TNU Journal of Science and Technology 231(01): 223 - 231

DOSE, SOURCE, AND TEMPORAL DYNAMICS OF GENE EXPRESSION IN LPS-INDUCED INFLAMMATORY RESPONSES IN FISH LEUCOCYTE CELL MODELS

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ARTICLE INFO

Received: 19/02/2025
Revised: 02/12/2025
Published: 02/12/2025

KEYWORDS

LPS
Cytokine
Inflammatory response
Cyprinus carpio
Immunostimulant

ABSTRACT

Lipopolysaccharides, endotoxins from Gram-negative bacteria, are widely used as immunostimulants to investigate inflammatory responses in fish. This study examined the effects of lipopolysaccharides dose, source, and exposure time on pro- and anti-inflammatory responses gene expression in head kidney leukocytes of common carp *Cyprinus carpio*. Leukocytes were exposed to lipopolysaccharides at 0, 10, and 100 µg/mL for 4 and 24h using two sources O11 and O26. Cell viability and the expression of *inos*, *tnf-α*, *il-1, il-6*, and *il-10* were measured. Results demonstrated a dose-dependent decline in viability, with significant reductions observed at 100 µg/mL. Lipopolysaccharides strongly induced pro-inflammatory cytokines, particularly *il-1* which peaked at 4h and remained elevated at higher doses after 24h. The anti-inflammatory cytokines *il-10* was also markedly upregulated at 4h, especially at 100 µg/mL. Lipopolysaccharides sources influenced response magnitude, with O11 eliciting stronger inflammation than O26. Overall, LPS triggered dose- and time-dependent cytokine regulation, highlighting a shift from robust pro-inflammatory activation to anti-inflammatory modulation.

LIỀU LƯỢNG, NGUỒN GỐC VÀ BIẾN ĐỘNG THEO THỜI GIAN CỦA BIỂU HIỆN CÁC GENE TRONG PHẢN ỨNG VIÊM DO LPS KÍCH THÍCH TRÊN MÔ HÌNH TẾ BÀO BẠCH CẦU Ở CÁ

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THÔNG TIN BÀI BÁO

Ngày nhận bài: 19/02/2025
Ngày hoàn thiện: 02/12/2025
Ngày đăng: 02/12/2025

TỪ KHÓA

LPS
Cytokine
Phản ứng viêm
Cyprinus carpio
Chất kích thích miễn dịch

TÓM TẮT

Lipopolysaccharide, nội độc tố từ vi khuẩn gram âm, được sử dụng rộng rãi như một chất kích thích miễn dịch để nghiên cứu các phản ứng viêm ở cá. Nghiên cứu này đánh giá ảnh hưởng của liều lượng, nguồn gốc và thời gian tiếp xúc với lipopolysaccharide lên sự biểu hiện gene liên quan đến phản ứng viêm và kháng viêm trong bạch cầu thận trước của cá chép (*Cyprinus carpio*). Bạch cầu được phơi nhiễm với lipopolysaccharide ở các nồng độ 0, 10 và 100 µg/mL trong 4 và 24 giờ với hai nguồn O11 và O26. Khả năng sống của tế bào và sự biểu hiện của các gene *inos*, *tnf-α*, *il-1*, *il-6* và *il-10* được xác định. Kết quả cho thấy khả năng sống của tế bào giảm phụ thuộc vào liều, với sự suy rõ rệt tại 100 µg/mL. Lipopolysaccharide gây cảm ứng mạnh các cytokine tiền viêm, đặc biệt là *il-1*, đạt đỉnh sau 4 giờ và vẫn duy trì ở mức cao hơn tại liều cao sau 24 giờ. Cytokine kháng viêm *il-10* cũng tăng cường biểu hiện đáng kể sau 4 giờ, đặc biệt ở nhóm 100 µg/mL. Nguồn lipopolysaccharide ảnh hưởng đến mức độ đáp ứng, trong đó O11 gây phản ứng viêm mạnh hơn O26. Nhìn chung, LPS kích hoạt điều hòa cytokine phụ thuộc liều và thời gian, thể hiện sự chuyển đổi từ đáp ứng tiền viêm mạnh sang điều hòa chống viêm.

