



EFFECT OF HIGH GLUCOSE-INDUCED HYPERGLYCEMIA ON VIRAL HAEMORRHAGIC SEPTICAEMIA VIRUS (VHSV) INFECTION IN ADULT ZEBRAFISH

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Abstract. Diets/nutrients have a significant effect on the biological process of all kinds of living organisms, especially high-fat diet or high glucose-induced obesity/diabetes affects hundreds of millions of people worldwide and considered as global epidemics. In this study, we conducted a high glucose immersion protocol to induce hyperglycemic zebrafish, later on, with the aim to evaluate the relationship between hyperglycemia and Viral Haemorrhagic Septicaemia Virus (VHSV) infection. As a result, zebrafish immersed in a 2000 ppm glucose solution for ten days, fasting blood glucose (FBG) has increased two times as compared to control. In addition, hyperglycemic zebrafish was more susceptible to the virus infection resulted in increased viral mRNA expression levels as well as the viral copy number as 1.7-log higher than control. The data also demonstrated that adult zebrafish fed high glucose/after a VHSV challenge highly increased in mRNA levels of inflammatory cytokines as well as in the expression level of endoplasmic reticulum stress. This knowledge has, in turn, allowed the future studies to understand the crosstalk between metabolic syndrome and infectious disease.

Keywords: diabetes mellitus, glucose, hyperglycemia, VHSV, zebrafish.

Classification numbers: 1.4.4, 1.5.4.

1. INTRODUCTION

Metabolic syndrome (MS) is an important and growing public health problem worldwide. Among MSs, obesity and diabetes are more popular and considered serious health problems, which increases the risk of other different chronic diseases [1, 2]. Additionally, diabetes is accompanied by numerous physiological changes that may directly or indirectly influence the immune system, which poses a significant risk of various diseases and certain types of cancer [3]. Viral hemorrhagic septicemia virus (VHSV), originally known as the Egtved virus, is a negative-

sense single-stranded RNA virus from the *Novirhabdovirus* genus that is responsible for outbreaks against several species of marine and freshwater fish around the world, causing large economic losses [4, 5]. On the other hands, the zebrafish (*Danio rerio*) is a tropical freshwater fish that has become one of the most popular vertebrate model organisms in biological research since zebrafish's organs and tissues show similarities to those of humans regarding their structure and function [2, 4, 6-9]. Recently, zebrafish has become a popular animal model for metabolic disorders, infectious diseases, toxicological studies, and especially drug screening [1, 4, 10-14]. In addition, glucose and lipid metabolism in zebrafish is very similar to that in humans [1]. Several authors have used zebrafish as a model for high-glucose exposure and diabetes [14-16]; as well as for the study of VHSV infections [4, 5, 17]. However, to our knowledge, there are still no reports on the effects of high glucose diet in relation to VHSV infection in zebrafish. Therefore, this study aimed to explore the effect of high glucose diet-induced hyperglycemia on the infection of VHSV using a zebrafish model with the purpose to understand the interplay between metabolic syndrome and infectious disease.

2. MATERIALS AND METHODS

2.1. Zebrafish care and experimental design

Adult zebrafish (*Danio rerio*) 200 – 300 mg in body weight at around 3 months old were purchased from a local aquarium shop and kept under a 14-h light/10-h dark cycle (turn on at 9 am and turn off at 11 pm) at a constant temperature of 28 ± 0.5 °C for a week prior to the experiment. The detailed experimental design and protocol are shown in Figure 1.

