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RESEARCH ARTICLE

Effect of dietary resveratrol on cell-mediated immunity and hepatocyte morphometry in the model organism medaka (*Oryzias latipes* Temminck & Schlegel)

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Abstract. The effect of dietary resveratrol (40 μg g^{-1} BW day^{-1}) on cell-mediated immunity (the activity of spleen phagocytes and the proliferative response of lymphocytes) and liver histology (hepatocyte morphometry, lipid vacuoles, and glycogen granules) in adult medaka (*Oryzias latipes*) (aged two years, body weight BW = 0.49 g) were tested after ten days of feeding with experimental diets. The fish were fed either a commercial diet (control group C) or this same diet supplemented with resveratrol (group R) three times daily at 3% of body weight (BW). Fish fed feed with resveratrol had significantly higher intracellular phagocyte killing activity than did those in the control group ($P < 0.05$). No differences in the sizes of hepatocytes or their nuclei were observed in the fish from groups C and R. The nucleo-cytoplasmic ratio ranged from 0.39 (± 0.03; group R) to 0.42 (± 0.03, group C) in adult medaka exposed to the two dietary treatments ($P > 0.05$). The results indicated that the diet supplemented with resveratrol at a dose of 40 μg g^{-1} BW day^{-1} had no impact on the liver tissues. Typical hepatocytes with lipids and glycogen were observed to the same extent in the vacuoles. Moreover, no mortality or pathological changes were noted in the fish studied. The diet containing resveratrol helped to maintain disease resistance without affecting the liver tissue after ten days of feeding.

Keywords: histology, immunity, medaka, Oryzias latipes, resveratrol

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Introduction

Nutrition plays a significant role in maintaining good health. This is why dietary supplements that support the immune response have been enjoying great interest recently. Supplements are used increasingly in stressful conditions that can potentially suppress immunity (Ashley 2007, Magnadóttir 2006). Resveratrol (trans-3,4',5-trihydroxystilbene) is a secondary plant metabolite that belongs to a polyphenol group. Polyphenols have potential antioxidant, anti-inflammatory, antimicrobial, and immunomodulatory effects in organisms. A commonly recognized polyphenol with antioxidant properties is resveratrol (Aggarwal et al. 2004). Recently, it has been documented to activate immune system responses (Kowalska et al. 2017) and to protect liver cells against oxidative stress (Sahin et al. 2012). Valenzano et al. (2006) also showed that it extends the short life cycles of vertebrates. Preliminary

studies on diets supplemented with resveratrol (in a range of 0-80 μg g^{-1} body weight day^{-1}, feeding time 14 days) indicated that, as a strong antioxidant, it had a wide range of effects not only on adult fish but also on the offspring of spawners fed feed that had been supplemented with it (Kowalska et al. 2017). The immunostimulatory effect was noted at doses ? 40 μg g^{-1} body weight day^{-1}.

While promoting the principles of the 3Rs (Replacement, Reduction, and Refinement) in scientific research, it is also desirable to design experiments using model organisms. Their use in research provides opportunities to test expensive, innovative supplements. The economic aspect is especially justified in pioneering research. Research that facilitates the development of aquaculture has long been performed on the model species medaka (*Oryzias latipes*) (Kinoshita et al. 2009). While medaka is a popular aquatic model organism, it is also regarded as an excellent model organism in various scientific fields including nutrition, reproduction, and evolution (Yang and Tiersch 2009). Medaka is a domesticated fish, and it can live for over two years in laboratory conditions. Techniques for conducting research, handling the fish, and observations are straightforward because of some of its features such as small body size and short life cycle (Kinoshita et al. 2009).

To date, no analyses have been performed on the impact a low, effective dose of resveratrol has on the immune system and the hepatocyte morphometry of this model species at the end of its life cycle. Therefore, it was justified to examine the effects supplementing the diets of medaka aged two years with 40 μg resveratrol g^{-1} BW for ten days had on the cellular immune response and liver histology, including hepatocyte morphometry.

Material and methods

The medaka model fish (inbred Hd-rR strain) were obtained from culture at the Inland Fisheries Institute (Olsztyn, Poland) from fertilized egg stock from the National Institute of Natural Science (Japan). Rearing, breeding, and feeding were conducted according to the requirements of this species (Kinoshita et al. 2009). The test animals (60 fish, body weight BW = 0.49 g ± 0.01, total length TL = 3.39 mm ± 0.20) were selected from among adult medaka (aged two years) and placed in six glass tanks (ten fish $tank^{-1}$). Each tank (30 cm × 40 cm × 30 cm, 15 L water volume) was equipped with a heater (AQ 25, Aquael, Warsaw, Poland). The fish were kept under a natural photoperiod at 27°C. Temperature variation was < 0.5°C among all the tanks. The fish were reared in Iwamatsu's balanced salt solution BSS (0.65% NaCl, 0.04% KCl, 0.02% $MgSO_4$ $7H_2O$, 0.02% $CaCl_2$ $2H_2O$) prepared with distilled water (Yamamoto 1975). Feces were siphoned off daily, and the water in each tank was replaced daily with freshly prepared BSS water (2 L per day). The dissolved oxygen and water pH were > 5.5 ± 0.4 mg O_2 L^{-1} and 6.0–6.5, respectively. The fish were held for ten days, and no mortality was noted during the experiment.

