IS THERE A ROLE FOR REACTIVE OXYGEN SPECIES IN
ZEBRAFISH EMBRYOGENESIS?

A thesis presented by
Vivek Krishnan

To the Department of Biology
In partial fulfillment of the requirements for the degree of
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Abstract of Thesis

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ABSTRACT

Reactive oxygen species (ROS) are generated by many exogenous and endogenous sources. They are highly reactive and can attack biomolecules such as proteins, lipids and DNA, causing damage to living cells. DNA is subjected to constant attack by many damage inducing agents including ROS, but constant repair and maintenance by multiple DNA repair pathways helps to maintain genomic stability. The cell is also protected from the effects of ROS by antioxidants. However, ROS are implicated in cell signaling as important primary and secondary messengers. Many critical transcription factors involved in embryonic development are sensitive to the redox condition in the cell and so reductive stress can be just as dangerous as oxidative stress. Indeed, this laboratory has shown that the position and type of oxidative damage can affect binding of at least one specific transcription factor to its consensus sequence. Based on this evidence, it is possible that ROS might have a role in embryonic development of zebrafish embryos. In the following thesis, we have attempted to answer some basic questions pertaining to the role of ROS in zebrafish embryogenesis.

We first asked whether some parts of the zebrafish embryo were more sensitive to ROS than others. We found that the head region of the 24 hours post fertilization (hpf) embryo showed a significant increase in 8-oxoG levels when treated with hydrogen peroxide ($\text{H}_2\text{O}_2$), but we were not able to detect any difference in 8-oxoG
levels or localization in earlier embryonic stages by standard fluorescence microscopy. Since AP endonuclease 1 (Apex1) is the main enzyme in the base excision repair (BER) pathway that repairs oxidative damage to DNA, we observed the effects of knocking down Apex1 and found that the knockdown embryos exhibited higher levels of 8-oxoG. Finally, we showed that antioxidants such as N-acetylcysteine (NAC) rescued neither peroxide-treated nor Apex1 knockdown embryos. In fact, NAC treatment sensitized embryos subjected to peroxide. NAC did not change 8-oxoG levels in peroxide-treated embryos. In conclusion, this thesis serves as a starting point for the investigation into the role of ROS in zebrafish embryonic development.
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# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abstract</td>
<td>2</td>
</tr>
<tr>
<td>Acknowledgments</td>
<td>5</td>
</tr>
<tr>
<td>List of Abbreviations</td>
<td>8</td>
</tr>
<tr>
<td>Introduction</td>
<td>10</td>
</tr>
<tr>
<td>DNA Damage and Repair Systems</td>
<td>10</td>
</tr>
<tr>
<td>ROS and Embryonic Development</td>
<td>16</td>
</tr>
<tr>
<td>Zebrafish as a Model Organism</td>
<td>22</td>
</tr>
<tr>
<td>Is there a role for ROS in zebrafish embryonic development?</td>
<td>25</td>
</tr>
<tr>
<td>Methods</td>
<td>26</td>
</tr>
<tr>
<td>Results</td>
<td>29</td>
</tr>
<tr>
<td>Figures</td>
<td>33</td>
</tr>
<tr>
<td>Discussion</td>
<td>41</td>
</tr>
<tr>
<td>Conclusion</td>
<td>47</td>
</tr>
<tr>
<td>Future directions</td>
<td>48</td>
</tr>
<tr>
<td>References</td>
<td>51</td>
</tr>
</tbody>
</table>
LIST OF ABBREVIATIONS

8-oxoG 8-oxoguanine
Apex1 Apurinic/apyrimidinic endonuclease 1
AP site Apurinic/apyrimidinic site
BER Base excision repair
Creb1 cAMP response element binding protein 1

dpf Days post-fertilization
dRP Deoxyribose-phosphate
Fen1 Flap endonuclease 1
hpf Hours post-fertilization
HR Homologous recombination
IHC Immunohistochemistry
Lig3, 4 DNA ligase 3, 4
Mbd4 Methyl-CpG-binding domain protein
MBT Mid-blastula transition
MMR Mismatch repair
MO Morpholino oligonucleotide
Mpg N-methylpurine-DNA glycosylase
NAC N-Acetylcysteine
NADPH Nicotinamide adenine dinucleotide
NER Nucleotide excision repair
NHEJ Non-homologous end joining
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ogg1</td>
<td>8-oxoguanine DNA glycosylase 1</td>
</tr>
<tr>
<td>Parp1</td>
<td>Poly(ADP-ribose) polymerase 1</td>
</tr>
<tr>
<td>PBST</td>
<td>Phosphate buffered saline with 1% Triton® X-100</td>
</tr>
<tr>
<td>Pms2</td>
<td>Postmeiotic segregation increased 2</td>
</tr>
<tr>
<td>Polb</td>
<td>DNA polymerase β</td>
</tr>
<tr>
<td>qrt-PCR</td>
<td>Quantitative real-time polymerase chain reaction</td>
</tr>
<tr>
<td>Rpa1</td>
<td>Replication protein A1</td>
</tr>
<tr>
<td>SS-MO</td>
<td>Splice-site morpholino oligonucleotide</td>
</tr>
<tr>
<td>Tdg</td>
<td>Thymine DNA glycosylase</td>
</tr>
<tr>
<td>TS-MO</td>
<td>Translation start site morpholino oligonucleotide</td>
</tr>
<tr>
<td>Udg</td>
<td>Uracil DNA glycosylase</td>
</tr>
<tr>
<td>Xpf, g</td>
<td>Xeroderma pigmentosum complementation groups F, G</td>
</tr>
<tr>
<td>Xrcc1</td>
<td>X-ray cross complementing protein 1</td>
</tr>
</tbody>
</table>
INTRODUCTION

DNA Damage and Repair Systems

DNA is subjected to constant damage by various exogenous and endogenous sources such as radiation, oxidizing agents, alkylating agents, replication errors, etc. If left unchecked, this damage could result in genomic instability with potentially serious consequences for the cell (1-3). In order to combat DNA damage, cells have evolved various DNA repair pathways such as nucleotide excision repair (NER), mismatch repair (MMR), non-homologous end joining (NHEJ), homologous recombination (HR), and base excision repair (BER). Each of these pathways recognizes specific types of damage but they also share many common steps and enzymes.

