Downregulation of basic fibroblast growth factor is associated with femoral head necrosis in broilers

P. F. Li, Z. L. Zhou,[1] C. Y. Shi, and J. F. Hou

College of Veterinary Medicine, Nanjing Agricultural University, Nanjing, Jiangsu 210095, China

ABSTRACT Femoral head necrosis (FHN) is a metabolic cartilage disease of rapidly growing broilers. The aim of the present study was to investigate the role of basic fibroblast growth factor (bFGF) in the apoptotic processes associated with FHN. Broilers were selected and categorized based on clinical examination in 3 groups: healthy, femoral head separation, or femoral head separation with growth plate lacerations. Hematoxylin and eosin staining showed fewer chondrocytes in the resting zone of the growth plates when FHN occurred. Moreover, the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay revealed a significant increase in chondrocyte apoptosis. Furthermore, immunohistochemical assays and real-time quantitative PCR analysis demonstrated a decline in bFGF expression. In addition, reduced Bcl-2 mRNA expression was observed along with a corresponding increase in Bax and caspase-3 mRNA expression in FHN samples. There was a correlation between bFGF protein expression and the proportion of TUNEL-positive cells and a correlation between bFGF mRNA expression and expression of Bax, and caspase-3. The results of the study suggested that the expression of bFGF was reduced in the process of chondrocyte apoptosis, which could play an important role in the pathogenesis of FHN in chickens.

Key words: apoptosis, basic fibroblast growth factor, broiler, chondrocyte, femoral head necrosis

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INTRODUCTION

Femoral head necrosis (FHN) occurs in rapidly growing poultry and is commonly associated with lameness, which gives rise to poor bird welfare and causes significant economic losses (Julian, 1998; McNamee and Smyth, 2000). Generally, FHN is characterized by the separation of the epiphysis from the articular cartilage, trabecular bone necrosis and ultimately fracture of the subchondral bone, and it can be divided into femoral head separation (FHS) or femoral head separation with growth plate lacerations (FHSL) depending on severity (Durairaj et al., 2009). Although the study of FHN in broilers has been in progress for many years, the etiologic basis of the disease is either not understood or, at best, understood to a limited extent. A number of pathophysiological mechanisms have been put forward for this disease including bacterial infections (McNamee and Smyth, 2000), lipid metabolism (Erken et al., 2012), mechanical stress (Julian, 1998; Wideman et al., 2012), etc. Recently, some laboratory and human clinical studies have proposed that FHN may be related to a distinct increase in chondrocyte apoptosis (Calder et al., 2004; Xu et al., 2014). However, the role of chondrocyte apoptosis in the etiology of FHN in the modern broiler has not been thoroughly examined.

Apoptosis, or programmed cell death, is strictly regulated by extracellular and intracellular signaling pathways (Thompson, 1995). As a member of the FGF family of proteins, basic fibroblast growth factor (bFGF, or FGF-2) takes part in the multiple cell signaling pathways underlying many significant physiological and pathological responses (Nugent and Iozzo, 2000; Jee et al., 2004; Neary et al., 2005; Skaletz-Rorowski et al., 2005). It has been reported that bFGF plays important roles in regulating cellular apoptosis and bone formation by suppressing the development of apoptosis via complicated pathways together with Bcl-2 and caspase families (Karsan et al., 1997; Miho et al., 1999; Kronenberg, 2003; Fromigue et al., 2005). Based on their roles in the intracellular signal conduction of apoptosis, Bcl-2 family proteins can be classified into 2 groups: the promoters (e.g., Bax and Bad) and the repressors (e.g., Bcl-2 and Bcl-XL) of apoptosis (Rios-Munoz et al., 2005). Bcl-2 is the target gene of bFGF at the transcriptional level (Menzel et al., 1996; Konig et al., 1997). The caspases are a family of cytoplasmic proteases that can trigger pro-apoptotic signals and cleave cellular substrates to induce programmed cell death (Wolf and Green, 1999). Among them, caspase-3 is an important mediator of apoptosis (Wolf et al., 1999; Agas et al., 2008; Ling et al., 2009). Many studies have shown that

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[1]Corresponding author: zhouzl@njau.edu.cn

bFGF up-regulates the expression of Bcl-2 while down-regulating that of Bax and caspase-3 during the apoptotic process (Konig et al., 1997; Agas et al., 2008; Kim et al., 2012). Based on these facts, we speculated that the bFGF signaling pathway may regulate chondrocyte apoptosis when FHN occurred in broilers.

