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Article

Effect of hypervitaminosis A in regenerating planaria: A potential model for teratogenicity testing

Eva Bennet[1], Kristen Murphy[1], Amrith Bennet[2]
[1]Hopkinton High School, Hopkinton, MA
[2]Alexion Pharmaceuticals Inc., Boston, MA

SUMMARY

According to the Centers for Disease Control and Prevention, a child with birth defects is born approximately every 4.5 minutes. The currently accepted method to determine the potential of new medicines and chemicals to cause birth defects (teratogenicity) involves testing in pregnant animals. Millions of mammalian animals are sacrificed annually for research, and scientists are searching for alternative models to reduce mammal use in research. This unique research study evaluated the potential use of the flatworm, brown planaria (*Dugesia tigrine*), as an alternative model for teratogenicity testing. There are similarities between mammalian embryonic development and regeneration of planaria during the early stages of development of the nervous system, including the eyes. In this study, we exposed amputated planaria to varying concentrations of a known teratogen, vitamin A (retinol), for approximately 2 weeks, and evaluated multiple parameters including the formation of blastema and eyes. Retinol delayed the formation of blastema and eyes in regenerating planaria in a concentration-dependent manner. At high concentrations of retinol, regenerating planaria also developed cyclopia, a well-known birth defect in humans, which has been linked to high levels of vitamin A in mammalian studies. The results from this study demonstrated that high concentrations of retinol caused defects in head and eye formation in regenerating planaria, with similarities to vitamin A related teratogenicity findings in mammals. Based on these results, regenerating brown planaria are a promising alternative model for teratogenicity testing, which can potentially be paradigm shifting as it can reduce cost, time, and pregnant animal use in research.

INTRODUCTION

Birth defects, otherwise known as congenital malformations or teratogenicity, are defects in the structure and function of the offspring that occur during fetal development (1). Most of the birth defects among humans can be diagnosed before or at the time of birth, while some of these defects are only evident later in life (1). According to the Centers for Disease Control and Prevention, birth defects occur in one in every 33 births in the United States of America, and approximately 7.9 million babies born yearly are affected worldwide (1). Birth defects are also a leading cause of death in infants, attributed to about 20% of the deaths in infants (2). Throughout history, birth defects have been recorded in humans and animals, and people have tried to understand the reason behind abnormal fetal development as early as 5th century BC, but the specific cause is not clearly understood for many birth defects (3).

Teratogens are substances or conditions that can lead to defective development, function, or death of a fetus if a pregnant animal is exposed to them. Teratogens can be classified into four major categories: (a) physical conditions, such as extremely high or low temperatures and radiation; (b) infectious diseases, such as Zika virus and herpes virus; (c) non-infectious diseases, such as thyroid disorders and diabetes; and (d) chemicals and medicines, such as alcohol, thalidomide, and vitamin A (3). To develop effective strategies for preventing or reducing the occurrence of birth defects, it is important to understand the teratogen associated with a specific birth defect. The World Health Organization has recommended certain public health measures to reduce the incidence of teratogenicity, such as dietary intake of appropriate amounts of vitamins and minerals, preventing maternal exposure to harmful substances, and adequate prenatal vaccination (2).

Understanding the teratogenic potential (or the ability to cause birth defects) of a new medicine is essential to determine if it can be safely prescribed to pregnant women. According to the current guidelines from health authorities of different countries who approve new medicines for human use (such as the U.S. Food and Drug Administration (FDA) and the European Medicines Agency), before administering a new medicine to women, its teratogenic potential should be tested in pregnant mammalian animals including evaluation of newborn offspring for any structural or functional defects, to understand if the medicine can cause abnormalities in fetal organ development (4,5). Currently, teratogenicity studies are conducted in pregnant laboratory animals such as rats, mice, rabbits, and monkeys. Since these animals take a long time to create offspring and some animals only have one offspring in each pregnancy, these studies are very long, expensive, and require the use of large numbers of animals. An estimated 26 million mammalian animals are used every year in the United States for research and development of new medicines (6). Scientists are searching for alternatives to reduce the mammalian animal use in teratogenicity testing of chemicals and medicines (6).

