STUDIES OF SEA LAMPREY (PETROMYZON MARINUS)

REPRODUCTION AND STERILITY ASSESSMENT

THESIS

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By

Tobie Dianne Burks Wolfe, B.S.

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Master’s Examination Committee:

Professor Kenrad Dabrowski, Ph.D., Adviser
Professor Joseph S. Ottobre, Ph.D.
Professor Paul C. Baumann, Ph.D.

Approved by

Adviser
School of Natural Resources
ABSTRACT

Current attempts to control sea lamprey (*Petromyzon marinus*) populations in the Great Lakes include the use of lampricides that target sea lamprey larvae, or ammocoetes, living in riverbeds, and sterilization of mature adult males and females with the chemosterilant, bisazir. The mutagenic actions of bisazir are not evident until the latter stages of embryonic development; therefore assays directed at estimating the extent of DNA damage to gametes would be an attractive alternative to *in vitro* fertilization tests. Single cell gel electrophoresis (comet assay) was optimized for lamprey spermatozoan cells to detect bisazir-induced DNA damage and compared with fertilization rates for control and bisazir-treated animals. Comet assay can be applied to sea lamprey sperm DNA fragmentation and used to describe the relationship between sperm DNA damage and sperm fertilizing ability. The assay can be used reliably and accurately, and unlike in the case of mammals, there is no need for additional steps related to improvement of efficacy of lysis and DNA decondensation. This agrees with the presence of histone proteins in lamprey sperm. An increase in DNA fragmentation was noted during short-term storage of milt (on ice, 0 - 4 d). The genotoxic effects of UV radiation and oxidative stress (exposure to hydrogen peroxide) were demonstrated and it was revealed that oxidative damage to sperm DNA was likely repaired after fertilization in the embryo. The repairing capacity of the oocyte toward sperm DNA lesions caused by UV was
restricted. While the *in vivo* toxic effects of bisazir may be associated with the induction of DNA-DNA and DNA-protein cross-linking, it is likely that the genotoxicity is related to other mechanisms of DNA degradation, such as oxidation. Additionally, 8-hydroxy-2'-deoxyguanosine was evaluated in sperm of control and treated animals as a measure of genetic mutation in response to oxidative stress. As well, activation and fertilization rates were analyzed with the surprising finding that bisazir may be significantly accelerating spermiation/ovulation. Finally, the levels of bisazir in treated animals were measured by high performance liquid chromatography (HPLC) 10 hours after injection of animals and on several days thereafter to record its persistence in the body. The understanding of the reproductive mechanisms of sea lamprey and the mutagenic action of bisazir are essential for optimizing the timing and dosage of bisazir injections and for evaluating the efficacy of current control programs as well as alternative sterilants.
Dedicated
to my
husband
Mark

♥
∞
†
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For God so loved the world that he gave his one and only Son, that whoever believes in him shall not perish but have eternal life. – John 3:16

But those who hope in the Lord will renew their strength. They will soar on wings like eagles; they will run and not grow weary, they will walk and not be faint. – Isaiah 40:31

With God all things are possible. – Matthew 19:26
VITA

Jan. 2002 – Mar. 2003 ............................................. Student Research Assistant, School of
Natural Resources, The Ohio State University

Dec. 2002 ................................................................. B.S. Molecular Genetics,
The Ohio State University

Mar. 2003 - Mar. 2005 ........................................... Graduate Research Associate, School of
Natural Resources, The Ohio State University

PUBLICATIONS

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CHAPTER 1

INTRODUCTION

1.1. Sea lamprey (*Petromyzon marinus*) life history

A member of the class Agnatha, the sea lamprey *Petromyzon marinus* has, in accordance with paleontological records, remained unchanged for 300 million years and is characterized as a jawless vertebrate belonging to the group of boneless fishes. Representatives of the lamprey group, fish with an elongated, eel-like shape, have a skeleton composed of cartilage and a long soft notochord that preceded the evolution of the backbone found in true bony fishes. Sea lamprey can attain lengths of up to three feet in the ocean and 12-20 inches in the Great Lakes. Having no paired fins, the lamprey instead utilizes a dorsal boneless fin and muscular contractions to move throughout water. The skin of the lamprey is smooth, gray-blue to brown in color, and devoid of scales. Glands in the skin secrete a mucous substance that provides a protective barrier. Many species of lamprey parasitize other fish using an oral disc, with replaceable “teeth” set in rows in a circular pattern, to provide a suction-cup like attachment to prey as the teeth cut into the prey’s flesh. A tongue-like structure in the center of the oral disc rasps into the prey’s skin while glands in the mouth secrete anticoagulants. Lampreys breathe
through a series of seven gill apertures located on each side of the body posterior to the eyes. Water is squeezed in a pulsatile motion in and out of the gill apertures that open into gill pouches where blood absorbs oxygen. A single nostril is located on the dorsal surface of the head.

As an anadromous fish, the sea lamprey lives as a mature adult in the Atlantic Ocean (or Great Lakes) but returns to freshwater tributaries to spawn and die. Lamprey larvae, called ammocoetes, hatch from eggs after an approximately 9-day incubation period and remain burrowed in the sediment of the riverbed for 3-17 years (or 3-6 years for the Great Lakes) where they feed by filtering microorganisms. Ammocoetes undergo a metamorphosis to the mature adult and migrate downstream to the ocean (or Great Lakes) to parasitize on other fishes.

With the opening of the St. Lawrence Seaway, the non-indigenous sea lamprey gained access to the Great Lakes in 1932. Native to the North Atlantic Ocean with a distribution from Northern Europe to Northwestern Africa, and from Canada to Northern Florida, the invasion of the sea lamprey in the Great Lakes had a devastating impact not only on lake trout, *Salvelinus namaycush*, but on other fish assemblages as well (Coble et al., 1990). From 1937 to 1947, the lake trout population in Lake Huron fell from a harvest of 7.5 million kg / year to virtually no catch. Lake Michigan suffered similar declines, experiencing a drop from 12.1 million kg / year in 1946 to 885 kg / year in 1953. Sea lamprey predation, in combination with factors such as over fishing and hybridization, also led to the extinction of three endemic species of the Great Lakes: the long jaw Cisco *Coregonus alpenae*, the deepwater Cisco *C. johanni*, and the black fin Cisco *C. nigripinnis*. Additionally, by virtually eliminating the top predator, lake trout,
another invasive species native to the Atlantic, the alewife, was able to colonize the Great Lakes during the 1940’s, further decimating already impaired fish populations.

1.2. Sea lamprey control efforts

The Great Lakes Fishery Commission (GLFC) was created in 1955 in direct response to the threat that sea lamprey presented to the commercial and sport fishery industries. The purpose was to control and eradicate sea lamprey at an estimated cost of 10 million dollars annually. Current, integrated management of sea lamprey involves United States and Canadian governmental agencies.

Current attempts to control sea lamprey are mainly (1) treatment of streams with lampricides to kill larvae, (2) collection and release of sterile males (sterilized with bisazir) to decrease spawning success, and (3) provision of river barriers. Lampricide treatments frequently resulted in reduced growth and biomass of salmonids co-inhabiting small streams with lamprey (DuBois and Blust, 1994).

Sterilization and release of male sea lampreys is a promising strategy to control sea lampreys in the Great Lakes. The pioneering work of (Hanson and Manion, 1980) provided the evidence that \( p,p\)-bis-(1-aziridynyl)-N-methylphosphinothioic acid (bisazir) is a very effective chemosterilant for male sea lamprey (Figure 1.1). This compound has been extensively tested, and experimental implementation of the Sterile Male Release Technique in sea lamprey management began in 1991 (Twohey et al., 2003). Sperm parameters of bisazir-treated sea lampreys, such as sperm concentration, motility, and fertilizing ability at the 2-cell stage do not differ from control animals (Ciereszko et al., 2000). However, high or complete mortality occurs at the later stages of embryonic
development and consequently no normal larvae are observed at hatching (Ciereszko et al., 2002; Ciereszko et al., 2000). This points to a genotoxic action of bisazir on sea lamprey spermatozoa, however, there is no evidence that bisazir effect on sperm DNA has ever been studied.

The sterile male release technique (SMRT) was implemented in 1991, involving sterilization in a specialized facility at Hammond Bay Biological Station (Millersburg, MI). Sea lamprey are trapped in tributaries during their spawning run and transported to Hammond Bay. Personnel wearing personal protective gear, including air-purifying respirators and Tyvek® suits, feed the males (and, more recently, females) into a mechanical apparatus with a robotic auto-injector that automatically weighs and injects each animal with the appropriate amount of bisazir at a position approximating 46% from the anterior end to ensure injection into the gonads, thus avoiding the liver (Twohey et al., 2003). The animals are released into holding tanks for 48 hours where water from Lake Huron is continuously pumped to exchange the tank water 1-2 times per hour (Twohey et al., 2003). Effluent from the tanks is then filtered through carbon to remove any remaining bisazir (Twohey et al., 2003).

1.3. Justification

The main goal of this study was to demonstrate that Comet assay could be applied to monitor sea lamprey sperm DNA fragmentation and used to describe the relationship between sperm DNA fragmentation and sperm fertilizing ability. The single-cell gel electrophoresis (SCGE), or Comet assay, is a rapid and sensitive fluorescent microscopic method for detecting primary DNA damage at the individual cell level (Singh et al.,
The Comet assay combines a biochemical approach to detect DNA strand breaks and/or alkali labile sites with a single-cell approach that is typical for cytogenetic assays. In this microgel electrophoresis technique, a small number of cells are embedded in agarose gel on a microscopic slide. The cells are then lysed, electrophoresed, and stained with DNA stain (SYBR green). Cells with increased DNA fragmentation display increased migration of DNA from the nucleus toward the anode, which resembles the shape of a comet. The Comet assay is a very sensitive analytical tool and is able to resolve DNA break frequencies up to a few thousand per cell (Collins et al., 1997; Green et al., 1996). The Comet assay has been successfully applied to monitor damage to mammalian sperm DNA and related to male infertility (Irvine et al., 2000; Morris, 2002). Sperm cells display a high interspecies variability that is particularly apparent in the presence of protamines, sperm nuclear proteins that constitute one of most heterogeneous group of known proteins (Kasinsky, 1989; Saperas et al., 1994). Due to this variability, differences in sperm lysis and decondensation can occur, therefore Comet assay needs to be modified for each species (Shen and Ong, 2000; Singh et al., 1989).

Testing the effectiveness of potential genotoxic compounds should be facilitated with the use of methods for evaluation of damage to sperm DNA. It is crucial to determine the usefulness of Comet assay for monitoring of sea lamprey sperm DNA fragmentation and to test relationships between sperm DNA fragmentation and sperm fertilizing ability. Applying Comet assay for the examination of the action of bisazir on sea lamprey spermatozoa DNA should determine if DNA fragmentation is part of the genotoxic mechanism of bisazir. Although bisazir is an effective sterilant for sea lamprey
males. It is a very hazardous substance and must be handled with extreme caution. Sterilization of sea lampreys may only occur in a specialized facility at the Lake Huron Biological Station (Twohey et al., 2003).

