) and gentamicin was provided from I. Ethem (Genta® 20 mg ampul, I. Ethem, Istanbul, Turkey). In all experiments, the solvent was evaluated for its effect on culture cells. PGT was dissolved in ethyl alcohol at 1/5 (0.2%) final concentration and the cells were incubated for a further 24 h.

Cell Culture

Neuroblastoma cells were proliferated in culture flasks with high glucose Dulbecco's Modified Eagle Medium (DMEM) with Glutamax-1, containing 5% fetal calf serum, 5% horse serum, 1% penicillin/streptomycin solutions (10000 U/10 mg) and 25 µg/mL gentamicin within the incubator humidified with 37°Cand 5% CO_2.

Cell Viability

The MTT assay, reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide to a purple formazan product, was used to estimate cell viability and growth. PGT at concentrations of 1, 3, 10 and 30 µM and CPS at 25 µM concentrations for measurement. Cell suspensions were first prepared at densities of 5×10^4/mL cells per each well of 96-well culture dishes and plated in triplicate for each concentration. Medium (100 µL) without PGT was used as a positive control, and only medium which did not contain any cells and PGT was used as a negative control.

Cells were treated with the concentrations previously mentioned for 24 and 48 h. Cells were incubated in humidified 5% CO_2 (in air) at 37°C with MTT in the last 4 h of the culture period tested. The medium was then decanted and 200 µL dimethylsulfoxide (DMSO, Sigma-Aldrich) was added to each well to ensure dissolving of the formazan salts. The absorbance was immediately determined at 570 nm in an UV-visible spectrophotometer multiplate reader (Versa Max, Molecular Device, Sunnyvale, CA) [10].

Measurement of Neurite Outgrowth

In order to measure neurite outgrowth, NB2a cells were plated in the proliferation medium on to 24 well culture plates at a cell density of 15.000 cells/mL. Twenty-four hours after, the cells were induced to differentiate and generate neurites in the presence of the PGT with the following method: the culture medium within each well were poured and replaced with serum-free medium plus 0.5 mM dibutyryl cyclic AMP containing PGT at concentrations of 1, 3, 10 and 30 µM and CPS at 25 µM concentrations for measurement of neurite outgrowth [11]. Ethanol was added in 1/5 (0.2%) final concentration and the cells were incubated for a further 24 h. Fixed cells with 4% (w/v) formaldehyde.in phosphate buffered saline (PBS) for 10 min at temperature of 24°C, then were stained for 3 min with Coomassie. Blue cell stain (0.6% [w/v] Coomassie Brilliant Blue G in 10% [v/v] acetic. acid, 10% [v/v] methanol, and 80% [v/v] PBS), washed with PBS. Three blinded observers took photograph of samples by using the Olympus BX-40 (Olympus, Tokyo, Japan) light microscope with a video camera (JVC-TK-C 601, Tokyo, Japan) for digital imaging. Image analyses were made by Image-Pro Plus image analyser (5.1.259, Bioscience Technology, Bethesda, MD, USA). Than, for drug and control, 10 different fields with approximately 10 cells were selected. A software routine was written using the functions of the image analyser to enable the automatic measurement of the total length of neurites (in pixels) for the cells in a given field and to express the results as the average.length of neurites per cell [11].

TUNEL Assay

Terminal deoxynucleotidyl transferase-biotin nick end-labelling (TUNEL) using the DeadEnd™ Colorimetric TUNEL system (Promega, Madison, WI, USA) were used to detect apoptotic cells. After application, cells were fixed in 4% paraformaldehyde for 30 min and rinsed three times in PBS for 5 min. After then cells were incubated with 20 µg/mL Proteinase K. for 10 min and washed three times again in PBS for 5 min. For endogenous activity inhibition, cells were treated with 3% hydrogen peroxide and rinsed in PBS. Afterward, cells which were treated with equilibration buffer for 5 min incubated with Tdt-enzyme for 60 at 37°C than were proceeded with 2×SCC solution for 15 min and then washed three times in PBS for 5 min. Streptavidin-peroxidase procedure was performed for 45 min, after which cells were rinsed in PBS and incubated with DAB; Mayer's hematoxylin was performed for counterstaining. Cells were than rinsed in distilled water and mounted in the mounting medium. TUNEL-positive staining was evaluated by the blinded observer under an Olympus BX40 light microscope [12]. Percentage and intensity of the immunostaining were scored with H-scoring and showed as the ratio of positively labelled cells to all cells in the chosen fields. An immunohistochemical score (HSCORE). was calculated as the sum of the percentages of positively stained epithelial cells multiplied by the weighted intensity of staining: HSCORE = $\sum Pi\ (I + 1)$, where "*I*" represents staining intensity (0=no expression, 1=mild, 2=moderate, and 3 = intense) and "*Pi*" is the percentage of stained cells for each intensity.

