ENVIRONMENTAL MONITORING OF CHINOOK SALMON

Dale Morris, Doug Boreham

McMaster University

Introduction

There is importance of monitoring non-human biota. The wew ICRP recommendations require monitoring of non-human biota. Monitoring of non-human biota will improve public image and ensuring the safety of the environment.

Radiation sensitivity is the cells' or organism's response to the detrimental effects of radiation. One species of bacteria Micrococus radiodurans can survive16,000 Gy, where hypersensitive human cells can only survive between 0.2 and 0.3 Gy. Even different tumours show differing sensitivities to radiation, making determination of the sensitivity before treatment very important. It is well known that some genes can greatly affect an individual's radiation sensitivity. Radiation sensitivity can be evaluated by many different laboratory techniques including; apoptosis detection, the micronucleus assay, spectral karyotyping, and cell survival and growth assays. All of the techniques are based on the cells' ability to correctly repair DNA double strand breaks. In the work here, we initially used the micronucleus assay to assess DNA repair capacity. Spectral karyotyping was subsequently used to better understand the fidelity of DNA repair. Apoptosis is the programmed cell death that eliminates compromised, damaged, virally infected or unneeded cells. Apoptosis can be initiated via the extrinsic pathway through cell suicide, a lack of survival signals, and cell murder, the receiving of external death signals.

The micronucleus technique is a relatively inexpensive and rapid method, to measure both chromosome loss and breakage (Fenech, 2000). The micronucleus assay allows the easy identification of genetic damage within a couple days, whereas other cytogenetic techniques are more time and labour intensive. Spectral Karyotyping (SKY) allows the easy identification of all the chromosomes by fluorescently colouring each pair a different colour using combinations of five different colour probes. With each chromosome appearing a different colour, it is relatively easy to identify abnormal chromosome. SKY is a very powerful tool for detecting aberrations caused by radiation between different chromosomes however SKY cannot detect all chromosomal rearrangements.

Materials and Methods

Cell Lines Culture Conditions

Human cell lines were maintained in DMEM F12 supplemented with 15% fetal bovine serum and 25mg/mL gentamicin (50 mg/ml). Rat cell lines were maintained in MEM-α

(supplemented with 10% fetal bovine serum and 1% Penicillin-Streptomycin ($1.0x10^5$ units of penicillin and $1.0x10^5$ µg of streptomycin per ml). All cell lines were maintained at 37°C in 5% CO_2 and 98% humidity. Phase contrast pictures were captured to ensure cell viability, while the cells were alive and growing on the surface of T75 flasks.

The Explant Technique

Chinook Salmon were euthanized using the MS222 in at concentration of 100mg/L. Using a scalpel a section of skin was cut and removed from the fish. The skin was gently scraped to remove underlying muscle and fat tissue. The skin was placed in a tube containing a special supplemented media; 420 ml RPMI 1640 media, 60mL fetal bovine serum, 12.5ml HEPES, 5ml L-Glutamine, 5ml Penicillin streptomycin amphotericin B, 1ml Hydrocortisone, 1ml Insulin, at 0°C for 5 minutes, the transferred to a second tube containing fresh media. The skin samples were washed in a Petri dish containing phosphate buffer solution (PBS) and the antibiotics penicillin, streptomycin, and amphotericin B (PSA) for 5 minutes, and repeated twice in fresh PBS and PSA. Put fresh media into a new Petri dish and segment the skin into smaller pieces about 3mm by 3mm. The skin pieces are places one piece into each T25 flask with just 1mL of RMPI media. The skin is placed in the middle of the flask scale side up, and the flasks are incubated at 19°C for 24 hours. Examine the growth ring of cells around the centre skin to see differ levels of cell growth. During high doses of radiation, cells will die, or stop dividing, resulting in smaller areas of growth at higher exposures.

Irradiation Dose Response Curves

Cell Line Irradiations

Cells were seeded into 9-cm^2 Nunc Glass Slide Flasks (Nalge Nunc International, Rochester, NY) at 2.0 x 10^5 cells per slide flaskette. While still attached in flaskettes and in 0°C PBS, cells were exposed to 0, 1, 2 and 4 Gy ^{137}Cs γ radiation at a dose rate of 0.1 Gy/min. Flasks were kept on ice for the duration of all irradiations. At the end of irradiation, the cold PBS was replaced with appropriate media warmed to 37°C. 1µL/mL of cytochalasin B (Sigma-Aldrich Canada Ltd., Oakville, Ontario) was added to each flask to block cytokinesis and cells were incubated for 24 hours to result in the formation of micronuclei.

