Influence of trans-resveratrol on macrophage and lymphocyte activity in rainbow trout (Oncorhynchus mykiss) – in vitro study

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Abstract

The objective of this study was to investigate the effect of trans-resveratrol, a potent antioxidant with anti-inflammatory and chemopreventive properties, naturally occurring in many fruits and plants on lymphocytes proliferation and also on macrophages metabolic and phagocytic activity. The aim of this study was to demonstrate the immunomodulatory effects of the compound on fish immunocompetent cells and determine the type of this interaction (immunosuppression or immunostimulation). Proliferative activity of lymphocytes was studied by MTT assay, and the respiratory burst was evaluated using the respiratory burst activity (RBA) test. Phagocytic killing was tested using the PKA test. The experiment have shown that trans-resveratrol suppressed blood B cells, while there was no significant influence on blood T lymphocytes. However, insignificant stimulatory effect occurred at the lowest concentration. In addition, the compound inhibited proliferation of T and B lymphocytes isolated from the organs. Importantly, trans-resveratrol caused stimulation of blood and organs macrophages phagocytic killing, and also increased the respiratory burst of macrophages isolated from organ. These results suggest a potential use of trans-resveratrol as an immunomodulator of innate immunity in fish. This is particularly important, as this kind of resistance plays leading role in protecting the body against infection. In comparison, adaptive immunity is slower and also much less precise.

Key words: trans-resveratrol, in vitro studies, immunomodulators, cellular immunity.

Introduction

Resveratrol (3,5,4’-trihydroxystilbene – RT) is a natural polyphenol and a member of the stilbene family. Its biological activity is associated mainly with its antioxidant properties, and it involves free radical scavenging and inhibition of free radical reactions. Resveratrol also exhibits oestrogenic activity and is classified as a phytoestrogen. Its chemical structure resembles that of the synthetic oestrogen diethylstilbestrol (trans-4,4’-dihydroxy-α,β-diethylstilbene). Resveratrol is produced by plants as part of a protective response to external stressors, including injury, cold stress, UV radiation, drought, heavy metal ions and pathogens (microorganisms and/or their metabolites).

3,5,4’-trihydroxystilbene was first isolated from the roots of white hellebore (Veratrum grandiflorum O. Loes) in 1939 by Takaoka [1]. The compound is also present in fruit, including grapes (Vitis vinifera) and red grapefruits (Citrus paradisi) [2]. Medicinal plants such as common knotgrass (Polygonum aviculare) and other knotgrass species are also a rich source of resveratrol. To date, the compound has been isolated from more than 72 plant species, and the search for other natural sources of resveratrol continues.

Resveratrol exists in two isomeric forms, cis and trans, which differ in biological activity. Trans-resveratrol (TRT) has four natural analogues: piceatannol (3,5,3’-trihydroxy-4’-methoxy-trans-stilbene), pinostilbene (3,3’,4’-trihydroxy-5-methoxy-trans-stilbene), pterostilbene (3,5-dimethoxy-4’-hydroxy-trans-stilbene) and rhapontigenin (3,5,3’-trihydroxy-4’-methoxy-trans-stilbene) [3]. It is considered to be a high biomedical activity compound [4, 5].

The metabolic activity of TRT was demonstrated in a study of rat leukocytes, which revealed that the analysed compound inhibits the production of eicosanoids responsible for inflammatory processes and thrombocyte aggregation [6]. Trans-resveratrol has cytoprotective, antiviral, antifungal, antibacterial and cytotoxic properties. It protects the circulatory system and prevents neurodegenerative processes. As a strong antioxidant, it delivers...
anti-inflammatory effects, and it is a nonselective inhibitor of cyclooxygenases COX-1 and COX-2. Much attention has been given to the chemopreventive activity of TRT and its ability to inhibit carcinogenesis in all three stages of inhibition, promotion and progression [4, 7].

Animal studies demonstrating the anti-aging effects of RT and its ability to lower the risk of obesity have set new directions for research. Trans-resveratrol extended the lifespan of mice fed a high-calorie diet and led to a general improvement in their health [7, 8]. There were found specific immunomodulatory effects of RT on the Sir2 gene expression family (SIRTs), enzymes with a very complex physiological role. The best known enzyme in this regard in this group is SIRT1, involved in many cellular processes by inhibiting the activity of transcription factors, e.g. nuclear transcription factor κB (NFκB). As a result, it shows anti-inflammatory, antitumor, and antiviral effects [9].

