Using Comet Assays to Analyze the Genetic Stress of Cells Containing the Damage Suppressing Gene Found in Tardigrades

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Tardigrades, also known as water bears, are microscopic multicellular organisms that can be found in about any environment. The creatures are known to be extremotolerant, and with well over 1000 different species, these stoic creatures can persist in dehydration, extreme temperatures, high pressures, and even the vacuum of outer space (Hashimoto). Tardigrades also hold the ability to withstand high levels of irradiation without extensive or irreputable damage to their DNA. While most mammals would find roughly 5-10 grays (unit for measuring levels of ionizing radiation) of ionizing radiation fatal, and some prokaryotes such as *E. coli* can survive roughly 50 grays, Tardigrades can survive up to 5,000 grays of radiation (Hashimoto). The Damage Suppressor Gene (Dsup), which is found exclusively in Tardigrades, does what the name suggest. The gene produces proteins that prevent damage to Tardigrade DNA from harsh environmental stresses. The proteins have been found to suppress both single strand and double strand breaks in DNA along with improving overall radiotolerance. What makes the gene even more impressive, however, is that it has been cultured in human cells and found to improve radiotolerance and viability of irradiated cells (Hashimoto).

The purpose of this research is to analysis how effective the Dsup gene at protecting genes in cells outside of tardigrades. Moreover, in the future test how effective the gene is at protecting other organisms from ionizing radiation damage. The primary method used to analysis the cells treated with different stresses, is the comet assay. The comet assay, also known as single cell gel electrophoresis (SCGE), was first developed in 1984 and is an uncomplicated but sensitive technique used to detect DNA damage in individual eukaryotic cells. The cells are mixed with low melting point agarose, lysed in extremely alkaline conditions, and stained with sliver nitrate to read DNA damage (Nandhakumar). DNA damage is determined based on the “comet trail” left by the cell from electrophoresis. The greater the trail left behind, the greater the damage to the DNA has been done.

Since there are over 1000 species of tardigrade, the genes that will be tested will be from the *Ramazzottius variornatus*, which is noted for not only being one of the most extremotolerant species, but also due to it genes having the most coverage of genetic analysis. The DNA holding the Dsup gene with be introduced to two primary stresses to test for breaks, the first being Hydrogen Peroxide to test for free radicals (another extreme which the gene can protect from), and the second being Ultra-Violent Radiation, which is the primary focus of the study.

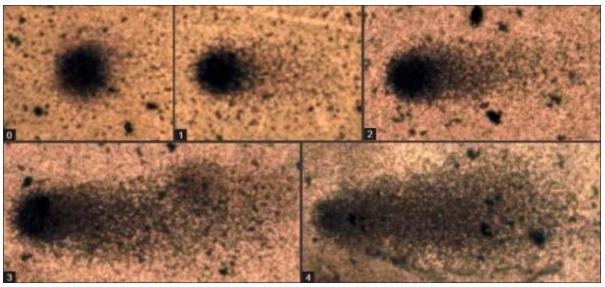

Fig. 1: Example of silver stained comet assay on human peripheral blood mononuclear cells.

IPTG Induction was done to ensure a group of cells contained the Dsup protein while a control group did not. Cells containing bacterial expression plasmids of Dsup (pCold-I-Dsup) were ordered from addgene and were subsequently cultured in a petri dish overnight. Slow IPTG induction was done under a protocol by GoldBio. In a 15-mL conical tube, 2 mL of Luria Broth (LB) and 2 µL of Ampicillin were added, and Dsup cells were swabbed from the dish into the solution. Cells were left to grow overnight at 37º C in a shaker. Sample was dilute 1:100 and left to grow again in another tube for 3 hours. For the uninduced sample 1 mL of the sample is removed to a 1.5 mL microcentrifuge tube, spun down, and stored at -20º C. For the induced sample, a separate tube is prepared with 1 mL of prewarmed LB + Ampicillin solution, along with 1 mM of IPTG. The remaining solution from the original tube is added to the new tube and left to shake for 16 hours at 20º C. After 16 hours, 1 mL is collected into a microcentrifuge tube, spun down, and stored at -20`° C. This process is repeated once more for a total of 2 uninduced samples and 2 induced samples. Cells were treated with hydrogen peroxide solution to test for genetic stress against free radicals. For both an induced and uninduced sample, four microcentrifuge tubes were

