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Running Title: Early Sea Urchin Embryos and Thalidomide

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Background: Clinical use of thalidomide has increased drastically, pushing the questions concerning the teratogenic mechanisms of this drug back to the forefront. Progress in understanding the teratogenic mechanisms has been slow, with the lack of non-primate vertebrate animal models susceptible to the classic reduction deformities remaining a concern. Sea urchin embryos have been used as model organisms for developmental studies for the last century. Like vertebrates they are deuterostomes and share similar developmental and signaling pathways suggesting they may be an effective system for thalidomide studies. Therefore, we tested sea urchin embryos to see if they were sensitive to the effects of thalidomide. **Methods:** Sea urchin embryos were obtained using standard spawning and fertilization techniques. Thalidomide dissolved in DMSO was added to embryo cultures either at fertilization or during early cleavage. Samples of the embryos were evaluated during specific development stages. **Results:** *Lytechinus pictus* embryos exposed to 400µM thalidomide at fertilization or within a window during early cleavage (2-6 hours post-fertilization) exhibit significant levels of abnormal embryos (60-82%) at the pluteus stage, compared to controls levels (≤10%). *Strongylocentrotus purpuratus* embryos exposed at initial fertilization or during early cleavage (2-6 hours post-fertilization) exhibit similar responses with significant abnormal levels ranging from (55-70%) at pluteus stage. **Conclusions:** Both species of sea urchin tested were susceptible to thalidomide induced teratogenesis during cleavage (4-16 cell stages). This response during cleavage stages warrants further study and indicates that sea urchin embryos may prove to be a useful tool for studying thalidomide effects early in development.

Key Words: thalidomide, sea urchin, teratogenesis, embryopathy, cleavage stage

Introduction

Thalidomide induced teratogenesis is probably one of the most well studied but least understood syndromes of the 20th century. Thalidomide, marketed as Contergan in Germany, gained wide popularity in Europe, Australia, Canada and Japan, where it is was prescribed as a sedative and sleep aid. Clinical testing in adults indicated that the drug had a very low level of acute toxicity when overdosed (Daemmrich, 2002; Lenz, 1985; 1988; Neubert R, 1997). At that time (the late 1950s and early 1960s), extensive

reproductive testing was not standard protocol for new drug development and the drug was assumed to be safe for use by pregnant women (Daemmrich, 2002; Neubert R, 1997; Warkany, 1988). The ensuing thalidomide tragedy resulted in the birth of approximately 10,000 children presenting clusters of congenital malformations which previously had been rarely seen. The most notable were reduction deformities of the limb where the proximal portion of the limb was greatly reduced or absent, while the more distal structures were less severely affected (Lenz, 1985; 1988; Newman, 1986). Thalidomide was removed from the German market in November 1961, never having been approved for distribution in the USA. Removal from other world markets such as Japan and Brazil did not occur until 1962 (Daemmrich, 2002; Neubert R, 1997). In 1995, thalidomide was re-introduced into the clinical pharmacopeia and gained FDA approval in 1998, specifically for the treatment of erythema nodosum leprosum. Additional off-label uses include treatment for Kaposi's sarcoma, primary brain malignancies, chronic graft versus host disease, Behcet's disease, systemic lupus erythematosus, the wasting syndrome associated with AIDS, multiple myeloma and other clinical conditions (Lewis, 2001; Melchert and List, 2007; Tseng et al., 1996). Unfortunately, recurrence of thalidomide syndrome has been reported pushing the need to understand thalidomide teratogenesis back to the forefront (Castilla et al., 1996; Pannikar, 2003).

Human embryo susceptibility to thalidomide has been estimated to range anywhere from 10% to 50% of the exposed pregnancies (Daemmrich, 2002; Lenz, 1985; 1988; Tseng et al., 1996; Warkany, 1988). Humans are an out bred wild population. The traditional laboratory animals used extensively in thalidomide research are derived from in-bred populations and could be limited in the range of responses which they exhibit to thalidomide. Indeed, work on the mechanism of thalidomide induced limb teratogenesis may be hampered by the fact that the proximal limb reduction malformations, particularly phocomelia, seen in humans are difficult to replicate non- primate species (Neubert et al., 1999; Neubert R, 1997) although limb truncations have been seen in the rabbit and chick (Hansen et al., 2002a; Knobloch et al., 2007; Somers, 1962).