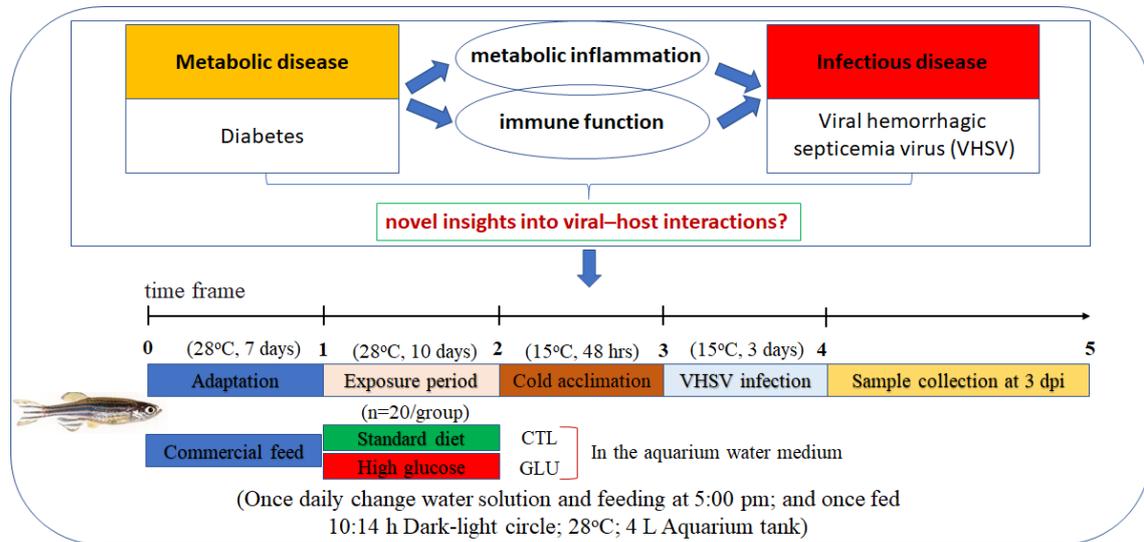


Figure 1. A simple diagram of the research objective and the experimental design for a short time high glucose diet and virus challenges protocol using adult zebrafish (*Danio rerio*).

Briefly, after acclimatization time, fish were randomly assigned into two experimental groups (20 fish/group; standard diet-fed group: CTL, High glucose diet: GLU) and maintained in a 4-L aquarium tank. During the experiment period, the fresh aquarium water was daily changed, and fish were fed once a day (at 10:00 am, for 10 days) with a commercial fish diet (Tetra Bits Complete fish food, Tetra GmbH, Herrenteich 78D-49324 Melle, Germany). For the high

glucose diet group, fish were immersed in the glucose solution (2000 ppm (mg/L), daily prepared). All experiments related to zebrafish were raised according to the zebrafish book (visit at https://zfin.org/zf_info/zfbook/zfbk.html).

2.2. Fasting blood glucose measurement

During 10 days of feeding period with a standard diet and high glucose diet, blood samples were collected at each time point (1 day, 5 days, and 10 days) to determine the fasting blood glucose levels. Firstly, fish were randomly selected (3 zebrafish/group), then transferred into a clean separate tank and kept overnight (about 12 hrs) without feeding. Blood samples from the individual zebrafish were collected from the dorsal artery and immediately measured using a glucometer (CareSens II Plus⁺, Informa Life Sciences) with twice measurement of a zebrafish.

2.3. VHSV preparation and infection to zebrafish

The viral stock of VHSV (Genogroup IV) was received as a kind gift from the Department of Aquatic Life Medicine at the Yeosu campus of Chonnam National University in Yeosu, South Korea. The titer of the obtained virus was $10^{8.8}$ TCID₅₀/mL (50 % Tissue culture Infective Dose). In order to replicate the virus for experimental infection, fathead minnow (FHM) cells were grown in a mixed media consisting of Leibovitz's L-15 Medium (Welgene, LM 003-01) with 10 % Fetal Bovine Serum (FBS; Merck, Lot #QVP1511200) and 1 % Penicillin-Streptomycin (PS; ThermoFisher Gibco, #15140-122). Viral stock was infected to FHM cells at the multiplicity of infection (MOI) of 0.2 in mixed media diluted 1:4 in L-15 medium supplemented with 2 % (FBS), 1 % PS. FHM infected cells were incubated at 15-20°C and allowed to replicate until cells were reduced to 10-15 % confluence, around 3 days. After viral replication, the virus in media was subjected to three freeze-thaw cycles to allow the virus to escape the cells. After centrifugation (10,000 rpm, 15 min at 4 °C), the supernatant containing the replicated virus was collected and stored at -80 °C until thawed for use in experimental infection.