*Nucleotide Excision Repair (NER)*

This is the main mechanism in the recognition and replacement of nucleotides damaged by UV radiation. There are two sub-pathways in NER damage recognition: global genome repair (GGR) and transcription coupled repair (TCR). TCR repairs lesions that block transcription in actively transcribed genes. GGR is a slower repair process that functions across the entire genome and repairs silent, transcribed and non-transcribed regions. Following damage recognition, these pathways converge on a common repair pathway in which the first step is the unwinding of DNA around the damage by DNA helicases. The multi-subunit
helicase TFIIH unwinds the lesion and several nucleotides on either side. Two nucleases, xeroderma pigmentosum, complementation groups F and G (Xpf and Xpg), cleave the strand with the damaged bases on the 3’ and 5’ sides, respectively and release a single stranded fragment of about 24-32 nucleotides. This newly formed gap allows DNA polymerase δ or ε to synthesize a new strand using the undamaged strand as a template, and the gaps are sealed by a DNA ligase (4, 5). Deficiencies in many of the several genes associated with the NER pathway have been shown to lead to the disease Xeroderma Pigmentosum in which the patient is sensitive to UV radiation and cancer prone (6, 7).

Mismatch Repair (MMR)

The MMR pathway is highly conserved and is responsible for the repair of errors introduced to the genome during normal semiconservative DNA replication that were not repaired by the replicative polymerases’ proofreading function (8). The exception is U:A, U:G, or T:G mispairs, which are repaired by the BER pathway (9). In MMR, faulty base pairing creates a lesion in the double-stranded DNA which is recognized either by the MutSα heterodimer, or the MutSβ heterodimer. The former demonstrates more activity when there is only a single mismatch, whereas the latter is preferentially activated when there are many consecutive errors. The enzyme mutL homolog 1 (Mlh1) then heterodimerizes with postmeiotic segregation increased 2 (Pms2) to cleave the daughter strand at one side of the
mismatch, while exonuclease 1 (Exo1) cleaves at the other side. Interaction with the Mlh1-Pms2 heterodimer is required for Exo1 to cleave 3’ to the error, while interaction with either of the MutS heterodimers and Rpa1 assist in 5’ cleavage. DNA polymerase δ fills in the gap, and DNA ligase 1 seals the nick (8).

Non-homologous End Joining (NHEJ) and Homologous Recombination (HR)

Double strand breaks caused by ionizing radiation or recombination errors are repaired by either NHEJ or HR. In NHEJ, the Ku heterodimer (formed by Ku70 and Ku80) tethers the two strands so that DNA ligase 4 (Lig4) can ligate the ends. If the ends have been modified by the break, such as by formation of 5’ hydroxyls and 3’ phosphates rather than the other way around, the ends must be repaired before Lig4 can seal the break. Polynucleotide kinase 3’-phosphatase, aprataxin, and some nucleases have been shown to modify these broken ends in preparation for NHEJ. DNA polymerases λ or μ can fill in gaps left behind by degradation of one of the strands by nucleases or the damage-causing event itself (10).

HR is a much more complicated process, which is comprised of a series of interrelated pathways in the repair of double strand breaks and interstrand crosslinks. It also provides critical support to DNA replication by helping recover stalled or broken replication forks. The key reactions of HR are catalyzed by the coordination of several proteins such as Rad50, Rad51, Rad52, Rad54, Rad55, Rad57, Rad-59, Rdh54, Mre11 and Xrs2 to twist the DNA in such a manner that
the original chromosomes are reformed. Classical HR is mainly characterized by three successive steps: 1) resection of the 5’ended DNA strand at break ends, followed by 2) strand invasion into a homologous DNA duplex and strand exchange, and 3) resolution of recombination intermediates. Depending on the ability of both double strand break ends to perform strand invasion and on the outcome of the strand invasion intermediate, different HR pathways can then complete double strand break repair (10, 11).

**Base Excision Repair**
The base excision repair (BER) pathway is the mechanism responsible for the repair of small, non-bulky lesions formed by both exogenous and endogenous agents. It also repairs spontaneously formed abasic sites and uracil residues inserted during replication or formed by deamination of cytosine, as well as T:G mispairs (3, 12).

Except for repair of AP sites, BER is initiated by one of the many DNA glycosylases that specifically recognize and remove the damaged base by breaking the N-glycosyl bond that bonds it to the deoxyribose backbone. This leads to the formation of toxic apurinic/apyrimidinic (AP) sites in DNA. Glycosylases include 8-oxoguanine DNA glycosylase (Ogg1), which removes 8-oxoguanine (8-oxoG); uracil-DNA glycosylase (Udg), methyl-CpG-binding domain protein 4 (Mbd4), and thymine-DNA glycosylase (Tdg), which all remove uracil depending on how it
was introduced to the DNA; and N-methylpurine-DNA glycosylase (Mpg), which removes hypoxanthine, the product of spontaneously deaminated adenine. Some glycosylases, such as Ogg1, also exhibit inherent lyase activity, so they can cut the backbone 3’ to the AP site in addition to removing the damaged base (3, 13). After the formation of AP sites, AP endonuclease 1 (Apex1) cleaves the phosphodiester bond 5’ to the lesion. In the case of glycosylases without lyase activity, cleavage of the AP site leaves behind a single strand nick sandwiched between a 5’ deoxyribose phosphate (dRP) and a 3’ hydroxyl group whereas in the case of glycosylases with lyase activity, it leaves a single nucleotide gap (3, 14). Poly(ADP-ribose) polymerase 1 (Parp1) then binds to the strand break, which induces the enzyme to attach branching chains of poly(ADP-ribose) to itself and nearby histones, and causes nucleosomes to unwind. This rearrangement of chromatin brought about by the poly(ADP-ribose)ylation of histone proteins is required for recruiting downstream enzymes to the damaged site (15-18). Following this, BER can proceed in one of two ways: short patch or long patch repair.