DOI: https://doi.org/10.34238/tnu-jst.12091

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1. Introduction

The immune system of fish, comprising innate and adaptive components, is fundamental to their defence against pathogenic challenges in aquatic environments. Fish rely more heavily on their innate immune system compared to mammals as adaptive immunity in teleosts is comparatively slower and less robust [1]. Innate immunity in fish includes mechanisms such as phagocytosis, mucosal defences, and the production of antimicrobial peptides and cytokines, which collectively form the first line of defence [2].

Lipopolysaccharides (LPS), endotoxins derived from the outer membrane of Gram-negative bacteria like *Escherichia coli*, serve as potent immunostimulants in fish immune studies. Research employing LPS has significantly advanced the understanding of fish immune mechanisms, aiding in the evaluation of immunostimulants and immune modulators relevant to aquaculture [3], [4]. The inflammatory response induced by LPS is mediated primarily through Toll-like receptor 4 (TLR4), which, in association with MD-2, activates downstream signalling pathways such as MyD88-dependent and TRIF-dependent cascades. These pathways regulate nuclear factor-kappa B (NF-κB) and interferon regulatory factors (IRFs), leading to the production of key pro-inflammatory cytokines like interleukin-6 (IL-6), interleukin-1 beta (IL-1β), tumour necrosis factor-alpha (TNF-α), reactive oxygen species (ROS), and nitric oxide (NO) [3], [5]. In teleost fish, LPS has been shown to elicit a strong upregulation of cytokines such as IL-1β, IL-6, IL-8, and TNF-α, as well as enzymes like cyclooxygenase (COX) and lipoxygenase (LOX), demonstrating its immunostimulatory potential [4]. Studies in *Oncorhynchus mykiss* macrophages demonstrated that LPS significantly increased TNF-α and IL-1β expression, highlighting its effectiveness as a pro-inflammatory agent [6]. LPS stimulation also modulates key molecular pathways associated with immune signalling. For example, the phosphorylation of TLR4 at tyrosine 672 has been shown to activate downstream pathways, including ERK1/2 and c-FOS, which enhance IL-6 and IL-12p40 production, demonstrating a precise regulatory mechanism of inflammatory signalling [7].

Fish exhibit a higher tolerance to LPS compared to mammals, which are highly sensitive to its toxic effects. While nanogram-level doses can trigger significant immune responses in mammals, fish such as stinging catfish *Heteropneustes fossilis*, common carp *Cyprinus carpio*, and *Mugil cephalus* often require much higher doses, typically in the range of 50 – 100 μg/mL, to elicit comparable responses [8] – [10]. In vitro studsies using fish-derived cell cultures, such as head kidney leukocytes (HKLs) and macrophages, provide valuable insights into the cellular and molecular mechanisms of immune responses. These models offer a cost-effective, controlled, and ethical alternative to in vivo experiments, aligning with the principles of the 3Rs (Replacement, Reduction, Refinement) [11].

In light of previous findings, the current study investigates the effects of LPS source, dose, and exposure time on the expression of genes involved in pro- and anti-inflammatory responses in *C. carpio* HKLs. By assessing cytokine expression profiles, this research aims to elucidate the molecular mechanisms underlying acute LPS-driven inflammation, providing insights into the optimisation of immunostimulant use in aquaculture.

2. Materials and methods

2.1. Fish and Husbandry

Healthy common carp (*Cyprinus carpio*), with an average body weight of 120.3 ± 12.4 g, were sourced from a commercial aquaculture facility. Upon arrival, fish were acclimatised for two weeks in the wet laboratory under controlled conditions (temperature 27.0 ± 1.0 °C; dissolved oxygen 7.5 mg/L; pH 7.5 ± 0.2).

2.2. Head kidney leukocyte isolation

Head kidney leukocytes were isolated using a protocol adapted from Nguyen et al. [4]. Head kidneys were aseptically excised and homogenised through a 100 µm nylon mesh (Corning® Cell Strainer, USA) into RPMI-1640 medium (Sigma-Aldrich, USA) containing 1% penicillin-streptomycin (1.0 - 3.0% streptomycin, 0.1 - 1.0% penicillin, P4333, Sigma-Aldrich, USA). The homogenate was centrifuged at 800× for 7 minutes at 25 °C. Red blood cells were lysed with a buffer (4.14 g NH_4Cl, 0.5 g $KHCO_3$, 0.018 g EDTA in 500 mL distilled water), and leukocytes were resuspended in RPMI-1640 medium at a concentration of 10^7 cells/mL for further experiments.

