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The Effect of Caffeine and Ethanol on Flatworm Regeneration.

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The Effect of Caffeine and Ethanol on Flatworm Regeneration

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by

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ABSTRACT

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Flatworms, or planarian, have a high potential for regeneration and have been used as a model to investigate regeneration and stem cell biology for over a century. Chemicals, temperature, and seasonal factors can influence planarian regeneration. Caffeine and ethanol are two widely used drugs and their effect on flatworm regeneration was evaluated in this experiment. Non-toxic levels of caffeine, a stimulant, and ethanol, a depressant, were determined. The tails of the flatworms were removed and the regeneration stage was analyzed every 3 days for 15 days to see the effect of these drugs alone and in combination on regeneration. For day 3 and day 6, there was a significant difference between the ethanol treatment and the other treatments (positive control, caffeine treatment, and combined treatment). The ethanol treatment showed a delay in the initiation of regeneration but caught up to the other treatments by day 15.

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CHAPTER 1

INTRODUCTION

For over a century, planarians have been used to investigate the principles of regeneration through surgical manipulations and cellular observations (Reddien and Alvarado 2004). Commonly found in freshwater streams and ponds, planarians, or flatworms, are bilaterally symmetric metazoans. Flatworms are of the phylum *Platyhelminthes* and are carnivorous, predominately feeding on living or dead animal matter (Ward's Scientific 2000). The species *Dugesia tigrina*, or brown planarian, was used in this study. To eat, flatworms extend their eversible pharynx to almost one-half of their body length. Flatworms have a high potential for regeneration and reorganization and are inexpensive to culture, maintain, and use for toxicological testing. (Inden et al. 2004)

During regeneration, neoblasts, which are undifferentiated stem cells and the only known proliferating cells in adult flatworms (Reddien et al. 2005), form a blastema, an aggregate of cells. These neoblasts make up possibly 30% of the cells of an adult worm (Gittins et al. 2003). Regeneration in planaria can be influenced by a number of factors including chemical, temperature, and seasonal factors. Flatworms are used as a classic model of regeneration and stem cell biology and help gain information about functioning of stem cells in all animals (Reddien et al. 2005). In most metazoans, stem cells are used in order to replace aged or damaged cells (Weissman 2000). Planarians can provide an insight into mechanistic investigation of in vivo stem cell regulation, which are likely to inform the functioning of stem cells in all animals including humans (Weissman 2000).

Caffeine and ethanol are two widely used drugs. Caffeine is a stimulating drug that could hinder the ability of the flatworm to regenerate completely. High doses of caffeine have shown to produce malformations in rodents (Christiani and Brent 2001). Caffeine increases release of catecholamines from the renal medulla. This increase may result in vasoconstriction in the uteroplacental circulation and lead to fetal hypoxia (Wisborg et al. 2003). The neoblasts might not be able to fully develop, which would not allow the blastema to form as normally as a neoblast that was untreated (Gittins et al. 2003). In contrast, ethanol is a depressant. This could also hinder the ability of flatworms to regenerate completely, similar to the caffeine treatment, however, in a different way. Alcohol is a teratogen and causes a broad variety of developmental abnormalities (Armant and Saunders 1996). Because caffeine and alcohol have opposing effects to the central nervous system, the interaction of caffeine and ethanol was looked at during this experiment.

Ethyl alcohol, also known as ethanol, is a flammable, colorless chemical compound and is one of the alcohols that is most often found in alcoholic beverages (National Institute for Occupational Health 2006). Ethanol has a depressing effect that decreases the responses of the central nervous system. The chemical formula for ethyl alcohol is C_2H_5OH, also written as C_2H_6O. The molecular weight of ethyl alcohol is 46.07 g/mol and it has a melting point of −114.3 °C (158.8 K) and a boiling point of 78.4 °C (351.6 K) (National Institute for Occupational Health 2006). Ethyl alcohol is fully miscible in water.

Injury to all organ systems in the human body can be seen from chronic alcohol use and has serious medical and public health implications (Shukla and Aroor 2006). For

humans exposed to ethanol during gestation, the effects can range from fetal alcohol syndrome in offspring to reduced average birth weight in offspring (Streissguth et al. 1980). Behavioral effects of women who consume two to three or more alcoholic beverages during pregnancy may range from mental retardation in children with fetal alcohol syndrome to milder developmental and behavioral effects in infants who were born to social drinkers. Exposure of animals to alcohol in utero may result in death, malformation, and growth deficiency (Streissguth et al. 1980). Also, behavioral and developmental abnormalities could result from exposure to alcohol in animals.

The placenta is permeable to ethanol and while the mother has ethanol dehydrogenase to remove ethanol from her blood the fetus has no such mechanism (Itthagarun et al. 2007). Due to the lack of this mechanism, the ethanol concentration in the fetus remains elevated for an extended period of time. Ethanol exposure in the fetus can lead to malformations in development. Alcohol intake during pregnancy causes birth defects, low birth weight, and deficits in the central nervous system. The fetus is not protected from the effect of alcohol because it does not have the enzyme to metabolize alcohol.

Caffeine, which can be found in beverages such as coffee, tea, soft drinks, products containing chocolate (cocoa), and some medications, is one of the most frequently ingested pharmacologically active substances in the world. Caffeine, which has the chemical formula, $C_8H_{10}N_4O_2$, has a molecular weight of 194.19g and is a stimulant to the central nervous system (O'Neil et al. 2001). Caffeine has been used as a cardiac and respiratory stimulant and as a diuretic (O'Neil et al. 2001). One gram of caffeine dissolves in 46ml of water at room temperature, 5.5ml of water at 80°, and 1.5ml

of boiling water (O'Neil et al. 2001). Caffeine has a LD50 orally in mice, hamsters, rats, and rabbits (mg/kg) of 127, 230, 355, and 246 in males respectively and 137, 249, 247, and 224 in females respectively (O'Neil et al. 2001).

Caffeine has been shown to cause adverse effects, which leads to the question "does caffeine have a deleterious effect on the developing tissues?" (Nawrot et al. 2003) Clearance of caffeine from the body is delayed in pregnant women (Santos et al. 1998). This delay occurs primarily in the second and third trimesters when it is decreased to one-half and to one-third the normal rate, respectively (Aldridge et al. 1979). Caffeine crosses the placental barrier, which means that the maternal blood levels of caffeine are virtually equal to fetal blood levels (Goldstein and Warren 1962). The enzymes needed for the metabolism of caffeine are absent in the fetus. So, when caffeine crosses the placental barrier it could lead to malformations in the fetus because the fetus lacks the enzyme needed for caffeine metabolism.