In short patch repair, the interaction of DNA polymerase β (Polb) with Apex1, automodified Parp1, and the scaffolding protein X-Ray cross complementing protein 1 (Xrcc1) fills in the gap by adding a single nucleotide. Polb lyase activity removes the 5’ dRP, so that DNA ligase IIIα (Lig3) can then seal the nick (19).
In long patch repair, the polymerase continues synthesis for an extra two to ten nucleotides, which results in the displacement of the original strand by the new strand (20, 21). To remove the displaced strand, flap endonuclease 1 (Fen1) cleaves the displaced DNA. Finally, DNA ligase I seals the nick to complete repair (22). Currently, it is not clear what factors are involved in influencing the cell to choose one pathway over the other (long patch over short patch or vice versa). One hypothesis suggests that the structure of the strand break plays the determining role. If the lyase activity of Polb can remove the 5’ dRP site left behind by Apex1, then BER will proceed by the short patch route. However, if the glycosylase possesses lyase activity, then the 3’ aldehydic end blocks Polb, so strand displacement is required to permit the modification’s elimination by Fen1 (23). The second hypothesis states that ATP concentration plays a role in the choice of pathway. Under normal or abundant ATP levels, Lig3 can seal the newly introduced nucleotide into the chromosome whereas during conditions of depleted ATP levels, long patch repair can work in conjunction with the other enzymes to create the ATP needed for the completion of BER (24).

As the eukaryotic cell’s most frequently used repair pathway (3), BER is responsible for repairing damage caused by both exogenous and endogenous sources. Given the pathway’s significance, it makes sense that a loss of activity in many of the enzymes can lead to highly deleterious effects such as mutagenesis,
tumorigenesis or death (20). Diseases such as Alzheimer’s, Parkinson’s and graft-versus-host have also been known to be associated with improper BER (25, 26). On the other hand, DNA damage repair genes are important targets for chemotherapy. Some tumor cells can survive the DNA damage inducing effects of drugs like cisplatin and oxaliplatin by repairing the damage. These cells can be eliminated by targeting them with BER inhibitors, thus making the drugs more effective (27-30).

The estimated numbers of single-strand breaks and spontaneous base losses in nuclear DNA are as high as $10^4$ per cell per day. Together with other types of spontaneous damage, the total may be close to $10^5$ lesions per cell per day (31). As BER is the eukaryotic cell’s most commonly used pathway (3), it is very important that we know more about it. Many studies have been carried out to learn more about the repair mechanisms of BER but it is also important to focus on the role of BER proteins in other critical processes such as embryonic development.

**Reactive Oxygen Species (ROS) and Embryonic Development**

*Reactive oxygen species*

A free radical is defined as any species that contains one or more unpaired electrons. ROS is a collective term that describes both oxygen radicals, such as superoxide ($O_2^-$), hydroxyl (OH·), peroxy (RO₂·) and hydroperoxy (HO₂·) radicals,
and certain nonradical oxidizing agents, such as hydrogen peroxide (H₂O₂), hypochlorous acid (HOCl), and ozone (O₃), that can be converted easily to into radicals (32). There is also another group called Reactive Nitrogen Species which consist of nitrogenous products such as nitric oxide (NO), nitroxyl (HNO), nitrosonium cation (NO⁺), higher oxides of nitrogen, etc. NO can react with O₂⁻ to form peroxynitrite (ONO'O⁻), a strong oxidant that reacts with most biomolecules and causes cell damage (33). The focus in this study, however, will be on ROS. Superoxide radical is formed when an oxygen molecule accepts a single electron. It has limited reactivity but can inactivate a few enzymes such as glutathione peroxidase and the reduced nicotinamide adenine dinucleotide phosphate dehydrogenase complex directly. H₂O₂ is a poorly reactive oxidizing agent. Unlike O₂⁻, however, it can pass through cell membranes easily. Although H₂O₂ and O₂⁻ exhibit limited chemical reactivity, they can generate the highly reactive OH⁻ radical, which reacts with almost every type of molecule found in living cells such as DNA, carbohydrates and membrane lipids. Transition metals like Fe (II) and Cu (I) can react with H₂O₂ to form OH⁻ through the Fenton reaction. 

\[
\text{Fe (II)} + \text{H}_2\text{O}_2 \rightarrow \text{OH}^- + \text{OH}^- + \text{FE (III)}
\]

Superoxide can reduce Fe (III) and Cu (II) to form Fe (II) and Cu (I) which can participate with H₂O₂ in the Fenton reaction. The combined reaction can be described as follows:
Fe/Cu

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

This is called the transition metal catalyzed Haber-Weiss reaction which accounts for at least a part of the damage that is caused to living cells by ROS (32).

**Sources of ROS**

ROS are generated by various endogenous and exogenous sources. Among the endogenous sources, oxidative metabolism is a key generator of ROS in the cell, with the mitochondria being a major site for the production of superoxide (34). In addition, enzymes such as NADPH-cytochrome P450 reductase, xanthine oxidase, NADPH oxidase, lipoxygenase, myeloperoxidase and cyclooxygenase generate ROS (32, 35). The other major endogenous source is the immune response produced by phagocytic cells that generate ROS such as \( \text{O}_2^- \), \( \text{H}_2\text{O}_2 \) and \( \text{HOCl} \), when stimulated appropriately (36). Exogenous sources of ROS include physical agents such as gamma ray, X-ray and UV irradiation and chemical agents. This diversity and availability of numerous sources of ROS underlines their potential contribution to cellular damage (37).

**Damage caused by ROS**

ROS can attack DNA, proteins and lipids. In DNA it can cause base damage, single strand breaks, abasic sites and double strand breaks. The most common form of damage seems to the 8-hydroxylation of guanine to form 8-oxoG (38, 39). The
poly unsaturated fatty acids in cell membranes are susceptible to attacks by ROS, leading to the destabilization of membranes. ROS can also attack proteins causing their oxidation which may lead to loss of function, binding and/or enzymatic activity (40). In general, the reducing environment inside cells helps to prevent free radical-mediated damage. This reducing environment is maintained by the action of antioxidant enzymes and substances, such as superoxide dismutase (SOD), catalase, glutathione peroxidase, glutathione, ascorbate (vitamin C), α-tocopherol (vitamin E), and thioredoxin (32).