One of the alternative animal species that has potential for teratogenicity toxicity is the fresh water flatworm, planaria, which has a remarkable ability to regenerate (7). Several studies have been conducted to understand the biology of regeneration in planaria and whether these regenerative properties can be applied in human medicine (8). Planaria can create offspring by both sexual and asexual (binary fission) reproduction, and it does not involve development of the offspring inside a uterus (9). However, gene expression profiles of regenerating planaria and mammalian embryos share similarities during the development of the nervous system and the formation of eyes, which are some of the key features affected by teratogens (10-13). Planarian neuronal stem cells and neurons have been used in studies to understand the molecular pathways involved in the regeneration of neurons(14,15) . The potential use of planaria for in vitro and in vivo teratogenesis studies was considered in the past(16,17). Our study adds scientific evidence to the suitability of planaria as a relevant alternative model for teratogenicity testing of new medicines.

Vitamins are considered essential for the proper development and functioning of human beings; however, consuming high doses of vitamins can also lead to toxicity like that caused by other medicines and chemicals (3). Vitamin A is known to have a critical role in cellular differentiation and is a key component of prenatal vitamins (18). However, if consumed at high doses (hypervitaminosis), vitamin A and its synthetic derivatives (such as retinoic acid and retinol) are teratogenic to animals and humans, and this is dependent on the dose and the duration of exposure (19,20). Due to the teratogenic nature, high doses of vitamin A are contra-indicated during pregnancy. Teratogenic findings associated with retinoids include malformations of the central nervous system, heart, thymus, and craniofacial defects, with an incidence rate that is comparable to thalidomide (21,22),.

The purpose of this research was to test whether regenerating planaria would be a good model for teratogenicity testing of new medicines. We hypothesized that retinol, a vitamin A derivative and a known teratogen at high concentrations, will cause defects in the regeneration of planaria supporting the use of planaria as a model to test the teratogenicity potential of new medicines. In our study, retinol caused defective head and eye development, including formation of cyclopia, in regenerating brown planaria in a concentration-dependent manner. Using planaria offers a fast and inexpensive teratogenicity testing model during early drug discovery, thereby potentially reducing the use of mammals in later stages of drug discovery.

RESULTS

Determining the lethal concentration of retinol to amputated planaria:

In a pilot experiment, we determined the lethal dose of retinol to planaria and a range of retinol concentrations that allowed continued survival of planaria during regeneration.

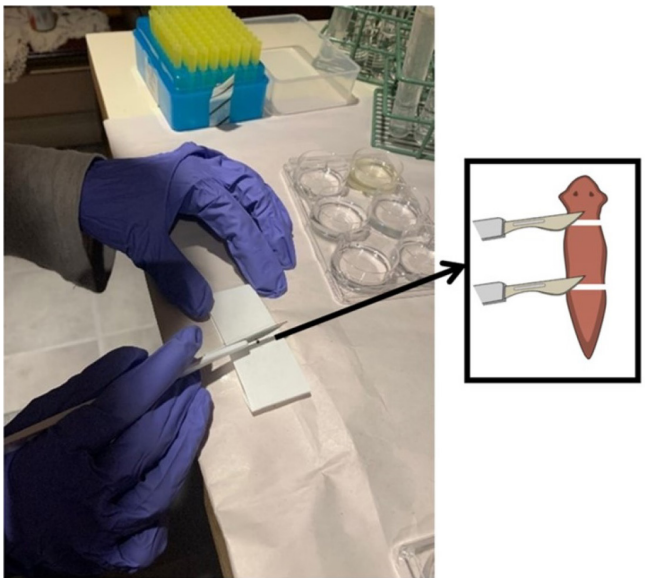

Figure 1: A representative image of the planarian amputation procedure. Planarian was placed on a histology slide wrapped in wax paper. A drop of spring water was added to the planarian to stretch it prior to amputation. Once stretched, the head of planarian was amputated using a scalpel, followed by its tail, before exposing it to test items.

