A Thesis

entitled

The Role of the Cytosolic Sulfotransferase SULT2 ST2 in Zebrafish Development

by

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Sulfonation is an important reaction in regulating the biological activities of a variety of endogenous and environmental compounds and is catalyzed by cytosolic sulfotransferases that use PAPS (3′-phosphoadenosine-5′-phosphosulfate) as the sulfonate (SO₃⁻) donor. SULTs are present in mammals and other vertebrates and play an important role in the detoxification of xenobiotics especially environmental estrogens. SULTs are also involved in the biotransformation of endogenous compounds (hormones, steroids) which might be a mechanism for maintaining the homeostasis of these compounds in-vivo. In this study we have attempted to assess the role of the hydroxysteroid sulfotransferase SULT2 ST2 in zebrafish (Danio rerio) development by knocking down the expression of the enzyme using a morpholino.

Zebrafish embryos were microinjected with a morpholino which had a sequence complementary to the sequence of the SULT2 ST2 gene. The injections were done when
the embryos were in the 1-4 cell stage with three concentrations of the morpholino: 0.5 ng/nL, 1.0 ng/nL and 2.0 ng/nL. The embryos were then observed for survival rates and abnormal phenotypes up to 144 hours post fertilization (hpf). The phenotypes observed were cardiovascular abnormalities such as cardiac edema and irregular heartbeat, abdominal edema, lordosis, notochord deformities, tail deformities and very few cases of craniofacial malformations. At the highest concentration of the morpholino, almost all knockdowns displayed mild to severe cardiac edema which was the specific phenotype at that concentration after the hatching period (48-55 hpf). The extent of knockdown was also determined by western blot experiment and it was found that the knockdown was partial and there was some enzyme present at measurable levels in the embryos.

In conclusion, morpholino knockdown of the SULT2 ST2 gene in zebrafish embryos caused several non-specific phenotypes. At the 2.0 ng/nL concentration mild to severe cardiac edema was observed in the most of the knockdowns after hatching which might indicate that SULT2 ST2 has a role to play in the cardiac development of zebrafish that involves maintaining steroid hormone homeostasis.
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1. INTRODUCTION

1.1 Sulfotransferases

Sulfate conjugation was first reported by Baumann in 1876. Sulfonation is an important pathway in the metabolism of numerous xenobiotics, drugs and endogenous compounds such as hormones, neurotransmitters, peptides and lipids. Sulfotransferases or SULTs are the group of enzymes that catalyze the transfer of a sulfonate group from the active sulfate, 3’-phosphoadenosine 5’-phosphosulfate (PAPS), to the substrate compounds containing hydroxyl or amino groups. For most xenobiotics and small endogenous substrates, more water-soluble products are formed aiding their excretion such as for drugs like acetaminophen. For xenobiotics such as N-hydroxy arylamines, polycyclic aromatic hydrocarbons, sulfonation is an activation process that leads to highly reactive electrophiles that are both mutagenic and carcinogenic. (Gamage et al., 2005)

There are two broad classes of SULTs: (1) Membrane-bound SULTs located in the golgi apparatus responsible for the sulfonation of peptides, lipids, and other macromolecules and (2) Cytosolic SULTs responsible for the metabolism of xenobiotics and small endogenous substrates such as steroids, hormones and other smaller molecules and are involved in the Phase II detoxification pathway (Suiko et al., 2005). On the basis of their amino acid sequence identity, it has been proposed that all cytosolic SULTs from
vertebrate animals belong to a gene superfamily which has been further classified into gene families. The two major families of cytosolic SULTs are the phenol SULT family (SULT1) and the hydroxysteroid SULT family (SULT2). The SULT1 family has at least four sub-families, SULT1A (phenol), SULT1B (thyroid hormone), SULT1C (hydroxyarylamine) and SULT1E (estrogen). The SULT2 family comprises of two sub-families, DHEA (dihydroepiandosterone) SULT (SULT2A) and cholesterol/pregnenolone SULT (SULT2B). The SULT2 sulfotransferases are involved in sulfonating both endogenous and exogenous steroids which is an important mechanism for homeostasis, bioactivation or transport of these compounds (Yasuda et al., 2006). SULTs belonging to the same family share at least 45% homology in amino acid sequence and the subfamilies share more than 60% homology (Yasuda et al., 2006).

SULT2 ST2 has been identified and characterized as a cytosolic SULT in zebrafish (Danio rerio) belonging to the DHEA sulfotransferase family. There are 864 nucleotides in the open reading frame of the sequence that code for a 287-amino acid polypeptide. This SULT contains sequences resembling so called “signature sequences” (YPKSGTxW in the N-terminal region and RKGxxGDWKNxFT in the C-terminal region) which are characteristic of SULTs. The N-terminal sequence has been shown by X-ray crystallography to be responsible for binding to the 5’-phosphosulfate group of PAPS and hence designated as the “5’-phosphosulfate binding motif”. The SULT also contains a “3’-phosphate binding motif” that binds to the 3’-phosphate group of PAPS. BLAST sequence analysis showed that the zebrafish SULT2 ST2 sequence displays 43% identity to human SULT2B1a and 40% identity to human SULT2A1. It displays a lower
% identity to other known SULTs. It also shows 87.5% identity to zebrafish SULT2 ST1 (Yasuda et al., 2006).

SULT2 ST2 exhibits strong activity toward DHEA and has an optimum activity at pH 6.5. It also shows sulfating activities toward pregnenolone, 17β-estradiol and estrone. However, it does not exhibit detectable activities toward endogenous compounds like L-DOPA, dopamine, hydrocortisone, progesterone, thyroxine and exogenous compounds like acetaminophen, n-napthol, p-nitrophenol (Yasuda et al., 2006).

SULT2 ST2 mRNA is significantly present in unfertilized eggs that indicate its maternal origin. After fertilization, the mRNA is not detected until 12 hpf during the neurula/segmentation period. The expression increases drastically at the larval stage and juvenile stage as well as being detected in adult male and female zebrafish (Yasuda et al., 2006).

1.2 Zebrafish as a model for biomedical research

Zebrafish (*Danio rerio*) is a tropical freshwater fish that belongs to the minnow family (Cyprinidae) and is named for five uniform, pigmented, horizontal blue stripes on the side of the body, extending to the end of the caudal fin. They can grow up to 2.5 inches in length. Males have a torpedo shaped body and have gold stripes and blue stripes. Females have silver stripes and a larger whitish belly. Adult females have a small genital papilla in front of the anal fin origin (Westerfield, 1995).