The analysed compound significantly increases the lifespan of simple organisms, including yeasts (Saccharomyces cerevisiae), roundworms (Caenorhabditis elegans) and fruit flies (Drosophila melanogaster). It prolongs the survival of short-lived fish such as Nothobranchius furzeri and improves their cognitive abilities and swimming efficiency. Trans-resveratrol is used in the production of dietary supplements and cosmetics with alleged anti-aging properties [7, 10-16].

Defence mechanisms and antioxidant potential are part of an organism’s immune system. The influence of RT on the immune system has been relatively poorly investigated, and this aspect has become the focus of recent research due to the growing interest in immune-based diseases. The results reported by various authors differ considerably; therefore, the data supplied by in vitro and in vivo studies have to be validated and systematized using various experimental models. Fish constitute a good animal model for investigating the influence of various factors on the immune system. In the evolutionary process, fish were the first class of vertebrates with a fully developed immune system containing elements of both innate and adaptive immunity. They were the first vertebrates to develop fully differentiated lymphatic organs whose functions and structure continued to evolve in other species. Therefore, in vitro as well as in vivo studies on immunocompetent cells are used to evaluate the ecotoxicity and immunotoxicity of pharmacological agents [17-20].

The aim of this study was to evaluate in vitro the influence of different concentrations of TRT on activity of macrophages and lymphocytes isolated from the blood and blood-forming organs of rainbow trout (Oncorhynchus mykiss).

Material and methods

Trans-resveratrol

A stock solution of trans-resveratrol (Sigma-Aldrich) with a concentration of 0.001 M in 1 M dimethyl sulfoxide – DMSO (POCH SA) was diluted to final concentrations of 1.25, 2.5, 10 and 20 µM with the RPMI-1640 medium (Sigma-Aldrich) on microtitre plates.

Fish

The experiment was performed on cells isolated from whole blood and blood-forming organs of four rainbow trout (Oncorhynchus mykiss) weighing 1-1.5 kg each. Fish were supplied by the Salmonid Breeding Farm of the Inland Fisheries Institute in Rutki (Poland). Fish were kept in 10 litre tanks with recirculated and oxygenated water at 15°C. Rainbow trout were anesthetized with Propiscin (INF – Inland Fisheries Institute in Olsztyn, Poland) at 0.2 ml·1·

Leukocyte isolation

Blood and organs (head kidney and spleen) were sampled aseptically. Blood was drawn from the tail vein into test tubes with heparin. Whole blood samples of 2 ml were combined with 2 ml of RPMI-1649 with L-glutamine and sodium carbonate (Sigma-Aldrich) and 1% antibiotic (Sigma-Aldrich). Blood-forming organs were isolated and homogenized with the addition of RPMI-1640 (Sigma-Aldrich) on sterile Petri plates. The homogenate was passed through a 25 µm mesh filter and centrifuged for 15 minutes at 2000 × g (4°C). The sediment was suspended in 4 ml of RPMI and layered on 3 ml of Gradisol G (Aqua-Medica, Łódź, Poland) or Gradisol L (Aqua-Medica, Łódź, Poland) (1 : 1 ratio) to isolate leukocytes by density gradient centrifugation. The samples were centrifuged for 40 minutes at 2000 × g (4°C), the interphase was separated, and the material was rinsed three times and centrifuged for 15 minutes at 2000 × g (4°C) each time. After the last centrifugation, the supernatant was discarded, and the sediment was suspended in 2 ml of RPMI with 10% fetal calf serum (FCS, Sigma-Aldrich). The same method was used to isolate blood cells. Tests were carried out in 96-well titre plates (Nunc, Denmark). Each well was filled with a suspension of 5 × 10⁶ cells·ml⁻¹. The plates were incubated at 22°C.

