1999

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THE USE OF THE DNA COMET ASSAY TO DETECT GENETIC DAMAGE IN RANA PIPIENS TAKEN FROM AFFECTED VERSUS NON-AFFECTED SITES

A THESIS

In Partial Fulfillment
of the Requirements for
the Degree Bachelor of Arts with Distinction
in the Department of Biology
of the College of St. Benedict/St. John’s University

by

Lisa Marie Hood

April 23, 1999
The Use of the DNA Comet Assay to Detect Genetic Damage in *Rana pipiens* Taken from Affected Versus Non-affected Sites

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Deformed frogs have been appearing frequently in Minnesota and throughout the United States. One hypothesis for the cause of deformities is genetic damage as a result of pesticides. This project will use the DNA Comet Assay to determine the extent of genetic damage in affected sites (sites with a greater number of deformities) versus non-affected sites (sites with fewer deformities). The DNA Comet Assay involves electrophoresis of cells in which the membranes have been solubilized and the DNA uncoiled. Electrophoresis spreads the genetic material. The length of the spread indicates the amount of genetic damage in the form of single and double strand breaks. The results indicate a difference in frogs collected from affected sites and non-affected sites.

**INTRODUCTION**

Deformed frogs have been appearing in our environment since as early as 1740 (North American Reporting Center for Amphibian Malformations). Reports of malformed amphibians have been reported in 35 states in the United States and 3 provinces in Canada since 1986 (http://www.pcastate.mn.us/hot/frog-faq.htm). In Minnesota approximately 66 counties out of 87 have reported malformed frogs since 1996 (http://www.pcastate.mn.us/hot/frog-faq.htm). Possible explanations that have been suggested for causing these deformities include ultra-violet light, habitat destruction including global warming, chemicals including pesticides and herbicides, and parasites. The Minnesota Pollution Control Agency has been intensively investigating the causes since 1993. The problems of deformities in Minnesota frogs have been steadily increasing. In 1996 the MPCA received 180 reports of deformed frogs (Associated Press, 1998). This is a problem that needs to be solved. These serious effects showing up in amphibians could be an indicator of conditions that could result in damage to the entire ecosystem including the human population.

There are several reasons why amphibians may serve as indicator species. One is that frogs are adapted to two different environments. They spend part of their life in water, where they can be subjected to pollutants from the soil and they spend part of their life on land where they are
subjected to pollutants that may be present in the air or soil. A second reason is that the skin of
gulls is fairly permeable, which may allow substances such as pesticides to enter the body more
easily than most other animals. Pesticides may be affecting gulls directly by the run-off from
agricultural fields that have been treated with the chemicals or by pollutants that gulls inhale
from the air. Several studies have been conducted near these types of fields.

One study was done on six wetland ponds that were sprayed with methoprene, which is a
pesticide used for mosquito control. Fourteen of ninety-one frogs and tadpoles collected
demonstrated deformities, while four of seventy-seven showed deformities from the control
ponds (Byron, 1998). Another study was conducted by the National Institute of Environmental
Health Sciences (NIEHS) and the Minnesota Pollution Control Agency (MPCA) on Minnesota
ponds. In September of 1997, these agencies issued a press release stating that water from two
Minnesota ponds where abnormal frogs were found also caused embryos of the African clawed
toad (Xenopus) to develop abnormally (Kaiser, 1997). Another study done by Jim Burkhart from
NIEHS presented data showing that when organic compounds and metals were filtered out of the
Minnesota water, it caused fewer deformities. Another group from NIEHS has tested water from
ten affected sites and from ten reference sites and found that deformities appear only in water
from the affected sites (Kaiser 1997).

