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Note

Characterization of histone gene expression in sevenband grouper, *Hyporthodus septemfasciatus* against nervous necrosis virus infection

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Recent studies revealed that histone proteins are involved in innate immune responses during pathogen invasion as well as DNA packing. This study characterized the histone genes (H2A.V) of sevenband groupers and analyzed gene expression in NNV-infected sevenband groupers. The open reading frame (ORF) of H2A.V is 387 bp which encoded 128 amino acid residues. The deduced amino acid sequence of H2A.V harbor a highly conserved domain for H2A/H2B/H3 and H2A_C binding domain. Quantitative real-time PCR analysis showed that H2A.V had a high gene expression level in the brain and blood after being NNV-infected. An increase in extracellular histone protein in the blood has been identified as a biomarker for vascular function in humans. More research is required to understand histone's immune response at the protein level or in aquatic animals.

Key words: Histone, nervous necrosis virus NNV, gene expression

Nervous necrosis virus (NNV) is a member of the Nodaviridae family which is further subdivided into alphanodavirus and betanodavirus. Alphanodavirus mainly infects insects while betanodavirus infects fish. The NNV genome consists of two single stranded positive sense RNA, RNA1 (about 3.1 kb) encodes RNA-dependent RNA polymerase required for viral genome replication, and RNA2 (1.4 kb) encodes capsid protein (Tan *et al.*, 2001). RNA3 is made from the 3'-terminus of RNA1 and encodes a non-structural protein, B2 protein (Mézeth *et al.*, 2009). Based on the phylogenetic analysis of the T4 variable region, which consists of 427 bases of RNA2 sequence, the fish nodaviruses are classified into four genotypes: tiger puffer NNV (TPNNV), striped jack NNV (SJNNV), barfin flounder NNV (BFNNV), and red-spotted grouper NNV (RGNNV) (Nishizawa *et al.*, 1995). NNV infected fish show abnormal swimming behavior, darkening, and vacuolization of nervous tissues (brain, spinal cord, and retina) (Hoeksema *et al.*, 2016, Grotmol *et al.*, 1997).

Groupers are important warm temperate water fishes that live in tropical, subtropical, and temperate water zones and have a high economic value in aquaculture (Kim *et al.*, 2012). The sevenband grouper, *Hyporthodus septemfasciatus*, is a highly prized marine finfish with significant aquaculture potential in Southeast Asia. Because of their scarcity of resources

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and high economic value, sevenband groupers have been considered candidate species for aquaculture. Although sevenband grouper aquaculture took off in Korea 20 years ago, its production amount is decreased with 292 tons produced in 2018, 145 tons in 2019, and 79 tons in 2020 (Lim, 2021). The decline of sevenband grouper production is due to an increase in larval mortality in hatcheries and cage cultured juveniles and adults by NNV infection.

Histone proteins are involved in DNA packing as well as regulation of gene expression and innate immune response to pathogens (Over and Michaeals, 2014). Histones are proteins found in eukaryotes and some archaea, and are classified into five types: H1/H5, H2A, H2B, H3, and H4. There have not been many studies on the innate immunity of histone proteins in fish. Histone H1-like protein of olive flounder (*Paralichthys olivaceus*) showed that strong antimicrobial activity against Gram positive and Gram-negative bacteria (Nam *et al.*, 2012). The H1 and H2B of European sea bass (*Dicentrarchus labrax*) and gilthead seabream (*Sparus aurata*) were found to relate to the immune response of NNV (Valero *et al.*, 2016). In *Carassius aurutus*, H2A was shown to play an important role in defense against invasion by *Aeromons hydrophila* (Kong *et al.*, 2017). Other studies have shown that histone mimics may not only potentiate the ability of the virus to interfere with the host, but also increase virus dependence on host transcription (Schaefer *et al.*, 2013). Furthermore, in the case of lytic infection by herpes simplex virus type 1, a study confirmed that histone strain H3.3 regulates gene expression (Plascek *et al.*, 2009). Antiviral activity of arginine-rich histone H3 against influenza A virus has been confirmed in studies (Hoeksema *et al.*, 2015). However, little study has been done on the role of histone in NNV-infected sevenband grouper.

In this study, we cloned and characterized histone gene H2A.V which is variant of H2A from sevenband grouper and analyzed the histone transcript expression level in each tissue of the sevenband grouper infected with NNV.