The objective of the present work was to investigate the role of bFGF in regulating the apoptosis of chicken chondrocytes with the presence of FHN in broilers. The mRNA expression of Bcl-2, Bax and caspase-3 was determined to assess the correlation between bFGF and the expression of these anti-apoptotic/pro-apoptotic genes.

MATERIALS AND METHODS

Birds and Tissue Collection

All animal experiments were conducted in accordance with the guidelines of the Nanjing Agricultural University ethical committee and the Chinese Government. One hundred newly-hatched broiler breeder chicks were housed in floor pens with a 23 h light and 1 h darkness cycle. A standard diet and water were given ad libitum. Birds were killed by carbon dioxide asphyxiation at 42 days old, and femoral integrity was examined (Rath et al., 2005). Four birds that spontaneously died of unknown causes during the course of the study were excluded. Femoral problems were categorized into a clean separation of the epiphysis (white cap of articular cartilage) from the underlying, macroscopically undamaged growth plate (FHS) and the separation of the epiphysis from the physis (growth plate), including macroscopic evidence of damage or laceration to the growth plate (FHSL) (Durairaj et al., 2009). The observation was replicated by an independent researcher. The femoral heads from half of the birds from each group of normal, FHS, or FHSL chickens were harvested, cut in half along the coronal plane and fixed overnight in 4% paraformaldehyde in PBS at 4°C. Growth plate tissues from the other half of the birds were shaved with the use of a 5-mm curette and snap frozen in liquid nitrogen.

Histopathology

After a thorough washing, the fixed tissues were decalcified in 4% ethylenediamine tetra acetic acid (pH 7.2) for approximately 3 weeks. To avoid under- or over-decalcification, decalcification was monitored by daily chemical testing. Then, tissues were embedded in paraffin and cut along the coronal plane into 4-μm-thick sections. The sections were processed with hematoxylin and eosin (H & E).

The tissues were examined and photographed at 100 × magnification with an NIS-Elements F optical microscope imaging system (Nikon Co., Ltd., Tokyo, Japan). Five high-quality sections were chosen (n = 4 chickens/group), and for each section the number of chondrocytes was counted in 10 randomly-chosen fields. FHN was identified by abnormal chondrocytes (with less chondrocytes) of the femoral head growth plates (Durairaj et al., 2009; Xiao et al., 2010). The observer was blinded when calculating the numbers of chondrocytes.

Apoptosis Detection

The sections were processed routinely to detect DNA fragments in apoptotic cells with a terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) detection kit (Boster Co., Ltd., Wuhan, China). The detecting procedures were strictly conducted according to the protocols of the kit.

Sections from the hypertrophic zone of femoral head growth plates were examined and photographed at 400 × magnification. Five high quality sections were chosen (n = 4 chickens/group), and for each section 50 positively immunostained nuclei per field were counted in 10 randomly-chosen fields. The proportion of apoptotic cells was quantitatively assessed by the percentage of the positively immunostained nuclei among the total nuclei in each field. A negative control was performed by eliminating TdT from the labeling mixtures. The observer was blinded to the identification of each sample when calculating the percentage of the positively immunostained nuclei.

Immunohistochemistry

The sections were processed with routine histological methods. After being immersed in phosphate-buffered saline (**PBS**) with 0.1% Tween 20 (pH 7.4) for 15 min, the specimens were incubated in 3% H_2O_2 for 10 min so that the endogenous peroxidase activity was inactivated. Tissues then were blocked with 5% bovine serum albumin (BSA; Boster Co., Ltd., Wuhan, China) at room temperature for 20 min, followed by a PBS wash. Then, the sections were incubated at 37°C for 2 h with the primary antibody anti-bFGF rabbit polyclonal IgG (diluted 1:100; PL Laboratories Inc., British Columbia, Canada). After being rinsed in PBS 3 times for 2 min each, the slides were allowed to react with goat anti-mouse IgG (Boster Co., Ltd., Wuhan, China) at 37°C for 20 min. The sections were then washed with PBS again and incubated at 37°C for 20 min with alkaline phosphatase-labelled streptavidin biotin-peroxidase (SABC-AP; Boster Co., Ltd., Wuhan, China) complex. Then, after the sections were washed in PBS four times for 5 min each, bFGF activity was determined using DAB as a chromogen.

