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TOXIC EFFECTS OF HYGROMYCIN B ON *KRYPTOLEBIAS MARMORATUS* EARLY DEVELOPMENT

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ABSTRACT

Kryptolebias marmoratus embryos were tested for susceptibility to hygromycin B under various concentrations prior to the late blastula stage of development. The concentrations arresting development to the point of lethality for 95% of *K. marmoratus* embryos within a 48 and 96 h period were 705 mg/l and 601 mg/l, respectively. Nearly half of all embryos were arrested with an effective concentration of 433 mg/l and 207 mg/l for 48 and 96 h, respectively. Data presented here are useful for selecting potential transgenic *K. marmoratus* (or their embryonic cells) by use of hygromycin B selection or for studying the midblastula transition during early development.

Keywords: *Kryptolebias marmoratus*, development, hygromycin B, toxicity, midblastula transition

INTRODUCTION

Hygromycin B is a potent product of *Streptomyces hygroscopicus* that acts as an antibiotic against both prokaryotic and eukaryotic organisms (1, 2). This aminoglycoside antibiotic inhibits protein synthesis and ribosomal subunit formation (2, 3). The hygromycin resistance gene hyg^R codes for a phosphotransferase protein that confers resistance through phosphorylation of position 7 of hygromycin's destomic acid moiety (4). Though resistance to hygromycin is normally used to screen prokaryotic and eukaryotic cells, it has also been demonstrated to effectively screen transgenic mice (5). We are interested in establishing *Kryptolebias marmoratus*, as a model organism for genetic and developmental studies due to its unique reproductive strategy as a hermaphroditic, self-fertilizing vertebrate (6). One mechanism for advancing developmental genetic studies in this fish species is the use of transgenic vectors for transforming genes into the germ-line. Although genetic transformation studies have not been demonstrated in *K. marmoratus* at present, the possibility exists for screening embryos that carry a hyg^R transgene, using hygromycin B as a selectable marker. As a preliminary step towards establishing this method of screening, we exposed *K. marmoratus* embryos (cleavage to early blastula stage) to various concentrations of hygromycin B. This stage of development was chosen because it is hypothesized to be prior to the midblastula transition (MBT) when the embryo begins to transcribe

zygotic genes, effectively halting development, as hygromycin B is a potent protein translation inhibitor.

MATERIALS & METHODS

Staging of Embryos

The *Hon9* clone used in this study was originally collected from Utila Islands, Honduras in 1996 and has been maintained at the Valdosta State University Aquatic Laboratory since 2001. *K. marmoratus* were individually placed in glass bowls with a lower layer of plastic mesh for isolation of newly self-fertilized eggs. Fish were fed freshly hatched brine shrimp *ad libitum* for one week followed by continued feeding and collection of embryos on a daily basis into the following week. At each collection embryos were sorted by developmental stage and photographed using an Olympus UTV1X-2/ CMAD3 camera mounted to an Olympus SZX12 dissecting microscope. Only cleavage and early blastula stage embryos were retained for further study.

Hygromycin B Exposure

A toxicity test was used to determine the levels of hygromycin B needed to effectively halt wild-type *K. marmoratus* embryonic development. For this test, K. marmoratus pre-blastula embryos between 0 – 9.5 h post fertilization were exposed to hygromycin B for a total of 96 h at concentration levels of 0, 100, 250, 350, 500, and 750 mg/l. Each of these hygromycin B solutions was prepared from a stock solution of 1,500 mg/l hygromycin B freshly dissolved in 18 m□ water. Ten replicate embryos were used for each test, with the exception of the 750 mg/l group in which eight embryos were tested. A single embryo was placed into each well of a 24-well cell culture plate containing 1 ml of the appropriate hygromycin B solution in 17 ppt artificial sea water (Instant Ocean® dissolved in 18 m□ water). The hygromycin solution was removed and replaced with fresh solution after 48 h of exposure. The embryos were observed for survival after 48 and 96 h of treatment. Both toxicity values and 95% confidence intervals were calculated using Probit Analysis with ToxCalc Software (Tidepool Scientific Software, McKinleyville, CA). Graphs were generated using SigmaPlot 8.0.