After 10 days of feeding period with normal diet and high glucose diet, fishes were randomly assigned to two different groups (1: MOCK, non-VHSV infected and 2: VHSV, Virus infected) and transferred into cleaned separate tanks. For viral infection, groups of 10 adult zebrafish were moved to 2.0 L tank and kept at 14 °C for cold acclimation. After 48 hrs, groups of 10 zebrafish were infected-by-immersion in 10^6 TCID₅₀/mL of VHSV at 14 °C during 2 hrs in 200 mL aerated bottles filled with aquarium water. After infection time, 10 zebrafish were then released to the 2.0 L tank and maintained at 14 °C, which is considered as the optimum temperature for virus growth. Samples were collected at each scheduled time points (MOCK and VHSV infected at 12 hpi and 72 hpi) for the analysis.

2.4. RNA extraction, cDNA synthesis, and quantitative real-time RT-PCR

Total RNA was extracted from the whole body of adult zebrafish using RNAiso Plus reagent (TaKaRa Bio, Shiga, Japan) according to the manufacturer's instructions. After RNA isolation, total RNA concentration was measured using the Nanodrop 1000 Spectrophotometer (Thermo Scientific, USA), and equalized to all the groups. The equalized RNA was used for cDNA synthesis using a TaKaRa PCR Thermal Cycler Dice Real-Time System as the following conditions: 63 °C for 10 min, 37 °C for 60 min and 95 °C for 5 min. Briefly, each 10 µL aliquot of equalized RNA was transcribed into cDNA using 1 µL random primer, 2 µL dNTP mixture, 0.5 µL recombinant RNase inhibitor (TaKaRa Bio), 1 µL MMLV RT, 4 µL 5X MMLV RT

buffer, and 2 μL 5X DTT (Beams Biotechnology, Seongnam, Korea). Finally, each 2 μL of those was used as a template for real-time qPCR. Real-time qPCR was carried out in TaKaRa Thermal Cycler Dice Real-Time System using SYBR Premix Ex Taq™ kit (TaKaRaBio). For the absolute quantification of viral copy number, the standards of serial ten-fold dilutions of VHSV concentration were prepared (10^4 , 10^5 , 10^6 and 10^7 copies of VHSV nucleocapsid/ μL , respectively) and used to create the calibration curve for absolute quantification. The copy number of VHSV N-gene was calculated according to the Ct value and then converted into Log numbers.

In the current experiment, we analyzed the several function genes, which target various biological indexes including inflammation and immune-related genes using gene-specific primers (Table 1). Quantitative real-time PCR was performed in a Thermal Cycler Dice Real-Time System (Takara Bio) using a 21 μL reaction mixture containing 10 μL SYBR Green Master Kit (TaKaRa Bio, Japan), 1 μL for each Forward primer and Reverse primer, 2 μL template cDNA, and 7 μL RNAase free water. The amplification conditions for the real-time PCR were as follows: 30-second hold at 95 °C, 45 cycles of denaturation for 05 seconds each at 95 °C, 10 seconds at 55 °C, and 20 seconds at 72 °C and 1 cycle of dissociation at 95 °C for 15 seconds, 60 °C for 30 seconds and 95 °C for 15 seconds. Beta-actin was used as the reference gene for normalization. The mRNA expression levels of target genes were determined by the $2^{-\Delta\Delta\text{Ct}}$ method ($\Delta\text{Ct} = \text{Ct}$ was for a target gene, Ct for reference gene; $\Delta\Delta\text{Ct} = \Delta\text{Ct}$ was for treat group – ΔCt for the control group). All the quantitative real-time PCR reactions were run in triplicate.