Sections from the hypertrophic zone of the femoral head growth plates were observed by optical microscopy and photographed at 400 × magnification. Five high quality sections were chosen (n = 4 chickens/group), and for each section 200 cells per field were counted in 10 randomly-chosen fields. bFGF protein was quantitatively assessed by determining the positively immunostained areas. Sections without primary antibody

processing were used as the negative control. The staining intensities of immunohistochemistry for bFGF on the images were represented by the mean density (integrated optical density/area) of the positive areas (Wang et al., 2009), which were quantified with Image-Pro Plus 6.0 software (Media Cybernetics, Baltimore, MD, USA). The observer was blinded when calculating the mean density of the positive-staining areas.

Total RNA Extraction and Real-time Quantitative PCR

Total RNA was extracted from the growth plates with TRIzol Reagent (Invitrogen, Shanghai, China) according to the manufacturer's protocol. The RNA concentration was determined spectrophotometrically. To assure the purity of total RNA, the OD260/280 value was 1.9–2.0. All samples were diluted to a concentration of 50 ng/μL and stored at -70°C.

Complementary DNA (**cDNA**) was synthesized from the extracted total RNA (2 μg) by reverse transcription (Takara Biotechnology Co., Ltd., Dalian, China) with the PrimeScript RT reagent Kit (Perfect Real Time, Takara Biotechnology Co., Ltd., Dalian, China) according to the manufacturer's instructions. Glyceraldehyde 3-phosphate dehydrogenase (**GAPDH**) was selected as a housekeeping gene for normalization purposes (Désiré et al., 2000; Jin et al., 2011). Primers were synthesized by Invitrogen (Shanghai, China) for the specific genes and designed based on sequences available in the NCBI GenBank (Table 1). By using SYBR Green PCR technology with the ABI PRISM 7300 HT sequence detection system (Applied Biosystems Inc., Foster City, CA, USA), RT- PCR was performed with the SYBR Premix Ex Taq^{T} (Tli RNaseH Plus, Takara Biotechnology Co., Ltd., Dalian, China). Reactions were conducted in a 20 μL reaction system, each containing 10 μL SYBR Premix Ex Taq (Tli RNaseH Plus), 0.4 μL PCR Forward Primer (10 μM), 0.4 μL PCR Reverse Primer (10 μM), 0.4 μL ROX Reference Dye (50 $\times$), 2 μL template, and 6.8 μL dH_2O (sterile distilled water). Reaction operations were performed as follows: pre-incubation at 95°C for 30 s, followed by 40 amplification cycles (95°C for 5 s, 60°C for 31 s). The relative gene expression level was calculated by the $2^{-\Delta\Delta C}{}_{T}$ method (Warnock et al., 2014). All PCR operations were performed in triplicate.

Statistical Analysis

Statistical analyses were conducted using IBM SPSS v11.0 (SPSS Inc., Chicago, IL, USA) for Windows. The differences among the groups were determined by 1-way analysis of variance (ANOVA, Dunnet's T3). Spearman's rank correlation coefficient was calculated to evaluate the correlations between bFGF activity and the proportion of apoptotic cells and the expression of bFGF and that of Bax and caspase-3. The sample sizes were 30 for correlation analysis. All data are presented as the mean ± SD. The level of statistical significance was set at $P < 0.05$.

RESULTS

Morphological Changes and Histological Analysis

During the course of the study, 3 chickens (later anatomical examination proved FHSL affected) could only move with great difficulty, using their wings to balance, while others had no apparent clinical symptoms. Of all of the necropsied birds, FHS- and FHSL-affected broilers accounted for a proportion of 16.7% and 4.17%, respectively. The rest of the birds were considered normal (Table 2).

Histology of the normal femoral heads showed that the resting zone chondrocytes present in the bone lacunae were regular (Figure 1A–C). And in FHS chickens (1657.00 ± 90.78; $P > 0.05$), there was no apparent difference in the number of chondrocytes compared with normal birds (1799.50 ± 90.52). However, FHSL-affected birds (1239.83 ± 138.91; $P < 0.01$) showed a decrease in the number of chondrocytes (Figure 1D).