**ROS in signaling and development**

Although high levels of oxidative damage by ROS are detrimental to the cell, it has been shown that at low levels, they are very important as primary and secondary messengers in regulating vital processes such as cell proliferation and differentiation (41-44). Many redox sensitive, critical transcription factors alter gene expression in the embryo, such as hypoxia inducible factor (HIF-1), nuclear factor κB (NF-kB), activator protein 1 (AP-1), nuclear factor (NF)-E2 related factor 1 (Nrf-1), wingless and integration site for mouse mammary tumor virus (Wnt), etc (45). Thus the redox status, which is determined by the concentration of ROS and antioxidants in a cell, plays an important role in the cell signaling pathways controlling embryonic development (45-47).
There are two ideas to describe the involvement of ROS in cell signaling: (a) Through modification of a protein target and (b) changes in redox state. The two are not mutually exclusive of each other. Some ROS such as H$_2$O$_2$ have been known to oxidize cysteine residues on proteins to modify their activity, which can be reversed through reduction by glutaredoxins, thioredoxins or glutathione. This can be considered as an instance where ROS are involved in signaling by protein modification. Redox state can be defined using redox potentials of redox couples (e.g. GSSG/GSH). A change in redox state means a change in the redox potential of redox buffers such as GSSG/GSH, NADP$^+/NADPH$, thioredoxins and glutaredoxins, and effects of these changes on cellular phenomena. Oxidation of protein thiol groups does imply a change in the redox potential of some individual protein species. The distinction between (a) and (b) is not easy to make and sometimes redox signaling is equivalent to ROS signaling. The difference between these terms depends on whether one detects redox signaling by measuring oxidative modifications of individual proteins or by measurements of changes in the redox environment in cellular compartments (43). Although the general perception in the field of biology and medicine is that antioxidants are good and ROS are bad, this is not always the case. In a study involving the chick embryo model, neuronal death was shown to be prevented using antioxidants. However, higher levels of these antioxidants were shown to be
detrimental (48), suggesting that a balance in the redox conditions is needed at critical periods of development and that reductive stress was just as dangerous as oxidative stress (47-49). Another study also showed that longevity in *C. elegans* mutant strains with elevated ROS levels was decreased when they were fed antioxidants (50), which further reinforces the importance of redox balance to the wellbeing of living cells.

As mentioned earlier, oxidative damage to DNA is repaired by the BER pathway in which the central enzyme is Apex1 (3). Previous studies in our lab have shown that Apex1 regulates cAMP response element-binding protein (Creb1) (51), an important transcription factor that regulates more than 100 pathways (52, 53). Electrophoretic mobility shift assays (EMSA) performed in our lab to elucidate the effect of oxidized bases on the binding of Creb1 to its consensus sequence have shown that binding can be enhanced or diminished with the type and position of the damaged base in the consensus sequence (S. P. G. Moore, manuscript in preparation). All of the above mentioned points support the argument that ROS may be both positively and negatively involved in the regulation of critical developmental processes. This highlights the need to delve deeper into the mechanisms by which ROS regulate embryonic development.
**Zebrafish as a Model Organism**

*Advantages of using the Zebrafish Model*

Zebrafish (*Danio rerio*) is the model organism used by the Strauss lab to explore the role of the BER pathway and its components in vertebrate embryonic development *(51, 54-56)*. Zebrafish exhibit most of the characteristics required for an ideal model system: large number of easily obtained embryos, small size, ease of maintenance and many genes and pathways shared with other vertebrates including humans. Zebrafish exhibit almost all the genes that are involved in the various DNA repair pathways in eukaryotes which makes them a particularly convenient model to study BER mechanisms *(55)*. Their embryos are transparent and develop relatively quickly which allows for detailed study of embryonic development and organogenesis in real time. In laboratory conditions, they are capable of breeding year round and a single female can lay dozens of eggs every week *(57)*. The zebrafish genome has also been fully sequenced and the latest annotations were released in 2013 *(58)*. This is also an excellent genetic model as gene expression can be modified easily. Microinjecting the embryos with the *in vitro* transcribed capped RNA of a particular gene results in the overexpression of the gene. To knockdown the expression of a particular gene, morpholino oligonucleotides (MO) can be microinjected *(59)*. MOs are nucleic acid analogs in which the bases are bound to a morpholine ring instead of a deoxyribose. They
function similarly to siRNA and shRNA in principle, as they are designed to be complementary to a small sequence of the RNA to be blocked. However, unlike these RNA antisense methods, MOs cannot be degraded and do not activate the cell’s RNAi machinery, and hence do not promote the degradation of the target transcript. Instead, translation of the proper protein is inhibited by either blocking the progression of the ribosome down the mRNA (translation start or TS-MO), or by sterically blocking spliceosome binding to the pre-mRNA, so retained introns cause the reading frame to shift (splice site or SS-MO). Knockdowns can be rescued by co-injection with the RNA of the MO target gene so long as the sequence to which the MO binds is absent or modified in the injected RNA (59).

Zebradish embryogenesis

The process of embryogenesis in zebrafish begins with fertilization of the egg and is typically completed by 3 days post fertilization (dpf). The process can be divided into seven stages during which the single initial cell grows, divides and differentiates into the various tissue types which make up the adult organism. After fertilization (stage 1), the cytoplasm of the zygote segregates to one end of the embryo to form the blastodisc and the maternally-deposited nutrients remain in the yolk on the opposite end. During cleavage (stage 2), which lasts from the second through seventh cell divisions, the blastodisc divides at regular intervals, typically every 15 minutes when the embryo is incubated at 28.5°C. The cells divide rapidly
and synchronously during the first half of the third stage, the blastula. After the
tenth cell division at the mid-blastula transition (MBT), the cell cycle lengthens
and becomes asynchronous as gastrulation begins (60). The gastrula period (stage
4) is characterized by epiboly, the flattening of cells into sheets that envelop the
yolk, a process which is typically completed in five hours. The germ layers also
begin to differentiate during this stage. The somites and other rudimentary organs
form during segmentation (stage 5). By the time the sixth stage begins at 24 hours
post fertilization (hpf), the embryo has a clearly defined head and tail, and the eyes,
brain, heart, kidneys, and other organs attain partial functionality. This period is
called the pharyngula, so named because of the development of the pharyngeal
arches, which will later grow into jaws and gills in the mature fish. The head and
tail straighten out, and pigmentation is produced, which darkens the eyes and the
body. The final stage, from 48 to 72 hpf, is the hatching period. As most organs
have matured, morphogenesis slows down noticeably. At some point during this
period, the embryo breaks out of the chorion and will later inflate its swim bladder
so that it can move freely through the water to catch prey. Full development and
sexual maturity are typically reached within three months after fertilization (60).
Is there a role for ROS in zebrafish embryonic development? - Studies in this thesis