Table 1. Details of the primer sequence used for qRT-PCR.

Primer	Description	Sequence (5' – 3')	References
<i>β-actin</i>	house-keeping gene	F: CTTCCCATCCATCGTGGGTC R: AGCCTCATCACCAACGTAGC	NM_181601.5
VHSV N-gene	VHSV genotype gene	F: CGCCATCATGATGAGTCGGATGCTG R: CTTCTCTGTACCTTGATCCCCTCC	GenBank: KJ768665.1
VHSV G-gene	VHSV genotype gene	F: GGACACATGATCACAGGGTG R: GACAGTTTCTTCGCTCCCCC	[5]
<i>IL-1β</i>	Interleukin 1 beta	F: GGCTGTGTGTTTGGGAATCT R: TGATAAACCAACCGGGACA	NM_212844.2
<i>IL-6</i>	Interleukin 6	F: TCAACTTCTCCAGCGTGATG R: TCTTTCCTCTTTTCCTCCTG	NM_001261449.1
<i>TNF-α</i>	Tumor Necrosis Factor-alpha	F: AAGGAGAGTTGCCTTTACCG R: ATTGCCCTGGGTCTTATGG	[18]
<i>HSPA5</i>	Heat shock protein 5 (glucose-regulated protein, 78kDa, GRP78)	F: AAGAGGCCGAAGAGAAGGAC R: AGCAGCAGAGCCTCGAAATA	NM_213058

2.5. Statistical analyses

Statistical analysis was carried out by using SPSS 21.0 statistical software. The results are expressed as mean \pm SD. Student's t-test and one-way ANOVA test are performed for analysis. GraphPad Prism 5 software (GraphPad, La Jolla, CA, USA) was used to draw figures. Differences were considered significant at $p < 0.05$ level.

3. RESULTS AND DISCUSSION

3.1. High glucose diet induces hyperglycemia and promotes higher virus replication in zebrafish

In order to assess the effect of high glucose diet in zebrafish, the body blood glucose level in two different groups including standard diet-fed group (CTL) and high glucose diet (GLU) was first tested, shown in Figure 2. As shown, during the feeding period, at 5 days (5 dpe) and 10 days (10 dpe) of the experiment, the GLU-treated zebrafish showed significantly higher increase in fasting blood glucose (FBG) than the CTL group, and the increasing level was remarkably higher with longer exposure time (10 dpe > 5 dpe > 1 dpe). However, no significant difference was observed in the CTL group during the feeding period. Similarly, the previous study reported the consistent result as the normal diet to remain unchanged blood glucose level in adult zebrafish up to 8 weeks of the feeding period. Meanwhile, diet-induced obesity showed significantly higher FBG than a normal diet as mentioned by Zang *et al.* [14]. In addition, Jung *et al.* [19] demonstrated that glucose level in embryo zebrafish was found much higher in the high glucose group than the control group. Our result demonstrated that after 10 days of glucose diet treatment, the FBG of treated zebrafish increased more than 2 times (114 mg/dL vs. 54 mg/dL) as compared to the control group. Therefore, the current model was succeeded to induce hyperglycemia in zebrafish with the lower glucose concentration in a shorter time as compared to the observation of Connaughton *et al.* [16].

