Ghrelin attenuates infectious bursal disease virus–induced early inflammatory response and bursal injury in chicken

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ABSTRACT Studies demonstrated that chicken ghrelin mRNA was expressed in immune organs of chicken. However, it was not known for its functions in chicken immune system. This study aimed to investigate the effects of ghrelin on infectious bursal disease virus (IBDV)-induced acute inflammatory and bursal injury. Chickens were divided into 4 groups. One group was used as control (“C”). The other three groups incubated with IBDV on the 19th d, of which 2 were injected intraperitoneally with 0.5 nmol (“LG”) or 1.0 nmol (“HG”) ghrelin/100g body weight from 18th to 22nd d, respectively, and one was injected intraperitoneally with PBS (“T”). Results showed that cytokines including interleukin (IL)-6, IL-1β, and IL-8 mRNA expression in I group were upregulated significantly after chickens infected with IBDV from 1 d post-infection (dpi) to 3 dpi (P < 0.05). However, the expression level of IL-6, IL-1β, and IL-8 mRNA in LG and HG groups was 7.3, ~43.3% as much as that of the I group at 2 dpi and 3 dpi (P < 0.05). Moreover, ghrelin administration attenuated significantly the bursal injury from 1 dpi to 7 dpi and prevents the reduction of bird weight gain at 5 dpi and 7 dpi, which were induced by IBDV (P < 0.05). The results indicated that ghrelin could play an important role in the immune system of chicken.

Key words: ghrelin, inflammatory response, bursal injury, IBDV, chicken

INTRODUCTION Infectious bursal disease virus (IBDV) is an immunosuppressive chicken Avibirnavirus of the family Birnaviridae. It mainly infects and destructs dividing B-lymphocytes and causes pronounced necrosis or apoptosis of B lymphocytes within the follicles of the bursa of Fabricius (BF) during 3–5 d post-infection (dpi) of IBDV (Jungmann et al., 2001). The loss of B lymphocytes leads to a suppressed primary antibody response, which decreases the susceptibility of chickens to other vaccines and resistibility to other disease and further results in the increase of chicken morbidity and mortality. Therefore, IBDV remains a threat to the commercial poultry industry. In addition, viral replication in the bursa is associated with the infiltration of inflammatory cells such as T lymphocytes, macrophages, and heterophils in the BF (Rautenschlein et al., 2002; Khatri et al., 2005; Palmquist et al., 2006; Li et al., 2018). These inflammatory cells produce cytokines such as interleukin 6 (IL-6), interleukin-1β (IL-1β), and interleukin 8 (IL-8), which are important in initiating the local inflammatory response at the site of infection. However, overproduction of proinflammatory cytokines may destruct tissue, retard recovery, and harm the host. Many studies suggest that excessive production of inflammatory cytokines that results from viral infection may lead to “cytokine storm” and then autologous cell and tissue destruction and further death of animals (O’Shea et al., 2002; Xie et al., 2018). Therefore, controlling the overproduction of proinflammatory cytokines and tissue injury from virus infection is important.