After being stocked into the glass tanks, the fish were acclimatized and fed commercial feed (TetraMin, Germany) for four days. Then the fish were divided into two feeding treatment groups in triplicate (30 fish in each group, n = 3). The fish were either fed the same commercial diet (control, group C) or this diet supplemented with resveratrol at a dose of 40 μg g^{-1} BW day^{-1} (group R). The resveratrol was administered with the dry food. The food was supplemented with resveratrol by dissolving it in ethanol, spraying it into Petri dishes, and immediately mixing it with the dry food. The control group (group C) was fed the commercial diet prepared as above but only with the ethanol solvent. After it had been mixed, the food was kept for two hours at room temperature to allow the ethanol to evaporate. The feed was prepared daily. The fish were fed three times daily (09:00; 12:00; 15:00) at 3.7% of BW.

After the fish had been fed the experimental diet for ten days, all of them were anesthetized on ice for 1 min and then sacrificed. Livers (n = 7; two/three fish $tank^{-1}$) and spleens (n = 30; ten fish $tank^{-1}$) were dissected and collected for immunological or

histological analyses. The livers were fixed in Bouin's solution and embedded in paraffin blocks, and sections were cut with a rotary microtome (Leica, Bensheim, Germany). These were then stained with hematoxylin and eosin with standard procedures. Histological observations were done under a light microscope (Nikon E600, Tokyo, Japan). The analysis and structural measurements were performed with the NIS-Elements Br v. 3.2 (Nikon, Tokyo, Japan) computer program. The sizes of the hepatocytes and their nuclei (± 0.01 μm) and the nucleocytoplasmic indexes (the ratio of nucleus diameter to hepatocyte diameter) were measured in tissue samples from each individual. Histological measurements were taken from 50 cells, and nuclei measurements were taken from tissue samples from each individual.

The spleens were removed aseptically from the fish and pressed through a 60 lm nylon mesh into RPMI-1640 medium (Sigma-Aldrich, St. Louis, Missouri, USA) with L-glutamine and heparin (Biomed, Warsaw, Poland). Cell-mediated immunity was examined by isolating leukocytes from the spleens according to the method described by Siwicki and Dunier (1990) and Małaczewska et al. (2014). The metabolic activity of spleen phagocytes was determined by measuring the intracellular respiratory burst activity (RBA) after stimulation with phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich, St. Louis, Missouri, USA). The method described by Siwicki and Robak (2011) was used to measure the potential killing activity (PKA) of spleen phagocytes. The proliferative response of pronephric lymphocytes stimulated with mitogen concanavalin A (ConA, Sigma, NY, USA) or lipopolysaccharide (LPS, Sigma, NY, USA) was determined using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliu m bromide] (Sigma, NY, USA) colorimetric assay (Mosmann 1983), with modifications described for fish species by Siwicki and Robak (2011).

Student's t-test was performed on immunological and histological values using the GraphPad Prism program (GraphPad Software Inc., USA) to detect the differences between the treatments.

Results

The effect was determined of dietary resveratrol on the cell-mediated immunity of adult medaka. The proliferative lymphocyte activity increased significantly in fish fed feed supplemented with 40 $\mu g\ g^{-1}$ BW day^{-1} for ten days ($P > 0.05$) (Table 1). However, the dose tested and the feeding period did not change the metabolic activity or intracellular phagocyte killing activity of adult medaka ($P > 0.05$) (Table 1). The livers in all the fish were pink, and they occupied one-fourth of the abdominal cavities. The hepatic tissue consisted of typical hepatocytes containing nuclei in all the experimental groups (Fig. 1). Lipids and glycogen were observed to the same extent in the vacuoles. Lipid vacuoles formed coalescing droplets and occupied most of the cytoplasm. Irregularly shaped glycogen granules were observed in all of the fish. No pathological changes such as swollen cells with cloudy, granular cytoplasms, small, pyknotic nuclei, or the excessive accumulation of fat in cytoplasms were noted in any of the fish. Resveratrol in the medaka diet slightly increased the size of hepatocytes

Table 1

Cell-mediated immunity of adult medaka (*O. latipes*) fed experimental diets for ten days (mean ± SD; n = 10)