The zebrafish was originally envisioned as a model to bridge the gap between fly/worm and mouse/human for understanding embryonic development (Barut and Zon, 2000). Recently, they have become one of the most popular model organisms in
Developmental biology and molecular genetics as well as in toxicology. Development in early life stages of all vertebrates is highly conserved, and therefore there are significant similarities in the morphology of all vertebrate embryos which allows the extrapolation of data from zebrafish to humans. With the advancement of technology, mutant zebrafish, high-throughput screening and bioassays for toxic and therapeutic end-points in zebrafish will become frequently used. Hence zebrafish can serve as a good model for toxicology studies (Hill et al., 2005).

There are a number of advantages of using zebrafish as a toxicological model. First, they are small in size which reduces husbandry costs and in contrast to larger species, lower dosing solution amounts reduce costs and also result in limited volumes of waste for disposal. Small embryos allow a large sample size to be used for parallel experimental treatments (Hill et al., 2005). Apart from their size, the zebrafish has the advantage of external development with optically transparent and non-sticky embryos. They have a relatively short generation time (2-3 months to maturity) and one pair of adult fish is capable of producing approximately 200-300 eggs per week (Barut and Zon, 2000). The basic body plan is laid out after 24hpf and embryos hatch approximately at 2-3 days post fertilization (dpf) and at 5 dpf, organogenesis of major organs is completed. Their transparency allows unobstructed observations of the main morphological changes up to and beyond pharyngulation (24-48 hpf). Furthermore, their optical clarity has facilitated identification of genes that are important in development by mutagenesis screens (Scholz et al., 2008). Another advantage is that whole mount larval staining can be performed rather than having to dissect the tissue or stain sections for histological tests. Also, embryological development can be continuously followed in live individuals.
rather than harvested embryos, unlike rodents. Additionally, zebrafish embryos that have malformations, missing organs or organ dysfunctions, can survive usually past the time in which those organs start to function in healthy individuals (Hill et al., 2005).

In contrast to cellular replacement methods, the zebrafish embryo model provides a complex, multicellular system that integrates the interaction of various tissues and differentiation process (Scholz et al., 2008). The zebrafish can also be used for screening of acute toxicity of drugs which helps better understanding of toxic effects of potential drugs on mammals. In a recent study, 12 compounds were tested in zebrafish embryos and the results were compared with the teratogenicity of those compounds in mammals. In case of non-teratogenic compounds the coincidence was observed in 75% while for the teratogenic compounds, there was 100% coincidence (Belyaeva et al., 2009). Since a lot of early developmental processes are conserved in all vertebrates, zebrafish embryos are used as a vertebrate model for screening of drugs influencing vital processes such as angiogenesis (Belyaeva et al., 2009). They are also used for determining total and organ-specific toxicity. Most human genes have homologues in zebrafish and functional domains in proteins like kinase ATP-binding domains share 100% homology. Since the binding of drugs occur at functional domains, the use of zebrafish for studying drug effects on humans is very reasonable. A very good example of drugs showing equivalent effects in zebrafish and humans is that drugs causing elongation of QT interval also caused bradycardia and AV blockade in zebrafish. After findings on silver nanoparticle toxicity in zebrafish, the model is being used for the evaluation of toxicity of phospholipid nanoparticle-based drug formulations (Belyaeva, et al., 2009).
Zebrafish embryo toxicity tests are also important in the risk assessment of environmental chemicals which cause serious effects such as disturbance of the immune defense in fish populations and endocrine disruption (Scholz et al., 2008). Of these, the effect of chemicals mimicking endogenous hormones has been intensively studied because of their association with numerous reproductive disorders. Some of them that affect estrogen-mediated responses have been the main focus and a major source is wastewater treatment facilities. Xenoestrogens like 17α-ethinylestradiol have been reported to cause vitellogenin induction, ovarestes, altered sex ratios and impaired reproductive capacity. Zebrafish has proven to be an excellent model for assessing the risks associated with environmental endocrine disruptors in the wildlife (Janz and Hill, 2002). Many mutant zebrafish models are useful for studying human diseases (Hill et al., 2005). Specially in hematopoietic, cardiovascular and kidney disorders zebrafish is amenable as a model system since these organ systems are very similar to higher vertebrates, thus providing a simple and accessible system for investigation (Dooley and Zon, 2000). There has also been use of metabolic models for disease states like diabetes, as the regulation of zebrafish glucose metabolism by insulin is similar to mammalian glucose metabolism. Studies of apoptotic mechanisms which can be utilized for treatment of tumor and degenerative diseases have also been developed (Belyaeva et al., 2009). All of these combined advantages make the zebrafish an excellent model for studying a myriad of research problems such as the genetic basis of development, toxicity testing of xenobiotics and drugs, ecotoxicology, metabolic pathways and various disease states (Dahm and Geisler, 2006).
1.3 Zebrafish embryonic developmental stages

Zebrafish embryo assays are more effective than using juvenile or adult fish since they provide diverse end points for toxicity evaluation. They are more sensitive to chemical stress than older fish (Oberemm, 2000). From an ethical standpoint too, embryos are preferred because most chemicals that would cause gross effects during early embryonic development will cause acute damage in adult fish (Oberemm, 2000). Hence, it is very important to know the different stages of normal embryonic development in zebrafish in order to correctly assess toxicity end points. There are seven broad periods of embryogenesis in zebrafish development- zygote, cleavage, blastula, gastrula, segmentation, pharyngula and hatching periods. These stages describe development for the first three days after fertilization (Kimmel et al., 1995). The zygote period occurs between 0 to 3/4 hour post fertilization (hpf), that is until the first cleavage occurs. After the first cleavage, the cells or blastomeres divide at about 15 minute intervals and so the cleavage period ranges from ¾ to 2.25 hpf which is until the 64 - cell stage. The blastula period stretches from 2.25 to 5.25 hpf where the embryo divides from 128 cells to about the 30% epiboly stage which produces a blastoderm of uniform thickness. Percent epiboly is defined as the fraction of the yolk cell that the blastoderm covers. Next is the gastrula period stretching from 5.25 to 10 hpf during which epiboly continues and primary germ layers as well as the embryonic axis are produced. During the segmentation period (10-24 hpf), a variety of morphogenetic movements occur – somites develop, rudiments of primary organs become visible and the embryo elongates as the tail extends. The pharyngula period (24 – 48 hpf) shows a well-developed notochord. The embryo continues elongating and the head straightens out. The brain gets
sculptured into five lobes. Other important features are the formation of fins and circulatory system, and tactile sensitivity appears. During the final period or the hatching period (48-72 hpf), morphogenesis of many organ rudiments is almost complete and slows down. Pectoral fin and protruding mouth development are the notable features of this stage (Kimmel et al., 1995).

