Alcohol Teratology of the Axolotl Embryo

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Abstract

It has been well documented that chronic alcohol ingestion during pregnancy is teratogenic to the developing fetus in humans and animal models. Little is known about the minimum amount of alcohol that can be ingested safely or about the length of time of alcohol ingestion that will cause significant defects. These experiments addressed these issues using axolotl embryos, *Ambystoma mexicanum*, as a model. As the embryos reached gastrulation, they were separated into different groups, and exposed to different concentrations of alcohol for varying periods of time. Embryos were staged and examined every day for defects which might be related to Fetal Alcohol Syndrome. They were also tested for behavioral problems. Acute exposure to 100, 200 and 400 mM ethanol for 24 hours suppressed embryo development ($p<0.001$). Exposure to 100 mM for 1, 2 and 4 days also suppressed embryo development ($p<0.001$). In these two experiments integrated dose x time exposure data were superimposable. Embryonic development was also suppressed in embryos exposed to 12, 25, 50 and 100 mM ethanol for 4 days ($p<0.001$). The axolotl embryo model is responsive to ethanol-associated developmental retardation and is worthy of further investigation as a research tool.

Introduction

It is written in the Book of Judges (13:7): "Behold, thou shalt conceive, and bear a son; and now drink no wine or strong drink." It has been known since Biblical times that alcohol ingestion in a pregnant woman is harmful to the fetus. Yet still, an average of seven cases of Fetal Alcohol Syndrome (FAS) are referred to the Genetics Department at the Children's Hospital in Winnipeg, MB each year (Chudley, 1991). There is a worldwide incidence of 1.9 per 1000 live births and in the western world FAS is the leading cause of mental retardation (Abel and Sokol, 1987).

Fetal Alcohol Syndrome is a totally preventable congenital disease caused by maternal ingestion of alcohol during pregnancy. It has been defined by Jones and Smith (1973) as a pattern of characteristic facial features, growth retardation and other birth defects, such as cardiac anomalies and mental retardation . The fetal alcohol study group of the Research Society of Alcoholism recommends the following minimum criteria for diagnosing FAS: prenatal or postnatal growth retardation or both (defined by a weight, height, or head circumference less than the 10th percentile for gestational age); signs of neurological abnormalities, developmental delay or intellectual impairment; at least two of: microcephaly with a head circumference less than the third percentile; microphthalmia or short palpebral fissures or both; poorly developed philtrum, thin upper lip and flattening of the maxillary area (Rosett, 1980). There may also be milder manifestations, such as learning disabilities, with a physically normal FAS child. Other abnormalities noted in FAS newborns are tremors, increased muscle tone, irritability, increased respiratory rate, seizure activity, arching of the back, abdominal distention and vomiting (Pierog et al., 1977). A graded dose-response relationship has been shown in both human and animal studies. Low doses tend to result in growth retardation while higher doses result in malformations and fetal death (Farrar and Blumeer, 1991).

The time at which alcohol is ingested relative to gestation of the pregnancy is an important factor. Studies have shown that ethanol is teratogenic during the stage of gastrulation and neural tube formation in the embryo. At this stage, morphogenetic cell movements appear to be inhibited, and these changes may manifest themselves as characteristic anomalies of FAS (Persaud, 1988). Later in the pregnancy, when tissue and organs types have differentiated, alcohol is more likely to cause cell depletion and growth retardation, although death can still occur (Beckman and Brent, 1984).

This study used axolotl embryos as a model and potential characteristics of FAS were examined in embryos exposed to alcohol. Although not all human characteristics of FAS can be demonstrated in axolotls, this study focused on the few that can be found in these embryos. It compared the alcohol-exposed embryos to controls, recording differences in

developmental rate, growth rate, morphological defects and behavioral abnormalities. These defects were characterized with different concentrations of alcohol and also different exposure times to the teratogen.

Materials And Methods

Freshly laid axolotl embryos were collected and staged, using a system initially proposed by Harrison for *Ambystoma mexicanum* (Bordzilovskaya et al., 1989). Stage 1 occurs when the fertilized eggs are laid. These experiments were started when embryos reached stage 10, the beginning of gastrulation. Neurulation starts at stage 13; neural folds are completely fused at stage 21, and hatching begins at stage 41. When embryos reached stage 10, they were randomly separated into groups of 30. The control group was placed in a covered rectangular glass dish containing 250 mL of modified Holtfreter's solution (Asashima et al., 1989). This solution was diluted with distilled water to 1/4 strength for use with embryos.