Caffeine and ethanol were used in this experiment in order to observe the effect that they had on the regeneration of flatworms. Both caffeine and ethanol can cross the placental barrier so they can cause adverse effects to growing fetuses. Caffeine and ethanol were chosen because they are both widely used and could have adverse effects on development. After non-toxic levels of caffeine and ethanol were determined, the tails of flatworms were removed and the regeneration process was analyzed in order to see what effects caffeine and ethanol had on regeneration and also any effect of caffeine and ethanol in combination. Comparing the effect of caffeine and ethanol on regeneration separately and also looking at the effect that caffeine and ethanol had on regeneration

when put in the same solution will allowed us to look at what effects these drugs had on stem cells.

CHAPTER 2

MATERIALS & METHODS

Preliminary Experiment

For the preliminary experiment, approximately 110 flatworms were used. Ethanol solutions of 1.0%, 0.1%, 0.01%, 0.001%, and 0.0001% and caffeine solutions of 0.1M, 0.01M, 0.001M, 0.0001M, and 0.00001M were prepared and tested in this experiment (See Figure 1). These solutions helped us find the appropriate concentration of caffeine and ethanol that we could use in the main experiment. Concentrations were chosen that would affect the worms but would not kill them, i.e., a non-toxic concentration. A Petri dish containing only water was used as the control. The effects of ethanol and caffeine treatments were compared to the control.

Approximately 10 flatworms were put in each of the 5 solutions of ethanol and 5 solutions of caffeine, 10 flatworms were also put in water alone and served as the non-treated control for this experiment. The flatworms were observed for 3 days and the number of surviving flatworms was recorded. The results were analyzed to see how various concentrations of caffeine and ethanol affected the flatworms. The ethanol and caffeine solutions were compared to the control to see if the effects of the two solutions differed from the effect of the control.

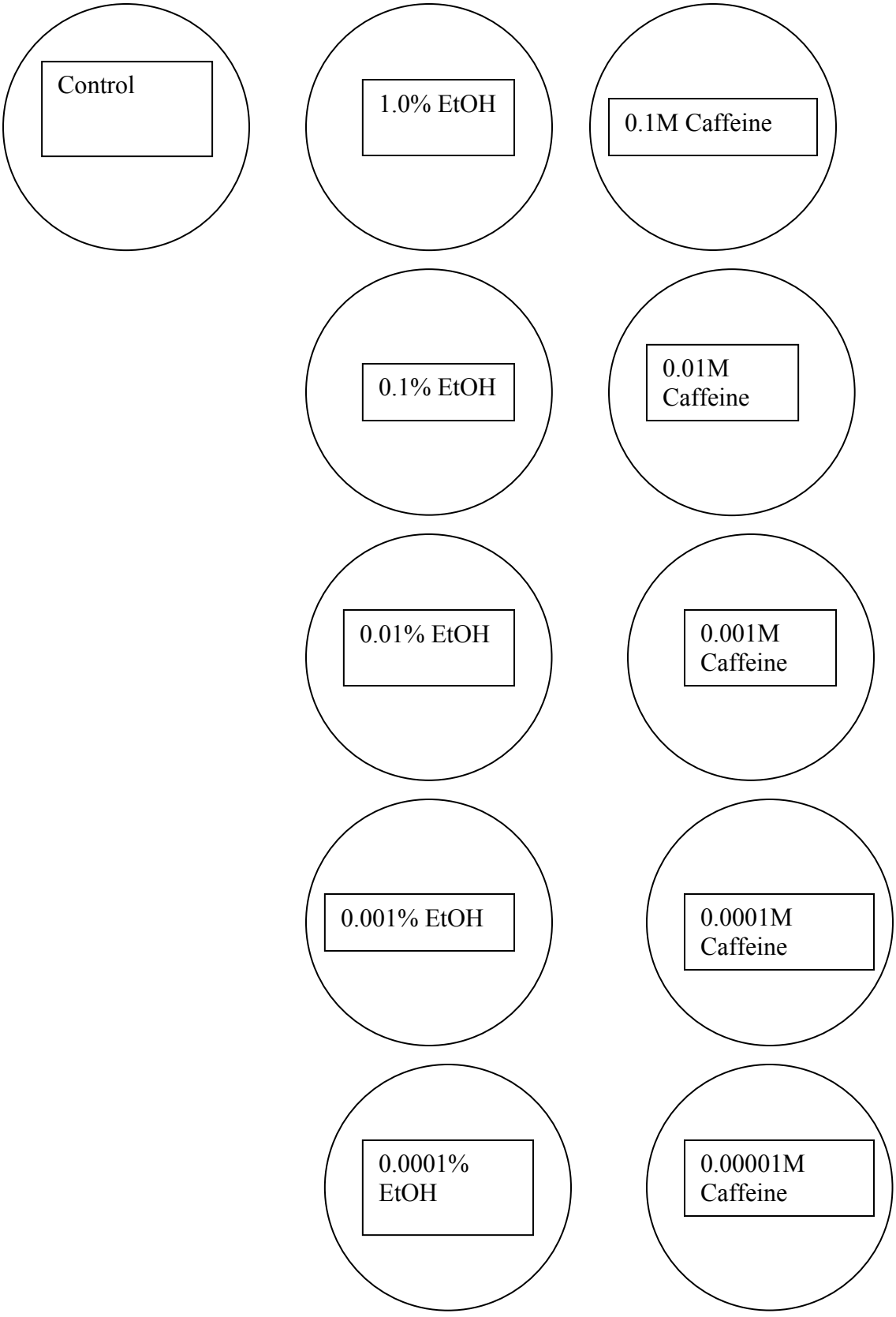

Figure 1 Preliminary Experiment Protocol

Main Experiment

For the main experiment, 175 flatworms were used. A facemask and powder-free gloves were worn to prevent any contamination from the person to the worms. In preparation for amputation of the tails, the flatworms were gently cooled in a refrigerator in order to slow their movement.

The cooled worms were cut with a sterile scalpel from one side of the worm to the other behind the eversible pharynx. Care was taken to remove only a very small portion of the tail (See Figure 3). Cutting a small portion of the tail of the flatworm behind the eversible pharynx enabled the worm to continue to eat, get rid of wastes, and regenerate normally. The tail portion of the flatworm was discarded.

