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EXPRESSION PROFILE OF TUMOR NECROSIS FACTOR ALPHA DURING SPRING VIREMIA OF CARP VIRUS INFECTION IN NILE TILAPIA

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ABSTRACT

Spring viremia of carp (SVC) is a contagious viral disease that causes high mortality among infected fish. The present study aimed to investigate the expression profile of the tumor necrosis factor alpha (TNF-α) gene following experimental infection of Nile tilapia (*Oreochromis niloticus*) with SVCV. Fish were exposed to SVCV (3.2×10^7 $TCID_{50}$/ml) by immersion for 4 hrs. Then, spleens of both infected and control fish were sampled at various time points (1 h, 12 h, 1 d, 3 d, 5 d, 7 d, 11 d, and 14 d) post-infection (pi). The expression of TNF-α gene at these time points was assessed using reverse transcription quantitative real-time PCR (RT-qPCR). The results revealed a significant upregulation of the TNF-α gene that started from 12 hrs pi and continued to reach its peak at the 3rd dpi recording a fold change of 2.3 and 6.1, respectively, compared to the control. Subsequently, TNF-α gene expression commenced to regress at 5th dpi until it became similar to its corresponding control at 11th and 14th dpi. To the best of our knowledge, this is the first study exploring one of the immune responses of Nile tilapia after SVCV infection.

***Keywords*:** Tumor necrosis factor alpha, SVCV, Nile tilapia

INTRODUCTION

Spring viremia of carp (SVC) is a contagious viral disease that necessitates early notification to the World Organization for Animal Health, Office International des Epizooties, OIE (Fijan *et al*., 1971; Ahne *et al*., 2002; Su and Su, 2018; OIE, 2021). Although common carp was initially considered the natural host of SVC, some studies proved the induction of the disease experimentally in other fish species including Caspian white fish (*Rutilus frisii kutum*) (Zamani *et al*., 2014), fathead minnow (*Pimephales promelas* Rafinesque), emerald shiner (*Notropis atherinoides* Rafinesque), and white sucker (*Catostomus commersonii* (Lacepede) (Misk *et al*., 2016). Few studies have dealt with isolation and characterization of the virus from Nile tilapia (Soliman *et al*., 2008; Gado *et al*., 2015). Yet, this information awaits consensus.

The immune system is a sophisticated system that protects the organism against pathogens or substances that might cause infection or

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disease. The immune system compromises two essential branches, innate and adaptive immune responses, which can recognize foreign structures and trigger various molecular and cellular mechanisms for antigen elimination (Secombes *et al*., 1996). The initial line of defense against pathogens and infections is innate immunity (Kimbrell and Beutler, 2001). Innate immunity can be triggered by numerous serious agents, like viruses, after being identified by pattern recognition receptors (PRRs), which are found within or on the surface of immune system cells. Through a number of conserved signaling pathways, PRRs initiate antimicrobial defense mechanisms (Broz and Monack, 2013) that eventually activate genes and cause molecules synthesis, including cytokines, chemokines, cell adhesion molecules, and immunoreceptors (Akira *et al*. 2006), which work together to coordinate the early host response to infection, while also providing a crucial link to the adaptive immune response (Mogensen, 2009). Furthermore, these molecules work in a harmonious environment to eliminate the viral infections via the inflammatory response. Some of these important pro-inflammatory molecules are interleukin-6 (IL6), interleukin-1 (IL1β), and tumor necrosis factor (TNF-α) which are transcriptionally activated by nuclear factor-κB (NF-κB) (Pang and Iwasaki, 2012). TNF-α is secreted by macrophages in response to mitogens, viruses, parasites, bacteria (lipopolysaccharides), and other cytokines that pose an immunological threat. It is both a regulator of the development of lymphoid organs and a pluripotent mediator of pro-inflammatory and antimicrobial defense mechanisms. (Frederick *et al*., 2004). TNF-α is a crucial inflammatory cytokine that is essential for homeostasis, inflammation, and autoimmune disorders (Balkwill, 2009; Chu, 2013). It has been found to play conserved roles in the regulation of inflammation, apoptosis, and homing, proliferation, and migration of leukocytes in bony fish (Zou and Secombes, 2016). Additionally, it increases the phagocytic activity of fish leukocytes (Zou *et al*., 2003; Garcia-Castillo *et al*., 2004). Several bony fish have been used to clone, describe, and identify TNF-α, including rainbow trout (Zou *et al*., 2003), gilthead seabream (García-Castillo *et al*., 2002), Japanese flounder (Hirono *et al*., 2000), mandarin fish (Xiao *et al*., 2007), goldfish (Grayfer *et al*., 2008), turbot (Ordás *et al*., 2007), tilapia (Praveen *et al*., 2006), catfish (Zou *et al*., 2003), carp (Saeij *et al*., 2003), and Chinese mitten crab (Huang *et al*., 2022). TNF-α has been demonstrated to increase the survival of healthy macrophages, while limiting the proliferation of bacteria in infected macrophages in zebrafish (*Danio error*) with *Mycobacterium marinum* infection (Clay *et al*., 2008). Programmed necrosis of mycobacteria and infected macrophages is caused by mitochondrial reactive oxygen species, which are produced when TNF-α is produced in excess (Roca and Ramakrishnan, 2013).