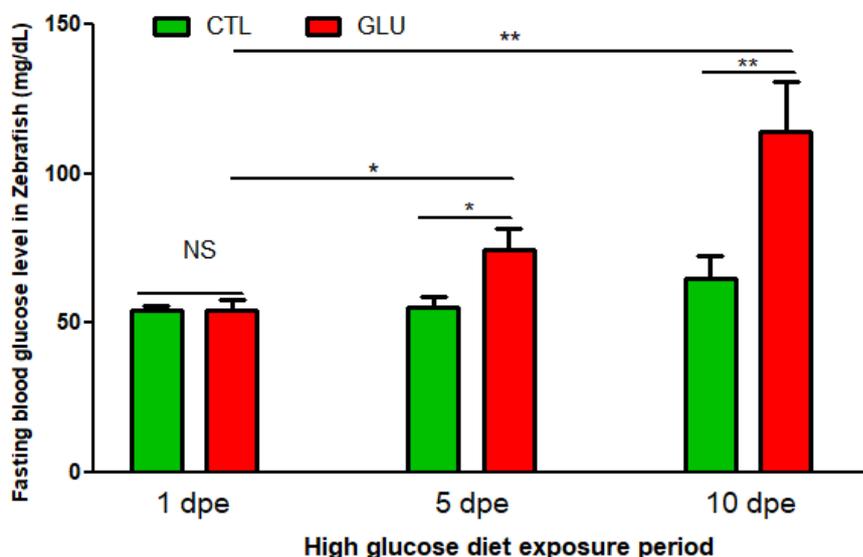


Figure 2. Effect of glucose exposure on blood glucose level in adult zebrafish (*Danio rerio*). Green indicates zebrafish fed standard diet (CTL); Red, with glucose diet (GLU). Results are means \pm SD of 3 - 4 replication. Asterisks and letters above bars indicate the level of significant difference from the groups determined using Student's t-test analysis (NS, not significance, * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$).