The flatworms were put into 5 separate Petri dishes. Spring water from a natural spring was used in all the solutions to provide the electrolytes that the worms needed to survive. A Petri dish with water containing the uncut, negative control worms and a Petri dish with just spring water containing amputated, positive control worms were also prepared. Another Petri dish with just caffeine, a Petri dish with just ethanol, and a Petri dish with caffeine and ethanol in it were also prepared (See Figure 2). The concentration of the solution of caffeine that was used in this experiment was 0.00001M. The concentration of the solution of ethanol that was used in this experiment was 0.01%. These two concentrations were found, during the preliminary experiments, to allow the flatworms to live but still be affected by the different drugs. The Petri dish with both the solutions in it was a mixture, in equal parts, of the concentrations of caffeine and ethanol

that were determined by the preliminary experiments. The anterior portion of the flatworms was put into the 5 different solutions in 5 aliquots.

Regeneration of the worms was observed for a period of 15 days. All the worms were fed regularly with macerated bovine liver. Regeneration was recorded by making photographs every 3 days using a Minivid digital photomicroscopic camera.

Every 3 days, when the flatworms were evaluated, they were given a score that represented their stage of regeneration. These scores are explained in the following figures (See Figures 4-11).

Data Analysis

The data were analyzed to determine how regeneration was affected by each solution. The caffeine, ethanol, and caffeine plus ethanol (combined) solutions were compared to the solution containing only water. The solution containing caffeine and ethanol was compared to the solutions containing only caffeine and only ethanol to determine whether there was a greater or lesser effect compared to the two solutions in one Petri dish. The extent to which there was interaction between caffeine and ethanol was also determined. All the solutions that contained cut worms were compared to the control solution that contained worms that had not been cut to compare the amount of time the worms survived.

The Kruskal-Wallis test, a non-parametric test, is a generalization of the rank-sum test to the case of $k > 2$ samples (Walpole et al. 1998). This analysis is used to test the equality of means in the one-factor analysis of variance when the experimenter wishes to avoid the assumption that the samples were selected from normal populations. The Kruskal-Wallis test was used to test each day interval for statistical significance. After

using the Kruskal-Wallis test to see which days had statistically significant data, the Mann-Whitney test was then used. The Mann-Whitney test is a non-parametric test that is used to test for differences between two independent groups.