Rabbits, particularly New Zealand White rabbits (Somers, 1962) and Himalayan strains (Neubert R, 1997) show a limb truncation response to thalidomide but not the classic amelia or phocomelia which is associated with thalidomide syndrome. Neubert and Neubert (1997) reviewed many of the early studies and concluded, that while effects were seen in rabbits, the doses required to elicit malformations often led to pronounced maternal weight loss and embryo mortality. More recently, Teo and colleagues (2004a) working in New Zealand White rabbits found no thalidomide related external effects on the fetuses of dams exposed prior to conception through the period of organogenesis. They did observe maternal weight loss and embryo lethality. In addition, thalidomide does not appear to impair reproductive capacity in treated male or female rabbits (Teo et al., 2004b). Rabbit embryo limb bud cultures circumvent the confounding factors of poor maternal health and embryo mortality. Consequently, they have been used for several mechanistic studies. Intracellular glutathione studies suggest that thalidomide teratogenicity may be linked to generation of specific reactive oxygen species or overall redox sensitivity (Hansen, 2006; Hansen et al., 2002a; Hansen et al., 2002b). It has been suggested that the differences in thalidomide responses between rats, mice and rabbits may be linked to the each species' ability to withstand oxidative stress (Knobloch et al.,

2008a; Wells et al., 2005; Wells et al., 2009). Studies using laboratory rodents besides the rabbit have been reported as well (Brent, 2004; Heger et al., 1988).

Work in the chick embryo also raised the question of changes in reactive oxygen species status which may be linked to changes in programmed cell death (Berson et al., 2008; Knobloch et al., 2008a; Knobloch and Ruther, 2008; Knobloch et al., 2008b), or BMP and the Wnt signaling pathway (Knobloch et al., 2007). Thalidomide induced inhibition of angiogenesis has been reported in a chick limb (Therapontos et al., 2009). The authors suggest that this anti-angiogenic response may be a possible mechanism for induction of the limb truncations. Like the rabbit, chick limb bud cultures have been used to address thalidomide teratogenesis as well (Knobloch et al., 2007; Stephens, 2009).

In most of the aforementioned studies thalidomide exposure has been during organogenesis, specifically during limb development. Few studies have looked at the effects of thalidomide exposure from the cleavage stages through early embryogenesis. Lucey and Behrman (1963) exposed rhesus monkeys to thalidomide from the onset of pregnancy and reported no live births from treated animals. They postulated that thalidomide exposure prior to implantation killed the embryos.

We have undertaken another approach to understanding thalidomide's mode of action, the developing sea urchin embryo. Adult sea urchins are readily obtainable and require minimum care beyond a large well functioning salt water aquarium. Since a single sea urchin spawning yields tens-of-thousands of gametes which can be fertilized *in vitro,* the number of animals which must be sacrificed to undertake these studies is minimal. The embryos readily develop in culture to the pluteus stage (Figure 1) and are translucent, making it possible to observe internal structures *in vivo* with light microscopy.

Reports of the sea urchin embryo as a model organism for toxicological studies can be found throughout the literature. Hagstrom and Lonning (1973) tested sea urchin embryos, *Parcentrotus lividus,* with a number of known toxicants including chloramphenicol, nicotine, chlorpromazine and to a limited extent thalidomide. They reported that all of these agents interfered with fertilization, cleavage and differentiation of the larva in drug specific ways. A sea urchin bioassay using the same species, looked at the developmental risks posed by the antifouling paints used in the maritime industry (Bellas, 2008). Bellas found synergistic effects when the agents from the paint were combined and tested, compared to being assayed as individual compounds. Glyphosphate based herbicides, similar to the commercially available herbicide Roundup, were tested in a *Sphaerechinus granularis* embryo assay. Marc and colleagues (2005) found that glyphosphate inhibited global transcription at the 16 cell stage which most likely resulted in the inhibition of hatching, the observed response to herbicide treatment. Exo-gastrulation has been documented in sea urchin embryos treated with estrogen compounds (Kiyomoto et al., 2008).