The question whether ROS have a role in zebrafish embryonic development is a very broad one and cannot be easily answered in a very short period of time by conducting a few simple experiments. In the following thesis, I have attempted to break down the overarching question into simpler, more specific ones to lay the foundation for deeper study into the topic. These are as follows:

1. Can we detect a ROS burst during early embryonic development? When? Where?
2. Might the burst be blocked with antioxidant treatment? Does antioxidant treatment itself affect development?
3. What happens when Apex1 is knocked down?

I hypothesize that ROS bursts occur at critical embryonic stages such as mid-blastula transition and 50% epiboly, which could affect development. It is likely that these bursts exhibit some kind of spatial localization. Antioxidant treatment may alter ROS levels which can disrupt ROS mediated signaling and thus affect normal development. Since Apex1 knockdown increases ROS levels, reducing ROS might alter development as well.
MATERIALS AND METHODS

Zebrasfish husbandry and breeding

Wild type zebrafish purchased from Aquatic Tropicals (Plant city, FL) were maintained at standard laboratory conditions (28.5°C on a 13 hour light, 11 hour dark cycle) (61). In order to breed, adult fish were placed in a small tank with a removable plastic barrier separating the sexes the night prior to mating. Just before the beginning of the light cycle the following morning, the tanks were set at a slight incline to create a depth gradient (57), the barrier was removed, and two green florets were added to the tank. Fish were allowed to mate for at least 30 minutes before collection of fertilized embryos.

Morpholino oligonucleotide and microinjection

TS-MO of zebrafish *apex1* mRNA was synthesized by GeneTools (Philomath, OR) and has been described previously (51, 54, 56). The sequence is 5’-GTT CTT CTT GGC TCT TTT GGG CAT G-3’. Embryos were no older than the 4 cell stage when they were injected with 0.15 mM TS-MO directly into the yolks to ensure equal distribution amongst all daughter cells. A 25% phenol red solution (in water) served as tracking dye. Controls were injected with the phenol red solution without morpholino. Injected embryos were then kept in a 29°C incubator until they reached the desired developmental stage for further experiments.
**Peroxide treatment at 24 hpf**

Embryos at the 24 hpf stage were placed in fish tank water with 0.44 M H₂O₂ (Sigma-Aldrich, St. Louis, MO) for an hour at 29°C (peroxide concentration and duration of exposure were determined using preliminary experiments), washed thoroughly 3 to 4 times, dechorionated using Dumont no. 5 forceps and fixed overnight at room temperature in 4% PFA-PBS to prepare for immunohistochemistry (IHC). Control embryos without the addition of H₂O₂ were processed in a similar fashion.

**Hydrogen peroxide (H₂O₂) and N-acetylcysteine (NAC) assays**

Fertilized embryos maintained at 29°C until 3 hpf were placed in fish water containing the indicated concentration of H₂O₂ for 30 minutes. The embryos were then washed thrice with fish water, following which they were incubated in fish water or the desired concentration of NAC for 24 hours at 29°C. Survival at 24 hours post treatment was recorded and the embryos were fixed overnight at room temperature in freshly prepared 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) in phosphate buffered saline (PFA-PBS) to prepare for IHC.

**Immunohistochemistry and fluorescence microscopy**

Dechorionated embryos fixed overnight in 4% PFA-PBS were placed in prechilled acetone at -20°C for 30 minutes. They were then washed and treated with 10 μg/ml
Proteinase K (New England Biolabs, Ipswich, MA) for five (embryos less than 24 hpf ) or ten (24 hpf or older embryos) minutes, after which they were post-fixed in 4% PFA-PBS for 30 minutes followed by another wash. The embryos were then blocked in a solution of 10% sheep serum-PBST(1% Triton® X-100 in PBS) for 4 hours, transferred to a 10% sheep serum-PBST solution containing the anti 8-oxodG antibody (Trevigen, Gaithersburg, MD) at a 1:250 dilution and incubated at 4°C overnight. The next day, the embryos were washed and placed in 10% sheep serum-PBST solution to which anti mouse IgG-TRITC conjugated antibody (Sigma-Aldrich, St. Louis, MO) was added at a 1:250 dilution and incubated overnight at 4°C. The embryos were washed for a final time and mounted in 70% glycerol for fluorescence microscopy using a Leica MZ 16 FA microscope (Leica Microsystems, Buffalo Grove, IL). All the washes were done thrice for 5 minutes each with 1% PBST.

Images were taken using the inbuilt cameras under visible and fluorescent wavelengths (~580 nm). Quantification of fluorescence was accomplished using ImageJ processing software (http://rsb.info.nih.gov/ij/) (62).
RESULTS

Hydrogen peroxide treatment at 24 hpf results in significant increase of 8-oxoG levels in the head region

Exposure to H$_2$O$_2$ increases ROS levels and consequently is expected to increase oxidative damage, especially conversion of guanine to 8-oxoG. To confirm the specificity of the antibody against 8-oxoG, and to observe whether different areas of the embryo exhibited different levels of sensitivity to ROS attack, IHC was performed on 24 hpf embryos treated with H$_2$O$_2$. The results of five independent experiments showed that there was a significant increase (57%, p<0.05) in the amount of fluorescence in the head region of treated embryos relative to controls. There was also an increase in the fluorescence of the tail region but it was not significant (63%, p>0.05) relative to control tails. By showing an increase in fluorescence with peroxide treatment, these results validated the specificity of the anti 8-oxoG antibody. They also showed that the head region was more sensitive to oxidative damage than the tail (Figures 1, 2). A DNAse digest step was added after the proteinase treatment step in the IHC protocol to determine if it decreased the amount of fluorescence in the embryos. However, the results for these experiments were not conclusive as there was a lot of variation.
No variation in 8-oxoG levels was detected between different embryonic stages or between different areas on the embryos.