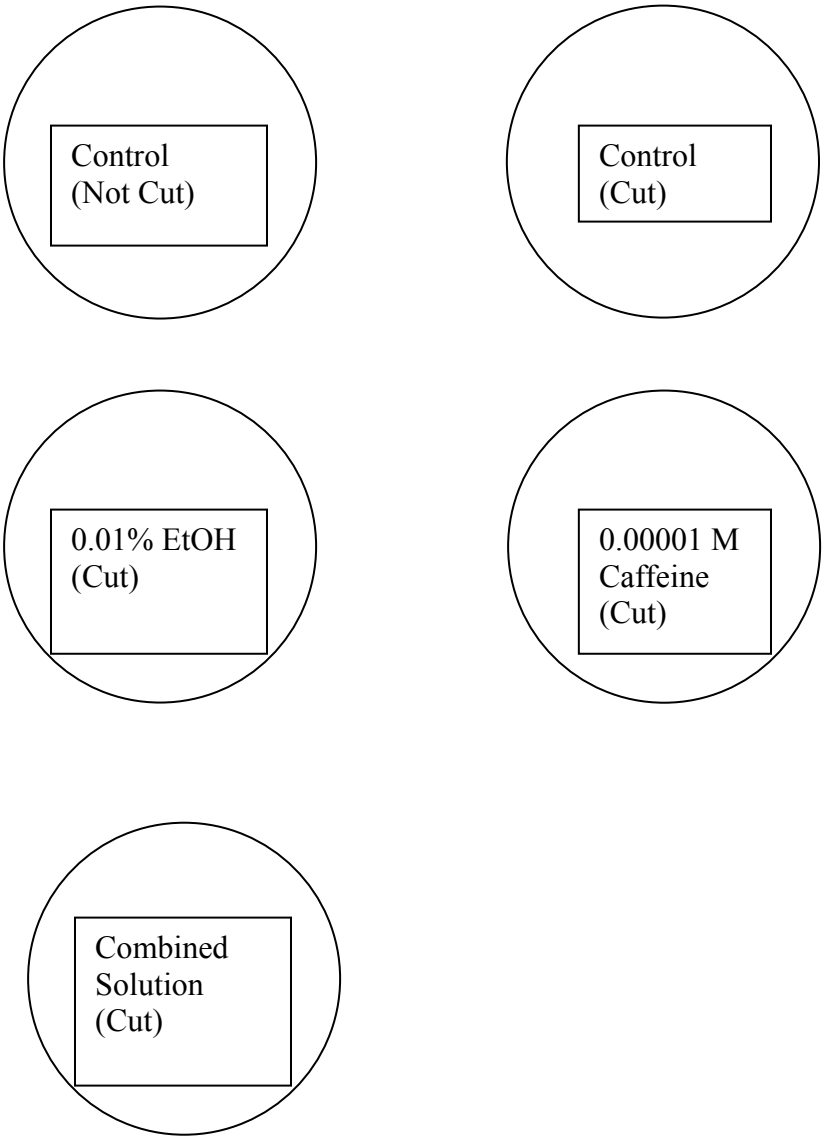

Figure 2 Main Experiment Protocol

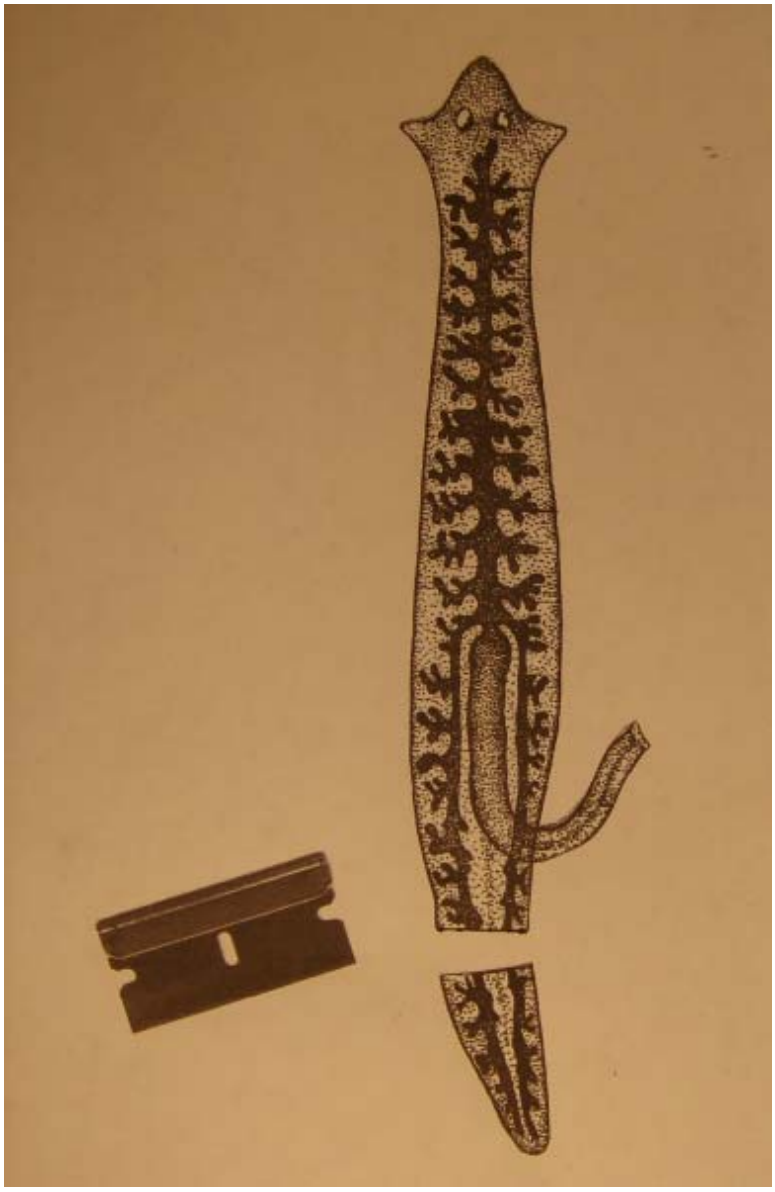

Figure 3 Worm Dissection. On day 0, the flatworms for the positive control, ethanol treatment, caffeine treatment, and combined treatment were cut from one side to the other behind the eversible pharynx. Cutting the flatworms behind the eversible pharynx enabled the worm to continue to eat, get rid of wastes, and regenerate normally.

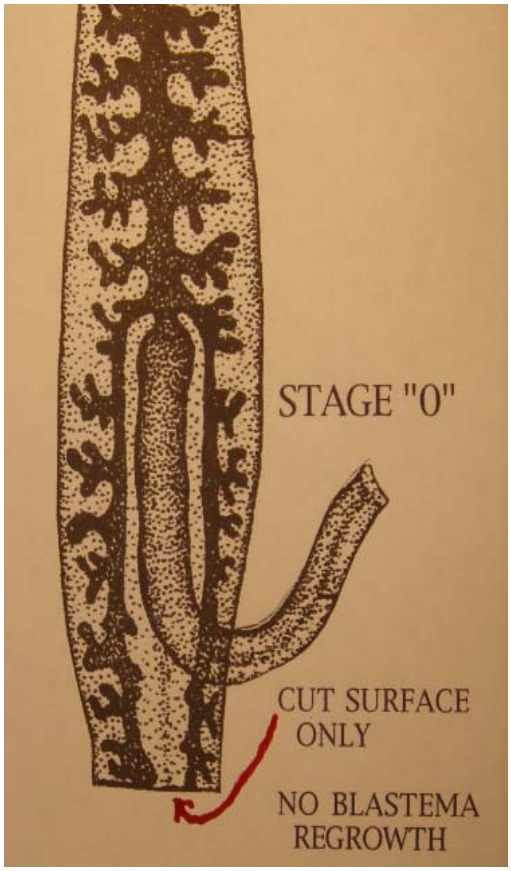

Figure 4 Stage 0 Worm Figure. In a Stage 0 flatworm, there is no blastema regrowth as shown in this figure and you see the cut surface only.

Figure 5 Stage 0 Worm Picture. In a stage 0 worm, there is no blastema regrowth as shown in this figure.

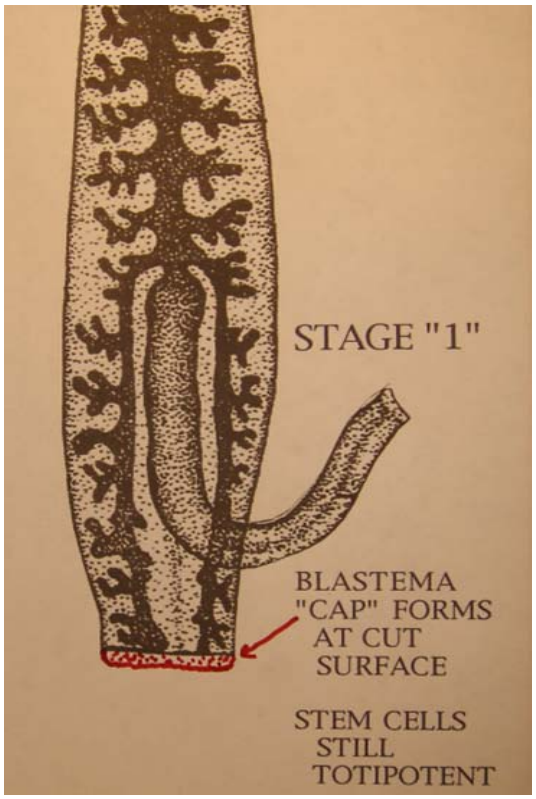

Figure 6 Stage 1 Worm Figure. In a Stage 1 flatworm, a blastema cap forms at the cut surface but the stem cells are still totipotent as shown in this figure.

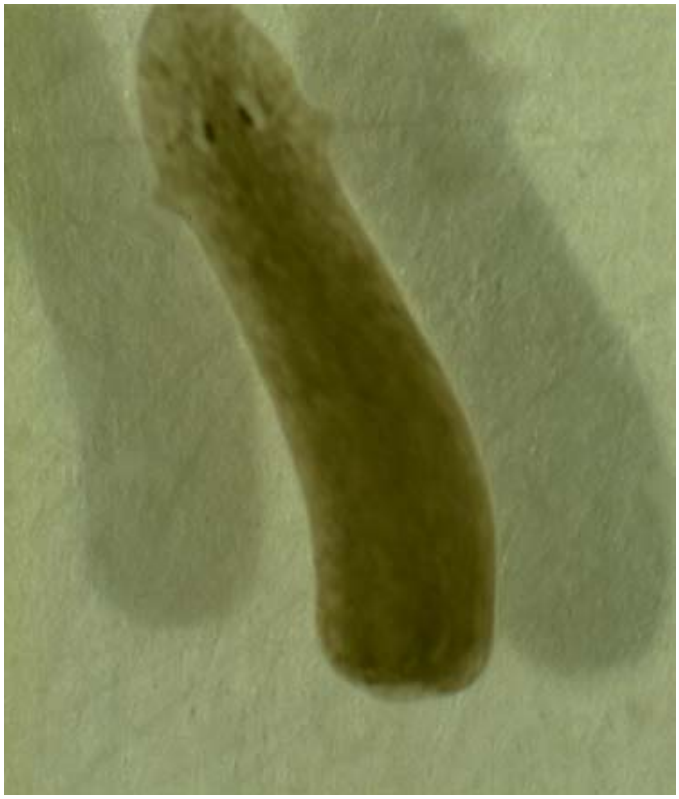

Figure 7 Stage 1 Worm Picture. In a Stage 1 worm, you see a blastema cap at the cut surface as shown in this figure.