Apoptotic Index

Apoptotic index was defined as the ratio of positively. labelled cells to all cells in selected fields. For TUNEL staining, each section was.counted for 100 TUNEL-positive cells from randomly chosen fields. The percentage of apoptotic cells were also checked out by a blinded observer as 0: no apoptosis, 1: 1%-10% apoptosis, 2:11%-25% apoptosis, 3: 26%-50% apoptosis, 4: 51%-75% apoptosis, and 5: more. than 75% apoptosis [12]. The apoptotic index was counted up as the percentage.of apoptotic cells relative to the total cell number.

Statistical Analysis

The results were analysed.usingGraphPad (GraphPad. Software, SanDiego, CA, USA) using one-way ANOVA with Tukey.post hoc testing and presented as mean ± SEM. Statistical significance was defined as $P \leq 0.05$ or $P \leq 0.001$.

RESULTS

Effect of Pioglitazone on NB2a Mouse Neuroblastoma Cells

NB2a cells were taken into the culture medium and left to proliferate. Then, we waited for further 24 h for the neurite outgrowth in the differentiation medium, and the cells were stained with Coomassie Blue *(Fig. 1)*. In the cell proliferation phase, PGT was applied, and analysis of neurotoxic effects was performed through MTT assay *(Fig. 2)*. PGT was left for neurite inhibition for 24 h after the differentiation *(Fig. 3)*. PGT did not show any neurotoxic effects nor caused neurite inhibition ($P > 0.05$).

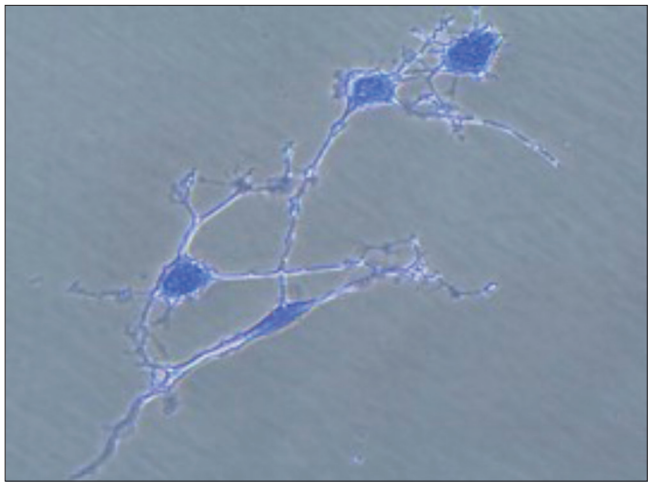


Fig 1. Differentiation NB2a cell to neurons by adding d-cAMP to serum free medium (Coomassie Blue stain, X400)