Three different experiments were performed: 1) Acute ethanol exposure for 24 hours. Embryos were placed in dishes containing 50 mM, 100 mM, 200 mM, and 400 mM ethanol mixed with diluted Holtfreter's solution. 2) Chronic ethanol exposure at a constant concentration. Embryos were exposed to 100 mM ethanol for 1, 2, and 4 days. 3) Embryos exposed for 4 days at lower concentrations of ethanol. They were placed in 12, 25, 50 and 100 mM of ethanol mixed with diluted Holtfreter's solution. The concentration of 12 mM approaches the human legal limit for intoxication, 17 mM. Every day the solutions of each group were changed to fresh solution. After each group of embryos was exposed to the ethanol for their designated periods of time, they were then switched to the diluted Holtfreter's solution for the remainder of the experiment. Each day the developmental stage and appearance of the embryos were noted.

After most of the embryos had hatched, behavior tests were performed. These consisted of swimming and eating tests. For the swimming test, a small container 2.6 cm in diameter, 2 cm high and containing water 1 cm in depth was used. The larvae were stimulated, by poking their tail with tweezers, to swim around the container. A normal response was recorded if the larvae swam forward and upright for at least a few millimetres.

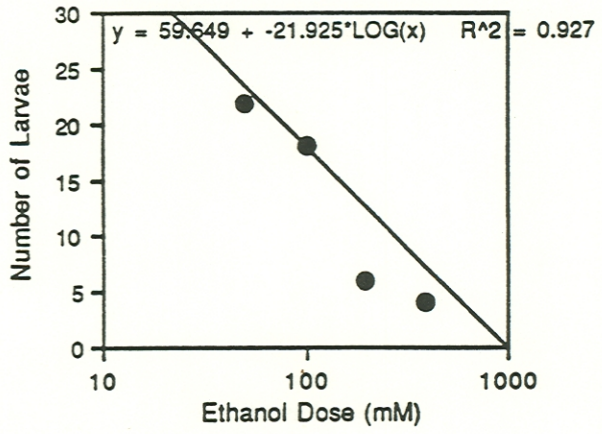


Figure 1: This figure illustrates the survival of embryos exposed to 50, 100, 200 and 400 mM ethanol for 24 hours, showing a classical log-dose response. Each group started with 30 embryos. There were 24 embryos left in the control group.

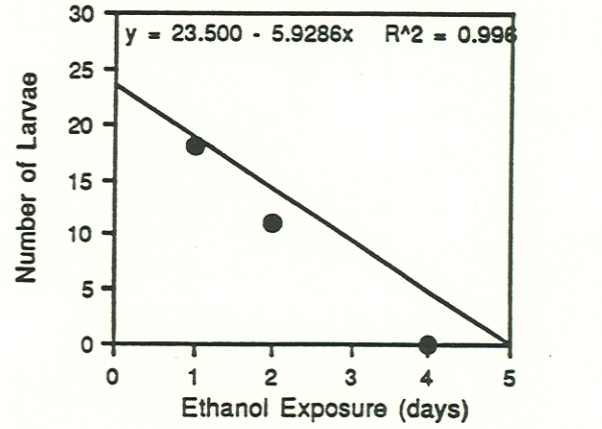


Figure 2: This figure illustrates the survival of embryos exposed to 100 mM ethanol for 1, 2, and 4 days. Each group started with 30 embryos.

The larva failed the test if after 5 trials it didn't react to stimuli, if it reacted to stimuli with a shivering motion, or if it had a normal response motion but didn't move forward more than a few millimetres. The eating test assessed the larvae's ability to hunt for and eat live brine shrimp. A few larvae at a time were placed in a container that held 1 litre of water to a 10 cm depth, along with a small amount of freshly hatched brine shrimp. They were observed for motions to catch and eat the shrimp. The larva's belly was orange if it had eaten. A failed test was defined as a larva that had no signs of having eaten after being in the container for a period of about 10 minutes.

