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The Effects of Betadine®, Polyvinylpyrrolidone (PVP), and Iodine on Regeneration in the Planarian *Dugesia tigrina*.

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The Effects of Betadine®, Polyvinylpyrrolidone (PVP), and Iodine on Regeneration in the Planarian *Dugesia tigrina*

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by

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ABSTRACT

The Effects of Betadine®, Polyvinylpyrrolidone (PVP), and Iodine on Regeneration in the Planarian *Dugesia tigrina*

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The freshwater planarian has a great capacity to regenerate and is an ideal animal model in the study of stem cell and regeneration biology. In this study planarian regenerating new tails were exposed to nonlethal doses of Betadine®, Polyvinylpyrrolidone (PVP), and Iodine. Betadine® is a topical antiseptic commonly used in the healthcare setting and may have a detrimental effect on wound healing. PVP is linked to iodine to create povidone-iodine, the active ingredient in Betadine®. Initially, a preliminary test was performed on the worms to determine the nonlethal concentrations of these chemicals . After this nonlethal concentration was determined, tails were amputated and a designated number of worms were placed in the Betadine, PVP, and Iodine solutions. Spring water was used as a control. This study determined that Betadine® and PVP showed no significant impact on regrowth rate and wound healing in the planarian, while Iodine did.

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CHAPTER 1

INTRODUCTION

Planarians are the simplest metazoans in which regeneration occurs and, as an experimental specimen, they are relatively easy to manipulate (Alvarado 2006). These flatworms belong to the phylum Platyhelminthes, a clade of about 50,000 species. This clade also includes the Lophotrochozoans (annelids and mollusks) and is a sister group to the Ecdysoza which includes arthropods and nematodes (Salo 2006).

Planarians average 2-8 mm in length and inhabit running freshwater communities (Kiefer 2006). Their primary food source includes insects, insect larvae, and other invertebrates. Planarians possess derivatives of all three germ layers: ectoderm, mesoderm, and endoderm (Reddien and Alvarado 2004). They are also among the simplest organisms to have bilateral symmetry and tissues with distinct organs, a characteristic that planarians share with chordates, arthropods, worms, and mollusks (Newmark and Alvarado 2002).

The body wall musculature of planarians contains longitudinal, diagonal, and circular muscle fibers that are used for negotiating obstacles. Locomotion is aided by mucous producing subepithelial gland cells. These cells also play a role in protection, adhesion, and prey capture (Newmark and Alvarado 2002; Reddien and Alvarado 2004). Food is ingested through a muscular extensible pharynx that serves not only as a mouth but also as an anus. Planarians lack a coelom (organ containing cavity). They also lack respiratory and circulatory systems (Reddien and Alvarado 2004). Oxygen is obtained via transdermal diffusion. Their excretory system consists of a network of flame cells with tufts of beating cilia that filter waste material into the system; osmoregulation also aides in removal of waste products. The planarian nervous system centers around a bilobed cerebral ganglia located at the anterior end of the organism with 2

longitudinal nerve cords underlying the ventral body wall musculature. Photoreceptors and chemoreceptors are also located at the anterior end and are responsible for generating direct responses. Planarians have a pair of eyes in the anterior portion of the body that consist of black pigment cells and visual neurons. Finally, planarians can reproduce sexually or asexually via fission (Newmark and Alvarado 2002; Agata 2003).

For over 100 years, scientists have been fascinated with the study of planarians. The first description of planarian regeneration was published in 1766 (Newmark and Alvarado 2002). During the early 20th century, T.H. Morgan and Charles Manner Child published numerous papers detailing planarian biology and thus laid the foundation for the modern scientific investigation into regeneration (Morgan 1934; Alvarado 2003). Harriet Randolph carried out important research on the migration of the neoblast in 1897 (Brondsted 1969). However, during the middle of the 20th century, a change in research priorities saw the study of planarian regeneration decline. Many scientists were looking at organisms more amenable to genetic analysis like *Drosophila* and *Caenorrhabditis elegans* (Salo 2006). However, the study of planarians and their regenerative abilities have made a comeback in recent decades. This is due in part to the fact that model systems such as *Drosophila* and *Caenorrhabditis* have limited or no regenerative ability (Newmark and Alvarado 2002). Unlike planaria, Urodele amphibians, which are also commonly used in regeneration studies, take up to a month to complete regeneration.

The ramifications of improving human health have motivated the recent surge of interest in planarians. Also, the study of these simple creatures offers insights into the basic questions in stem cell biology and regenerative medicine. Free-living planarians and mammals possess similar biochemical and physiologic organization such as using some of the same neurotransmitters and having sensitivity to similar toxins. Therefore, some researchers consider

planarians a good system for the study of teratogens (Best and Morita 1982). In addition, regeneration studies provide a largely unexplored area for understanding molecular and cellular processes of stem cells (Alvarado 2006).

Regeneration can be defined as "….the ability of the fully developed organism to replace lost fragments by growth or remodeling of somatic tissue (Salo 2006)." Most asexually reproducing invertebrates are renewal organisms and can undergo some degree of regeneration. For these invertebrates, regeneration is directly related to survival and represents an essential part of their life cycles (Grant 1978). In humans regeneration exists but in a more subdued form. It is true that humans cannot regrow a lost arm or leg, but many of the tissues in the human body can undergo regeneration when injured; and is the mechanism behind human wound healing. Liver cells have great regenerative power, with each cell undergoing mitosis until healing is complete. For example, 70% of a rat's liver can be removed and still be regenerated. Connective tissues such as bone, tendons, bone marrow, and fibroblasts can regenerate, as can muscular tissue in a limited capacity (Majno and Joris 2004).

Like embryogenesis, regeneration involves the assembly of newly made tissues. On the other hand, regeneration differs from embryogenesis in that it also involves the integration of newly made parts into older pre-existing tissues (Alvarado 2006). The driving force behind regeneration is the presence of stem cells, which individually, or as a population, can produce both differentiated progeny and reproduce themselves (Slack 1991). Stem cells can be described as being totipotent or pluripotent. Totipotent cells are able to contribute to the production of all tissue types, while pluripotency describes the ability of the cell to contribute to only certain tissue types in the developing organism (Newmark and Alvarado 2002). Planarian stem cells are able to respond to environmental factors such as nutrient status by undergoing cell cycle arrest or

activation (Alvarado and Kang 2005). Despite the recent increase in stem cell research, little is known about the molecular mechanisms by which stem cells work. Planarians possess a large and experimentally accessible population of stem cells (Alvarado 2007). The great therapeutic potential of stem cells for curing degenerative disease and repairing injuries, as well as gaining a further understanding of stem cell behavior *in vivo*, makes planarian regenerative studies a worthwhile endeavor.

The undifferentiated stem cells found in planarians are called neoblast cells (Subramoniam 2002). Neoblast cells are small, about 5-10 μm in diameter, and are embedded in the parenchyma (mesodermal tissue) that fills the space between the epidermis and the gut of the worm (Rossio et al. 2008). They constitute 20-30% of the total number of cells in the planarian body. With the exception of spermatogonia and oogonia, all cells that exhibit mitotic division in the planarian body are defined as neoblasts (Newmark and Alvarado 2002; Subramoniam 2002). The division progeny of the neoblasts generate the approximately 40 different cell types found in adults, including germ cells (Ladurner et al. 2000 ; Alvarado 2006). The normal everyday function of the neoblast cells is to regenerate replacement cells for those lost during daily physiological turnover (Newmark and Alvarado 2002). However, if a serious injury occurs such as an amputation of the head or tail, the neoblast cells differentiate into new structures after accumulating in the blastema (Subramoniam 2002).

Regeneration consists of 2 events in the planarian. After the initial cut, a thin layer of epithelium will cover the wound within 30 minutes (Reddien and Alvarado 2004). Groups of undifferentiated cells, neoblast cells, occur here and give rise to what is called a regeneration blastema (Subramoniam 2002). The source of these cells is not completely known, but 2 different hypotheses have been proposed. The dedifferentiation hypothesis states that cells in the

blastema are derived from dedifferentiation of cells in the wound vicinity. On the other hand, the neoblast hypothesis supposes that the neoblasts are undifferentiated cells (totipotent) and proliferate in response to injury (Newmark and Alvarado 2002). Planarians can restore bilateral symmetry even when cut into irregularly shaped fragments. A small fragment of a planarian (4000 to 10,000 cells) will regenerate a complete individual with all needed structures in the correct proportions in about 7-15 days (Egger et al. 2006 ; Reddien et al. 2007; Gonzales-Estevez et al. 2007).