1.4 Morpholino knockdown strategies

The zebrafish genome project has provided available DNA sequence information that is useful in gene identification, expression and full length cDNA isolation. The function of many of these genes is not deducible from just their sequences and hence the need for the development of tools to investigate the roles of genes (Boonanuntanasarn, 2008).

There are several efficient methods for the addition or inhibition of gene expression to study the role of genes. Creating transgenic fish are a valuable means since it allows the detection of a phenotype that has been altered by adding a function. But this method is time consuming and so is the gene knockout technique. Gene knockdown is an alternative technique where an antisense agent is introduced that has a complementary nucleotide sequence to target mRNA in living cells to inhibit gene expression in a specific manner. The role of such a gene can be determined by phenotypic comparison. It seems to be the most effective technique in fish since it does not require specialized facilities and temporary effects can be determined within one generation (Boonanuntanasarn, 2008).
Morpholino oligonucleotides are antisense P-chiral analogs of nucleic acid that have morpholine rings instead of ribose rings and linked through phosphorodiamidate groups instead of phosphodiester linkage (Summerton and Weller, 1997). They have shown high stability in living cells, excellent solubility in any transfection or microinjection solution, low cellular toxicity, and resistance to repeated freezing and thawing (Boonanuntanasarn, 2008). The backbone makes them resistant to digestion by nucleases and also since they have no significant ionic charge at neutral pH they are less likely to interact non-selectively with cellular proteins (Bill et al., 2009). Convincing proof of their specificity and efficacy comes from the fact that morpholino injection results in phenotypes that are identical to those observed from null mutations (Gilmour et al., 2002). They knock down gene expression by an RNaseH independent mechanism (Eisen and Smith, 2008).

Morpholinos act by two main mechanisms: splice blocking and translational blocking. The mechanism of splice blocking is thought to be binding and inhibiting pre-mRNA processing by the inhibition of splicesome components (Bill et al., 2009). With this mechanism the efficacy of the morpholino could be quantified such as in the knockdown of zebrafish Islet1 protein which proved that the protein is important for motorneuron differentiation (Eisen and Smith, 2008). Translational blocking morpholinos act by binding complementary mRNA sequences at about the first 25 bases past the AUG translation start codon (within the 5’ UTR), hindering ribosome assembly (Bill et al., 2009). For example, they have been used to establish the role of Notch signaling in zebrafish neurogenesis (Eisen and Smith, 2008). Morpholinos also act by inhibiting maturation of miRNA (Moulton and Yan, 2008). In zebrafish, morpholinos are
introduced into the yolks of 1-4 cell stage embryos since the cytoplasmic bridges connecting these early embryonic cells allow rapid diffusion of hydrophilic morpholinos, resulting in uniform delivery. The new phenotypes are usually identifiable within the first three days of development, but effects have also been observed at 5 days post-fertilization (Bill et al., 2009). Morpholinos can be used to eliminate the function of genes acting early in development, including maternally localized genes. Weaker phenotypes can be observed by injecting lower amounts suggesting that the method can be used to generate the equivalent of an allelic series. Some batches of morpholinos show increased toxicity due to the presence of residual acid from the synthesis procedure and such injections may result in high lethality rates. However, toxic batches can be identified by using phenol red which turns yellow if the solution is acidic (Gilmour et al., 2002). Although an even distribution of morpholinos have been seen in zebrafish after injection, it is important to note that phenotypes may vary considerably in severity and specificity (Hill et al., 2005). The most common non-specific effects are delayed development and cell-death. For the developmental effects, it is more important to stage-match than age-match the experimental embryos with the control group. Cell death is sometimes restricted to the brain and sometimes widespread, causing early lethality. In some cases, it could be shown that the particular phenotype is due to that morpholino by rescuing the phenotype with the wild type mRNA of the gene of interest (Mullins, 2005).

Morpholinos can be delivered into the cytosol or nuclear compartment of cells by various methods. An early method was scrape loading. This technique never delivered oligos to all cells in culture and has been replaced by more reproducible methods. The most widely used method for delivering morpholinos is microinjection directly into the
cytosol. Another standard technique of morpholino delivery is electroporation where the cells become transiently permeable for the oligos to diffuse across the plasma membrane. Endo-Porter is another method adopted to deliver morpholinos to the cytosol through an endocytic pathway. It is an amphiphilic peptide that becomes cationic at a low pH and morpholinos are co-endocytosed with it. Other techniques of morpholino delivery include peptide conjugation of which arginine peptides are the most published and vivo-morpholinos which have an octaguanidinium group attached to the 3’-end used for delivering splice-inhibiting antisense activity to the nuclei of cells by intravenous administration (Moulton and Yan, 2008).

Morpholinos also have applications in developing models of human disease such as hepatoerythropoietic porphyria (HEP) that is caused by a defect in heme biosynthesis (Ekker and Nasevicius, 2000). They can be used to target viral RNA such as hepatitis C, dengue virus, SARS virus. Radioisotopes can be delivered to target tissues using morpholinos for imaging or therapeutic applications while exposing the organism to lower doses of radiation away from the targeted region (Moulton and Yan, 2008).

