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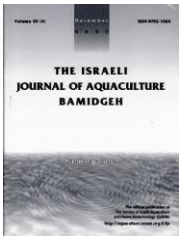

Cyclopamine Induced Expression of Immune-related Genes in Rainbow trout (*Oncorhynchus mykiss*) Head Kidney Leukocytes

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Keywords: cyclopamine; rainbow trout; leukocyte; immune responses; gene expression

Abstract

This study investigated the effect of cyclopamine, a hedgehog signaling inhibitor, on immune-related gene expression in rainbow trout head kidney leukocytes *in vitro*. At 1h sampling time COX 2, IFN-2, TNF-α, C3, MHC-II, Il-1β, IL-12, IL-10, and IL-6 increased significantly in groups exposed to cyclopamine at 30 µg/ml. However, in groups exposed to 10 and 20 µg/ml cyclopamine, the expression of these genes decreased significantly. At 4h sampling time, levels of COX-2, IFN-1, IFN-2, TNF-α, TGF-β, IgT, and MHC-II in groups exposed to 20 µg/ml cyclopamine increased. At 8h sampling time, COX2, IFN-Reg, and TNF-α expression in groups exposed to 10 and 30 µg/ml cyclopamine increased significantly compared to those in the controls. Conversely the expression decreased significantly for almost all other genes. At 12h sampling time, almost all genes increased significantly in groups exposed to 10 µg/ml cyclopamine compared to those in the controls. Notably, after 24h, the gene expression in all groups significantly decreased compared to the controls. Our results suggest that the activation of cyclopamine can be a useful tool for the examination of immune-related gene expression in the rainbow trout.

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Introduction

Cyclopamine is a steroidal alkaloid with antitumor activity capable of inhibiting hedgehog signaling (Chen, 2002). It is also a teratogen (Chen, 2016), inducing malformation or mutation in sheep (Siebert et al., 1991), mice (Chiang et al., 1996), and humans (Roessler et al., 1996). In the aquaculture industry, the use of medicinal plants has dramatically increased owing to their importance as alternatives to chemicals and antibiotics. *Veratrum* species, which contain cyclopamine, have immunostimulant and antimicrobial properties (Namba, 1993); however, to the best of our knowledge, *Veratrum* spp. has not been reported as an immunostimulant in fish. Owing to this lack of information this study focused mainly on *in vitro* transcriptomic analysis of pro-inflammatory cytokines and other immune-related genes in rainbow trout (*Oncorhynchus mykiss*) head kidney leukocytes.

Materials and Methods

Fish samples

Rainbow trout (weight, 53 ± 2.2 g; n = 3) were acquired from Kastamonu University, Fresh Water Fish Species Research and Innovation Center. Before the start of the experiment, the fish were acclimatized in a recirculation system in tanks held at 16°C and fed with a commercial diet for two weeks.

Leukocyte collection and RNA-cDNA analyses

All experiments were conducted according to the protocols in Kuhadyek (2017.09). Head kidney cells were collected according to Bilen et al. (2014). Leukocytes were separated using a two-layer (1050 and 1065) Percoll gradient and centrifuged at 1500 rpm for 50 min. Separated cells were transferred into a culture media RPMI 1640 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 5% fetal bovine serum and 1% streptomycin/penicillin (Invitrogen)] and adjusted to 1×10^7 cells/ml. The leukocyte cells were seeded into 24-well plates treated with 10, 20, and 30 µg/ml cyclopamine, and the plates were incubated for 1, 4, 8, 12, and 24 h at 25°C. A control was maintained for each time point. Each treatment and control had three replicates. Incubated cells were harvested at the time points stated above, and total RNA was extracted from the stimulated leukocyte cells using a BIOLINE kit (ISOLATE II RNA Mini Kit) according to the manufacturer's instructions. The concentration of all RNA samples was analyzed quantitatively and qualitatively using a multiscan spectrophotometer. Extracted total RNA was used for cDNA synthesis via reverse transcription from 1 µg mRNA using a BIOLINE kit (SensiFAST ™ cDNA Synthesis Kit). The primer sequences of all genes used in qPCR are listed in Table 1. The qPCR reaction was performed using SensiFAST ™ Probe NO-ROX Kit for each sample run in triplicate, as per the protocol described by Kinoshita et al. (2014). The comparative threshold cycle (C_T) method ($2^{-\Delta\Delta CT}$ method; Schmittgen and Livak, 2008) was used to analyze the expression level of genes using β-actin as the internal control gene.