We also evaluated whether polarity (regeneration of head in place of an amputated head and regeneration of tail in place of an amputated tail) would be affected by retinol. Since retinol was purchased as a solution in squalane, appropriate dilutions of squalane in spring water were used as a control to identify retinol-related effects on planarian survival. After the amputation of head and tail (**Figure 1**), brown planaria were exposed to five different concentrations of retinol (35 fM, 35 pM, 35 nM, 35 µM and 35 mM) or the corresponding concentrations of squalane and spring water (control groups) for up to 14 days (**Figure 2A**). Using a USB digital microscope, we evaluated the viability of individual planaria by their response to touching with a paint brush. Planaria exposed to 35 mM of retinol or squalane were found dead one day post-amputation. Planaria exposed to 35 µM retinol were found dead 11 days post-amputation, while the planaria exposed to the corresponding concentration of squalane were alive until the end of the experiment (13 days post-amputation). The death of planaria in the 35 mM of retinol was considered to be unrelated to retinol, since the planaria exposed to the same concentration of squalane also died and is likely due to the oily nature of squalane. The death of planaria exposed to 35 µM retinol was due to their exposure to retinol, since the planaria exposed to the same concentration of squalane were alive. Planaria at concentrations of retinol ≤ 35 nM were alive for up to 14 days post-amputation. There were no retinol-related changes in polarity observed in any of the surviving planaria. Based on these results, 35 µM was identified as the concentration of retinol that was lethal to amputated planaria.

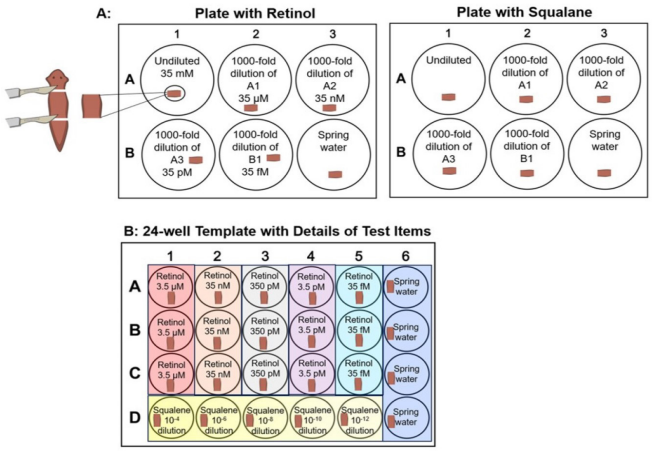

Figure 2: A) Illustration of the planarian amputation procedure and the setup of plates in the pilot experiment to identify the lethal concentration of retinol. Concentrations of retinol and squalane in each well are marked on the plates labeled with each test item. After amputation of head and tail, the middle region of the planaria was exposed to retinol, squalane or spring water as indicated in the illustration. **B) Illustration of the planarian amputation procedure and the setup of plates in the definitive experiment.** Concentrations of retinol and squalane in each well are marked in plates labeled with each test item. Each concentration of retinol, squalane and spring water are identified by different colors; 3.5 µM (red), 35 nM (light red), 350 pM (white), 3.5 pM (purple), 35 fM (light blue), water (blue) and squalane (yellow). After amputation of head and tail, the middle region of the planaria was exposed to retinol (n = 3/dose level), squalane (n=5) or spring water (n=4) as indicated in the illustration.

Retinol delayed blastema formation in regenerating planaria:

We evaluated the effect of retinol on the early stages of head regeneration in planaria by assessing the timing of blastema formation. Blastema is a "bud" like structure formed at the site of amputation and consists of a group of unpigmented, undifferentiated cells that can differentiate into organs or structures. We exposed brown planaria (after the amputation of the head and tail), to five different concentrations of retinol (ranging from 35 fM to 3.5 µM) or corresponding concentrations of squalane or spring water (control groups) for 14 days (**Figure 2B**). We examined the planaria daily for the formation of blastema (**Figure 3**) using a USB digital microscope without removing them from treatment wells. Blastema was visible in all the planaria exposed to spring water, squalane, or retinol at the concentration of 35 fM at 4 days post-amputation. Blastema was visible in one planarian at 3.5 pM of retinol at 4 days post-amputation while the blastema was visible in the other 2 planaria at that concentration on 5 days post-amputation. Blastema was visible in all the planaria at ≥ 350 pM retinol at 5 days post-amputation (**Figure 4**). Overall, there was a statistically significant (p-value < 0.001) delay in blastema formation in regenerating planaria exposed to retinol at concentrations ≥ 3.5 pM compared to water and squalane control groups (**Figure 4**). These observations showed that retinol significantly delayed blastema formation in