There is a lack of literature concerning SVC in Nile tilapia and the immune responses following viral infection. Thus, the present study was designed to investigate the expression profile of the TNF-α, as one of the pro-inflammatory genes, in Nile tilapia after experimental infection with SVCV.

MATERIALS AND METHODS

Ethics statement

Fish used in the current study were handled and treated strictly in accordance with procedures in the Guide of the Use of Experimental Animal Welfare Committee of Faculty of Veterinary Medicine, Assiut University, Assiut Egypt. The methods used in the in vivo experiments were approved by the committee (Code No. 06/2023/0046). Fish were not exposed to any unnecessary pain or sacrification.

Fish

Nile tilapia (n=144), with average body weight of 2.7 ± 0.8 g and total length of 5 ± 0.3 cm, were kept in a flow-through for at least 2 weeks of acclimation. The temperature was adjusted to 14±0.5°C. Fish were fed a commercial diet containing 32% protein twice daily. To ensure their freedom from SVCV, random fish samples were tested following the method described by Shimahara *et al*. (2016).

Experimental infection

Fish were divided into two groups. The first group was immersed in Minimal Essential Medium (MEM) containing SVCV (3.2×10^7 $TCID_{50}$/ml) for 4 hrs., and the second group (control) was immersed in sterile MEM containing no virus for the same period. Thereafter, fish of both groups were transferred to clean aquaria supplied with chlorine-free freshwater containing neither virus nor MEM. The experiment was carried out in triplicates. Subsequently, at various time points (1h, 12 hrs., 1d, 3 d, 5 d, 7 d, 11 d, and 14 d) post infection (pi), 9 fish were randomly sampled from each of the infected and control groups. Following fish euthanasia using MS-222, spleens were sampled, immersed in RNA*later* (Ambion, Invitrogen) (1:5 wt/vol) and stored at -80°C until RNA extraction.

RNA extraction and reverse transcription

Total RNA was extracted from the spleens (30 mg/sample) using the RNeasy mini kit (Qiagen, Germany) as per manufacturer's protocol. The purity and concentration of RNA was measured using a nanophotometer (Implen GmbH, Germany). To produce 20-µl volume of cDNA from each sample, about 1 µg of total RNA was utilized using the RevertAid cDNA synthesis kit (Thermo Scientific, Germany) following the manufacturer's instructions.

Quantitative real-time PCR (RT-qPCR) and data analysis

RT-qPCR was performed using Maxima SYBR Green qPCR kit (Thermo, USA) and the QuantStudio™ real-time qPCR detection system (Applied Biosystems, USA). The expression of TNF-α was detected using the primer set (Table 1). The cycling profile was as follows: an initial activation step at 94 °C for 5 min followed by 40 cycles of 94 °C for 10 s and 55 °C for 30 s as an annealing step and 72 °C for 40 s (extension). Fluorescent data were collected during the extension step. The obtained melting curve analysis verifies the specificity and identity of PCR products. Tilapia β-actin and Elongation factor (EF1α) genes were used as housekeeping genes (internal control for cDNA normalization). According to Livak and Schmittgen (2001), the delta-delta Ct ($2^{-(\Delta\Delta Ct)}$) approach was used to calculate the fold change in expression of TNFα, and then the results were subject to statistical analysis.

Statistical Analysis

Two-way analysis of variance (ANOVA) was used to analyze data of relative expression of the TNFα gene. All analyses were performed using programmed Graph Pads Prism® 8 Software (version 8.4.3). The *p*-value of each data analysis was calculated.