Respiratory burst activity test

The metabolic activity of monocytes and macrophages was determined in the respiratory burst activity (RBA) test after stimulation with phorbol myristate acetate (PMA, Sigma-Aldrich) according to the method described by Chung & Secombes [22] and modified by Chettri et al. [23]. Each well in a 96-well plate was filled with 100 µl of the cell suspension (from blood or organs) and incubated for 2 h at 22°C. Non-adherent cells were discarded by rinsing with RPMI-1640. Trans-resveratrol solutions were added, and the plates were incubated for 24 h. After incubation, 100 µl of PMA (1 µg ml⁻¹) with 0.1% nitro blue tetrazolium (NBT, Sigma-Aldrich) was added to
each well, and the mixture was incubated for 30 minutes at 24°C. Absolute alcohol was added to stop the reaction, and the plates were twice rinsed with 70% ethanol. The plates were dried at room temperature, and 120 µl of KOH and 140 µl of DMSO were added to each well to dissolve the produced formazan. Absorbance was measured at 620 nm with the Sunrice absorbance reader (Tecan, Austria). The results were expressed as mean extinction of PMA-stimulated cells relative to the control (non-stimulated) sample.

**Potential killing activity test**

The phagocytic activity of monocytes and macrophages was determined in the potential killing activity test (PKA) where NBT is reduced to insoluble formazan by phagocytic cells. The PKA test was conducted based on the procedure proposed by Rook *et al.* [24] and modified by Małaczewska *et al.* [25]. Each well in a 96-well titre plate was filled with 100 µl of cell suspensions (from blood or organs) and incubated for 2 h at 22°C. Non-adherent cells were discarded by rinsing with RMPI-1640. *Trans*-resveratrol solutions were added and the plates were incubated for 24 h. Suspensions of live *Aeromonas hydrophila* (1 × 10⁶ cells) in the amount of 100 µl were added with 0.1% NBT to each well containing 100 µl of cell suspension. The plates were incubated for 30 minutes at 24°C, and the contents were poured out. Absolute alcohol was added to stop the reaction, and the plates were twice rinsed with 70% ethanol. The plates were dried at room temperature, and 120 µl of KOH and 140 µl of DMSO were added to each well to dissolve the produced formazan. Absorbance was measured at 620 nm with the Sunrice absorbance reader (Tecan, Austria). The results were expressed as mean extinction of phagocytic cells relative to a control (non-stimulated) sample.

**Statistical analysis**

The results were processed statistically in the GraphPad Prism 6 application. Data were subjected to one-way ANOVA or its non-parametric equivalent. Statistical significance at \( p < 0.05 \) was estimated using Dunnett’s test or Dunn’s test (non-parametric analysis). The significance of differences are marked on charts with stars, according to

![Fig. 1. The influence of TRT on the metabolic activity of macrophages isolated from the blood and blood-forming organs of rainbow trout](image1)

![Fig. 2. The influence of TRT on the phagocytic activity of macrophages isolated from the blood and blood-forming organs of rainbow trout](image2)
Results

In the RBA test, the metabolic activity of PMA-stimulated blood monocytes was not statistically significant (Fig. 1). An analysis of the PKA activity test of blood monocytes incubated with different doses of TRT and suspensions of *Aeromonas hydrophila* in NBT demonstrated a significant increase in killing activity at 20 µM \( (p < 0.05) \) (Fig. 2).

In this concentration, TRT stimulated the RBA of macrophages from fish immunological organs \( (p < 0.05) \) (Fig. 1). The PKA of macrophages was stimulated within a broader range of TRT concentrations at 5 µM \( (p < 0.05) \), 10 µM \( (p < 0.05) \) and 20 µM \( (p < 0.01) \) (Fig. 2).

An increase in blood ConA-stimulated T cell proliferation was noted in response to the lowest dose of *trans*-resveratrol (1.25 µM), but not at the remaining concentrations (2.5 µM, 5 µM, 10 µM, 20 µM) \( (p < 0.05) \) (Fig. 2).

The proliferative capacity of LPS-stimulated B cells from the blood of rainbow trout was significantly lowered only in response to *trans*-resveratrol dose of 10 µM \( (p < 0.001) \). Proliferation was less suppressed at a dose of 2.5 µM \( (p < 0.05) \) (Fig. 3).