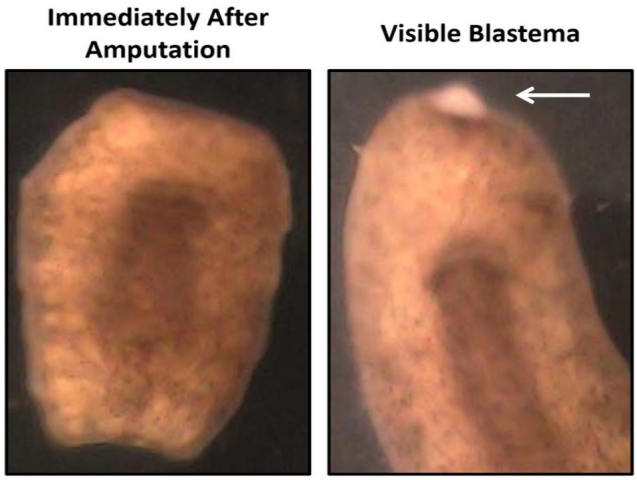

Figure 3: A representative image of blastema under a USB digital microscope. On the left is an image of a regenerating planarian immediately after amputation without any visible blastema. On the right, is an image 4 days after amputation in which a visible blastema can be seen. Blastema is identified by a white arrow. These representative images were from a planarian exposed to water.

regenerating planaria in a concentration-dependent manner.

Retinol delayed eye development in regenerating planaria:

We next examined the effects of retinol on eye development in regenerating planaria. Similar to the experiment evaluating the effect of retinol on blastema formation, we exposed head and tail-amputated brown planaria to retinol (concentrations ranging from 35 fM to 3.5 µM) or corresponding concentrations of squalane or spring water (control groups) for 14 days.

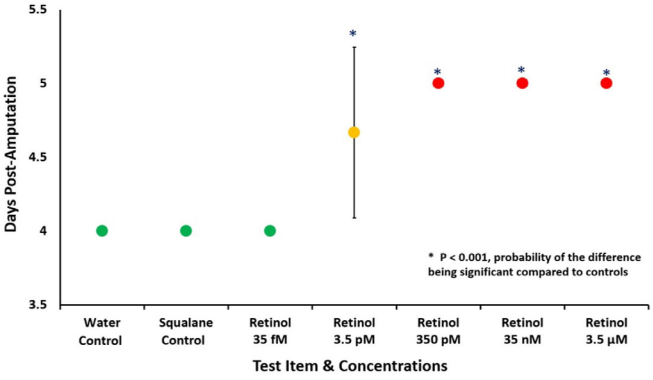

Figure 4: Effect of retinol on the timing of blastema formation in regenerating planaria. Graph shows the day of blastema formation (mean ± SD) post-amputation in planaria exposed to increasing concentrations (35 fM, 3.5 pM, 350 pM, 35 nM and 3.5 µM) of retinol (n = 3/concentration), corresponding concentrations of squalane (n = 5) or spring water (n = 4). There are no error bars for groups where the blastema formation occurred on the same day for all planaria within the group. Student's t-test was performed between each retinol group and the combined water and squalane control groups. * = statistically significant difference between the control groups and retinol group with a p-value of <0.001.