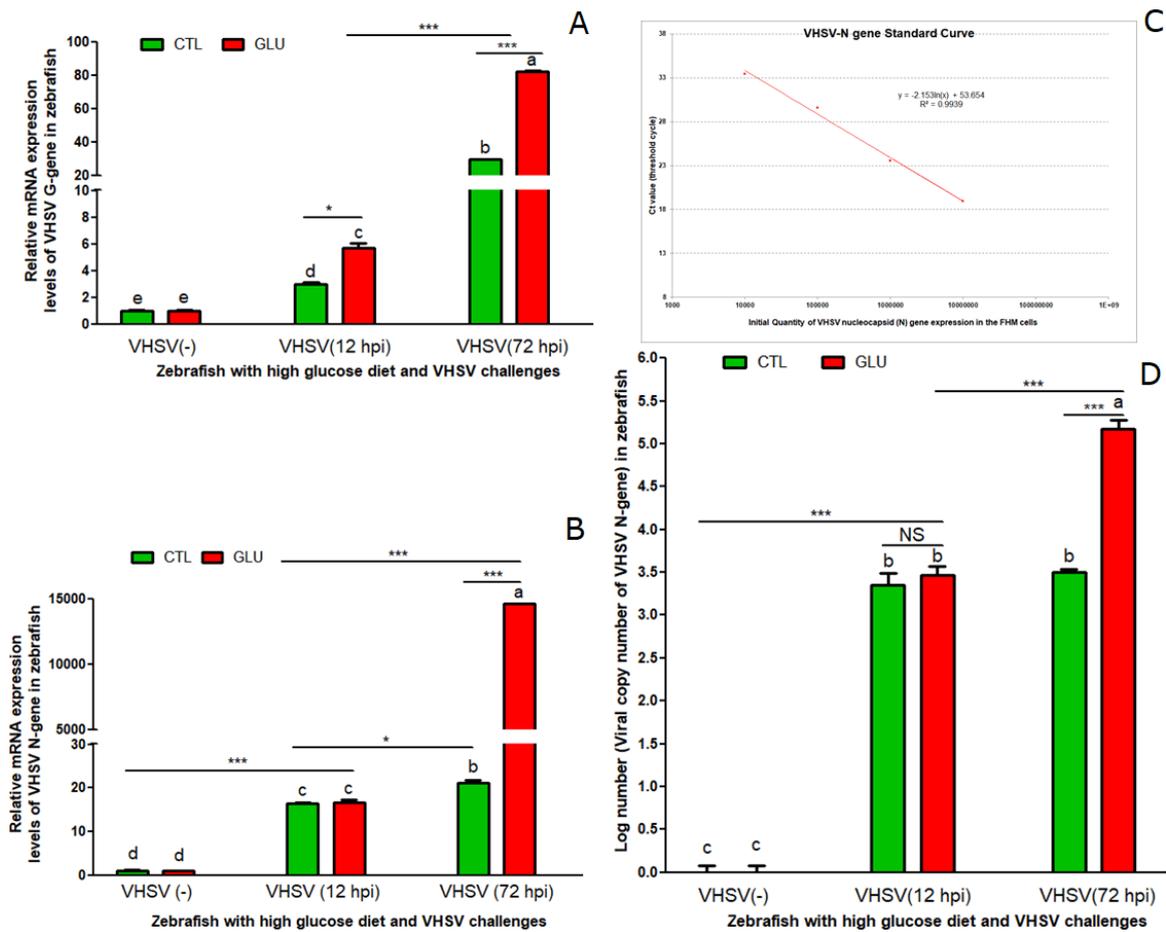


Figure 3. Effects of glucose exposure and/or viral haemorrhagic septicemia virus (VHSV) infection on the expression of VHSV N gene (A), VHSV G gene (B), the calibration curve of VHSV N-gene (C) and the absolute quantification expressed as Log viral copy number of N-gene (D). Green indicates zebrafish fed standard diet (CTL); Red, with glucose diet (GLU). Results are means \pm SD of 3 - 4 replication. Asterisks and letters above bars indicate the level of significant difference from the groups determined using Student's t-test analysis (NS, not significance, * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$).

In addition, to evaluate the effect of a high glucose diet on the infection of VHSV in adult zebrafish, we analyzed the mRNA expression levels of VHSV N-gene and VHSV G-gene using qRT-PCR analysis by comparing GLU/CTL for mock and virus infections. As the results are shown in Figure 3A and 3B, after virus infection, the mRNA expressions of VHSV N-gene and G-gene gradually increased from 12 to 72 hpi (hours post-infection) in zebrafish. Moreover, it is clearly shown that in glucose-treated zebrafish resulted in highly increased viral mRNA expression when compared to control zebrafish. In fact, there was a significant expression of G-gene at 12 hpi ($p < 0.05$, Figure 3A), but not statically different in N-gene expression at the same sampling time (Figure 3B) between GLU and CTL zebrafish. However, at 72 hpi, there were remarkably increases in both G-gene and N-gene expression ($p < 0.001$). Additionally, to estimate the infectivity of the VHSV infection in two groups of zebrafish, we performed the VHSV standard curve to calculate the viral copy number based on Ct value (threshold cycles)

and the quantity of VHSV nucleocapsid (N) gene (shown in Figure 3C), the results were expressed as Log number. Similarly, the results revealed that at 12 hpi the VHSV N-gene copy number reached 3.4 - 3.5-log, and was no difference observed between GLU and CTL group (Figure 3D). However, at 72 hpi, we found that the copy number significantly rose up to 5.2-log in GLU-treated group but still almost unchanged as the log value of 3.5 in the CTL group (Figure 3D). In similarity, previous reports have observed that VHSV is highly infected to adult zebrafish [4,17]. Furthermore, the current result was consistent with the findings of *Castroet et al.* [20] and *Kim et al.* [5] also reported that at 1 dpi of VHSV, the transcription levels of viral gene remained undetected or showed a weak band, meanwhile significantly expressed at 2 dpi or 3 dpi.