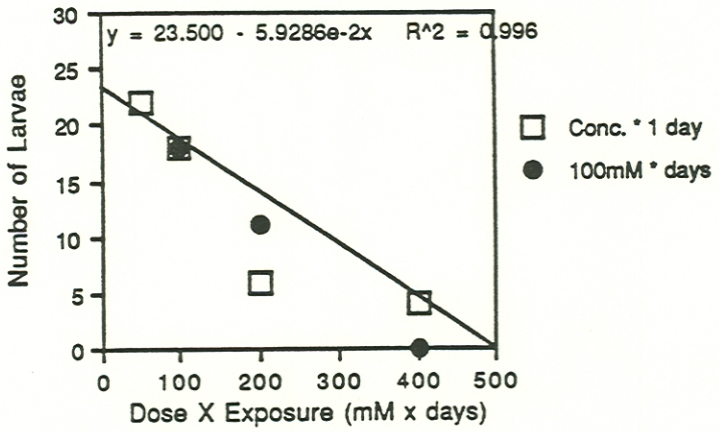


Figure 3: Comparison of survival of embryos exposed to increasing ethanol concentration for 24 hours and embryos exposed to 100 mM for increasing time.

The embryos that were exposed to 0, 12, 25, 50 and 100 mM ethanol for 4 days were preserved after the larval stage was reached and the behavior tests had been performed. Smith's preserving fluid was used to fix the larvae; they were then placed in absolute alcohol. Once preserved, several measurements were recorded, including eye span (centre to centre), head width, body width, length and the height at the abdomen. Volume was calculated using body width x length x height. Data were compared for significant differences by the appropriate ANOVA model. Multiple comparisons were completed with Tukey's HSD test. A significant difference was accepted when $p < 0.05$.

Results

Morphological defects were produced in some embryos in each experiment. Examples of these were failure for the neural tube to completely fuse, twisted and curved spines, large distended bellies, and small size. Some embryos failed to hatch. In every case, defective embryos failed to survive.

In the first two experiments, survival decreased with increasing ethanol concentration and exposure time. Figure 1 illustrates the survival of embryos exposed to increasing ethanol concentrations for 24 hours. The data were consistent with the classical log-dose response relationship with an apparent deleterious effect with 50 mM ethanol. Figure 2 is an illustration of the survival of embryos exposed to a constant concentration of ethanol for increasing amounts of time. The survival of embryos exposed to 200 mM ethanol for 24 hours is comparable to that of embryos exposed to 100 mM for 2 days. Likewise, survival for embryos exposed to 400 mM ethanol for 24 hours and embryos exposed to 100 mM for 4 days are also comparable. This is illustrated in figure 3. Survival was not affected in the third experiment.

Neither behavior test performed showed differences among survivors of control and treatment groups in any of the three experiments. The results from these tests are shown in Table 1.

There were also no differences detected among control and treatment groups for any of the morphological measurements completed, the results of which are shown in Table 2.

When the embryos that were exposed to ascending doses of ethanol for 24 hours were staged, it was found that control was different from all treatment groups ($p<0.001$). Forty-eight hours after the experiment was started no difference in development of the treatment groups was detectable. After 96, 144 and 168 hours, the 200 and 400 mM ethanol groups were less well developed than the 100 mM group ($p< 0.001$). In summary, the highest ethanol doses were associated with slower embryological development than the lower doses.

In the experiment exposing embryos to 100 mM ethanol for 0, 1, 2 and 4 days, there was also a treatment effect ($p< 0.001$). After 48 hours there was no difference among treatment groups. The group exposed to ethanol for 4 days did not survive past 96 hours. The embryos exposed to ethanol for 1 day were at

Table 1: Summary of behavior test data on axolotl larvae studied for effects of dose and exposure time to ethanol during embryological development.

Ethanol Dose (mM)	Exposure Time (Days)	Test Failures	
		Swimming	Eating
0		2/24	0/24
50	1	3/22	0/22
100	1	2/18	2/18
200	1	2/6	2/6
400	1	0/4	0/4
0		2/24	0/24
100	1	2/18	2/18
100	2	1/11	2/11
0		0/26	0/26
12	4	1/25	2/25
25	4	1/25	3/25
50	4	0/25	0/25
100	4	0/24	0/24

a higher average developmental stage than those exposed for 2 days, when studied after 96 ($p< 0.001$), 144 ($p= 0.003$) and 168 hours ($P<0.001$)