Explant Irradiations

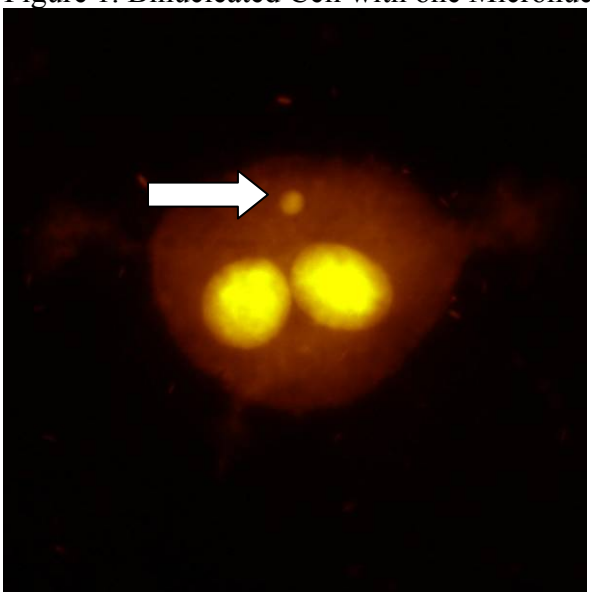
T25 flasks were removed from the incubator, and placed into a styrofoam container. While still attached in flask and at room temperature, explants were initially exposed to 0, 0.5, 1, 5 and 10 Gy ^{137}Cs γ radiation at a dose rate of 0.5 Gy/min. Later the experiment was repeated with higher dose up to 30Gy. At the end of irradiation, the media was discarded and replaced with fresh media. The flasks were placed back at 19°C and grown for another 5 days. 1µL/mL of cytochalasin B (Sigma-Aldrich Canada Ltd., Oakville, Ontario) was added to each flask to block cytokinesis and cells were incubated for 24

hours. Later experiments irradiated live fish first then followed the explant procedure to examine the effects of in vivo irradiation.

Micronucleus Assay

After 24 hours of incubation in cytochalasin B, the media was removed and cells were incubated in a hypotonic solution of 0.075M KCl for 5 minutes. The hypotonic solution was removed and the cells were then fixed in acetic acid:methanol (1:3, v/v) for 15 minutes. Slides were stained with 40µg/ml acridine orange in distilled water for 30 seconds, then washed for 2 minutes in distilled water. Acridine Orange stained binucleated rat cells containing micronuclei are shown in Figures 1A and 1B. Micronuclei were scored according to *Fenech 2001*, using a Zeiss Axioplan 2.0 imaging fluorescence microscope. See example figure 1. The more micronuclei observed the greater the exposure.

Figure 1. Binucleated Cell with one Micronucleus. The arrow points to a micronucleus.



Spectral Karyotyping

Metaphase chromosomes for all cell lines were prepared. Cells were detached using Trypsin/EDTA and treated with a 1.398g/L KCl solution for 20 minutes at 37°C. This was followed by fixing in methanol:acetic acid (3:1 v/v). Metaphase spreads were prepared using the Hanabi Metaphase Spreader to control humidity, temperature and drying time to ensure proper chromosome spreading. Slide pretreatment, hybridization, posthybridization washes, and detections were performed according to manufacturer's instructions. Spectral images were acquired and analyzed with a DM 230025 Applied Spectral Imaging spectral cube attached to an Olympus BX51 microscope using Acquisition Expo (ver. 3.0.1; ASI Ltd.) and analyzed by use of SKYVIEW® (ver. 2.1.1; ASI Ltd.) software. Chromosome aberrations were detected using DAPI staining with