	Group C	Group R
Cellularity (psc $fish^{-1}$)	6.57^{a} ± 0.90	6.33^{a} ± 0.93
Metabolic activity of spleen phagocytes (RBA; OD 620 nm)	1.43^{a} ± 0.30	1.63^{a} ± 0.38
Potential killing activity of spleen phagocytes (PKA; OD 620 nm)	2.29^{a} ± 0.32	2.30^{a} ± 0.21
Lymphocyte proliferation stimulated by ConA (LP-ConA; OD 620 nm)	1.48^{a} ± 0.25	2.20^{b} ± 0.37
Lymphocyte proliferation stimulated by LPS (LP-LPS OD 620 nm)	1.23^{a} ± 0.27	1.11^{a} ± 0.13

[a]Fish fed diets without supplementation (group C) and with resveratrol supplementation at a dose of 40 $\mu g\ g^{-1}$ BW day^{-1} (group R)

Different superscript letters on the same line denote significant differences ($P < 0.05$)

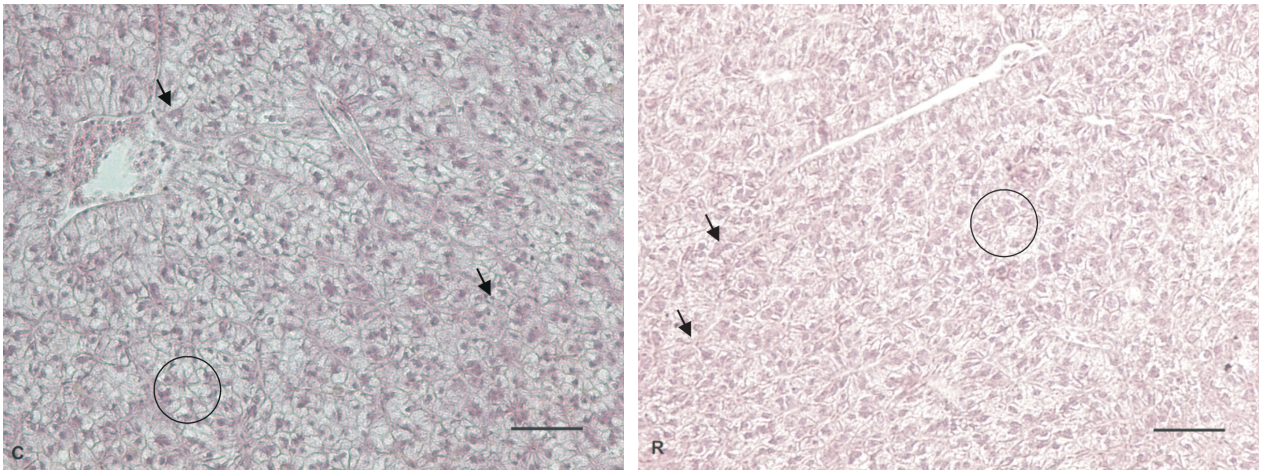

Figure 1. Histological picture of the liver of medaka (*O. latipes*) fed the control diet without supplementation (C) and the diet supplemented with resveratrol at a dose of 40 μg g^{-1} BW day^{-1} (R); arrows indicate small glycogen granules and lipid vacuoles of hepatocytes; the circle indicates the characteristic arrangement of liver cells (scale bar 50 μm).

(9.47 to 9.65 μm) (P > 0.05; Table 2). Moreover, no statistically differences were noted in the sizes of hepatocyte nuclei or in the value of the nucleo-cytoplasmic ratios (P > 0.05; Table 2) between dietary treatments.

Table 2
Comparison of hepatocyte morphometric characteristics of adult medaka (*O. latipes*) fed experimental diets for ten days (mean ± SD; n = 7)

	Group C	Group R
Size of hepatocyte (μm)	9.47 ± 0.96	9.65 ± 0.67
Size of nuclei (μm)	3.73 ± 0.20	3.69 ± 0.16
Nucleo-cytoplasmic ratio N/C	0.42± 0.03	0.39 ± 0.03

Fish fed diets without supplementation (group C) and with resveratrol supplementation at a dose of 40 μg g^{-1} BW day^{-1} (group R).

Discussion

The results of the experiment indicated that the ten-day dietary supplementation with resveratrol at a dose of 40 μg g^{-1} BW day^{-1} stimulated the immune response of medaka aged two years. It was confirmed that this dose increased lymphocyte proliferation by more than 30%. A longer feeding period (14 days) of feed supplemented with resveratrol (≤ 40 μg g^{-1} BW day^{-1}) also effected increased metabolic activity and intracellular phagocyte killing activity, and increased percentages of macrophages and their mean fluorescence intensities in medaka of reproductive age (9 months) (Kowalska et al. 2017). Our previous studies indicated that while increased cell-mediated immunity in medaka was proportional to the resveratrol dose in the diet, the effect of small quantities was not statistically significant. The immunostimulatory effect of resveratrol thus depended on the dose and the length of the period during which it was applied as a dietary supplement. A similar dependency was also noted in higher vertebrates, in which, depending on the concentration, it promoted lymphocyte proliferation and simultaneously induced the production of certain interleukins (Feng et al. 2002).

