Are Your Eggs Broken? A Study of Chromosomal Breakages in Irradiated Chicken Embryos.

By

Renee Rheinecker

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Renee Rheinecker

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ABSTRACT

Chromosomal breaks can be provoked by ionizing radiation which could result in diseases such as cancer. The objective of this experiment is to quantify the amount of chromosomal damage that occurs from using diagnostic x-ray procedures. The range of radiation exposure will be increased by approximately 15% starting at 40 kVp and ending at 90 kVp. These numbers are used because they are the upper amounts of diagnostic x-ray exposure and some therapeutic levels, such as CT scans. The degree of chromosomal fragmentation will be determined by quantifyng DNA repair enzyme activity on whole cell preparations of early chicken embryos. To quantify the increase in γH2AX, a DNA repair enzyme at chromosomal breaks, following the x-ray, I will use tagged antibodies that can be visualized under microscopic analysis. The number of stained nuclei will correlate to the amount of DNA damage associated with diagnostic x-ray (FitzGerald et. al). After analyzing 100 cells per 20 slides received, there were a total of 11 positively stained cells and 1989 negatively stained cells for γH2AX. Through this experiment, our hypothesis has been rejected. There is no correlation between 15% increases in kVp and mAs with double strand DNA breaks. Because we saw some positive staining, we are leaning towards the conclusion that the diagnostic x-ray levels didn't cause significant damage to the chicken embryos.

Keywords: γH2AX, double strand DNA breaks, immunohistochemistry

INTRODUCTION

To begin with, we must first understand what DNA damage actually is. There are three main types of damage including strand breaks, mutations, and chromosome instability. Strand breaks can occur either as a double strand or single strand breaks. These refer to whether both or one strand of the DNA double helix is severed. Double-stranded breaks can occur for many different reasons including exposure to ionizing radiation, particular drugs like bleomycin, or even when DNA polymerase "runs into an unrepaired nick in the DNA" (Goodsell). A mutation is any change in the nucleotide sequence. Generally, they arise due to unrepaired DNA or RNA damage, errors from replication, or insertions or deletions in DNA segments. Chromosomal instability is generally a broad subject. According to David Gisselsson, it can "be defined as a state of continuous formation of novel chromosome mutations, at a rate higher than in normal cells" (Gisselson).

Now that we know what DNA damage is, we need to examine what causes the DNA to be damaged. Mutagens, or agents that change DNA, work both physically and chemically to change the DNA. Chemical mutagens include "aflatoxin (from mold), caffeine (found in coffee and colas), LSD (lysergic acid diethylamide; a hallucinogenic drug), benzo(a)pyrene (found in cigarette and coal smoke), Captan (a fungicide), nitrous oxide (laughing gas), and ozone (a major pollutant when in the lower atmosphere)" ("Mutation"). Physical mutagens encompass polymerase inserting the wrong base, incorrect chromatid separation, and radiation. DNA polymerase is the enzyme used in DNA replication. Their main job is to make DNA molecules from nucleotides in the cell during replication. Sometimes they insert the wrong base which will code for different proteins in the organism. During mitosis or meiosis, when cells are replicating and dividing, a stage called anaphase occurs. In this stage, the chromosomes are split and sister

chromatids move to opposite sides of the cell. Sometimes, the chromatids don't move to opposite sides causing one cell to have no genetic information while the other cell has both sets of information.

Next we need to understand the role of radiation in DNA damage. To begin with, we need to look at alpha and beta particles. Alpha particles are positively charged particles with high energy. Because of their high energy, they do more damage to cells, but are short-lived. One good thing about these particles is that they don't have enough energy to break past the skin cells, therefore creating no immediate concern. Similarly, beta particles have very low energy, but are negatively charged. Like alpha particles, there is no immediate concern with beta particles since they can be stopped by a substance as thick as one millimeter, including clothes. However, our main concern is gamma and x-rays. They are forms of ionizing radiation. Gamma and x-rays are very similar except where they come from, their wavelengths, and their photon energy. Gamma rays come from the atomic nucleus, have a wavelength of about 10 pm to 100 fm, and have photon energy around 100 keV to 10 MeV. X-rays come from electrons, have a wavelength of about 10 nm to 10 pm, and have photon energy around 100 eV to 100 keV. Most diagnostic x-rays occur at a wavelength between 100 to 10 pm and photon energy between 10 to 100 keV. One may think, how could something so small and seemingly harmful affect my body? When absorbed, radiation causes the atoms to become excited. Once excited, the molecules increase in reactivity which can cause secondary and tertiary reactions to take place creating damaged DNA. The radiation can also affect one's body indirectly by causing cellular injury. One of the main occuring injuries includes the formation of free radicals from water. This can affect processes such as homeostasis and lead to cellular death. According to x-ray safety, there are many factors that can lead to more or less damage including "total dose, dose rate, presence

of oxygen, sex, nutritional status, and other physiological factors which affect the body" ("Damage Mechanisms"). They go on to mention the regions exposed to radiation also affect the outcomes since our body includes radiosensitive organs, which are those high in mitotic activity and relatively undifferentiated.