Recently, strides have been taken in the advancement of planarian regeneration studies. Many of these studies focus on the neoblast cells themselves. BrdU (bromodeoxyuridine) labeling has been used to study the distribution, migration, and differentiation of neoblast cells (Newmark and Alvarado 2000). With BrdU labeling, up to 45 hours of regeneration have been traced, including differentiation of the neoblasts into epithelial, muscle, neuron, rhabite, and flame cells (Salo 2002). Along with anti-phos-H3 double labeling, researchers have determined that almost all mitotic cells are located along the lateral sides of planarians and that this is independent of body size (Nimeth et al .2004). Also, it has been demonstrated that neoblast movement is not passive but migratory (Newmark and Alvarado 2000).

Electron microscopic studies have revealed relevent morphological characteristics of neoblasts. They were found to be smaller than any other differentiated cells, with a high nucleus/ cytoplasm ratio and ovoid in shape. Also, the cytoplasm was shown to include large numbers of free ribosomes, mitochondria, and structures called chromatoid bodies that resemble germ granules of other animals (Sato et al. 2006).

In this study the effects of Betadine® , PVP, and Iodine on wound healing and regeneration were assessed. The antibacterial properties of the halogen iodine have been used

for over 150 years (Durani 2008). Iodine is only slightly soluble in water but very soluble in many organic solvents and iodine solutions. The many applications of iodine include animal feed supplement, dye/ink manufacture, iodized table salt, sanitation, and cleaning compounds. Pharmaceutical manufacturers use iodine in expectorant compounds, X-ray contrast, and most commonly in antiseptics (Iodine 2009).

When iodine is linked to PVP, it creates a water-soluble, chemically stable, nonirritating solution (Gilmore 1977). This combination is known as povidone-iodine. PVP is an inert, hydrophilic, synthetic polymer with low toxicity (Kramer 1999). It has a molecular weight of 111.142 g/mol and the formula C_6H_9NO (Information about PVP and Betadine 2009). Also, it is soluble in water, forming a colloidal solution (Budavari 1996). Due to these factors, PVP is commonly used in pharmaceuticals, biomedical sciences, food, beverages, and cosmetics, as well as a blood plasma extender/substitute. In food, it can be used as a binder for vitamin and mineral tablets (Adachi et al. 2003). Betadine® is frequently used in the healthcare setting as a topical antimicrobial and sporicidal solution used pre- and postoperatively (Kramer 1999). The active ingredient in commercially sold Betadine® is 10% povidone-iodine, with the inactive ingredients purified water and sodium hydroxide (Betadine® Microbicides 2008). It is especially attractive due to its broad antimicrobial spectrum with no known bacterial resistance (Goldenheim 1993).

Normally, there are 3 phases involved in wound healing. The inflammatory phase is initiated within 4 hours of trauma and begins with neutrophils recruited to the immediate area, killing bacteria. Macrophages digest the damaged tissue and recruit fibroblasts. During the proliferative phase, fibroblasts establish, maintain, and repair tissues by producing collagen. Granulation tissue fills the defect and epithelial cells migrate from the margins of the wound to aide in closure during the maturation phase (Kramer 1999; Lin 2003). Some wound treatments,

such as Betadine® may retard this healing regimen.

Research has shown that Betadine® when introduced directly into the wound has an inhibitory effect on normal wound healing. Betadine® at full strength (10%) has been shown to cause irritant dermatitis (Kara et al .2007). Successful tissue repair depends on the viability of the cell types involved, namely fibroblasts and kerotinocytes. Previous studies have demonstrated that cultures of human fibroblasts were killed by 10% povidone-iodine, and only at dilutions as low as 1:1000 did no toxicity occur (Lineweaver et al. 1985a ; Wilson et al. 2005). Further investigations have shown that povidone-iodine concentrations of greater than 0.05% were toxic to granulocytes and monocytes and 0.004% concentrations were toxic to keratinocytes (Burks 1998). Another study assessed the cytotoxicity of 4 common antiseptics: 1% povidone-iodine, 0.25% acetic acid, 3% hydrogen peroxide, and 0.5% sodium hypochlorate. All were shown to be cytotoxic and adversely affect wound healing in animal models (Lineaweaver et al. 1985b). In clinical studies, patients were treated with different antiseptics pre- and postsurgically. Betadine® (10% povidone-iodine) was applied to sub cutis and skin edges, and in 24% of the group wound healing was compromised (Kothuis 1981). Moreover, topically applied Betadine® was shown to increase inflammation in rat wounds (Aramwit and Sangcakul 2007). In 1 study, peritonitis was experimentally induced in dogs and 4 -8 ml/kg doses of 1% povidone-iodine solution caused 100% mortality (Lagarde et al. 1978). As for PVP, there is little evidence that it causes such detrimental effects. Several case reports detail incidences of allergic contact dermatitis to PVP, while repeated subcutaneous injections of PVP in aqueous solution to rats led to local sarcomas (N-vinyl-2-pyrrolidone and polyvinyl pyrrolidone 1987; Adachi et al. 2003; Quartier et al. 2006;).

In contrast to the studies demonstrating the harmfulness of using Betadine®, other

studies maintain that there is no noticeable detrimental effect (Oliveira and Santos 2007; Leaper and Durani 2008). One study found that a 1% solution was safe and still maintained the antimicrobial effect (Viljanto 1980). Another study analyzing rat, guinea pig, rabbit, and pig wounds concluded that there was no significant difference in healing between 1%, 5% , and 10% povidone-iodine solution (Goldenhein 1993). Additionally, povidone-iodine was shown to reduce mortality of mice and rats with bacterial peritonitis (Gilmore 1977). In human clinical trials, topical Betadine® treatment was shown to be effective in preventing the progression of skin ulcers (Daroczy 2006). Finally, the FDA reported that its studies did not indicate any deleterious effect of povidone-iodine on wound healing (Burks 1998).

The aim of this experiment was to determine if Betadine® or either of its main components has a negative effect on wound healing and regeneration in the planarian *Dugesia tigrina*. Based on other research, it may be inferred that the application of Betadine® directly on the amputated area of the worm may have a detrimental effect on healing by being toxic to the cells responsible for regeneration.

CHAPTER 2

MATERIALS AND METHODS

Preliminary Experiment

The preliminary experiment was a lethality test using the following solutions of Betadine®, PVP and Iodine: Betadine 1%, 0.1%, 0.01%, 0.001%, 0.0001%, 0.00001%; PVP 1g/100ml, 0.1g/100ml, 0.01g/100ml, 0.001g/100ml; and Iodine 0.01%, 0.001%, 0.0001%, 0.00001%, 0.000001%, 0.0000001%. The Betadine® was acquired from Purdue Pharma (Stamford, CT); PVP from the US Biochemical Corporation (Cleveland, OH); and the Iodine (0.1000 Normal) from the Hartman-Leddon Company (Philadelphia, PA). The purpose of the dose response experiments was to determine at what concentrations the Betadine®, PVP and Iodine were lethal to the flatworms and the highest concentration that still permits survival. The appropriate concentrations were used for the main experiment. The species *Dugesia tigrina,* a brown planarian acquired from Ward's Natural Science (Rochester, NY), was used for this experiment and approximately 245 worms were used.

The flatworms were placed in large, sterile, disposable, polystyrene petri dishes (100 x 15mm) with the ambient temperature maintained at 72˚F or below. Fifteen worms were placed in each of the 6 Betadine® solutions (with the exception of the 1% Betadine). Five worms were placed in the 1% Betadine® solution due to the expected lethality. Fifteen worms were placed in each of the 4 petri dishes of PVP; and 15 in each of the 6 petri dishes of Iodine. As a control, 15 worms were placed in a petri dish of natural spring water (see figure 1). The worms were observed daily for a period of 7 days and the number of survivors was recorded. The effect of the Betadine®, PVP and Iodine treatments was compared to the control. Analysis of the number of survivors provided the suitable concentration values for each treatment, which was necessary

for the study of the effects that these substances could have on regeneration and wound healing (see Tables 1-3; Figures 3-5).