All sea urchins species are similar in their early embryonic development (Gilbert, 2006; Giuduce, 1986; Wilt, 1987). They undergo radial holoblastic cleavage and the pattern of the first seven cleavage divisions remains the same for all individuals of the same species. Cleavage divisions become less regular after the seventh cleavage and the number of divisions that encompasses each stage of embryonic development differs between the various species (Ernst, 1997; Gilbert, 2006; Giuduce, 1986; Wilt, 1987). The blastula stage typically begins around the 128-cell stage, after the blastocoel forms. Soon

after blastocoel formation, the blastulas break free of the fertilization envelope, referred to as hatching, and become free-swimming. It is during this time that the primary mesenchyme cells (PMCs) ingress into the blastocoel. (Figure 1) Following a period of migration the PMCs organize into the synctial ring, produce the skeletogenic matrix and biomineralize to form the calcareous skeleton (McClay et al., 2004). Gastrulation ensues shortly after hatching and is marked by the formation of the archenteron and morphogenesis into the prism shape. Growth of the arms, which are supported by skeletal rods, signals the formation of the pluteus and marks the end of early development and the beginning of the larval stage. Sea urchin plueti are bilaterally symmetric (Figure 1). Morphogenesis from pluteus stage to the adult includes a change from bilateral symmetry to the radial symmetry found in the adult (Ernst, 1997; Gilbert, 2006; Wilt, 1987)

Sea urchins like vertebrates are deuterostomes. Deuterostomes exhibit many developmental pathways, signaling mechanisms and gene regulatory networks which are evolutionarily conserved. The sequencing of the genome from the sea urchin *Strongylocentrotus purpuratus* (Sodergren et al., 2006), and the subsequent work on the endoderm-mesoderm developmental gene regulatory network (Longabaugh) indicate that the sea urchin does indeed exhibit many developmental pathways and signaling systems that are also found in vertebrates. Recent studies using NanoString® technology and perturbation modeling have started to delineate a gene regulatory network for specifying the oral/aboral ectoderm in the sea urchin embryo (Su et al., 2009). Nodal and BMP signaling, important in vertebrate axial development (De Robertis and Kuroda, 2004; Little and Mullins, 2006), were found to contribute to the oral/aboral ectoderm axis specification of the sea urchin embryo (Duboc et al., 2004). Chordin, which is involved in neural and axial patterning in *Drosophila* and vertebrate embryos (De Robertis and Kuroda, 2004), contributes to the development of synaptogamin B-positive neurons in the sea urchin embryo. However, chordin does not appear to be involved in axial patterning (Bradham et al., 2009). Activation of the transcription factor β-catenin plays a role in sea urchin endomesoderm specification during the fourth to sixth cleavage. Later, the endomesoderm cells activate Notch which plays a role in secondary mesenchyme cell specification with consequences for pigment cell migration and later gastrulation (McClay et al., 2004).

There are some differences in echinoderm development compared to vertebrates. In vertebrates, gastrulation becomes modified from the sea urchin pattern to accommodate large amounts of yolk or extra-embryonic membranes. Similarly, the position of the mouth may be differentially affected by BMP signaling in sea urchins compared to chordates. In chordates, BMP signaling seems to promote mouth development but antagonizes it in echinoderms and hemichordates (Christiaen et al., 2007). Despite the differences, strong similarities remain between sea urchin and vertebrate embryos in terms of the underlying regulators of differentiation and morphogenesis (Gilbert, 2006; McClay et al., 2004).

The goal of these initial studies was to document the sensitivity of sea urchin embryos to thalidomide exposure. Sea urchin embryos are indeed sensitive to thalidomide. They exhibit a window of vulnerability to this drug during early cleavage. We propose that the sea urchin embryo can be a viable model system in which to study thalidomide effects very early in development.

Materials and Methods

Wild adult sea urchins, *Lytechinus pictus* and *Strongylocentrotus purpuratus* were purchased from Marinus Scientific, Garden Grove, CA and maintained in salt water aquaria. Gravid *L .pictus* adults are generally available from April thought October and *S. purpuratus* from late November through March. Thalidomide (98% purity) and dimethyl sulfoxide (DMSO) were purchased from Sigma St. Louis, MO. Instant Ocean®, distributed by Carolina Biological Supply Burlington, NC was used to prepare artificial seawater for the aquaria and experimental protocols.

Spawning and fertilization- Spawning was induced in both species by injecting the sea urchins with 0.5 to 2.0 mL of 0.5M KCL at multiple sites around the animal's Aristotle's lantern, the mouth apparatus on the ventral surface of the sea urchin. Successful spawning occurred when the gametes were extruded through the gonadopore on the aboral surface. The orange eggs were collected by inverting the females on top of a small beaker filled with 18°C artificial seawater. Males extruded white sperm, which were collected clean and dry, by inverting the male over an empty small beaker.