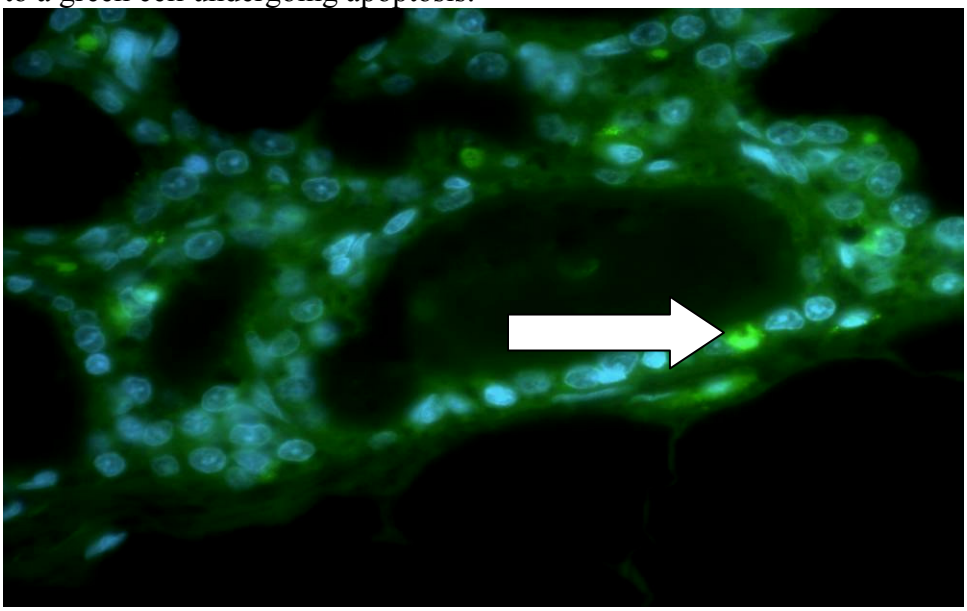
final image inverted so the chromosomes appear similar to G-banding, as well as the classification pseudocolor assignments. All the metaphases on each slide were captured and analyzed with a minimum of at least 50 metaphases. See example figure 2. The more chromosome rearrangements observed the greater the exposure.

Figure 2 Human Karyotype, showing both DAPI and pseudo coloured Chromosomes

Apoptosis Detection

The Apoptag® TUNEL assay was used to mark apoptotic cells in tissue slices.
The Procedure has 5 main steps; paraffin removal, protein digestion, enzyme binds to all 3’ nucleotide ends, created during apoptotic nuclear degradation, enzyme stained with flourescein antibody (green colour), and a second stain DAPI, binds to all DNA (blue colour). Slight changes where made in the procedure in order to reduce the amount of background Fluorescein fluorescence including reducing the amount of TdT enzyme used, and increasing the number and length of PBS 1x washes after the application of the Fluorescein conjugate. The end result of the procedure is all nuclei, including apoptotic nuclei, are stained blue with the DAPI stain. All apoptotic nuclei are stained green with the Fluorescein. See example figure 3. The more apoptotic cells observed the greater the exposure.

Figure 3. Slice of Tissue stained with Apoptag® TUNEL assay. The white arrow points to a green cell undergoing apoptosis.



Results

Dose response curves were constructed for Human cells, Rat cells, and Chinook Salmon cells. These graphs were made using lust the micronucleus data, because the human and rat cells cannot be examined using the explant procedure. The results for the fish explant experiments have not been completely finished. Given data is meant to show the expected results based on other experiments in our lab.

Figure 4. Human Cell Dose Response Curve

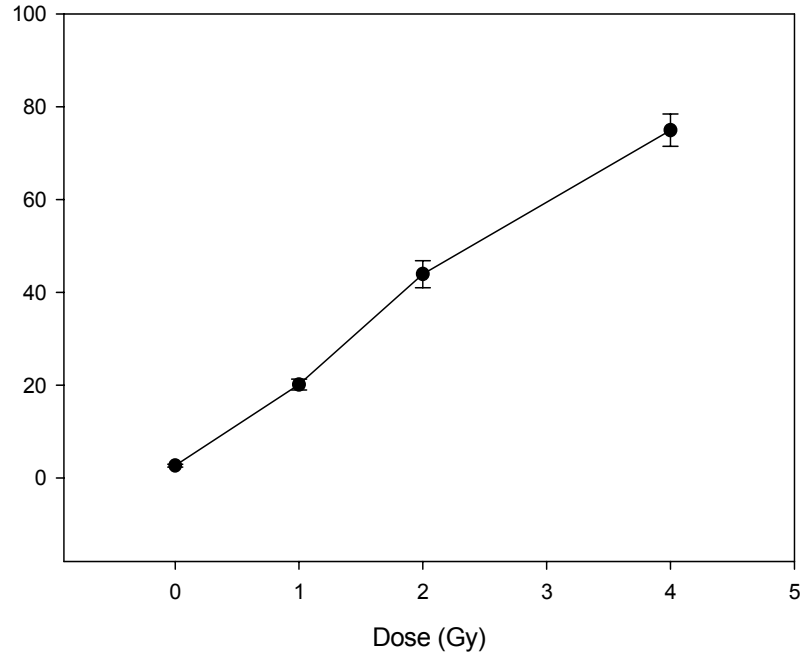
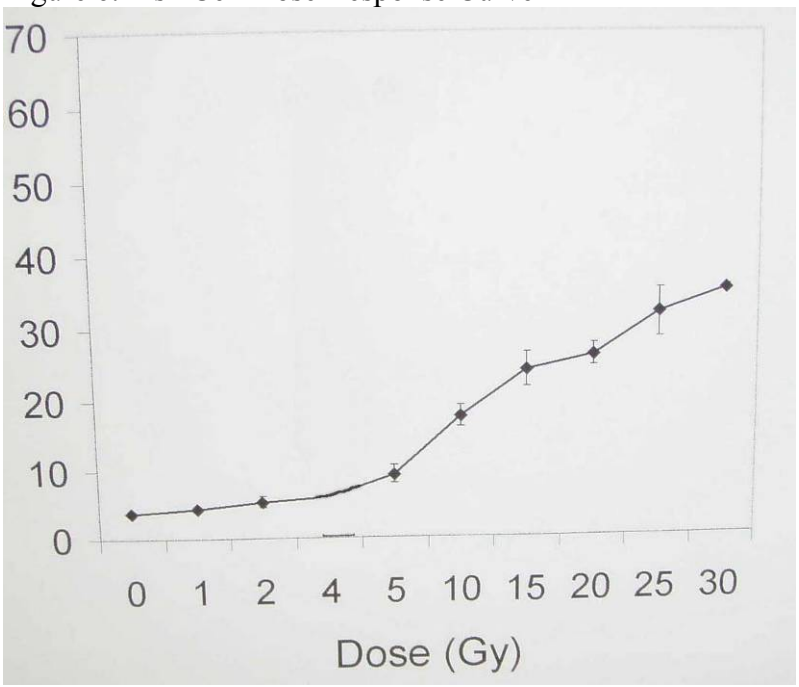