Resveratrol has a wide range of effects on the immune systems of fish. Castro et. al (2008) demonstrated that it inhibited inflammatory response, including cell migration, respiratory burst activity, prostaglandin (PG) synthesis, and the activity and expression of intra- and extracellular peroxidase activity in neutrophils. Moreover, resveratrol added as adjuvants to vaccines inhibited the expression of genes involved in inflammatory response including gene expression in kidney leukocytes of fish (Domínguez et al. 2013). Similarly in higher vertebrates, resveratrol blocked the synthesis and release

of pro-inflammatory mediators produced by monocytes and trombocytes (Das and Das 2007, Chen et al. 2014). The effect of resveratrol as an immunomodulator that inhibits inflammatory response has been determined. Our data suggested that resveratrol strongly modulated phagocyte and lymphocyte activity in medaka (Kowalska et al. 2017, this study). Simultaneously, the positive effect of resveratrol at a dose of 40 μg g^{-1} BW on the immune system did not distort hepatocyte structure in old medaka.

Supplementing feed with resveratrol did not affect the structure of liver tissue. Hepatocyte vacuolation and the storage of glycogen grains were similar in the histological picture of fish livers from both experimental groups. Differences in the sizes of fish hepatocytes were influenced by such factors as fatty acid and glycogen storage (Gravel and Vijayan 2006). This is probably why similar hepatocyte morphometric parameters were noted in the present experiment. To date, it has not yet been studied whether resveratrol as an inhibitor of enzymes associated with the synthesis of estrogens (Egan et al. 2004) involved in the process of vitellogenesis in fish (Verslycke et al. 2002) affects the size of female hepatocytes. Triebskorn et al. (2004) and Flippin et al. (2007) demonstrated such a relationship, i.e., the effect on hepatic growth in female medaka, but on other inhibitors of the enzymes above. However, even higher doses of resveratrol (80 μg g^{-1} BW day^{-1}) tested for a longer period (14 days) did not affect the hepatosomatic index of female medaka (Kowalska et al. 2017). It can be assumed that higher doses of resveratrol administered for longer periods do not effect a reduction in the number of glycogen granules in the hepatocytes. The lack of pathological changes in the livers and also the hepatocyte morphometry (this study) confirmed that resveratrol in the dose tested and for the period administered in the model organisms was not a factor of contaminant detoxification in the livers (Gravel and Vijayan 2006).

Resveratrol, as a polyphenol, has strong antioxidant properties and reduces the amount of free radicals in the body (Kohnen et al. 2007, Castro et al. 2008). These reactive oxygen species (ROS) are formed in naturally functioning cells during the process of respiration. However, a number of external biotic and abiotic factors, particularly environmental stress, intensifies the release of ROS by cells (so-called oxidative burst). Reacting with various cell structures, ROS violate the permeability of cell membranes, interfere with the transport of substances, and cause metabolic disorders. Phagocytes in fish produce large amounts of ROS after stimulation. Elevated ROS production during respiratory burst plays a protective role and can kill pathogens while simultaneously inducing oxidative stress (Reynolds et al. 2007). Resveratrol causes reductions in the production of ROS in leukocytes induced by PMA (phorbol myristate acetate) in fish (Castro et al. 2008). Therefore, ROS neutralization protects the body against their excessive activity and adverse changes in the body. It was found that decreased amounts of ROS in fish leucocytes incubated with resveratrol have a scavenging/protective effect on internal organs (Castro et al. 2008). As a polyphenol, resveratrol breaks the chain of adverse reactions in the body. The above examples are an advantage especially for individuals in the final stage of ontogenesis. Short term exposure to resveratrol beneficially effects medaka throughout ontogenesis; however, these effects are dependent on the concentration of resveratrol (this study, Kowalska et al. 2017).

Conclusions

The diet supplemented with resveratrol at a dose of 40 μg g^{-1} BW appeared to distinctly improve lymphocyte proliferation after ten days of feeding in medaka aged two years. This dose did not cause pathological changes in the liver. Moreover, resveratrol diet supplementation had no effect on any aspect of hepatocyte morphometry.

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Author's contribution. A.K. designed and performed the experiment, performed and analyzed the histological data, wrote the paper; J.M. performed and analyzed immunological data.

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