Resveratrol alters the rate of calcium incorporation during early bone development in *Danio rerio*

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Abstract

Resveratrol, a naturally occurring phytochemical found in grapes and red wine has been shown to have many health benefits as well as increase bone growth in some mammalian species. Resveratrol is thought to exert many of these health effects (including those on bone growth) through its ability to weakly bind and activate the estrogen receptor. Little is known, however, about the effects of resveratrol on bone ossification in *Danio rerio* (zebrafish). This study investigated the effects of resveratrol on vertebrae ossification in *D. rerio*. Embryos were exposed to increasing concentrations of resveratrol (10^{-6} M -10^{-4} M) for 12 days. To determine the effects of resveratrol on ossification, embryos were then stained with calcein dye from day 6 to day 12. Calcein is a fluorescent dye that adheres to calcium ions, thereby serving as a marker for ossified bone. Specimens were imaged using confocal microscopy and vertebrae were scored according to their degree of ossification. Results indicate that resveratrol accelerates ossification of vertebrae at all concentrations beginning at day 6 compared to untreated controls supporting the hypothesis that resvertatrol alters early bone development in *D. rerio*.

Introduction

Our research project focused on the field of developmental biology and specifically used a zebrafish model. The compound utilized in the project was resveratrol, a chemical found in red wine and plants. Resveratrol is commonly used for strengthening the circulatory system, lowering cholesterol, and for its anti-cancer effects. Recently, however, given that resveratrol can act as an estrogen agonist, opening the possibility that it may also modify the rate of bone development. This possibility, has led to our hypothesis that resveratrol exposure can increase the rate of bone ossification during early development.

Resveratrol (3.5.4'-trihydroxystilbene) is a neutraceutical commonly found in red wine and Japanese knotweed. Interestingly, resveratrol is though to have played a role in the French Paradox, an apparent contradiction between the low incidence of coronary heart disease and the high saturated fat diets of the French. This compound has received increased attention in recent years for its many potential benefits on human health. This specific compound is thought to be an estrogenic substance, which suggests that resveratrol can increase the production of estrogen in vertebrates. Resveratrol is also known to have antioxidant properties that are good for one's heart.

Zebrafish embryos at fertilization were exposed to increasing concentrations of resveratrol. After the fish were subjected to the compound for a specific period of time, we used a diluted form of the stain, calcein. Calcein is known to attach to calcium and causes cartilaginous and bony structures in the fish to fluoresce. This fluorescent stain allowed us to physically observe any effects that resveratrol had on bone calcium incorporation in the embryo.

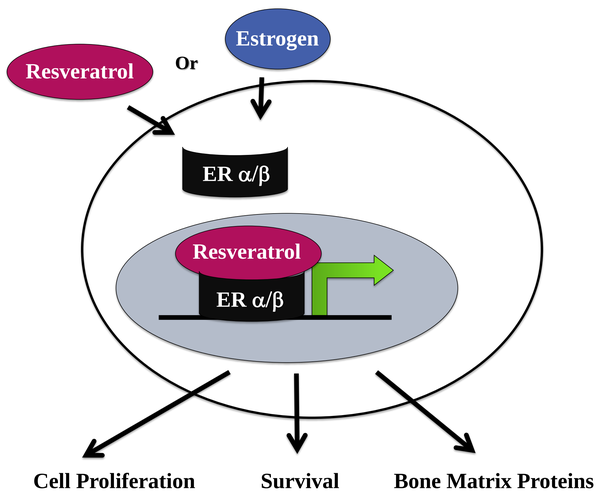


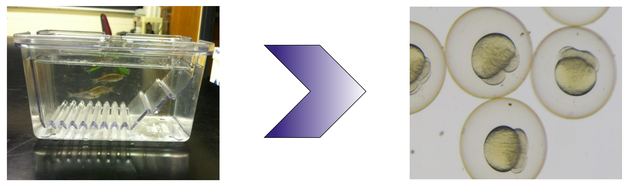
Figure 1. Proposed resveratrol signaling activation

Materials and Methods

Experimental Procedure

Day 0: Set up breeding pairs in the afternoon

Day 1: At 8am, remove breeding tank divider allowing males and females to interact. Harvest embryos 1hr-2hrs later and set up 8-10 embryos per well in a 12-well plate. Add treatment regimen to the water in each well.