Statistical analysis

One-way analysis of variance followed by DUNCAN tests were used to detect differences in gene expression between different stimulations at each sampling time using SPSS for Windows v. 23.0 program (SPSS Inc., Chicago, IL, USA). The accepted level of significance was $P < 0.05$.

Table 1. Gene-specific primers used for real-time qPCR analysis.

Gene	*Primer sequence*	*Amplicon size (bp)*	*References*
β-Aktin	F5′ ATGGAAGGTGAAATCGCC 3′ R5′ TGCCAGATCTTCTCCATG 3′	186	Sigh et.al. 2004
IL-1β	F5′ ACCGAGTTCAAGGACAAGGA 3′ R5′ CATTCATCAGGACCCAGCAC 3′	181	Awad et. al. 2011
IL-8	F5′CACAGACAGAGAAGGAAGGAAAG3′ R5′ TGCTCATCTTGGGGTTACAGA 3′	162	Awad et. al. 2011
TGF- β	F5′ AGATAAATCGGAGAGTTGCTGTG 3′ R5′ CCTGCTCCACCTTGTGTTGT 3′	275	Awad et. al. 2011
IL-12 Beta	F5′ GAACCCAGACGACGATGATT3′ R5′ GTTCAAACTCCAACCCTCCA 3′	190	Komatsu et. al. 2009
TNFα1	F5′ CAAGAGTTTGAACCTTGTTCAA 3′ R5′ GCTGCTGCCGCACATAGAC 3′	181	Panigrahi et. al. 2007
IL-10	F5′ CGACTTTAAATCTCCCATCGAC 3′ R5′GCATTGGACGATCTCTTTCTTC 3′	70	Raida et. al. 2011
COX-2	F5′ GGGCTTTGACATCCTCAACA R5′ CATCGGACAAGAACCCTTGA	73	Chettri et al 2011
IL-6	F5′ACTCCCCTCTGTCACACACC R5′ GGCAGACAGGTCCTCCACTA	91	Chettri et al 2011
TLR5	F5′ GGCATCAGCCTGTTGAATTT R5′ATGAAGAGCGAGAGCCTCAG	89	Raida ve Buchmann 2008
C3	F5′AGCTTGCTGACTGGCTTTGT R5′TCATAAACGGTGACCCCAAC	227	Sigh et al 2004
IGM	F5′AGTTCCACAGCGTCCATCTG R5′TACTGGGCCATGCATCTCTG	399	Sigh et al 2004
MHC-II	F5′ATGTCGATGCCAATTGCCTTCTA R5′TGTCTTGTCCAGTATGGCGCT	336	Sigh et al 2004
iNOS	F5′CGAATGGAGCTATCGTCAGACC R5′CGGGAACGTTGTGGTCATAATACC	234	Sigh et al 2004
IgT	F5′ AGCACCAGGGTGAAACCA R5′GCGGTGGGTTCAGAGTCA	72	Raida ve Buchmann 2008
IFN1	F5′AGAATGCCCCAGTCCTTTTCC R5′GACTTTGTCCTCAAACTCAGCATCA	71	Ooi et al 2008
IFN2	F5′GTTGAGGGCCATGGATGTG R5′TCCAGCCCATCAAGCAGAA	68	Ooi et al 2008
IFN-Reg	F5′ACACCGACTACTGGTCACTGACAAC R5′CAAGAAGTGGGCATGTGATCTGT	76	Ooi et al 2008

Results

Transcriptional responses analyzed by qPCR assay for 17 different immune response genes in head kidney leukocytes of the rainbow trout are given in Figs. 1, 2, 3, and 4. There were increases in the expression of IFN-2, TNF-α, C3, MHC-II IL-1β, IL-12 IL-10, and IL-6 genes compared to the controls at 1h sampling time in the 30 μg group ($P < 0.05$). At 4h sampling time, the expression of COX 2, IFN-1, IFN-2, TGF- β, TNF-α, IgT, and MHC-II genes increased in groups exposed to 20 μg/ml cyclopamine. At 12h sampling time, the expression of all genes significantly increased in groups exposed to 10 μg/ml cyclopamine compared to those in controls, with the exception of of IFN-2, iNOS, and TLR5. Notably, at the end of the study (24-h sampling time) gene expression in the experimental groups was significantly lower than that in the controls ($P < 0.05$).