Materials and Methods

Fish and virus

Juvenile sevenband groupers were purchased from aquafarm where VNN (Viral nervous necrosis) had never occurred. Fishes were acclimated to the laboratory recirculating seawater system at 25℃ for two weeks and were fed twice daily before experimental manipulation. To confirm the absence of NNV, representative samples were screened by real-time PCR before use in experiments according to previous study (Kim *et al.*, 2016). In this study, NNV isolate SGYeosu08 (Kim *et al.*, 2012) belonging to the RGNNV was used. NNV was propagated in SSN-1 cell line, which had been established from whole fry tissue of striped snakehead Ophicephalus striatus (Iwamoto *et al.*, 2000). To propagate NNV, SSN-1 cells were grown in 75-㎠ cell culture flasks until 90-95% confluence and NNV was inoculated onto SSN-1 cell monolayer. Once an extensive complete cytopathic effect(CPE) was observed, cell lysate was collected and centrifugated at 3,300 x g, for 15 min at 4℃. After centrifugation, the supernatant was aliquoted and stored at -80℃ as virus sample.

Cloning and characterization of Histone H2A.V gene

The cDNA sequences, which indicated homology to known histone genes (H2A.V), were identified from the previous study (Kim *et al.*, 2017) and primers for cloning and quantitative PCR were designed based on the sequences (Table 1). The PCR amplified products were cloned and re-sequenced to evaluate the open reading frame (ORF). Total RNA was extracted from the brain of *H. septemfasciatus* using RNeasy® Plus Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. cDNA was synthesized using SuPrimeScript RT-premix (GeNet Bio, Daejeon, Korea). Then, cDNA was amplified us-

Table 1. Primers used in this study

Primer	Sequence(5'-3')	Used for
H2A.V_for	ATGGCTGGTGGCAAGGCAG	cloning
H2A.V_rev	CTATGCAGTCTTCTGCTGGCCC	cloning
H2A.V_qPCR_for	AGCGGTGAGGTACTCAAGGA	qPCR
H2A.V_qPCR_rev	GGTCGTATCCACAGGCACTT	qPCR
EF1a_qPCR_for	CGAGAAGTTCGAGAAGGAAGC	qPCR(reference)
EF1a_qPCR_rev	GATGAGCTGCTTCACACCAAG	qPCR(reference)

ing Table 1 primer set. The PCR reaction was performed in a 20 μl volume containing AccuPower™ PCR Premix (Bioneer, Daejeon, Korea), 1 μl of each specific primer pair, 2 μl cDNA and 16 μl ddH20. The PCR conditions were 95℃ for 10 min, followed by 30 cycles of 95℃ 30 sec, 60℃ 30 sec, 72℃ 1 min. PCR product was eluted from the agarose gel with AccuPrep™ PCR/Gel Purification Kit (Bioneer, Daejeon, Korea) and ligated with pGEM®-T Easy Vector of TA cloning kit (Promega). Ligation mixture was incubated for overnight at 4℃ and transformed into BioFACT™ competent Cell(DH5α) (BioFACT, Daejeon, Korea) by heat shocked transformation. The transformants were placed on MacConkey agar plate containing ampicillin (50 mg/ml) and incubated at 37℃ for 18 hours. The single colony was inoculated to LB broth containing ampicillin (50 mg/ml) and plasmid DNA was extracted with AccuPrep™ Plasmid Mini Extraction Kit (Bioneer, Daejeon, Korea). Plasmid DNA was sequenced by Bioneer (Daejeon, Korea). Conserved domains and signal peptides of the histones H2A.V polypeptide were analyzed using the PROSITE database searching tool. Multiple-sequence alignment of the reported histones gene sequence was constructed by ClustalW and a phylogenetic tree was constructed using the MEGA 11 software based on maximum likelihood method with 1,000 bootstrap replicates (Tamura *et al.*, 2021).