During the early stages of embryogenesis, cells divide rapidly without differentiation until the gastrulation period (~5.25 hpf). In order to determine if differences in 8-oxoG levels could be detected, IHC was performed on embryos fixed at 2 cell, 4 cell, 8 cell, high, sphere, dome, 30% epiboly and 50% epiboly (~5.3 hpf) stages. At least 3 embryos were observed per stage per independent experiment. A total of four independent experiments were performed. No differences in the levels of 8-oxoG were detected by qualitative analysis using standard fluorescence microscopy (Figure 3).

Apex1 knockdown caused 8-oxoG levels to increase in early stage embryos

Apex1 is the central enzyme of the BER pathway which is primarily responsible for repairing oxidative damage to DNA including the presence of 8-oxoG. In order to observe the effects of Apex1 knockdown on 8-oxoG levels, fertilized embryos, no older than the 2 cell stage were injected with the Apex1 TS-MO (0.15 mM) and fixed at 2 cell, 4 cell, high, dome, sphere and 30% epiboly stages. IHC was performed and fluorescence intensities were quantified. Embryos injected with only phenol red dye instead of the MO acted as the control. From the results, it was clear that the knockdown of Apex1 caused an increase in the 8-oxoG levels in the
embryos (Figure 4). At least 4 embryos were examined per stage per independent experiment. A total of 2 independent experiments were performed.

NAC treatment does not rescue but decreases survival of hydrogen peroxide treated embryos

Antioxidants can counter the effects of pro-oxidants by maintaining a reducing environment (32). In order to determine whether NAC treatment could rescue the embryos from the effects of H$_2$O$_2$ treatment, survival assays were carried out. To begin with, a H$_2$O$_2$ killing curve was generated by treating 3 hpf embryos with 0 mM, 30 mM, 60 mM, 90 mM and 150 mM H$_2$O$_2$ for 30 minutes. Peroxide concentration of 90 mM was chosen for further studies, as there was ~50% survival at 24 hpf after treatment with this concentration (Figure 5A). Similarly, a NAC killing curve was generated by incubating 4 hpf embryos in 0 mM, 1 mM, 2 mM, 3 mM and 5 mM NAC for 2 hours (Figure 5B). A concentration of 1 mM was chosen as the working concentration for NAC to determine whether it could rescue H$_2$O$_2$ treated embryos.

For the rescue experiment, embryos were treated with different concentrations of H$_2$O$_2$ (0 mM, 9 mM, 18 mM, 30 mM, 45 mM, 60 mM and 90 mM) at 3 hpf followed by continuous exposure to 1 mM NAC or fish water starting at 4 hpf. Contrary to expectations, the results showed that at higher concentrations of H$_2$O$_2$ (>30 mM), NAC treatment reduced the survival with respect to control (Figure 6).
**NAC treatment does not decrease 8-oxoG levels in peroxide treated embryos**

Even though NAC failed to increase viability of embryos exposed to peroxide, I examined whether NAC might still reverse the increase in fluorescence arising from increased 8-oxoG levels in DNA.

Embryos treated with either fish water, 30 mM \( \text{H}_2\text{O}_2 \), 1 mM NAC or both 30 mM \( \text{H}_2\text{O}_2 \) followed by 1 mM NAC were fixed for IHC to determine the effect of these treatments on 8-oxoG levels. Although NAC is an antioxidant and was expected to decrease 8-oxoG levels, the results showed that a decrease did not occur. In fact, there seemed to be an increase in 8-oxoG levels with NAC treatment. However, this may not be significant due to the large variance (Figures 7 and 8).

**NAC treatment did not rescue Apex1 knockdown embryos**

In order to determine whether Apex1 knockdown embryos could be rescued by NAC, fertilized embryos no older than 4 cell stage were microinjected with 0.15 mM Apex MO and incubated at 29°C until 4 hpf. They were then incubated in 1 mM NAC for 24 hours at 29°C and survival was recorded. Three independent experiments were performed and NAC treatment either had no effect or decreased survival of Apex1 knockdown embryos but did not rescue them (Data not shown).
FIGURES

Figure 1: Peroxide treatment of 24 hpf embryos causes an increase in 8-oxoG levels. Comparison image showing the fluorescence intensities of control and peroxide treated embryos after IHC. A and B are bright field and fluorescence images of the control whereas C and D are the bright field and fluorescence images of the H$_2$O$_2$ treated embryo. The areas highlighted by the white lines indicate the head and tail areas considered for fluorescence quantification.
Figure 2: Graph showing the fluorescence ratios of peroxide treated embryos relative to control embryos in head and tail regions. At least four embryos were observed per group per independent experiment. A total of six independent experiments were performed. Statistical analysis revealed that only the head region exhibited a significant increase in 8-oxoG levels (One tailed, unpaired t-test). Error bars indicate standard error of the mean.
Figure 3: None of the early embryonic stages or regions showed a detectable difference in 8-oxoG levels. Embryos were fixed at required stages (2 cells, 4 cells, 8 cells, high, sphere, dome, 30% epiboly and 50% epiboly) and then subjected to IHC. The regions outlined in white are the cell mass. At least three embryos were examined per stage per independent experiment. A total of four independent experiments were performed.
Figure 4: Knockdown of Apex1 leads to increase in 8-oxoG levels in early embryonic stages. Apex knockdown embryos (bottom panels) exhibited increased fluorescence (arrows) compared to control embryos (top panels) at various stages (2 cells, high, dome, 30% epiboly). At least four embryos were viewed per stage per independent experiment. Images below are representative of two independent experiments.
Figure 5: A) Peroxide killing curve generated by treating embryos with indicated concentrations of H$_2$O$_2$ at 3 hpf for 30 minutes. Three independent experiments were performed. Error bars indicate standard error. B) N-acetylcysteine killing curve generated by incubating 4 hpf embryos at indicated concentrations of NAC. Error bars indicate standard error (except for 2 and 3mM NAC where error bars indicate range). Four independent experiments were performed.
Figure 6: N-acetylcysteine (NAC) treatment decreases survival in peroxide treated embryos. Embryos were treated with indicated concentrations of peroxide for 30 minutes followed by 0 or 1 mM NAC. Results were generated from three independent experiments. Open circles and blocked squares represent individual point values from the three experiments. The lines are drawn to intersect the average values.