We should now understand what DNA damage is and what creates it, but how can we fix it? Since molecular pathways are so intricate, systems of enzymes and proteins are in place to try to fix any DNA problems. When examining single stranded breaks, there are two types of repair: homologous recombination and nucleotide excision repair. Homologous recombination is where the nucleotide sequences between two similar or identical molecules of DNA are exchanged. Nucleotide excision repair occurs when a small area of the damaged strand of DNA is removed. The gap that is left is then filled with the proper base with the help of DNA polymerase and DNA ligase.

Double stranded breaks are similar to single stranded breaks in the use of homologous recombination as a mechanism for repair. However, it can also use non-homologous end-joining as another form of repair. In this process "two DNA ends are joined directly, usually with no sequence homology…" (Poplawski and Blasiak). Both 53Bp1 and γH2AX are proteins that are actively recruited to early DNA breaks and are necessary for repair through this mechanism of non-homologous end joining (Redon et. al). Because of this, the damage is kept localized until other repair proteins are recruited and have time to perform their respective functions.

At this point, you're probably wondering why we should even care about DNA damage and repair. However, DNA codes for genes and gene products. It is virtually what codes for the development and function of all living things. The smallest of damage to the code can lead to mutant proteins. Mutant proteins lead to a disruption in the cellular cycle. When the cell cycle is

dysregulated, a variety of events can take place including blockage of cellular checkpoints. Without these checkpoints, cells can undergo many mutation and uncontrolled cellular growth which then can lead to cancer. Damaging DNA's code can also lead to problems with cellular differentiation. Under normal circumstances, sometimes a less specialized cell can undergo changes and maturation causing it to have a more distinct form and function. When this process is damaged, cells usually don't mature and function properly. Through this experiment, we hope to determine the amount of double strand breaks caused by diagnostic x-rays in an avain model. We expect that low levels of exposure will have fewer breaks, and high levels of exposure will have more breaks as quantified by localization of double stranded break repair proteins.

METHODS

Obtaining eggs and irradiation

I obtained 48 fertilized chicken eggs, approximately 1-2 days old, from the Tyson Chicken Hatchery in Monett, Missouri. The male was a Hubbard M99 and the female was a Cobb 500. Each egg was then labeled with a number (1-48) which it would keep through the duration of the experiment. The eggs were also marked with an "x" on one side and an "o" on the other for rotation purposes. All of the eggs were then placed in a Farm Innovators still-air incubator (model 2100) at a temperature of 99.5 degrees Fahrenheit and humidity 55%. Three times a day at approximately 7:30 am, 12:30 pm, and 5:30 pm, the eggs were rotated 180 degrees. Eggs were candled each evening to check for growth and abnormalities. Eleven of the 48 eggs had no development, looked to be progressing slower than the other eggs, or were cracked and were removed from the study.

After seven days of incubation, the eggs were removed from the incubator and transported to the Health & Science Building on the campus of Missouri Southern State

University in Joplin, Missouri. After being weighed, the eggs were irradiated under the supervision of Dr. Alan Shiska. Individually, the eggs were placed on the x-ray table 14" from table top with a field of view of 8" by 8" and exposed to x-ray radiation, with the lowest setting of 40 kVp and a 200 mAs. These doses were increased by 15% for each subsequent group, to a maximum of 90 kVp and 634 mAs. A total of three eggs were exposed to seven settings (Table 1). Three eggs were mock-irradiated as a control.

Extraction, fixation and mounting slides

Following irradiation, the eggs were transported to Reynold's Hall for embryonic isolation. One at a time, the eggs were cracked into a shallow dish. Using foreceps, the embryo was isolated from all membranous sacks. The embryo was then decapitated with a scalpel blade and placed into a coincidingly numbered vial containing formalin. There was an average of 82 minutes between the time irradiated to the time placed in formalin. Once all embryos were extracted and in formalin, they were refridgerated at 4 degrees Celcius overnight. Approximatedly 24 hours later, the embryos were then placed in a solution of 70% ethanol and stored at room temperature. They were then packaged and shipped to the University of Arkansas-Little Rock to be sectioned and mounted onto slides.