Maintenance of DNA integrity is critical for preservation of sperm quality. Sperm DNA is tightly packed in order to reduce the volume of spermatozoa, to minimize damage by exogenous agents, and to keep the genome transcriptionally inactive (Singh et al., 2003). Additional protection of sperm DNA is exerted by seminal plasma antioxidants (Ciereszko et al., 1999). Despite all these defenses, sperm DNA damage does occur and is linked to male infertility (Irvine et al., 2000). Lack of repair mechanisms makes sperm DNA damage irreversible. Main factors involved in the etiology of DNA damage in the spermatozoa have been identified as oxidative stress, deficiencies in natural processes such as chromatin package and abortive apoptosis (Sharma et al., 2004). Testing DNA integrity is important for evaluation of sperm fertilizing potential and may help in evaluation procedures designed to keep the sperm integrity intact (such as sperm cryopreservation studies) or to monitor effectiveness of induction of DNA damage (such as sterilization studies).

Assessment of sterilization success and efficacy of bisazir (especially in relation to state of sea lamprey spermiation/ovulation) may increase the likelihood of achieving the goals of lamprey control programs. Such studies may lead to the development of new or modification of existing lamprey control procedures. Unfortunately, short-term biological fertilization tests cannot be used for evaluation of bisazir effects because its mutagenic action is observed only during later stages of embryonic development (Ciereszko et al., 2000). Ova exposed to spermatozoa from normal and bisazir-injected
males successfully activate and commence cleavage within 5 hours. However, bisazir affected embryos fail to survive to the pre-hatching stage, approximately 9 days after oocyte activation. Attractive alternatives to biological tests are assays directed at estimating the extent of damage to DNA. The goal would be the development of these assays as primary evaluation screening protocols for examining the potential effectiveness of alternative chemosterilants. Due to the addition of females to the GLFC sterilization program, studies on bisazir effect on female reproduction are also necessary. Procedures have been developed by which the success of fertilization at the 2-cell stage can be extrapolated to determine hatching percentages (normal controls, gossypol) (Ciereszko et al., 2000) and set criteria for evaluation of normal (control) and bisazir-treated sperm of sea lamprey, the latter of which can only be evaluated at the pre-hatched larvae stage (Ciereszko et al., 2000). Therefore, based on these procedures, the mutagenic actions of bisazir on sea lamprey spermatozoa was evaluated as an indicator of general physiological condition using single cell-electrophoresis (Comet assay) and compared with fertilization and hatching rates.

The 8-hydroxydeoxyguanosine (8-OHdG) assay was employed to quantitatively measure the oxidative DNA adduct, 8-hydroxy-2'-deoxyguanosine (8-OHdG), which forms under conditions of oxidative stress. DNA, or more specifically 2-deoxyguanosine, reacts with a hydroxyl free radical to form 8-hydroxy-2'-deoxyguanosine, which is a product of oxidative stress. The 8-OHdG assay is based on extraction and digestion of DNA for the purpose of estimating 8-OHdG concentrations resulting from DNA damage due to oxidative stress. 8-OHdG is a commonly studied biomarker of oxidative sperm damage due to its high specificity, potent mutagenicity, and relative abundance in DNA
Past studies have established a relationship between high levels of 8-OHdG in mammalian sperm and male infertility or poor semen quality (Fraga et al., 1991; Kodama et al., 1997).

1.4. Objectives

Objectives of the study were: (1) to evaluate usefulness of Comet assay performed according to the protocol provided by Trevigen Inc. (Anonymous, 2001) in sea lamprey spermatozoa and compare the results with mammalian and teleost fish sperm; (2) to analyze DNA fragmentation in relation to sperm short-term storage; (3) to evaluate effects of UV radiation and hydrogen peroxide on lamprey sperm DNA and fertilizing ability; and (4) to measure DNA fragmentation of sperm in bisazir-injected lampreys.

Additionally, to optimize the effectiveness of bisazir treatment in sea lamprey, in terms of timing and dosage, (5) the level of bisazir in sea lamprey tissues was estimated several hours after injection, three to four days after treatment, and at the time of ovulation or spermiation.

The sixth objective (6) was to establish a protocol for measuring 8-hydroxy-2’-deoxyguanosine (also abbreviated 8-hydroxydeoxyguanonsine, or, 8-OHdG) in sea lamprey spermatozoa and examine the relationship between the genotoxic actions of bisazir and the formation of the hydroxyl free radical adduct of deoxyguanosine, 8-OHdG.
Figure 1.1. The chemosterilant $P, P$-bis (1-aziridinyl)-$N$-methylphosphinothioic amide, or, bisazir. Chemical formula, $C_5H_{12}N_3PS$. Bisazir is a known alkylating agent.
CHAPTER 2

METHODS

2.1. Source and maintenance of animals

Summer 2002

Sea lampreys originating from the Cheboygan River in Michigan were obtained on four separate occasions from Hammond Bay Biological Station, Millersburg, Michigan (located on Lake Huron) during the 2002 summer spawning season. Some fish were injected intraperitoneally with bisazir (100 mg/kg) at the station and some were kept as controls (non-injected). Three batches were transported by ground from Hammond Bay to The Ohio State University Aquaculture Facility in the School of Natural Resources, Columbus, Ohio (June 1, July 12 and August 9, 2002). Fish were separated by sex and into control and bisazir-injected groups and transported in plastic bags filled with ice water and oxygen for 8-10 hours. Fish were acclimated to 17°C upon arrival to the aquaculture facility. Accelerated maturation of the second batch of animals (n=6 male and 5 female controls) was attempted by injecting animals intraperitoneally with prime and resolving doses of luteinizing hormone releasing hormone analogue (LHRHa, Sigma Chemical Company, St. Louis, Missouri). Animals not injected with LHRHa were kept as negative controls and injected with 0.6% physiological saline, NaCl (0.1 ml/100g body
weight). Animals were maintained at 15-20°C for 25-40 days in 4 circular 400-liter re-circulating tanks, with bisazir and control animals separated.

Prior to all hormone injections, organ harvests, and stripping of sperm/ova, animals were anesthetized with 0.1% tricaine methanesulfonate (MS-222, Argent) buffered in 0.3% sodium bicarbonate (Langille and Hall, 1988) for approximately 5 minutes.

**Summer 2003**

Similar protocols were used in the following year as described for 2002 but for a few modifications. Four batches of lampreys were obtained, transported, and maintained as described previously. Animals were collected from the Cheboygan River, Michigan and transported to the Hammond Bay Biological Station (Millersburg, Michigan) where they were injected intraperitoneally with bisazir at a dose of 100 mg/kg (with the exception of the August shipment, which consisted of control non-injected animals only) and immediately prepared for transport to OSU (June 17, July 10, July 24, August 7).

As lamprey approach spawning maturity, they undergo deterioration of body organs and a shutdown of several functions in preparation for complete investment in gonads and reproduction, with death naturally succeeding spawning. During the summer 2002 maintenance of animals in the Aquaculture facility, this natural deterioration of skin coupled with mucus secretions and mechanical problems with the water chilling system contributed to increased water turbidity and, subsequently, animal mortality. In order to reduce water turbidity, an activated carbon filter was constructed to fit in the chilling tank. The filter significantly decreased water turbidity and improved lamprey survival.
The chilling system was repaired and water circulating to the bisazir-treated tanks was not allowed to re-circulate but was drained out of the system through the overflow as freshly chilled water was continually added. Control tanks were allowed to re-circulate their water through the chiller. Additionally, LHRHa injections were administered soon after arrival of males and females to re-evaluate the response, i.e. spermiation and/or ovulation.

2.2. Sperm/ova collection, evaluation and fertilization

Male lampreys were anesthetized with 0.1% tricaine methenesulfonate (MS-222, Argent) buffered in 0.3% sodium bicarbonate (Langille and Hall, 1988) for approximately 5 minutes. Semen was gently stripped from spermiating males by running a thumb down the abdomen of the animal to push the gametes out and collect them in a plastic specimen cup. Care was taken to expel any urine before collection of sperm to prevent contamination. Sperm was stored on ice and evaluated under a light microscope for relative motility (% motile) and concentration (Ciereszko et al., 2002). The latter was measured using a Double Neubauer Counting Chamber.

Females were anesthetized with 0.1% tricaine methenesulfonate (MS-222, Argent) buffered in 0.3% sodium bicarbonate (Langille and Hall, 1988) for approximately 5 minutes. The eggs were gently stripped from ovulating females by running a thumb down the abdomen of the animal to push the gametes out and collect them in a plastic bowl where they were weighed and placed on an inverted plastic container, elevating the specimen approximately 10 cm above the surface of the ice. Eggs were used the same day for fertilization trials.
Two replicates were performed for each fertilization (or activation) including controls. Eggs (0.3 g) were fertilized in a plastic Petri dish with 30 ul of sperm and activated with 10 ml dechlorinated city water. The dish was gently swirled for a few seconds and allowed to rest at room temperature until the first cleavage appeared (~5 hours). Fertilization success was assessed in terms of percent reaching the 2-cell stage by manually counting the zygotes under a dissecting microscope:

**Dividing zygotes**

Dividing zygotes + Non-dividing eggs

All samples were transferred to egg baskets (~8 cm diameter each), placed in California hatching trays and maintained at ~14°C until the pre-hatching stage (8-9 days after fertilization). Embryos were counted manually under a dissecting microscope and the percent reaching the pre-hatching stage assessed:

**Live embryos**

Live embryos + Dead eggs

### 2.3. Single Cell Gel Electrophoresis (SCGE) (Comet assay)

Sperm cells to be evaluated by Comet assay were diluted with cold phosphate buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄·7H₂O, 1.4 mM KH₂PO₄, pH 7.4) to a final concentration of 250,000 cells per ml and used on the day of collection for Comet assay and fertilization trials.

A Comet assay reagent kit for single cell gel electrophoresis (SCGE, Trevigen, Inc., Gaithersburg, MD) was used. Cells were combined with molten (42°C) 1.0 M Agarose at a ratio of 1 to 10 (v/v) and immediately pipetted onto one of two sample cavities on Comet Slide microscope slides (Trevigen, Inc., Gaithersburg, MD). Slides were
incubated at 4°C in the dark for 30 minutes in order to solidify and adhere the agarose to the slide. The slides were then immersed in cold lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris base, 1% sodium lauryl sarcosinate, 1% Triton X-100, and 1% DMSO) for 60 minutes (4°C) followed by immersion in alkali solution (300 mM NaOH, 1 mM EDTA) at room temperature in the dark. After DNA unwinding, slides were subjected to alkali electrophoresis for 30 minutes (300 mM NaOH – 1 mM EDTA, pH > 13). Electrophoresis was performed in a Sub-Cell BT cell, 15 x 15 cm gel (Bio-Rad, Hercules, California). Current parameters were 25 V, 300 mM. A maximum of 12 slides could be electrophoresed concurrently. After electrophoresis, samples were immersed in 70% ethanol for 5 minutes and allowed to dry on a slide warmer set on low. Once the slides showed signs of drying, they were removed from the heating plate, allowed to air dry in the dark overnight, and then placed in a slide storage box with a desiccant. DNA was stained with diluted SYBR Green (Trevigen, Inc., Gaithersburg, MD) for epifluorescence microscopy. The excess dye was tapped off the slide and an anti-fade solution (UVDE FLARE Assay Kit, Trevigen, Inc., Gaithersburg, MD) was applied to each circle. The slides were mounted, sealed with nail polish and viewed immediately.