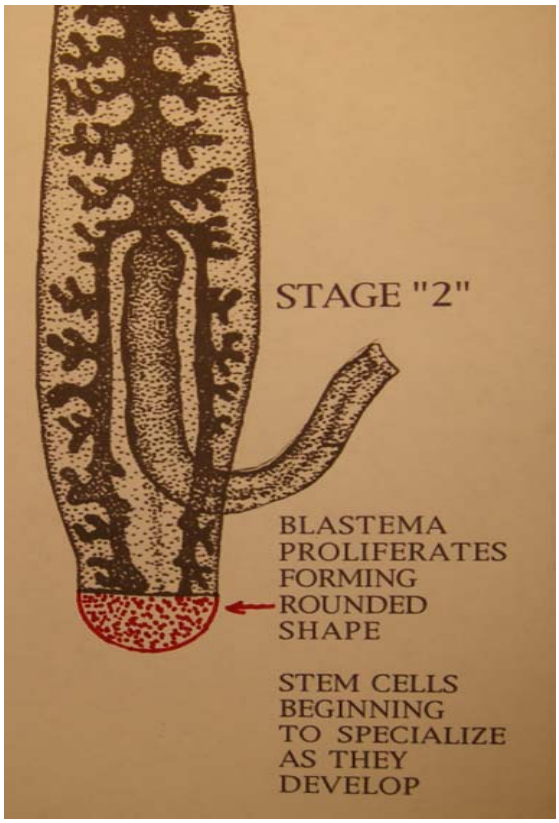

Figure 8 Stage 2 Worm Figure. In a Stage 2 flatworm, the blastema has proliferated forming a rounded shape as shown in this figure and the stem cells are beginning to specialize as they develop.

Figure 9 Stage 2 Worm Picture. In a Stage 2 worm, the blastema has formed a rounded shape as shown in this figure.

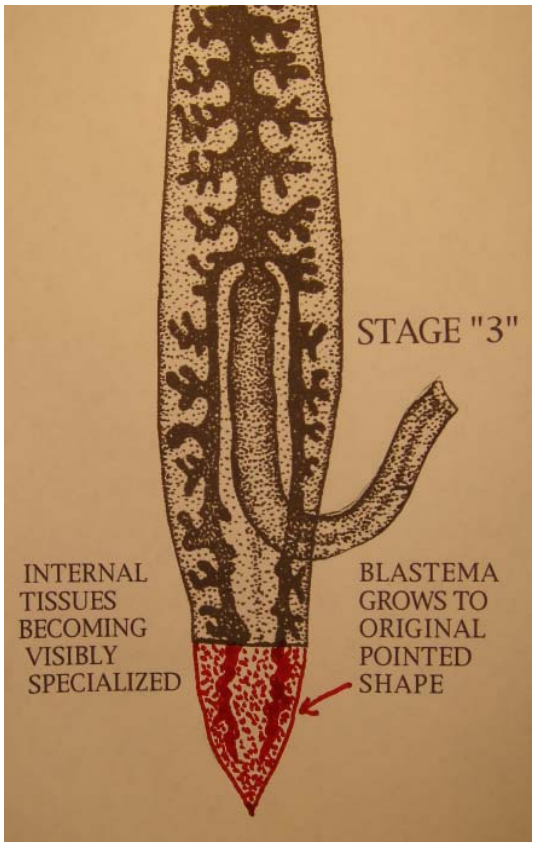

Figure 10 Stage 3 Worm Figure. In a Stage 3 flatworm, the blastema has grown to the original pointed shape and the internal tissues have become visibly specialized as shown in this figure.

Figure 11 Stage 3 Worm Picture. In a Stage 3 worm, the blastema has grown to the original pointed shape and the internal tissues have become visibly specialized as shown in this figure.

CHAPTER 3

RESULTS

Preliminary Experiment

The results for the preliminary experiment are tabulated in Table 1.

Table 1 Number of Flatworms Alive

	Number of Flatworms Alive			
Solution	Day 0	Day 1	Day 2	Day 3
Control	10	10	10	9
0.1 M Caffeine	11	0	0	0
0.01 M Caffeine	10	1	0	0
0.001 M Caffeine	10	4	4	4
0.0001 M Caffeine	10	6	6	5
0.00001 M Caffeine	10	10	10	10
1% ETOH	11	6	4	3
0.1% ETOH	11	9	9	9
0.01% ETOH	11	9	9	9
0.001% ETOH	10	9	9	9
0.0001% ETOH	10	10	10	10

The findings for the preliminary experiment indicated that the lowest concentration of caffeine (0.00001M) would be the working concentration of caffeine. 0.01% ETOH was determined to be the working concentration of alcohol for the main experiment. These concentrations showed an effect on the worms but did not kill them.

The results are also plotted in graphical form (see next page). (Figure 12 shows the effects of caffeine compared to the control and Figure 13 shows the effects of ethanol compared to the control.) The t-test determined that none of the groups was statistically different from the others.