In the following research we have attempted to use morpholino knockdown technique to assess the role of the cytosolic sulfotransferase SULT2 ST2 in the development of Danio rerio.
2. OBJECTIVES

Over the past decade there has been considerable concern about the potential effects of endocrine disruptors, especially environmental estrogens (such as bisphenol A, nonylphenol and 17α-ethinylestradiol), on humans, wildlife as well as in fish populations. Their major source is wastewater treatment facilities and they have been shown to have effects in humans such as effects on male reproductive capacity and breast cancer in women (Safe et al., 2001) in addition to impaired reproductive capacity, ovatestes and altered sex ratios in fishes (Hill and Janz, 2003). Cytosolic sulfotransferases (SULTs) are a class of enzymes in vertebrates that are responsible for the sulfonation leading to detoxification or bioactivation of environmental estrogens as well as endogenous hormones and steroids. The role of the hydroxysteroid SULT SULT2 ST2 in zebrafish development was investigated by morpholino knockdown of the SULT2 ST2 gene and an attempt was made to relate the changes caused by the reduced expression of the enzyme and its effect on hormone/steroid homeostasis in zebrafish.
3. MATERIALS AND METHODS

3.1 Zebrafish breeding and husbandry

The AB strain of zebrafish (*Danio rerio*) was obtained from the Zebrafish International Resource Center (ZIRC) at the University of Oregon. They were maintained in the circulating water system AHAB from Aquatic Ecosystems at 28°C in the Department of Laboratory Animal Research. A water pH of 7.6 was maintained. For best breeding results, procedures were followed as instructed in the Zebrafish Book. The fish were fed 3-4 times a day with a diet consisting of live brine shrimp obtained from Brine Shrimp Direct and Tetramin® Flakes (Blacksburg, VA). For the purpose of breeding, ten gallon tanks were used which were scrubbed and cleaned thoroughly to prevent any contamination, prior to breeding. The tanks were filled with 20 L RO water along with 60 mg/L of Instant Ocean sea salt. The water was heated to 28°C by a submersible heater and maintained at that temperature. To ensure maximum egg production, a group of 6 fish were used for breeding with a female to male ratio of 2:1. A light/dark cycle of 14 hours of light and 10 hours of darkness was maintained. The fish were fed before putting them into the breeding tanks. Glass marbles were laid at the bottom of the tanks right before the lights turned off, which acted as a substrate for laying eggs and prevented the fish from consuming the fertilized eggs. The fish started breeding in the morning at the first light and since microinjections need to be done in the 1-4
cell stage embryos, eggs were collected as soon as they were laid by siphoning. They were then placed in egg water (RO water with 60mg/L salt) in Petri dishes at 28°C.

3.2 Morpholino preparation and Microinjection

For the purpose of knocking down the SULT2 ST2 gene, the corresponding morpholino (antisense oligonucleotide) was ordered from Gene Tools, LLC, with the sequence 5’CCGAATACAGCTCCGATTCAGTCAT 3’. The morpholino stock solution was prepared according to the guidelines by Gene Tools. The 300 nanomoles of the lyophilized oligonucleotide was dissolved in 0.30 mL sterilized water to give a concentration of 1 mM to the stock solution. Dilutions were made from this solution to 0.5 ng/nL, 1.0 ng/nL and 2.0 ng/nL concentrations for the injections. This was done by diluting the stock solution with 0.5% Phenol red (from Sigma) and Danieau Solution using stock solutions of 5M NaCl (MCB SX420), 3M KCl (Fisher Scientific P217-500), 100mM MgSO$_4$.7H$_2$O (Acros Organics 423905000), 100 mM Ca(NO$_3$)$_2$.4H$_2$O (Fisher Scientific C109-500), and 1M Hepes (Fisher Scientific BP310-100). The final Danieau solution was 58 mM NaCl, 0.7 mM KCl, 0.4 mM MgSO$_4$, 0.6 mM Ca(NO$_3$)$_2$ and 5 mM Hepes, with a final pH of 7.6.

The final morpholino solution was then microinjected into 1-4 cell stage zebrafish embryos using a Narishige microinjector (Model: IM-9A) and micromanipulator (MN-151). Glass capillary tubes, 6 inches long and 1.0 mm in diameter (World Precision Instruments, Inc TW100F-6) were used as injection needles. The needles were pulled on a P-87 Flaming / Brown micropipette puller (Sutter Instrument Co.) with the following settings: Heat-365, Pull-70, Velocity-80, and Time-200. A scalpel was used to cut the
tips and bevel them. The needle was then loaded onto the micromanipulator and mineral oil was pushed to the tip. Approximately 5 µL of the injection fluid was drawn into the tip of the needle by applying negative pressure through the injector. Prior to loading the needle with the morpholino solution, it was heated for 10 minutes at 65°C in a thermal cycler (Eppendorf Mastercycler) and vortexed for a few seconds.

The embryos were lined up on the injection slide with their vegetal pole facing the tip of the needle. The micromanipulator was employed to gently push the needle into the vegetal pole, piercing the chorion and approximately 1 nL of morpholino solution was injected. The injection volume was estimated to be 1 nL which fills up approximately 1/5th of the total volume of the embryo (Rosen et al., 2009). This injection volume allowed 0.5 ng, 1.0 ng and 2.0 ng of the morpholino delivered for the three concentrations respectively. The same procedure was repeated for all the lined up embryos, removing any embryos crushed or damaged in the process. The injected embryos were then rinsed off the slide into a Petri dish with egg water. The microinjection needle was changed and another set of embryos was injected with the control solution (0.5% phenol red and Danieau buffer). They too were rinsed off into a Petri dish with egg water. Both sets of embryos were incubated at 28°C in egg water. Approximately, 80 knockdowns and 40 controls were injected per experiment. The embryos were observed under a Zeiss microscope (Axiovert 25) at 12 hpf, 24 hpf, 32 hpf, 55 hpf, 80 hpf and 144 hpf (according to Oberemm, 2000) to determine if any phenotypic abnormalities could be observed and pictures were taken using a Sony Cybershot camera.
3.3 Extraction of protein from zebrafish embryos for SDS-gel analysis and Western blot

The knockdown and control embryos were prepared for SDS-gel analysis and western blot experiment for the purpose of detecting the presence/absence of SULT2 ST2 according to the protocol described in the Zebrafish Book. The embryos (approximately 20 from each group) were dechorionated at 24 hpf and rinsed several times in Danieau solution in a 1.5 mL centrifuge tube. Excess liquid was removed from the tubes and the embryos were flash-frozen in liquid nitrogen. 50 µL of SDS sample buffer containing 63mM Tris-HCL, pH 6.8, 10% glycerol, 5% beta mercaptoethanol, 3.5% sodium dodecyl sulfate was added to the samples. Microcentrifuge pestles (Kontes Pellet Pestle) were used to homogenize the mixture. The samples were then boiled for 5 minutes in a dry bath incubator (Fisher Scientific) and were ready to be run on SDS gel.