Comets were scored using Komet 5.5 (Kinetic Imaging, Bromborough, Wirral, United Kingdom) software. Approximately 25-50 comets were measured per sample (i.e., slide circle). Previous studies investigating comet measurement were performed by manually scoring comets using a 5-type scale (0-4) related to the extent of DNA damage (Green et al., 1996). Zero damage was scored as 0 and maximal damage (induced by 100 μM H₂O₂) was scored as 4. While limited by the parameters that can be measured, this method is also prone to inconsistency based on “scorer bias”. Additionally, subtle effects
of mutagens may go undetected by the human eye when scoring comets visually. Therefore, Komet 5.5 software (Kinetic Imaging, Wirral, United Kingdom) was purchased and tested as an unbiased tool to measure various parameters of the comet and to detect damage to DNA that may be missed by visual scoring. Emphasis was placed on optimizing the capabilities of the software to provide quantitative measurements of comet parameters for direct evaluation between treatments. Following is a descriptive list of parameters that Komet 5.5 measures (Kinetic Imaging, Wirral, United Kingdom):

Comet intensity profile: mode, mean, std. dev., extent, distributed moment, inertia, skew (i.e. asymmetry), coefficient of variation, optical intensity

Head parameters: mode, mean, std. dev., extent, distributed moment, inertia, skew, coefficient of variation, optical intensity

Tail parameters: mode, mean, std. dev., extent, distributed moment, inertia, skew, coefficient of variation, optical intensity

Global Comet Parameters: head % DNA, Tail % DNA, comet optical intensity, tail length, tail from center, tail/head, Olive tail moment = (tail mean- head mean)\*tail %DNA/100, extent tail moment = tail length * tail % DNA/100

2.4. Alkali Comet assay of sperm from negative control and bisazir-treated sea lamprey

Semen was collected as described in section (§) 2.2 from eleven control and eight bisazir-treated males and stored on ice for use the same day. Sperm motility and
concentration were evaluated (Ciereszko et al., 2002). DNA fragmentation of sperm cells was quantified following a Comet assay that was performed on sperm cells from four of the eleven control males and four of the eight bisazir-injected males. Results were compared to experiments from 2000 and 2001 (data of all years including 2002 were evaluated by different investigators).

2.5. Effect of hydrogen peroxide (H$_2$O$_2$) concentrations on DNA fragmentation of sea lamprey spermatozoa

Hydrogen peroxide is an oxidizing substance causing fragmentation of DNA in fish cells (Michelmore and Chipman, 1998). For this reason, it can be used as a positive control for Comet assay. Spermatozoa differ from somatic cells in having more compactly packed DNA and different nuclear proteins. This may modulate the yield of comet production and their shape.

Semen from eight males was collected as described in § 2.2 and stored on ice for use the same day. Spermatozoa were suspended in ice-cold PBS containing 0, 100 and 1000 µM (0.1 mM and 1 mM) H$_2$O$_2$. Suspensions were incubated on ice for 30 minutes immediately followed by the Comet assay procedure as described in § 2.3.
2.6. Effect of short-term ice storage on motility and DNA fragmentation of sea lamprey spermatozoa

Sperm was collected by stripping (§ 2.2) and stored on ice in a cooler for 0, 1, 2 and 4 days. Following each respective incubation period, cells were counted using a hemacytometer and diluted to 250,000 cells ml\(^{-1}\) for Comet assay. Comet assay was performed as described in § 2.3.

2.7. Effect of UV irradiation on DNA fragmentation of sea lamprey spermatozoa

Sperm was collected by stripping and stored on ice (§ 2.2). Samples of 150 µl of sperm were diluted with 850 µl of sea lamprey Ringer’s solution (137 mM NaCl, 12 mM KCl, 1 mM CaCl\(_2\), 10 mM HEPES, pH 7.4) (Ciereszko et al. 2000). Part of the diluted sample (500 µl) was placed on a glass Petri dish sitting on ice and an 8 mm magnetic stir bar was placed in the dilution. The Petri dish was placed on a magnetic stirrer and irradiated using the Stratalinker (Stratagene, La Jolla, California). Sperm was irradiated for 0, 0.1, 0.2, 0.3 or 0.6 minutes. The Petri dish was covered with a “black-painted” lid to prevent photoactivation before and after UV exposure. Eggs (0.3 g) were immediately inseminated with an excess of irradiated sperm (200 µl) and covered with 5 ml dechlorinated city water. Irradiated sperm (10 µl) was pipetted into 10 ml of ice cold PBS, counted with a hemacytometer, and diluted to final concentrations of 250,000 cells ml\(^{-1}\) for Comet assay (§ 2.3).
2.8. Effect of potassium permanganate (KMnO₄) on DNA fragmentation of sea lamprey spermatozoa

Sperm was collected by stripping and stored on ice for use the same day (§ 2.2). Stock solutions of KMnO₄ were prepared by mixing 25 μl of PBS (negative control), and 25 or 250 μM KMnO₄ with 1.325 ml ice cold PBS. Ten microliters of each stock solution was added to 90 ul of sperm and incubated on ice for 30 minutes. Relative sperm motility was then assessed. Samples of 0.3 g of eggs were fertilized with 200 ul of diluted sperm and covered with 5 ml of dechlorinated city water. Sperm was counted on a hemacytometer and diluted to 250,000 cells ml⁻¹ for Comet assay (§ 2.3).

2.9. Analysis of bisazir in fish tissues using high performance liquid chromatography (HPLC)

Blood and other tissues (liver, kidney, gonads, and muscle) were collected from male and female, bisazir-treated sea lamprey within 9-12 hours after injection with bisazir, or at the onset of ovulation (females/some males).

Bisazir in transport and holding water of sea lamprey was separated by HPLC with modification of the method described by (Scholefield et al., 1997). No extraction steps were applied for the water sample. The water sample was directly filtered through a syringe filter (0.45 μm, Whatman Inc., Clifton, NJ, USA) and injected into the HPLC system (Beckman, San Ramon, CA, USA. Model 406 System Gold system controller with 110B solvent delivery system, 7725i Rheodyne injector, System Gold 166 programmable detector, and 202 Peak Simple data system). The column used for the separation was a Phenomenex Luna C18 column, No. 00G-4252-EO (Phenomenex,
Torrance, CA). Individual liver samples, weighing approximately 0.5 g each, were combined with 1.5 ml of an extraction solvent (50% methanol in water), homogenized (Model: Omni 5000, Omni International, Inc., Marietta, GA, USA) for 30 s, and then centrifuged (Beckman, model J2-21, Fullerton, CA) for 30 min at 10,000 x g at 4°C. After centrifugation, an aliquot of the supernatant was diluted 1:10 with demineralized distilled water to obtain a desirable concentration for detection. The diluted sample extract was filtered with the syringe filter and injected into the HPLC system. The HPLC system consisted of two delivery system pumps (Model 506A, Beckman Instruments Inc., San Ramon, CA, USA) equipped with a 20 µl injection loop connected to a 4.6 mm × 150 mm Phenomenex Discovery C-18 column (Phenomenex USA, Torrance, CA, USA) packed with octadecyl-bonded porous silica gel (5 µm). The UV detector (Programmable detector module 166) was purchased from Beckman Instruments Inc., San Ramon, CA, USA. The UV wavelength was set to 210 nm. The flow-rate was set to 1.0 ml/min. The mobile phase was composed of water and methanol (1:1, v/v). An external standard curve of bisazir was made for the calculations. Detection levels for bisazir was < 1.0 ng/20 µl with a signal-to-noise ratio of 3 for UV detection at 210 nm.

Individual gonad and muscle samples were also weighed out to approximately 0.5 g and treated exactly like the liver samples. Kidney samples were treated similarly with the exception of individual sample sizes, which were approximately 0.3 g each. Urine samples were diluted 1:5 with demineralized distilled water to obtain a desirable concentration for detection prior to filtration and injection into the HPLC system.
2.10. Effects of bisazir injection on gonadal maturation, hatching and larval viability of progenies

Both genders, on average 15 males and 15 females per treatment (control and bisazir injected), were injected with luteinizing hormone releasing hormone analog (LHRHa) (Sigma, St. Louis, Missouri) at a dose of 200 µg/kg as a primary dose. All males received only one LHRHa injection while females were administered a resolving dose of 500 µg/kg. Females were checked for signs of ovulation by palpating the abdomen and observing any release of eggs in tanks, or by anesthetizing and attempting to strip the eggs (§ 2.2). When the first females were ovulating, males were checked for sperm release by gentle massage of the abdomen.

Fertilization trials were performed in Petri dishes using 0.3 g of eggs (~200 eggs) and an excess of spermatozoa corresponding to ~50,000 spermatozoa per egg or 30 µl of milt per dish. Sperm samples were composed of a mixture of 3-5 control males (non-bisazir). Bisazir-injected males were examined individually for fertilizing ability. Gametes in all experiments were activated with water (10 ml) and incubated first at room temperature for 5 h (2-cell embryo stage), then transferred to California-type hatching trays for 9-10 days (pre-hatching stage) at 14-17°C.
2.11. Optimization of 8-hydroxy-deoxyguanosine (8-OHdG) assay of sea lamprey sperm as a measure of DNA damage due to oxidative stress (Protocol based on (Huang et al., 2001))

DNA Extraction

Milt from sea lamprey was collected by gentle stripping and placed on ice. Sperm motility of each individual was assessed and sperm concentration estimated microscopically using a Double Neubauer Counting Chamber. The milt was centrifuged and the resulting supernatant (seminal plasma) was measured and removed leaving behind a sperm pellet. The sperm pellets were stored in Eppendorf tubes at -70°C until the initiation of DNA extraction. All buffers and reagents used for DNA extraction, DNA digestion, and HPLC/EC analysis were prepared with C18 triple filtered HPLC water and filtered through a 0.2 μm filter.

Sperm pellets were removed from storage, placed on ice, and diluted with 1 ml of room temperature phosphate buffered solution (PBS, 10 mM) to restore previously measured sperm concentrations. The tubes were gently tapped and aspirated with a pipette to facilitate dispersal of all cells. Persistent cell clumps were carefully disrupted with a hand-held, battery-powered mini-homogenizer fitted for 1.5 ml Eppendorf tubes. A volume of sperm + PBS corresponding to a total of 75 million cells was pipetted into a 15 ml tube. Each tube received 6 ml of Cell Lysis Solution (CLS) (Puregene, Gentra Systems, Minneapolis, MN) and 36 ul of diethylenetriamine pentaacetic acid (DTPA). DTPA prevents artificial DNA oxidation by chelating iron that may be released from red blood cells (RBC) or cytoplasm. The contents of each tube were mixed by pipetting up and down. Next, 240 ul of 1M dithiothreitol (DTT) (Sigma, St. Louis, MO) solution and
30 ul of Proteinase K (20 mg/ml) (Puregene, Gentra Systems, Minneapolis, MN) were added to each sample. The tubes were inverted 25 times and incubated at 55°C for 1 hour in a water bath. Tubes were inverted 25 times after 30 minutes of lysis.