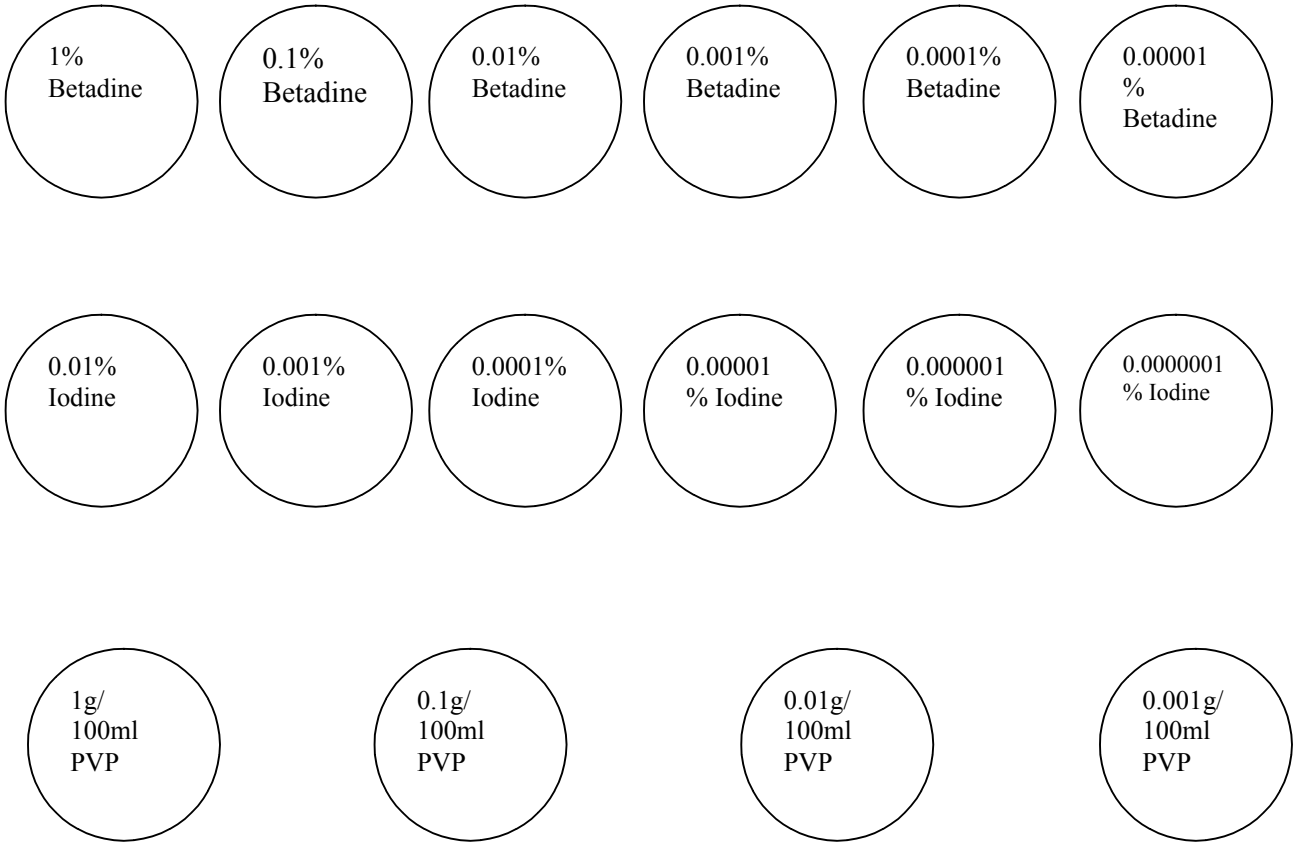

Figure 1.Preliminary Experiment Protocol

Main Experiment

By analyzing the number of survivors in each treatment solution of the preliminary experiment, appropriate nonlethal concentrations of Betadine®, PVP and Iodine were determined for the main experiment. The following treatment concentrations were used: 0.1% Betadine, 0.01g/100ml PVP, and 0.001% Iodine. Approximately 250 worms of the species *Dugesia tigrina* were used with 50 worms in each of the 5 petri dishes of treatment solution (see Figure 2).

The worms were slightly chilled in order to reduce movement. With a sterile scalpel, a section of the tail was amputated. The zone of amputation was located just behind the pharynx and was approximately the same for each worm. Amputated tails were discarded and the amputees were placed in 5 separate petri dishes, each containing the appropriate treatment concentration. Two controls were used; a positive control with cut worms in spring water and a negative control with uncut worms in spring water.

For a period of 15 days, the tail regeneration of the flatworms was observed. Every 3 days, the flatworms were fed a diet of macerated chicken livers. Furthermore, control spring water and test solutions were changed every 3 days. Ambient temperature was controlled at 72˚ F or below throughout the period.

At 3-day intervals, the flatworms were evaluated. The results were photographically recorded visually using a Minivid® digital photomicroscopic camera. At the end of the 15-day observation period, the tail photos representing the regeneration stages were evaluated and scored by a "blind" observer in order to get the best objective interpretation of the results. Each score represented the stage of regeneration (see Figures 16-19). To evaluate the degree of regeneration the stages were: stage 0 (day 0; no blastema formation), stage 1 (blastema cap

forming), stage 2 (rounded blastema), and stage 3 (complete regeneration; original tail structure) (Collins 2007).

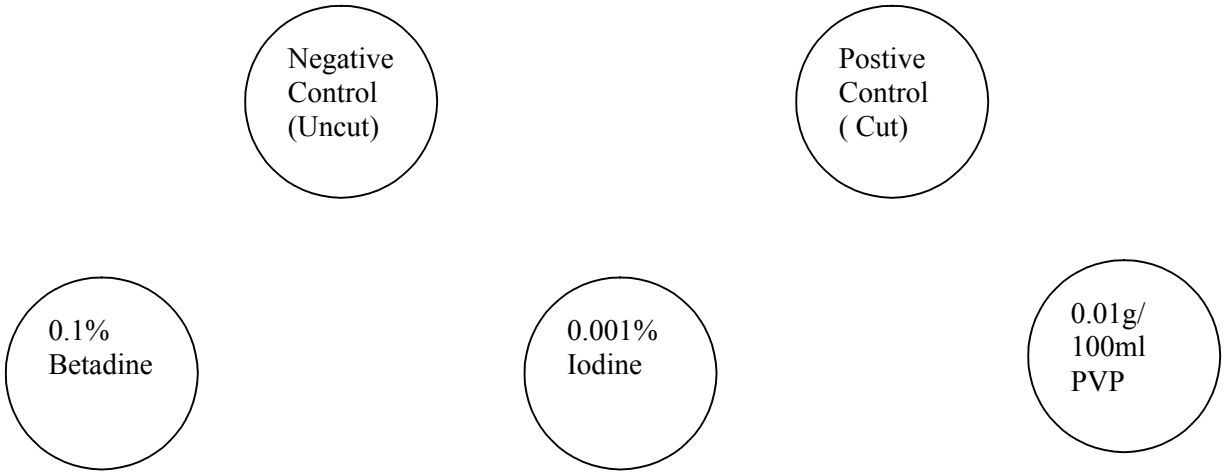

Figure 2. Main Experiment Protocol

Data Analysis

The data were analyzed statistically to determine how the Betadine®, PVP, and Iodine solutions affected regeneration in the planaria. Each of the solutions was compared to the spring water treatment to ascertain if any relationship exists between wound healing (regeneration) and treatment.

The Chi-Square test was used to statically analyze the data. This test is a nonparametric statistical technique that is used to determine if the distribution of observed frequencies is different from the expected frequencies. It uses nominal or categorical data (QMSS 2009; Mamahlodi 2006). The Chi-Square test was employed to determine if there exists a statistical difference between the treatments at each day interval. The statistical software used was Minitab.

CHAPTER 3

RESULTS

Preliminary Experiment

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

Table 1. Surviving Number of Flatworms in Varying Concentrations of Betadine® Solutions.

Solutions	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
Control	15	18	19	22	22	24	26	28
Betadine 1%	5	0	0	0	0	0	0	0
Betadine 0.1%	15	7	7	7	8	10	10	10
Betadine 0.01%	15	15	15	15	19	19	18	20
Betadine 0.001%	15	15	19	20	21	21	21	24
Betadine 0.0001%	15	15	20	22	23	24	29	31
Betadine 0.00001%	15	15	15	20	20	20	22	23

The findings for the preliminary experiment with Betadine indicate that the 0.1% solution would provide a high enough concentration to possibly show an effect but remain low enough to be nonlethal. Only 5 flatworms were initially used for the 1% Betadine solutions because of expected lethality. Furthermore, the day 7 counts were greater that day 0 because of reproduction (fission) by the flatworms.

Table 2. Surviving Number of Flatworms in Varying Concentrations of PVP Solutions.

Solutions	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
Control	15	18	19	22	22	24	26	28
PVP 1g/100ml	15	15	15	7	0	0	0	0
PVP 0.1g/100ml	16	16	16	16	14	14	14	12
PVP 0.01g/100ml	15	15	17	20	20	21	21	21
PVP 0.001g/100ml	15	15	18	19	19	19	23	23

The findings for the preliminary experiment with PVP indicate that the 0.01 g/100ml solution would provide a high enough concentration to possibly show an effect, but remain low enough to be nonlethal.

Table 3. Surviving Number of Flatworms in Varying Concentrations of Iodine Solutions.

Solutions	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
Control	15	15	15	15	15	15	15	15
Iodine 0.01%	15	0	0	0	0	0	0	0
Iodine 0.001%	15	15	15	15	15	15	16	16
Iodine 0.0001%	15	15	15	15	15	15	15	15
Iodine 0.00001%	15	15	15	15	15	15	15	15
Iodine 0.000001%	15	15	15	15	15	15	15	16
Iodine 0.0000001%	15	15	15	15	15	15	15	15

The findings for the preliminary experiment with Iodine indicated that the 0.001% solution would provide an effect while remaining nonlethal.