2.3. Lipopolysaccharide (LPS) source trial

To assess the effect of different LPS sources, HKLs were cultured in RPMI-1640 medium supplemented with 1% phytohaemagglutinin (Gibco™), 10% fetal bovine serum (FBS; Sigma-Aldrich, USA), and 1% HEPES (20 mM). LPS compounds from *E. coli* strains O111:B4 and O26:B6 (Sigma-Aldrich, USA), named as O11 and O26 treatments, were prepared as a 10 mg/mL stock solution in phosphate-buffered saline (PBS). Cells were adjusted to a concentration of 1×10^7 cells/mL in RPMI-1640 and exposed to LPS concentrations of 0, 10, and 100 µg/mL (named Ctrl, LPS10, and LPS100) for 4h at 27 °C. After exposure, cells were harvested for gene expression analysis, and viability was assessed using the MTS assay (CellTiter 96® Aqueous One Solution, Promega). The LPS source with the most consistent results was selected for subsequent experiments.

2.4. LPS dose-response and time-course trial

HKLs were adjusted to a concentration of 10^7 cells/mL in RPMI-1640 medium following the method of Nguyen et al. [4]. Cells were exposed to LPS (O111:B4) at doses of 0, 10, and 100 µg/mL (named as Ctrl, LPS10, and LPS100) at 27 °C. Samples were collected at 4- and 24-hours post-exposure. Cells were then harvested by a centrifugation at 2000 × g for 10 minutes at 4 °C and subsequently processed for RNA extraction and gene expression analysis.

2.5. Gene expression analyses

The expression of candidate genes was evaluated following the methodology outlined by Nguyen et al. [4]; in which, total RNA was individually extracted from HKLs obtained from a batch of six fish for each experimental condition. Primers specific for the target genes including *inos* (Inducible nitric oxide synthase), *il-8* (interleukin 8), *il-1* (interleukin 1), *tnf-α* (tumour necrosis factor-alpha 1), *il-6* (interleukin 6), and *il-10* (interleukin 10) were designed and validated using Primer3 and Amplifx software, based on sequences available for *Cyprinus carpio* in GenBank. Primer sequences, including forward and reverse strands, and their functions are detailed in Table 1. The amplification efficiency of each gene was confirmed prior to analysis. Each primer pair was tested for target specificity using conventional PCR. PCR products were analyzed by agarose gel (1%) electrophoresis to confirm clear, specific bands of the expected size. Housekeeping genes, specifically 40s and 18s [12], were used to normalise gene expression. PCR amplification was conducted in triplicate for each sample using the SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA). The qPCR procedure adhered to the protocol described in Nguyen et al. [4]. For quantification, a standard curve generated from pooled cDNA across all samples was utilised to calculate PCR efficiency and normalise transcript levels. Relative RNA levels for each gene were quantified using densitometry by measuring photostimulated luminescence values, with analyses performed using StepOne Software v2.1. Ratios of target gene expression to housekeeping gene expression were calculated for each sample, enabling the comparison of expression levels between experimental groups.

Table 1. *Primer sequences for amplification of genes involved in pro- and anti-inflammatory response in common carp*

Genes	Function	GenBank No.	Primer Sequence
il-8	Interleukin 8	EU011243	Fw: TTGAAACAGAGAGCCAACGCATT Rv: GCTGGTGTTTTGTTGGCAATGA
il-1	Interleukin 1	AJ245635	Fw: ACAGTAAGACCAGCCTGACCT Rv: AGGCTGTGCTTCCTTTTGTG
tnf-α	Tumour necrosis factor-alpha 1	AJ311800	Fw: GTGATGGTGTCGAGGAGGAA Rv: TCCGCCTTCCTGATTGTTCT
inos	Inducible nitric oxide synthase	XM_019116975	Fw: TGTTGTCACCAGCACCTTTG Rv: CAGCGCTGCAAACCTATCAT
il-6	Interleukin 6	KC858890	Fw: TCTTCCTGTCTGCCGTACTG Rv: AACCTCGTCCCCAGATGTTT
il-10	Interleukin 10	JX524551	Fw: GCGCTTTTACTTGGACACCAT Rv: TCCCGCTTGAGATCCTGAAA
18s	18S ribosomal RNA	FJ710826	Fw: GAGTATGGTTGCAAAGCTGAAAC Rv: AATCTGTCAATCCTTTCCGTGTCC
40s	40S ribosomal protein	AB012087	Fw: CCCAAGGCCAACAGGGAAA Rv: AGGGCGTAACCCTCGTAGAT

