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Dietary resveratrol increases mid-life fecundity of female *Nothobranchius guentheri*

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ABSTRACT

The decline of female reproductive function is an early phenotype of aging in humans, occurring only midway through the lifespan. Yet the number of women delaying pregnancy continues to rise in industrialized societies due to personal or socioeconomic circumstances, often resulting in subfertility or difficulty conceiving. There are few defined mechanisms associated with this etiology, and equally few effective therapies. To combat this problem, we used a novel emerging model, *Nothobranchius guentheri*, that recapitulates the age-associated spectrum of changes that adversely affect human fertility. We hypothesized that resveratrol (RSV), which activates SirT1 as an oxidative stress sensor and longevity assurance enzyme, would improve female fecundity in mid-life. RSV, a polyphenol found in grapes and red wine, has been touted as an anti-aging dietary supplement due to its ability to prolong both lifespan and health span. SirT1 is an NAD + dependent histone deacetylase, whose activity is regulated by the nicotinamide to NAD + salvage pathway, especially the rate-limiting enzyme NAMPT. We found that female *N. guentheri* fed 600 μg RSV/g food into mid-life (~20 weeks), beginning at sexual maturity, showed increased embryo production compared to those on Control diet. Furthermore, the RSV-fed fish had significantly increased NAMPT. This suggests that dietary RSV has a positive effect on female fertility, and that it may become an effective therapy to regulate sirtuin activity and combat reproductive senescence.

1. Introduction

Women's ability to delay pregnancy and preserve fertility is an important reproductive freedom. In the U.S., the number of women delaying their first pregnancy even into their mid-40s has risen 4-fold from 1985 (Matthews and Hamilton, 2014). While older first-time mothers generally have more education and financial resources, the biological consequences of midlife pregnancy can include reduced reproductive success, increased gestational complications that often require hospitalization, and marked elevations in spontaneous abortions. Despite the social and public health burdens from lowered reproductive success, there are few defined mechanisms associated with this etiology, and equally few effective therapies. In part, this stems from the paucity of short-lived animal models that recapitulate the age-associated spectrum of changes that adversely affect human fertility.

For just over a decade, research on the *Nothobranchius* genus of fishes has established that they are excellent models for performing longitudinal studies of age-related deterioration and disease (Cellerino et al., 2016). *N. furzeri* of the Gona-*Re*-Zhou (GRZ) strain is the shortest-lived vertebrate kept in captivity, with a median lifespan of approximately 9–11 weeks (Terzibasi et al., 2008; Valdesalici and Cellerino, 2003; Valenzano et al., 2006). The related species *N. guentheri* typically lives 10–14 months (Yu and Li, 2012), which is still much shorter than other vertebrate models such as rodents. Furthermore, *Nothobranchius* shares traits common with mammalian aging such as the shortening of telomeres, cellular senescence, and an accumulation of cellular damage (Genade et al., 2005; Harel et al., 2015; Hartmann et al., 2011, 2009). While *N. furzeri* GRZ do not show a marked decline in female fertility with age, other strains of *N. furzeri* and other *Nothobranchius* species that live longer than GRZ's 9 weeks do experience these changes, which are characterized by atrophied ovaries, interstitial fibrosis, and follicular atresia (Blažek et al., 2017; Di Cicco et al., 2011). Recently, *N. guentheri* ovaries were shown to degenerate by or before 12 months of age (Liu et al., 2017).

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The principal determining factor in female reproductive aging is the ovarian lifespan. In humans, follicular reserve gradually declines with age until an insufficient number remains to sustain the hormonal cycle required for menstruation and menopause is reached. Ovarian aging is represented by loss of both follicular quantity and quality. Loss of quality is seen in spindle aberrations, telomere shortening, chromosome misalignment, and mitochondrial dysfunction, which contribute to aneuploidy and infertility (Dorland et al., 1998). The reasons for this loss of quality before oocyte depletion is thought to mirror age-related changes in other organs, and may result in large part from an age-related disruption in the ovarian redox state, resulting in increases in oxidative stress, decline in antioxidant gene expression, and the subsequent accumulation of oxidative damage (Agarwal et al., 2005; Fujii et al., 2005).

Members of the sirtuin family (silent information regulator 2 (Sir2) proteins) have recently been demonstrated to act as sensors of redox state in oocytes and ovarian granulosa cells (Tatone et al., 2015). Sirtuins are NAD + dependent enzymes with deacetylase and mono-ADP-ribosyltransferase functions and are known to decline markedly with age (Imai and Guarente, 2014), along with the pool of available NAD + and the rate-limiting enzyme in the nicotinamide to NAD + salvage pathway, NAMPT (Yoshino et al., 2011). Sirtuins also appear to play a key role in ovarian and follicular aging, as a decline in both protein level and activity of at least one member – SirT1 – is seen with age (Braidy et al., 2011; Gong et al., 2014; Thompson et al., 2014). SirT1 is conserved across the animal kingdom, and is highly expressed in the gonad of teleost fishes (Pereira et al., 2011). Furthermore, recent work suggests that beneficial effects on ovarian lifespan seen in caloric restriction occurs via activation of SIRT1 and that downregulation of SIRT1 leads to accelerated follicle loss (Wang et al., 2014). This suggests that pharmacological sirtuin induction could benefit ovarian health over the reproductive lifespan. One of the most potent in vitro SIRT1 activators found to date is resveratrol (RSV), a polyphenol found in grapes, wine, and other botanicals. Experimental overexpression of sirtuins (particularly SirT1) in animal models mimics the anti-aging health benefits of resveratrol (Araki et al., 2004; Parker et al., 2005). RSV has also been shown to increase levels of NAMPT, thus providing sufficient NAD + to maintain sirtuin activity (Huang et al., 2015). RSV provides a multitude of benefits, to include reducing inflammation, inducing anti-carcinogenic effects, protecting mitochondrial function, enhancing telomerase activity, and inhibiting cell senescence. It is touted as an anti-aging dietary supplement due to its ability to prolong both lifespan and health span in many model species (Valenzano and Cellerino, 2006). RSV is also safe and beneficial in humans; it is currently being used in clinical trials in men and women of all ages for prevention of cardiovascular disease and diabetes (clinicaltrials.gov).