The results for the preliminary experiment have also been plotted graphically.

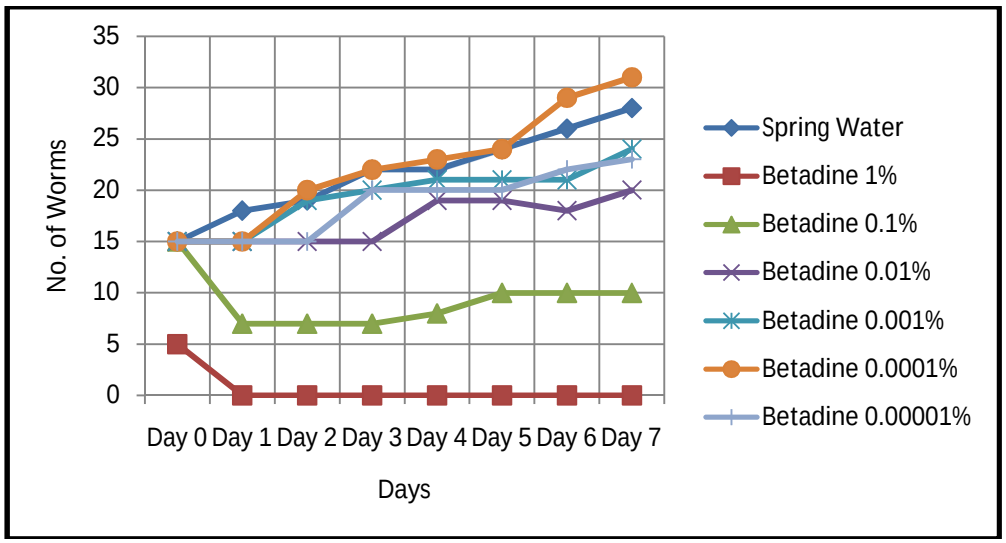

Figure 3. Daily Survival of Flatworms in Varying Concentrations of Betadine® for Preliminary Experiment.

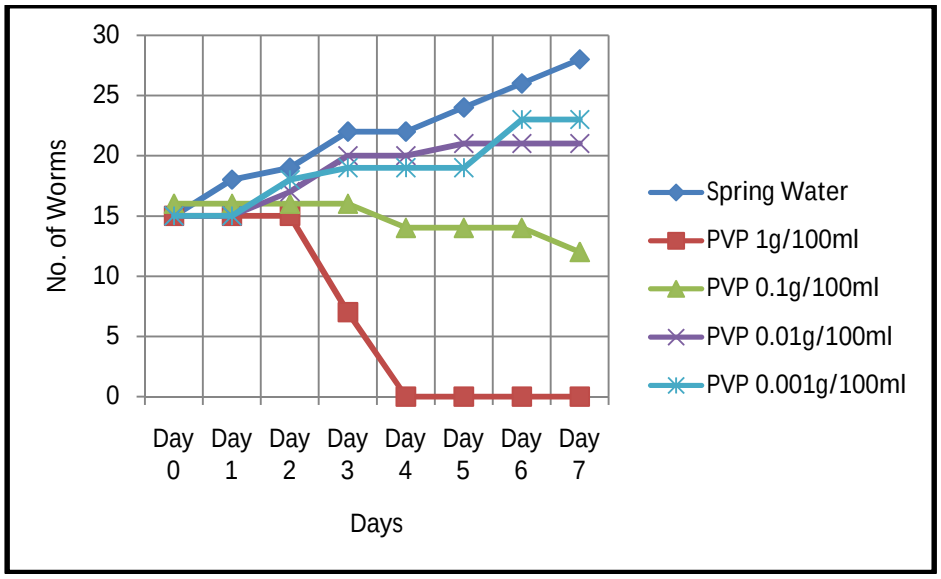

Figure 4. Daily Survival of Flatworms in Varying Concentrations of PVP for Preliminary Experiment.

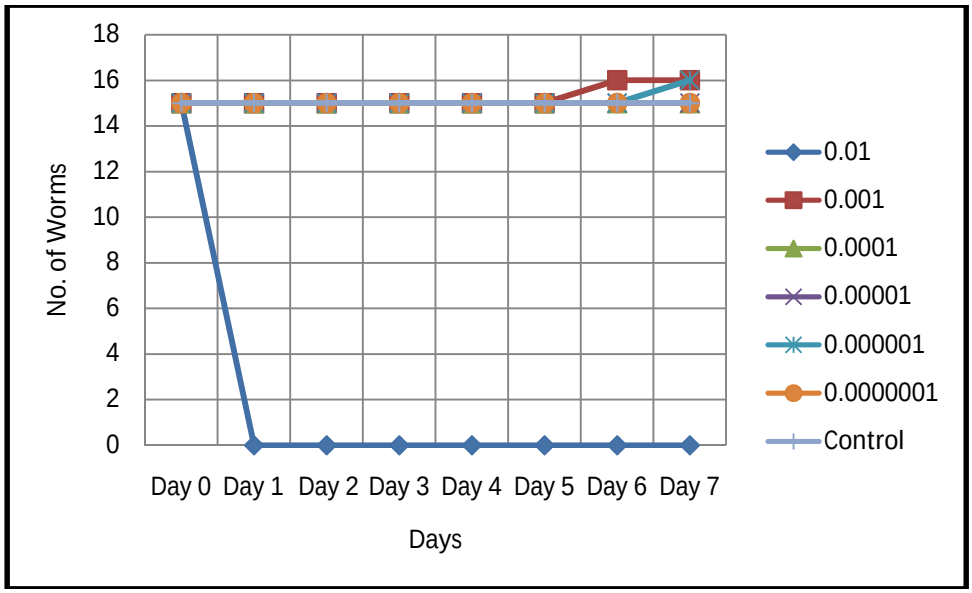

Figure 5. Daily Survival of Flatworms in Varying Concentrations of Iodine for Preliminary Experiment.

Main Experiment

The mean scores for regeneration results are presented in Table 4 and shown graphically in Figs.6-9.

Table 4. Mean Scores of Flatworm Regeneration

Solutions	Day 0	Day 3	Day 6	Day 9	Day 12	Day 15
Control	0.00	0.98	1.73	2.58	2.68	2.78
Betadine 0.1%	0.00	0.88	1.88	2.33	2.70	2.89
PVP 0.01g/100ml	0.00	0.81	1.70	2.41	2.68	2.80
Iodine 0.001%	0.00	0.96	1.48	2.12	2.35	2.42