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**Metabolic activity of macrophages isolated from blood and organs**

Trans-resveratrol did not exert a significant influence on phagocytes isolated from blood. At lower concentrations (1.25, 2.5 and 5 µM), the studied compound had a somewhat inhibitory effect on the respiratory burst, but it acted as a stimulant at higher doses (10 and 20 µM). The metabolic activity of macrophages isolated from blood-forming organs was significantly stimulated only at a concentration of 20 µM, whereas the remaining concentrations produced results similar to those noted in the control sample. The inhibitory influence of TRT on the respiratory burst could be attributed to its antioxidant properties and the ability to scavenge free radicals. However, the predominant immunostimulatory effect of trans-resveratrol observed in this study is not consistent with published findings. Already at a dose of 1 µM, the analysed compound inhibited the respiratory burst of human leukaemia U937 cells differentiated with retinoic acid and vitamin D 

Similar observations were made when THP-1 cells infected with *Chlamydia pneumoniae* were exposed to TRT concentrations of 1 and 10 µM [32]. A study of mouse peritoneal macrophages treated with TRT doses of 3 and 30 µM delivered similar results [33]. The stimulation of the respiratory burst in rainbow trout macrophages could be attributed to the fact that free radicals also play a positive role by controlling various immune responses, stimulating T cells and inducing the adhesion of leukocytes to vascular endothelium, thus promoting leukocyte transport from the circulatory system to the site of inflammation.

**Phagocytic activity of macrophages isolated from blood and organs**

This experiment demonstrated that at concentrations of 2.5-20 µM, TRT stimulated the phagocytic activity of macrophages isolated from blood and immunological organs, but its stimulatory effect was statistically significant only at 20 µM for blood and 5, 10 and 20 µM for organs. Our results differ from the findings reported in other studies where the stimulatory influence of TRT was noted only at concentrations of 1-10 µM, whereas concentrations of 10 µM and higher had an inhibitory effect on phagocytic activity. The stimulatory influence of low concentrations of TRT was reported in a study on the intracellular killing of *Candida albicans* by human macrophage-like cells. Phagocytosis was inhibited when TRT was applied at a dose of 10 µM. The phagocytic activity of peritoneal exudate macrophages directed against *Kluyveromyces lactis* was inhibited by concentrations of 10-100 µM [34]. The phagocytosis of *Escherichia coli* and *Staphylococcus aureus* was inhibited by THP-1 cells of the human monocytic leukaemia cell line when exposed to a trans-resveratrol dose of 10 µM [35]. The results of our study suggest that in fish, the tested concentrations of TRT do not exert an immunosuppressive influence on macrophages. This is a very important observation because the immune system of fish relies mainly on non-specific responses and the phagocyte system. TRT could stimulate Toll-like receptor (TLR) signalling pathways through pathogen-associated molecular patterns (PAMPs) or by increasing TLR expression. The analysed compound could also regulate the expression of other families of pathogen recognition receptors (PRRs) such as scavenger receptors (SRs) and C-type lectin receptors (CLRs) C [35]. The examined polyphenol could also intensify chemotaxis by stimulating the release of chemotactic factors.

**Proliferative activity of lymphocytes isolated from blood and organs**

An analysis of TRT’s effect on the proliferation of rainbow trout lymphocytes revealed that the examined compound is a weak immunomodulator for T cells isolated from blood. Despite an absence of statistically significant results, TRT’s influence on this subpopulation of lymphocytes cannot be completely ruled out. This lack of statistical significance could be attributed to the fact that specific immune responses in fish are slower and less targeted than in mammals. In a study by Boscolo *et al.* [27], trans-resveratrol doses of 0.1 and 10 µM had only a minor stimulatory effect on human T cells. In our study, the lowest concentration of TRT (1.25 µM) weakly stimulated lymphocytes isolated from the blood of rainbow trout, and it exerted a minor suppressive effect at the remaining concentrations. The immunostimulatory effect of the analysed compound could be attributed to the release of TH1 cytokines such as IL-2 and IFN-γ, which intensifies cell proliferation [28], as well as enhancing production of IL-1, which also stimulates T cell proliferation. The inhibitory influence of TRT could result from its ability to suppress ribonucleotide reductase and DNA polymerase, the key enzymes involved in DNA synthesis, as well as its ability to arrest the cell cycle in phase S/G2 due to the accumulation of cyclins E and A [36-38]. The anti-proliferative properties of TRT could also be attributed to its antioxidant activity because reactive oxygen species participate in cell responses to cytokines and growth factors [29]. The inhibitory influence of the analysed compound could result from its ability to suppress the expression of NF-κB, a compound that controls the transcription of genes encoding the immune response, in stimulated T cells [39].