Ghrelin is a peptide hormone with many physiological functions. In the last 20 yr, studies have demonstrated that ghrelin plays a vital role in the immune system of humans and animals. For example, ghrelin promotes the age-associated changes in thymic architecture and thymocyte numbers and enhances the proliferation of murine CD4+ primary T cells and a CD4+ T-cell line through upstream activation of phosphatidylinositol-3-kinase.
and protein kinase C (Dixit et al., 2007; Lee et al., 2014). Reports have also demonstrated that ghrelin inhibits proinflammatory cytokines IL-6, IL-1β, and tumor necrosis factor alpha expression and promote anti-inflammatory cytokines interleukin-10 (IL-10) expression (Dixit et al., 2004; Waseem et al., 2008; Ziko et al., 2018; de Paula Silva et al., 2019). In addition, ghrelin has been considered as a potent anti-inflammatory mediator in mammals (Baatar et al., 2011). In some animal models of diseases such as colitis and sepsis, the administration of ghrelin attenuates the inflammatory and autoimmune response (Gonzalez-Rey et al., 2006; Zeng et al., 2015). Ghrelin effectively suppresses expression of tumor necrosis factor alpha–induced inflammatory factors including intercellular adhesion molecule-1, vascular cell adhesion molecule 1, monocyte chemoattractant protein-1, and IL-1β (Zhang et al., 2019). Moreover, many reports suggest that ghrelin protects organs from damage induced by burn, medicine, and diseases in different animal models (Dembinski et al., 2003; Kiang et al., 2014; Zeng et al., 2015). Recently, Martinez et al. (2016) found that a novel synthetic growth hormone secretagogues A233 (ghrelin-like) could promote the expression of antiviral protein complexes such as mitochondrial antiviral-signaling protein, enhance superoxide anion production and the interferon-γ level in murine macrophage J774A.2 cells, and reduce viral load in a dengue virus mouse model of infection, which suggested that ghrelin also had the antiviral activities.

Chicken ghrelin consists of 26 amino acids and has high homology with mammals (Kaiya et al., 2002), and its mRNA is also expressed in different organs including the bursa and spleen (Ma et al., 2015). However, whether chicken ghrelin has the same function as mammals in immune system is not known. In the previous study, we demonstrated that plasma ghrelin levels increased sharply after chickens were infected with IBDV with robustly increasing cytokines level (Yu et al., 2019). Moreover, this increase positively correlated with the inflammatory cell infiltration in bursa of chicken (Yu et al., 2019). This observation prompted us to speculate that ghrelin may modulate chicken acute inflammatory response induced by IBDV. Thus, in the present study, we performed an investigation to determine the effect of ghrelin administration on the early inflammatory responses and whether ghrelin attenuates the bursal injury of chicken infected with IBDV.

MATERIALS AND METHODS

Experimental Design and Sample Collection

One hundred and eighty specific pathogen-free Hyline brown laying chickens (presented by HULAN BIO COMPANY, Xinxiang, Henan) were raised in negative-pressure isolators (GJ-1; Suzhou Fengshi Laboratory animal equipment Co., Ltd.) at a specific pathogen-free animal laboratory on the hatch day. The fodder was purchased from Merial Vital Laboratory Animal Technology Co., Ltd. (Beijing, China), and sterile water was given ad libitum. At the 18th d of raising (D18), the chickens were weighed, and those weighing approximately 270 g were selected and randomly divided into 4 groups (n = 21 + 21). From day 18 (D18) to D22, one group of chickens (LG group) was injected intraperitoneally with 0.5 nmol of ghrelin/100 g body weight (BW) (Abcam, ab120231) according to previous studies (Furuse et al., 2001; Geelissen et al., 2006). The second group of chickens (HG group) was injected intraperitoneally with 1.0 nmol of ghrelin/100 g BW. At D19, the third group of birds (I group), LG group, and HG group were inoculated with the virulent IBDV strain BC6/85 (purchased from China Institute of Veterinary Drug Control, Beijing, China) at a dose containing 10^5.23 EID50/0.1 mL each through ocular-nasal. The fourth group (C group) was mock treated with sterile PBS (pH = 7.38). Chickens of each pen in I group, LG group, and HG group were raised in different negative-pressure isolators in one room, and chickens of 2 pens of the C group were raised in different negative-pressure isolators in another room. From 1 dpi to 3 dpi, six chickens were randomly collected from each group (3 chickens per pen), weighed, and then euthanized with cervical dislocation under deep nembutal anesthesia (45 μg/g of BW, intraperitoneal injection; Shanghai Chemical Factory, Shanghai, China). The BF was quickly collected from each chicken. One part of each BF was fixed in 4% paraformaldehyde (Solarbio, P1110, Beijing, China) for hematoxylin and eosin (HE) staining, and the other part of BF was frozen in liquid nitrogen and stored at −80°C for total RNA isolation. The remaining chickens of different groups were weighed at 4, 5, and 7 dpi and then euthanized to collect BF. The BF was fixed in 4% paraformaldehyde for HE staining. All animal experiments in the study were approved by the scientific ethical committee of the Henan Institute of Science and Technology.