After all of the eggs had settled to the bottom of the beaker the seawater was decanted and discarded. To remove the jelly coat, the eggs were passed through several layers of moistened cheese cloth and allowed to settle in the bottom the beaker. After a second passage through the cheesecloth, they were transferred to a 50mL plastic centrifuge tube to settle into the tip. To insure a consistent uniform suspension, the seawater was removed and the loosely packed eggs re-suspended at 0.1mL of packed eggs/ 25mL of seawater. Sperm were prepared for fertilization by suspending a drop of the spawned sperm into 10mL of sea water and gently mixing to obtain a uniform suspension. Two to five mL of this diluted suspension were used to fertilize 25mL of egg suspension. Successful fertilization was indicated by presence of a fertilization envelope surrounding the egg. Following standard protocol, if a fertilization rate of 95% was not achieved within the first 10 minutes, more sperm solution was added to the eggs. The fertilization rate was reevaluated following 5 minutes incubation. After the fertilized eggs settled to the bottom of the tube, the water containing the sperm was removed and the fertilized eggs re-suspended in fresh sea water. Nine milliliters of fertilized egg solution were placed in small glass finger bowls containing 91mL of fresh sea water creating a final volume of 100mL.

Thalidomide treatment- Fertilized eggs were added to all of the dishes at the same time which was defined as 0 hours. Thalidomide, dissolved in DMSO, was prepared as a 100mM solution. Four hundred microliters of this solution were added at various time points post-fertilization for a final bowl concentration of 400μM. Control dishes, which contained seawater alone or seawater plus DMSO, were prepared at the same time points. The culture dishes were loosely covered with aluminum foil and incubated at 18°C. All experimental and control cultures were performed in triplicate for each experiment.

Thalidomide removal- At six hours post-fertilization, the contents of each culture dish were split between two plastic 50mL centrifuge tubes. The embryos were centrifuged for 3-5 minutes in a Fisher Centrific Centrifuge (Model 225 set at 4) until they formed a soft pellet at the bottom of the tube. The liquid in each centrifuge tube was aspirated and the embryos were re-suspended in fresh seawater and placed back in their respective culture dishes, which had been rinsed and refilled with 100ml of untreated sea

water. For these experiments the controls were also centrifuged and re-suspended in fresh seawater.

Thalidomide dose response L. pictus- Four hundred microliters of DMSO or a thalidomide solution were placed in the culture dishes at initial culturing. Stock solutions of thalidomide were adjusted so that final concentrations in the culture dish were either, 100µM, 200µM, 300µM, 400µM, or 500µM. The culture dishes were loosely covered with aluminum foil and incubated at 18°C. All experimental and control cultures were performed in triplicate. The cultures were maintained for 72h and the data recorded.

Data collection and statistical analyses- The embryos were observed at 24, 48, and 72 hours post-fertilization using light microscopy. A sample of one hundred live embryos was counted per replicate at each time point with three replicate dishes being the standard for most experiments. Embryos which had been removed from the culture dish for evaluation were discarded following sampling. Each experiment was routinely repeated at least three times. Consequently, a minimum of 900 embryos per condition was usually evaluated. The number of abnormal embryos per 100 embryo sample was recorded for each individual culture. Data were analyzed using Student's T-test on Microsoft Excel or the program StatView was used to run ANOVA and unpaired T-tests for analyses of the data. The standard error of the mean (SEM) is represented in Figures 6 and 7 as barred lines above and below the data points.

Results

Lytechinus pictus – general description of abnormalities and qualitative observations for embryos treated with 400µM thalidomide

Twenty-four hour observation point- At this observation point control embryos were found to be between the late blastula and early gastrula stages (Figure 1). The embryos still in late blastula stage had an easily identified vegetal plate. Gastrulation was considered to have started if some invagination of the archenteron had occurred. Embryos were counted as structurally abnormal if the vegetal plate was malformed or if the early developing archenteron was not in the proper position. Embryos which were markedly smaller were scored as abnormal. In several experiments the thalidomide treated embryos appeared to have progressed into gastrulation which normally is expected around 48 hours. These embryos were scored as abnormal as well. There was no evidence, of this faster development rate or markedly smaller embryos, in either the sea water or DMSO control cultures.

Forty-eight hour observation point- Control embryos during this timeframe were consistently found to be in the late gastrula stage and early prism morphology (Figure 2). Treated embryos exhibited characteristic structural abnormalities including malformations of the archenteron with or without abnormal prism morphology. Abnormal archenteron formation included incomplete archenteron formation, an archenteron which was not centrally located or one which was curved. Exo-gastrulation was also observed. Embryos were characterized as having abnormal prism morphology if there was no evidence of spicule formation or if the spicules had not organized into the characteristic angular prism shape seen in the control embryos. In addition some embryos were categorized as abnormal if their developmental stage was different from the controls. In most instances the embryos had not progressed to the same stage as the control cultures.