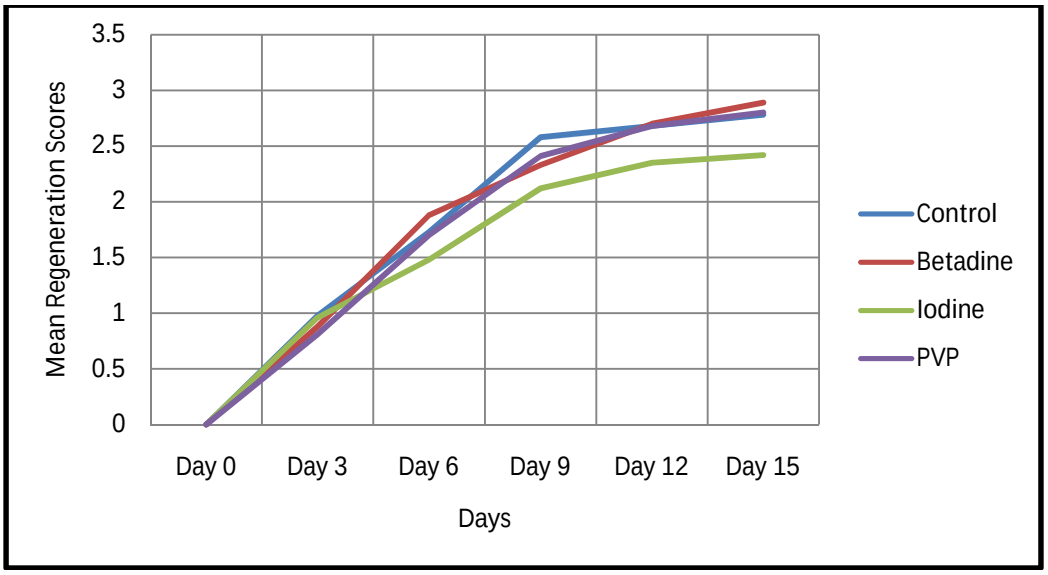

Figure 6. Mean Scores of Flatworm Regeneration for all Solutions

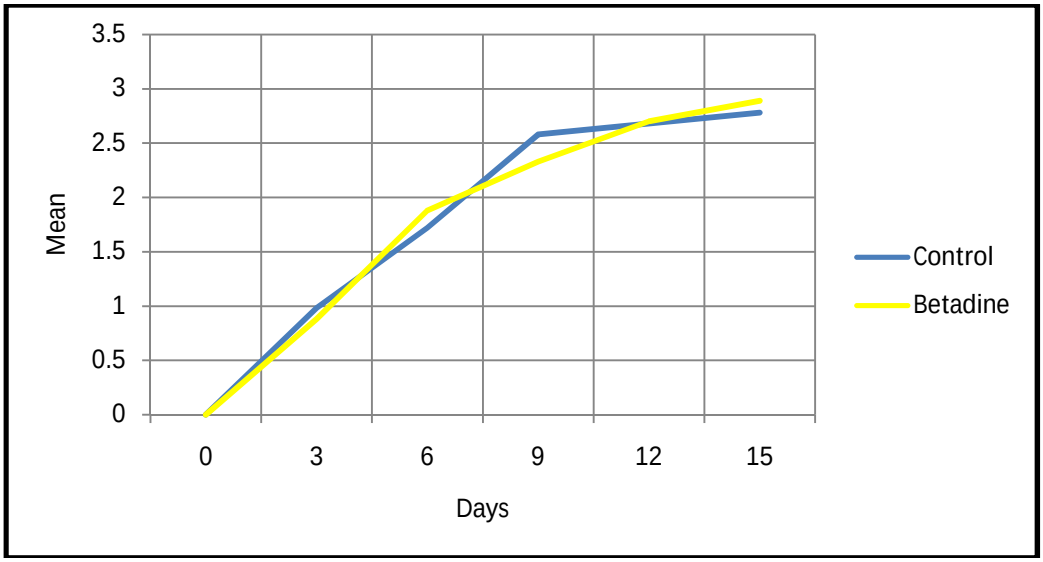

Figure 7. Mean Scores of Flatworm Regeneration for Control vs. 0.1% Betadine

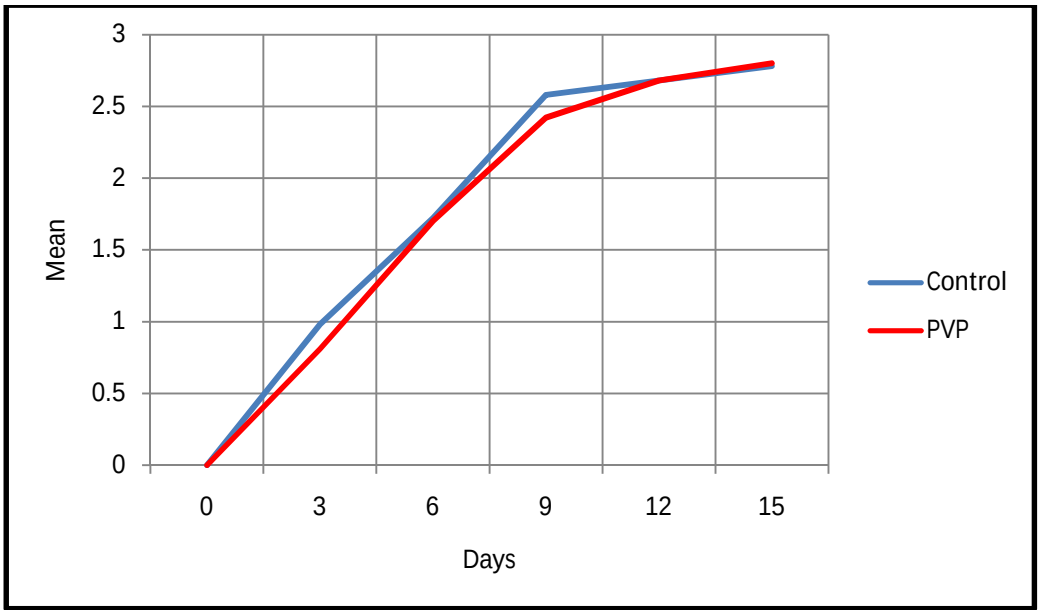

Figure 8. Mean Scores of Flatworm Regeneration for Control vs. 0.01g/100ml PVP

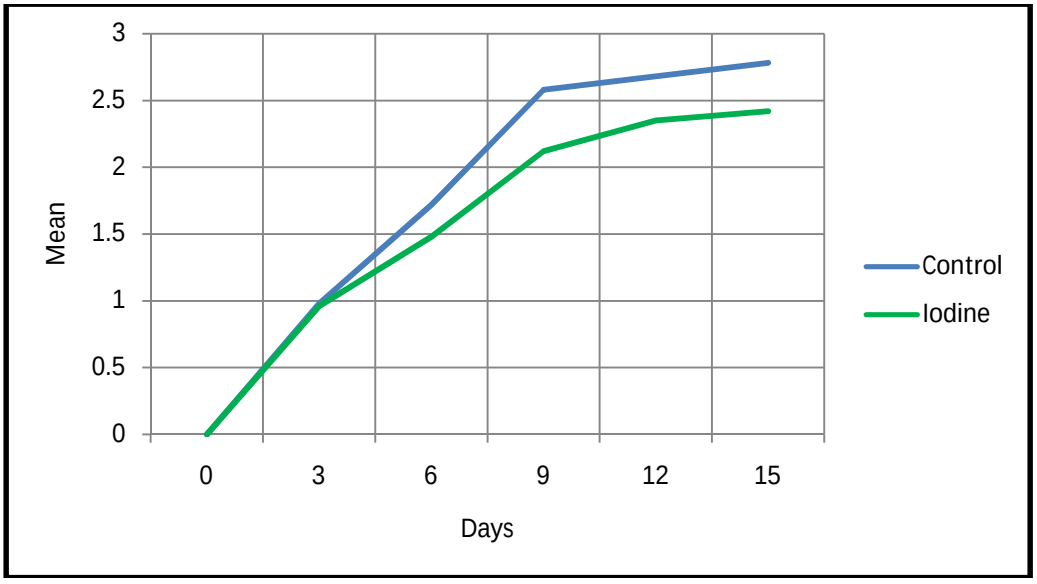

Figure 9. Mean Scores of Flatworm Regeneration for Control vs. 0.001% Iodine

The number of worms in each stage (stage 0- stage 3) are tabulated in Table 5.

Table 5. Number of Flatworms in Each Stage Per Day.

Day 0				
Solutions	Stage 0	Stage 1	Stage 2	Stage 3
Control	50	0	0	0
Betadine	50	0	0	0
PVP	50	0	0	0
Iodine	50	0	0	0
Day 3				
Solutions	Stage 0	Stage 1	Stage 2	Stage 3
Control	1	50	0	0
Betadine	6	44	0	0
PVP	13	30	4	0
Iodine	4	44	2	0
Day 6				
Solutions	Stage 0	Stage 1	Stage 2	Stage 3
Control	0	14	37	0
Betadine	0	6	44	0
PVP	0	15	30	1
Iodine	2	22	26	0
Day 9				
Solutions	Stage 0	Stage 1	Stage 2	Stage 3
Control	0	1	19	30
Betadine	0	1	31	17
PVP	0	2	22	21
Iodine	0	2	41	8
Day 12				
Solutions	Stage 0	Stage 1	Stage 2	Stage 3
Control	0	1	14	35
Betadine	0	0	15	35
PVP	0	0	15	32
Iodine	0	2	29	20
Day 15				
Solutions	Stage 0	Stage 1	Stage 2	Stage 3
Control	0	1	9	40
Betadine	0	0	5	40
PVP	0	2	5	39
Iodine	0	4	22	26