There was also a treatment effect in the experiment exposing embryos to 12, 25, 50, and 100 mM ethanol for 4 days ($p< 0.001$). There was no difference among treatment groups after 24 hours. The 12 and 25 mM groups were at higher average developmental stages than the 50 and 100 mM groups after 48 hours ($p<0.001$). After 72 hours the 100 mM ethanol group was less well developed than all other groups ($p<0.001$). The 50 mM group was at a lower average stage than the 12 and 25 mM groups and the 100 mM group was once again less well developed than the other groups, after 96 hours ($p<0.001$). After 120 hours the 100 mM group was at a lower stage than all others and the 50 mM group was less well developed than the 25 mM group ($p<0.001$). The 100 mm ethanol group was at a lower developmental stage than all others after 144 hours ($p<0.001$) and lower than the 25 mM group after 168 hours ($P<0.001$). The development rates for the treatment groups followed normal development for the first 24 hours. See figures 4-6. After that point, there was a delay in the rate of development of exposed embryos. Even after the embryos were removed from ethanol, their development rates never paralleled the control rate. Only the rate of embryos exposed to 100 mM for 24 hours was suggestive of catch-up growth within the first 184 hours. There was evidence from the morphological measurements of the embryos from the third experiment that these larvae eventually caught up physically to the controls.

Table 2: Results of measurements performed on axolotl larvae exposed to low doses of ethanol for four days. The data is presented as mean +/- S.D. Volume was calculated using the body width.

Treatment	Eye Span (mm)	Head Width (mm)	Body Width (mm)	Length (mm)	Height (mm)	Volume (mm3)
Control	1.7±0.1	3.0±0.1	2.1±0.3	13.1±0.7	2.7±0.3	71.8±20.2
12 mM	1.7±0.2	3.0±0.2	1.9±0.3	13.1±1.1	2.7±0.3	68.2±14.0
25 mM	1.7±0.1	3.1±0.2	2.0±0.2	13.3±0.8	2.7±0.2	72.8±9.8
50 mM	1.7±0.1	3.0±0.1	2.0±0.3	13.2±0.8	2.8±0.3	75.5±21.1
100 mM	1.6±0.1	3.0±0.1	1.9±0.2	12.7±0.7	2.6±0.2	64.0±10.3

Figure 4. Embryos exposed to 100, 200, and 400 mM ethanol for 24 hours.

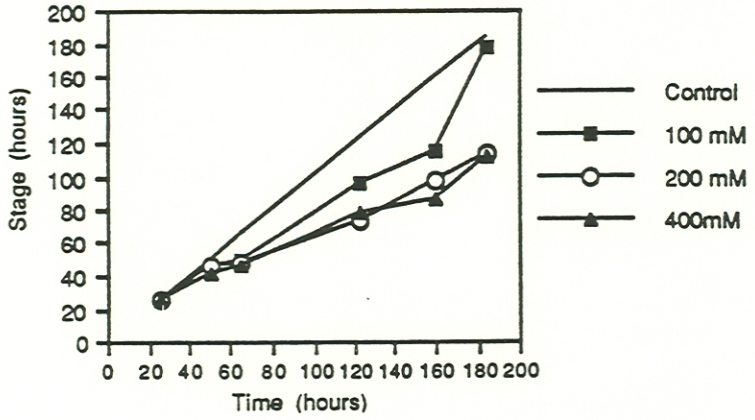


Figure 5. Embryos exposed to 100 mM for 1, 2, and 4 days.

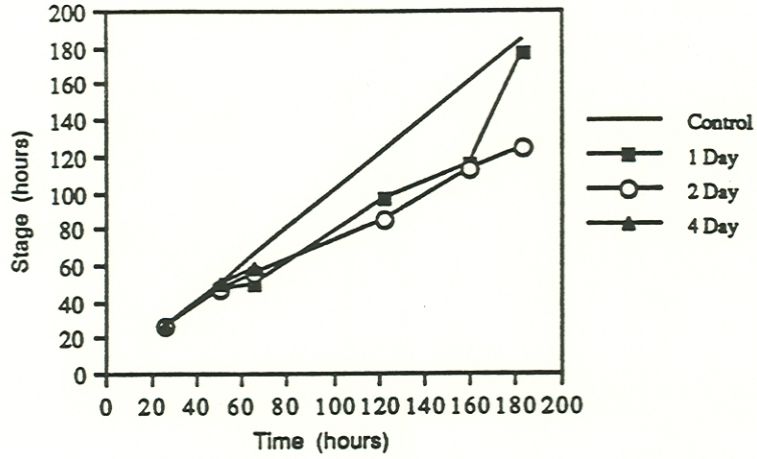
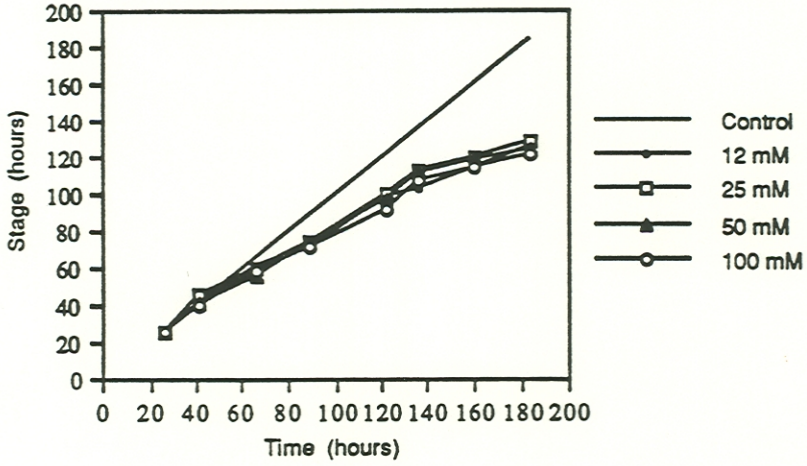