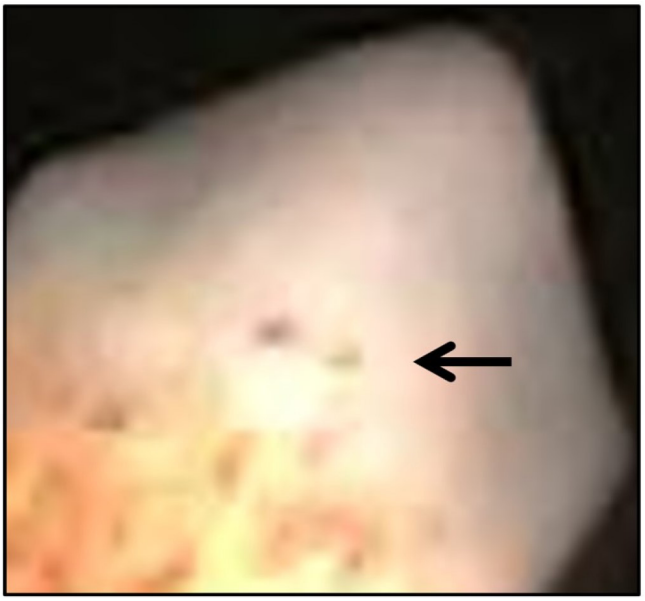

Figure 5: A representative image of eyes on a regenerating planaria under a USB digital microscope. Eyes were observed in regenerating planarian head starting 8 days post amputation in control groups. Regenerating eyes are identified by black arrow.

Using a USB digital microscope, the development of eyes was examined daily without removing them from treatment wells. Both eyes were visible in one planarian exposed to spring water at 8 days post-amputation (**Figure 5**). Eyes were visible in the remaining planaria exposed to spring water and all the planaria exposed to squalane or retinol at concentrations ≤ 3.5 pM at 9 days post-amputation (**Figure 6**). Eyes were visible in all the planaria at 350 pM retinol and one planaria at 35 nM retinol on 10 days post-amputation. Eyes were visible in the 2 remaining planaria at

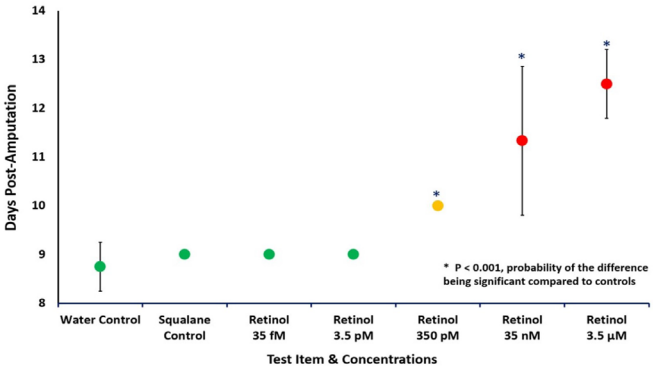

Figure 6: Effect of retinol on the timing of eye development in regenerating planaria. Graph shows the day of eye development (mean ± SD) post-amputation in regenerating planaria exposed to increasing concentrations (35 fM, 3.5 pM, 350 pM, 35 nM and 3.5 µM) of retinol (n = 3/concentration), corresponding concentrations of squalane (n = 5) or spring water (n = 4). There are no error bars for groups where the eye development occurred on the same day for all planaria within the group. Student's t-test was performed between each retinol group and the combined water and squalane control groups. * = statistically significant difference between the control groups and retinol group with a p-value of <0.001.

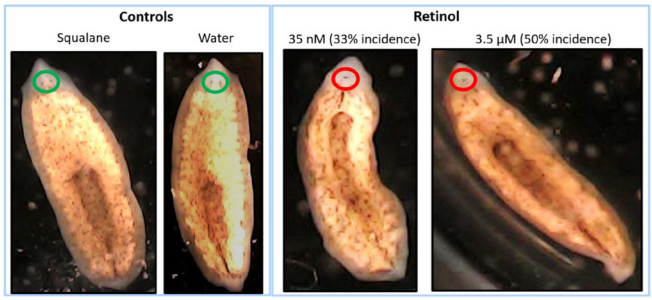

Figure 7: Retinol at concentrations ≥ 35 nM caused cyclopia in regenerating planaria Representative images of normal eyes from planaria in control groups (left, green circles) and planaria with cyclopia in retinol groups (right, red circles). In planaria exposed to control treatments (water or squalane), two distinct eyes were clearly visible on either side of the mid-line. In planaria exposed to retinol at concentrations ≥ 35 nM, there was cyclopia, a centrally placed single eye.