TUNEL Assay for Chondrocyte Apoptosis

The nuclei of the apoptotic cells were positively stained brownish yellow with the TUNEL reagent, and

Table 1. Sequences of primers used to amplify specific mRNAs by real-time quantitative PCR.[1]

Target gene	Oligonucleotide	Primer sequence (5′–3′)	Accession number
GADPH	Forward	GAACATCATCCCAGCGTCCA	NM_204305
	Reverse	CGGCAGGTCAGGTCAACAAC	
Bcl-2	Forward	CACCTGGATGACCGAGTACC	NM_205339
	Reverse	GTCCAAGATAAGCGCCAAGA	
Bax	Forward	CCGGAGTACAGGCATTTGTT	XM_422067
	Reverse	CTTGCAGGACGATGGACAC	
Caspase-3	Forward	GAAGCAAGCAGTGGACCAGA	NM_204725
	Reverse	GTTCAAGTTTCCTGGCGTGT	
bFGF	Forward	AAACCGCTTTCTGGCTATGAA	NM_205433
	Reverse	AGTGCCACATACCAATCAGAG	

[1]GAPDH, glyceraldehyde 3-phosphate dehydrogenase; bFGF, basic fibroblast growth factor.

Table 2. Sample size and morphological changes in femoral head for different birds.[1]

	Normal birds	FHS birds	FHSL birds
Sample size	76	16	4
Percentage (%)	79.2	16.7	4.17
Morphological changes	Free of the leg problems	A clean separation of the epiphysis from underlying, macroscopically undamaged growth plate	Separation of the epiphysis from the physis, including macroscopic evidence of damage or laceration to the growth plate

[1]FHS, femoral head separation; FHSL, femoral head separation with growth plate lacerations.

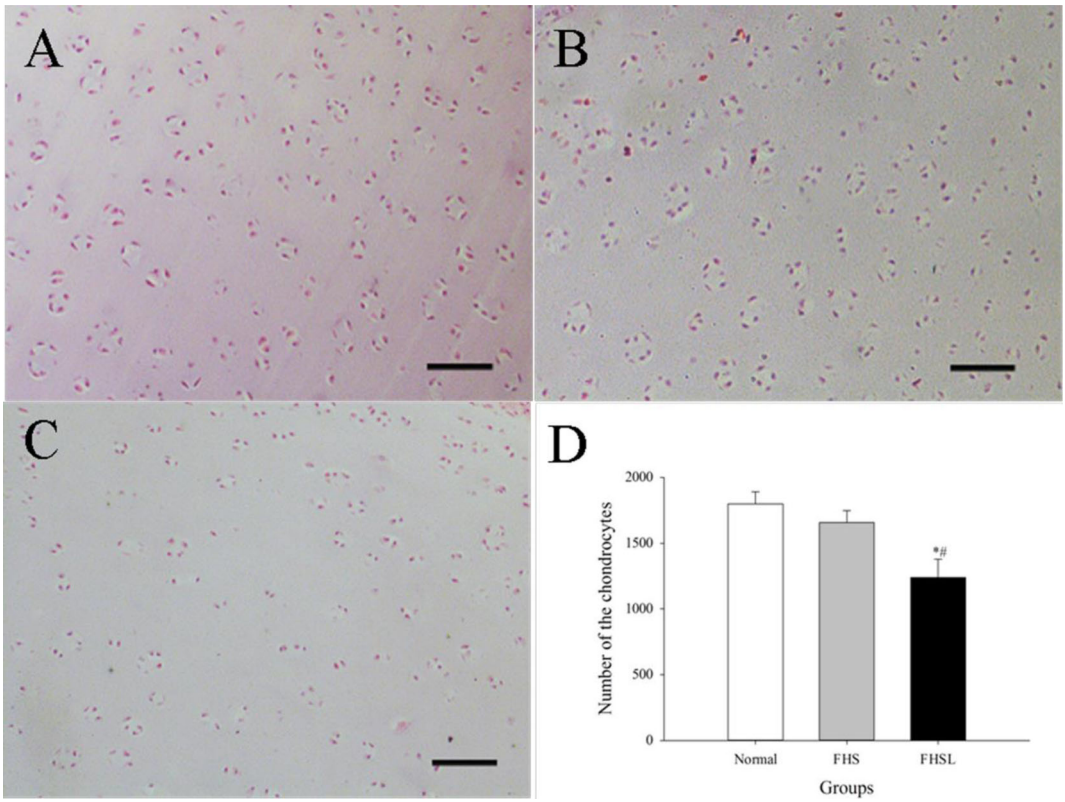

Figure 1. Histological analysis of the resting zone of femoral head growth plates. A: Chondrocytes were regular in normal birds. B: For FHS broilers, there was no apparent difference in the chondrocytes compared with normal birds. C: FHSL-affected birds showed a decrease in the number of chondrocytes. Bar = 100 μm. FHS: femoral head separation; FHSL: femoral head separation with growth plate lacerations.