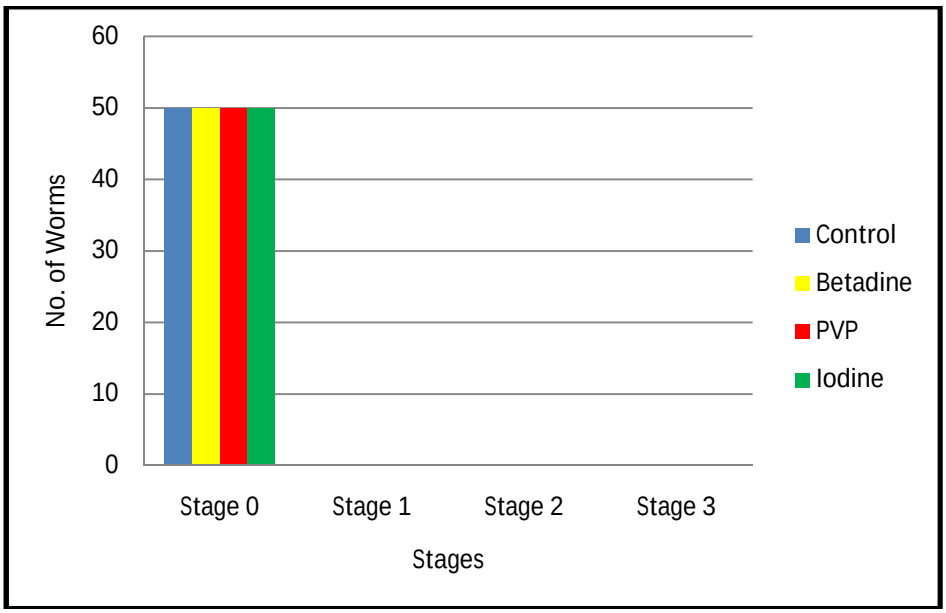

Figure 10. Number of Worms in Each Stage Day 0

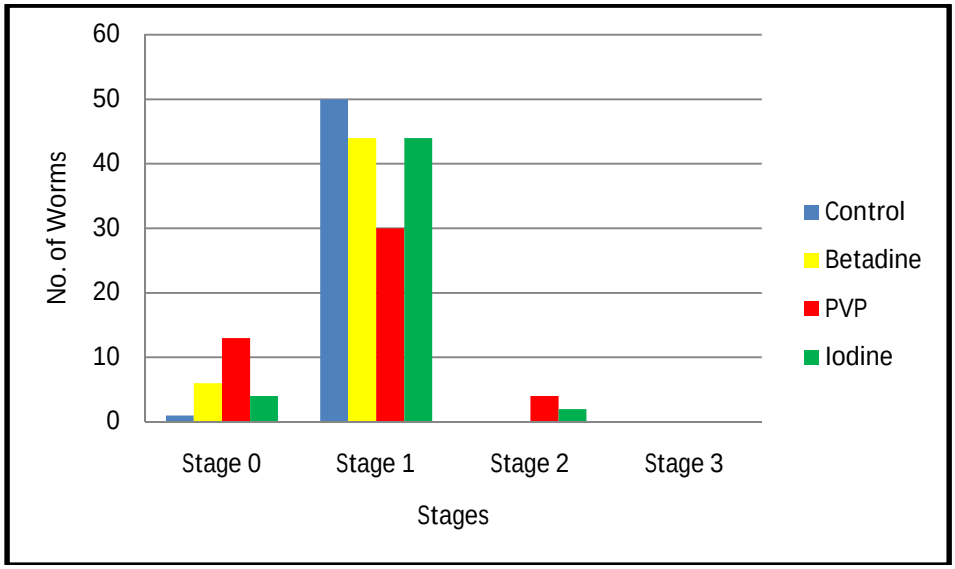

Figure 11. Number of Worms in Each Stage Day 3

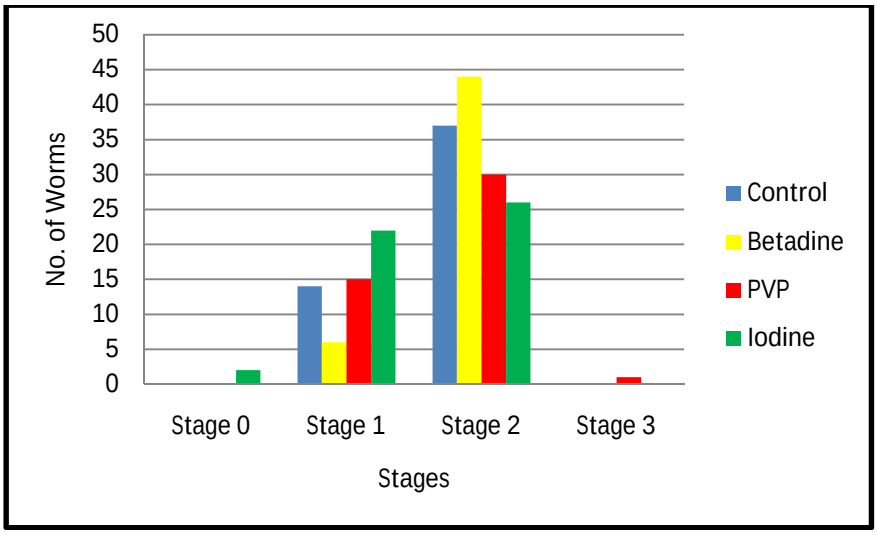

Figure 12. Number of Worms in Each Stage Day 6

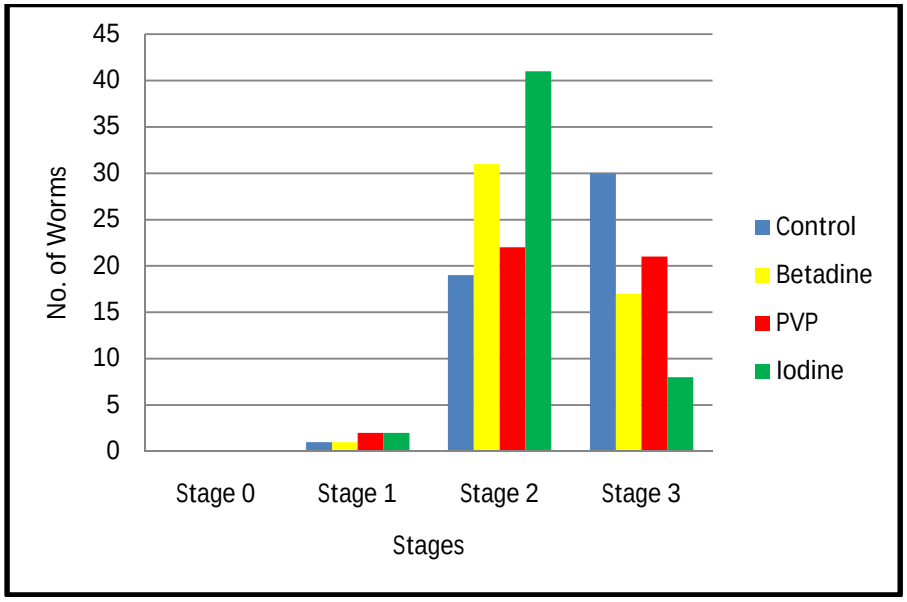

Figure 13. Number of Worms in Each Stage Day 9

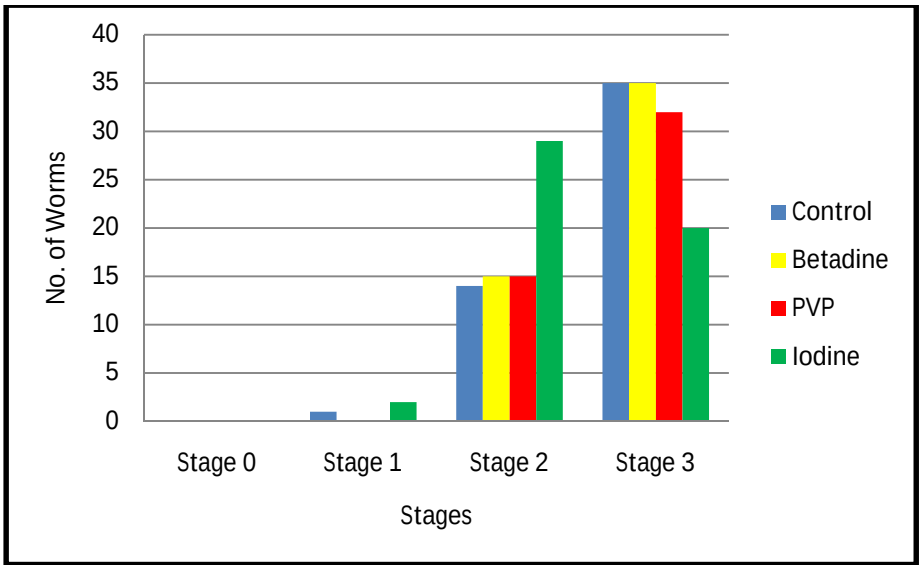

Figure 14. Number of Worms in Each Stage Day 12

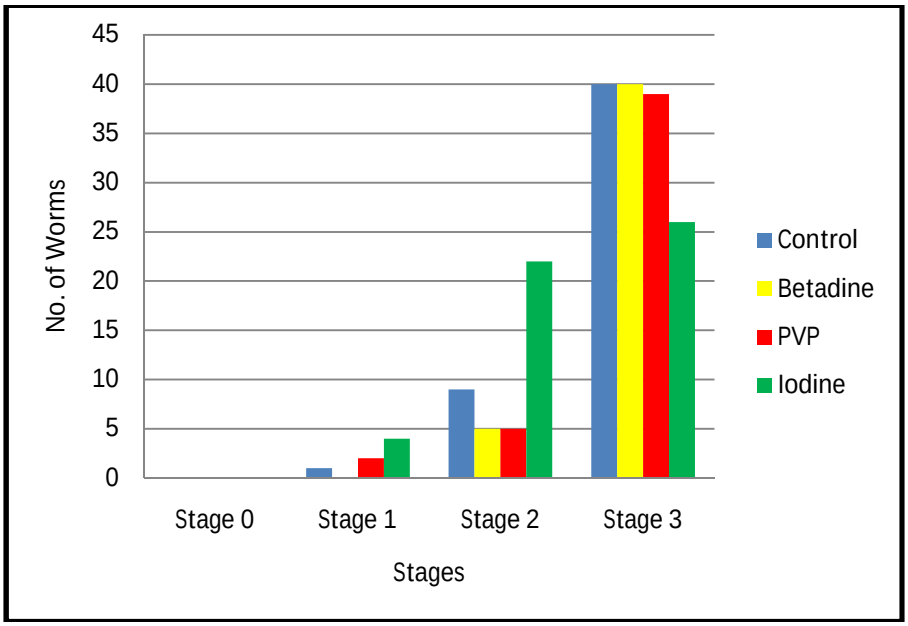

Figure 15. Number of Worms in Each Stage Day 15

Figure 16. Stage Zero (0) Worm Picture. On Day 0, the flatworms for the positive control, Betadine, PVP, and Iodine treatments were cut just behind the eversible pharynx. This allows the worms to eat and eliminate waste. There is no blastema regrowth.

Figure 17. Stage One (1) Worm Picture. Blastema cap at the cut surface is evident.

Figure 18. Stage Two (2) Worm Picture. The blastema has formed a rounded shape.

Figure 19. Stage Three (3) Worm Picture. The blastema has grown to the original pointed shape.