Samples were removed from the water bath, inverted 25 times, and 30 ul of RNase-A Solution (Puregene, Gentra Systems, Minneapolis, MN) was added to each tube. The samples were mixed by inverting 25 times and incubated at 37°C in a water bath for 30 minutes. After cooling the samples to room temperature, 2 ml of Protein Precipitation Solution (Puregene, Gentra Systems, Minneapolis, MN) was added to the RNase-A treated cell lysate and vortexed vigorously at high speed for 20 seconds. Samples were cooled on ice for 5 minutes and centrifuged at 2000 x g for 10 minutes to precipitate all proteins. The supernatant containing suspended DNA was carefully poured off the protein pellet into a clean 15ml tube containing 100% isopropanol (HPLC grade, Fisher Scientific, Pennsylvania) and 6 ul of glycogen (Puregene, Gentra Systems, Minneapolis, MN). Glycogen increases DNA yields by acting as a carrier. The tubes were gently mixed on a rocker for 1 minute and centrifuged at 2000 x g for 3 minutes, precipitating the DNA into a small white pellet. The supernatant was poured off and the tubes were drained on a paper towel.

DNA was washed twice with 70% isopropanol, centrifuging after each washing. The isopropanol was poured off the pellet and the DNA was dissolved in HPLC grade water. The absorbance of the DNA was measured at 260 nm to determine the ug of DNA successfully isolated (Beckman 640 DU Spectrophotometer) and the 260 / 280 nm ratio determined.
Preparation of Standards

(Performed at New York University School of Medicine, Department of Environmental Medicine. NY. NY, courtesy of Dr. Krystyna Frenkel’s lab)

Nucleosides (2’-deoxycytidine (dC), 2’-deoxyinosine (dI), 2’-deoxyguanosine (dG), thymidine (dT), and 2’-deoxyadenosine (dA)) (“cigta”) (Sigma, St. Louis, MO) were weighed and added to triple filtered HPLC grade water at a concentration of 6 mM, except for dA, which was prepared to a concentration of 3 mM. The solutions were incubated at 37°C for 5 minutes and vortexed to ensure complete dissipation. A 1 mM concentration of total nucleosides was prepared by mixing 1 ml each of dC, dI, dG, dT and 2 ml of dA. Aliquots of 200 ul of the mixture were pipetted into Eppendorf tubes, dried under vacuum centrifugation (SpeedVac, Savant, Holbrook, NY) and stored at -70°C. The concentration of 8-hydroxy-2’-deoxyguanosine (8-OHdG) (Sigma, St. Louis, MO) in the standard was measured by dissolving ~1 g of 8-OHdG in 1 ml of triple filtered HPLC water, measuring the UV absorbance at 248 nm and dividing by the extinction coefficient of 12,300. 8-OHdG was diluted to a final concentration of 1 uM and aliquoted into 100 ul portions, dried and stored at -70°C. Standards were prepared for HPLC analysis by adding 1 ml of HPLC water to the 8-OHdG tube and 200 ul of water to the “cigta” tube. The solutions were allowed to sit for 20 minutes prior to HPLC and then combined in equal amounts for a final concentration of 0.5 mM cigta and 0.05 uM 8-OHdG. During HPLC sampling, 5, 10, 20 and 40 ul of the standard (corresponding to 2.5-20 nmoles nucleosides and 0.25-2 pmoles 8-OHdG) were used to prepare a standard curve.
Deoxyribonucleic acid from salmon testes (ST DNA) (Sigma, St. Louis, MO) was hydrated in triple filtered HPLC water at a concentration of 5 ug/ul. Thirty ug (6 ul) of ST DNA was aliquoted into one set of Eppendorf tubes and 100 ug (20 ul) of ST DNA was aliquoted into another set of tubes to serve as DNA standards. The portions were stored at -70°C until needed for use as digestion controls.

**DNA Digestion and HPLC Separation**

*(Performed at New York University School of Medicine, Department of Environmental Medicine, NY, NY, courtesy of Dr. Krystyna Frenkel’s lab)*

Isolated and hydrated DNA samples from sea lamprey were aliquoted in ~40 ug DNA portions into Eppendorf tubes for digestion. One tube containing 30 ug of ST DNA and one tube of 100 ug ST DNA were pulled from storage for digestion. HPLC water was added to the 100 ul mark on all sample tubes. Each tube was gently tapped to ensure even dispersal. The following solutions, previously prepared, were added to each tube: NaCl, 2 ul 5M; MgCl₂, 10 ul 100mM; Tris, 1 ul 1M (pH=7.4); DTPA, 2 ul 2mM; and DNase I, 2 ul 20 units/ul (Sigma, St. Louis, MO). Each tube was gently tapped to create a homogenous mixture, quickly spun in a mini-centrifuge and incubated in a 37°C water bath for 30 minutes. Samples were acidified with 1 ul 3M (pH=5.2) acetate buffer and digested with 1 ul 1 unit/ul NP1 (Sigma, St. Louis, MO). After 1 hour of incubation, the PH was raised with Tris, 10 ul 1 M (pH=8.0) and 1 ul 1 unit/ul AP added for digestion. Following 30 minutes of incubation, 1 ul each of PDE I (0.1 unit/ul) and PDE II (0.01 unit/ul) were added to ensure complete digestion of DNA and incubated another 30 minutes.
Samples were transferred to filter tubes with a molecular weight cut-off of 5,000 (Sigma, St. Louis, MO) and spun at ~12,000 x g for 20 minutes. The filtered samples were transferred to HPLC sample collection tubes and 60 ul of DNA hydrolysates were analyzed by HPLC (Beckman Coulter Inc., Fullerton, CA) coupled with UV (Model 166) and EC (Coulochem II, ESA Inc., Chelmsford, MA) detectors, via an autosampler (Model 507e). The system utilized 2 pumps with 4-way solvent programming (Model 126). Data from the UV and EC detectors was collected by Gold Nouveau Software (Beckman Coulter Inc., version 1.6) installed on an IBM computer. The mobile phase incorporated 50 mM sodium acetate buffer (pH 5.2) in 6% aqueous methanol with a flow rate of 1 ml/min. Normal nucleosides (dC, dT, dG, dA) were detected by UV absorption at 254 nm and the EC detector using analytical cell 1 at 400 mV and 20nA identified 8-OHdG. The column used on the HPLC system was a Rainin ODS C_{18} protected by an Ultrasphere ODS guard column. A standard curve was created for normal and oxidized nucleosides and was used to quantify the levels of each compound in the samples. The degree of DNA damage was expressed as 8-OHdG per 10^6 total normal nucleosides or as 8-OHdG per 10^6 dG.
CHAPTER 3

RESULTS

Sampling

Sperm was obtained in 2002 from only 1 of 6 T4 males, 8 days following LHRHa injection, and from 6 T6 males, 5 and 8 days after the T4 group received hormone injections (See Table 3.1 for treatment key). Eggs were obtained from 1 of 5 T4 females 8 days after LHRHa injection, and from 4 T6 females, 11 and 14 days after the T4 group received hormone injections. The poor response to hormone injection as seen in 2002 could be attributed to poor water quality and segregation of the sexes for both control and bisazir-treated groups. The following season (2003) yielded significant increases in survival and number of animals reaching achieving ovulation/spermiation. To cite a representative example; of the control animals collected on 7/24/03 (Table 3.2), 8 T1 and 6 T2 males spermated while 1 T1 and 8 T2 females ovulated. Of the bisazir group, 6 T3 and 8 T4 males spermated while 4 T3 and 9 T4 females ovulated.
3.1. Alkali Comet assay of sperm from negative control and bisazir-treated sea lamprey

As part of an ongoing sterile-male-release program to control sea lamprey in the Great Lakes, males are sterilized with injections of bisazir. Sterility of these lampreys was confirmed in previous in vitro studies (Ciereszko et al. 2002).

The current study sought to determine if sterility of sperm might be correlated to the alkali Comet assay intensity of nucleus fragmentation and measured by Komet 5.5. Manual scoring has previously shown very low levels of damage compared to the maximal damage of the positive control (H₂O₂ effect).

According to the manufacturer’s instructions, alkali electrophoresis will detect single-stranded DNA breaks, double-stranded DNA breaks and the majority of AP (apurinic, apyrimidinic) sites as well as alkali labile DNA adducts (e.g., phosphoglycols, phosphotriesters). AP sites result from the loss of a purine or pyrimidine residue from the DNA while the alkali labile adducts are chemical compounds formed by an addition reaction that are unstable under alkaline conditions. Representative comets of fish spermatozoa are shown in Figures 3.1-3.5.

Sperm motility and concentration were recorded for each treatment group and are shown in Table 3.3. Mean sperm concentration for the control males selected for Comet assay was 1.14 x 10^9 ml⁻¹. Likewise, mean sperm concentration for the bisazir-treated males was 0.95 x 10^9 ml⁻¹.

Sperm from the negative control group of males demonstrated no DNA damage. Comet appearance for this group was described as an intact head (or, nucleus/nucleoid) with no trailing DNA fragments. These comets were brightest, as all DNA was still
within the “head” and, therefore, fluorescence was concentrated in this region. Bisazir treated spermatozoa demonstrated no visibly detectable DNA damage. Heads were intact and bright with large diameters and none-to-very small tails (Figure 3.2 A, B).

Results of the computerized analyses confirmed results obtained earlier by visual scoring (Ciereszko et al. 2002). A small but significant difference in the percentage of head and tail DNA was found between control and bisazir-injected males (Figure 3.6). No significant differences in the other comet measurements were found, presumably due to high variability among bisazir-injected males (n=4).

The same analyses were repeated the following year, 2003, and although the same trends in differences between the control and bisazir-injected males were revealed, no statistically significant differences were found, likely due to high variability between individual bisazir-injected males, as quantified by measurement of various comet parameters of (Figure 3.7) (n=8-10). These data suggest that the response of individual males to bisazir may vary. Interestingly, the fertilizing ability of bisazir-injected animals in 2003 was unusually high when compared to previous years. Perhaps lower DNA fragmentation revealed by Comet assay may substantiate the lower efficacy of bisazir for sterilization in 2003. Additionally, variability between individual males may be attributable to programming errors within the bisazir auto-injector at the SMRT facility. It is feasible that some animals did not receive enough bisazir sufficient for sterilization.
3.2. Effect of hydrogen peroxide (H$_2$O$_2$) concentrations on DNA fragmentation of sea lamprey spermatozoa

The objective of this experiment was to determine the usefulness of epifluorescence microscopy and Komet 5.5 software for determination of damage to DNA of sea lamprey spermatozoa.