the proportion of the positively immunostained cells was regarded as the degree of apoptosis. For normal chickens, apoptotic cells were rare in hypertrophic chondrocytes (Figure 2A), whereas they were distributed over a considerable area in FHS and FHSL birds (Figure 2B, C). The mean apoptosis in FHS birds (16.3 ± 4.24%; $P < 0.01$) was much higher compared with that in normal birds (5.16 ± 2.21%). FHSL birds (25.1 ± 2.97%; $P < 0.01$) had the greatest increase of apoptosis (Figure 2D).

Expression of bFGF Protein in the Growth Plate

Immunohistochemistry showed that bFGF protein was mostly expressed in the cytoplasm of the hypertrophic chondrocytes. The degree of protein expression was assessed by determining the positively immunostained areas (shown in Figure 3A–C). Quantitative image analysis illustrated that there was a significant decrease in bFGF protein expression in FHS broilers (0.253 ± 0.0319; $P < 0.01$) in contrast to normal broilers (0.179 ± 0.0255). FHSL birds (0.314 ± 0.0588) demonstrated a more marked inhibition of bFGF activity than normal ($P < 0.01$) or FHS birds ($P < 0.05$; Figure 3D).

mRNA Expressions of Relative Genes

As shown in Figure 4, in contrast to the normal chickens, there was a considerable decrease in bFGF and Bcl-2 mRNA expression ($P < 0.01$) and an apparent promotion of Bax and caspase-3 mRNA expression ($P < 0.01$) in FHS broilers. In FHSL birds, the largest decrease in bFGF ($P < 0.01$) and Bcl-2 ($P < 0.01$) mRNA