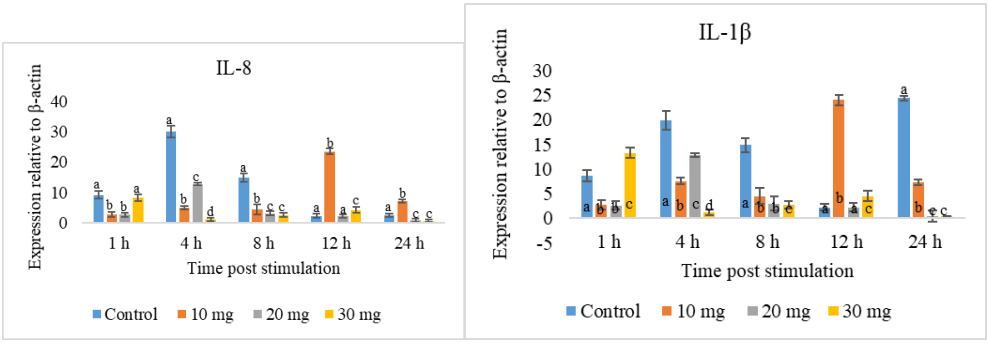

Fig. 1. Expression levels of pro-inflammatory cytokines related genes

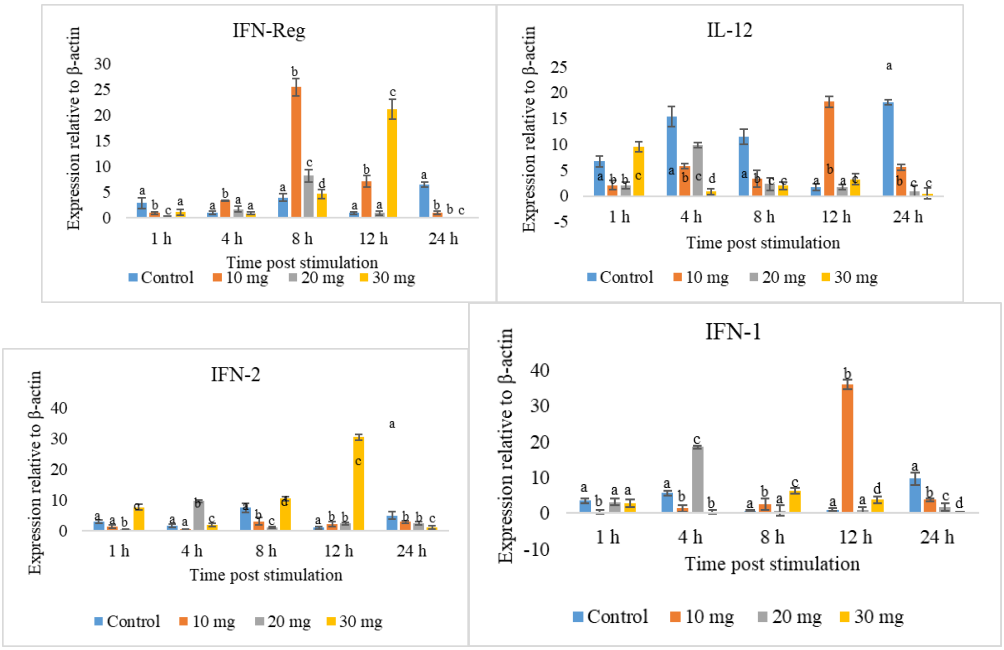

Fig. 2. Expression levels of IL-12 and IFN genes expressed for protection against parasites, viruses and bacteria