2.6. Statistical analysis

Mean values of all variables were first evaluated for homogeneity using univariate tests. Statistical analyses included both one-way and two-way analyses of variance (ANOVA), depending on the experimental design. One-way ANOVA was used to compare treatments within LPS dose or exposure time, while two-way ANOVA was performed to examine interactions between LPS dose and exposure time. Post-hoc comparisons were conducted using Fisher's Least Significant Difference (LSD) test to identify significant differences between treatments. Each experimental condition used the fish replicate as the statistical unit (n = 6). Statistical significance was set at $P < 0.05$. All analyses were carried out using STATISTICA 10.0 software (StatSoft, Inc., Tulsa, OK, USA).

3. Results and discussion

3.1. Results

3.1.1. Cell viability

The results demonstrated a dose-dependent decrease in cell viability, with statistically significant differences observed between treatment groups (Figure 1, $P < 0.05$). However, no significant differences were found between LPS sources in each experimental dose ($P > 0.05$).

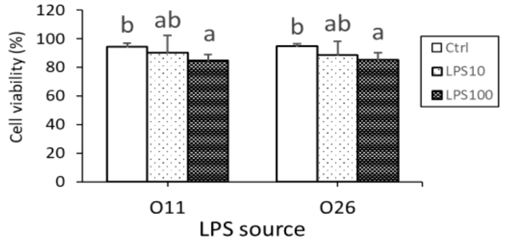
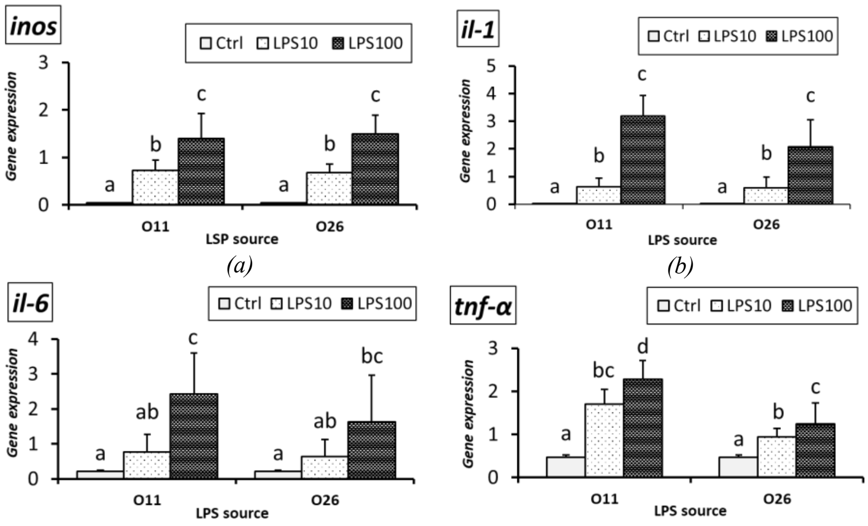


Figure 1. *Effect of LPS sources at 0, 10, and 100 µg/mL on the viability of head kidney leucocytes (HKLs) from Cyprinus carpio. Bars represent mean ± SD, and different letters indicate significant differences between treatments (p < 0.05)*

Cells treated with 0 µg/mL O11 and O26 LPS (Ctrl) showed the highest viability (94.2 and 94.6%, respectively), which was significantly higher than the 100 µg/mL LPS treatment group. The 10 µg/mL O11 and O26 LPS group exhibited intermediate viability (90.1 and 88.5%, respectively) with no statistically significant difference compared to the control group as well as 100 µg/mL groups (84.8 and 85.1%).

3.1.2. LPS source trial

The expression of pro-inflammatory genes (*inos, il-1, il-6,* and *tnf-α*) in HKLs was significantly influenced by the source and dose of LPS ($P < 0.05$, Figure 2). Treatment with LPS induced a robust upregulation of these genes in a dose-dependent manner, with notable differences observed between the two LPS sources.