Seventy- two hour observation point- All control cultures at this observation point exhibited mature pluteus development (Figure 3). Abnormal embryos exhibited malformations which included variations in the shape of the animal anterior region. In many cases, the anterior region was extremely pointed or elongated. The angle at which the arms extended from the anterior region varied as well. Often the arms appeared to be set at an angle much wider than expected. Variations in the structure of the arms were also noted. Most often observed were abnormal arm lengths and asymmetry in arm development. Abnormal archenteron formation was also evidenced usually in conjunction with skeletal abnormalities. Developmental delay where embryos had not progressed to the pluteus stage was also noted.

***Lytechinus pictus*- dose response comparison of several thalidomide concentrations within a single experiment.**

The experimental concentration of 400µM thalidomide had been determined empirically through the course of several seasons of sea urchin experimentation (internal data). The graph (Figure 4) represents the data from a single experiment comparing the percentage of abnormal embryos obtained by treating cultures with a range of thalidomide concentrations at initial culturing. The lower treatments 100µM and 200µM yielded levels that were indistinguishable from control data with the maximum number of abnormal embryos seen as 72 hours. At blastula stage (24h), treatments of 300µM through 500µM resulted in numbers of abnormal embryos that were 6 to 12 times higher than control levels. This trend continued through gastrulation (48h) with the 500µM treatment resulting in more than 80% abnormal embryos. By pluteus stage (72h) the 400µM treatment had also reached levels of 80% abnormal embryos. The 500µM treatment group at 72h had no surviving embryos although the control cultures were quite healthy.

***Lytechinus pictus*- effect of variation in the times at which thalidomide was added to the cultures.**

Initial experiments focused on adding thalidomide at 0, 6 and 12 hours post-fertilization (data not shown). Those studies demonstrated that the timing of the addition of thalidomide affected the number of abnormal embryos observed at the 24h, 48h, and 72h observation points. When the thalidomide was added at 12h, the percentage of abnormal embryos was generally lower by about one third at all observation points compared to when thalidomide was added at initial culturing. Subsequent experiments focused on adding thalidomide at various points during cleavage, up to 6 hours post fertilization. Those data indicated that timing differences of as little as 2 hours consistently altered the number of abnormal embryos observed later in development. Differences between control and thalidomide treated cultures were noted as early as the late blastula/early gastrula stages (24h). Addition of thalidomide at 2h increased the percent of abnormal embryos by at least 10% compared with thalidomide addition at 6h or at initial culturing (Figure 5). By gastrula stage (48h) the difference had narrowed to slightly less than 10% and remained consistent at pluteus stages as well (72h). When thalidomide was added at 4h the percentage of abnormal embryos consistently fell between the highest levels found in the 2h condition and the lower levels found at the addition at either initial culturing or 6h. In summary, the addition of thalidomide at very

early cleavage (2h) resulted in highest number of abnormal embryos compared to addition at all other time points, specifically addition at initial culturing (0h) or slightly later during cleavage (4h and 6h).

Lytechinus pictus- Effect of addition of thalidomide during early cleavage and removal at 6 hours post-fertilization.

The final phase of these experiments focused on more closely identifying this early window of vulnerability during cleavage stages. Thalidomide was added at either initial culturing, 2 hours, or 4 hours post-fertilization and removed at 6 hours post-fertilization. The number of abnormal embryos for all control cultures remained below 10% at all time points (Figure 6). The embryos treated at initial culturing with thalidomide removal at 6 hours exhibited percentages of abnormal embryos ranging from 55% at 24h to 72% at 72h. *L. pictus* embryos treated with thalidomide at initial culturing exhibited statistically significant higher levels of abnormalities ($p \leq .0001$) compared to the two control conditions, seawater only or seawater plus DMSO at all observation points. When thalidomide was added at 2 hours and removed at 6, the highest numbers of abnormal embryos were observed at all observation points compared to any other treatments or controls. At 24h this group exhibited 66% abnormal embryos with a final level of 81% at 72 h. These data are not only highly significantly different ($p \leq .0001$) from control data but also from the other experimental conditions, thalidomide added at fertilization, or at 4 hours (Figure 6.) All embryos which were either malformed or at a developmental stage different from the sea water controls were considered abnormal for statistical analyses in the above experiments. The physical abnormalities fall into two general categories, defects in gastrulation and defects in skeletogenesis (Table 1). Interestingly, a group of the treated embryos at 24 hours appeared to be close to completing gastrulation. This more rapid rate of development was never seen in control cultures which were usually at the late blastula or very early stages of archenteron formation. In contrast, by 48 and 72 hours there were clearly embryos in the treated groups which had not progressed beyond gastrulation and prism formation.