RESULTS

Expression profiles of TNF-α

The generated PCR products showed a melting curve with a single peak, which confirms the specificity of the used primers and the identity of PCR products (Fig. 1, 2, and 3).

Compared to the control, TNF-α was significantly upregulated starting from 12 h pi and reached the highest expression level 3 d pi, then continued its significant upregulation, though with lower values, till the seventh d pi. Thereafter, it showed downregulation to the control level at the days 11 and 14 pi. The fold changes in the expression of TNF-α at the different time points (1 h, 12 h, 1 d, 3 d, 5 d, 7 d, 11 d, and 14 d) following SVCV infection, in comparison to control, were 1.0, 2.3, 4.0, 6.1, 3.9, 2.5, 1.2, and 0.9 respectively (Fig. 4).

Table 1: Primers used for the detection of Tumor Necrosis Factor (TNFα) gene expression in the spleen of Nile tilapia, experimentally infected with spring viremia of carp virus (SVCV)

Primer	Sequence (5`→3`)	Reference
EF1α-E1F EF1α-E1R	CTACGTGACCATCATTGATGCC AACACCAGCAGCAACGATCA	(He *et al.*, 2014)
β-actineF1 β-actineR1	CAGCAAGCAGGAGTACGATGAG TGTGTGGTGTGTGGTTGTTTTG	(J. C. Pang *et al.*, 2013)
TNFα F TNFα R	GCTGGAGGCCAATAAAATCA CCTTCGTCAGTCTCCAGCTC	(Selim & Reda, 2015)

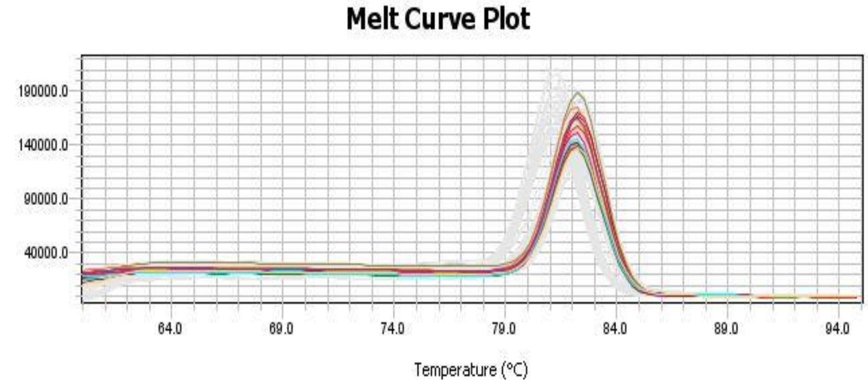

Fig. 1: Melting curve of Tilapia β-actin gene. The generated PCR products showed a melting curve with a single peak.

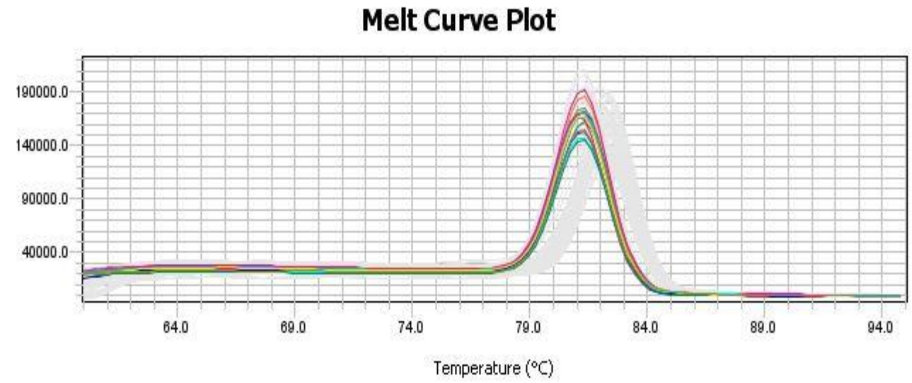

Fig. 2: Melting curve of Tilapia elongation factor 1α gene. The generated PCR products showed a melting curve with a single peak.