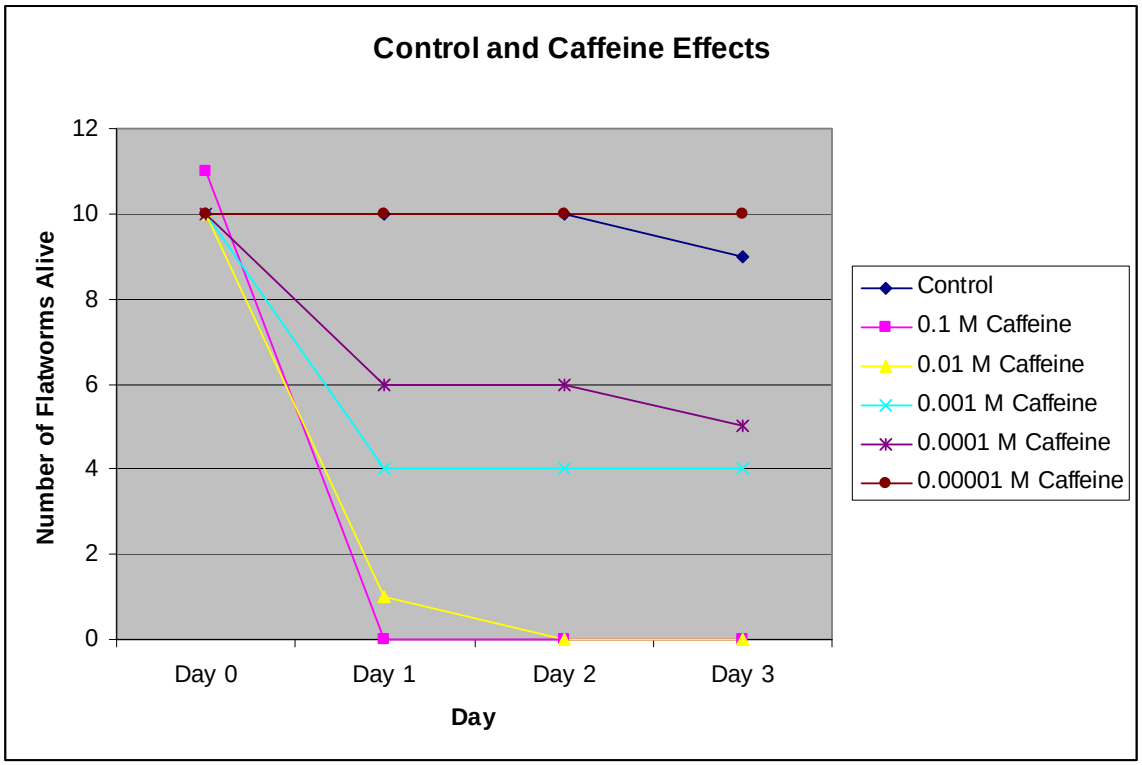

Figure 12 Control vs. Caffeine Effects in Preliminary Experiment. The effects of the caffeine solutions were compared to the control solution. The lowest concentration of caffeine (0.00001M) was determined to be the suitable working concentration for the main experiment because it was shown to be a non-toxic concentration.

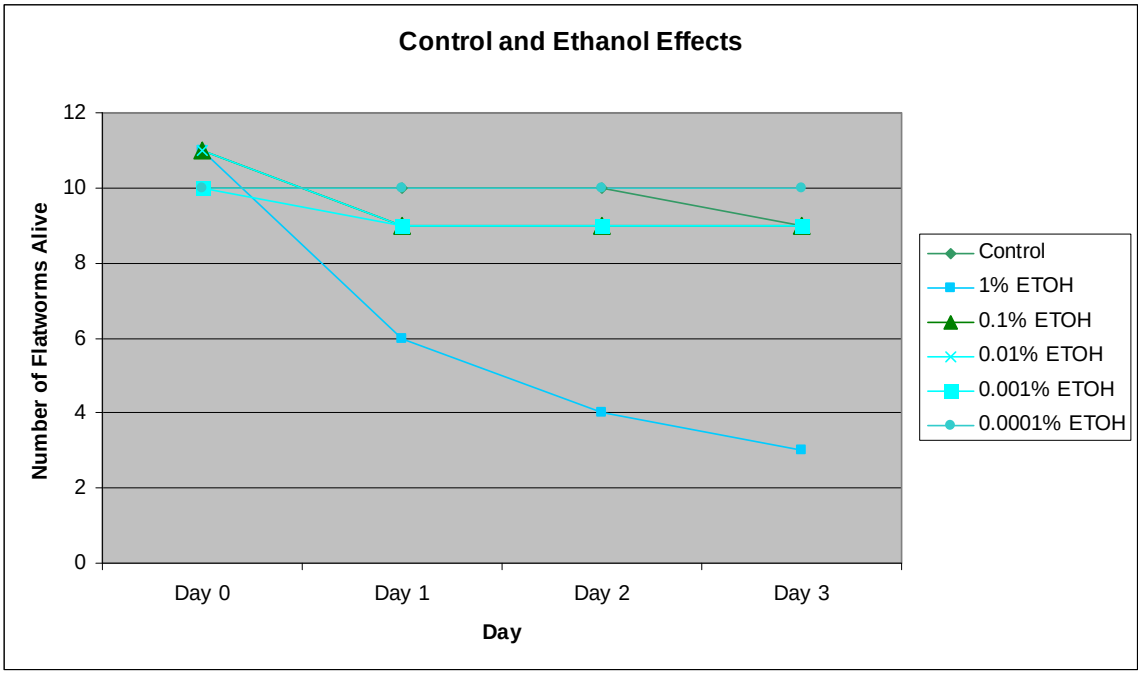

Figure 13 Control vs. Ethanol Effects in Preliminary Experiment. The effects of the ethanol solutions were compared to the control solution. The 0.01% ETOH solution was determined to be the suitable working concentration for the main experiment because it was shown to be a non-toxic concentration.

Main Experiment

The results of the worm survival data for the main experiment are tabulated into Table 2 and shown graphically in Figure 14. The increase in number of flatworms alive in some of the solutions was due to splitting, which is the process by which flatworms reproduce.