Strogylocentrus purpuratus- effect of addition of thalidomide during early cleavage

Many of the genome sequencing and developmental gene regulatory network studies use the sea urchin species, *S. purpuratus.* It was of interest to determine if *S. purpuratus* displayed patterns of sensitivity to thalidomide early in development similar to those we observed in *L. pictus*. A more limited series of experiments were performed with this species.

S. purpuratus exhibited sensitivity to thalidomide during early cleavage as well. We compared adding thalidomide at initial culturing with cultures where thalidomide was added during cleavage, 2h, 4h, or 6h post-fertilization (Figure 7). In these experiments thalidomide remained in the cultures through the course of the experiment. Control cultures, DMSO and sea water, remained fairly constant at all time points with approximately 10% abnormal embryos. The condition where thalidomide was added at initial culturing resulted in levels of abnormal embryos which ranged from 43% at 24 hours to 56% at 72 hours. Embryo cultures treated with thalidomide at initial culture exhibited statistically significant increases ($p \leq .0001$) in abnormal embryos compared

with seawater and DMSO controls (Figure 7). The highest percentages of abnormal embryos were observed when thalidomide was added during early cleavage (2h or 4h post fertilization). Addition of thalidomide at 4 hours resulted in 5% more abnormal embryos than either the 2h or 6h treatment groups at 24 hours, and 10% more at 48 hours. The difference was even greater when addition at 4 hours was compared with addition at initial culturing (Figure 7) for the 24 and 48 hour observations These levels differed significantly from the condition when thalidomide was added at initial culturing ($p \leq .0001$) for both the blastula/early gastrula stages (24h) and the gastrula-early prism stage (48h). At gastrulation, we noted three general classes of abnormalities: abnormal archenteron formation alone, abnormal archenteron formation with poor, but recognizable, prism morphology, and a third group with abnormal archenteron formation and what appeared to be very poor spiculogenesis resulting in embryos that did not display recognizable prism morphology. The pluteus stage (72h) displayed abnormalities most of which involved the arms of the pluteus. Frequently, the arms were abnormally wide, uneven in length or shortened. In addition, abnormal archenteron formation was also observed. In our laboratory the *S. purpuratus* cultures were difficult to maintain with high levels of viability through 72 hours. We were not always able to maintain three replicate dishes for each condition through the end of the experiment. Consequently, we decided not undertake statistical analysis of the 72 hour data for *S. purpuratus* but rather present that data points as indications of the probable trends.

Discussion

Like humans, the sea urchins embryos we study represent an out bred wild population. Marinus Scientific collects the adult sea urchins from different locations throughout the course of the season and from season to season (personal communication). One could expect that there would be a range of responses exhibited by embryos resulting from the random mating of out bred wild populations, which is indeed what we observed. In fact, while limb reduction deformities came to define thalidomide syndrome in humans, a range of anomalies was reported which included several organ systems (Lenz, 1985; 1988; Neubert and others, 1999; Newman, 1986). Forty years after the thalidomide tragedy the teratogenic mechanism of this drug remains an enigma. Although, numerous theories have been proposed, no one has yet completely unraveled the puzzle of thalidomide's teratogenic effects, particularly in humans (Argiles et al., 1998; Finnell et al., 2002; Knobloch and Ruther, 2008; Knobloch et al., 2007; Koch and Czejka, 1986; Neubert et al., 1999; Stephens and Strecker, 1983; Therapontos et al., 2009; Wells et al., 2009). Our studies on the effects of thalidomide during early sea urchin embryo cleavage approach this puzzle from a different perspective.