RESULTS

Fifty-eight early stage *K. marmoratus* embryos were selected from approximately 90 individuals based on distinguishable stages of early embryonic development prior to the MBT (Figure 1). Non-fertilized embryos were easily distinguished by the absence of the perivitelline membrane space between the egg cell membrane and egg shell chorion (Figure 1A). The non-fertile eggs were discarded, while recently fertilized embryos with the characteristic perivitelline membrane space were retained (Figure 1B). Slightly older embryos exhibiting early cleavage stages such as one and four-cell embryos along with early, high blastula stages were also retained (Figure 1C-E). Embryos equal to or older than late blastula were discarded as they were easily identifiable as

flattened blastula having > 100 cells (Figure 1F). Therefore, the approximate range of embryos used in this study was from recently fertilized to the characteristic high blastula stage, which is equivalent to 0 – 9.5 h post-fertilization and is hypothesized to be prior to the MBT.

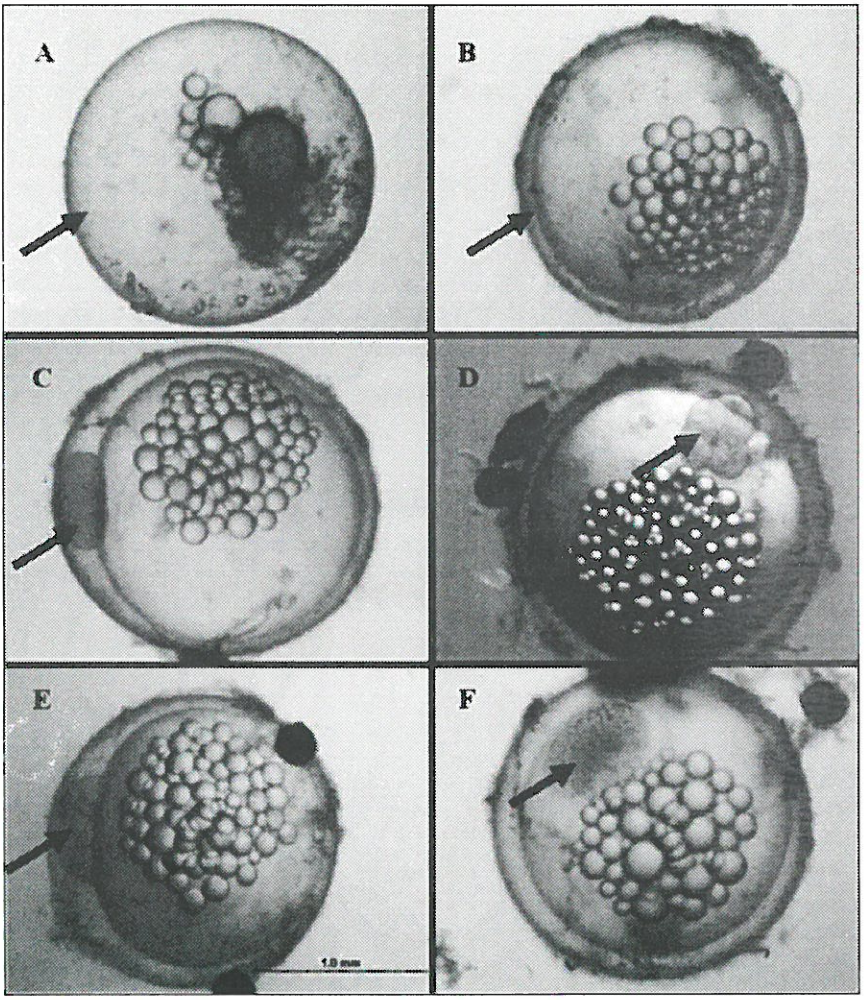

Figure 1. Early developmental stages of *K. marmoratus* embryos. A. Non-fertilized embryo with perivitelline membrane space absent. B. Recently fertilized embryo (< 2.5 h) with perivitelline membrane space present. C. One-cell stage embryo (2.5 h). D. Four-cell stage embryo (4 h). E. High Blastula stage embryo (9.5 h). E. Late Blastula stage embryo (10.5 h). Dark arrows indicate descriptions above. Many translucent oil droplets are present on the interior of the egg. The opaque balls on the exterior of the embryos are brine shrimp eggs carried over from collections.

After 48 h of hygromycin B exposure, the no observed effect concentration (NOEC) was 350 mg/l and the lowest observed effect concentration (LOEC) was 500 mg/l (Figure 2). All of the embryos exposed to 750 mg/l were under complete developmental arrest leading to death after 48 h of exposure (Figure 2). The effective concentration at which 50% of the fish were developmentally arrested (EC50) was 432.4 mg/l and the EC95 was 704.9 mg/l (Table I).