(a) *(b)*

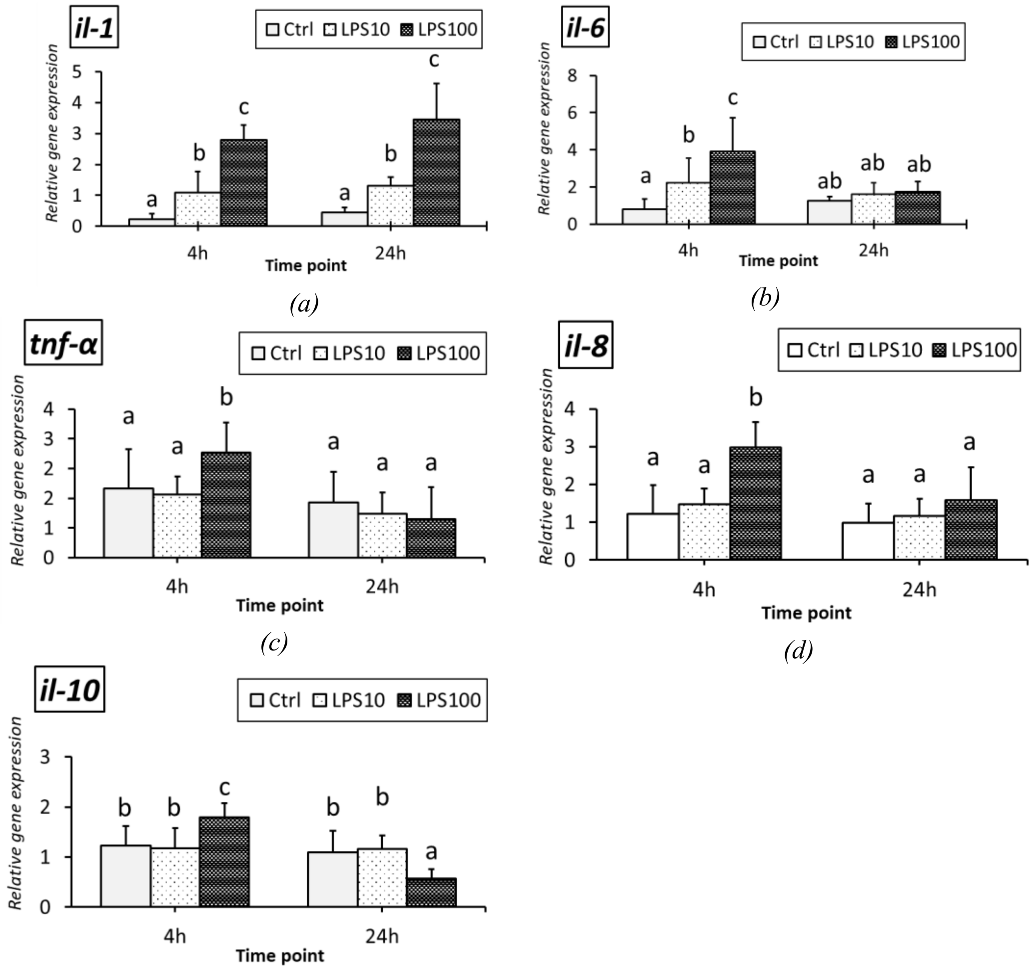
(c) *(d)*

Figure 2. *Expression of target genes involved in pro-inflammatory response in HKL including inos (a), il-1 (b), il-6 (c), and tnf-α (d) exposed to different LPS sources (O11 and O26) at 0, 10, and 100 µg/mL. Bars represent mean ± SD, and different letters indicate significant differences between treatments ($P < 0.05$)*

For *inos*, both LPS sources significantly increased expression ($P < 0.05$) at 10 µg/mL (0.72 and 0.68) and 100 µg/mL (1.39 and 1.49) compared to the control (0.04) but no differences were found between LPS sources. Expression value of *inos* in 100 µg/mL O11 and O26 LPS treatments (1.39 and 1.49) was also higher ($P < 0.05$) than 10 µg/mL one (0.77). Similar observations were recorded in *il-1* expression. The highest values of *il-1* expression were recorded in 100 µg/mL O11 and O26 LPS treatments (3.18 and 2.07), following in 10 µg/mL ones (0.63 and 0.60), and the lowest values belonged the control (0.01). *Il-6* expressions increased significantly with both sources of LPS (O11 and O26) at 100 µg/mL (2.42 and 1.63, respectively) while no differences were found at 10 µg/mL LPS treatments (0.77 and 0.64) compared to the control (0.21). The expression of *tnf-α* followed a similar trend, with significant upregulation at both 10 µg/mL and 100 µg/mL compared to the control ($P < 0.05$). At 100 µg/mL, O11-treated cells showed significantly higher ($P < 0.05$) *tnf-α* expression (2.28) compared to O26-treated cells (1.25). These results demonstrate that while both LPS sources elicit strong pro-inflammatory responses in HKLs, O11 induces higher expression of pro-inflammatory genes, particularly at the higher dose of 100 µg/mL.