There have been few reports in the literature of the effects of thalidomide when it is administered during early cleavage. Rhesus monkeys treated from the onset of pregnancy failed to produce any live infants. The authors concluded that the embryos died before implantation which in this species is at the blastocyst stage (Lucey and Behrman, 1963). Sea urchin embryos continue to develop despite the early insult. A previous report using the sea urchin species, *P. lividus*, mentioned that when thalidomide was added at fertilization, some moderate effects on cleavage and gastrulation were observed with more pronounced effects at the pluteus stage. Particularly noted were those affecting the skeletal system (Hagstrom and Lonning, 1973). The authors provided only

qualitative evidence and did not indicate how many embryos were affected. In general, we concur with their findings regarding the addition of thalidomide at fertilization. However, we found that adding thalidomide, during the first through fourth cleavages, increases the likelihood of abnormal embryogenesis and larval development. Both *L pictus* and *S. purpuratus* are vulnerable to thalidomide exposure during early cleavage. The levels of abnormal embryos range between four and eight times the levels seen in control conditions when the embryos are examined at gastrulation or the pluteus stag (Figures 6 and 7). The treated embryos observed at 48 and 72 hours exhibited abnormal archenteron formation and abnormal skeletogenesis, suggesting that early exposure to this drug is disrupting processes that are needed for normal morphogenesis of both endoderm and mesoderm derived tissues later in development. The apparent slight shift between the time of maximum sensitivity in *L. pictus* (2h) compared to *S. purpuratus* (4h) is most likely a reflection of different rates of embryonic development of these two species in our laboratory. Analysis of the stage of development compared to time post-fertilization indicated that the early window of sensitivity may actually be occurring at similar stages during embryogenesis. *L. pictus* embryos reach the 16 cell stage approximately two hours earlier than *S. purpuratus* (internal data). Adjusting for the differences in the rates of development, it becomes clear that in both species, early embryos respond maximally to thalidomide teratogenesis at the 4 to 16 cell stages.

Thalidomide is unstable in aqueous solutions and hydrolyzes rather quickly in solutions with a pH higher than 6. The reported half-life at pH 7.4 and 37ºC is between 2.4 and 5 hours (Neubert R, 1997). Instant Ocean® is slightly alkaline (internal data). Even though the sea urchin embryos are incubated at slightly cooler temperatures 17-18ºC, one would expect hydrolysis to occur within a timeframe similar to the one reported by Neubert and Neubert (1997). In our studies, when thalidomide is added at initial culturing, the percentage of abnormal embryos is lower than when thalidomide is added at either 2 or 4 hours. This may indeed be a reflection of thalidomide's relatively short half-life in slightly alkaline aqueous solutions. Consequently, when thalidomide is added at initial culturing there is an effectively lower concentration of the drug by the 4-16 cell stage when the embryos are most vulnerable.

Most intriguing is that treatment during early cleavage results in increasing numbers of observable abnormal embryos as development progresses, even when cleavage appears to be progressing normally. For example, in *L. pictus* (Figures 5 and 6) the percentage of structurally abnormal embryos at blastula stage (24 hours) is approximately 60%. Depending on the treatment, by pluteus stage (72h) the number of abnormal embryos has increased to 80%, even when the thalidomide was removed at 6 hours (later stage cleavage). This trend holds for *S. purpuratus,* although the numbers are slightly lower (Figure 7). There has been extensive work on the sea urchin developmental gene regulatory network. Most of that work examines development after six hours. By six hours post fertilization, the PMCs start to differentiate. Involved in this process are maternally derived transcription factors, genes in the Wnt family and other ubiquitous factors found in the egg and zygote (Longabaugh). Additional genes have been identified some of which exhibit expression in the endoderm that appears to be up regulated by 8 or 9 hours (Su et al., 2009).

It can be argued that thalidomide interferes with a process, or processes, upstream which may be required for normal gastrulation or skeletogenesis later. For example,

thalidomide could be interfering with maternally derived transcription factors such as β-catenin or any other maternal or zygotic signaling factors which may become active during cleavage (Longabaugh; Su et al., 2009). The early cleavages in sea urchin embryos are critical to the establishment of cell fate. By the time the eight cell stage has been reached the embryo has established an animal and vegetal half of four cells each. The animal cells contribute to the ectoderm of the embryo while the vegetal cells give rise to the endo-mesoderm. Uneven cleavage occurs at the fourth cleavage stage. This uneven cell division results in animal pole macromeres and vegetal pole micromeres (Gilbert, 2006). β-catenin interaction is involved with specifying the vegetal derived micromeres from which the PMCs will later arise. Disruption of development at this point could be critical, since the PMCs act as an important signaling center for the rest of the embryo (Cameron et al., 2009; McClay et al., 2004; Sodergren et al., 2006; Wikramanayake et al., 2004). The abnormalities we see in archenteron formation and skeletogenesis suggest involvement of endomesoderm differentiation systems (Table 1).