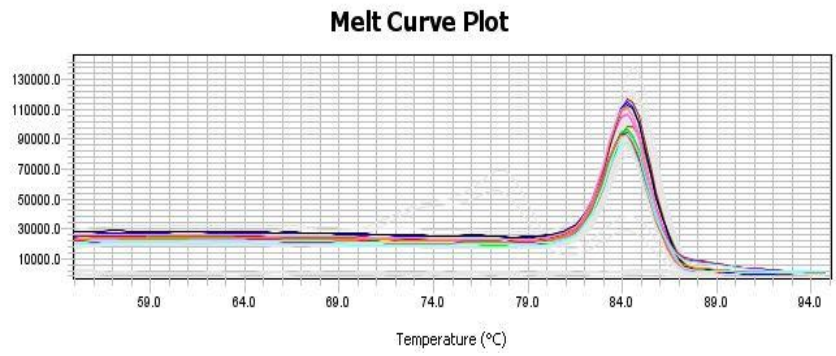

Fig. 3: Melting curve of TNF-α gene. The generated PCR products showed a melting curve with a single peak.

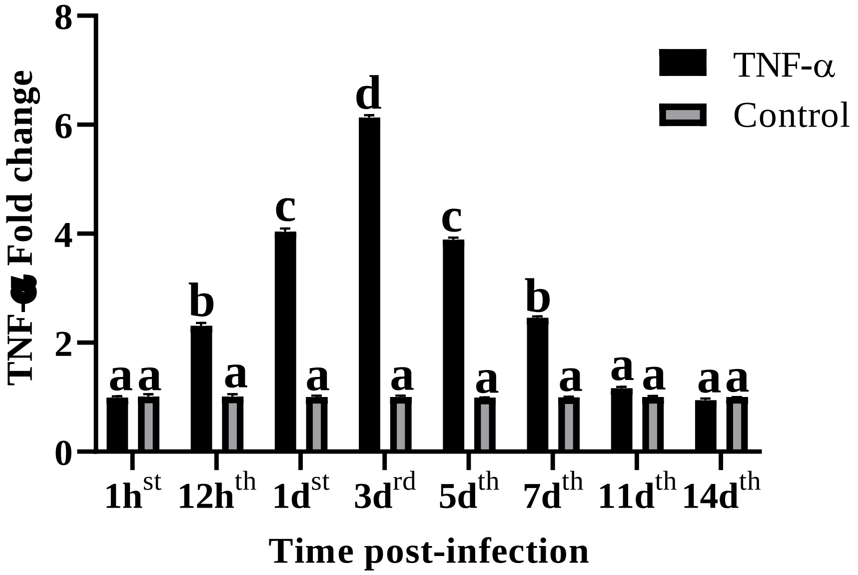

Figure 4: Expression profile of TNF-α in spleen of SVCV-experimentally infected Nile tilapia at various time points (1 h, 12 h, 1 d, 3 d, 5 d, 7 d, 11 d, and 14 d) post infection. Fish were immersed in 3.2×10^{7} $TCID_{50}$/ml of SVCV for 4 hrs. Data are expressed as means (n=9) ± SEM. Different letters denote significant differences ($P < 0.05$).

DISCUSSION

In the present study, the expression profile of TNF-α gene in the spleen of Nile tilapia infected with SVCV by immersion was investigated to understand the host-pathogen interaction in terms of fish responses at the molecular level during infection.

The spleen was chosen as the target organ for this study because it is considered a primary lymphoid organ, and almost all gnathostomes possess it. It is the organ in which adaptive immune responses are generated (Flajnik, 2018). As in other vertebrates, it is the main filter of blood-borne antigens and performs immune-poietic functions. In teleost fish, it is involved in hematopoiesis and may have immune functions comparable to lymph nodes in mammals (Hitzfeld, 2005). Additionally, it contains a significant number of resident macrophages and lymphocytes that, when stimulated by pathogens, secrete a large amount of TNF-α (Zhu *et al*., 2016).

Following virus detection and NF-κB activation, the inflammatory response led to the release of pro-inflammatory cytokines and the stimulation of innate immune cells, which are implicated in antiviral defensive mechanisms (Rakus *et al.*, 2020). Since TNF-α expression levels have been linked to a variety of infections, and a previous study demonstrated that SVCV-infected EPC cells showed activation of the TNF-α signal (Yuan *et al*., 2014), it is probable to use it to understand how the host reacts to infection (Jantrakajorn and Wongtavatchai, 2016; Zhi *et al*., 2018).