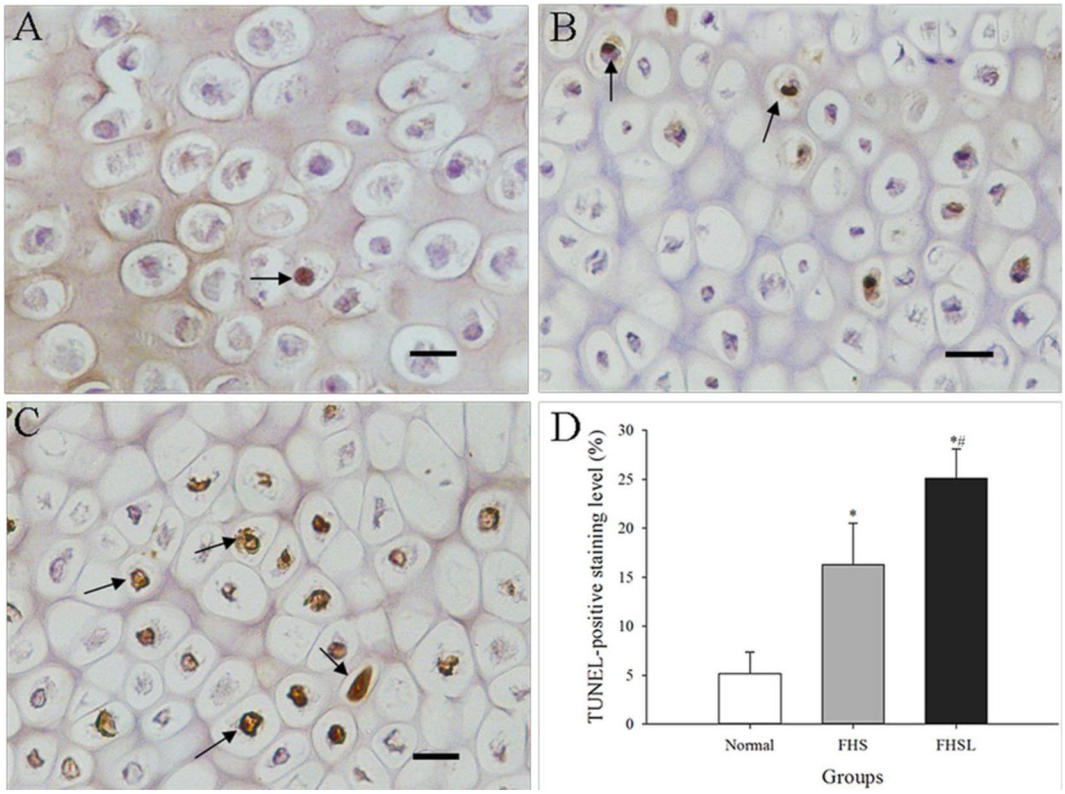

Figure 2. TUNEL assay for chondrocyte apoptosis. TUNEL-positive cells in the hypertrophic zone are shown with black arrows in the images above (A–C). A: Only a few positive-staining cells were observed within the normal chondrocytes. B: In FHS birds, positive-staining chondrocytes were clearly increased compared to the normal birds. C: There was a significant promotion of positive-staining cells in FHSL chickens, and considerable nuclear condensation was exhibited in the sections. D: There was a significant elevation of the chondrocyte apoptosis rate with the presence of FHS. Bar = 25 μm. Data are expressed as the mean ± SD. $^{*}P < 0.05$ versus the normal birds; # $P < 0.05$ versus the FHS birds. FHS: femoral head separation; FHSL: femoral head separation with growth plate lacerations.

expression and the greatest promotion of Bax and caspase-3 mRNA expression ($P < 0.01$) were recorded.

Correlation Analysis

Spearman's rank correlation coefficient revealed that there were significant negative relationships between the level of bFGF protein expression and the proportion of TUNEL-positive cells ($r = -0.635$; $P < 0.001$) and the mRNA expression of bFGF and Bax ($r = -0.751$; $P < 0.0001$) and caspase-3 ($r = -0.546$; $P = 0.002$; Table 3).

DISCUSSION

FHN is a common skeletal problem in birds undergoing rapid growth (Julian, 2005). In the worst cases, the birds are only able to move by using their wings to balance (Bradshaw et al., 2002). Sometimes the birds can walk easily and there are no apparent clinical symptoms, but anatomical examination shows that the lesion has already occurred, which is why our data reveal a high incidence (20.8%) of FHN. Some researchers tried to investigate the pathogenesis of FHN in various experimental models; however, the exact etiology is still inconclusive (Knowles et al., 2008; Durairaj et al., 2009; Olkowski et al., 2011; Erken et al., 2012).

Abnormal conditions of chondrocytes are considered one of the characteristic signs of FHN at the microscopic level (Durairaj et al., 2009; Xu et al., 2014). The pictures obtained (Figure 1C, D) showed fewer chondrocytes in FHSL-affected chickens, suggesting that the femoral head was undergoing necrosis. Apoptotic cells exhibit considerable nuclear condensation, volume contraction, and activation of an endonuclease that cleaves DNA into oligonucleosomes of 180–200 bp (Charriaut-Marlangue and Ben-Ari, 1995; Weinstein et al., 2000). It was previously reported that the pathology of FHN predominantly involves the apoptosis of chondrocytes in humans and rodents (Weinstein et al., 2000; Calder et al., 2004; Kerachian et al., 2011; Tian et al., 2013; Xu et al., 2014); however, little literature has provided information on chondrocyte apoptosis in broilers with FHN. The normal growth plate represents a highly dynamic sequence of quiescent stem cells/primordial chondrocytes (resting zone), rapidly dividing chondrocytes (proliferating zone), maturing and swelling chondrocytes (prehypertrophic zone), and dying chondrocytes (hypertrophic zone) that then are transformed into the dead chondrocytes of the calcifying zone (Chung et al., 2001). In this study, TUNEL was conducted on bone sections, and the existence of typical signs of

