

# ***Biology***

## **Expression of JNK3 Apoptotic Protein in SH-SY5Y Cells After Acute Exposure to Paraquat**

Sponsoring Faculty Member: Dr. Melinda Pomeroy-Black

Robby Williams and Melinda Pomeroy-Black

### **Introduction**

Pesticides are used around the world to protect crops and organophosphorous (OP) compounds are some of the most commonly used pesticides. There has been extensive research conducted on the correlation between chronic OP exposure and neurodegenerative diseases, including Parkinson's Disease (PD).<sup>5,8</sup> Parkinson's Disease is the second most common idiopathic progressive neurological disease in humans with only 10% of cases are related to genetic factors.<sup>3,7,8</sup> The OP compound 1,1'-dimethyl-4,4'-bipyridinium dichloride (paraquat) is used worldwide as a pesticide and recent studies have linked workers such as orchardists, pesticide applicators, and farmers who use paraquat to an increased incidence of PD.<sup>1,4,5,8</sup> One mechanism by which paraquat may lead to PD is inducing apoptosis mediated by the endoplasmic reticulum (ER) in dopaminergic neurons.<sup>5,8</sup> The SH-SY5Y cell contains phenotypic characteristics of human dopaminergic neurons and has been used in many studies for dopaminergic pathologies.<sup>6</sup> In vitro studies have shown that paraquat may induce ER stress-mediated apoptosis through the signaling cascade initiated by activation of c-Jun N-terminal protein kinase (JNK) in SH-SY5Y cells.<sup>2,5,8</sup> Evidence indicates that paraquat stimulates the phosphorylation of the JNK proteins in SH-SY5Y neuroblastoma cells.<sup>5,8</sup> Therefore I hypothesize that SH-SY5Y neuroblastoma cells exposed to the OP compound paraquat will show an increase in concentration of inactivated JNK proteins prior to their phosphorylation, thereby increasing the efficiency of apoptosis.

### **Materials and Methods**

Human SH-SY5Y neuroblastoma cells were grown in flasks at 37°C with 5% CO<sub>2</sub>. The cells were maintained using growth media changed every three to five days. The SH-SY5Y neuroblastoma cells were grown to 80-90% confluency and differentiated with 10<sup>-7</sup> M retinoic acid for three days.

The cells were then exposed to 5x10<sup>-1</sup> mM, 5x10<sup>-2</sup> mM, and 5x10<sup>-3</sup> mM of paraquat diluted in media for 8, 12, and 24 hours. The cells were lysed using a lysis buffer. To lyse cells, they were first treated with trypsin and placed into sterile 15

ml tubes and centrifuged at 1200 RPM for 7 minutes at room temperature. The pellet in the 15 ml tube was rinsed twice with 1 X PBS and centrifuged at 1200 RPM for 7 minutes at room temperature. The cells were then treated with a lysis buffer (1:200 Protease inhibitor Cocktail III to 5 ml RIPA Lysis Buffer). The lysate was added to a 1.7 ml tube and disrupted with a 21 gauge needle to ensure shearing of the cell membrane. Any mucoid aggregate was removed using the needle. The 1.7 ml tubes were then incubated on ice on a shaker for 30 minutes. The tubes were then centrifuged at 10,000 x g for 10 minutes at 4°C and the lysate was transferred to new sterile cold 1.7 ml tubes. The respective lysates were stored at -20° C. A standard BCA protein curve was run to determine the total protein concentration in each lysate. The lysate was diluted 1:1 with sample buffer. From these protein concentrations, 50 µg of protein was loaded onto a gel and gel electrophoresis was conducted. Proteins were then transferred to a nitrocellulose membrane using a wet transfer method.

After transfer the membranes were treated for one hour with Tween-milk (5g dry milk, 3 µl Tween 20, and 10 0ml TBS) to block unbound proteins. Following the incubation in Tween-milk, the membranes were exposed to the primary antibody, anti-JNK3 (1:200 in Tween-milk). The antibody was poured around the membranes and membranes were incubated at 4°C on a gentle rocker over night.

The following day the membranes were rinsed with 1X TBS and exposed to the secondary antibody (horseradish peroxidase-conjugated goat anti-mouse IgG) diluted 1:200 in Tween-milk. The membranes were then left at room temperature on a gentle rocker for an hour.

Membranes were then treated with an HRP color detection reagent in a dark room and incubated at room temperature on a gentle rocker for 5-10 hours until bands were obtained. The membranes yielded in this experiment were analyzed visually for thickness and darkness of the bands corresponding to the molecular weight of JNK3 protein. The darkness and thickness of the bands was used as a measure of JNK3 concentration where the darker bands indicated a higher concentration of JNK3 protein.

## Results

The JNK3 bands were darkest in the cells exposed to the  $5 \times 10^{-1}$  mM paraquat for 8h, 12h, and 24h. This was consistent for all three replicates. Of all the bands corresponding to the  $5 \times 10^{-1}$  mM paraquat concentration, two were darker compared to the rest. These were the wells containing the cells exposed to  $5 \times 10^{-1}$  mM paraquat for 24h in the second and third replicate. The membranes also contained faint bands in the control cells, and in the cells exposed to  $5 \times 10^{-2}$  mM paraquat and  $5 \times 10^{-3}$  mM paraquat. The cells exposed to  $5 \times 10^{-2}$  mM paraquat, and  $5 \times 10^{-3}$  mM paraquat for 12h in the second replicate had darker bands than the control cells exposed for 8h, 12h, and 24h in the second replicate.

## Conclusion

The data support the hypothesis that SH-SY5Y neuroblastoma cells will show an increased concentration of the JNK3 apoptotic protein after acute exposure to paraquat. Cells exposed to the  $5 \times 10^{-1}$  mM paraquat concentration expressed the highest concentration of the JNK3 apoptotic protein compared to the controls (which received strictly growth media), and cells exposed to  $5 \times 10^{-2}$  mM and  $5 \times 10^{-3}$  mM paraquat concentrations. A distinguishable difference in darkness of bands of cells exposed to  $5 \times 10^{-2}$  mM and  $5 \times 10^{-3}$  mM concentrations were seen on only one membrane. As the other two membranes did not have a distinguishable difference in band density, this suggests that cells exposed to these concentrations did not express the JNK3 protein at levels equal to cells exposed to the  $5 \times 10^{-1}$  mM paraquat. This could mean that paraquat concentrations of  $5 \times 10^{-2}$  mM and  $5 \times 10^{-3}$  mM paraquat concentrations are not high enough to cause an increased up-regulation of the JNK3 protein.

The expression of the JNK3 apoptotic protein suggests that these cells were entering the process of programmed cell death (apoptosis). One method of apoptosis occurs if the JNK3 protein is phosphorylated. Therefore the data suggest that there is an initial up-regulation of the JNK3 protein prior to phosphorylation, which is then followed by apoptosis in neurons. The up-regulation of JNK3 protein in nervous tissue, as seen in this experiment, could be a reason that workers using paraquat show an increased incidence of PD.

## References

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