3.1.3. Temporal dynamics of inflammatory and anti-inflammatory responses

The expression of pro-inflammatory (*il-1, il-6, tnf-α,* and *il-8,* Figure 3a, b, c, and d) and anti-inflammatory (*il-10,* Figure 3e) cytokines in HKLs exposed by O11 LPS was significantly influenced by LPS dose and exposure duration (Figure 3). *Il-1* expression increased significantly ($P < 0.001$) at both 10 µg/mL (1.10 and 1.32, respectively) and 100 µg/mL (2.80 and 3.44) after 4h and 24h compared to the controls (0.23 and 0.44). On the other hand, at 4h, *il-6* expression peaked at 100 µg/mL (3.92, $P < 0.01$) and decreased at 24h across both 10 and 100 µg/mL (1.63 and 1.76, respectively). Regarding *tnf-α* expression, at 4h, *tnf-α* expression was significantly elevated at 100 µg/mL (2.83, $P < 0.001$) compared to 10 µg/mL (1.70) and control (1.86) treatments. At 24h, *tnf-α* level in 100 µg/mL reduced to the basal levels as other groups (1.45, 1.18, and 1.03 for Ctrl, LPS10, and LPS10 treatments, respectively). Similar observations were recorded for *il-8* expression.



(a) *(b)*

(c) *(d)*

(e)

Figure 3. *Expression of inflammatory cytokines (a, b, c, and d) and anti-inflammatory one (e) in HKL exposed to different O11 LPS doses (0, 10, and 100 µg/mL) after 4h and 24h. Bars represent mean ± SD, and different letters indicate significant differences between treatments ($p < 0.05$)*

The expression of *il10* (Figure 3e), an anti-inflammatory coding gene, exhibited a similar response as pro-inflammatory cytokines indicating that the activity of anti-inflammatory factors occurs simultaneously with pro-inflammatory ones. The expression of *il-10* in LPS100 treatment (1.79) was also higher ($P < 0.05$) than LPS10 (1.18) and control (1.23). The downregulation of this treatment to basal level was also recorded after 24h of treatment.

3.2. Discussion

Pro-inflammatory cytokines represent the first line of immune defence, enabling rapid responses to pathogenic stimuli and facilitating innate and adaptive immune crosstalk. In the present study, we observed a dose- and time-dependent upregulation of key pro-inflammatory cytokines, including *inos, tnf-α, il-1*, and *il-6*, following LPS exposure in head kidney leukocytes (HKLs). The findings underline the marked responsiveness of teleost immune cells to endotoxins, particularly at higher doses and highlight temporal dynamics, with peak expression occurring at 4h. This is consistent with studies in rainbow trout macrophages, where a 3-fold increase in *tnf-α* expression was recorded following LPS stimulation at similar time points [11].

