Effects of IIIM1 on Rat Astrocytes

Tahreem Fatima, Marta Sidoryk-Wegrzynowicz, Yingchun Yu, Uri Wormser, Michael Aschner

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BRIEF. Designed to study a peptide's ability to protect cells exposed to manganese chloride.

ABSTRACT. This project tests the ability of a small peptide called IIIM1 to protect the brain from manganese (Mn) toxicity. High Mn levels can cause brain dysfunction, leading to Mn poisoning known as manganism. We hypothesized that treatment with IIIM1 would protect against the detrimental effects of increased doses of Mn. Cells treated with IIIM1 in the presence of Mn showed less cell death and improved health compared to Mn exposed cells that were untreated. This supports future testing of IIIM1 for prevention of premature cell death and protection against cellular stress. We also intend to repeat experiments with varying exposure times to better understand the mechanisms associated with IIIM1's protection.

INTRODUCTION.

Manganese, an essential element for the human body, is necessary for bone development and utilization of key nutrients through activation of enzymes (substances used to assist chemical reactions) that are imperative in digestion and metabolism [1]. However, high levels of manganese in the brain can lead to severe brain disorders. Of particular concern is manganism, which shares multiple symptoms with Parkinson's disease. In addition, manganese has been known to accumulate in certain nervous system cells, mainly astrocytes [2]. Astrocytes have a number of important functions, such as regulating the nutrients that come in contact with the brain, and providing physical support to other important brain cells, the neurons [3].

If the accumulation of manganese in astrocytes occurs, it may result in oxidative stress that may lead to the damage of cell structures over time [4]. Oxidative stress can be assessed using F_2-Isoprostanes, which are biomarkers within a cell that become elevated when a cell undergoes stress. F_2-Isoprostanes were chosen because they are stable, easy to measure, and are normally present in the cells [5].

Previously [6], it was discovered that a small peptide referred to as IIIM1 had the greatest reduction in inflammation in an animal model injected with carrageenan, a well-known irritant. This led to another study [7] testing the effects of IIIM1 on three different groups of astrocytes. It was noted that the group treated only with the IIIM1 experienced the greatest reduction in oxidative stress and this was thought to have been brought about by the presence of this peptide.

In view of these studies, this experiment was designed to test IIIM1's effect on astrocytes and whether it helps reduce manganese-induced toxicity. In order to investigate this, assays were performed to examine the number of cells alive as well as their physical condition and the amount of stress they experienced after exposure to high levels of manganese. It was hypothesized that IIIM1 would attenuate the manganese-induced toxicity and help the cells better survive stressful conditions.

IIIM1 had a proliferative effect on T-regulatory cells among other positive effects, and *Shapira et. al* deigned the peptide to be appropriate for human testing because of its positive outcomes time after time. The authors also hinted at IIIM1's ability to induce production of another peptide, UBE, which had proven beneficial effects, like reduced renal damage, on mice with systemic lupus erythematosus (SLE), a chronic autoimmune disease that affects multiple organs [8]. With attention to the above mentioned study, organ transplants are a good example for possible applications for this research; the IIIM1 used in conjunction with organ cells would increase their chances of survival. This research can also be applied to diseases caused by premature cell death or excessive amounts of heavy metals, such as Parkinson's disease, where IIIM1 can be a possible treatment for degenerative symptoms caused by oxidative stress [9].

MATERIALS AND METHODS.

Cell Culturing.

Primary astrocytes were isolated from cerebral cortices of post natal day-1 Sprague-Dawley rats as performed by *Milatovic et al* [10]. A bacterial neutral protease (dispase, *Invitrogen*) was used to digest the tissue. The astrocytes were maintained in minimum essential medium (MEM) supplemented with 10% heat-inactivated horse serum, 100 μg/mL of streptomycin and 100 U/mL of penicillin. The cells were grown for at least two weeks in plates in the incubator, which was set at 37˚C and 5% CO_2.

Astrocyte Treatment.

