New *In Vitro* Strategy of Astrocytoma (Astrocytes-like) Cells Treated with Pioglitazone (PPAR gamma agonist) Offers Neuroprotection Against Nanoparticles-Induced Cytotoxicity

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ABSTRACT

Recently, we and others have developed several cell models for the investigation of the molecular mechanisms underlying the cytotoxicity of nanoparticles of metallic and non-metallic oxides. These models allow us to develop a set of strategies whereby critical mammalian cell types (e.g., neural cells) can be protected from the cytotoxicity induced by nanoparticles and other nanomaterials. We have continued to develop cell models *in vitro* to further elucidate and exploit the putative neuroprotective properties of astrocytes-like astrocytoma cells against the cytotoxicity induced by nanomaterials. We hypothesized that nanoparticle-induced cytotoxic stress can elicit and/or enhance the neuroprotective effects of astrocytes through the activation of astrocytic signaling and alteration of astroglial function. Furthermore, the neuroprotection can be enhanced with pioglitazone. Thus, our results may have pathophysiological implications in neuroprotection and nanotoxicological research.

Key Words: cytotoxicity, nanoparticles, co-culture, neuroprotection, pioglitazone, nanotoxicology

Topic Area: Neurology Nanotech: Regulating Neural Cell Properties (Under Medicine & Biotech)

1 INTRODUCTION

Astrocytes (non-nerve, glial cells) in the central nervous system are known to play vital roles in protecting neurons (nerve cells) against various assaults although the putative protective mechanisms are poorly understood [1].

Alzheimer's disease (AD) is associated with complication of neuronal cell death and the key symptom of memory loss is one of many chronic neurodegenerative diseases [2]. AD remains to be the top ten leading cause of death in the elderly population in the United States due to the ineffective current treatments and absence of a known cure for this disease. Thus, there is an urgent need to discover new strategies to cure AD. One promising line of investigation is to elucidate neural factors that protect neurons from degenerative assaults. Astrocytes have been known to protect neurons from such assaults although the mechanisms mediating this protection have not been fully elucidated. Consequently, it is imperative to delineate the key factors by which astrocytes exert their protective effects on neurons.

Our study aims to investigate the putative neuroprotective properties offered by astrocytes to protect neurons employing the recently modified *in vitro* co-culture cell models. Our previous and ongoing studies focus on the development of models to elucidate the cytotoxic and inflammatory mechanisms associated with the initiation and progression of neuronal cell damage and cell death [4-7]. Previously, we have demonstrated the usefulness of co-culture cell model of U87 astrocytoma and SK-N-SH neuroblastoma cells *in vitro* because of their close functional resemblance to normal astrocytes and neurons, respectively. We have demonstrated U87 cells protect SK-N-SH cells from different pathological assaults [4-7].

The present studies have tested the hypothesis that nanoparticles-induced cytotoxic stress can elicit and/or enhance the neuroprotective effects of astrocytes through the activation of astrocytic signaling and alteration of astroglial function. Furthermore, the neuroprotection can be enhanced in the presence of pioglitazone.

2 MATERIALS AND METHODS

2.1 Cell cultures of U87 and SK-N-SH cells

The human astrocytoma (U87) and neuroblastoma (SK-N-SH) cell lines were obtained from (ATCC; Manassas, VA, USA) and cultured in Minimum Essential Medium Eagle (MEM) supplemented with 10% (v/v) fetal bovine serum (Atlanta Biologicals; Lawrenceville, GA), 1% (w/v) sodium pyruvate (Sigma Aldrich; St Louis, MO), 0.292 g/L L-glutamine (Sigma), 1.5g/L sodium bicarbonate (Sigma) and 1% (v/v) antimycotic (Atlanta Biologicals; Lawrenceville, GA). Cells were maintained at 37°C and 5% (v/v) CO_2 as described previously [4-5].

2.2 Treatment of U87 cells

The U87 cells were cultured in T-75 tissue culture flasks to ~50% confluent in 20 ml of 10% FBS-containing MEM. Then the MEM was discarded and changed to 20 ml of 5% FBS-containing MEM and the cells were incubated for 24 hours. After that, the MEM was replaced with 20 ml of MEM containing 0% FBS and the cells were incubated for an additional 24 hours. Cells were then treated with zinc oxide nanoparticles (ZnO), pioglitazone (PZ) or a combination of both as described previously [7].

2.3 Cell Survival (MTT) assay

The neuroprotective effects of U87 cells on SK-N-SH cells were determined in different combinations of regular medium (RM) and conditioned medium (CM) collected from U87 cells cultured under various conditions (see above) employing the MTT assay [5,7].