Pesticides are believed to cause genetic damage by damaging specific chromosomes or by
causeing chromosomes to malfunction. It is important to be able to detect the amount of
chromosomal damage in individual cells. In order to accomplish this, a DNA Comet Assay can
be used. This assay involves a microgel electrophoresis technique. The cells that have more
chromosomal damage will have longer tails. Basically there will be more migration of the DNA
away from the nucleus.
The alkaline DNA Comet Assay was first introduced by Singh et al. in 1988 (Belpaeme et al., 1996). This technique is capable of detecting a wide range of DNA damage including: single strand DNA breaks, any lesion capable of being transformed into a single strand DNA break at the alkaline pH used, DNA crosslinks, and incomplete excision repair events (Belpaeme et al., 1996). This type of technique is useful in studies of environmental toxicants, cancer and aging because these studies are tissue and cell-type specific (Singh et al., 1988). The Comet Assay involves a microgel electrophoresis technique during which cells are embedded in agarose gel on microscope slides. They are then lysed by detergents and high salts, and electrophoresed for a short period of time under alkaline conditions. It is important to have an alkaline buffer because this allows evaluation of DNA damage in single-stranded breaks rather than only double-stranded breaks. Once the DNA has been allowed to unwind, electrophoresis is performed on the fluorescent stained blood cells. Electrophoresis causes the genetic material to spread out. The more fragments of DNA there are, the longer the tail because fragments of DNA will be shorter than intact chromosomes and will move more rapidly in the gel. The length of the spread will be proportional to the amount of genetic damage. The more genetic damage, the more broken the DNA will be and the longer the spread.

There have been some recent findings suggesting that pesticides may be causing genetic damage which results in deformities. A study conducted by Martin Ouellet, a Canadian researcher, found a definite link between pesticides and deformities. Ouellet’s latest findings are from research conducted on about 30 ponds in the St. Lawrence River valley. The control ponds were those near abandoned farmland or land being used mainly for grazing with no recent pesticide use. This area contained frog populations with 0 to 2 percent deformities. Ponds near land that was being farmed and that was subject to a variety of pesticides, insecticides, and
fungicides had frog populations with an average of about twenty percent deformities (Meersman 1998). Most of the deformities that were found from this study were in the hind limbs (Meersman, 1998). It is believed that pesticides are causing genetic damage. These chemicals may be affecting the frogs early in development. The earlier in development a frog is affected, the greater the genetic impact could be on the phenotypic expression (Meersman, 1999). This is due to the fact that cells are going through mitosis at a much faster rate at an early stage in development than they are at a later stage in development.

I will attempt to find if there is a difference in the extent of genetic damage in *Rana pipiens* taken from several sites in Minnesota. I will be comparing two different groups of frogs. One group, the control, will be those *Rana pipiens* that have been taken from sites where few deformities have been found. The second group, the experimental, will be those *Rana pipiens* taken from sites where more deformities have been found. I will take blood from the heart of each frog and perform a DNA Comet Assay on the cells. By examining the tails of the electrophoresed cells, I can compare the two sites and determine if the frogs at these sites have red blood cells that produce tails of different lengths.
METHODS

The *Rana pipiens* tadpoles and frogs used for this experiment were collected from six different sites around the state of Minnesota by the Minnesota Pollution Control Agency. The three control sites, sites where fewer deformities have been found, were signified as BUR, LMS, and MHL. The three experimental sites, sites where deformities have been found, were designated as NEY, ROI, and CWB. The Minnesota Pollution Control Agency made collections on July 8, July 14, July 20, August 3, August 4, and August 19, 1998.

First a tube of the solidified 0.75% agarose and a tube of the 0.5% low melting point agarose were warmed to melting. Three hundred microliters of 0.75% agarose was placed on each of two slides and small coverslips were added. These slides were then placed at four degrees Celsius for ten minutes to allow the agarose to solidify. The slides were removed and the coverslips were carefully loosened and removed. A heparin coated syringe was used to collect blood. The heart of the *Rana pipiens* was punctured and its blood was drawn into the syringe. This blood was then mixed with the 0.5% low melting agarose. One hundred microliters of this blood/agar mixture was added to the slides with coverslips and again placed at four degrees Celsius for five minutes. The coverslips were removed and a top layer (100 microliters) of 0.5% low melting agarose was added along with coverslips and they were again placed at four degrees Celsius for five minutes. The coverslips were removed and the slides immersed in a lysing solution (1% sodium sarcosinate, 2.5M NaCl, 100mM Na EDTA, 10mM Tris, pH 10, and 1% Triton X-100 added fresh). The jar containing the slides was incubated at four degrees Celsius for one hour to lyse the cells and allow for DNA unwinding.