Histopathological Examination and Analysis

The deparaffinized tissue sections were stained with HE for histopathological examination. A semi-quantitative histopathological assessment was based on the following scoring system (Table 1) according to a previous study (Shaw and Davision, 2000). Three histological features of the BF were scored from 0 to 3, including bursal follicle, fibro in bursa interstitium, and infiltrated inflammatory cells, and the total score was the last score. All the samples in each group were assessed, and the data were shown as means ± SD.

Quantification of IL-6, IL-1β, and IL-8 in BF

For quantitative real-time PCR, total RNA was extracted from BF with TRIzol reagent (Life Technologies, NY), and cDNA was synthesized by using a PrimeScriptRT reagent kits with gDNA eraser (TakaRa, Dalian, China) as previously described (Ma et al.,
Three pairs of primers screened were used to detect the expression of IL-6 (forward: AAATCCCTCCTCGCCAATCTG, reverse: CCTCACGGTCTTCTCCATAAACG, HM179640), IL-1β (forward: ATGTCGTGTGTGATGAGCGGC, reverse: AGGCGGTAGAAGATGAAGCGG, NM_204524), IL-8 (forward: GCTGCTCTGTCGCAAGGTAGG, reverse: ACATCTTGAATGGATTTAGGGTGG, HQ008779), respectively, and β-actin (forward: TTGTGATGACTGGTGATGGTG, reverse: TTCTCTCTCGGCTGTGGTGGTG, NM_205518) was applied as an internal standard. The primer pairs for IL-6, IL-1β, and IL-8 and β-actin were synthesized. The real-time PCR procedure was described briefly as follows: an initial incubation for 30 s at 95°C, 40 cycles of 5 s at 95°C, 20 s at 58°C, and 20 s at 72°C. The quantification was based on the fluorescence detection by real-time PCR with SYBR Premix Ex Taq II (Takara, RR820 A, China). The specificities of the PCR products were assessed by dissociation curve analyses. Each sample was repeated in triplicate. The data were analyzed with the QuantStudio 5 (Applied Biosystems, Thermo Fisher Scientific, Marsiling, Singapore) using the $2^{-\Delta\Delta CT}$ approach.
method. The relative expression of cytokines was that target gene relative to the housekeeping gene.

**Statistical Analysis**

All the data in the present experiments were analyzed with multiple comparison (LSD based on proc anova) by using SAS software (version 8.01; SAS Institute, Cary, NC) to measure the differences. A $P$ value of less than 0.05 was considered as statistically significant.