Virus challenge and quantitative PCR

Juvenile sevenband grouper (average weight 12.3 ± 0.7 g) were intramuscularly injected with NNV at a dose of 10^4 $TCID_{50}$/100 μl/fish, whereas negative control fishes were injected with L15 medium at 100 μl/fish. After the challenge, animals (n = 3 from each tank) from infected and control groups were collected at five time points (1 day, 2 day, 3 day, 4 day, 5 day) post challenge. Tissues (spleen, heart, gill, brain, eye, liver, kidney, and blood) were collected aseptically from the sampled fishes. Total RNA was extracted sevenband grouper spleen, heart, gill, brain, eye, liver, kidney and blood with TransZol Up (Transgenbiotech, Beijing, China). cDNA was synthesized using M-MLV Reverse Transcriptase (Bioneer, Daejeon, Korea). Quantitative real-time PCR was performed to determine expression levels of histone in different tissues on Exicycler™ 96 Real-Time Quantitative Thermal Block (Bioneer, Daejeon, Korea). The PCR reaction was performed in a 20 μl volume containing 10 μl AccuPower 2X Greenstar qPCR Mastermix, 1 μl of each specific primer pair, 2 μl cDNA, 6 μl ddH_20. The PCR conditions were 95℃ for 5 min, followed by 40 cycles of 95℃ 30 sec, 60℃ 30 sec, 72℃ 30 sec. Melting curve analysis of amplification products was performed at the end of each PCR to confirm that only one product was amplified. The expression level of histones was analyzed using the comparative threshold cycle method ($2^{-\Delta\Delta CT}$) with EF1α as an internal reference. EF1α was amplified as an internal control using primers EF1α_F and EF1α_R (Krishnan *et al.*, 2019), while histones amplify used primers. The primers used, specific for the histone H2A.V were designed using the Primer3Plus tool (Primer3Plus - Pick Primers) and are shown in Table 1.

Statistical analysis

Data in figures are represented as mean values ± standard error (SEM) of each group. Statistical significance was analysed by one-way ANOVA using Prism software (Ver 8.2.1).

Results and Discussion

Histones are basic proteins that have been conserved throughout evolution and are found in all eukaryotic cells. They play an important role in the organization and regulation of DNA as an important component of chromosomes (Luger *et al.*, 1997). In addition, histones have been also linked to innate immune response being their role as antimicrobial peptides (AMPs) which were first characterized in mammals a long time ago (MacMillan and Hibbitt, 1969). They act as functional barricades to cells exerting various antimicrobial actions, including penetration into the membrane, binding to bacterial lipopolysaccharide (LPS) in the membrane, neutralizing the toxicity of bacterial LPS, and entrapping pathogens as a component of neutrophil extracellular traps (NETs) (Tsourouktsoglou *et al.*, 2020).

The histone genes H2A.V of *H. septemfasciatus* were cloned and the characteristics of the gene were determined using sequence analysis. The amino acid sequence of H2A.V had an H2A/H2B/H3 binding domain as well as modification sites (Fig. 1). Multiple alignments with other species confirmed that H2A.V has the same amino acid sequence as fish and mammals, indicating that the interspecies sequence is very conservative (Fig. 2). There were no significant differences in phylogenetic analysis due to the amino acid sequences were highly conserved in various species (Fig. 3).

Histone gene expression in healthy *H. septemfasciatus* tissues was investigated (Fig. 4). H2A.V genes were most expressed in the heart. When the results

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   1  GGATGGGTGAGTGCTCTGCAAGCTGCTCGGCGATTGGTCGAAACAGAGATTCGAAGTAAC  60
          M  A  G  G  K  A  G  K  D  S  G  K
  61  AAAACGCACCCTGATTGGCTGTCCAGACTCCCACGAGGGGTCGCATGCAAAAGATTTGCG  120
 121  AAAGGGTTTATTGTTTCTTTGCTTTCGGGAGCCGAAATTGTTTGCTATCTCCTCAGTTCT  180
 181  ACAATCAAATAACGACACCGAAAAATGGCTGGTGGCAAGGCAGGAAAAGACAGTGGCAAA  240
      A  K  A  K  A  V  S  R  S  Q  R  A  G  L  Q  F  P  V  G  R
 241  GCCAAGGCGAAAGCAGTGTCTCGCTCCCAGAGGGCTGGGCTGCAGTTCCCAGTGGGTCGT  300
      I  H  R  H  L  K  T  R  T  T  S  H  G  R  V  G  A  T  A  A
 301  ATCCACAGGCACTTGAAGACTCGCACAACCAGCCACGGGCGCGTAGGAGCCACAGCAGCT  360
      V  Y  S  A  A  I  L  E  Y  L  T  A  E  V  L  E  L  A  G  N
 361  GTGTACAGTGCAGCTATCCTTGAGTACCTCACCGCTGAAGTACTAGAGTTGGCGGGTAAT  420
      A  S  K  D  L  K  V  K  R  I  T  P  R  H  L  Q  L  A  I  R
 421  GCCTCCAAAGACTTGAAGGTGAAGCGTATCACTCCCCGTCACTTGCAGCTGGCCATCCGT  480
      G  D  E  E  L  D  S  L  I  K  A  T  I  A  G  G  G  V  I  P
 481  GGTGACGAGGAGTTGGACTCCCTTATCAAGGCAACAATTGCTGGAGGAGGTGTCATTCCC  540
      H  I  H  K  S  L  I  G  K  K  G  Q  Q  K  T  A  *
 541  CACATCCACAAATCCCTCATTGGGAAGAAGGGCCAGCAGAAGACTGCATAGACGCCATGT  600
 601  TGACCAGAAATTTCGGGGCTTGGGCTTTGATCGGACCAGGAGTTCACATTTGTTCTTTTT  660
 661  TATTATTAATATTATAGCAAACGAAGAGTGATTGTTAGGATTTGTGTTGTAATATTTTCC  720
 721  CATAACTAGAAAAAAAAGTACAGAAAACCCCTTAACGACAAAAAGAAAAAAAAAACTTTG  780
 781  TATGTTATTGTAGAACGTTTGACTGGGGAGTTCATTATTCTTTCAAATGGTGACCGTGGA  840
 841  TTTGTTTGATTGGCCTGGGACTCTGTAATGTTTTATACTGAAAGAAATTGTTAAAACCGT  900
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Fig. 1. The cDNA and deduced amino acid sequence of *H.septemfasciatus* histone H2A.V. H2A.V gene consists of a 387 bp ORF encoding 128 amino acids. Histone H2A/H2B/H3 binding domain is boxed and H2A_C binding domain is in shadow. The ubiquitination site is circled.