Figure 5. Rat Cell Dose Response Curve

Figure 6. Fish Cell Dose Response Curve



Discussion

Fish seem to be insensitive to the damaging effects of ionizing radiation. There are possible causes for these differences. Radiation is attenuated through the water, as a result the fish may be receiving less radiation, however the amount of water needed to cause these differences is far more than was used during irradiation. The fish swim underwater where there is reduced oxygen, and as such there is a lessen oxygen effect. Irradiation of tissue in the presence of oxygen produces damaging reactive oxygen species, which causes complex DNA damage that is difficult for cells to repair. The human and rat cells are grown in presence of higher than normal levels of oxygen, while the fish grow in lower levels of oxygen. Fish are cold blooded, and have a lower metabolic rate than the human and rat cells. Higher metabolic and cell division rates don't allow for as much time for the cells to repair before the cell divides. This is especially true for the human and rat cells used in this experiment, these cells were dividing at a higher than normal rate due to their culture conditions. A final reason is that fish may have better DNA repair mechanisms than mammals. Whatever the reason for the fish being more resistant to radiation damage, the different techniques can be compared to highlight their advantages and disadvantages for monitoring environmental exposures to radiation.

		Advantages	Disadvantages
Explant Growth area	–Examines difference between cell growth rates	–Doesn't require expensive lab equipment –Simple to examine	–Explant growth may not correlate with low radiation exposures –Requires sterile procedures
Spectral Karyotyping	–Examines chromosomes for incorrect DNA repair	–Can show effects from low doses –Very detailed level of DNA damage can be observed	–Requires very expensive lab equipment and training –Requires dividing Cells –Only available for humans, mice, and rats
Micronucleus Assay	–Examines cells for incorrect DNA repair	–Works for most animals including yeasts –Has been widely used for many toxicology studies –Simple to examine	–Requires expensive lab equipment –Requires dividing Cells
Apoptosis Detection	–Examines tissues for dying cells	–Works for most multi-cellular animals –Does not require a live animal –Simple to examine	–Requires expensive lab equipment –Many cells undergo apoptosis naturally

The micronucleus assay and apoptosis detection methods require the use of a fluorescence microscope to view the results. The spectral karyotyping also requires the use of a fluorescence microscope, but also additional equipment and software to examine the data.

Conclusion

Fish are less sensitive to radiation than both rats and humans. Current water effluent limits are more than enough to protect the fish, so salmon are not in any danger from any minor release. These procedures can be repeated for other biota in the environment, such as frogs, turtles. From the techniques used to monitor genetic damage in organisms, would be to use the micronucleus assay with the explant procedure if live specimens are obtainable. If live specimens cannot be obtained the apoptosis detection method would be the only method left to use. All of these techniques monitor genetic damage whether or not it is due to a minor radiation release or other possible chemical agents, such as nearby pesticide use. For this reason it is very important to use control animals of known exposures monitor other chemical agents that may be affecting your environment.