![Graph](image-url)
Figure 7: NAC treatment does not decrease 8-oxoG levels in peroxide treated embryos. Panels A, B, C and D show the embryos after IHC. At least four embryos were observed per group per independent experiment. Two independent experiments were performed.

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Figure 8: NAC treatment does not decrease 8-oxoG levels in peroxide treated embryos. Graphical representations of the head and tail fluorescence intensities quantified by ImageJ to measure 8-oxoG levels. Error bars indicate range. At least four embryos were examined per group per independent experiment. Two independent experiments were performed.
DISCUSSION

DNA is the molecule which encodes the genetic blueprint of life. However, it is under constant attack by many endogenous and exogenous sources of damage which can be fatal to the living cell (3). The only reason cells can survive this onslaught of DNA damage is because of the presence of multiple, efficient repair mechanisms such as NER, MMR, NHEJ, HR and BER. Among these pathways, BER is the most commonly occurring one as it is responsible for repairing the most common form of damage – 8-oxoG, which results from ROS attack (3, 63). ROS are known to be highly reactive and can damage biomolecules and thus harm the living cell. Exposure of DNA and other biomolecules to ROS is a very common event as ROS are produced constantly by many endogenous and exogenous sources. While BER can repair DNA damage, the cell also protects itself from ROS with antioxidants which maintain a reducing environment inside the cell (32). Although ROS are harmful to the cell at high concentrations, they also serve as intracellular messengers in important signaling pathways at low concentrations (43, 44). It has been shown that a redox balance is required in the cell for it to function properly and many important transcription factors which play important roles in cell proliferation and differentiation during embryogenesis are redox sensitive (45, 46). Reductive stress, which manifests as an increase in
NADH/NAD+ ratio and/or increase in reduced glutathione (GSH) levels \((64, 65)\), has been shown to be just as dangerous as oxidative stress \((50, 66)\). Redox status is important for DNA itself as well as the protein that bind/recognize DNA. Studies in our lab have also shown that the oxidative damage to DNA can itself modulate binding by transcription factors both positively and negatively. For instance, oxidative damage to selected sites in the consensus sequence of Creb1, which regulates one fourth of the mammalian genome, can affect the binding of the Creb1 protein to its sequence (S.P.G. Moore, manuscript in preparation) both positively and negatively. Based on all this evidence, it is clear that ROS may have an important role in embryonic development.

To understand this role further, I chose to use the zebrafish system, the model organism employed by the Strauss lab. The goal of my project was to understand if there was a role played by ROS during zebrafish embryogenesis. Specifically, I wanted to find out if there were any stages during early embryogenesis which showed increased levels of oxidative damage \((8\text{-}oxoG)\). I also wanted to examine selective regions of the embryo which showed unusual levels of oxidative damage at early developmental stages. I also tried to determine whether embryos treated with \(H_2O_2\) could be rescued by antioxidant treatment. I knocked down the central BER enzyme Apex1 using a MO in order to observe its effects on oxidative damage levels. I made the following observations from my experiments:
1. Peroxide treatment of 24 hpf embryos resulted in significantly increased 8-oxoG levels in the head region but not the tail region.

2. No differences in 8-oxoG levels were detected between early stages or regions of the early embryo (prior to ~5.3 hpf) using standard fluorescence microscopy.

3. Apex1 knockdown resulted in increased 8-oxoG levels in early (prior to ~5.3 hpf) embryonic stages.

4. NAC treatment did not rescue peroxide treated embryos but sensitized them instead.

5. No significant differences in 8-oxoG levels were detected between control and peroxide + NAC treated embryos using standard fluorescence microscopy.

*Head region of the embryo was found to be more sensitive to peroxide treatment than tail region*

After differentiation begins at the gastrula stage, different regions of the embryo develop at different rates (60) and the genes that are transcribed and expressed at the different regions of the embryo may also vary. If ROS have a role in regulation of gene expression, then there should be a difference in the sensitivity of different regions of embryo to oxidative stress. To test this hypothesis, embryos were treated with 0.44 M H₂O₂ for one hour at 24 hpf, when there is a distinct difference
between the head and tail region (60). The results showed that both the head and
tail regions were sensitive to H$_2$O$_2$ treatment. However, only the change in
fluorescence in the head was significantly different from control. Even though the
tail region showed an increase in 8-oxoG levels, it was not consistent between
repeats and was not significant (p>0.05).

*Differences in 8-oxoG levels between different stages and regions of the embryo
could not be detected*

Zebrafish embryo development at the early stages is fast and dynamic. There is
rapid cell proliferation for the first ten divisions, followed by differentiation (60).
Hence, it was essential to look at physiological 8-oxoG levels during these stages
without the addition of an external ROS source. In order to do this, early stage
embryos were fixed for IHC. Although no differences in 8-oxoG levels were
detected either between the different stages or in different regions of the embryo
using standard fluorescence microscopy, we cannot say conclusively that the
differences are absent because these differences could be too subtle to be observed
using standard microscopy.

*Apex1 knockdown results in increased 8-oxoG levels in early stage embryos*

Apex1 is the central enzyme of the BER pathway and its complete knockdown is
embryonic lethal by the mid blastula transition in zebrafish. Microinjection of
lower concentrations of MO lead to improper brain and cardiac development and
eventually to death by 7 dpf (56). Previous studies in our lab have shown that Apex1 regulates the expression of the critical transcription factor Creb1 (51) and Apex1 knockdown embryos exhibit elevated 8-oxoG levels at 24 hpf (Pei, D.S., manuscript in revision). Hence, it is important to look at the effects of knocking down Apex1 on 8-oxoG levels in earlier embryonic stages. This was achieved by microinjecting embryos before they reached the 4 cell stage with 0.15 mM Apex1 TS-MO and fixing them for IHC at the required stages. As expected, the results showed that knocking down Apex1 resulted in an increase in 8-oxoG levels at the 2 cell, high, sphere, dome and 30% epiboly stages.