Day 2: Monitor embryo viability

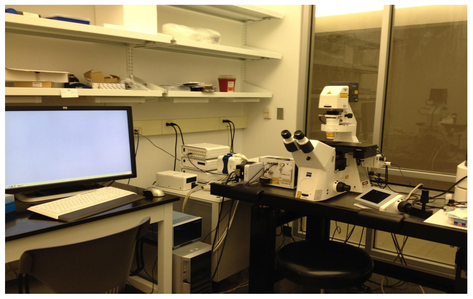
Day 3: Monitor embryo viability and change water (repeating the treatment regimen daily)

Day 4: Monitor embryo viability

Day 5: Monitor embryo viability, change water (repeating the treatment regimen as performed on Day 1),

Day 6-7: Monitor embryo viability

Day 8-10: Monitor embryo viability, stain 2 embryos per treatment condition with 1% Calcein dye for 20min, wash 3x, and score vertebrae on a Zeiss spinning disk confocal



Results

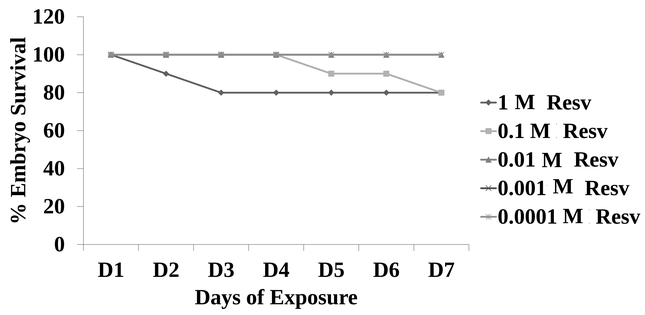
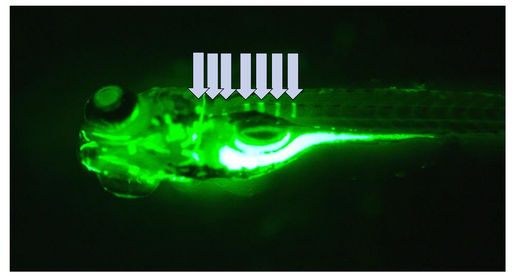


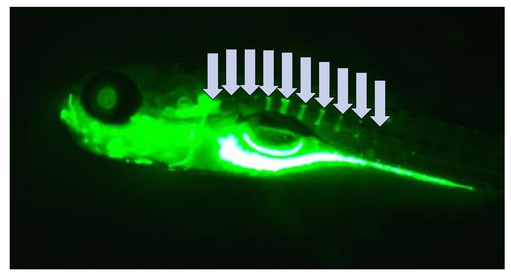
Figure 2. Titrations to determine maximum tolerated concentration of resveratrol. Embryos were plated into indicated concentrations of resveratrol and monitored for seven days. All compounds were dissolved in DMSO and diluted so that each well contained the indicated concentrations of resveratrol and a final concentration of 0.01% DMSO. Embryos viability was standardized to a DMSO treated embryo control at each time point.

Results

a) 8dpf DMSO treated control stained with calcein dye



b) 8dpf Resveratrol (1e-4 M) treated and stained with calcein



c)

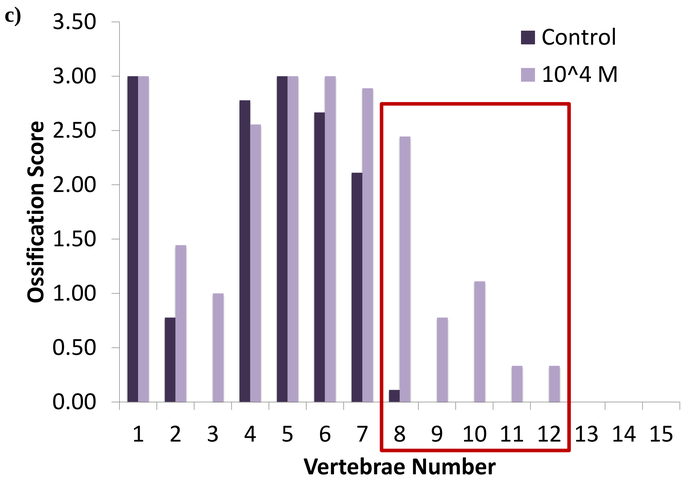


Figure 3. Calcein staining and scoring of vertebrae at 8dpf

Embryos were harvested at fertilization and treated with either DMSO (control) or 1e-4 M Resveratrol daily for 8 days. The embryos were then stained with calcein dye (fluoresces when bound to calcium), anesthetized in MS-222 and imaged on a Zeiss spinning disk confocal microscope. a) and b) show representative images of 8dpf DMSO or Resveratrol treated and imaged embryos. Four embryos per time point and treatment regimen were imaged. The images were blinded, randomized and scored for the number of vertebrae visible and the intensity of staining per vertebra (0 = no stain, 1 = less than 25%, 2 = 25-75% and 3 = greater than 75%)

Conclusions

- Resveratrol is non toxic to developing zebrafish embryos at doses equal to and lower than 1e-2M
- 1e-4M resveratrol increases the rate of calcium incorporation into the vertebrae of developing zebrafish embryos

References

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- Du SJ, V Frenkel, G Kindschi, and Y Zohar. Visualizing Normal and Defective Bone Development in Zebrafish Embryos Using the Fluorescent Chromophore Calcein. Developmental Biology 238, 239–246 (2001)
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