A landmark study by Valenzano et al. using RSV in GRZ *N. furzeri* showed significant lifespan extension (Valenzano et al., 2006). As the GRZ strain of females continued to produce fertile eggs throughout their lives, for our study we chose to use *N. guentheri*, due to its representative age-related ovarian decline, to test the benefits of RSV on reproductive success. With this information, we can pinpoint future strategies for using pharmacological agents to preserve fertility. In this study, we fed *N. guentheri* females 15 μg RSV/fish/day for up to 5 weeks during early mid-life. We tracked fecundity as measured by the number of fertilized eggs over time. We also measured the effects of dietary RSV on ovarian SirT1 and NAMPT levels. Over time, RSV significantly increases female fecundity and body size. RSV also increased NAMPT protein in the ovary.

2. Materials and methods

2.1. Fish model and husbandry

Nothobranchius guentheri, Zanzibar strain, were hatched and maintained using the methods of Genade et al. (Genade, 2005) with a modification in housing. In brief: Embryos were kept on peat moss in a humid environment for several months until ready to hatch. Hatching was performed by flooding the peat moss with reverse osmosis water at laboratory room temperature (21 °C), waiting overnight, and pouring the fry-containing water into a shallow dish for raising. Dishes were floated at the top of static aquariums held at 25 °C and 20% of water was changed daily. Fry ate freshly hatched *artemia nauplii*. Juveniles (~2 weeks of age) were transferred into static tanks with sponge filters and half-strength brackish water (1000 microSiemens or 0.5 g/L) using Crystal Sea Bioassay Formula salt from Aquaneering (San Diego, CA) and transitioned from eating *artemia nauplii* to chopped frozen bloodworms (Hikari Aquatic Diets, Hayward, CA) at ~4 weeks of age. Beginning at 8 weeks of age, juveniles were sorted by size and housed in groups of 2–4 in an Aquaneering recirculating zebrafish rack in 2.8 L tanks at 27 °C. At 10–15 weeks of age, when sex determination was definite, breeding pairs were housed together in order to enable egg counts from individual females during breeding sessions. At this time, specialized diet was begun. Diet was whole bloodworms with test or control compound as described below. Recirculating water was maintained at 2000 microSiemens to reverse osmosis water and the room was maintained on a 14:10 light/dark cycle. All animal work was approved and in accordance to IACUC guidelines (Assurance Number: A3229–01). The AAALAC-accredited Laboratory Animal Resources Center (LARC) provided management and veterinary care.

2.2. Specialized diet and feeding

Study fish were fed twice daily between 8:00–9:00 AM and 5:00–6:00 PM. Resveratrol solution (6.0 mg/mL) was made by dissolving RSV in 95% water/5% ethanol. Control solution was 95% water/5% ethanol. Bloodworms were soaked in RSV and Control solutions for 2 h at 4 °C, then made into pellets with 5% gelatin and stored at −20C. Concentration of RSV was 600 μg/g food. Fish ate ~25 mg/day food for a total of 15 μg RSV/fish/day.

2.3. Breeding and egg collection

Breeding was performed in the style of Polačik et al. (Polačik et al., 2016) with slight modification. Plastic dishes containing biofilter beads (Aquaneering, San Diego, CA) as a substrate were used for breeding. Fertilized eggs were collected and counted Mondays, Wednesdays, and Fridays; nonviable eggs were scored separately.

2.4. Sacrifice and dissection

For each dietary group, we sacrificed three fish that had been on the diet for 3.5 weeks, beginning at 10.5 weeks of age (sacrifice age: 14 weeks) and three fish that had been on the diet for 5 weeks, beginning at 15 weeks of age (sacrifice age: 20 weeks). Euthanasia was performed by overdose of buffered tricaine methylsulfonate (MS-222) at 500 ppm. Fish were patted dry, weighed, and measured. Tissues were removed, weighed, and flash frozen. Ovaries were dissected to remove mature oocytes before storage.

2.5. Immunoblotting

Commercial antibodies to *Nothobranchius* proteins are not available, but there is precedent for immunoblots in this genus (Genade and Lang, 2013; Ripa et al., 2017). We selected ours by comparing the epitopes of the antibodies to the GenBank sequences for *N. guentheri* or *N. furzeri*. The full genome is not yet reported for *N. guentheri* as it is for *N. furzeri*, but the proteins we were looking for have a high homology across vertebrate species. Antibodies made to the following proteins were used: Visfatin/NAMPT (ab58640, Abcam, USA) with 68% sequence identity immunogen to *N. furzeri*; SirT1 (AV32386) with 84% sequence identity, and Actin (A5441) with 85% sequence identity, both from