3.4 SDS-PAGE and Western blot analysis

SDS PAGE was run using 12 % running gel in an electrophoresis system from Fisher Biotech (FB 400). The gel contained 12 mL Acrylamide-Bisacrylamide (30:0.8), 7.5 mL of 1.5M Tris (pH 8.8), 0.15 mL of 20% SDS, 10.2 mL of H₂O, 0.15 mL of 10% ammonium persulfate and 0.02 mL of TEMED. A 1X electrophoresis running buffer consisting of 25 mM Tris, 250 mM glycine (pH 8.3) and 0.1% SDS was used (Towbin et al., 1979; Laemmli, 1970). The samples were loaded into the wells and the gel was run at constant amperage of 35mAmps for 30 minutes. The molecular weight marker used for sizing purposes was Prestained Protein Molecular Weight Marker (Fermentas Life
Sciences #SM 0441). After gel electrophoresis was accomplished the gel was extracted from the cassette and prepared for western blot experiment.

Western blot experiment was run in a wet blot transfer apparatus from IDEA Scientific Co. It was filled with western transfer buffer with the composition of 39 mM glycine, 48 mM Tris base and 0.037% SDS and a pH of 8.3. A PVDF (Polyvinylidene difluoride) membrane (Immobilon P Transfer Membrane from Millipore) was used. Before the transfer started the membrane was washed with methanol, milliQ water and then agitated gently in the western transfer buffer for 5 minutes. The cassette was set up in the following order: screen, plate electrode, screen, three layers of sponge wetted by western blot buffer, two layers of 3 mm filter paper, gel, membrane (care was taken to roll out all the air bubbles between the gel and the membrane), three layers of filter paper, three layers of sponge (wetted by buffer), screen and electrode. The cassette was then fitted into the apparatus. The transfer process was run overnight at 150 mAmps and 10 Volts.

Once transfer was complete, blocking of non-specific detection was accomplished by floating the membrane in 25 mL of 5% dry milk solution for 1 hour with gentle agitation. Following this, 10 µL of antibody to SULT2 ST2 was added to the solution and incubated for 1 hour with gentle agitation. The milk solution was then discarded and 25 mL of 1X PBS was added to the membrane for wash away any unbound antibody. The washing was done 5 times for 5 minutes each. Blocking was carried out the same way with 5% dry milk solution for 5 minutes. The membrane was then probed with a secondary antibody conjugated with horse radish peroxidase for 1 hour with gentle agitation taking care to keep the membrane in dark during this process. The membrane
was washed as before with 1X PBS five times. It was then covered with approximately 1
mL of substrate solution (Pierce ECL Western Blotting Substrate from Thermoscientific
#32109) for 5 minutes. Next, the membrane was placed in a Kodak X-Ray exposure
holder and a RPI X-ray film (for autoradiography/chemiluminiscence) was pressed firmly
against the membrane in dark for 1 minute. The film was then processed in a medical
film processor (Konica Minolta SRX-101A). Finally, the bands on the film were
compared to the Fermentas marker labeling to mark the positions of the detected protein
bands according to their size.
4. RESULTS

The control and the SULT2 ST2 knockdown zebrafish were observed at the following critical developmental time points: 12 hpf, 24 hpf, 48 hpf, 55 hpf, 80 hpf and 144 hpf. Their survival rates as well as hatching rates and heart rates were noted. Any phenotypic mutations or morphological abnormalities were also noted. Observations were made for microinjected embryos at all three concentrations: 0.5 ng/nL, 1.0 ng/nL and 2.0 ng/nL.

4.1 Observation of lethality in Zebrafish embryo and larvae

At 0.5 ng of SULT2 ST2 morpholino injection, no specific trend could be found. There was some non-specific embryo lethality and after the initial cell death till 12 hpf no further lethality in the knockdowns was observed. No significant difference in the survival rates of the knockdowns and controls was found.

The concentration of the morpholino was then increased to 1.0 ng/nL. At this concentration there was significant lethality in the embryos and the larvae of both the control and the knockdown groups. The average survival rates of the controls and the knockdowns are depicted in Table 1. Controls and knockdown embryos showed similar embryo lethality in this group. There was a high lethality rate till 12 hpf and survival rates seemed to stabilize after that in the controls. In the knockdowns there was high embryo lethality until 24 hpf and survival rates reached a plateau after that time point.
The uninjected group kept in just egg water also showed similar survival rates. Figure 4 graphically represents the data in Table 1.

At 2.0 ng of morpholino injection, a slightly higher lethality was observed in the knockdowns than the controls. The controls showed average survival rates of 38%, 38%, 36%, 32%, 32%, 32% and 32% at 12 hpf, 24 hpf, 48 hpf, 55 hpf, 80 hpf and 144 hpf respectively. The fish in the knockdown group showed a lower average survival rate, almost half of that of the controls at those time points: 20%, 18%, 18%, 18%, 18%, 16% and 6%. Table 2 depicts the survival rates versus time of both the knockdown and the control groups at 2.0 ng/nL morpholino concentration. Figure 5 graphically represents the data in Table 2. It is interesting to note that there was a high rate of mortality in the embryos till 8-12 hpf and the lethality rate seemed to stabilize somewhat after that time and the progressive death rates were low after the hatching period for both injection concentrations and for the control embryos. However, there was a slight drop in the survival rate of the knockdowns again at 80 hpf.

4.2 Observations of the Hatching rates

The hatching rates of the controls and the knockdowns for both the morpholino concentrations were assessed at 55 hpf by counting how many embryos completely shed their chorions. Table 3 depicts the hatching rates of the control and knockdown embryos injected with both 1.0 ng/nL and 2.0 ng/nL concentrations at various time points. Hatching rates were calculated based on the number of embryos still living after 55 hours post fertilization. The embryos in the control group had a hatching rate of 94% while the embryos injected with 0.5 ng of morpholino had a 93% hatching rate. The knockdown
embryos injected with 1.0 ng of SULT2 ST2 morpholino had a hatching rate of around 91% which was not much different from the controls. The knockdowns injected with 2.0 ng of the morpholino had a hatching rate of about 75%.