Staining and analysis

Once the slides were received, a series of staining occurred using a Vectastain ABC kit. Each section/slide was deparafiniezed in xylene for 5 min, followed by rehydration in decreasing concentations of laboratory grade ethanol (100-50% for 5 min each), and a final rehydration in 30% ethanol for 1 hour. Following rehydration, the slides were prepared with an antigen unmasking solution for antigen retrieval. Afterwards, the slides were treated with a blocking solution. This step is used to ensure that the primary antibody does not connect with any protein

other than the γH2AX attached to double strand chromosomal breaks. A primary antibody of mouse anti-gammaH2AX (1:500 dilution in 1% goat serum in PBS) was used and then stored in a moist chamber at 4 degrees Celcius overnight. The same process was used for the secondary antibody of goat anti-mouse (1:200 dilution in 1% goat serum in PBS). Afterwards, a combination of the Vectastain ABC and PBS are added to the slides and incubated for 30 minutes. Once finished, the sections were incubated in a peroxidase solution until the appropriate stain intensity appeared. A counterstain of hematoxylin was also added until the appropriate stain intensity appeared. Finally, the stains underwent dehydration in increasing concentrations of laboratory grade ethanol (30-95% for 5 min each) and a final rehydration of 100% ethanol for 1 hour.

After staining was complete, each slide was observed under the microscope and a picture was taken. Using the picture, 100 cells were randomly counted and marked as positive for γH2AX (dark brown) or negative for γH2AX (blue or light brown).

Embryo number	Weight (g)	kVp	mAs	Exposure length (s)
2	65.5	40	200.91	0.6
3	67	40	201.03	0.6
5	67.2	40	201.06	0.6
6	65.4	46	251.19	0.5
7	57.3	46	251.31	0.5
9	71.3	46	251.04	0.5
10	70.7	53	321.23	0.5
11	67.8	53	321.46	0.5
13	69.8	53	321.64	0.5
14	64	61	401.76	0.6
15	58.2	61	402.22	0.6
16	64.8	61	402.16	0.6
18	65.2	70	502.78	1.2
20	57.9	70	502.91	1.2
21	63.1	70	502.89	1.2
23	63.4	80	633.75	2.5
24	60.5	80	633.74	2.5

26	72.6	80	633.66	2.5
28	63.6	90	634.53	2.9
29	73.7	90	634.46	2.9
30	67.4	90	634.46	2.9
45	63.3	n/a	n/a	n/a
46	66.8	n/a	n/a	n/a
48	60.3	n/a	n/a	n/a

Table 1: The settings used for 15% increases of kVp and mAs.

RESULTS

After analyzing 100 cells per 20 slides received, there were a total of 11 positively stained cells and 1989 negatively stained cells for γH2AX. The corresponding data can be seen in Table 2.

Increasing energy 	Brown (positive for γH2AX)	Blue (negative for γH2AX)
	0	100
	0	100
	0	100
	0	100
	3	97
	3	97
	0	100
	0	100
	3	97
	0	100
	1	99
	0	100
	0	100
	0	100
	1	99
	0	100
	0	100
	0	100
	0	100
	0	100
Totals	**11**	**1989**

Table 2: Embryo numbers corresponding to the data collected on presence of γH2AX.

DISCUSSION

Through this experiment, our hypothesis has been rejected. There is no correlation between 15% increases in kVp and mAs with double strand DNA breaks. We suspect these results could be caused by one of two things: the staining analysis didn't work, or the x-rays didn't do very much damage. Because we saw some positive staining, we are leaning towards the conclusion that the diagnostic x-ray levels didn't cause significant damage to the chicken embryos.

For further studies, I would recommend a few differences from this experiment. One could perform the same experiment, but instead of exposing the embryo still encased in its shell to diagnostic x-ray levels, one could cut a small window in the shell. This would give the embryo direct exposure in case the shell absorbed the bulk of x-rays. Another possible change could be to use embryos at a different embryonic age; perhaps embryos 7 or 8 days old not as sensitive to x-rays as embryos 4 or 5 days old. Changing the class or type of animal may also result in a different outcome. Would a mammal or reptile embryo yield the same results as our avian embryo? Is a Robin embryo more or less susceptible to x-rays than the chicken embryo? The last suggestion for further research would be to use a different assay for finding γH2AX. Using an assay that is more sensitive to the presence to γH2AX could generate different results.

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