3.2. Changes in inflammation and the immune system by high glucose diet and VHSV infection in zebrafish

In order to understand why zebrafish with high glucose diet-induced hyperglycemia promotes the higher replication of virus after a VHSV challenge, we analyzed the expression level of inflammatory genes serve as biomarkers for inflammation including interleukin 1 β (*IL-1 β*), interleukin 6 (*IL-6*), and tumor necrosis factor- α (*TNF- α*) as well as the endoplasmic reticulum chaperone (*HSPA5*, also is called heat shock protein 5). Since excessive inflammatory cytokin eproduction is a common pathogenesis of many chronic diseases, including cardiovascular and bowel diseases, diabetes, arthritis, infectious diseases, and cancers [21-23].Therefore, it is important to explore the role of these genes and how it linked from metabolism to virus replication in the present study. The results revealed that after 10 days of high glucose diet challenge, GLU-treated zebrafish group highly stimulated inflammatory response genes (*IL-1 β* and *IL-6*), which were increased 7-fold as compared to CTL-group, shown in Figure 4. This is suggesting that a high glucose diet has a major impact on the immune system via the production of inflammatory cytokines as such *IL-1 β* and *IL-6*, which may be the major players in inflammatory signaling pathways could induce hyperglycemia in zebrafish. This is supported by several studies, which have been stated that the overexpression and production of pro-inflammatory cytokines such as *IL-1 β* , *IL-6* are risk factors that cause insulin resistance [22, 24, 25]. Similarly, after VHSV challenge (72 hpi) resulted in gradually increased expressions of *IL-1 β* and *IL-6* in both CTL and GLU group. Additionally, in the GLU-treated group in combination with VHSV-infected provoked up to more than 9-fold and 18-fold increase when compared to control zebrafish (CTL diet + non-infected) in mRNA levels of *IL-6* and *IL-1 β* , respectively. However, there were no statistical relevant changes in *TNF- α* gene by high glucose-treated and/or VHSV-infected. It seems that the transcription of *TNF- α* is not so much affected by glucose treatment and/or virus infectioninthe present observation. It is also possible that while there is no upregulation of mRNA expression, there could still be an increase expression of TNF- α protein, also could depend on the different response to the type of viruses or possible differ from the sampling time at early or later response after virus infections. Our results are consistent with other studies that have also shown the role of the inflammatory response in VHSV infection, such as the increase in proinflammatory *IL-1 β* , *IL-6* and *IL-8* during early infection in rainbow trout [20, 26]; but there was no induced expression of *TNF- α* following infection with either infectiouspancreatic necrosis virus (IPNV) or infectious salmon anaemia virus (ISAV) in Atlantic salmon [27].