4.3 Observation of the Heart rates

Observations were made for heart rates (beats per minute) starting from 24 hpf when the heart beat first develops until 80 hpf. No observations were made for 144 hpf time point as the pigmentation was too intense to observe anything and the fish were swimming around. Beats were counted for 10 seconds and then converted to beats per minute. Neither any significant changes nor any specific trends were found in the heart rates of the knockdowns. All control embryos showed regular heartbeat. Some of the most severely deformed knockdowns were observed to have an irregular heartbeat and hence it was hard to count the beats per minute.

4.4 Phenotypic observations of the injected Zebrafish

The controls and the knockdowns were observed at 12 hpf, 24 hpf, 48 hpf, 55 hpf, 80 hpf and 144 hpf for any phenotypic changes and pictures were taken with a Sony Cybershot camera. Observations were made for any craniofacial abnormalities, structure of the tail, jaw formation, pigmentation, cardiovascular abnormalities such as presence of edema in the cardiac sac, edema in the yolk sac and poor swim bladder inflation. For the embryos injected with 0.5 ng of morpholino hardly any mutations were seen and most of the knockdowns looked and developed similarly to the control embryos.
With the increased concentration of 1.0 ng/nL, some phenotypic changes started to appear but not until after hatching (55 hpf). The embryos in the control group mostly showed normal development with a regular heartbeat except for one or two that had mild cardiac edema and lordosis. In the knockdown group, around 50% of the larvae showed abnormalities like cardiac and abdominal edema, lordosis, and underdeveloped or abnormal tail structures. The number of larvae was based on how many were living at that particular time point. Some of the larvae with knocked down levels of mRNA lived until the 80 hpf time point while some lived until 144 hpf. However, the knockdowns with normal development continued to live up to and beyond 144 hpf. Except for the embryos with severe malformations, all the rest showed spontaneous movements and swam around. Figures 6, 7 and 8 depict the various phenotypic mutations observed with 1.0 ng/nL morpholino injection concentration at different time points.

The knockdown embryos injected with 2.0 ng of SULT2 ST2 morpholino also did not start manifesting the phenotypic abnormalities until after the hatching period (55hpf). The mutations were similar to the ones seen in the 1.0 ng knockdown group and again approximately 50% of the knockdowns living at that time point showed those abnormalities. The controls showed normal development except for one or two that showed mild edema and mild lordosis. A small number of knockdown embryos had some phenotypic mutations at the 24 hpf time point such as craniofacial abnormalities. The knockdowns with malformations usually lived until the 80 hpf or 144 hpf and then died with necrosis in the tissues. However, it was seen that at this concentration almost all the knockdowns had mild to severe cardiac edema. Figures 9, 10, 11 and 12 depict the
various phenotypic observations of the animals injected with 2.0 ng/nL morpholino injection concentration at various time points.

### 4.5 Western Blot

Western blot analysis was performed to quantify the SULT2 ST2 protein production in the control and the knockdown embryos. The experiment was performed on 20 embryos each from the control group and the knockdown embryos injected with 2.0 ng/nL morpholino solution concentration. The embryos were homogenized for this purpose with SDS sample buffer as described in the methods. After SDS gel electrophoresis and transfer to a PVDF membrane the protein was identified using a SULT2 ST2 antibody. Figure 13 represents the X-ray film that was processed after contact with the membrane for 2 minutes. The mass values of the bands for the marker are labeled with arrows from 117 kDa to 26 kDa. Lane 1 contained the band for the control embryos and Lane 2 contained the band for the knockdown embryos. The band for SULT2 ST2 was found to be positioned at the 33 kDa position in both the lanes as it was expected (Yasuda et al., 2006). The band in Lane 2 was found to be lighter in intensity than the band in Lane 1 which might indicate that that the SULT2 ST2 gene was at least partially knocked down but not completely.
5. DISCUSSION

Environmental estrogens have presented some concerns over the past few years as humans and wildlife alike are getting exposed to them on a daily basis mainly through the water supplies. They have been reported to cause a reduction in human male fertility and increased risk of breast cancer (Safe et al., 2001) in addition to reproductive problems in fish populations (Hill and Janz, 2003). Sulfotransferases (SULTs) are a class of enzymes that catalyze the sulfation/sulfonation of hydroxyl or amino groups on various endogenous compounds such as steroids, hormones, proteins and environmental estrogens. The cytosolic SULTs are a part of the Phase II detoxification pathway (Suiko et al., 2005; Gamage et al., 2005; Ohkimoto et al., 2003). The zebrafish cytosolic sulfotransferase SULT2 ST2 is a hydroxysteroid SULT belonging to the DHEA SULT sub-family. It has been shown to display the highest activity with DHEA which is an important precursor for the production of several sex steroids (Yasuda et al., 2006).

The zebrafish has been proven to be an excellent model for developmental toxicity and knockdown studies. This model was chosen for its high fecundity and transparent embryos that allow researchers to observe the all of the embryonic development. Morpholinos were used to knock down the expression of the zebrafish cytosolic sulfotransferase SULT2 ST2 by using microinjection technique. The morpholinos were injected into the yolk sac early in development (2-4 cell stage) so as to allow its effective diffusion into the cells. Three concentrations of the morpholino were
chosen for the injections: 0.5 ng/nL, 1.0 ng/nL and 2.0 ng/nL and the injection volume was 1 nL for each embryo allowing to inject 0.5 ng, 1.0 ng and 2.0 ng of morpholino respectively. This volume is the usual standard for injections and fills up about 10% of the egg volume. These concentrations were chosen since delivering up to 1-2 ng is the usual procedure in such experiments (Rosen et al., 2009). Too high of a concentration might be toxic to the embryo and too low of a concentration might not produce any effect at all.