2.4 Western Blot Analysis

Western blot analysis was employed to assess protein expression in cell lysates to determine the effects of media, ZnO and PZ. SDS-PAGE electrophoresis was performed as previously described [7-8].

2.5 Reactive Oxygen Species (ROS) Detection

SK-N-SH cells were subjected to treatment with RM/CM depicted above for 48 hrs; then carboxy H_2DCFDA (2',7'dihydrodichlorofluorescein diacetate) was used to determine production of ROS by the treated cells. The fluorescence of the oxidized form of the dye was measured at excitation wavelength of 492 nm and emission wavelength of 521 nm using a microplate reader (Biotek Synergy HT, Winooski, VT, USA).

2.6 Lactate Dehydrogenase (LDH) Release by Cells as Indicator for Cell Damage

Cell damage was determined by monitoring LDH release from cells into the culture medium as previously described [9] because LDH release is a marker of necrotic cell damage or cell death.

3 RESULTS AND DISCUSSION

The ever-increasing applications of nanomaterials in the industrial settings have raised health concerns as the exposure of nanomaterials leads to their accumulation in various organ systems in studies with animals and studies with cell models *in vitro* have demonstrated cytotoxicity induced by several types of nanoparticles of metallic and nonmetallic oxides in human neural cells [6]. These important findings have raised the awareness for the need to derive a set of strategies whereby mammalian cell types (e.g., neural cells) can be protected from the cytotoxicity induced by nanomaterials. As part of our response to the need to

devise a strategy to protect critical cell types against the cytotoxicity induced by nanomaterials, we have continued to develop cell models *in vitro* to further elucidate and exploit the putative neuroprotective properties of astrocytes-like astrocytoma cells (U87). We developed a newly modified co-culture strategy which allows the characterization of the putative "protective effects" proferred by U87 cells to protect SK-N-SH cells in a non-contact cell culture set up.

Zinc is an essential trace metal although its excess intake leads to expression of toxic symptoms in mammals. However, the putative toxicity of nanoparticles of zinc compounds in mammalian cells is largely unknown. Thus, there is a need to breach this knowledge gap [3].

Our previous studies demonstrated the cytotoxicity induced by ZnO nanoparticles in both U87 cells and SK-N-SH cells [3]; however, the underlying mechanisms have not been fully elucidated. Therefore, zinc oxide nanoparticles (ZnO) were used to induce cytotoxicity in cells while peroxisome proliferator activated receptor gamma (PPARγ) agonist, pioglitazone (PZ), was used to block the inflammation because inflammation is usually unregulated in AD [10]. In the present study, we utilized ZnO nanoparticles and PZ in addition to serum depletion to further elucidate the putative protective mechanism(s) of U87 cells on SK-N-SH cells. Serum depletion and drug treatment for U87 cell cultures were prepared as shown in the flow chart below (Fig. 1). U87 cells cultured in a medium containing 10% FBS were used as control (not shown in Fig. 1). Medium containing 0% FBS were defined as serum free (SF) medium.

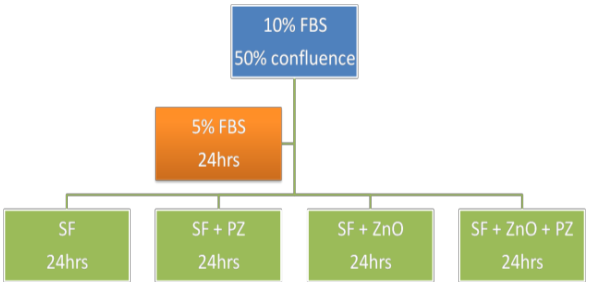

Figure 1. Experimental design of U87 cells treated with ZnO, PZ or in combination. The U87 cells were grown to ~50% confluent in 10%-FBS containing medium and subsequently discarded and changed to 5% FBS-containing medium and incubated for 24 hours. After that, the medium was replaced with medium containing 0% FBS (SF) and incubated for an additional 24 hours. Cells were treated with ZnO nanoparicles or PZ or a combination of both for another 24 hours of incubation.

Oxidative stress and environmental stress are known to induce morphological and functional changes/adaption in astrocytes *in vitro* and *in vivo* and we observed these morphological and associated protein level changes previously [7]. In this study, we introduced treatment with ZnO nanoparticles and FBS depletion to induce cytotoxicity and environmental stress, respectively, in U87 cells and access the morphological, functional changes/adaption and the modulation by PZ thereon. Employing light microscopy, we found that treatment with SF medium and ZnO nanoparticles induced U87 cells to put out multipolar processes (data not shown).