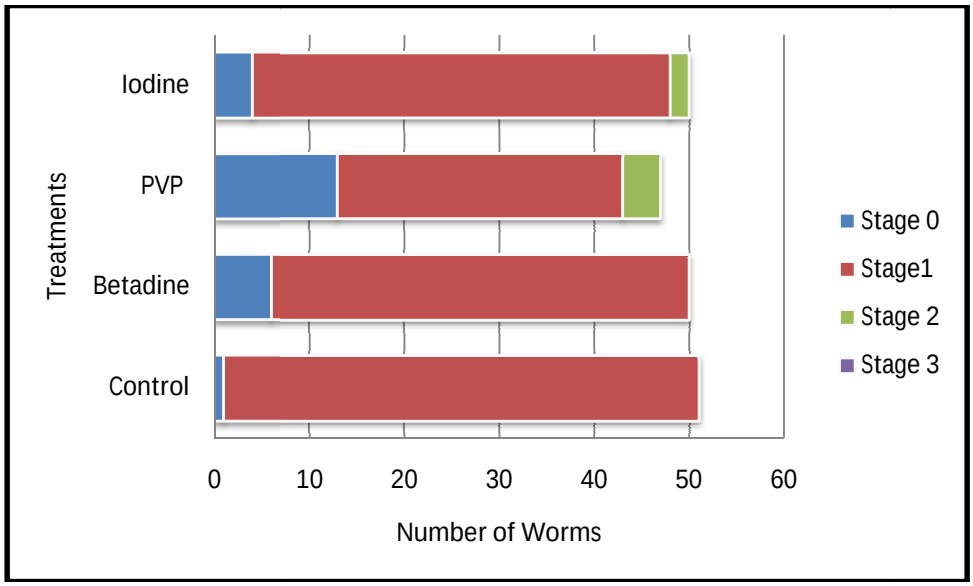

Figure 20. Number of Worms in Each Stage Per Treatment for Day 3

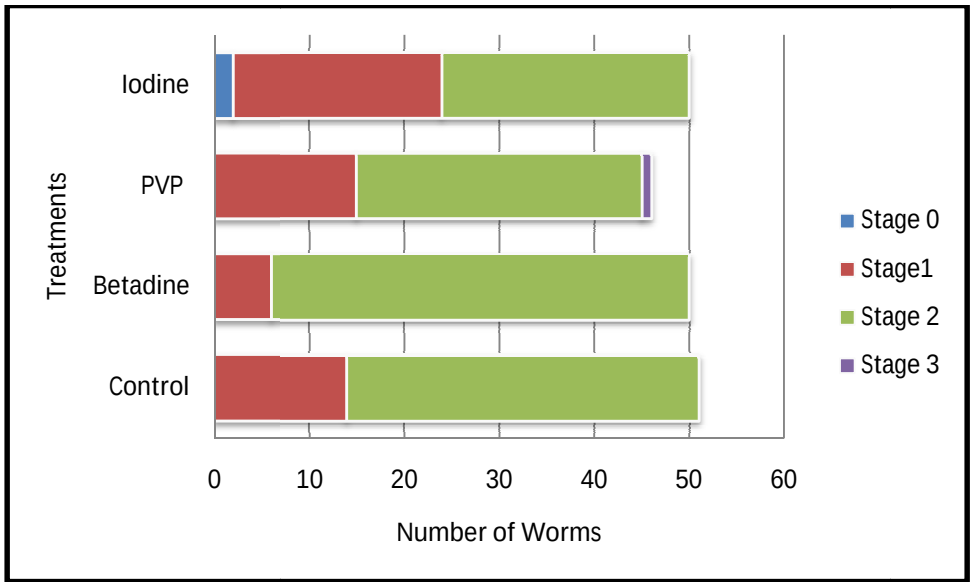

Figure 21. Number of Worms in Each Stage Per Treatment for Day 6

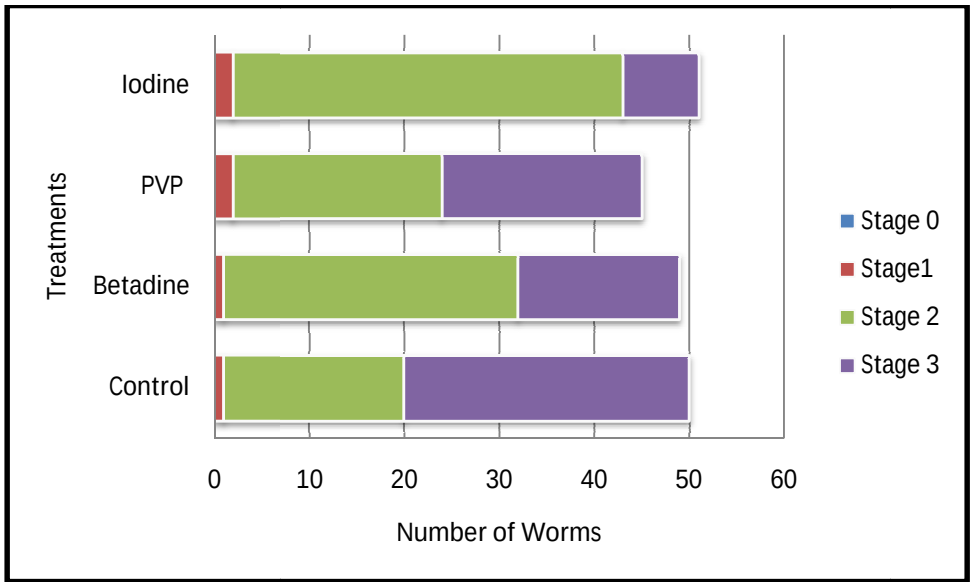

Figure 22. Number of Worms in Each Stage Per Treatment for Day 9

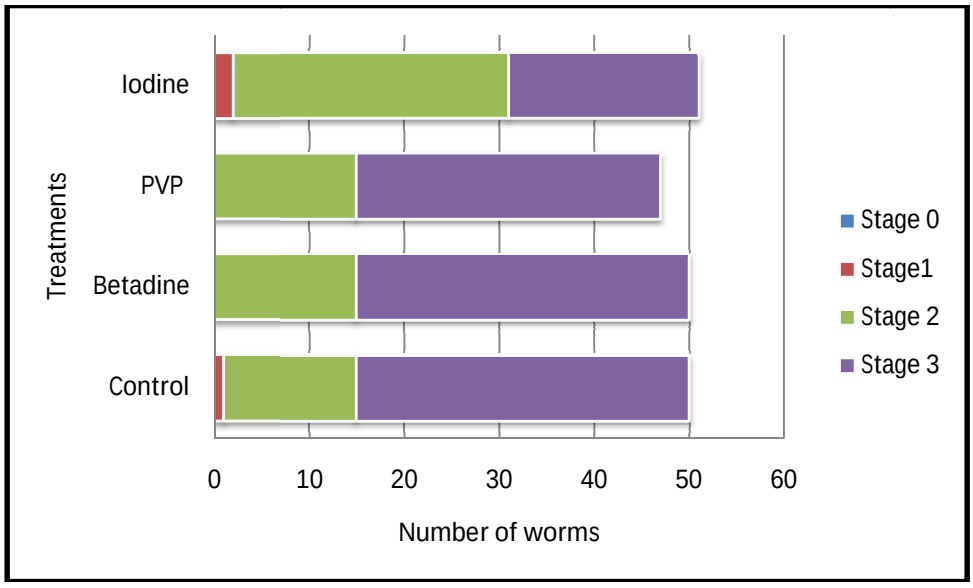

Figure 23. Number of Worms in Each Stage Per Treatment for Day 12

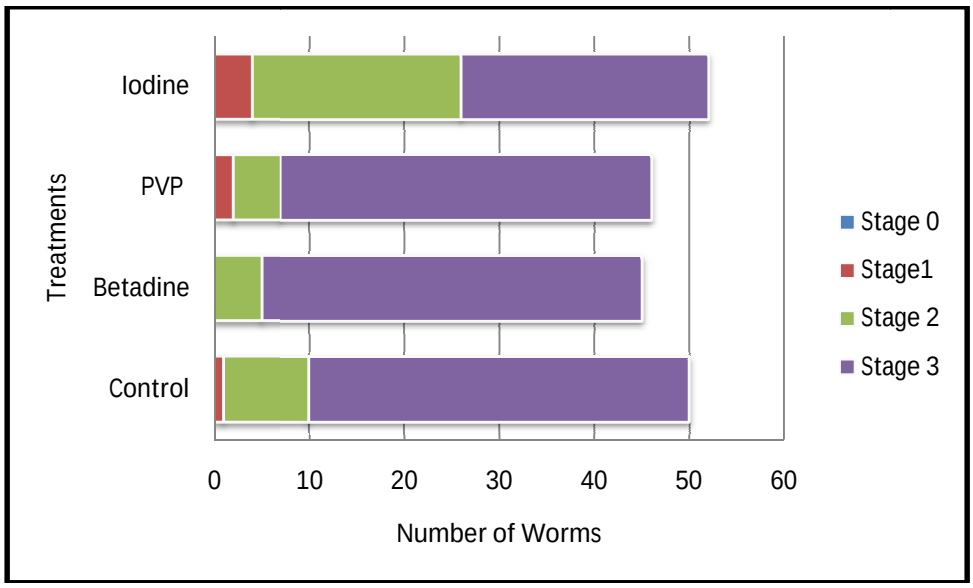

Figure 24. Number of Worms in Each Stage Per Treatment for Day 15

Table 6. P-values From Chi-Square Statistical Analysis of Flatworms

Solutions	Day 0	Day 3	Day 6	Day 9	Day 12	Day 15
Control vs. Betadine	NA	P=0.047 +/-	P=0.051 +/-	P=0.012 +	P= 1.000 -	P=0.235 -
Control vs. PVP	NA	P= 0.000 +	P=0.580 -	P=0.193 -	P=0.838 -	P=0.540 -
Control vs. Iodine	NA	P= 0.162 -	P=0.033 +	P=0.000 +	P=0.002 +	P=0.003 +

Significant (+) values were considered to have a p value < 0.05; while p>0.05 is not significant (-). +/- are border-line in significance.

The Chi-Square test was applied to compare the three treatments, Betadine®, PVP, and Iodine, and the control. The null hypothesis (H_0) assumes that there is no association between the variables and therefore no significant difference between the control and the treatments. A chi-square analysis was performed using Minitab Statistical Software on each day, individually comparing each treatment with the control.

Day zero (0) was not included in the analysis because all of the worms are at a stage 0 and there exists no difference. Beginning at day 3, only stage 0 and stage 1/2 are considered in the analysis because there are very few individuals representing stage 2 and stage 3. By omitting theses stages we ensure a more accurate analysis because having many low frequencies compromises the adequacy of the test. This is a similar case for subsequent analyses for days 6, 9, 12, and 15, where only stage 2 and stage 3 were considered. In some cases, the data were collapsed, thereby adding the few outlying individuals to the dominant stage of regeneration. This creates a negligible change to the p-value.

For day 3 and day 6, the control compared to the Betadine® treatment is of borderline significance because $p= 0.047$ and $p=0.051$, respectively, $p< 0.05$ is significant. Day 9 is significant with $p=0.012$, while day 12 and 15 are not significant. Control compared to PVP only showed a significant difference during day 3, the initial stages of regeneration. Iodine was significantly different from the control for days 6, 9, 12, and 15.

CHAPTER 4

DISCUSSION

Research using different substances on planarians has shown differing effects on healing and regeneration. From a historical perspective, planarian researchers have been intrigued by what substances and conditions act to promote regeneration and ones that are inhibitory. In 1927, experiments were performed that determined higher temperatures made heads regrow faster than lower temperatures. Research in the 1950s and 1960s demonstrated that riboflavin and colchicine were both shown to accelerate regeneration. On the other hand, substances like nicotine amide were found to inhibit regeneration and reduce the number of mitotic cells. Interestingly, iodine was analyzed and found to affect eye pigmentation (Brondsted 1969).

More recently, certain substances like neuropeptides have been shown to actually aide in regeneration (Hori 1997). However, many substances demonstrate deleterious effects. Accumulation of methyl mercury in the planarian body has been shown to lead to delayed formation of photoreceptor organs, suppression of regeneration, and death (Medvedev and Komov 2005). Studies have shown that both *Cannabis sativa* oil and cocaine are toxic to regenerating worms (Fournier et al. 1978). Retinoic acid disrupts anterior, but not posterior regeneration by interfering with pattern formations (Romero and Bueno 2001). Another study observed the rate of cell division in the blastema of planarians under the influence of such mutagens as dimethyl sulfate, urethane, 2-thiouracil, and 5-bromouracil; the results were a mixture of inhibitory and stimulatory effects (Aditya et al. 1994). Lastly, X-irradiation of planarians results in loss of proliferative cells such as neoblasts and therefore death of the organism.