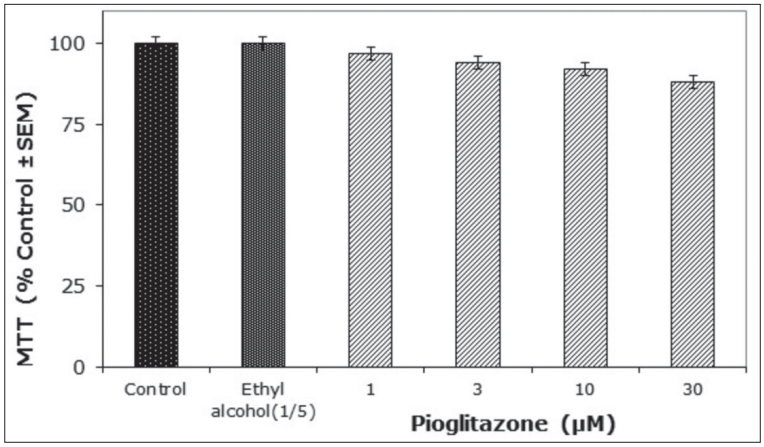
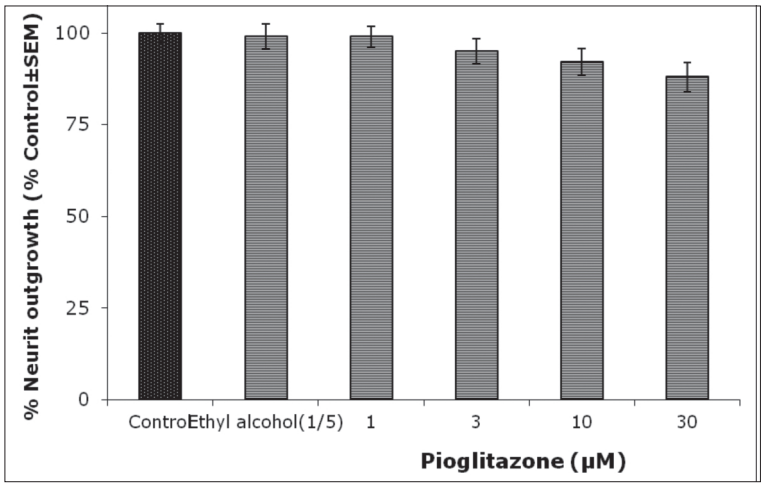


Fig 2. MTT measurements with different concentrations of PGT in NB2a cell culture Results are expressed as mean percentage compared the controls ± SEM (P>0.05)

Fig 3. The effects of PGT at different concentrations the neurite outgrowth measured by image analysis. Results are expressed as mean percentage compared the controls ± SEM (P>0.05)



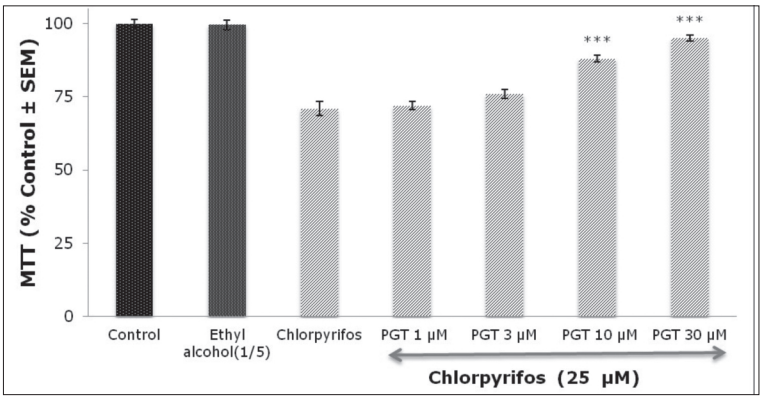


Fig 4. The effect of Chlorpyrifos administration (25 µM) with PGT at different concentrations on NB2a cells on MTT metabolism. Results are expressed as mean percentage compared the controls ± SEM (*** P<0.001)

The Neuroprotective Effect of Pioglitazone on Chlorpyrifos Induced Neurotoxicity in NB2a Cells

CPS was found to reduce the cell viability and proliferation, based on to the MTT assay results. PGT began to prevent the CPS-dependent reduction in cell proliferation at and above 10 µM concentration in the MTT assay (P<0.001) *(Fig. 4)*. PGT started to prevent the neurite outgrowth caused by CPS significantly 3 µM concentration (P<0.05) *(Fig. 5)*.

Pioglitazone is Protective Against Chlorpyrifos Induced Apoptosis in NB2a Cells

No significant difference was observed in the apoptotic cell index of PGT usage, which was analysed by TUNEL, compared to the control group of apoptosis. Depending on the dose, PGT was observed to reduce the apoptotic cell count, which was significantly increased (P<0.001) when CPS was added at a concentration of 25 µM in the culture *(Fig. 6,7)*.