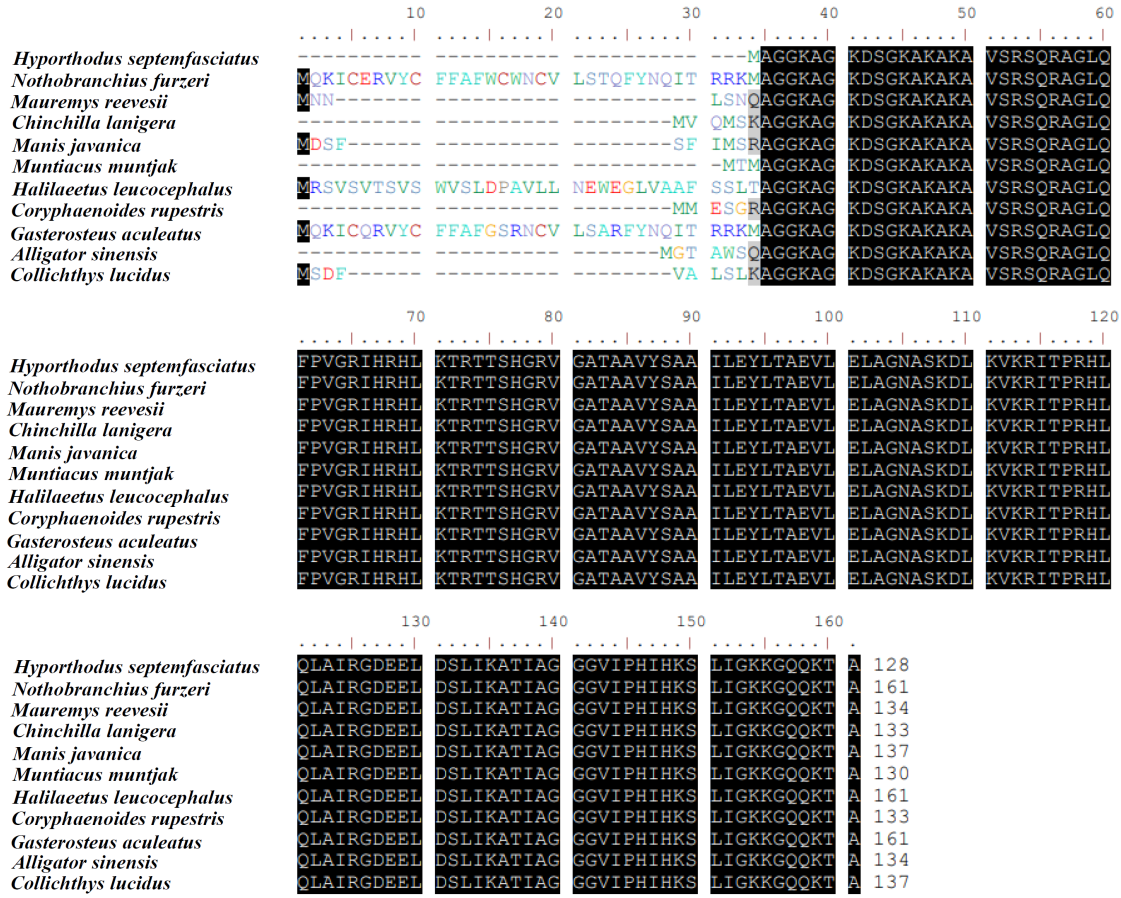

Fig. 2. Comparison of the sevenband grouper H2A.V amino acid sequence with other known H2A.V proteins.

of *H. septemfasciatus* histone expression was compared to the results of European seabass and gilthead seabream histone expression, it was confirmed that there was a difference in expression amount for each tissue. H2B gene of European sea bass did not expressed constitutively in brain, skin, spleen while all tissues in gilthead seabream are showing constitutive expression level of H2B gene (Valero *et al.*, 2016). Four histones (H1, H2AX, H3, H3.3) exhibited relatively high expression levels in immune organs (blood, head kidney, and liver) of naive olive flounder (Wang et al., 2020). This suggests that differences in the amount of histone gene expression by tissue may exist between fish species.