35 nM retinol at 11 days or 13 days post-amputation. At the highest concentration of retinol, 3.5 µM, eyes were visible only in 1 of the 3 planaria at 12 days post-amputation and in another planaria at 13 days post-amputation. Eyes were not visible by 13 days post-amputation in one remaining planaria at 3µM (**Figure 5**). Overall, there was a statistically significant (p-value < 0.001) delay in the development of eyes in regenerating planaria exposed to retinol at concentrations ≥ 350 pM (**Figure 6**). These observations show that retinol significantly delayed the development of eyes in regenerating planaria in a concentration-dependent manner.

High concentrations of retinol caused cyclopia in regenerating planaria:

In addition to the delayed development of eyes, one planarian each at retinol concentrations of 35 nM and 3.5 µM had only one eye. The single eye in these planaria was located on the midline of the head (**Figure 7**) compared to the normal appearance of eyes on either side of the midline. The presence of centrally placed single eye in planaria was similar to cyclopia, a well-known birth defect in humans, which has been linked to high levels of vitamin A in animal studies (20,22).

DISCUSSION

The current model for safety assessment of chemicals and medicines involves the use of very expensive and lengthy studies that use large numbers of pregnant mammalian animals, raising ethical concerns. The three Rs principle (Reduction, Replacement, and Refinement) encourages biomedical scientists to reduce animal use and develop alternative models for animal research (6). Planarian head and eye regeneration shares similarities in gene expression with mammalian embryonic development of the nervous system and eyes (10–13). Using flatworms such as planaria instead of mammals for teratogenicity risk assessment fits with the replacement principle of 3Rs.

Vitamin A is an essential nutrient for animals and humans,

with a critical role in the formation and maintenance of healthy teeth, bones, skin, and good vision, especially in low light (18). However, high doses (such as 10,000 IU) of vitamin A derivatives such as retinoic acid are known to cause teratogenicity in humans, including craniofacial defects (including cleft palate, cleft lip, and eye defects) and malformations of nervous system (21). In a rat study comparing the teratogenicity of nine different retinoids, there were defects involving the central nervous system, craniofacial area, and the urogenital system, and the 35,000 IU of Vitamin A was associated with toxicity (22). This indicated that vitamin A derivatives are useful reagents to test whether planaria can be a good model to detect teratogenicity.

We showed that retinol clearly demonstrated defects in the regeneration of head and eyes in a concentration-dependent manner. These included a statistically significant delay in the development of blastema and eyes as well as the formation of cyclopia, similar to the teratogenic effects caused by derivatives of vitamin A in animals (22), demonstrating the value of regenerating planaria as a promising alternative model for teratogenicity testing.

The duration of regeneration of head and eyes of planaria in control groups in this study was consistent with previous reports (9). The doses of vitamin A associated with teratogenicity in humans is ≥ 10,000 IU daily (21). The retinol concentrations that caused delay in head and eye formation in regenerating planaria in this study were ≥ 3.5 pM to 350 pM. In a developmental biology study, retinoic acid was associated with a similar delay in the regeneration of the anterior region from the tail segment of Girardia tigrina at a concentration of 500 µM (23). Chemicals and medicines can have differences in toxicity across species. This may be due to the differences in the molecular pathways involved in the development and function of different species and how they differ in absorbing and using chemicals and medicines. Further research to understand the molecular mechanism of how retinol causes defects in the regeneration of planaria will be helpful to further characterize planaria as an alternate teratogenicity testing model for medicines for human use.