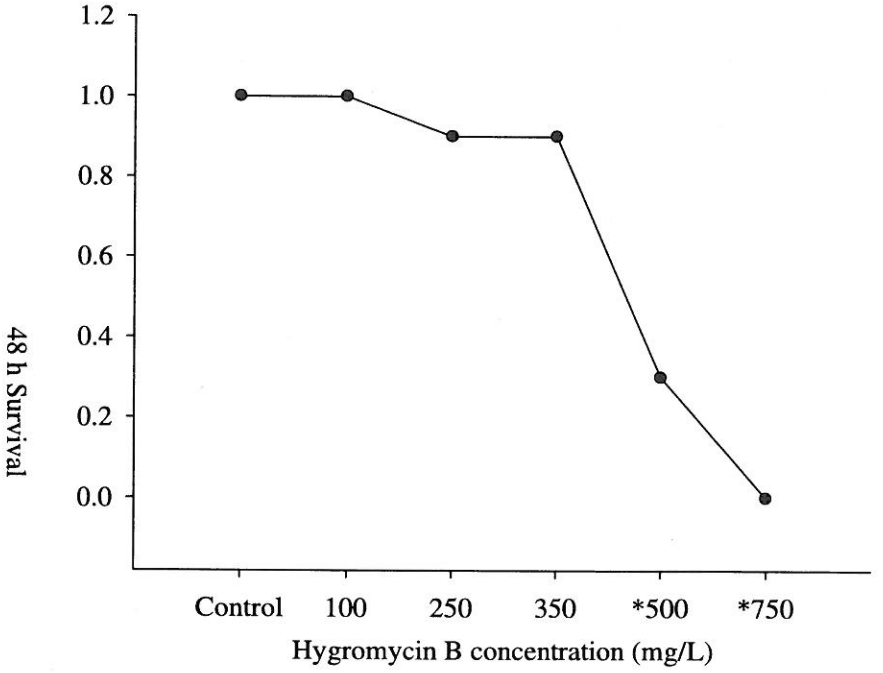

Figure 2. Percent survival of *K. marmoratus* embryos exposed to increasing concentrations of hygromycin B for 48 h. * Indicates statistically effective concentration.

Table I. Effective concentrations (EC) of early staged *K. marmoratus* embryos exposed to hygromycin B for 48 h.

Point	Probits	mg/l	95%	Confidence Interval
EC01	2.7	216.7	102.6	280.8
EC05	3.4	265.3	154.4	325.1
EC10	3.7	295.5	191.0	353.2
EC15	4.0	317.9	219.7	374.9
EC20	4.2	336.8	244.7	394.4
EC25	4.3	353.9	267.6	413.3
EC40	4.7	401.1	328.5	474.5
EC50	5.0	432.5	364.6	525.5
EC60	5.3	466.3	398.5	591.0
EC75	5.7	528.4	450.5	736.8
EC80	5.8	555.3	470.2	808.8
EC85	6.0	588.4	493.0	904.0
EC90	6.3	632.8	521.9	1042.8
EC95	6.6	704.9	565.6	1293.5
EC99	7.3	863.1	653.2	1951.1

After 96 h, the NOEC was 100 mg/l and the LOEC was 250 mg/l (Figure 3). All of the embryos tested at 500 mg/l were completely developmentally arrested or dead after 96 h of exposure (Figure 3). The 96 h EC_{50} was 206.6 mg/l and the 96 h EC_{95} was 601.2 mg/l (Table II).