After infecting *H. septemfasciatus* with nervous necrosis virus, the expression of histone genes in each tissue was compared and analyzed over time (Fig. 5). At 2 dpi (day post infection), H2A.V confirmed a high expression volume in the brain and blood, which then gradually decreased. Considering NNV causes necrosis of the brain and retina (Lin *et al.*, 2021), the increased expression of H2A.V genes in the brain, eye suggests that histone might be involved in innate immune response against NNV infection. The amount of expression in liver was lower than in brain and blood, but it increased at 48 hpi and then gradually decreased. A biomarker indicating vascular dysfunction, such as severe trauma or sepsis, is an increase in the amount of extracellular histone in the bloodstream (Villalba *et al.*, 2020). Increased histone ex-

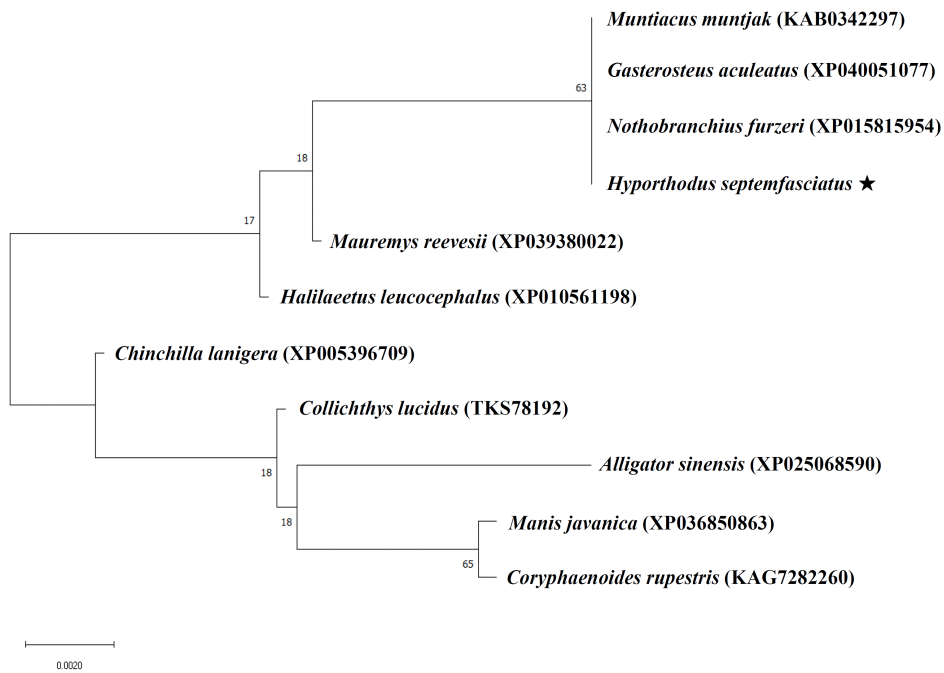

Fig 3. Maximum-Likelihood tree of complete amino acid sequence of H2A.V. The bootstrap value shown at the node of the tree (1,000 bootstrap)