Milt collected from eight male lampreys had sperm concentrations of: 0.66, 1.17, 1.59, 1.08, 0.96, 1.57, 0.25, and 2.635 x 10$^9$ ml$^{-1}$; mean concentration was 1.24 x 10$^9$ ml$^{-1}$. A large effect of H$_2$O$_2$-induced damage to DNA of sea lamprey sperm was observed in all treatments as compared with the control (Figure 3.8). Control spermatozoa had minimal damage. Sperm exposed to 100 and 1000 μM (0.1 and 1.0 mM) H$_2$O$_2$ demonstrated high and maximally damaged spermatozoa, respectively. Comet shape was characterized by round heads with a few trailing DNA fragments. With increased damage, the shape of the tails became elongated and oval in appearance, with some separation between head and tail observed in some samples. Maximal damage to sperm DNA was characterized by the absence of a comet head (Figure 3.2 C). All or most DNA was concentrated in the tail. No sperm remnants were observed.

Increased concentrations of H$_2$O$_2$ caused a significant decrease of sperm DNA in the head and an increase in the tail as well as in tail length (Figure 3.8). Both “extent tail moment” (tail length * tail % DNA/100) and “Olive tail moment” ((tail mean- head mean)*tail %DNA/100) increased with an increase in H$_2$O$_2$ concentrations. These data suggest that all major comet measurements are useful for evaluation of H$_2$O$_2$ effects on sea lamprey spermatozoa degradation. Additional studies measuring comet scores for a
series of H$_2$O$_2$ concentrations between 0 and 0.1 mM is needed to establish a progressive range of DNA damage as affected by H$_2$O$_2$.

3.3. Effect of short-term ice storage on motility and DNA fragmentation of sea lamprey spermatozoa

Over the course of this study, it was logistically necessary to store sperm of sea lamprey on ice prior to activation of eggs. This experiment was performed to test whether progressive DNA damage occurs as storage of sperm on ice is prolonged.

A significant decrease in head and an increase in tail DNA was found during short-term storage of sea lamprey milt (Figure 3.9). Those differences paralleled the observed decrease in sperm motility (60 ± 4, 37 ± 12, and 14 ± 9%, for 0, 2, and 4-day storage, respectively). These results indicate for the first time that an increase in DNA fragmentation may be associated with sea lamprey sperm storage on ice.

3.4. Effect of UV irradiation on DNA fragmentation of sea lamprey spermatozoa

UV irradiation is a known DNA mutagen. To further test the efficacy of the Comet assay and Komet 5.5 program, an additional positive control was tested (H$_2$O$_2$ was the initially tested positive control). This was deemed necessary to further evaluate the Komet 5.5 software since the observed damage to bisazir treated spermatozoa is very low.

Comet assay appears to be a useful tool for monitoring of UV effects of sea lamprey sperm DNA. Dose-dependent effects of UV on DNA fragmentation were indicated for most comet characteristics (Figure 3.10). Those changes paralleled dose-
dependent decreases in sperm fertilizing ability measured at pre-hatching stage (71.4 ± 5.2, 29.6 ± 2.3, and 15.3 ± 1.6%, for 0, 0.1, and 0.2 min exposure, respectively). No changes in sperm motility were observed in relation to UV irradiation (data not shown).

3.5. Effect of potassium permanganate (KMnO₄) on DNA fragmentation of sea lamprey spermatozoa

KMnO₄ was tested as an additional positive control, although it was suspected that this treatment could not induce as severe changes as ultraviolet light radiation or H₂O₂. To develop accurate procedures for measuring bisazir treated spermatozoa, it is necessary to test a wide range of agents with known impact on DNA.

Representative comets from these experiments are shown in Figure 3.5. Potassium permanganate, tested as an additional positive control, did not induce significant DNA damage as measured by Comet assay.

3.6. Analysis of bisazir in fish tissues using high performance liquid chromatography (HPLC)

The HPLC system effectively separated bisazir based on chromatograms produced (Figure 3.11). In all chromatograms, bisazir peaks were distinct, occurring at the same retention time.

Bisazir treated animals were segregated into male and female groups and held in a closed water system for twelve hours during transport from Millersburg, Michigan to Columbus, Ohio. Ice in plastic bags was used for cooling. Upon arrival at The Ohio State University, Columbus, Ohio, water and tissue samples were collected. Liver samples
from four bisazir treated males contained an average of 174.7 μg/g of bisazir and liver samples from four bisazir treated females contained an average of 129.2 μg/g of bisazir (Figure 3.12). The water in which the male animals were held during one collection trip contained an average of 3.21 ± 1.77 μg/ml of bisazir 12 hours after the lamprey were injected and, on another trip, 5.9 ± 3 μg/ml after 9-10 hours. The water housing the females contained an average of 2.90 ± 1.24 μg/ml of bisazir after one trip and, on another collection trip, 7.7 ± 2.6 μg/ml after 9-10 hours (Figure 3.13). Animals were held in a closed water system with ice for 9-12 hours, depending on the travel time for an individual collection date.

In 2003 (data for 2002 was too sparse due to high animal mortality), bisazir concentrations were measured in liver, kidney, gonad, and muscle tissues (Figure 3.13; Tables 3.4 -3.5). Bisazir concentrations in both liver and water samples of male lamprey were 54% and 10%, respectively, higher than those found in females while average whole body weights of males exceeded females by only 55.3 g, or 22% (Tables 3.4 -3.5). There appears to be a trend indicating that females may be eliminating bisazir from the liver more rapidly than the males but are not excreting the chemical from their bodies as quickly. It is likely that females accumulate and hold bisazir in their gonads, as female gonads account for ~22% of the animal’s whole body weight while male gonads account for less than 2% of a male’s total body weight (Table 3.7). The highest concentrations of bisazir were found in female gonads (Figure 3.13). The percent of total injected bisazir sequestered in the female gonads 9-10 hours after bisazir injected was 8.5% as compared to only 0.7% in the male. Perhaps bisazir tends to preferentially accumulate in the gonads. However, it is important to emphasize that no statistical significance concerning
preferential organ accumulation of bisazir was found. The high variability between individual animals underlines the need for additional data. Additionally, it is noteworthy that the SMRT facility switched to a different bisazir manufacturer between 2002 and 2003. The possibility of quality control variabilities between manufacturers’ chemicals must be presented as a possible explanation for the difference in liver accumulation of bisazir as estimated for these two years (Figures 3.12 and 3.13).

3.7. Effects of bisazir injection on gonadal maturation, hatching and larval viability of progenies

The results indicate that both genders of T4 experienced accelerated spermiation/ovulation over the T2 group. Bisazir females ovulated on average 4.3 ± 0.58 days after the initial injection with LHRHa while control females ovulated on average 7.5 ± 1.87 days after the initial LHRHa injection (Figures 3.14 and 3.15). The bisazir significantly accelerated ovulation by 3.2 ± 2.19 days (P < 0.01). Five of the 11 control females were injected a second time, 4 days after the initial LHRHa injection. This may have accelerated ovulation of these control females over those injected only once (Figures 3.14 and 3.15). However, until this observation was made, the intention of the study was not to compare the effect of LHRHa, but solely to “synchronize” ovulation in lamprey.

All males received only one LHRHa injection and were checked for spermiation when the first ovulating females were detected. The males were not systematically checked for the first signs of spermiation, therefore, the effect of bisazir on accelerated male maturation cannot be analyzed independently. The first ovulating T4 females were found
in both experiments (July and August) exactly 4 days after the initial LHRHa injection. Despite having no record of earlier spermiation, when males were checked at this time (4 days), 100% of the T4 males were spermiating compared to 50% of T2 and T3 males (Figure 3.16). Males were checked the second time at 6 days after the initial LHRHa injection. All of the T4 males were spermiating as well as 100% of the T3 males (Figure 3.16). The percentage of T2 spermiating males increased to 66% and the percentage of T1 males spermiating remained the same at 44% on both sampling days (Figure 3.16). Bisazir significantly accelerated spermiation in males (P < 0.01).

Fertilization experiments were performed to test the viability of eggs obtained from bisazir treated females. Milt from control males was used to test the effectiveness of bisazir to prevent egg fertilization. Likewise, milt from individual bisazir treated males was tested against control, untreated females. Fertilization (or, as in the case of bisazir-affected spermatozoa, activation) rates were assessed at the 2-cell and pre-hatching stages. The activation rate for bisazir males with control females at the 2-cell stage was high but then decreased significantly by the pre-hatching stage (Table 3.6). The fertilization rate for bisazir females with control males was 66.3 ± 34.0 at the 2-cell stage and also decreased significantly in the pre-hatching stage (Figure 3.17, Table 3.6). However, females did not achieve complete sterilization as in the case of males. Females successfully fertilized, as evidenced by viable embryos at the pre-hatching stage, may be less susceptible to the chemosterilant effects of bisazir. The two control females who did not have any viable progeny by the pre-hatching stage may have had eggs that were either not completely ovulated, or were over-ripe, at the time of fertilization (Figure 3.17).
3.8. Optimization of 8-hydroxy-deoxyguanosine (8-OHdG) assay of sea lamprey sperm as a measure of DNA damage due to oxidative stress

A commercial kit was successfully utilized for isolation of sperm DNA and preparation of a digest of lamprey sperm for nucleoside chromatography (Fig. 18). More specifically, the degradation product of 8-hydroxy-deoxyguanosine was identified and measured. There were no significant changes in nucleoside concentrations between bisazir-treated and control lamprey sperm (Figure 3.19). However, bisazir treated males had significantly greater concentrations of 8-OHdG than controls, and when 8-OH-dG was expressed in terms of total dG or total nucleosides, the data were statistically significant for bisazir treated males (Figure 3.20). These results need to be viewed in the context of the earlier analysis of bisazir concentrations in testes (Tables 3.4 and 3.5) and sperm viability (hatching rate, Table 3.6). Subsequently, further analysis is required to compare the level of 8-OH-dG with individual fertility. Results of 2003 analyses indicated that bisazir concentrations varied in testes by a factor of 8-10 suggesting that treatments (doses) were extremely variable between individual fish. This may be attributable to dose accuracy errors occurring in the auto-injector. Secondly, some bisazir treated males provided sperm with comparably high fertility. Therefore, we can conclude that despite the relatively high complexity of 8-OH-dG analysis, it may offer a very reliable measure of DNA degradation (oxidation) resulting in sperm infertility when physiological inferiority (motility, insemination capacity, i.e., percentage of 2-cell embryo) was not observed.
<table>
<thead>
<tr>
<th>Treatment</th>
<th>Treatment Abbreviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control + Saline</td>
<td>T1</td>
</tr>
<tr>
<td>Control + LHRHa</td>
<td>T2</td>
</tr>
<tr>
<td>Bisazir + Saline</td>
<td>T3</td>
</tr>
<tr>
<td>Bisazir + LHRHa</td>
<td>T4</td>
</tr>
<tr>
<td>Control only</td>
<td>T5</td>
</tr>
<tr>
<td>Bisazir only</td>
<td>T6</td>
</tr>
</tbody>
</table>