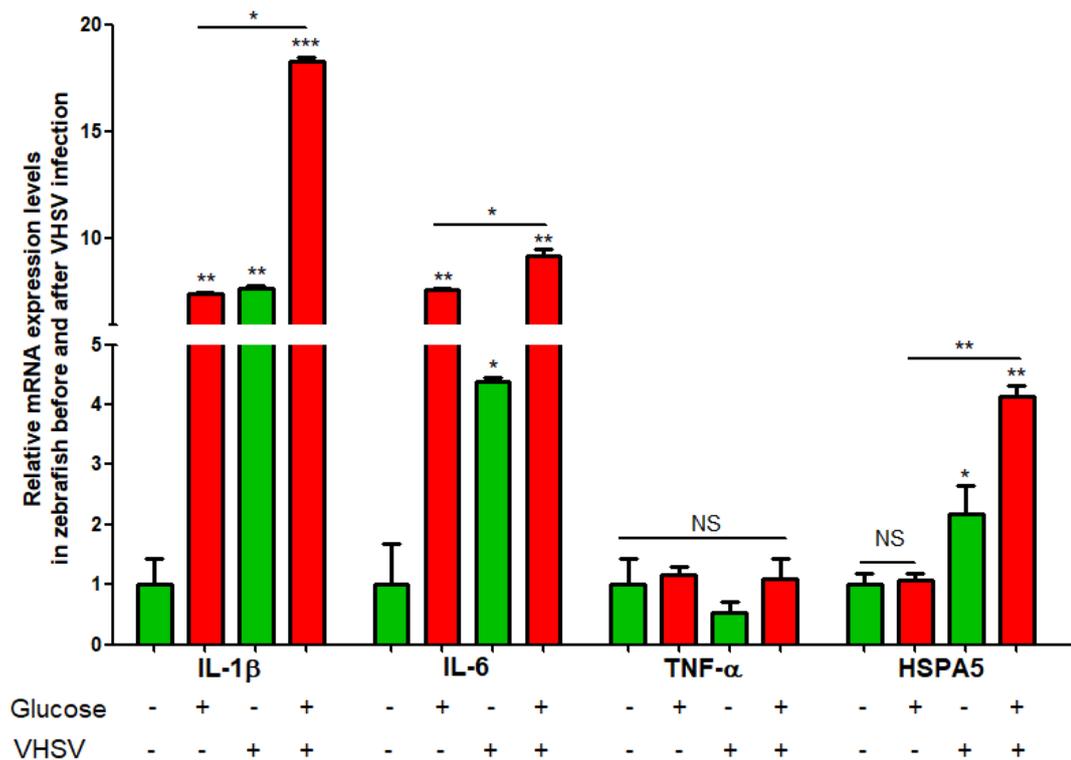


Figure 4. Effects of glucose exposure and/or viral haemorrhagic septicemia virus (VHSV) infection on mRNA expression of cytokine-related genes and immune response using qRT-PCR. All expression levels of mRNA of target genes were determined by the $2^{-\Delta\Delta Ct}$ method ($\Delta Ct = Ct$ was for a target gene, Ct for reference gene (β -actin); $\Delta\Delta Ct = \Delta Ct$ was for treat group – ΔCt for the control group). Green indicates zebrafish fed standard diet (CTL); Red, with glucose diet (GLU). Results are means \pm SD of 3 - 4 replication. Asterisks and letters above bars indicate the level of significant difference from the control and non-VHSV-infected zebrafish determined using Student's t-test analysis (NS, not significance, * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$).

On the other hands, to determine the effect of high glucose diet and/or VHSV infection on the endoplasmic reticulum (ER) stress in zebrafish, the heat shock protein 5 (*HSPA5*) was considered as a biomarker since heat shock proteins (HSPs) have been highly responsive to a variety of stresses, specifically promote metabolic disease progression [28, 29]. As shown in Figure 4, no significant difference was observed between the CTL and GLU group after the feeding period (10 days). However, after VHSV challenged, there was a significantly upregulated up to a 2-fold in the CTL group, whereas remarkably increased up to a 4-fold in GLU group. It was not surprising that many studies have shown that the expression of chaperon immune-related genes, such as HSPs family is significantly induced after infectious pathogens. Cha *et al.* [30] reported that there was a significant induction of HSPs including Hsp10, Hsp40A4, Hsp40B6, Hsp40B11, Hsp60, Hsp70, glucose-regulated protein 78 (Grp78), Hsp90a, Hsp90b and Grp94 after *Streptococcus parauberis* infection in the flounder. Therefore, the increased expression of *HSPA5* could be due to the stimulus of the innate immune response against VHSV as *HSPA5* is related to immune response genes as stated above.

Taken all together, our results suggested that the overexpression of two major pro-inflammatory cytokines (*IL-1 β* and *IL-6*) in hyperglycemic zebrafish could induce risk factors

that cause a higher virus replication. Moreover, results indicate that the treatment of co-incubation of glucose and VHSV would cause a synergistic effect on the production of cytokines (*IL-1 β* and *IL-6*) as well as the activation of the immune response via *HSPA5* expression. These 3 genes that were differentially regulated between GLU/CTL and virus/mock could be considered as the potential biomarkers of the relationship between hyperglycemia and VHSV infection in zebrafish.

4. CONCLUSION

The current study is the first to report a relationship between high glucose diet and VHSV infection in zebrafish. The results revealed that high glucose diet-induced hyperglycemic zebrafish resulted in increased replication of VHSV. Moreover, high glucose diet and/or VHSV infection highly induced inflammatory production and the expression level of ER stress in adult zebrafish. It has also suggested that the high glucose-based diet modulated metabolism syndrome may be connected to the immune system via inflammation which impairs the immunity in zebrafish thereafter promotes higher infectivity. Thus, it can serve as a platform for further studies to give a novel insight into metabolic syndromes and infectious diseases.

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