Among cytokines, TNF-α facilitates the recruitment of neutrophils and monocytes through chemokine induction and promotes localised vascular permeability, enabling leukocyte extravasation into infected tissues. Similar trends have been reported in carp HKLs [13]. Furthermore, in zebrafish, Novoa et al. [8] observed rapid activation of *tnf-α* upon LPS challenge, showing its conserved function across teleost species. The prolonged elevation of *tnf-α* at 24h in the current study, particularly at higher LPS concentrations, suggests the involvement of feedback loops mediated by NF-κB signalling. Studies in gilthead seabream (*Sparus aurata*) have indicated that NF-κB activation drives the transcription of secondary pro-inflammatory mediators, amplifying the inflammatory response [9]. However, sustained *tnf-α* expression may contribute to immunopathology, including tissue damage and chronic inflammation, necessitating a balance between activation and resolution. The observed upregulation of *il-1* highlights its critical role in amplifying the inflammatory response through the recruitment and activation of immune cells. As a key pyrogenic cytokine, Il-1β not only induces fever but also enhances the expression of adhesion molecules on endothelial cells, facilitating leukocyte migration. Swain et al. [3] reported similar findings in rainbow trout, where *il-1β* expression peaked within 4h of LPS exposure, reflecting its rapid induction during the acute phase of inflammation. Mechanistically, *il-1β* is activated through the inflammasome pathway, where pro-IL-1β undergoes cleavage by caspase-1. The upregulation observed in this study indicates that LPS-induced inflammasome activation is conserved in teleosts. The expression of il-6 shows its multifunctional role in modulating immune responses. *Il-6* is a key driver of acute-phase protein production in hepatocytes and promotes the differentiation of naïve T cells into Th17 or regulatory T cells, depending on the cytokine milieu. The sustained elevation of *il-6* at 24h aligns with findings by Antonopoulou et al. [9], where *il-6* expression in seabream was maintained during prolonged LPS stimulation. Interestingly, the dose-dependent dynamics observed in our study suggest that higher LPS concentrations may enhance il-6 stability through post-transcriptional modifications. It was reported that the activation of STAT3 downstream of *il-6* signalling creates a positive feedback loop that amplifies its expression, particularly under high endotoxin loads [13]. The upregulation of inos after 4h, highlights its role in mediating the cytotoxic effects of nitric oxide (NO) during infections. NO acts as an antimicrobial effector, targeting bacterial enzymes and disrupting cell membranes. Similar trends were observed in trout macrophages, where inos expression correlated with elevated NO production in response to LPS [14]. While NO plays a protective role, its overproduction can lead to oxidative stress and tissue damage. The temporal regulation observed in our study, with *inos* levels declining at 24h, reflects a balance between effective pathogen clearance and the prevention of collateral damage, a phenomenon well-documented in teleost immunity [11].

Anti-inflammatory cytokines play a vital role in resolving inflammation and preventing tissue damage caused by prolonged immune activation. In this study, the upregulation of *il-10* and other regulatory mediators was observed predominantly at 24h, particularly at 100 μg/mL LPS. This temporal pattern reflects the delayed activation of anti-inflammatory pathways necessary to counterbalance the robust pro-inflammatory response induced by LPS [4], [15].

The interplay between pro- and anti-inflammatory cytokines observed in this study shows the complexity of immune regulation in teleosts. While pro-inflammatory mediators such as *tnf-α* and *il-1* are essential for pathogen clearance, their excessive expression can lead to immunopathology, including oxidative damage and impaired tissue function. Anti-inflammatory cytokines, particularly *il-10* act as counter-regulators to suppress inflammation and facilitate healing. This balance is critical for maintaining immune homeostasis. These findings were similar to the observation in study of [11] in rainbow trout.

The findings of this study have significant implications for aquaculture, where immunostimulants such as LPS are used to enhance disease resistance. The dose- and time-dependent dynamics of cytokine expression observed in HKLs suggest that careful optimisation of LPS dosing is necessary to achieve a balance between effective immune activation and the prevention of immunopathology. High doses of LPS, while effective in inducing robust immune responses, may carry the risk of tissue damage due to sustained pro-inflammatory activity. Conversely, suboptimal doses may fail to elicit a sufficient protective response. Future studies should focus on exploring the long-term effects of LPS stimulation on fish health, including potential trade-offs between immune activation and growth performance. Additionally, understanding the molecular pathways underpinning cytokine regulation, particularly the cross-talk between TLR4 signalling and downstream transcriptional networks, could provide novel insights into immune modulation in teleosts.

4. Conclusion

This study elucidates the dose-, source-, and time-dependent modulation of cytokine expression in *Cyprinus carpio* head kidney leukocytes following LPS stimulation. High-dose LPS (100 µg/mL) markedly reduced cell viability and elicited a robust pro-inflammatory response, notably *il-1* at 4h, followed by increased *il-10* expression indicative of regulatory feedback. Differential responses to LPS sources (O11 > O26) further highlight the influence of structural variation on leukocyte activation. These findings advance our understanding of innate immune regulation in teleosts and inform the rational use of immunostimulants to enhance host defense while controlling excessive inflammation in aquaculture settings.

Acknowledgements

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors. This work was supported by project ARES-CCD, a cooperation program between Belgian Government and Vietnam National University of Agriculture (VNUA), with the Belgian Development Cooperation Fund.

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