**Table 3.1.** List of treatment abbreviations used throughout text. Control groups received no bisazir injections and no bisazir placebos (saline). Bisazir groups received one bisazir injection at a dose of 100 mg/kg of body weight. Groups were given LHRHa, or a hormone placebo (physiological saline, 0.6% NaCl, 0.1 ml/100g body weight), or no hormone placebo.
Table 3.2. Initial number (n) of animals at each collection period (indicated by date). Animals are separated by date of collection, sex (M/F = number of males/number of females) and treatment (see Table 1 for treatment definitions). Numbers for 2002 are not shown. High mortalities were experienced in 2002 due to inadequate filtration and mechanical problems with the water chilling system.
<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Sperm concentration (x $10^9$ sperm/ml)</th>
<th>Sperm motility (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control males (n=11)</td>
<td>1.02 ± 0.47</td>
<td>36.0 ± 28.1</td>
</tr>
<tr>
<td>Bisazir-treated males (n=8)</td>
<td>0.70 ± 0.41</td>
<td>46.9 ± 24.9</td>
</tr>
</tbody>
</table>

**Table 3.3.** Mean (± SD) characteristics of sperm from control and bisazir-treated males. Number of individuals sampled (n) is indicated. Sperm concentration was estimated using a hemacytometer. Motility was evaluated by visual examination under a light microscope.
<table>
<thead>
<tr>
<th>Sex</th>
<th>Code</th>
<th>Liver (µg/g)</th>
<th>Kidney (µg/g)</th>
<th>Gonad (µg/g)</th>
<th>Muscle (µg/g)</th>
<th>Blood plasma (µl/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female</td>
<td>9</td>
<td>17.26</td>
<td>10.87</td>
<td>43.76</td>
<td>6.34</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>17.17</td>
<td>15.95</td>
<td>61.40</td>
<td>11.25</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>23.89</td>
<td>16.79</td>
<td>8.62</td>
<td>15.43</td>
<td>-</td>
</tr>
<tr>
<td>Mean ± SD (n=3)</td>
<td></td>
<td>19.44 ± 3.85</td>
<td>14.54 ± 3.20</td>
<td>37.93 ± 26.87</td>
<td>11.01 ± 4.55</td>
<td>-</td>
</tr>
<tr>
<td>Male</td>
<td>7</td>
<td>15.31</td>
<td>11.49</td>
<td>26.38</td>
<td>9.15</td>
<td>19.87</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>57.14</td>
<td>37.68</td>
<td>41.27</td>
<td>34.27</td>
<td>66.00</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>3.30</td>
<td>3.213</td>
<td>5.47</td>
<td>2.42</td>
<td>8.00</td>
</tr>
<tr>
<td>Mean ± SD (n=3)</td>
<td></td>
<td>25.25 ± 28.26</td>
<td>17.46 ± 17.99</td>
<td>24.37 ± 17.98</td>
<td>15.28 ± 16.79</td>
<td>31.29 ± 30.64</td>
</tr>
</tbody>
</table>

One-way analysis of variance revealed no significant differences in bisazir concentration of various tissues between females and males.

**Table 3.4.** Bisazir (individual and mean ± SD) concentration in tissues 10 hours after injection with bisazir (6-17-03). Animals were contained within a closed water system with ice for 10 hours prior to tissue collection. Excreted bisazir was confined to the system and thus available for repeated uptake.
<table>
<thead>
<tr>
<th>Sex</th>
<th>Code</th>
<th>Liver (μg/g)</th>
<th>Kidney (μg/g)</th>
<th>Gonad (μg/g)</th>
<th>Muscle (μg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female (242.3 ± 9.6 g)</td>
<td>20</td>
<td>12.54</td>
<td>6.44</td>
<td>111.55</td>
<td>2.19</td>
</tr>
<tr>
<td></td>
<td>21</td>
<td>17.47</td>
<td>9.27</td>
<td>1.04</td>
<td>5.54</td>
</tr>
<tr>
<td></td>
<td>22</td>
<td>13.97</td>
<td>6.89</td>
<td>2.83</td>
<td>5.35</td>
</tr>
<tr>
<td>Mean ± SD (n=3)</td>
<td></td>
<td>14.66 ± 2.54</td>
<td>7.53 ± 1.52</td>
<td>38.47 ± 63.30</td>
<td>4.36 ± 1.88</td>
</tr>
<tr>
<td>Male (317.3 ± 32.0 g)</td>
<td>17</td>
<td>67.84</td>
<td>42.94</td>
<td>198.66</td>
<td>22.02</td>
</tr>
<tr>
<td></td>
<td>18</td>
<td>25.02</td>
<td>12.05</td>
<td>14.85</td>
<td>11.60</td>
</tr>
<tr>
<td></td>
<td>19</td>
<td>21.06</td>
<td>18.56</td>
<td>45.37</td>
<td>9.11</td>
</tr>
<tr>
<td>Mean ± SD (n=3)</td>
<td></td>
<td>37.97 ± 25.94</td>
<td>24.52 ± 16.28</td>
<td>86.29 ± 98.50</td>
<td>14.24 ± 6.85</td>
</tr>
</tbody>
</table>

One-way analysis of variance revealed no significant differences in bisazir concentration of various tissues between females and males.

Table 3.5. Bisazir (individual and mean ±SD) concentration 9 hours after injection with bisazir (7-10-03). Animals were contained within a closed water system with ice for 9 hours prior to tissue collection. Excreted bisazir was confined to the system and thus available for repeated uptake.
<table>
<thead>
<tr>
<th></th>
<th>Males</th>
<th></th>
<th>Females</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Bisazir</td>
<td>Control</td>
<td>Bisazir</td>
</tr>
<tr>
<td></td>
<td>N</td>
<td>5</td>
<td>7</td>
<td>16</td>
</tr>
<tr>
<td>2-cell embryo</td>
<td>Survival (%)</td>
<td>73.4 ± 21.3</td>
<td>74.2 ± 25.8</td>
<td>81.8 ± 18.8</td>
</tr>
<tr>
<td>Pre-hatching</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N</td>
<td>5</td>
<td>7</td>
<td>14</td>
<td>12</td>
</tr>
<tr>
<td>Survival (%)</td>
<td>72.9 ± 21.7</td>
<td>8 ± 18.6</td>
<td>71.7 ± 23.3</td>
<td>21.4 ± 30.5</td>
</tr>
</tbody>
</table>

**Table 3.6.** Sea lamprey survival rates at 2-cell zygote and pre-hatched larvae stages. Data was combined from three batches of fish obtained from Lake Huron Biological Station, Millersburg, Michigan. The first two batches of fish originated from the Cheboygan River. All others originated from the St. Mary’s River. Survival expressed as mean ± SD.
<table>
<thead>
<tr>
<th></th>
<th>Female (n=6)</th>
<th>Male (n=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average total body weight (g)</td>
<td>247.4 ± 57</td>
<td>302.7 ± 37.3</td>
</tr>
<tr>
<td>Average total gonad weight (g)</td>
<td>53.7 ± 13.3</td>
<td>3.6 ± 0.3</td>
</tr>
<tr>
<td>Gonadal proportion of total body weight (%)</td>
<td>21.7</td>
<td>1.2</td>
</tr>
<tr>
<td>Average concentration of bisazir in gonads (ug/g)</td>
<td>38.2 ± 43.5</td>
<td>55.3 ± 71.8</td>
</tr>
<tr>
<td>Average total amount of Bisazir in gonads (mg)</td>
<td>2.1</td>
<td>0.2</td>
</tr>
<tr>
<td>Estimated amount of bisazir Initially injected at 100 mg/kg (mg)</td>
<td>24.7</td>
<td>30.3</td>
</tr>
<tr>
<td>Proportion of injected bisazir sequestered in the gonads (%)</td>
<td>8.5</td>
<td>0.7</td>
</tr>
</tbody>
</table>

**Table 3.7.** Characteristics of sea lamprey injected with bisazir and calculated values of bisazir deposited in the gonads. Males and females were injected with bisazir at a dose of 100 mg/kg body weight. n = number of animals sampled. Weights and average concentration expressed as mean ± SD.
Figure 3.1. A typical comet showing DNA migrating away from the sperm nucleus/nucleoid (head), forming a “tail” of DNA fragments. The tail length is positively correlated with the extent of DNA damage. Likewise, high fluorescence intensity indicates high concentration of DNA in a specific region.

Figure 3.2. Comets of sperm of bisazir-treated fish (A), non-bisazir-treated fish (B, negative control), and sperm treated with hydrogen peroxide (C, 1 mM H₂O₂, positive control).
Figure 3.3. Comets of sperm after two (A) and four days (B) storage on ice.

Figure 3.4. Comets of sperm exposed to increasing duration of ultraviolet light radiation, 0.1 min (A), 0.2 min (B), 0.3 min (C), and 0.6 min (D).