**RESULTS**

**Ghrelin Reduces the Expression of Chicken Pro-inflammatory Cytokines Induced by IBDV**

In the present study, cytokines in the BF were first detected to explore whether exogenous ghrelin could modulate chicken pro-inflammatory cytokines induced by IBDV (Figures 1A–C). At 1 dpi, the IL-6, IL-1β, and IL-8 mRNA expression levels increased in the I group, which were 6.19-, 2.19-, and 1.86-fold higher than those of the C group, respectively ($P < 0.05$). However, in the LG and HG groups, the IL-6, IL-1β, and IL-8 mRNA expression levels were significantly lower than that of the I group ($P < 0.05$). However, the pattern of IL-6, IL-1β, and IL-8 mRNA expression at 3 dpi in different treatment groups were significantly lower than that of the C group ($P < 0.05$) that were 140.04-, 268.97-, and 112.51-fold higher than those of the I group ($P < 0.05$). However, the IL-6, IL-1β, and IL-8 mRNA expression level in the BF increased abruptly in the I group, which were 140.04-, 268.97-, and 112.51-fold higher than that of the C group ($P < 0.05$) but significantly higher than those of the C group except for IL-8 mRNA expression, which was significantly lower than that of the C group. At 2 dpi, the IL-6, IL-1β, and IL-8 mRNA expression level in the BF increased abruptly in the I group, which were 140.04-, 268.97-, and 112.51-fold higher than that of the C group. At 3 dpi, the IL-6, IL-1β, and IL-8 mRNA expression levels in the I group were 7.30 to 43.34% lower than that of the I group ($P < 0.05$), although they were all significantly higher than that of the control. At 3 dpi, the IL-6, IL-1β, and IL-8 mRNA expression level in the BF in different treatment groups decreased compared with that of 2 dpi. However, the pattern of IL-6, IL-1β, and IL-8 mRNA expression at 3 dpi in different treatment groups was similar to that of 2 dpi, of which was the IL-6, IL-1β, and IL-8 mRNA expression levels in the I group which were significantly higher than those of the C, LG, and HG groups ($P < 0.05$) and significantly lower in the C group than those of the LG and HG groups ($P < 0.05$). Therefore, IBDV induced high expression of pro-inflammatory cytokines in chicken, and ghrelin administration downregulated their high expression evoked by IBDV.

**Ghrelin Attenuates the Bursal Injury of Chicken Induced by IBDV**

Reports suggested that excessive production of inflammatory cytokines, which resulted from viral infection, might lead to tissue destruction. Hence, we investigated whether ghrelin attenuated the injury of the chicken bursa induced by IBDV. Histological examination of bursal tissues revealed that bursal injury induced by IBDV varied over time and depending on administration of ghrelin (Table 2). The bursal follicle of chicken in the C group was intact, the demarcation of the cortex and medulla was clear, and size of interstitium was normal (Figures 2A, E, I, M, Q, U), and therefore, the scores were 0 during the entire experiment (Table 2). However, when chickens were inoculated with IBDV, the bursal follicle of the chicken bursa became smaller, the demarcation of cortex and medulla disappeared, and interstitial size became bigger in the I group at 1 dpi (Figure 2B), and according to the scoring system, the score was 2.00 ± 0.00. While in the LG and HG groups, only connective tissue emerged in bursal interstitium (Figures 2C, 2D), and the score was 1.00 ± 0.00 (Table 2). At 2 dpi, the number of lymphocytes in bursal follicular depleted, but <50%. Connective tissue emerged in the bursa interstitium. And, a large number of inflammatory cells infiltrated into both bursa interstitium and bursal follicle except in the LG group (Figures 2F–2H). According to the scoring system, the score of LG was lower significantly than that of the I and HG groups ($P < 0.05$; Table 2). At 3 dpi, still <50% of lymphocytes in bursa follicle depleted; infiltrated inflammatory cells distributed mainly in bursal interstitium in the I, LG, and HG groups. However, connective tissue took up >50% of bursal interstitium in I and HG groups (Figures 2J–2L). Therefore, the pathological scores were 5.67 ± 1.53, 4.00 ± 1.00, and 4.33 ± 0.58 in the I, LG, and HG groups, respectively, and bursa injury was less severe in the LG and HG groups than in the I group ($P < 0.05$; Table 2). At 4 dpi, >50% of lymphocytes in bursa follicle depleted, a

| Day post infection | C Group | I Group | LG group | HG group |
|-------------------|---------|---------|----------|----------|
| 1                 | 0.00 ± 0.00a | 2.00 ± 0.00b | 1.00 ± 0.00c | 1.00 ± 0.00d |
| 2                 | 0.00 ± 0.00a | 4.00 ± 1.00a | 2.33 ± 0.57b | 4.00 ± 1.00a |
| 3                 | 0.00 ± 0.00a | 5.67 ± 1.53a | 4.00 ± 1.00a | 4.33 ± 0.58b |
| 4                 | 0.00 ± 0.00a | 7.67 ± 0.58a | 3.67 ± 0.58a | 3.33 ± 0.58a |
| 5                 | 0.00 ± 0.00a | 8.00 ± 1.00a | 5.33 ± 1.15a | 5.00 ± 1.00a |
| 7                 | 0.00 ± 0.00a | 6.00 ± 1.00a | 5.00 ± 1.00a | 5.33 ± 1.15a |