_NAC treatment sensitized peroxide treated embryos instead of rescuing them_

Antioxidants have been claimed to reverse the effects of ROS on living cells and increase their survival (67) but some studies claim that antioxidant treatment is actually deleterious (48, 50). In order to test the effects of antioxidant treatment on zebrafish embryos and to observe whether this treatment could rescue embryos from the effects of peroxide treatment, 3 hpf embryos were treated with peroxide followed by incubation in NAC and survival was recorded 24 hours later. The results quite clearly showed that NAC did not rescue peroxide treated embryos but sensitized them even more. These results could be explained using the evidence from a study which showed that, GSH mediated reductive stress induced by NAC
treatment could actually trigger mitochondrial oxidation and increase cytotoxicity even at low levels of ROS (64).

**Peroxide +NAC treatment did not cause any detectable changes in 8-oxoG levels**

Since NAC did not rescue embryos exposed to $\text{H}_2\text{O}_2$, we were curious about whether levels of 8-oxoG changed with NAC treatment. The results showed that NAC was unable to reverse peroxide-induced increase in 8-oxoG levels. One way of explaining these results is by using the argument that increased reductive stress can trigger mitochondrial oxidation. It is possible that this leads to increased ROS production, which might be the reason for 8-oxoG levels not decreasing with NAC treatment. An alternate explanation is that NAC might inhibit enzymes of the BER pathway. We could not find any studies supporting this in normal cells, but a study conducted using cells from patients with Battens disease, which is characterized by increased BER activity, showed that NAC treatment reduced Ogg1 and DNA polymerase beta message and protein levels (68). There is also the possibility that the acidic pH of 1mM NAC (pH 3.3) might have been responsible for the decreased viability. The decreased pH had little effect on viability when embryos were treated with NAC alone, which is evident from the killing curve data (~92% survival) (Figure 5B). However, in conjunction with peroxide treatment or Apex1 knockdown, the acidic pH may have a role in decreasing viability.
**Conclusion:**

Studies have shown ROS to be a double edged sword. At high concentrations, they can attack most types of biomolecules and cause damage to the living cell. However at low concentrations, they have been implicated as messengers involved in cell signaling during cell proliferation and differentiation. The redox balance in the cell is known to be critical for its wellbeing; furthermore, reductive stress could be just as dangerous as oxidative stress (45, 46, 50, 66). In this thesis, I have attempted to answer some basic questions pertaining to the role of ROS in zebrafish embryonic development.

I have shown that both the head and tail regions were sensitive to H₂O₂ treatment. However, only the change in fluorescence in the head was significantly different from control which could mean that the gene expression in the head region may be partly regulated by redox status. This hypothesis is further supported by studies which show that redox sensitive transcription factors such as HIF-1, Nrf-1 and Wnt (45) are involved in brain development and transcriptional regulation (69-71).

I was not able to detect any differences in 8-oxoG levels or localization between early stage embryos. As mentioned earlier, this might be due to the limitation of optics rather than the lack of any differences.

I have also shown that Apex1 knockdown caused an increase in 8-oxoG levels in early stage (until ~5.3 hpf) embryos. This reinforces the argument that Apex1,
which is a regulator of oxidative damage as the main enzyme of the BER pathway and a regulator of the critical transcription factor Creb1, has a major role to play in regulating embryonic development of zebrafish.

Finally, I have shown that NAC treatment does not rescue but sensitizes peroxide treated embryos, which might be due to the triggering of increased mitochondrial oxidation and cytotoxicity by GSH mediated reductive stress induced by NAC. This result lends further credence to the importance of maintaining appropriate redox balance in the embryo. It also suggests that we should not blindly encourage the increasing usage of antioxidants as beneficial health supplements until there is a deeper understanding of the concepts of oxidative stress, reductive stress, redox homeostasis and their roles in cellular processes.

**Future directions**

The main limitation in this project was the limited optics available for imaging. Due to time and logistical constraints, I was not able to utilize a more powerful optical system to try and detect subtle changes in 8-oxoG level. This would be the logical next step to my studies, which would surely provide a clearer and more specific representation of the change in 8-oxoG levels.

Another caveat in this project was that I was unable to show that fluorescence levels decreased in embryos when digested with DNAse during IHC, which means that there is potential for artifact in the form of non-specific binding. One way to
overcome this would be to perform a control IHC experiment without the primary antibody to determine the fluorescence resulting from non-specific binding of the secondary antibody and subtract it from the experimental data as background fluorescence. One could also use a different antibody against 8-oxoG, where the specificity can be verified, or use other indicators of oxidative stress such as FapyG or nitric oxide synthase levels.

IHC experiments give us a snapshot of the 8-oxoG level at the stage when the embryos were fixed. However, this might not be the most accurate representation as the changes may be transient. Using a real-time quantification system such as ARQiv (Automated Reporter Quantification in vivo), which can monitor changes in the levels of 8-oxoG continuously and quantify the fluorescence of various regions simultaneously (72), would be much more effective.

In the experiments involving antioxidant treatment, pH of 1 mM NAC was found to be acidic (3.3). Although this did not adversely affect viability of embryos when they were treated only with NAC (Figure 5B), there is a chance that it might do so when NAC treatment is combined with peroxide treatment or Apex1 knockdown. In order to rule out this possibility, the pH of NAC could be neutralized before treatments.

Knocking down other proteins of the BER pathway, such as Ogg1 and Polβ, and the critical transcription factor Creb1 in order to observe the effects on 8-oxoG levels
might also provide insight as to how the BER pathway is involved in ROS mediated regulation of embryonic development. Finally, antioxidants and pro oxidants other than NAC and H$_2$O$_2$ can be used to perform similar damage/rescue experiments to see if they have a different effect on the embryos. In conclusion, my project is one of the first ones to question the role of ROS, redox balance and the effect of antioxidant treatment on the embryonic development of the zebrafish system. I believe that it can act as a starting point for future studies which can build upon it with better optics and real-time quantification systems.
REFERENCES


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