Figure 3.5. Comets of sperm exposed to increasing concentrations of potassium permanganate (KMnO₄), 25 μM (A), and 250 μM (B).
Figure 3.6. Sea lamprey sperm comet measurements from control and bisazir-treated males (Data from 2002). Number of individual animals evaluated is indicated (n). For each individual, two replicates of 25-50 comets (i.e. nucleoids) were evaluated resulting in a total of 50-100 comets scored for each animal. Mean number of comets scored per individual = 75. Extent tail moment = tail length * tail % DNA/100. Olive tail moment (OTM) = (tail mean - head mean)*tail
Figure 3.7. Sea lamprey sperm comet measurements from control and bisazir-treated males (Data from 2003). Number of individual animals evaluated is indicated (n). For each individual, two replicates of 25-50 comets (i.e. nucleoids) were evaluated resulting in a total of 50-100 comets scored for each animal. Mean number of comets scored per individual = 75. Olive tail moment (OTM) = (tail mean - head mean) * tail %DNA/100.
Figure 3.8. Effect of hydrogen peroxide ($\text{H}_2\text{O}_2$) on sea lamprey sperm measurements (Data from 2002). Number of individual animals evaluated is indicated (n). For each individual, two replicates of 25-50 comets (i.e. nucleoids) were evaluated resulting in a total of 50-100 comets scored for each animal. Mean number of comets scored per individual = 75. Extent tail moment = tail length * tail % DNA/100. Olive tail moment (OTM) = (tail mean - head mean)*tail %DNA/100.
Figure 3.9. Effect of storage time on sea lamprey sperm comet measurements. Number of individual animals evaluated is indicated (n). For each individual, two replicates of 25-50 comets (i.e. nucleoids) were evaluated resulting in a total of 50-100 comets scored for each animal. Mean number of comets scored per individual = 75. Extent tail moment = tail length * tail % DNA/100. Olive tail moment (OTM) = (tail mean - head mean)*tail %DNA/100.
Figure 3.10. Effect of UV irradiation on sea lamprey sperm comet measurements. Number of individual animals evaluated is indicated (n). For each individual, two replicates of 25-50 comets (i.e. nucleoids) were evaluated resulting in a total of 50-100 comets scored for each animal. Mean number of comets scored per individual = 75. Extent tail moment = tail length * tail % DNA/100. Olive tail moment (OTM) = (tail mean - head mean)*tail %DNA/100.
Figure 3.11. Chromatogram of bisazir. Bisazir standard (A), bisazir in holding water of treated male sea lamprey 12 h post-injection (B), bisazir in liver of treated sea lamprey female 12 h post-injection (C), and spiked with bisazir standard (D). “B” designates bisazir.
Figure 3.12. Bisazir concentration in liver of sea lamprey 12 h post-injection as estimated by HPLC (Data from 2002). Number of individual animals evaluated is indicated ($n$). Mean concentration of bisazir in male livers: $174.7 \pm 50 \mu$g/g. Mean concentration in female livers: $129.2 \pm 69.6 \mu$g/g. $n=4$ for each group.
Figure 3.13. Bisazir concentration in various tissues of sea lamprey at 9-10 hours post-injection. No detectable bisazir levels were observed seven days after injection. Four control sea lampreys (non-bisazir injected) had no detectable levels of bisazir. Number of individual animals evaluated is indicated (n). One-way analysis of variance revealed no significant differences between sexes and between dates of sampling (6/17/03, 7/10/03) (See also Table 3.7). Water samples taken from transport bags 12 or 9-10 hours after injection with bisazir (June 2003 or July 2003, respectively): Males, $3.21 \pm 1.77 \mu g/ml$, and $5.9 \pm 3 \mu g/ml$, respectively; Females, $2.90 \pm 1.24 \mu g/ml$, and $7.7 \pm 2.6 \mu g/ml$, respectively.
Figure 3.14. Number of ovulating sea lamprey females, treated with bisazir or untreated controls, following LHRHa injection on July 10, 2003. All of the bisazir females that ovulated received only a priming dose of LHRHa, whereas controls were injected with the resolving dose on the 4th day after the priming dose because they had not reached the stage of ovulation. First injection: n=18 T2; n=17 T4. Second injection: n=16 T2; n=4 T4. None of the control females ovulated before the day the resolving dose was administered. Of the bisazir females that received a resolving dose, none achieved ovulation. T2 = Control / LHRHa, T4 = Bisazir / LHRHa (Table 1). Chi-square analysis determined that the time of ovulation is significantly different between the two treatment groups. Degrees of freedom: 2, Chi-square = 10, p < 0.01.
Figure 3.15. Number of ovulating sea lamprey females, treated with or without bisazir, following LHRHa or saline injection on July 28, 2003. Only animals determined to be non-ovulating on day 4 received the resolving dose of LHRHa. First injection: n=19 T2; n=16 T4; n=13 T3. Second injection: n=10 T2; n=1 T4; n=6 T3. T2 = Control / LHRHa, T3 = Bisazir / saline, T4 = Bisazir / LHRHa (Table 1). Zero females out of 16 T1 achieved ovulation (data not shown in figure). Chi-square analysis determined that the time of ovulation is significantly different between the control and bisazir groups (but not within the two control or bisazir sub-groups). Degrees of freedom: 6, Chi-square = 20.6, p < 0.01.
Figure 3.16. Number of spermiating sea lamprey, treated with or without bisazir, following LHRHa or saline injection in July 2003. All males received only a priming dose on day 0. n=10 control/saline injected; n=7 control/LHRHa injected; n=7 bisazir/LHRHa injected; n=8 bisazir/saline injection. Chi-square analysis determined that the time of spermiation is significantly different between the control and bisazir groups (but not within the two control or bisazir sub-groups). Degrees of freedom: 6, Chi-square = 20.2, p < 0.01.
Figure 3.17. Sea lamprey survival rates at 2-cell zygote and pre-hatch larvae stages for individual control (A) and bisazir treated (B) females. Sperm from 3-4 control males was used in all experiments. n = 16 control females, 13 bisazir females. Viability of embryos at pre-hatching stage of bisazir-injected females (solid bars) suggests ineffective action of bisazir in these females.
Figure 3.18. Representative HPLC-UV (A) and EC (electrochemical detector) (B-D) chromatograms showing peaks of total nucleosides in standard solution (A) and 8-hydroxydeoxyguanosine (8-OH-dG) (arrows) in DNA digests from a standard (salmon testes DNA) (B), sea lamprey sperm from bisazir-treated male (C), and sea lamprey sperm from non-treated control (D).
**Figure 3.19.** Concentration of individual nucleosides (dC, dI, dG, dT, and dA) and total nucleosides in control and bisazir-treated animals. The number of animals sampled for evaluation, n = 4 control and 4 bisazir. No significant differences were observed between treatments.
Figure 3.20. Levels of 8-OHdG in control and bisazir-treated males. First two bars measure total 8-OHdG in pmoles per treatment group (control and bisazir). The middle two bars and the two far right bars measure the ratio of 8-OHdG per dG, and 8-OHdG per total nucleosides, respectively, per treatment group. The number of animals sampled for evaluation, n = 4 control and 3 bisazir. Measurements between treatment groups are significantly different.
CHAPTER 4

DISCUSSION

Bisazir is an aziridinyl compound whose genotoxicity is related to its alkylating properties (Devi and Reddy, 1985). Alkylation of DNA results in creation of DNA adducts, DNA-DNA and DNA-protein cross-links, sister chromatid exchanges, chromosomal aberrations, and DNA strand breaks (Coddington et al., 2004). The results of this study as determined by Comet assay indicate that DNA strand breaks are not the primary damage to sea lamprey sperm exerted by bisazir because fragmentation of spermatozoa of bisazir-injected sea lampreys was much smaller than fragmentation caused by hydrogen peroxide or UV radiation exposure. The mechanism of the genotoxic action of bisazir seems to be similar to other alkylating agents, such as cyclophosphamide. Rat spermatozoa exposed to cyclophosphamide also exhibits modest Comet assay fragmentation patterns (Coddington et al., 2004). These data suggest that the genotoxicity of bisazir is primarily related to DNA cross-linking and, to a lesser degree, to DNA fragmentation. This implies that alkylating agents, less hazardous than bisazir, can be potential replacements of bisazir. It is known that alkylating agents can destabilize sperm chromatin through alkylation of nuclear proteins (Evenson et al., 1993).
However, it is not clear if this mechanism is involved in the sterilization mechanism of bisazir. Further studies are needed to establish the exact mode of action of bisazir.

Bisazir concentrations in both liver and water samples (~10 hours after intraperitoneal injection of bisazir) of male lamprey were 54% and 10% higher, respectively, than those found in females (Tables 3.4, 3.5 and 3.7). However, the average whole body weights of males exceeded females by only 22%. The females appear to be eliminating bisazir from the liver more rapidly than the males but are not excreting the chemical from their bodies as quickly. HPLC separation of bisazir from the gonads reveals that females are sequestering 8.5% of the bisazir in the ovaries, while males are holding only 0.7% of the bisazir in the testes (Table 3.7).

Alkali Comet assay of sea lamprey sperm, unlike in the case of mammals (Singh et al., 1989), can be successfully performed according to the protocols developed for somatic cells with no additional steps, such as hydrolysis with proteinase K, dithiothreitol or diiodosalicylate treatments needed. Comet assay was evaluated for its effectiveness in determining DNA lesions in sperm cells, revealing more DNA damage to cells obtained from bisazir treated animals than from controls (non-treated). Likewise, microscopic evaluation of bisazir spermatozoa revealed sub-standard concentrations and impaired motility. This data affirms the mutagenic action of bisazir to gonadal cells (spermatozoa). However, this damage was less dramatic than the maximal damage induced by H₂O₂, where no “head” remained. Bisazir damage was represented by slightly enlarged heads (over control) with small amounts of DNA spilling out into a tail. Further studies on the effects of bisazir on tissue cells from organs such as the liver, kidney, muscle and even heart may prove helpful in fully understanding the mode of action of bisazir. This could
prove important in elucidating the effects of bisazir on the health, performance, and mortality of sea lamprey; all critical factors when evaluating the ability of treated animals to compete for spawning with non-treated males.

Comet assay also revealed dramatic increases in sea lamprey sperm DNA damage caused by hydrogen peroxide and, in a dose-dependent manner, by UV radiation. Maximal damage to sea lamprey spermatozoa was induced by exposing control spermatozoa to concentrations of H$_2$O$_2$ up to 1 mM. This data served as a positive control with which to compare control and bisazir treated cells. DNA damage was evaluated by using an unbiased, computer-based scoring program (Komet 5.5). A large increase in damage to DNA of sea lamprey sperm was observed upon exposure to all concentrations of H$_2$O$_2$ (0.1 and 1 mM), by all the major comet measurements, thus validating the usefulness and accuracy of the scoring program. As maximal damage was induced at the lowest concentration of H$_2$O$_2$, further studies are needed to establish a progressive increase in the DNA fragmentation pattern of cells exposed to H$_2$O$_2$ in the concentration ranges 0-0.1 mM.

Contrary to the effects of UV, no dramatic decrease in percentage of pre-hatched larvae was observed after exposure of sperm to H$_2$O$_2$. These results indicate that, despite a similar profile of fragmentation, damage to DNA caused by UV was detrimental, but by H$_2$O$_2$ was not. Spermatozoan DNA lacks effective repair systems, therefore recovery of sperm cells exposed to H$_2$O$_2$, resulting in high progeny survival following activation of the oocyte, must be attributed to the ability of the oocyte to repair damage to sperm DNA. The existence of such repair mechanisms has been well-documented in both mammalian and fish oocytes (Ahmadi and Ng, 1999; Ashwood-Smith and Edwards, 1996; Brandriff
and Pedersen, 1981; Kopeika et al., 2004). However, sea lamprey milt may have some anti-oxidant mechanisms capable of partially neutralizing H$_2$O$_2$ before damage can occur. The doses of H$_2$O$_2$ required to elicit fragmentation of DNA differ in direct relation to sperm concentration (data collected in 2001, from Ciereszko et al., in print). Further studies should be conducted to investigate the possible mechanisms responsible for an anti-oxidant defense mechanism in sea lamprey milt.

The Comet assay proved of further usefulness for detecting UV-induced damage to DNA. Dose-dependent effects of UV on DNA fragmentation were clearly illustrated by several major comet parameters. These changes parallel the dose-dependent decrease in sperm fertilizing ability as measured at pre-hatching stage. The ability of the oocyte to repair DNA damage caused by one type of stressor (H$_2$O$_2$), but not another (UV), may be due to the different mechanisms involved in UV-induced DNA damage versus DNA damaged induced by H$_2$O$_2$. UV radiation directly damages the DNA molecules by inducing three major base modifications: cyclobutane-type pyrimidine dimers, pyrimidine-pyrimidone (6-4)-photoproducts, and thymine glycols (Griffiths et al., 1998; Mitchell et al., 1990; Sancar, 2000).