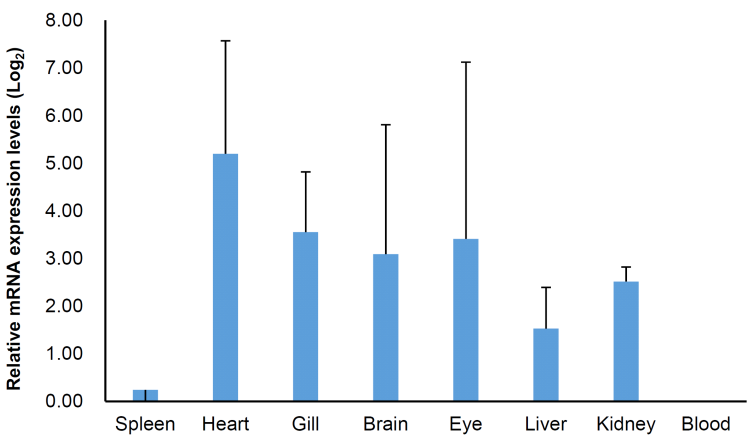

Fig. 4. Relative expression of Histone H2A.V mRNA in various tissues of healthy *H. septemfasciatus*. Each value represents a mean value ± SD of replicates (n=3).

pression in the blood suggests that histones play a role in innate immune responses. Wang et al. (2020) also mentioned that the high expression of flounder histones in immune organs including blood indicated their involvement in anti-pathogenic infection. H2A from Gold fish (*Carassius aurutus*), was shown to play a critical function in defense against invasion by *Aeromons hydrophila* (Kong *et al.*, 2017). In case of NNV, Valero et al. (2016) characterized two histones (H1 and H2B) from gilthead seabream and European sea bass and suggested that both histones are related to immune response against NNV. Indeed, other studies explained that histone mimics lead to the expression of critical regulatory genes associated with inflammatory and viral diseases (Schaefer *et al.*, 2013).

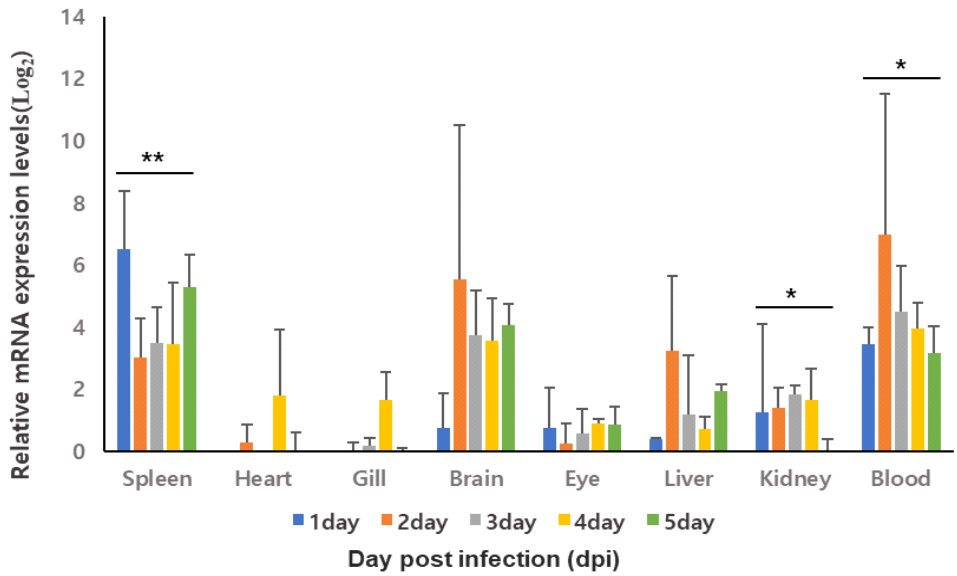

Fig. 5. Relative expression of Histone H2A.V mRNA in various tissues of *H. septemfasciatus* after NNV infection. Each value represents a mean value ± SD of replicates (n=3). Significant differences within tissues were determined by one-way ANOVA and marked by asterisks ($^{*}p < 0.05$, $^{**}p < 0.01$).

In conclusion, this study identified and analyzed characteristic of the *H. septemfasciatus* histone gene (H2A.V). Furthermore, H2A.V genes were found to be involved in the immune response of *H. septemfasciatus* infected with NNV by analyzing the histone gene expression of each tissue by infection time. More research is needed to identify histone's innate immune response mechanism and to explain the immune response at the protein level of the histone gene.

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