Lethality observations were made for all three concentrations with no significant mortality at 0.5 ng of morpholino in the knockdowns compared to the controls. Both the groups had high embryo lethality rates until 8-12 hpf and all the rest of the living embryos survived until hatching and beyond up to 144 hpf (since they were observed only until 144 hpf) without any observable deformities. The high embryo lethality till the 8-12 hpf time point could indicate infertility issues as a similar lethality rate was also observed in the uninjected control embryos. For 1.0 ng of morpholino solution concentration, both the experimental and control groups showed a high mortality rate until 12 hpf where almost half of the embryos died. Again, this could be attributed to infertility issues. After the 24 hpf time point, the knockdowns showed a slightly lower survival than the controls which became lower progressively until 144 hpf when around 50% of the knockdown embryos alive at 24 hpf were alive. For controls, around 90% of the embryos alive at 24 hpf survived from 55 hpf to 144 hpf. The progressive decrease in the survival of the knockdowns compared to the controls from 24 hpf onward could be a non-specific effect of the morpholino as the most reproducible phenotype for all translation-blocking morpholinos is cell death (Bill et al., 2009). For 2 ng of morpholino
injection, the knockdown and the control embryos showed a drastic fall in survival at 12 hpf as before. However, this time the knockdowns had a survival rate of almost half of that of the controls at 24 hpf and the survival progressively decreased until 144 hpf when the final survival was 6% for the knockdowns and 32% for the controls as seen in Figure 5. This observation could be due to the higher concentration of the morpholino and non-specific widespread cell death as a consequence. At 80 hpf and 144 hpf, some of the larvae died from necrosis of tissues. It has been found that some morpholinos activate p53 and upregulate the p53 apoptosis pathway but it is not known by what mechanism (Bill et al., 2009; Eisen and Smith, 2008). This characteristic of the morpholino could also be a reason for the non-specific cell death observed in the two higher concentrations. One other reason could be that knocking down the SULT2 ST2 gene caused misbalances in hormone levels in the fish body (since SULTs help maintain the homeostasis of several steroids/hormones) and may have contributed to some of the mortality. More studies are needed to confirm this hypothesis.

The phenotypic malformations started to appear after hatching period (48-55 hpf) in the embryos injected with 1.0 ng/nL of morpholino concentration (Figure 6). SULT2 ST2 expression starts manifesting from the neurula/segmentation period (12 hpf) but it increases radically once the animal reaches the larval stage which is 48-55 hpf (Yasuda et al., 2006). This might explain the appearance of deformities at the onset of the larval stage. The most common abnormalities observed at this stage were cardiac and yolk-sac edema, deformities of the tail and lordosis. These malformations were seen in approximately half of the knockdown embryos. It was also observed that the severely affected larvae had irregular/slow heart rates. There wasn’t much change in the larvae
once they reached 80 hpf and 144 hpf. Since several phenotypes were observed in about half of the knockdowns, it could be ascribed to „off-target” effects of the morpholino. Morpholinos have the ability to influence the production of completely irrelevant gene products and the observed phenotypes may only partially be a result of the gene under study or in the worst case have nothing to do with the gene (Eisen and Smith, 2008). However, this conclusion cannot be made until further studies confirm it. The reason for observing various phenotypes could also be that the knockdown was incomplete either resulting from compensative effects of other related enzymes or the concentration of the morpholino.

Embryos injected with 2 ng of morpholino started showing some craniofacial malformations at 24 hpf as depicted in Figure 9 although the number of embryos having them was very low. These embryos did not survive beyond 55 hpf or 80 hpf. The rest of the knockdowns did not start manifesting phenotypic abnormalities until after hatching (48-55 hpf). Again, this could be due to reason that SULT2 ST2 expression increasing dramatically after hatching (Yasuda et al., 2006). The craniofacial abnormalities at 24 hpf could be explained by the fact that the concentration of the morpholino was doubled and it could have been a result of the toxicity from the morpholino itself or it could have been something inherently wrong with the embryos. SULT2 ST2 is known to have highest activity against DHEA which acts as a neuro- steroid playing an important role in the functioning of the neuroendocrine system. The fact that the enzyme is first expressed at the neurula/segmentation period (12 hpf) which coincides with the beginning of the development of the nervous system, has led to the question if the enzyme is involved in the regulation/homeostasis of DHEA (Yasuda et al., 2006). This could also explain the
appearance of craniofacial abnormalities in the zebrafish embryos. Further work is needed to verify this assumption. The morpholino concentration could be increased to see if it elicits the same phenotype in most of the embryos or challenge the knockdowns with DHEA or similar steroids and notice whether it produces the same phenotypes. The phenotypes observed after hatching included edema, lordosis and notochord deformities. The malformations actually worsened at 80 hpf and 144 hpf as depicted in Figures 11 and 12. This observation might point to the possibility of lower levels of the enzyme causing increased expression of steroid hormones over the time that could lead to the worsening of malformations in the larvae, although exposure studies on the knockdown embryos are needed to reaffirm this explanation. The occurrence of different phenotypes randomly at different stages of development could again be a result of off-target effects of the morpholino since morpholinos are usually known to produce a single phenotype in most of the experimental embryos. This logic yet again has to be confirmed by more experimental analyses such as an mRNA rescue experiment. An interesting fact to be noticed here is that almost all the knockdown embryos at this concentration expressed mild to severe cardiac edema at stages after hatching and the embryos displaying severe edema had an irregular heartbeat. This could be due to decreased level of the enzyme due to the knockdown or could be one of the off-target effects since the degree of edema was not equal in all knockdown embryos. Research has shown that Nr2F5, a member of the COUP-TF family of nuclear receptors, when overexpressed causes cardiac toxicity including edema in zebrafish. They are involved in the transcription regulation of steroid hormone receptors (Chen et al., 2008). The low expression of SULT2 ST2 could have altered steroid hormone homeostasis in a way that might have led to the overexpression
of the Nr2F5 gene and resulted in the observed cardiac edema. This hypothesis needs to be explored in detail to reach a conclusion. The varying degrees of edema in different larvae might be ascribed to the possibility that the knockdown was not complete or uniform in all experimental embryos. It may also point to the fact that in spite of the knockdown of the enzyme, there could be another protein compensating for the function of the enzyme (Moulton and Yan, 2008). Previous research has shown that the cytosolic sulfotransferase SULT2 ST1 displays the highest activity against DHEA (Sugahara et al., 2003). Hence it can be assumed that SULT2 ST1 or some other related enzyme might be compensating for the decrease in the levels of SULT2 ST2 and this could be one of the main reasons for observing a low/varying expression of the phenotype in the knockdown embryos.