Lesions of DNA produced by UV usually are repaired via nucleotide excision repair (NER). NER consists of two steps, first the damaged site is incised/excised and a DNA single-strand break is created and then repair synthesis and ligation completes the repair process (Balajee et al., 1998; Lankinen et al., 1996; Sancar, 2000; Wood, 1996). A low survival of embryos after activation with UV-irradiated lamprey sperm suggests that either the sea lamprey oocyte NER does not operate efficiently to repair sperm DNA lesions or the damage to sperm DNA was beyond the ability of NER to repair damaged
DNA. Data collected from the Comet assay study (fragmentation) and from the observation of relative sperm motility (no effects on motility observed) support the known genotoxic effects of UV on spermatozoa. The use of UV radiation to inactivate sperm DNA of higher and lower vertebrates is well-documented (Bordignon and Smith, 1999; Lin and Dabrowski, 1998; Tompkins, 1978) and this study further confirms the usefulness of UV radiation for inactivation of the fish sperm genome.

Exposure of DNA to oxidative stress leads to more than 20 different types of base damage, producing oxidized and ring-fragmented nitrogen bases (Slupphaug et al., 2003). The most prevalent damage to purines is 8-oxoguanine, while the most common damage to pyrimidines is the formation of thymine glycol. Hydrogen peroxide is believed to cause DNA strand breakage by generation of the hydroxyl radical (OH) close to a DNA molecule, via the Fenton reaction.

\[
\text{H}_2\text{O}_2 + \text{Fe}^{2+} \rightarrow \text{OH}^+ + \text{OH}^- + \text{Fe}^{3+}
\]

Base excision repair (BER) is the primary defense mechanism against DNA lesions caused by oxidative damage of DNA (Annet et al., 2004; Brozmanova et al., 2001). The major lesions repaired by BER include de-aminated, oxidized, alkylated and missing bases (Memisoglu and Samson, 2000). Initiation of BER includes recognition of damaged or abnormal bases by specific glycosylases and cleavage of the glycosylc bond that links the base to the sugar phosphate backbone. Abasic sites are formed at this point and are further processed via short or long patch pathways (Dianov et al., 2003). The
results of this study suggest that BER, but not NER, is efficient in sea lamprey oocytes and embryos, and is therefore capable of repairing serious lesions caused by hydrogen peroxide.

KMnO4 was investigated in vitro as a possible DNA mutagen. KMnO4 affected damage to sea lamprey spermatozoa as detected by Comet assay. The damage incurred by the cells more closely paralleled the conservative damage seen in bisazir treated cells. Further exploration of the fertilizing ability of spermatozoa following exposure to KMnO4 is needed. However, the consistent fragmentation patterns established for the various treatments (bisazir, UV, H2O2) studied makes Comet assay a perfect validation assay for quality control purposes of bisazir treatments.

Storage of sea lamprey sperm on ice was tested to screen for the possibility of spurious DNA damage due to the effects of storage. During short-term storage of sperm on ice, a steady increase in DNA fragmentation occurred, paralleling a decrease in sperm motility. These data confirm earlier results (Ciereszko et al. 2002) indicating a decrease in sperm motility after 1-day storage and lower quality of spermatozoa after 2-day storage. A lower extent of damage to DNA as compared to sperm motility can explain previous findings (Ciereszko et al., 2000) indicating that if loss of motility is compensated (by an increase of sperm number) an increase in fertilization success can be expected, because some damage to sea lamprey sperm can be repaired by the oocyte. Therefore, it is imperative that cells targeted for DNA studies are immediately prepared for evaluation following collection or be stored at -70°C until further studies can be conducted.
In an attempt to synchronize ovulation and spermiation of sea lamprey, animals were injected intraperitoneally with luteinizing hormone releasing hormone analogue (LHRHa). LHRHa accelerated the rate of maturation and onset of ovulation and spermiation of bisazir treated animals over controls. Furthermore, preliminary data strongly suggests that bisazir is significantly accelerating the spermiation/ovulation of sea lamprey. However, the mechanism by which this may be occurring is as yet unknown. It is possible that bisazir may be acting to potentiate the estradiol-hypothalamic positive feedback loop, thus, stimulating the release of gonadotropic hormones. It is clear that bisazir is not as effective a sterilant in females as it is in males and may actually be enhancing the reproductive performance of these females and the probability of producing viable larvae. Treating both males and females with bisazir sets the stage for early maturation of both genders and, therefore, early fertilization of bisazir females by control (normal) males. This selection theoretically enhances fertilization rates with less competition from control (normal) females. Conversely, bisazir treated lamprey males may spermiate earlier and die before control (normal) females become ready to spawn (ovulate). This may compromise the competitiveness of bisazir treated males against control (normal) males for activation opportunities of control (normal) female eggs.

While the intention of this project was not to study the effects of LHRHa, these results clearly warrant future studies on the effects of bisazir on gonadal maturation of males and females. Current sterilization protocols targeting injection of both males and females with bisazir may be assisting these animals in reaching spermiation/ovulation
sooner than their control counterparts, thus reducing competition between treated and controls. This finding should be considered a priority research goal in “lamprey control” programs.

The assay for determination of 8-OHdG in tissues was successfully implemented in the laboratory following DNA isolation and digestion. Spermatozoan DNA from bisazir-treated lamprey contained higher ratios of 8-OHdG per dG (2’-deoxyguanosine) when compared to controls. This is indicative of DNA damage induced by oxidative stress. Again, this procedure, although time consuming and more expensive than Comet assay, can be used for quality control of bisazir treatment in lamprey sperm.

This study investigated DNA fragmentation, measured by Comet assay, of sea lamprey spermatozoa subjected to different stressors. The Comet assay protocol developed for somatic cells was shown to be successful when applied to sea lamprey spermatozoa. This may be explained by the presence of histone proteins in lamprey sperm. These histone proteins do not contain significant amounts of cysteine, which creates additional disulfide bridges and, thus, cross-linking, in mammalian sperm DNA (requiring additional unwinding steps using thiol reagents). DNA fragmentation was established as a possible cause of decline in sperm quality during short-term storage, positively correlating with increased storage time. Additionally, the genotoxic effects of UV radiation and oxidative stress (hydrogen peroxide exposure) were demonstrated with reversibility of the latter found, most likely due to a restricted repairing activity of the oocyte directed to sperm DNA. Mechanisms of bisazir sterilization action were investigated and its alkylating properties were pointed out as possible mechanisms of genotoxicity. The genotoxicity of bisazir is most likely related to DNA cross-linking
and, to a lesser degree, to DNA fragmentation. Alkylating agents less difficult to handle than bisazir may reveal a potential replacement for bisazir in the chemosterilization of sea lamprey. Additionally, the Comet assay and 8-OHdG assay were successfully tested as potential primary evaluation screening protocols, in lieu of biological fertilization tests, for testing the effectiveness of genotoxic agents. While the cost of the 8-OHdG assay may be prohibitive for most laboratories, the reliability, low cost, and rapid performance of the Comet assay make it useful for monitoring DNA damage in sea lamprey spermatozoa.
CHAPTER 5

CONCLUSIONS

Neutral and alkali comet assays were evaluated for their effectiveness in determining DNA lesions in sperm cells and erythrocytes. Both assays revealed more DNA damage to cells obtained from bisazir treated animals than from controls (non-treated). Likewise, microscopic evaluation of bisazir spermatozoa revealed sub-standard concentrations and impaired motility. This data affirms the mutagenic action of bisazir to gonadal cells (spermatozoa) and to cells not directly involved in reproduction (erythrocytes). Further studies on the effects of bisazir on tissue cells from organs such as the liver, kidney, muscle and even heart may prove helpful in fully understanding the mode of action of bisazir. This could prove important in elucidating the effects of bisazir on the health, performance, and mortality of sea lamprey; all critical factors when evaluating the ability of treated animals to compete for spawning with non-treated males.

Maximal damage to sea lamprey spermatozoa was induced by exposing control spermatozoa to concentrations of H$_2$O$_2$ up to 1 mM. This data served as a positive control with which to compare control and bisazir treated cells. DNA damage was evaluated by using an unbiased, computer-based scoring program. A significant increase
in damage to DNA of sea lamprey sperm exposed to \( \text{H}_2\text{O}_2 \) was observed in all the major comet measurements, thus validating the usefulness and accuracy of the scoring program.

Spermatozoa from bisazir treated animals exhibited greater DNA damage when compared to controls. The damage displayed itself less dramatically as the maximal damage induced by \( \text{H}_2\text{O}_2 \), illustrated by its long tails. Bisazir damage presented slightly enlarged heads (over control) with small amounts of DNA spilling out into a tail. This, however, makes comet assay a perfect validation assay for quality control purposes of bisazir treatments.

Storage of sperm on ice was tested to screen for the possibility of spurious DNA damage due to the effects of storage. An increase in DNA fragmentation was indeed detected in direct relation to storage time. It is therefore imperative that cells targeted for DNA studies are immediately prepared for evaluation following recovery, or be stored at \(-70^\circ\text{C}\) until further studies can be conducted.

Comet assay proved further usefulness for detecting UV-induced damage to DNA. Dose-dependent effects of UV on DNA fragmentation were clearly illustrated by several major comet parameters. These changes parallel the dose-dependent decrease in sperm fertilizing ability as measured by viable pre-hatched larvae.

Furthermore, \( \text{KMnO}_4 \) was investigated \textit{in vitro} as a possible DNA mutagen. \( \text{KMnO}_4 \) affected damage to sea lamprey spermatozoa as detected by comet assay. The damage incurred by the cells more closely paralleled the conservative damage seen in bisazir treated cells. It would be interesting to further explore the fertilizing ability of spermatozoa following exposure to \( \text{KMnO}_4 \).
Bisazir concentrations in both liver and water samples (~10 hours after intraperitoneal injection of bisazir) of male lamprey were 54% and 10% higher, respectively, than those found in females. However, the average whole body weights of males exceeded females by only 22%. The females appear to be eliminating bisazir from the liver more rapidly than the males, but are not excreting the chemical from their bodies as quickly.

In an attempt to synchronize ovulation and spermiation of sea lamprey, animals were injected intraperitoneally with luteinizing hormone releasing hormone a (LHRHa). LHRHa significantly accelerated the rate of maturation and onset of ovulation and spermiation of bisazir treated animals. While the objective of the study was not aimed at investigating the effects of LHRHa, it should be noted that in light of these results, further studies of the effects of bisazir on gonadal maturation of males and females should be conducted. Current sterilization protocols targeting injection of both males and females with bisazir may be assisting these animals in reaching reproductive maturity sooner than their control counterparts, thus reducing competition between treated and controls. This finding should be considered a priority research goal in lamprey control programs.

The assay for determination of 8-OHdG in tissues was successfully implemented in the laboratory following DNA isolation and digestion. Spermatozoan DNA from bisazir-treated lamprey contained higher ratios of 8-OHdG per dG (2'-deoxyguanosine) when compared to controls. This is indicative of DNA damage due to oxidative stress. Again, this procedure, although time consuming and more expensive than comet assay, can be used for quality control of bisazir treatment in lamprey sperm.
LIST OF REFERENCES