After the lysis, the slides were placed horizontally on an electrophoresis box. The electrophoresis box contained a fresh alkaline buffer (1mM Na -EDTA and 300 mM NaOH).
The pH of this buffer was thirteen. The box was filled with the buffer until the level was approximately 0.25 centimeters above the slides. The cells were then exposed to the alkaline treatment alone for forty minutes. The lights were dimmed and the box was in an ice bath throughout this whole process. The cells were then electrophoresed using 20 millivolts for ten minutes. Once this was complete, each slide was washed twice in 0.4M Tris, pH 7.5 for five minutes. Each slide was then removed from the Tris and fifty microliters of ethidium bromide (2 ug/mL) and coverslips were placed on each slide. Each slide was viewed under a fluorescent microscope using an objective lens with a magnification of 10x and twenty cells from each slide were measured from the center of the nucleus to the end of the tail. In order to measure the nuclear fragmentation, the cells were projected onto a video monitor and measured in centimeters using a ruler. Two slides were made for each Rana pipiens so forty cells were measured for each frog.
STATISTICAL ANALYSIS

The data collected from the experimental and control groups were compiled and a 2-tailed t-test was used to test for significance of differences between the two groups. A 2-tailed t-test is appropriate because I am testing to determine whether there is a significant difference between the means of the two groups. It is conceivable that the experimental group could have tails that are either longer or shorter than the control group. The null hypothesis being tested is that there is no difference between cells from control populations and experimental populations in the mean tail length of the nucleus. In order to test for a significant difference between the variances, a F distribution was used. The null hypothesis being tested here is that there is no difference between the variances of the control populations and the experimental populations.

RESULTS

The results indicate a significant difference in the mean nucleus tail length between the control and experimental sites. According to the 2-tailed t-test, the mean tail lengths of the two groups are significantly different. The t value is 2.175 with p<0.05. According to the F distribution test, the variances of the two groups are not significantly different. The calculated F statistic is 1.19 and at a 0.05 level of significance the critical value is 2.055. Since 1.19<2.055, the decision is to fail to reject the null hypothesis.

Table 1 The combined tail length means and variances for the experimental and control groups.

<table>
<thead>
<tr>
<th>Group</th>
<th>Mean tail length (cm)</th>
<th>Mean variance</th>
<th># of frogs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.067</td>
<td>0.133</td>
<td>19</td>
</tr>
<tr>
<td>Experimental</td>
<td>2.230</td>
<td>0.112</td>
<td>25</td>
</tr>
</tbody>
</table>
The mean tail length for the experimental group is larger than that of the control group (Table 1).

Table 2 Results of 2-tailed t-test.

<table>
<thead>
<tr>
<th>T-value</th>
<th>degrees of freedom</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.175</td>
<td>36.995</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

The results of the 2-tailed t-test indicate there is significantly more nuclear fragmentation in the experimental group compared to that of the control.

Table 3 Results of F distribution.

<table>
<thead>
<tr>
<th>Calculated F statistic of the variance</th>
<th>degrees of freedom</th>
<th>Critical value of F distribution (alpha=0.05)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.19</td>
<td>18, 24</td>
<td>2.055</td>
</tr>
</tbody>
</table>

According to the F distribution test there is not a significant difference between the variances of the experimental and control groups.

However it may be worth noting that the data collected for the nuclear tail lengths of the red blood cells in the control group, although not statistically significant, show greater variability than these same measures in the experimental group (Figures 1 and 2).

Figure 3 is a plot of mean DNA comet tail length in each frog versus variance of tail length in that frog. The experimental frogs show a much tighter clustering.
Figure 4 is a plot of the data collected for the red blood cells in the control and the experimental groups together. This plot shows the greater variability in the controls relative to the experimentals.
Figure 1  This figure shows the nuclear tail length for all the red blood cells counted in this experimental group of frogs.
Figure 2 This figure shows the nuclear tail length for all the red blood cells counted in this control group of frogs.
Figure 3 This figure shows the mean tail length for each frog versus the variance. The experimental values are yellow and the control values are blue.
Figure 4. This figure shows the nuclear tail length for all the red blood cells counted in the experimental group and the control group. The experimental values are yellow and the control values are blue.
DISCUSSION

In experiments done using the DNA Comet Assay, significant results have been found. When cultured hepatocytes were treated with either DMSO or dimethyl nitrosamine (NDMA), tails were produced that were much longer than control cells not exposed to these chemicals (Ashby et al., 1995). In another experiment, the comet assay was used to detect genetic damage in the erythrocytes of brown trout exposed for 3, 9, and 14 days to polychlorinated biphenyls (PCB’s). PCB’s are a type of pollutant that can be found in almost every area of terrestrial and aquatic ecosystems (Belpaeme et al., 1996). This test used Ethyl methanesulphonate at a concentration of 25 mg/L as a positive control. Results from this experiment showed that EMS induced a significant increase of single strand breaks in the comet assay (Belpaeme et al., 1996). As shown by these experiments, the comet assay is capable of detecting genetic damage.