The same letter represented no significance ($P > 0.05$), and different letters represented significant difference ($P < 0.05$) among different groups at the same day.
large number of infiltrated inflammatory cells distributed in bursal interstitium and connective tissue took up >50% of bursal interstitium in the I group (Figure 2N), and the pathological score was 7.67 ± 0.58. While in the LG and HG groups, many lymphocytes came back to the bursal follicle and demarcation of cortex and medulla emerged; many infiltrated inflammatory cells were distributed in the bursal interstitium. Connective tissue also took up >50% of bursal interstitium (Figures 2O, 2P). Therefore, the total scores were 3.67 ± 0.58 and 3.33 ± 0.58 in the LG and HG groups, respectively (Table 2). At 5 dpi, in the I group, depletion of lymphocytes in bursa follicle was very severe, and only the follicular outlines remained; connective tissue nearly took up all the bursal interstitium, and a large number infiltrated inflammatory cells were found distributed in the bursal interstitium (Figure 2R) and the total score was 8.00 ± 1.00 (Table 2). However, in LG and HG groups, the injury became severe again compared with the LG and HG groups at 4 dpi. Demarcation of cortex and medulla disappeared, and more but <50% of lymphocytes in bursal follicle depleted. Connective tissue still took up nearly all the bursal interstitium, and many of the infiltrated

Figure 2. Bursal histopathology of chickens in different treatment groups. A, E, I, M, Q, and U represent the bursal follicle in the C group at 1, 2, 3, 4, 5, and 7 dpi; B, F, J, N, R, and V represent bursal follicle in the I group at 1, 2, 3, 4, 5, and 7 dpi; C, G, K, O, S, and W represent the bursal follicle in the LG group at 1, 2, 3, 4, 5, and 7 dpi; D, H, L, P, T, and X represent the bursa nodules in the HG group at 1, 2, 3, 4, 5, and 7 dpi. Abbreviations: b, bubble; BF, bursal follicle; c, cortex; f, fibro; inc, inflammatory cells; m, medulla.
inflammatory cells were found distributed mainly in the bursa interstitium (Figures 2S, 2T). Therefore, the pathological scores were 5.33 ± 1.15 and 5.00 ± 1.00 in LG and HG groups, respectively (Table 2). At 7 dpi, in the I group, a few of the lymphocytes came back into the bursa follicle and the follicular outlines were more clear, connective tissue continued to take up all the bursa interstitium and a large number infiltrated inflammatory cells were found distributed in bursal interstitium (Figure 2V), and the total score was 6.00 ± 1.00 (Table 2). In the LG and HG groups, <50% of lymphocytes in bursa follicle depleted, connective tissue took up nearly all the bursa interstitium, and the infiltrated inflammatory cells were found distributed mainly in the bursa interstitium (Figures 2W, 2X). Therefore, the pathological scores were 5.00 ± 1.00 and 5.33 ± 1.15 in the LG and HG groups, respectively (Table 2). From these results, ghrelin administration could attenuate the bursa injury of chicken induced by IBDV infection significantly.