Trans-resveratrol did not stimulate the proliferation of LPS-stimulated B cells isolated from blood. The analysed compound significantly inhibited cell proliferation at a concentration of 10 µM. Similar results were reported in another study where the proliferation of B cells from the blood of healthy volunteers was inhibited at the same concentration of trans-resveratrol. The above could be attributed to an increase in the activity of caspase 3, an
enzyme involved in cell apoptosis [40] and responsible for the degradation of numerous substrates (e.g. topoisomerase I) and DNA.

Trans-resveratrol had a much stronger immunomodulatory effect on lymphocytes isolated from organs. In fish, the head kidney and the spleen are the major lymphatic organs. Younger individuals are protected mainly by blood cells and macrophages from external integuments, whereas in adults, the head kidney and the spleen constitute the backbone of the immune system. In the present study, all concentrations of TRT significantly inhibited the proliferation of T cells isolated from organs. Similar results were reported by Sharma et al. [41], who found that TRT at concentrations of 1, 5, 10 and 20 µM had an inhibitory effect on splenocyte proliferation in mice. Those findings could suggest that TRT suppresses the immune response by stimulating macrophages to produce IL-10 (suppressor cytokine), which prevents lymphocyte activation. The cited authors also demonstrated that TRT can reduce the expression of the molecule CD28 on the surface of Th cells. The said molecule stimulates Th cells and initiates the second activation signal by interacting with the molecule CD80 on an antigen-presenting cell (APC) whose expression was also reduced by TRT. This is an important consideration since when the second activation signal is not generated, lymphocytes become anergic and they can no longer be stimulated by antigens. According to some reports, mouse splenocytes are stimulated by low doses of TRT in the range of 0.75-6 µM [28], 6.25 µM and 12.5 µM [29], but the above effect was not confirmed in our study of rainbow trout.

Trans-resveratrol suppressed the proliferation of B lymphocytes isolated from fish organs and blood. The above effect was observed at all concentrations, but it was statistically significant only at a dose of 5 µM. In the work of Sharma et al. [41], TRT inhibited the proliferation of mouse splenocytes at concentrations of 1, 5, 10 and 20 µM. Similarly to T cells, this reduction in proliferative activity could be attributed to enhanced production of IL-10. The tested compound could also lower the expression of costimulatory molecules CD40 that participate in the activation process, as demonstrated in mouse macrophages. Therefore, the same mechanism of action could be expected in B cells from fish [41]. Similarly to B cells isolated from blood, TRT could also enhance the activity of caspase 3 [40].

Conclusions

The results of our in vitro study to evaluate the influence of TRT on lymphocytes and phagocytes isolated from the blood, spleen and head kidney of rainbow trout (Oncorhynchus mykiss) lead to the following conclusions:

Trans-resveratrol did not induce a dose-dependent response in the immunocompetent cells of rainbow trout.

Trans-resveratrol suppressed in vitro the proliferation of B cells isolated from the blood of rainbow trout and the proliferation of T and B cells isolated from the blood-forming organs of rainbow trout.

The analysed compound did not exert a significant influence on T lymphocytes isolated from fish blood. It had a minor stimulatory effect on T cells at lower concentrations and a somewhat inhibitory effect in the remaining doses. It did not significantly enhance the respiratory burst of blood phagocytes, but it exerted a somewhat stimulatory effect at higher concentrations.

This compound stimulated the killing activity of phagocytes isolated from blood and organs and the metabolic activity of macrophages isolated from blood-forming organs.

Our results pave the way for further research into the influence of TRT on the immune system of fish. It is the first comprehensive study examining the activity of immunocompetent cells from blood and organs relative to the immune system of fish. Trans-resveratrol influences both non-specific (stimulation) and specific (suppression) immune responses, and it could stimulate non-specific defence mechanisms that play a key role in fish immunity. The adaptive immune system is less prominent due to slow lymphocyte proliferation, limited immunological memory, inhibited affinity maturation and immunoglobulin secretion in fish. The results of this study suggest that fish constitute a good model for research into the efficacy of TRT in the prevention and treatment of immune deficiencies. The immunomodulatory properties of the analysed compound could be used to stimulate disease resistance in both fish and mammals.

The authors declare no conflict of interest.

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