To test the effects of IIIM1 on the astrocyte cultures, one group was treated with the following concentrations of manganese-chloride ($MnCl_2$): 0, 50, 100 and 500 μM. Another group was pretreated with 5 μg/mL of the IIIM1 for an additional 24 hours before $MnCl_2$ was added (in the same concentrations as before), making the total treatment time for this group 48 hours.

Lactate dehydrogenase (LDH) and 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide (MTT) assays were performed as soon as the treatments were ready. In order to prevent sample degradation, samples for F_2-Isoprostane analysis were frozen in liquid nitrogen prior to being stored at -80˚ C.

F_2-Isoprostanes.

F_2-Isoprostanes were used to assess oxidative stress. Methanol with butylated hydroxytoluene (BHT) and 15% KOH was added to 200 μL of sonicated samples. Then the samples were placed in 37˚C water bath for 30 minutes. The sample's pH was adjusted to 3, after which $^4H_2$-labeled 15-$F_{2\alpha}$-IsoP (internal standard) was added. The cells were then purified by C_{18} and Silica Sep-Pak extraction and by thin layer chromatography. Analysis took place via gas chromatography and negative ion chemical ionization mass spectrometry [10].

MTT.

MTT, a yellow tetrazolium salt, is used to measure cell viability. MTT is reduced to formazan crystals by metabolic enzyme activity, resulting in a color change. *In vitro* toxicology assay kit, (MTT based, *Sigma*) was used for this purpose and the absorbance was measured at 570 nm. The results were derived from subtracting the background absorbance (690 nm) from the primary absorbance (570 nm).

Statistical Analysis.

Statistical analysis examining dose effect within each group was carried out by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test with statistical significance set at $p<0.05$. Each treatment within the manganese only group and the combination of IIIM1 and manganese group was compared to the control and IIIM1 only treatment, respectively. All analyses were carried out with GraphPad Prism 4.02 for Windows (GraphPad Software, San Diego, CA, USA).

RESULTS.

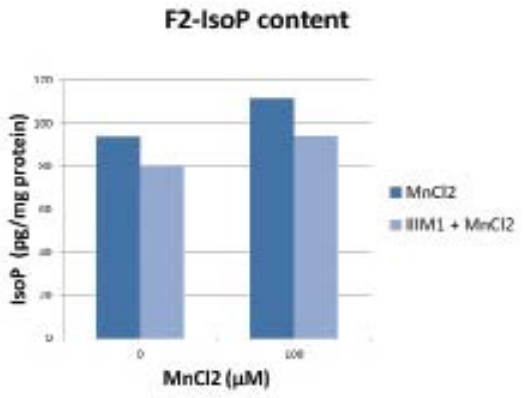

Figure 1. F_2-Isoprostane (marker of oxidative injury) levels (obtained from peak area) found in the control, 5 μg/mL IIIM1, 100 μM $MnCl_2$ and 100 μM $MnCl_2$ + 5 μg/mL IIIM1 treatments. $MnCl_2$ induces stress, while the IIIM1 lowers it. For each treatment, n = 1.

As evidenced in Figure 1, the IsoP levels displayed a reduction in stress levels in the IIIM1 treated groups. The more F_2-Isoprostanes present, the higher the level of oxidative stress and both IIIM1 groups had lower oxidative stress as opposed to their manganese treated counterparts. The IsoP level for the control treatment was 93.732 pg/mg protein, while IIIM1 treated cells had a IsoP. level of 80.236 pg/mg protein. When IsoP levels were compared between the 100μM manganese treatment (111.377 pg/mg protein) to the IsoP levels in the combination of 100 μM manganese plus IIIM1 treatment group (93.981 pg/mg protein), once again, a IIIM1 pre-treated group had the lower amount (Figure 1). This indicates that cells treated with IIIM1 experienced lower levels of oxidative stress.