Western blot analysis was used to verify whether the SULT2 ST2 gene was knocked down and if it was, then to what extent that was accomplished. Translation-blocking morpholino was designed for this study. In case of translation-blocking morpholinos the activity is measured by western blot using antibodies to the protein under consideration (Eisen and Smith, 2008; Moulton and Yan, 2008). Figure 13 depicts the western blot on a PVDF membrane. Both Lane 1 and 2 contain a single band at ~33 kDa which is the molecular weight of the enzyme SULT2 ST2 (Suiko et al., 2006), thus proving that the band represented the enzyme under study. The analysis was done using knockdowns injected with 2 ng of morpholino. Lane 1 consisted of the protein from the control embryos whereas Lane 2 consisted of the protein from knockdown embryos. By looking closely at the X-ray of the western blot, one could observe that the band from the control embryos was darker in intensity than the band from knockdown embryos. This
could indicate that the SULT2 ST2 gene was knocked down but not completely and there was still some protein present at detectable levels in the morpholino injected embryos. Such an observation might be due to the fact that most morpholino knockdowns are incomplete and result in the production of small but measurable amounts of the protein. Long half-life proteins could reach a functional non-phenotypic concentration through its accumulation in spite of the morpholino knockdown (Bill et al., 2009). The incomplete knockdown could also explain the occurrence of various phenotypes distributed unevenly in both the experimental concentrations. Another reason for the low level of knockdown could be that the concentration of the morpholino was not accurate or high enough to knock the gene down completely or it might not have been thoroughly dissolved. For this study 2.0 ng/nL was chosen as the highest concentration as it was found to be the most toxic concentration for the embryos and caused high mortality.

In conclusion, morpholino knockdown of SULT2 ST2 gene resulted in some non-specific phenotypes and lethality. Most of the embryos injected with 2 ng of morpholino displayed mild to severe cardiac edema after the hatching period. These effects could be attributed to the decrease in the enzyme levels that might have eventually altered hormone homeostasis in the organism that might have a role to play in the cardiac development in zebrafish. The phenotypes also might have been partially influenced by the compensative effect of other cytosolic sulfotransferases belonging to the same family/subfamily. Additional knockdown studies are essential to correctly assess the role of this enzyme in the development of *Danio rerio*. 
6. FUTURE INVESTIGATIONS

I. Perform rescue experiment by introducing synthetic mRNA encoding the enzyme into the morpholino injected embryos as well as control embryos and compare the phenotypes of both sets to be sure that the observed phenotypes are resultant of the morpholino knockdown only.

II. Simultaneous delivery of another non-overlapping translation-blocking morpholino to elicit a phenotype at a lower concentration without causing much toxicity.

III. Co-injecting a morpholino targeted toward a related SULT (SULT2 ST1) with similar function to prevent the compensative effect from masking the effect of the knockdown.

IV. Exposing the morpholino injected embryos to DHEA and/or environmental estrogens to observe any specific toxic effects.

V. Determination of the physiological expression of the enzyme in zebrafish by injecting GFP-tagged morpholino.

VI. Injecting a splice-inhibiting morpholino targeting the same gene independent of the translation-blocking morpholino to ensure that they produce the same phenotype.

VII. Injecting higher concentrations of the morpholino to get a specific phenotype in most of the experimental animals.
VIII. Characterization of the developmental ontogeny of SULT2 ST2 at the protein level in zebrafish.

IX. Studying the role of the Nr2F5 gene in regulating steroid hormone homeostasis.
7. REFERENCES


**Figure 1:** Structure of DHEA, the main substrate for SULT2 ST2

**Figure 2:** Structure of a morpholine ring and a morpholino oligo
Figure 3: Zebrafish (*Danio rerio*) developmental stages up to hatching

From: Kimmel et al. Stages of embryonic development of the zebrafish Dev. Dyn. 203:253-310, 1995
Figure 4: Survival rates for control and knockdown embryos injected with 1.0 ng/nL SULT2 ST2 morpholino. Survival rates were calculated by counting how many embryos/larvae were alive at the specific hours post fertilization (hpf) and calculating the percent survival out of the total number of embryos at the beginning of the experiment. The figure graphically represents this data.
Figure 5: Survival rates for control and knockdown embryos injected with 2.0 ng/nL SULT2 ST2 morpholino. Survival rates were calculated by counting how many embryos/larvae were alive at the specific hours post fertilization (hpf) and calculating the percent survival out of the total number of embryos at the beginning of the experiment. The figure graphically represents this data.
Figure 6: Abnormalities at 1.0 ng/nL at 55 hpf. (A) Control larvae (B) Cardiac and yolk sac edema (C) Abdominal, cardiac edema and malformed tail (D) Malformed tail and edema (E) cardiac edema and lordosis.
Figure 7: Abnormalities at 1.0 ng/nL at 80 hpf. (F) Cardiac edema and kinked tail (G) Malformed tail and notochord.

Figure 8: Abnormalities at 1.0 ng/nL at 144 hpf. (H) Edema and lordosis (I) Notochord deformity
Figure 9: Abnormalities at 2.0 ng/nL at 24 hpf (J) and (K) Craniofacial defects

Figure 10: Abnormalities at 2.0 ng/nL at 55 hpf (L) Cardiac edema (M) Notochord deformity
Figure 11: Abnormalities at 2.0 ng/nL at 80 hpf
(N) Edema and lordosis
(O) Edema and severe deformation
(P) Severe edema and malformations in the tail.

Figure 12: Abnormalities at 2.0 ng/nL at 144 hpf
(Q) Severe edema and lordosis.
Figure 13: X-ray film depicting the bands for SULT2 ST2 protein from control and knockdown embryos. Lane 1 contains the band representing the protein from the control embryos and Lane 2 contains the band representing the protein from the knockdown embryos injected with 2.0 ng/nL morpholino concentration solution.
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Table 1: Survival rates for control and knockdown embryos injected with 1.0 ng/nL SULT2 ST2 morpholino. Survival rates were calculated by counting how many embryos/larvae were alive at the specific hours post fertilization (hpf) and calculating the percent survival out of the total number of embryos at the beginning of the experiment.
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**Table 2:** Survival rates for control and knockdown embryos injected with 2.0 ng/nL SULT2 ST2 morpholino. Survival rates were calculated by counting how many embryos/larvae were alive at the specific hours post fertilization (hpf) and calculating the percent survival out of the total number of embryos at the beginning of the experiment.
<table>
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<td>75%</td>
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**Table 3: Hatching rates for the control and knockdown embryos injected with 1.0 ng/nL and 2.0 ng/nL concentrations of SULT2 ST2 morpholino.** The hatching rates were determined by counting how many embryos had completely shed their chorions at 55 hpf and the percent rate calculated based on the total number of embryos used at the beginning of the experiment.