In the current work, early upregulation of TNF-α 12 hpi and its long-lasting upregulation until the 7th dpi can indicate its vital role in the initiation and regulation of inflammation and immune response, such as phagocytosis and respiratory burst activity, as was previously explained (Pleić *et al*., 2014). TNF-α promotes the recruitment and activation of phagocytes (Garca-Castillo *et al*., 2004). The present results are consistent with the findings of Varela *et al*. (2014) who observed that TNF-α was induced in zebrafish larvae as a result of systemic infection with SVCV. Furthermore, the initial upregulation of cytokine expression might be associated with the recognition of SVCV by TLRs, followed by a second upregulation of cytokine with a consequent inflammatory response (Negash *et al*., 2013). This inflammatory response also triggered the response of TNF-α which was induced from the 12th to 24th hpi (Varela *et al*., 2014).

TNF-α reached its expression peak 3 dpi, which can be explained by the development of splenomegaly at that time of SVCV infection. Espn-Palazón *et al*. (2016) stated that TNF-α can exacerbate SVCV infection by improving SVCV replication and pathogenesis, which, in our study, reflects the appearance of signs and the beginning of mortalities. Though induced by a different cause, similar results have been reported by Deshmukh *et al*. (2013), who found that during the third day after infection, the TNF-α gene in *Y. ruckeri*-infected rainbow trout was significantly up-regulated in the spleen and kidney, suggesting that the higher bacterial load was responsible for inducing this immediate response. Finally, in the current study, the expression of TNF-α in the spleen reached a normal level at the 11th dpi, and this may be correlated to the observed reduction of inflammatory signs in SVCV-surviving fish. It is known that TNF-α prevents viruses infection by direct antiviral or covert immunomodulatory mechanisms (Herbein and O'brien, 2000). However, other authors reported that TNF-α interferes with autophagy-mediated clearance of SVCV by host-infected cells, which is an effective antiviral strategy in response to several viral infections, including SVCV (Yu *et al*., 2009; García-Valtanen *et al*., 2014; Espín-Palazón *et al*., 2016). In addition, TNF-α has potent effects on endothelial cells of fish, which suggests that it might promote the propagation of SVCV (Roca *et al*., 2008). Interestingly, due to the key role of TNF-α in host protection against viral infections, some viruses have developed different ways to interfere with the TNF-α pathway (Herbein and O'brien, 2000). Thus, it seems that a few viruses might utilize the host produced TNFα to their benefit.

CONCLUSION

The present study addresses the innate immune response after SVCV infection in Nile tilapia. To our knowledge, this is the first study presenting TNF-α gene expression in SVCV-infected Nile tilapia. Further studies are still needed to investigate the expression of the other immune-related genes following the SVCV infection in Nile tilapia.

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نمط التعبير لجين عامل نخر الورم-ألفا أثناء العدوي بفيروس حمي المبروك الربيعية في أسماك البلطي النيلي

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يعد مرض فيروس حمي أسماك المبروك الربيعية من الأمراض الفيروسية التي تسبب وفيات مرتفعة في الأسماك المصابة. ولذا فقد هدفت الدراسة الحالية إلى توضيح نمط التعبير لجين عامل نخر الورم-ألفا أثناء العدوي بفيروس حمي المبروك الربيعية في أسماك البلطي النيلي المصابة إصطناعياً. وتعرضت أسماك البلطى لجرعة بلغت ٣٫٢×١٠^٧ من الجرعة نصف المميتة بواسطة التغطيس لمدة أربعة ساعات. ثم تم تجميع الطحال من الأسماك المصابة و كذلك الأسماك المستخدمة كضابط للتجربة بعد ساعة، إثنى عشر ساعة، يوم، ثلاثة أيام، خمسة أيام، سبعة أيام، أحد عشر يوماً وأربعة عشر يوماً من وقت العدوى. وتم تقييم معدل التعبير الجينى باستخدام تفاعل البلمرة المتسلسل الكمي. أوضحت النتائج أنه كان هناك ارتفاعا معنوياً فى معدل التعبير لجين عامل نخر الورم-ألفا في الساعه الثانية عشر بعد الإصابة حيث وصل إلى ٢٫٣ ضعف نظيره في الأسماك المستخدمة كضابط للتجربة وأظهر أعلي تعبير له عند اليوم الثالث بعد الإصابة، حيث وصل إلى ٦٫١ أضعاف ثم بدأ التعبير الجيني فى الانخفاض بدءاً من اليوم الخامس ليصل لنفس المستوى في الأسماك المستخدمه كضابط للتجربة في اليوم الحادي عشر والرابع عشر بعد العدوى. تعد هذه الدراسة هي الأولى التي توضح أحد الاستجابات المناعية في أسماك البلطي النيلي بعد الإصابة بفيروس حمى أسماك المبروك الربيعية.