Glutamine synthetase (GS) is an important enzyme which prevents neurons from excitotoxicity by converting neurotoxic glutamate and ammonia to non-toxic glutamine. Employing western blot analysis, we found GS protein expression was upregulated after treatment with SF medium and ZnO nanoparticles with or without PZ, suggesting that upregulation of GS expression could also be involved in the cell survival mechanism.

We collected media in which we grew U87 cells under the conditions specified in Figure 1 above and assessed their ability to influence survival of SK-N-SH cells. Our results suggested that exposure of SK-N-SH cells to various combinations of SF media allowed them to survive better than when cultured in 10% FBS medium (control condition) alone (data not shown). Moreover, exposure to PZ enhanced this protective effect, even under conditions of cytotoxic stress. Therefore, we access the putative mechanism of cell protection activated by PZ. Exposure of U87 cells to PZ enhanced this protection, namely through the suppression on lactate dehydrogenase (LDH) release and reactive oxygen species (ROS) generation (Figures 2 & 3).

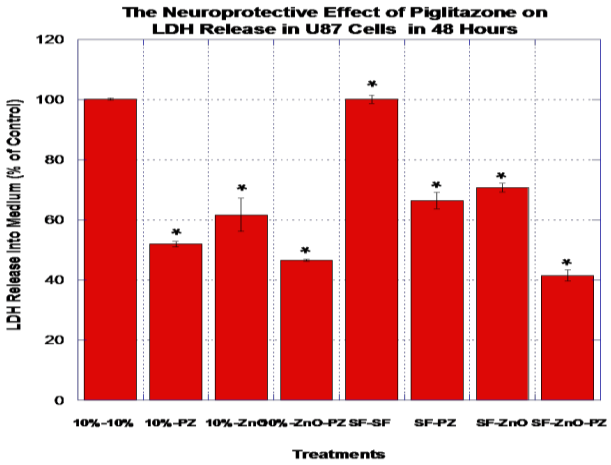

Figure 2. Treatment effects on LDH release by U87 cells. Values are expressed as % of the control and are given as mean ± SEM of three separate determinations. *p<0.05 versus control.

4 CONCLUSIONS

When taken together, our results suggest astrocytes-like U87 cells changed their cell shape and morphology when cultured under serum-free media with and without drug treatment. Moreover, exposure of SK-N-SH cells to

various combinations of SF media allowed them to survive better than when cultured in 10% FBS medium (control condition) alone and exposure to PZ enhanced this protective effect, even under conditions of cytotoxic stress. Thus, our results provide additional evidence that cytotoxicity induced by ZnO nanoparticles can be reduced with the addition of PZ. Our results may assume pathophysiological importance in neuronal survival/death mechanisms in neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease. Clearly, this is an area that merits further study.

5 ACKNOWLEDGMENTS

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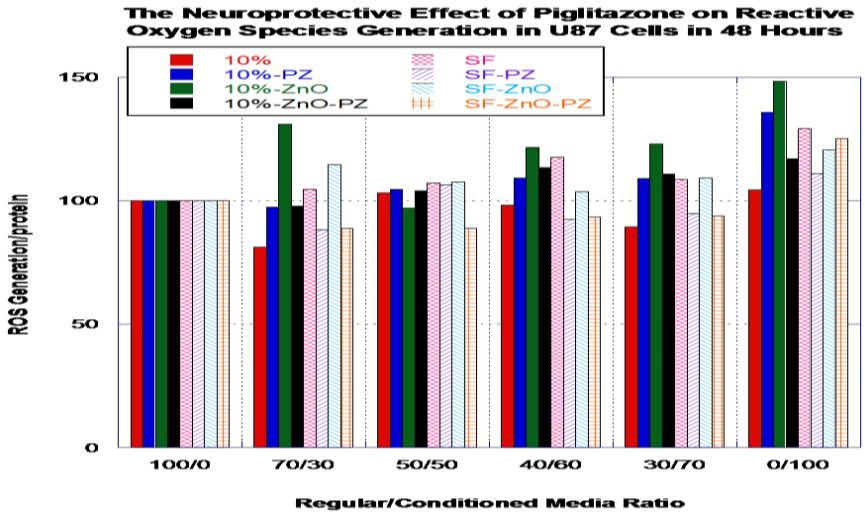

Figure 3. Treatment effects in U87 cells on ROS generation in SK-N-SH cells. Values are expressed as % of the control.