By convention the appendages on the sea urchin pluteus are referred to as arms. We do not mean to imply that these are homologous structures to vertebrate and specifically mammalian limbs. The pluteus skeletal system is derived from the PMCs which ingress into the blastocoel at gastrulation (Gilbert, 2006; McClay et al., 2004). The pattern for skeletal formation appears to be autonomously specified in the PMCs with integration of positional cues from the ectoderm of the embryo (McClay et al., 2004). Treatment of the embryo with thalidomide, around the time of micromere formation but before PMC differentiation, could alter skeletogenesis by affecting the ability of the PMCs to fully differentiate or by disrupting their ability to read the positional cues from the ectoderm. Understanding the nature of thalidomide's disruption of sea urchin development could lead to greater understanding of the patterns of abnormal development seen in vertebrates, particularly by indicating the types of molecules, signaling pathways or positional cues that are sensitive thalidomide (Davidson, 2006). Of special note would be those associated with skeletogenesis such as the Wnt pathway (Cameron et al., 2009; Longabaugh) and BMP like signaling molecules (Angerer et al., 2000). Knobloch and colleagues found that 15%-20% of the chick embryos they treated at the primitive streak stage exhibited limb truncations including amelia. They concluded that thalidomide creates increased oxidative stress, which up regulates BMP and DKK1. BMP and DKK1 work agonistically to the canonical Wnt/β-catenin pathway, consequently, this up regulation leads to increased apoptosis and eventual limb truncation (Knobloch and Ruther, 2008; Knobloch et al., 2007).

Sea urchin embryos provide a different view of the interactions between thalidomide and the embryo. Indeed, both species studied responded to thalidomide exposure by exhibiting abnormal development. When exposure occurred during early cleavage stages, we observed significant structural abnormalities including abnormal gut and skeletal system formation, suggesting that exposure during cleavage has the potential to disrupt gastrulation and skeletal morphogenesis. Future studies undertaking more detailed analyses of specific abnormalities involving morphogenesis and development are planned. We believe that the developing sea urchin embryo may be a useful tool to further unravel the questions associated with thalidomide exposure and embryogenesis.

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Figure and Table Legends

Figure 1. *Lytechinus pictus* embryos demonstrating the typical of stages defined as blastula, gastrula/prism, and pluteus, usually observed at 24, 48 and 72 hours post-fertilization respectively. Some of the key anatomical and developmental features have been labeled.

Figure 2. Typical abnormal *Lytechinus pictus* embryos at 48 hours post-fertilization compared with a normal embryo. Similar abnormalities were observed in *S. purpuratus.*

Figure 3. Abnormal *Lytechinus pictus* embryos at 72 hours post-fertilization compared with a normal embryo. Similar abnormalities were observed in *S. purpuratus.*

Figure 4. Response of *L .pictus* embryos to a range of thalidomide concentrations. Thalidomide was added at initial culturing and the percent of abnormal embryos evaluated at 24, 48 and 72h. No data point is included for the 500µM dose at 72 hours since embryos in all three replicate cultures did not survive. This graph represents a single experiment in which all doses were tested simultaneously.

Figure 5. Percent of *L .pictus* embryos that were abnormal after the introduction of thalidomide at initial culturing or 2, 4 or 6 hours post-fertilization. Addition of thalidomide at 2 hours resulted in higher percentages of abnormal embryos compared to all other conditions.

Figure 6. Percent of *L. pictus* embryos that were abnormal after the addition of thalidomide at time 0 and removed after 6 hours, added at 2h removed at 6h, or added at 4 hr and removed at 6. All thalidomide data points were significantly different from controls. Each of the thalidomide conditions were significantly different from each other at 24h, 48h and 72h again at the same high level $p<.001$. For each data point a minimum of 900 embryos was observed and recorded as either normal or abnormal. Error bars represent the SEM.

Figure 7. Percent of *S .purpuratus* which were abnormal following the addition of thalidomide at 0, 2, 4 and 6 hours post-fertilization. For each data point a minimum of 900 embryos was observed and recorded as either normal or abnormal. Note; statistical analyses of the data collected at 72 hours were not undertaken due to the poor quatilty of the cultures at that time point. Error bars at 24 and 48hs represent the SEM.

Table 1. Distribution of abnormal embryos from a single experiment in *L*. pictus. Thalidomide was added at the time indicated on the table and removed by the standard wash-out procedure at 6 hours. *Exo-gastrulated embryos were also counted in the Abnormal Progress category for 24 hours since control embryos at 24h normally have not progressed beyond slight archenteron invagination.