Table 2 Number of Surviving Flatworms

Solution	Day 0	Day 2	Day 3	Day 5	Day 6	Day 7	Day 9	Day 11	Day 12	Day 15
Water (no cut)	29	28	28	29	29	29	29	29	29	29
Water (cut)	42	42	42	42	42	42	42	43	43	43
0.01%EtOH (cut)	40	36	36	36	36	36	36	36	36	37
0.00001M Caffeine (cut)	35	35	35	34	34	34	34	32	32	33
Combined Soln (cut)	28	28	28	28	28	27	27	30	30	26

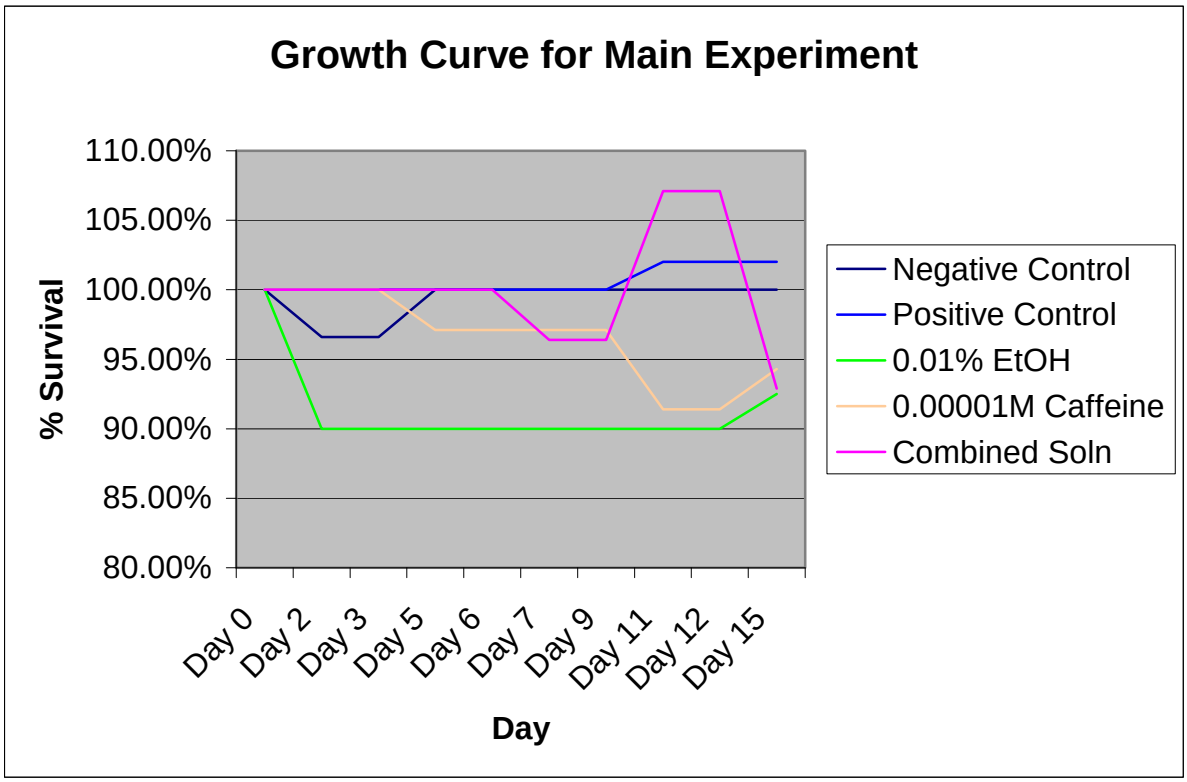

Figure 14 Growth Curve for the Main Experiment. The growth curve for the main experiment shows the survival of the different solutions compared to the control solutions.

The mean score for regeneration results are tabulated in Table 3 and show graphically in Figure 15.

Table 3 Mean Scores

Solutions	Day 0	Day 3	Day 6	Day 9	Day 12	Day 15
Control	0	1.2	1.86	2.38	2.57	2.57
EtOH	0	0.2	1.28	1.92	2.47	2.61
Caffeine	0	1.06	2.08	2.09	2.28	2.41
Combination	0	1.04	2.11	2.22	2.47	2.69

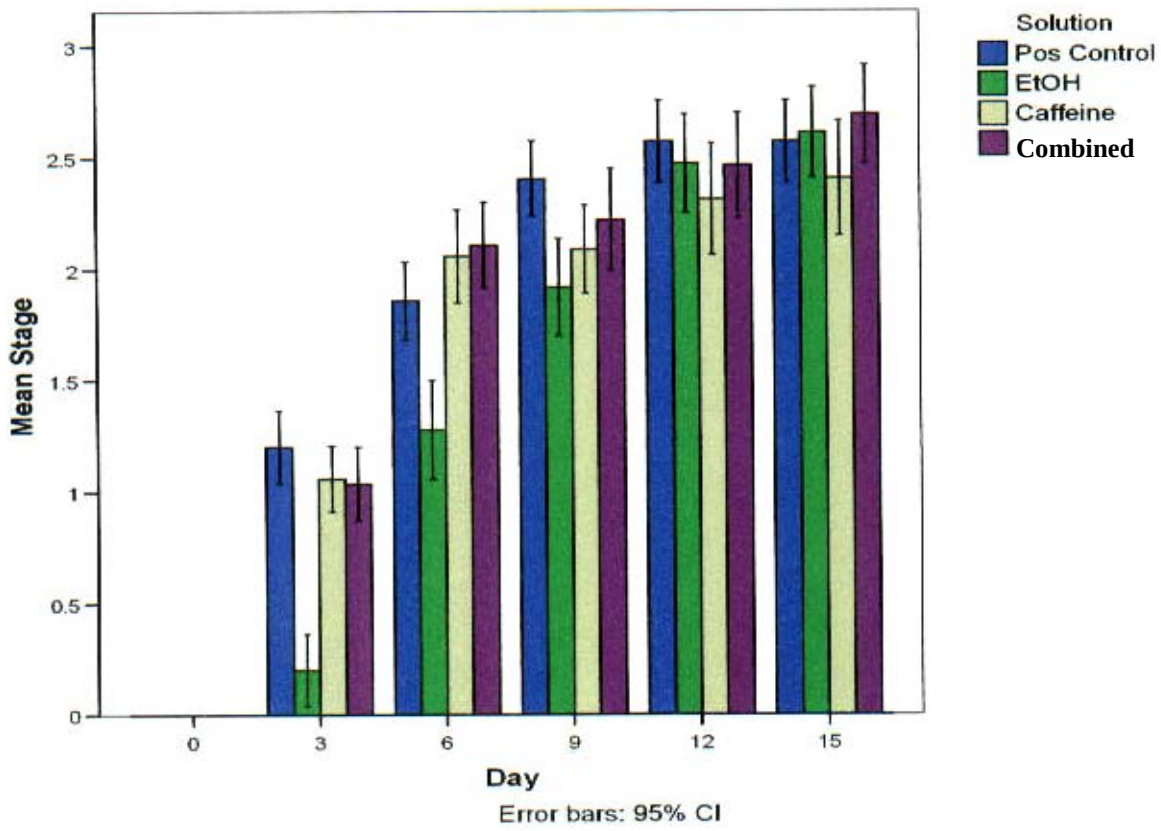

Figure 15 Mean Scores for the Main Experiment. The mean scores for the main experiment show the different effects of the solutions compared to the positive control and the error bars for each solution.