DISCUSSION

OP is an important health problem, and there is not sufficient information about its chronic toxicity [13]. Information on possible chronic effects could be obtained only with the tests to be performed in the culture [14,15]. PGT is used in

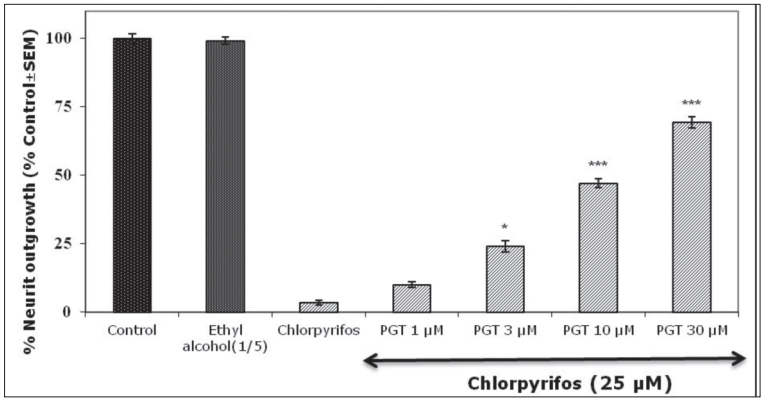
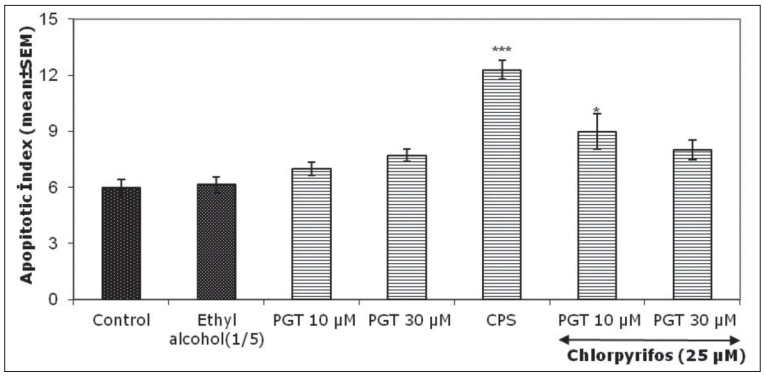


Fig 5. The effects of PGT at different concentrations on the neurite outgrowth measured by image analysis on Chlorpyrifos (25 µM) on NB2a cells. Results are expressed as mean percentage compared the Chlorpyrifos ± SEM (* P<0.05, *** P<0.001)

Fig 6. NB2a cells were observed to have quite healthy morphology with neurite outgrowths at 10 and 30 µM concentrations under PGT influence and without marked apoptosis. No statistically significant difference was observed between PG 10 and 30 µM concentrations (P>0.05), Chlorpyrifos (25 µM) produced significant apoptotic cells which were significantly (*** P<0.001) reduced by PGT at 30 µM concentrations compared to that of control