**Ghrelin Prevents the Reduction of Bird Weight Gain Induced by IBDV**

Weight increase is an important index to measure the health of animals. Therefore, the weight increase of chicken was demonstrated in the next study. The results showed that the average weight of chickens in different treatment groups had no significant difference compared with that of the C group from 1 dpi to 4 dpi. However, at 5 and 7 dpi, the weight of chickens in the I group was 19.6 and 12.7% lower than that of the C group, respectively (Table 2). The average weight of chickens in the LG and HG groups had no significant difference with that of the C group at 5 dpi and 7 dpi (P > 0.05), although they were slightly lighter than that of the C group, indicating that ghrelin prevented the reduction of bird weight gain (BWG) of chickens induced by IBDV.

**DISCUSSION**

Studies demonstrated that proinflammatory cytokines increased quickly after chickens were infected with IBDV, and as a result, acute inflammation occurred (Liu et al., 2010; Rasoli et al., 2015). In the present study, mRNA expression of cytokines such as IL-6, IL-8, and IL-1β was upregulated by significantly from 1 to 3 dpi in the I group compared with that in the C group. Considerable evidences showed that exogenous ghrelin could modulate the release of inflammatory cytokines in mammals, as reviewed by Baatar et al. (2011). In addition, our previous study has shown that the plasma ghrelin level and ghrelin mRNA expression levels in the BF increased sharply after chickens were infected with IBDV, which was accompanied with high level of cytokine expression and correlated positively with the inflammatory cell infiltration in the BF of chicken (Yu et al., 2019). Therefore, we hypothesized that ghrelin might modulate chicken acute inflammation induced by IBDV. The results showed that mRNA expression of cytokines IL-6, IL-1β, and IL-8 in the BF in the LG and HG groups was significantly lower than that of the I group during the early stage of chicken infection with IBDV. Thus, for the first time, our study has proven ghrelin plays a role in regulating cytokine expression in chicken similar to that of mammals. Further investigation should be performed using different avian disease models and different methods in vivo and in vitro.

In general, the overproduction of proinflammatory cytokines that resulted from viral infection might lead to tissue damage and harm of the host. Therefore, we tested whether downregulating cytokines expression induced by ghrelin would alleviate bursal injury compared with that of the I group. In mammals, a number of studies demonstrated that ghrelin alleviated organ injury in different disease models. For example, ghrelin protected the heart against ischemia/reperfusion injury (Wang et al., 2017), ameliorated acute lung injury after hemorrhagic shock (Zhang et al., 2019), and regulated sepsis-induced rat acute gastric injury (Li et al., 2019). Our results indicated that ghrelin could protect the chicken bursa from damage similar to that of mammals through decreasing the production of cytokines in the bursa evoked by IBDV.

In the present study, chickens infected by IBDV had diarrhea, depression, feather ruffling, and loss of appetite, resulting in the slow increase of weight. However, in the LG and HG groups, these clinical signs of chickens became lighter and weight increased faster than in the I group, which suggested that ghrelin administration prevented the reduction of BWG of chickens induced by IBDV. This seemed paradoxical with previous studies’ reports that ghrelin inhibited chickens’ feeding (Furne et al., 2001; Geelissen et al., 2006; Ocloń and Pietras, 2011; Kaiya et al., 2013). One explanation was that IL-1β and IL-6 could control food intake and energy.
homeostasis through acting on the central nervous system (Dantzer, 2001). Loss of appetite in IBDV-infected chickens might be mediated via proinflammatory cytokines. Therefore, a reduction of the IL-1β and IL-6 levels in ghrelin-treated chickens might be the cause of a comparatively maintained BW and feeding in acute inflammation.

In conclusion, our study investigated the effect of ghrelin administration on the proinflammatory cytokine increase and bursa injury evoked by IBDV. The results showed that ghrelin attenuated the early inflammatory response and bursal injury of chickens induced by the virus. This study was the first direct evidence that ghrelin could regulate cytokine expression and protect organs from damage in chicken, which suggested that ghrelin played a vital role in the innate immune system of chicken. The primary data would lead us to perform further investigation on other functions of ghrelin in the chicken immune system.

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