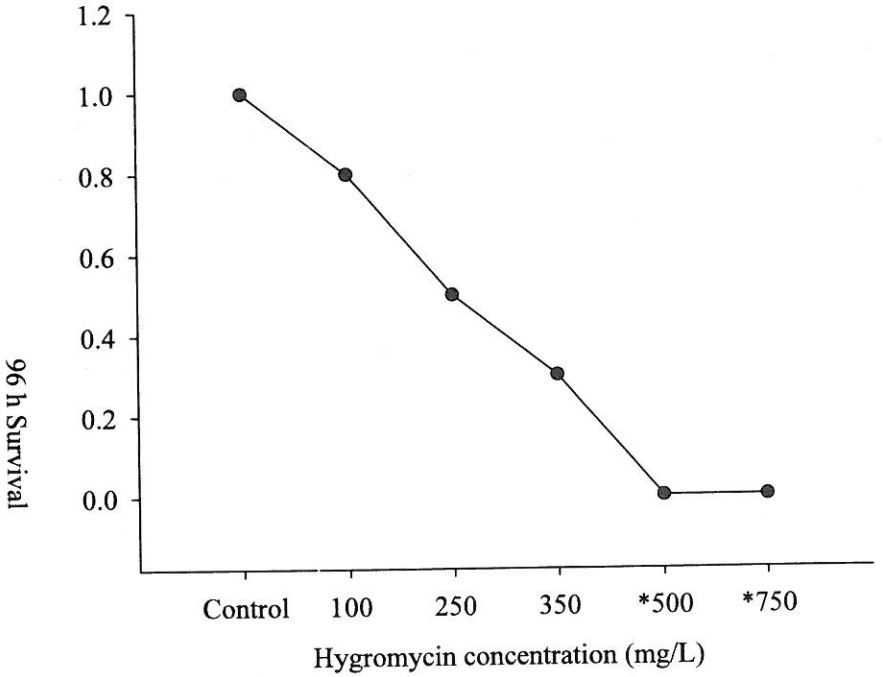

Figure 3. Percent survival of *K. marmoratus* embryos exposed to increasing concentrations of hygromycin B for 96 h. * Indicates statistically effective concentration.

Table II. Effective concentrations (EC) of early staged *K. marmoratus* embryos exposed to hygromycin B for 96 h.

Point	Probits	mg/l	95%	Confidence Interval
EC01	2.7	45.6	7.7	85.9
EC05	3.4	71.0	18.2	117.2
EC10	3.7	89.9	28.6	139.0
EC15	4.0	105.4	38.8	156.3
EC20	4.2	119.6	49.3	172.0
EC25	4.3	133.3	60.3	187.3
EC40	4.7	175.3	98.8	235.7
EC50	5.0	206.6	130.5	276.0
EC60	5.3	243.5	168.1	331.1
EC75	5.7	320.2	238.3	481.8
EC80	5.8	356.9	267.2	572.7
EC85	6.0	405.0	301.6	709.4
EC90	6.3	474.9	346.4	941.3
EC95	6.6	601.2	418.3	1455.8
EC99	7.3	935.9	580.2	3387.3

Finally, the dose response is faster in the 48 h treatments at the higher hygromycin concentrations versus the equivalent 96 h treatments, as would be expected (Figure 2 & 3). However, much lower statistically effective concentrations of hygromycin B between 250-500 mg/l are required for the 96 h versus 48 h treatment (Figure 2 & 3). All of the developmentally arrested embryos observed in this study eventually died, while their sibling controls developed normally, indicating the toxic effects of hygromycin B were lethal as they disrupted early development in an irreversible manner. Most of these developmentally arrested embryos died during late blastula to early gastrula while some made it through gastrulation, but terminated development at the characteristic shield stage of development where an obvious anterior to posterior axis had formed.

DISCUSSION

A simple protocol for collecting early developmentally staged *K. marmoratus* embryos and subsequent selection by hygromycin B is described here. The early stages of embryonic development displayed in Figure 1 confirm those reported by Koenig & Chasar (7). The most effective conditions for halting 95% of development in these embryos is an exposure of approximately 600 mg/l hygromycin B for 96 h (Table II).

It is now feasible to use hygromycin B selection of potential hyg^R transgenic fish. For example, putative transgenic early stage embryos could be exposed to the EC_{95} and identified as survivors. Or such embryos might be exposed to approximately 200 mg/l hygromycin B for 96 h (EC_{50}) followed by identification of transgenic fish as healthier, faster developing embryos as compared to non-transgenic siblings. Following this logic, various concentrations might be chosen for embryo selection with 95% confidence intervals across a range of hygromycin B concentrations at either 48 or 96 h exposures (Table I & II).

Additional modifications could also be made to this protocol to reduce the amount of hygromycin B required for a similar toxic effect or transgenic selection scheme. One modification would include exposure of embryos in fresh water to reduce the effect of salt on hygromycin B activity. A second modification might include addition of pronase during the 48 or 96 h incubations with hygromycin B which helps breakdown the protective chorion covering the embryo. The later technique was used for efficient exposure of *K. marmoratus* embryos to 17α-methyl testosterone for directed sex determination into male progeny (8).

The MBT has not been characterized in *K. marmoratus*. However, results presented here indicate the MBT is likely to be similar to other teleost fish as hygromycin B was effective at disrupting normal development in cleavage to early blastula stage embryos prior to late blastula (Figure 1E). In zebrafish the MBT begins by the tenth cellular division of early embryonic development which is equivalent to a late blastula of about 1000 cells (9). In both medaka and *Fundulus heteroclitus*, the MBT is a little later than the tenth cellular division, but is equivalent to late blastula, based on molecular and cellular motility data (10, 11). Further characterization of the MBT in *K. marmoratus* by microscopic and molecular techniques would complement and confirm the data presented here.

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