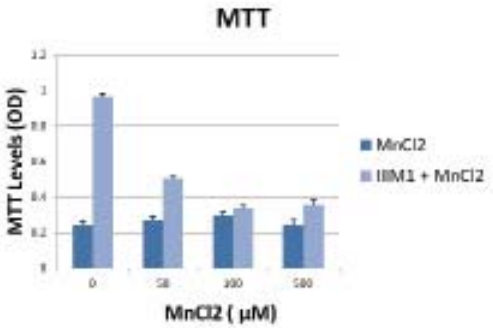

Figure 2. MTT (measures cell viability based on Optical Density) content for astrocytes treated with $MnCl_2$ and IIIM1+$MnCl_2$. Treatments with IIIM1 experience greater cell viability than the treatments with $MnCl_2$ only. For each treatment, n = 3. Error bars represent SEM.

Note: All IIIM1+$MnCl_2$ treatments were the same concentration of IIIM1 (5 μg/mL), but different concentrations of $MnCl_2$. Dose effect was tested using one-way ANOVA (followed by Tukey's multiple comparison test), where each treatment within the manganese only group and the combination of IIIM1 and manganese group was compared to the control and IIIM1 only treatment, respectively. There were no statistically significant differences found.

The IIIM1 treated groups, when tested for cellular activity with the MTT assay, performed better than the manganese alone groups (Figure 2). The more enzymatic activity that occurs as the MTT compounds are reduced to formazan dye, the higher the OD. The control treatment (no IIIM1 or manganese exposure) had an average value of 0.242 optical density (OD) ($p>0.05$), while the IIIM1 treatment had an average of 0.964 OD ($p>0.05$). Likewise, the 500 μM manganese treatment group had an average of 0.245 OD ($p>0.05$) while the 500 μM manganese treatment with the IIIM1 had an average of 0.358 OD ($p>0.05$). All treatments in the IIIM1 group had higher OD levels, thus higher cellular activity, than any of the manganese only treatments. One-way analysis of variance (ANOVA) was performed for the MTT assay, followed by Tukey's multiple comparison test, determining dose effect within each group. There were no statistically significant differences found.

DISCUSSION.

This study has demonstrated that treatment with IIIM1 can reduce oxidative stress, thus increasing cellular viability. The results of this study are supported by the fact that the mitochondria are suspected of having an important role in mediating heavy metal- induced neurotoxicity [6].

As expected, the F_2-Isoprostanes and MTT assays were indicative of IIIM1's potential to reduce oxidative stress; in the future, IIIM1 can be used to reinforce membrane integrity when paired with anti-depressants, like imipramine, that induce lymphocyte apoptosis, or fluoxetine, another anti-depressant known for reducing lymphocytes. It also shows promise of being an efficacious treatment for patients of Crohn's disease, an inflammatory bowel disease [11], as well as a host of other disorders and diseases like Parkinson's disease, manganism, and hepatic encephalopathy, a condition where brain functioning is disrupted due to various liver disorders [2].

An additional experiment was performed to assess membrane integrity (refer to supplemental section); however, the findings appear anomalous and require further analysis.

Our next step is to conduct this experiment with the same variables, but at different time points to examine the difference between our two groups more clearly and to check how long it takes the cells to be effected after exposure to the peptide. We also hope to repeat similar experiments with other recently synthesized peptides like GHYAERVG and KGHYAERV in hopes of finding other protective peptides. These peptides had shown, along with IIIM1, a significant reduction in swelling in the animal models upon injection of carrageenan, which was used as an irritant in this study [6].

Our research indicates IIIM1's ability to combat manganese toxicity in astrocytes by reducing oxidative stress, and as a result, increasing cell viability. These results suggest that IIIM1 may be beneficial in helping people with neurodegenerative diseases or any disease in general associated with oxidative stress, inflammation or accumulation of heavy metals, such as Parkinson's disease, to reduce the symptoms upon treatment with this peptide.

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SUPPORTING INFORMATION.

Supplementary Methods

Supplementary Results

Supplementary Discussion

Figure S1. LDH content for astrocyte treatments

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Tahreem Fatima is a student at Martin Luther King Jr. Magnet High School and enrolled in the School for Science and Math at Vanderbilt.