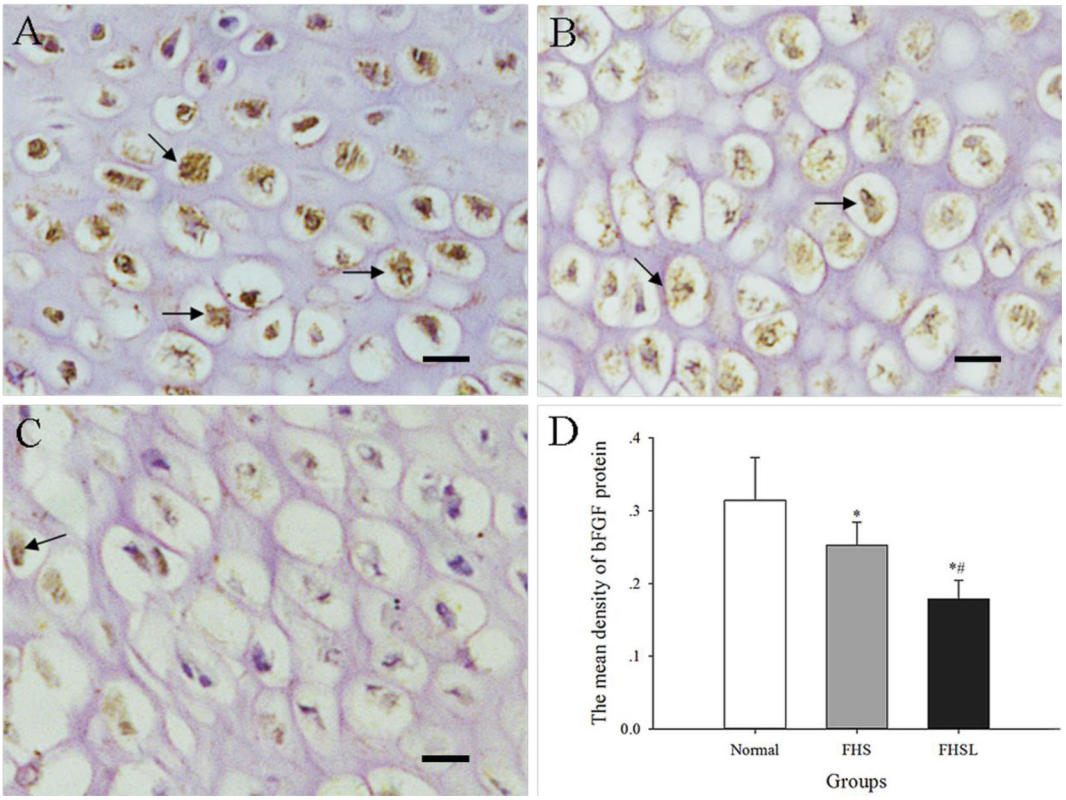

Figure 3. Immunohistochemical analysis of bFGF. Positive-staining cells in the hypertrophic zone are shown with black arrows in the images above (A–C). A: Intense staining was observed in the cytoplasm of normal chickens. B: In FHS birds, positive staining was much less intense compared to the normal birds. C: There was a significant reduction in the intensity of positive staining in FHSL chickens compared with other birds. D: There was a marked reduction in bFGF protein expression with the presence of FHS. Bar = 25 μm. Data are expressed as the mean ± SD. $^{*}P < 0.05$ versus the normal birds; # $P < 0.05$ versus the FHS birds. FHS: femoral head separation; FHSL: femoral head separation with growth plate lacerations; bFGF: basic fibroblast growth factor.