This is the first study that evaluated the regeneration of head and eyes in brown planaria after dual amputation of head and tail to understand whether planaria can be an appropriate model for teratogenicity testing of new medicines. There is a high bar for testing the safety of medicines and this new model may not entirely replace pregnant animal use for teratogenicity testing right away, but it can serve as a useful initial test to reduce pregnant animal use to identify drugs that cause defects in head and eye development. After the tier 1 test in planaria, pregnant mammals can then be used only as the final test for drugs that did not cause head or eye development defects in planaria. Planaria can also be used as a quick, inexpensive, and effective screening tool in drug discovery research when there are many drug candidates with similar properties, allowing advancement of only the non-teratogenic compounds into the drug development phase. Currently, rat embryos, zebra fish embryos, and mouse stem cells are used as alternate models to detect changes in morphology and expression of genes that are known to be associated with teratogenicity (24-26). A major limitation for the currently available alternative models that can detect morphological changes in embryos is their inability to develop into functional organisms in an in vitro system. This leaves a gap in understanding the impact of the observations in an early-stage embryo on a fully developed offspring. Compared to these currently used alternative models, planaria are a value-added model that can be useful to detect changes in the structure and appearance during regeneration and in a fully developed organism. In addition, regenerating planaria only require spring water for maintenance and do not require specialized in vitro medium that is necessary for the maintenance of embryos and stem cells. Similar to other alternative in vitro models, planaria also only require small amounts of drug for testing, which can be especially useful during the drug discovery phase in which compound availability is limited. Acceptance of alternative models in biomedical research by health authorities requires validation of these models. Future studies using known teratogens and non-teratogens will be necessary to understand the sensitivity and specificity of regenerating planaria in detecting the teratogenic potential of new medicines.

Health authorities like the FDA have published regulatory guidelines on the nonclinical toxicology testing including what tests needs to be conducted, how, and when these need to be done in relation to different phases of clinical development (4,5). For teratogenicity testing, these guidelines currently only include pregnant mammalian animal studies prior to enrolling women of child-bearing potential. A potential limitation of the planarian model is that they do not grow offspring in a uterus, which is a typical consideration for a traditional mammalian teratogenicity test system. The findings in this study are consistent with the published vitamin A related teratogenicity in humans and animals (18-21). However, the regulation of developmental processes cannot be generalized across species, and the underlying mechanisms which led to the teratogenicity findings could be different between mammals and a flatworm such as the planarian. Therefore, further research to understand the molecular mechanism of how retinol causes defects in the regeneration of planaria will be helpful to further characterize planaria as an alternate teratogenicity testing model for medicines for human use. The predictive toxicology principles published by authors from the FDA and the pharmaceutical industry outline the use of multiple alternative methods to improve prediction of human outcomes while reducing the cost, time, and the use of mammals in toxicity assessments (27,28). Consistent with this predictive toxicology approach, the inherent limitations of the planarian model need to be balanced by using it as part of a synergistic panel of currently existing in vitro models to identify the potential teratogenic hazard of drug candidates in early drug discovery.

In summary, retinol, a vitamin A derivative, caused defective head and eye development, including formation of cyclopia, in regenerating brown planaria in a concentration-dependent manner. The defects in regenerating planaria were similar to the teratogenic effects of vitamin A derivatives in animals and humans, and regenerating planarian model could be an innovative and highly valuable tool in developmental toxicology research and in pharmaceutical drug discovery and development. Further investigations to understand the molecular mechanism behind these observations in regenerating planaria will add value. For health authorities to accept regenerating planaria as a new alternate teratogenicity testing model for new medicines, it will be important to validate the model using other known teratogenic and non-teratogenic medicines.

MATERIALS AND METHODS

Maintenance and amputation of planaria:

Brown planaria (Carolina biologics, Cat# 132954) were kept in commercially available spring water (Poland springs) in clean glass or plastic containers with a loose-fitting lid for air. Planaria were maintained on a weekly diet of hard-boiled egg yolk. For feeding, egg yolk was mixed with spring water. After 24 hours of feeding, they were transferred to a clean container with spring water. Planaria were fed 2 days before each experiment and were not fed for 14 days during the experiment. For dual amputation of head and tail, individual planaria were transferred from the container using a clean paint brush (crafter's square, Greenbrier International) and placed on a glass histology slide (Carolina Biologics) wrapped in wax paper (Greenbrier International). Then, a drop of spring water was placed on the planaria, allowing it to stretch, which allowed quick amputation of the head and tail. Once stretched, the head of planaria was amputated using a scalpel, followed by its tail, before exposing it to test items (**Figure 1**).