In flatworm regeneration studies, a layer of epithelial cells will normally cover the wound

after the initial cut. A regeneration blastema is formed with 3 cell types residing here: undifferentiated cells, differentiated cells, and regenerative cells (Hori 1997). Wound healing generally begins to occur in less than 1 hour. Definitive determination of lost structures takes place during the first 2 days of regeneration (Baguna et al. 1994).

The results of this study showed that there are significant differences between Betadine®, Iodine, and spring water, occurring at different intervals of regeneration. One reason why healing may have become delayed in planarians exposed to Betadine® and Iodine was that the initial growth of the epithelial layer may have been delayed by the toxicity of the solution. In a study performed on wounded rats, Betadine® treatment caused wounds to not be fully epithelialized, a decrease in collagen, greater inflammation, and a general unevenness of the epidermis (Aramwit and Sangcakul 2007).

Overall, PVP produced no significant difference from the control in regards to regeneration. This is somewhat of a contradiction from the original preliminary experiment in which the PVP solutions seemed to be more lethal and presented limited splitting by the planaria as compared to the control, Betadine®, and Iodine test solutions. It appears that PVP would pose no harmful effects on wound healing in its role as a solubilizing agent for Iodine.

The Chi-Square analysis(see table 6) confirmed that significant effects were seen initially in days 3, 6, and 9 for the 0.1% Betadine® test solution. However, regeneration in Betadine® was not significantly different from the control in days 12 and 15. This leads to the conclusion that Betadine's® influence on wound healing and regeneration did not seem to cause longterm deleterious effects.

Analysis of the 0.001% Iodine treatment data showed a significant difference between the Iodine treatment and the control. During the initial period of healing (day 3), there was no

significant difference. However, days 6, 9, 12, and 15 were all significantly different from the control. The Iodine treatment appeared to have a complete opposite effect as compared to the Betadine® treatment. Iodine appeared to adversely affect long-term healing, whereas Betadine® affected only initial healing.

One of the first antiseptic preparations of Iodine was Lugol's solution. Application of this solution proved to be painful and irritating. In commercial antiseptic solutions, free Iodine is carried by an iodophore (i.e. PVP) and is slowly dispersed into solution. Iodine concentrations in PVP-I preparations varies between 9.0% and 12% (Cooper 2007). Published reports about the cytotoxicity of Iodine and its formulations (Betadine ®) are both positive and negative. Of the 3 test solutions in this study, Iodine alone appeared to have the most negative and obvious impact on overall wound healing. This is not only evidenced in the Chi-Square analysis but also in a comparison of the overall mean regeneration scores and number of worms representing each stage per day.

While previous literature findings debate the use of Betadine® as a safe surgical antiseptic, this study found no significant difference in regeneration between Betadine® and the control, spring water. By using the planaria as a model organism for tissue regrowth and wound healing, this experiment showed that Betadine® was not toxic to regenerating cells and had no effect on wound healing.

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