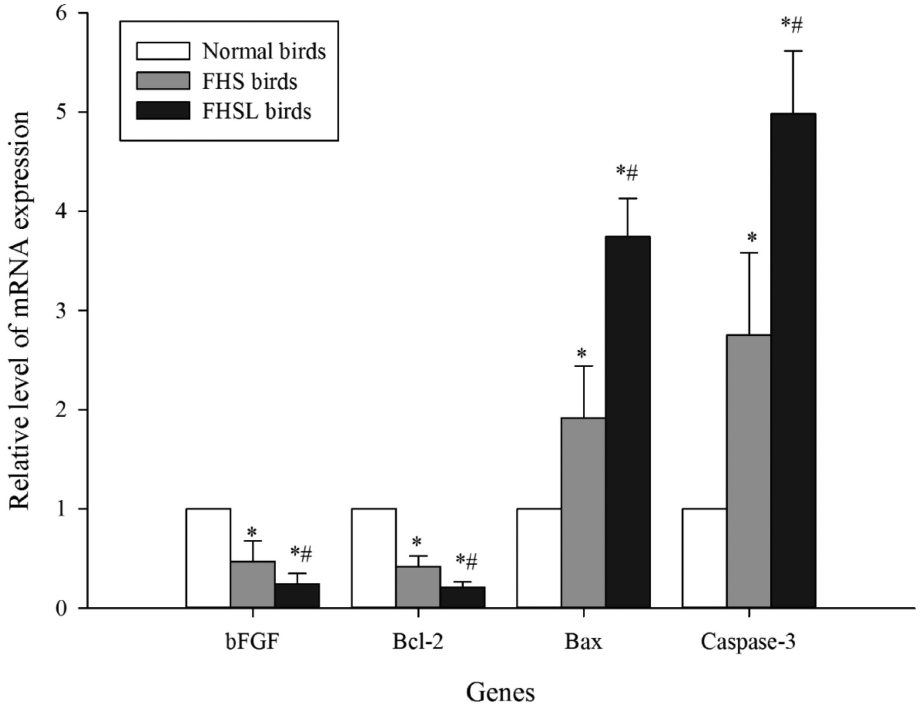

Figure 4. mRNA expression of chondrocyte apoptosis relative genes. Along with the aggravation of FHN, there was a considerable decrease in bFGF and bcl-2 mRNA expression and an apparent promotion of bax and caspase-3 mRNA expression. Data are expressed as the mean ± SD. $^{*}P < 0.05$ versus the normal birds; # $P < 0.05$ versus the FHS birds. FHS: femoral head separation; FHSL: femoral head separation with growth plate lacerations; bFGF: basic fibroblast growth factor.

Table 3. Results of Spearman's rank correlation coefficients analysis.[1]

Correlation variables	n	r	P
bFGF protein expression × TUNEL-positive cells proportion	30	−0.635	<0.001
bFGF mRNA expression × Bax mRNA expression	30	−0.751	<0.001
bFGF mRNA expression × caspase-3 mRNA expression	30	−0.546	0.002

[1]bFGF, basic fibroblast growth factor.

apoptosis in hypertrophic chondrocytes was confirmed by positive labeling. Our study showed marked differences in the proportion of apoptotic cells between normal birds and FHN-affected chickens, indicating that chondrocyte apoptosis was promoted in the FHN affected birds. Interestingly, we found that there was no significant decrease of chondrocyte numbers in FHS chickens, which indicated that apoptosis might precede FHS. In the more advanced FHSL birds, a significant drop in chondrocyte numbers was observed (Figures 1 and 2). According to the data in this study, we cannot determine whether FHS actually drives chondrocyte apoptosis and more studies were needed to clarify causality.

The Bcl-2 family has been described as an important apoptosis-regulating family and includes both pro-survival molecules, such as Bcl-2, and pro-apoptotic members, such as Bax (Hotchkiss et al., 1999; Chang et al., 2007). In the caspase family, caspase-3 is a marker and mediator of apoptosis and takes part in the process of apoptosis through various mechanisms (Park et al., 2008). In this study, Bcl-2, Bax and caspase-3 mRNA expression was detected in growth plate chondrocytes. When FHS occurred, Bcl-2 mRNA expression decreased greatly, but, in contrast, there were different degrees of promotion of Bax and caspase-3 mRNA expression. These findings suggested that Bcl-2, Bax and caspase-3 were involved in the process of chondrocyte apoptosis in FHN birds.

As a member of the FGF family, bFGF can regulate chondrocyte apoptosis in humans and rodents (Karsan et al., 1997; Konig et al., 1997; Miho et al., 1999; Ma et al., 2001; Fromigue et al., 2005; Rios-Munoz et al., 2005). However, the role of bFGF in the apoptotic process in chickens and its exact mechanism are still not understood (Konig et al., 1997; Sabbieti et al., 2009; Kim et al., 2012). In the present work, we attempted to investigate whether bFGF is related to FHN in broilers. Immunohistochemistry demonstrated that the mean density of the bFGF-positive area declined sharply in FHN birds. There was a significant negative correlation between bFGF protein expression and the proportion of TUNEL-positive cells. We also observed that bFGF mRNA was significantly decreased in FHS and FHSL chickens in contrast to the normal birds. The correlation between the mRNA expression of bFGF and its downstream regulatory factors (Bax and caspase-3) was analyzed. The results showed that the relationship between bFGF and both of the apoptotic effectors was very strong, which suggested that the decrease in bFGF expression may play critical roles in the process of hypertrophic chondrocyte apoptosis through the Bax and caspase-3 pathways in FHN chickens. The birds used in the present study were classified into normal or FHN birds according to the gross morphological changes. However, these apparently normal birds may be at an early asymptomatic pathological stage. Nevertheless, our findings were indicative that bFGF participated in the pathogenesis of FHN, though more studies are needed to evaluate the exact mechanistic pathway.

CONCLUSIONS

In conclusion, our results provided evidence that chondrocyte apoptosis in the growth plate was greatly promoted in chickens with femoral head necrosis. The mechanisms are possibly related to bFGF expression inhibition and the activation/inactivation of related apoptotic effectors. However, we cannot discount the possibility that mechanisms driving chondrocyte apoptosis result from FHS, and further studies are needed to clarify causality.

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