Figure 6. Embryos exposed to 12, 25, 50, and 100 mM ethanol for 4 days.



Figures 4-6: These figures illustrate the developmental rates of ethanol-exposed embryos as compared to control, at 19 °C (Bordzilovskaya et al., 1989).

Discussion

The experiments performed showed that ethanol exposure had an effect on axolotl embryos. Several types of defects were produced in exposed embryos. These embryos failed to survive and so the incidence of defects was reflected in the survival numbers. Survival numbers decreased with increasing concentration and exposure time to ethanol. In the first two experiments integrated dose X time exposure data were superimposable. Exposure to high concentrations of ethanol for a short period of time is just as detrimental to embryonic survival as to a lower concentration for a longer exposure times. The third experiment, using lower concentrations of ethanol, did not reveal significant decreases in survival. Behavior tests did not differentiate between controls and the treatment groups; either most of the larvae that survived had no behavioral deficits, or the tests performed were not sensitive enough to detect them. Ethanol significantly suppressed embryonic development in all three experiments.

These experiments provide a baseline for further experimentation with axolotls and alcohol. Extensions of the study that could be considered include testing for alcohol dehydrogenase activity in the axolotl. If activity is present, an inhibitor of ethanol metabolism, such as 4-Methylpyrazole, could be added to determine whether it is the ethanol that causes the defects, the metabolites of ethanol, or a combination of both. Other studies may include histological examination of exposed embryos for heart anomalies and other organ damage and also time lapse microscopy of the gastrulation wave (Gordon et al., 1994).

Acknowledgements

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References

Abel, E.L. and Sokol, R.J. 1987. Incidence of fetal alcohol syndrome and economic impact of FAS related anomalies. Drug-Alcohol-Depend **19**:57-70.

Asashima, M., Malacinski, G.M., and Smith, S.C. 1989. Surgical manipulation of embryos. In *Developmental Biology of the Axolotl.* Armstrong, J.B. and Malacinski, G.M., eds. Oxford University Press, New York, p. 256.

Beckman, D.A. and Brent, R.L. 1984. Mechanisms of teratogenesis. Annual Reviews of Pharmacology and Toxicology **24**:483-500.

Bordzilovskaya, N.P., Dettlaff, T.A., Duhon, S.T., and Malacinski, G.M. 1989. Developmental stage series of axolotl embryos. In *Developmental Biology of the Axolotl.* Armstrong, J.B. and Malacinski, G.M., eds. Oxford University Press, New York, pp. 203-214.

Chudley, A.E. 1991. Fetal alcohol syndrome, a preventable cause of birth defects and mental retardation. Manitoba Medicine **61**:53-56.

Farrar, H.C., and Blumeer, J.L. 1991. Fetal effects of maternal drug exposure. Annual Reviews of Pharmacology and Toxicology **31**:525-547.

Gordon, R., Bjorklund, N.K., and Nieuwkoop, P.D. 1994. Dialogue on embryonic induction and differentiation waves (invited). Int. Rev. Cytol., in press.

Jones, K.L. and Smith, D.W. 1973. Recognition of fetal alcohol syndrome in early pregnancy. Lancet **2**:999-1001.

Persaud, T.V.N. 1988. Fetal alcohol syndrome. CRC Crit. Rev. Anat. Cell. Biol. **1**:277-325.

Pierog, S., Chandavasu, O., and Wexler, I. 1977. Withdrawal symptoms in infants with the fetal alcohol syndrome. J. Pediat. **90**:630.

Rosett, H.L. 1980. A clinical perspective on the fetal alcohol syndrome. Alcoholism **4**:119-122.