prepared. 0.4 mL of ddH_20 was added to each tube to resuspend sample, and 0.1 mL of 3% hydrogen peroxide was added to one tube of each sample. Serial dilutions were performed on the remaining centrifuge tubes. Cells were left to incubate at room temperature for 10 minutes. Cells were treated shortly before performing comet assay. Afterwards, samples were resuspended in 0.5 mL of ddH_20 in a microcentrifuge tube. Samples were added into an ELC-500 Light Exposure System and were left to radiate for 5 minutes. Irradiated samples were collected for comet assay. UV Light treatment was performed shortly before comet assay. A Comet assay was performed to identify damage done to the plasmids of the samples. A protocol by *The Journal of Pharmacology and Pharmacotherapeutics* was followed for the comet assay. Solutions that were not available were made in the Hampden-Sydney Biology Department. Plain, clean microscope slides were used for electrophoresis. 0.5% low melting point agarose (LMPA) and 0.75% normal melting point agarose (NMPA) were prepared in PBS and microwaved for 1 minute. 100 µL of NMPA was dropped onto a slide and spread with another slide. Slide was dried at 37ºC for 10 minutes. Once dried, 60 µL of LMPA was mixed with 20 µL of sample and dropped onto the slide and covered with a cover slip. Slide was left to solidify at 4ºC for 15 minutes. After refrigeration, the cover slip was removed and 75 µL of LMPA was dropped on slide. Cover slip was added again, and slide was gently set into cold lysis solution for up to 24 hours at 4ºC. Electrophoresis was carried out after lysis. Slides were set on opposite sides of the electrophoresis tank, near the electrodes, and cold electrophoresis buffer was gently poured to fill the tank. Slides stayed in the tank 30 minutes prior to electrophoresis to allow for the DNA to unwind. Electrophoresis was carried out for 30 minutes at 300 mA. After electrophoresis, cells were gently lifted and placed into a tray containing neutralizing tris buffer (pH 7.4) for 5 minutes. Slides were then washed with water several times. Slides were dried at room temperature overnight. Sliver Staining was performed to allow visualization of cells and comets. Once neutralized, cells were placed in fixative solution for 10 minutes then washed in water several times. Slides were left to dry at 37º C overnight. Once dried, slides were placed into an amber staining box and 32 mL of staining solution A, and 68 mL of solution B, were gently poured into the box. The box was then put on a platform rocker to shake at a slow speed for 10 minutes, and the process was repeated 3 more times. Slides were gently removed and placed in stopping solution for 5 minutes. The slides were then washed in water and left to dry overnight at an inclined position at room temperature. Once dried, slides were finally observed under a microscope.

Unfortunately, due to various factors, most of the results seemed to be inconclusive. However, much was learned between each attempt to improve the next. The first comet assay was performed using the hydrogen peroxide treated samples, but the samples were only made and used in the assay once. The main reason the peroxide samples were created and used once was due to UV treated samples being the priority for the comet assay.

Fig. 2: The uninduced sample (left, 2a) demonstrates how the cells were torn during the electrophoresis, while the induced sample (right, 2b) demonstrates the significant trails during electrophoresis.

Based on the results, though the cells were in fragments (figure 2a), and no comets showed, one could visibly see were the cells traveled during electrophoresis (figure 2b). Therefore, it could be concluded that this method of comet assay can properly function under proper conditions.

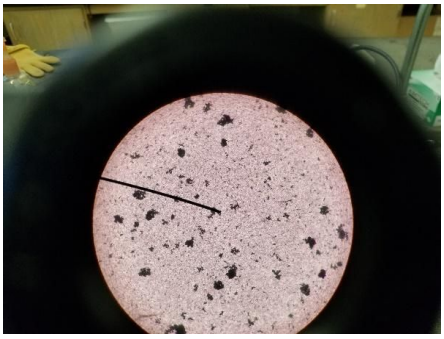

Fig. 3: The image shows the induced sample with cells mostly intact, but with a lack of comet trails. The uninduced sample is much the same.

Two more comet assays were performed using samples that were treated with UV light. The first of the two assays gave cells that were mostly whole within the gels (figure 3), possibly due to the cells not being affected by the peroxide. However, after staining there were no comet trails to be observed. It was soon realized that cells were not being left to unwind in the buffer for the allotted time required. Moreover, for the silver staining process, one of the solutions was found to use silver nitrate,

which is light sensitive, and the staining had to be done in a low light setting.

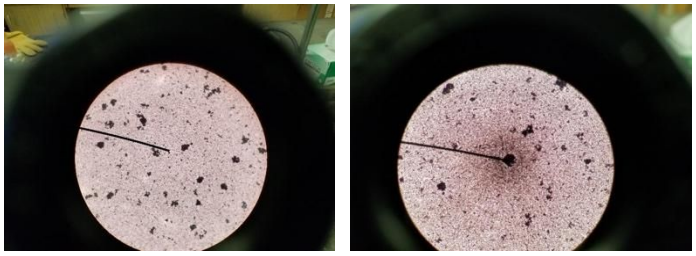

Fig. 4: The induced sample (left, 4a) holds cells which a intact with no trace of comets, but the uninduced sample (right, 4b) shows a cell with a light halo indicating some form of DNA damage.