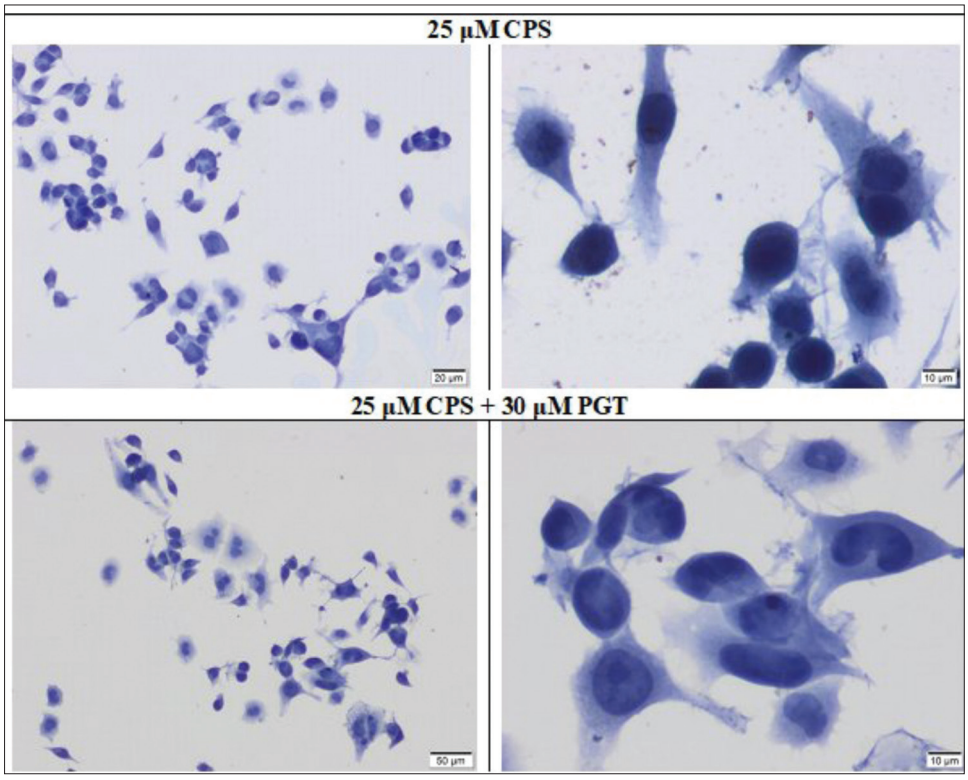


Fig 7. An increase in the number of apoptotic cells under the influence of Chlorpyrifos (25 µM) on NB2a cells was seen at low (x100) and high (x400) magnification and there was a significant decrease by PGT at 30 µM with similar magnification

the treatment of diabetes mellitus, and it is thought to have beneficial effects as well as neuroprotective effects, particularly in peripheral neuropathies, in patients with diabetes [16]. In the culture medium, OP toxicity on the neurons was shown with MTT assay. PGT showed neuroprotective effect against this toxicity. TUNEL showed that neurons underwent apoptosis in response to toxic effects, and the antiapoptotic effect was detected for PGT. MTT assay revealed that CPS provided neurite inhibition, as well as apoptosis by toxic effect, and it was blocked

by PGT. Protective effects of PGT treatment against antiproliferative, antiapoptotic and neurite retraction were revealed for the neurons in culture.

If the cell is exposed to a toxic effect, degeneration will started due to the synapse deterioration as a result of perineurium being directly affected or loss of trophic factors. The damage is determined by the severity, duration and persistence of the toxicity. Degeneration process may occur quickly or slowly depending on the mechanism. The delayed neurotoxic effect can be shown by measuring the enzyme level of neuropathy target esterase (NTE) in a culture medium [17-19]. İn our results we didn't see any toxic effect.

Neurite outgrowth, which is the primary function.of neurons *in vitro* medium, is associated with critical cellular events, such as axonal transport. The neurite outgrowth depends on specific structural elements such as the neurite-like microtubule binding protein and neurofilament protein. Neurite outgrowth can be inhibited by biological, chemical and environmental toxic substances. The neurite-developing factor, neurotropic factor and glial maturation factor have roles in the process of neurite outgrowth. Thus, monitoring of neurite outgrowth could be used to investigate the neurotoxic activities of the new molecules [11]. The mechanism by which OP reduced neurite outgrowth, whether alone or in combination is their ability to interact with acetylcholinesterase might play a role, as acetylcholinesterase has a trophic role influence in developing cells. However, this role is unrelated to the inhibition of hydrolysis of acetylcholine and therefore the relative potencies of the OP to inhibit neurite outgrowth would not necessarily be related to their short-term toxicities [15]. Our results showed that PGT did not show any significant toxic effect to our cells.

Some OP compounds, such as CPS, have neurotoxic effects in susceptible species and cause neurodegenerative changes in peripheral nerves. Accordingly, they cause organo-phosphate-induced delayed neurotoxicity. Typically, this syndrome begins with impaired coordination one or a few weeks after exposure to organic phosphorous.compounds and can progress to full paralysis of the hind legs. This condition tends to affect the distal parts of the long axons of nerve cells and manifests itself with a Wallerian-like degeneration. It is believed that there is a relationship between NTE inhibition and organophosphate-induced delayed neuropathy [20].

The main determinants in alcohol-induced neurotoxicity are mechanisms which play roles in excitotoxicity and neuroinflammation, and the protective effects of PPARy agonist seem to be associated with the inhibition of pro-inflammatory cytokines [21]. In the MPTP mouse model of Parkinson's disease, PGT reduces neuronal damage through a mechanism that involves the inhibition of the MAO-B enzyme [22]. In several studies, PPARy activation has been shown to lead to growth inhibition, apoptosis, and differentiation of a number of tumor cells [23,24].