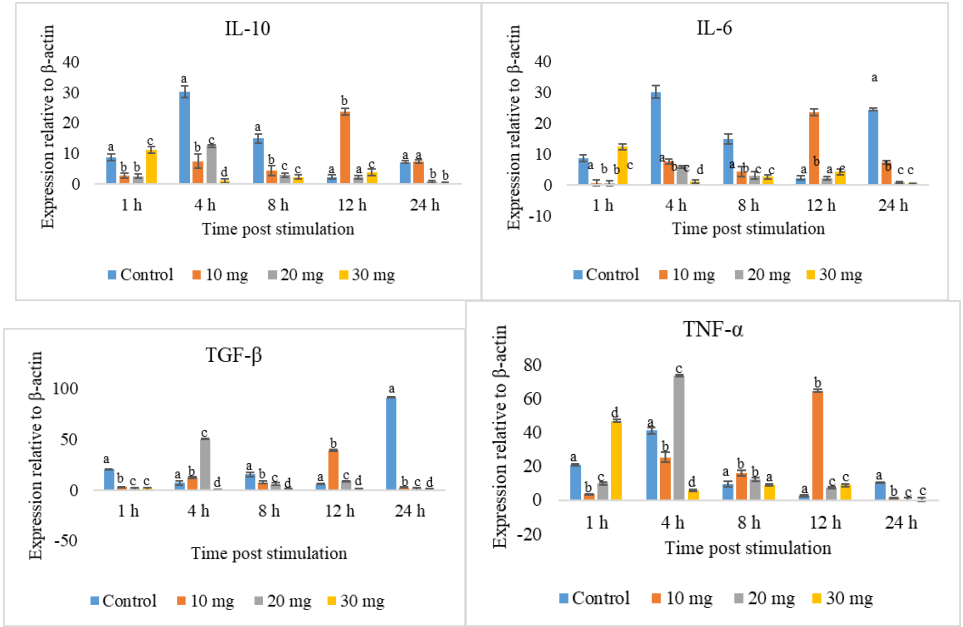

Fig. 3. Expression level of respiratory burst activity, pleiotropic cytokine, multifaceted cytokine-related genes

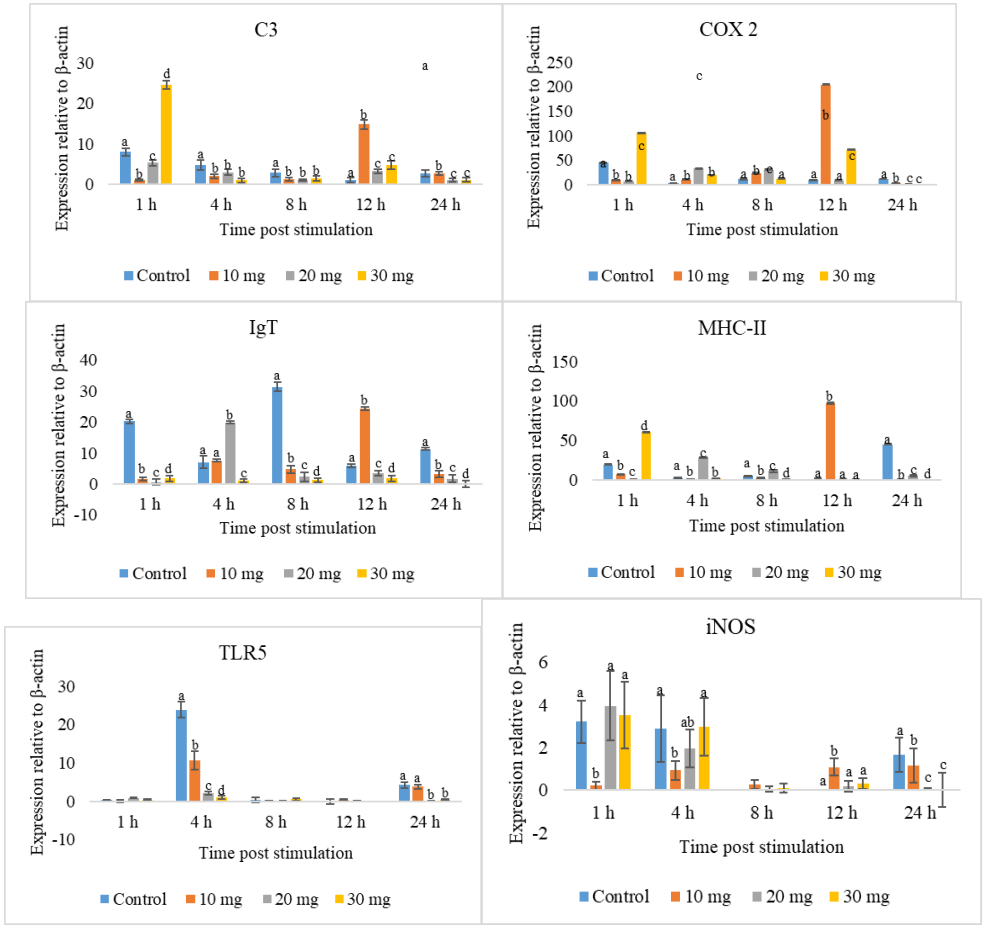

Fig. 4. Expression levels of other immune-system-related genes

Relative expression (mean ± SE; n = 3) of cytokines and other immune-related genes at different time points in rainbow trout head kidney leukocytes stimulated with cyclopamine (10, 20, and 30 µg/ml). Bars with different superscript letters indicate a significant difference in expression levels between stimulated cells and unstimulated control cells at a time point (α = 0.05).