The data for day 3, with a P value of 1.47×10^{-13}, and day 6, with a P value of 4.66×10^{-7}, were found to be statistically significant by using the Kruskal-Wallis test. After the Kruskal-Wallis test determined which days were statistically significant, the Mann-Whitney test was used to determine if there was differences between the treatment groups. For day 3, there was a significant difference between the control and ethanol solutions, the ethanol and caffeine solutions, and the ethanol and the combined solutions all with a P value of < 0.0001. For day 6, there was a significant difference between the control and ethanol solutions (P value=0.0005) and between the ethanol and caffeine solutions and ethanol and combined solutions with a P value of < 0.0001.

The biometric analysis showed a statistically significant difference on day 3 and day 6 between ethanol and the other three treatments; the control treatment, the caffeine treatment, and the combined treatment. So, the regeneration in the ethanol treatment group of worms was significantly lower than the other three treatments on both day 3 and day 6. The ethanol treatment showed a retarded growth initially but after day 6, the regeneration in the ethanol treatment caught up to regeneration in the other treatments. There were no significant differences between the regeneration in the treatments on day 9, day 12, and day 15. Throughout the experiment, the caffeine treatment and the combined treatment did not have significant effects on the regeneration of the flatworms compared to the control. So, the stimulating effects of caffeine did not speed up the regeneration significantly. The combined solution, which contained caffeine and ethanol, did not have the antagonistic effect that was predicted.

CHAPTER 4

DISCUSSION

The results of this study showed that ethanol had an initial delayed effect on the regeneration of the flatworms. This delay could be caused by a variety of factors. Identification of exactly what caused this delay was not possible. One explanation of this delay is that the flatworms might have acclimated to the ethanol in the first 6 days, followed by normal development and then continued to develop normally. The ethanol treatment had a statistically significant effect on flatworm regeneration compared to the other treatments on day 3 and day 6 of this experiment. The caffeine treatment and the combined treatments had no statistically significant effects on the regeneration throughout the experiment. The regeneration in the combined treatment was similar to the control and the caffeine treatment. There seemed to be a protective effect seen from caffeine in the combined treatment. The caffeine in the combined treatment seemed to protect the worm from the delayed effect that ethanol produced in the ethanol treatment.

Knowing how caffeine and ethanol affect regeneration can help in the understanding of how these drugs could affect fetal development and the effect on stem cells. Caffeine and ethanol are commonly used drugs and could be used by pregnant women leading to adverse effects to the fetus. The flatworm is a model organism and the results of this study can be compared to how these drugs would affect fetal development and the effect on stem cells. From this study, we conclude that ethanol has a delayed effect on regeneration, which could mean that there could be a delayed effect on the development of a human fetus. The delay that was seen in the first 6 days of this experiment in the ethanol treatment did not affect the overall regeneration in this

treatment. The worms in the ethanol treatment regenerated normally after this delay. In fetal development this delay could be during an important time in development when the central nervous system, the brain, major organs, and other important features start to develop. So, this delay could cause serious problems in the fetus. The protective effect seen in the combined treatment in this study suggests that if you drink caffeine to offset the delayed effects of ethanol, there would be no effect on growth or development.

Although this is what happened in this study of flatworms, the extension to pregnant women and fetal development should not be taken literally. The flatworm is just a model organism that helps us gain insight into what might happen in fetal development. The results of this study would not correlate directly to fetal development. The effects that ethanol has been shown to have on fetal development would not be worth the risk of trying to see in the fetus the results that were seen in this study. Further research of the effect of caffeine and ethanol could be beneficial in order to learn more about the mechanisms by which these drugs affect development.

Fetal alcohol syndrome (FAS) affects 9.1 in 1000 births in America and is characterized by facial and growth abnormalities and can severely impact the central nervous system (Green 2007). The specific facial deformities associated with FAS are a smooth philtrum (the vertical ridge between the nose and upper lip), a thin vermilian border (a thin upper lip), and small palpebral fissures (small slanted eyes) (Morantz 2006). Other characteristics of children with FAS are that they have a small head circumference, are underweight, and have a short stature (Morantz 2006). Intrauterine exposure to alcohol can greatly affect the development of the brain and these effects can range from mild to severe mental retardation (Green et al. 2007). The risk of

spontaneous abortions doubles in women who drink 30mL of alcohol twice a week (Itthagarun et al. 2007). Difficulties in planning, organizing, and attention are seen in people who have FAS and they often have memory deficits (Green 2007). Psychiatric disorders such as depression, anxiety disorder, obsessive-compulsive disorder, and bipolar disorder can be a result of prenatal alcohol exposure.

Caffeine readily crosses the placental membrane and the enzyme responsible for its degradation is absent in the fetus and neonate (Christiani and Brent 2001). Due to the absence of this enzyme, caffeine has been researched as a possible hazard during pregnancy that could cause fetal growth retardation, increased likelihood of spontaneous abortion, congenital malformations, preterm delivery, intrauterine growth retardation, sudden infant death syndrome, still birth, and infant death during the first year of life (Dlugosz and Bracken 1992). The half-life of caffeine in pregnant women is "increased to 10 hours at 17 weeks of gestation and 18 hours in the third trimester" (Bracken et al. 2003). Alteration of cellular development due to inhibition of phosphodiesterase, which increases levels of cyclic adenosine monophosphate, is an effect of caffeine, and this may lead to modification in hormone expression in both the mother and the fetus (Dlugosz and Bracken 1992).

Caffeine has been investigated in numerous studies to evaluate its effect on low birth weight, spontaneous abortion, sudden infant death syndrome, and congenital malformations. There are paradoxical results from these studies. Some studies show that caffeine intake during pregnancy increases the risk of these birth defects but other studies found no association between caffeine intake and birth defects. In several rodent studies, high doses of caffeine have been seen to cause fetal resportion, low birth weight,

miscarriage, malformations, and stillbirth. Although these rodent studies cannot be extrapolated to humans because the pharmacokinetics (degradation of the xenobiotic agent by the body) is different in humans and animals (Fenster et al. 1991, Pastore and Savitz 1995). Further research is needed in order to determine exactly how caffeine affects regeneration and development in animals and humans and how they are related to one another.

Continued research into regeneration in flatworms can help us learn more about the effects of other drugs and compounds on stem cells. Some of the drugs that could be used in regeneration experiments could be alkaloids other than caffeine such as nicotine, cocaine, morphine, and codeine. These alkaloids have different effects; nicotine and cocaine are stimulants and morphine and codeine are analgesics.

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