The experimental group, sites where more deformities had been found, showed a higher degree of nuclear fragmentation than the reference group, which represented sites where fewer deformities had been found. This result demonstrates that there is more genetic damage in the experimental group than the control group. In addition to finding a significant difference in tail length, another interesting observation involves the pattern of comet tail lengths in the red blood cells.

Each frog has a population of red blood cells that varies in character. Some frogs have a higher mean with a tight variance, therefore signifying a population of cells that consistently show a higher level of nuclear fragmentation. Some frogs have a lower mean and a tight variance signifying a population of cells that consistently show nuclei with little fragmentation. Some frogs have a mixed population of cells with both high and low levels of fragmented DNA. In other words the variance is higher for these frogs. I would anticipate a healthy frog having a
low mean with a tight variance. In an experiment done on rat hepatocytes using the comet assay, the controls had a small variance and a low mean tail length in comparison to the experimentals (Ashby et al., 1995). This situation is not the case for most of the control frogs used in this study. Many control frogs have higher means with low variances and others have high variances. This suggests that the control sites, although not producing as many deformities, may have frogs that are acquiring genetic damage as evidenced by the presence of some cells with long DNA comet tails. According to Dr. Judy Helgen from the MPCA (personal communication), more deformities have currently been found at these reference sites.

Since the control group of frogs had a trend of producing frogs with more variance in their tail lengths than the experimental group, it is important to realize that the reference sites may also be sites where frogs are being exposed to the same stresses as the frogs from our experimental sites. It is difficult to obtain a control group that has not been affected by something in the environment. One possible way to obtain better controls would be to raise our own frogs in the laboratory. This approach would allow us to control the frog's environment.

Another aspect which could increase the precision of the data would be to draw blood from each frog at the same stage of their development. One researcher believes that pesticides could be damaging amphibian embryo DNA which would result in abnormalities during metamorphosis (Kaiser, 1997). It is not known at which stage of metamorphosis those results would be apparent. Some frogs were fully metamorphosed at the time of the blood draw, while others were only half-way through their metamorphosis. This may have caused a difference because the full extent of genetic damage may not have been truly detectable until the full metamorphosis. Frogs that were fully metamorphosed may have shown more genetic damage than those that were only half-way through their development simply because they are older and
have had more time to acquire the damage. Metamorphosis is a stressful stage in development. Possibly only the most healthy frogs survive metamorphosis. Younger frogs may be better able to tolerate higher levels of genetic damage that would show up as longer DNA comet tails because they have not yet been required to endure the test of metamorphosis. Therefore older frogs may be a selected population of better fit frogs. They may not have as much genetic damage because they would not have survived to this stage. This lack of uniformity in the developmental stage of frog subjects may also have contributed to the trend of more variation in the results.

There are several things that could be done to continue this research project. The first is to have a larger sample size. This would enable us to make site by site comparisons. The control and experimental sites were geographically paired. Since there was not enough data to test for significance between the paired sites, the data was pooled. Also with a larger sample size, the sites could be monitored over time to see if the level of DNA fragmentation would be increasing as evidenced by a higher mean comet tail length and tighter variance. Another idea would be to raise our own control frogs. This would allow us to minimize the effects the environment may have on these frogs. Since the control frogs we used were collected from their natural environments, they had been exposed to some UV light and pesticides, which may have led to genetic damage in their red blood cells. If we raised our own controls, we would be able to limit what each frog was exposed to and probably be able to draw more definite conclusions. Finally, another important idea would be to draw blood from the frog’s heart at a certain stage in the metamorphosis process. This may produce more precision in the data because the sampling stage would be the same for all of the frogs. All of these ideas could be used to obtain more information about the extent of genetic damage in Rana ples.
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