The primary effect of thiazolidinedione (TZD) agents is to decrease the peripheral insulin resistance. They show their primary effects by activating specific receptors called PPARs. It has three subtypes: PPARα, PPARβ (δ) and PPARγ. Antidiabetic effects of TZDs have been found to be closely related to the ability to bind to and activate the PPARγ [4]. TZDs have effects on the vascular.system. Most of their vasculoprotective effects are independent from their effect on relieving the insulin resistance or antihyperglycemic effects [25]. A common function of PPAR subtypes is the suppression of oxidative stress and inflammatory processes. In this way, PPAR agonists have been shown to have neuroprotective effects in various disorders of the central nervous system [26,27]. We showed that PGT may protect the cells from toxic effect of CPS.

AT1 receptor blockage activates the nuclear receptor PPARγ, which is an important neuroprotective system. Telmisartan or losartan are thought to exert neuroprotective effects by PPARγ -activations [28,29]. Telmisartan has a neuroprotective effect in apoptosis through AT1 receptor blockage and PPARγ activation [29]. Additionally, PPARγ activation may promote neuroprotection against glutamate-mediated neurotoxicity and may also reduce neuronal damage in neurodegenerative diseases [27].

The modulation of PPARγ activity and peroxisomal function for PGT was found to relieve the NO, hydrogen peroxide-mediated neuronal damage and axonal damage. It has been suggested that PGT is a new therapeutic approach for the neurodegenerative changes associated with neuro-inflammation [30]. In the MPTP-induced rodent model of Parkinson's disease, the possible neuroprotective effect of fenofibrate and PGT has been shown to be mediated by NF-κB activation, which plays a role in neuroinflammation. MPTP treatment has been shown to activate caspase-3, resulting in apoptosis-associated neuronal death. On the other hand, treatment with neuroprotective drugs has been shown to inhibit caspase-3 activity and reduce neuronal damage [5]. PGT has been shown to be neuroprotective against decreased locomotion and rearing frequencies and to reverse hypolocomotion following intranigral infusion of MPTP. Administration of PGT at a dose of 30 mg/kg has been shown to elicit a partial neuroprotective effect against the neurotoxic effect of MPTP [31]. These results and mechanisms also support our results for neuroprotection of PGT.

Lee Et al reported that CPS attenuated PPAR-γ expression and NF-κB played a proapoptotic role in CPS-induced neuroblastoma cells death [32]. PGT is PPAR-γ agonist which has also neuroprotective effects and is able to inhibit the proinflammatory factors. These anti-inflammatory effects might.be involved in preventing the neuronal cell death caused by CPS.

AMP-activated protein kinase (AMPK) regulates signalling pathways related to cell survival and apoptosis and plays a role in increasing hippocampal neurogenesis [33]. It has been argued that AMPK activation directly inhibits β-amyloid accumulation *in vitro* [34,35]. PGT, by AMPK induction, has been shown.to improve neuronal apoptosis and β-amyloid accumulation in monosodium glutamate (MSG) neurotoxicity, which develops secondary to AMPK depletion by [36]. Our TUNEL results were similar with these studies.

Intracellular antioxidant activity caused by TZDs is important and responsible for some of the cellular protective effects. They do not have a direct antioxidant effect on free radicals. However, they exhibit protective effects by blocking the mechanism involved in the formation of the several hyperglycemic conditions causing oxidative stress [37,38]. In a study performed on hippocampal neurons, Calcium channel functions of which were improved in the culture medium, were shown to live.much longer than known. TZDs modulate Calcium dependent pathways in the brain and have different inhibitory profiles on two major Calcium sources, potentially conferring neuroprotection to an area of the brain that is particularly vulnerable to the effects of ageing and/or Alzheimer's disease. TZD may have potential applications in conditions associated with impaired learning and memory [39]. Neuroprotective effect of PGT has been.observed at doses in the range of 5-10 uM. This range is comparable.to plasma concentration of PGT when it is used in humans [40]. These studies also support for the effect of PGT which could be use for similar disease.

In vitro methods are known to be effective in demonstrating in-vivo neurotoxicity. This property can also be used to determine if an agent may be protective and preventive in case of acute damage. Neurite inhibition is an important indicator that could provide information about the long-term consequences of moderate toxicity-related damages. MTT, on the other hand, indicates severe toxic effects on cells. Using these methods, we found a significant protective effect of PGT in CPS-induced neurotoxicity and that apoptosis was involved in this damage, which can be alleviated by PGT. Absence of an adverse effect of PGT in culture environment and presence of neuroprotective effects highlights the efficacy and safety of PGT in the clinical use for many disease.

Conflict of Interest

The authors declare no conflict of interest

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