From the results, an inverse relationship between dosage and time is evident. At the beginning of the study, gene expression was elevated in groups exposed to 30 µg/ml cyclopamine, but with time, the expression decreased; in contrast, in groups exposed to 10 µg/ml cyclopamine, gene expression increased over time.

Discussion

This study demonstrates that cyclopamine affects rainbow trout head kidney leukocytes, and our findings provide a comparative understanding of the immunomodulatory effects of different doses of cyclopamine in rainbow trout.

In the process of infection and inflammatory response the kidney, as an immuno-competent organ, transcripts pro-inflammatory cytokines, IL-1β, IL-6, IL-18, TNF-α and TNF-N. Cytokines play various roles in the immune system against bacterial or viral diseases. IL-1β and IL-8 are a pro-inflammatory cytokine and activating the lymphocytes during immune response (Low et al., 2003). IL-6 is a pleiotropic cytokine and plays a regulatory role in processes, such as immunoglobulin synthesis, T-cell differentiation, acute phase reaction and haematopoiesis (Øvergård et al., 2012). IL-10 is a multifaceted cytokine that is produced by, and affects a variety of cell populations, including macrophages and T, B, and NK cells (Harun et al., 2011). IL-12 controls cell-mediated immune responses and produces IFN 2 (IFN-γ) from NK cells to provide immune protection against parasites, viruses and bacteria.

In our study, IL-1β and IL-8 gene expression level increased on 12h sampling time in the all experimental groups compared to control. IL-6, IL-10, and IL-12 gene expression levels were higher than the control only on 12h (Fig. 1). IL cytokines gene expression level showed a similar finding which has been reported in trout fed caper (Bilen et al., 2016a), whereas a decrease in IL-1β gene expression in rainbow trout fed black cumin was observed by other authors (Altunoglu et al., 2017).

IFN-reg, IFN-1, and IFN-2 are responsible for the generation of immune response against viral infections (Ooi et al., 2008). In this study, a significant increase in expression of all IFN genes was observed compared to that in the control group on 1h, 4h, 8h, and 12h sampling time-related IL-12 gene expression levels (Fig. 2).

Along with its essential roles, such as those in lymphocyte proliferation and differentiation, TGF-β maintains immune system tolerance. TGF-β regulates immune responses by controlling chemotaxis in NK cells, dendritic cells, lymphocytes, mast cells, macrophages, and granulocytes (Li et al., 2006). TGF-β gene expression increased significantly on 4h and 12h sampling time in the 20 and 10 µg/ml cyclopamine groups, respectively. These results show that cyclopamine has a stimulating effect on the immune system of rainbow trout. TNF-α is another cytokine that regulates respiratory burst activity. Increases in its activity were remarkable on 1 h sampling time in 30 µg/ml group, 4 h sampling time in 20 µg/ml group, and 12 h sampling time 10 µg/ml group when compared to control in the present study (Fig. 3). Increased TNF-α expression was reported in response to Fenugreek (*Trigonella foenum graecum*) in gilthead sea bream *Sparus aurata* L. (Awad et al. 2015). TLR5 gene expression level was low in all experimental groups compared to the control group. Complement factor C3 markedly increased on 30 µg/ml group compared to that in the control and other experimental groups (Fig. 4).

Cyclooxygenase gene COX-2 catalyzes the initial reactions in prostanoid biosynthesis and produces the common prostanoids precursor and the increase in COX2 level is a sign of the struggle with secondary infections (Ishikawa et al., 2007). It generally plays a role against parasitic infections (Holland et al., 2003). Similarly, C3 activates the complement system in generating immune response against parasites (Sigh et al., 2004). Inducible nitric oxide synthase (iNOS) plays a role in production of nitric oxide against bacteria (Campos-perez et al., 2000).

Cyclopamine, especially in the 20 and 30 µg/ml groups at 1h and 4h, elevated iNOS gene expression when compared to control and consequently could indicate an increased activity against the bacteria. Major histocompatibility complex (MHC) molecules are the key players in initiating immune responses towards invading pathogens. In the present study, the increased MHC II gene expression showed that the groups receiving 10, 20, and 30 µg/ml had an elevated immune response until 12h (Fig. 4).

[Th]is study, which was performed in vitro, demonstrated the effect of cyclopamine on immune system-related genes. Our results suggest that cyclopamine effectively increased immune gene expressions levels on rainbow trout, and that natural plant extracts containing cyclopamine can be used as a natural immunostimulants.

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