The final assay was performed under corrected criteria with better results. The induced sample was observed with whole cells that lacked any signs of silver stains (Figure 4a), which indicated that the cells that produced Dsup protected the plasmid inside the cells. Moreover, the uninduced sample produced a light halo of sliver stain indicating the cells did not produce any Dsup leaving the plasmids vulnerable (figure 4b). Unfortunately, the halo is meant to be a comet, and the lack of a trail implies that the cells may not have traveled during electrophoresis, which possibly means the amps for the process were not high enough.

Most of the time spent on the research was done preparing the materials needed to run the comet assay. The assays themselves took roughly a week to perform, and even after multiple attempts getting comet trails on the samples were not completely possible with the time given to do the research. Moreover, analyzing the trails to get a quantitative result would have required a program to analyze and measure the trails along with a proper camera to take pictures of the samples. Though there were no definitive results, the multiple assays did imply that with more attempts the eventual results would have given consistent data that could have been properly analyzed.

More attempts with peroxide treated samples would be idea, as there were no results to imply that the Dsup gene protects against free radicals. Moreover, though the UV light treated samples eventually gave results that implied Dsup protects against it, further testing should be done to further expose the cells to UV light. It is suggested that thinner and smaller centrifuge tubes be used to push the Dsup proteins extremes, much like they may be exposed to in tardigrade cells. The idea also applies to the peroxide treatment with higher concentrations of peroxide per assay.

One of the biggest issues that persisted in the first comet assay was during the washes. The LMPA agarose layer that contained the sample would wash off the slide leaving only the few stray cells on the NMPA agarose. This explains why the first assay looks comparatively barren of cells compares to the later assays. It would be recommended to use a higher volume of agarose on the slides to anchor the samples better to the slides.

The initial idea when going into the research was to also perform site directed mutagenesis to disrupt certain nucleotide sequences. The sequences are believed to be critical in the function of Dsup proteins. A comet assay would be performed on mutated plasmids to observe if any trails are present where they are not meant to be. That being reiterated, a lot was learned from this study and more experimentation could lead to more appealing data.

REFERENCES

"Comet Assay." *Wikipedia*, Wikimedia Foundation, 18 May 2018, en.wikipedia.org/wiki/Comet_assay#Background

European Bioinformatics InstituteProtein Information ResourceSIB Swiss Institute of Bioinformatics. "Damage Suppressor Protein." *Dsup - Damage Suppressor Protein - Ramazzottius Varieornatus (Water Bear) - Dsup Gene & Protein*, 22 Nov. 2017, www.uniprot.org/uniprot/P0DOW4.

Hall, Jessica. "Tardigrade DNA Inserted into Human Cells Gives Them X-Ray Resistance." *ExtremeTech*, 23 Sept. 2016, www.extremetech.com/extreme/236180-tardigrade-dna-inserted-into-human-cells-gives-x-ray-resistance.

shimoto, Takuma, and Takekazu Kunieda. "DNA Protection Protein, a Novel Mechanism of Radiation Tolerance: Lessons from Tardigrades." *Life*, MDPI, June 2017, www.ncbi.nlm.nih.gov/pmc/articles/PMC5492148/.

Hashimoto, Takuma, et al. "Extremotolerant Tardigrade Genome and Improved Radiotolerance of Human Cultured Cells by Tardigrade-Unique Protein." *Nature News*, Nature Publishing Group, 20 Sept. 2016, www.nature.com/articles/ncomms12808.

Nandhakumar, S., et al. "Evaluation of DNA Damage Using Single-Cell Gel Electrophoresis (Comet Assay)." *Advances in Pediatrics.*, U.S. National Library of Medicine, 2011, www.ncbi.nlm.nih.gov/pmc/articles/PMC3127337/.

"New DNA Degradation-Suppressant Protein Found in Extremotolerant Tardigrade Genome." *GenomeWeb*, 20 Sept. 2016, www.genomeweb.com/sequencing/new-dna-degradation-suppressant-protein-found-extremotolerant-tardigrade-genome#.WrANb6jwaM8.

“ Tardigrade Protein Helps Human DNA Withstand Radiation.” *Nature News*, Nature Publishing Group, www.nature.com/news/tardigrade-protein-helps-human-dna-withstand-radiation-1.20648.

Turk, Victoria. “Scientists Identify Gene That Protects Tardigrades From Radiation.” *Motherboard*, 20 Sept. 2016, motherboard.vice.com/en_us/article/53dkz5/scientists-identify-gene-that-protects-tardigrades-from-radiation.