Preparation of test items:

Retinol is a commercially available liquid form of vitamin A that is safe for use to conduct experiments at home under the Coronavirus Disease (COVID-19) pandemic restrictions. Retinol was commercially available as 1% solution (35 mM) in squalane (The Ordinary Company). This stock solution of retinol was further diluted in spring water to achieve appropriate dilutions, and the solubility was assessed by microscopic evaluation for the presence of potential precipitates. There were no precipitates at any concentration used in the study. To identify the highest non-lethal concentration of retinol in the pilot experiment, the stock solution was serially diluted 1000-fold up to a final concentration of 35 fM (**Figure 2A**). In the definitive experiment to evaluate the effects of retinol on regeneration in planaria, 100-fold dilutions of retinol from a starting concentration of 3.5 µM were performed up to a final concentration of 35 fM (**Figure 2B**). Control groups included spring water and corresponding concentrations of squalane, which was diluted in a similar manner as retinol. These test items at various concentrations were considered as the independent variables.

Exposing planaria to test items:

The middle section of planaria without head and tail was immediately transferred into either a 6-well or 24-well plate (Carolina Biologics) for incubation with different concentrations of retinol or squalane or spring water. One planarian was incubated in each well. In the pilot experiment to identify the highest non-lethal concentration, an n = 1 per concentration of retinol or squalane was used (**Figure 2A**). In the definitive experiment to identify the effects of retinol on planaria regeneration, an n = 3 was used for each concentration of retinol with an n = 1 for each concentration of squalane, and an n = 4 for spring water control (**Figure 2B**). Microscopic examination of planaria after amputation: Amputated planaria exposed to test items were examined once daily for 14 days under a Universal Serial Bus (USB) digital microscope (Duety) without removing them from the treatment wells. The following parameters (dependent variables) were evaluated to identify the effect of retinol on regeneration of planaria.

Viability:

The planaria were touched gently with a paint brush to check if they were alive. If the planaria was able to move portions of its body, then it was considered alive. The day when planaria did not respond was recorded as the day when the planaria died.

Formation of blastema:

Blastema is the non-pigmented structure formed at the site of amputation in regenerating planaria and is one of the early stages of head regeneration (**Figure 3**). The earliest day on which blastema was detectable was recorded for each regenerating planaria. This parameter was evaluated to determine if retinol had any effects on the start of head regeneration in planaria.

Development of Eyes:

High concentrations of vitamin A affect the development of eyes in humans and animals. The earliest day on which eyes were detectable in each regenerating planaria was recorded (**Figure 5**). This parameter was evaluated to identify if retinol caused delay or defects in the regeneration of eyes in planaria.

Statistical analysis:

Statistical analysis was performed using the Microsoft Excel program. Mean and standard deviation were calculated for the day of blastema formation and eye formation for each group. Frequency of cyclopia in regenerating planaria was expressed as a percentage of the number of planaria with a single eye, with the total number of planaria with eyes in

the group. Statistical analysis was performed between the water control group and the squalane (vehicle control) group. Since there was no statistically significant difference between these groups, the combined mean for both water and squalane groups were used as the mean for control group, increasing the number of observations in control groups and improving the statistical power for comparison. The individual observations in some groups were the same as the group mean, thus lacking variability. Therefore, the standard deviation (SD) was calculated for all the 24 wells used in the experiment. The relationship of the effects observed in each retinol concentration groups to that observed in control groups was evaluated by performing the student's t-test using the following equation. Each retinol concentration was compared against the combined water and squalane controls to get the t-value (t = (mean of each retinol group – mean of control groups) ÷ (SD/square root of number of observations used to calculate SD)). The probability of difference between the compared groups (p-value) was calculated in a 2-tail distribution and degrees of freedom value of 23 (total number of observations – 1).

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