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RESEARCH ARTICLE

Changes of bone remodeling, cartilage damage and apoptosis-related pathways in broilers with femoral head necrosis

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Highlights

- Although regulatory factors related to bone remodeling are changed in all broilers with femoral head necrosis, significant changes in bone biomechanics occur only when severe femoral head necrosis (femoral head separation with growth plate lacerations) occurs.
- The severity of femoral head necrosis and the level of HIF-1α are both closely associated with cartilage damage and apoptosis in broilers.

Abstract

Femoral head necrosis (FHN) is a common leg disorder in the poultry industry often leads to significant cartilage damage. The mechanism behind abnormal apoptosis in FHN broilers, leading to cartilage damage, remains unclear; although endoplasmic reticulum stress (ERS) has been found to play a role in glucocorticoid-induced FHN broilers. In this study, we collected samples from broilers with femoral head separation (FHS) and femoral head separation accompanied with growth plate lacerations (FHSL) in a broiler farm. The aim was to investigate the potential association between the severity of FHN, bone remodeling and cartilage damage. Additionally, primary chondrocytes were treated with methylprednisolone (MP) to construct an *in vitro* FHN model, followed by inhibition or activation of ERS or *hypoxia inducible factor-1α* (*HIF-1α*) to further investigate the mechanism of apoptosis in cartilage. The results suggested that cartilage appeared to be the appropriate tissue to investigate the potential mechanisms of FHN, as the degree of cartilage damage was found to be closely related to the severity of the disease. Bone quality was only affected in FHSL broilers, although factors related to bone metabolism were significantly altered among FHN-affected broilers. In addition, cartilage in FHN-affected broilers exhibited high levels of apoptosis and upregulated expression of ERS-related and HIF-1α, which was consistent with both *in vivo* and *in vitro* findings after MP treatment. The results were further supported by treatment with *HIF-1α* or ERS inhibition or activation. In conclusion, bone remodeling and cartilage homeostasis were affected in FHN broilers, but only cartilage damage was significantly exacerbated with FHN development. Moreover, activation of ERS or *HIF-1α* resulted in apoptosis in cartilage, thus exhibiting a significant correlation with FHN severity.

Keywords: femoral head necrosis, bone remodeling, cartilage homeostasis, apoptosis-related pathways

1. Introduction

The broiler industry has experienced significant growth in recent decades as a result of increasing demand for animal protein in developing countries (Raehtz *et al.* 2018). Advances in genetic selection, nutrition and management have significantly improved the feed conversion and body weight (BW) in meat-type broilers (Havenstein *et al.* 2003). However, the BW gain has played a part in the increasing incidence of leg disorders like femoral head necrosis (FHN), resulting in significant financial losses in the global commercial broiler industry annually (Cook 2000). FHN is a common skeletal disorder in broilers characterized by separation of the epiphysis from the articular cartilage, necrosis of trabecular bone, and eventual occurrence of bone fractures. This disease can cause lameness and reduce performance in broilers due to difficulty in accessing feed and

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water. FHN typically occurs at 4–6 wk of age and coincides with a steady increase in BW from 1 to 6 wk of age in broilers (Packialakshmi *et al.* 2015b). Since the etiopathology of FHN in broilers remains unclear, additional studies are needed to investigate its pathogenesis.

Initially, it was identified that infections caused by bacteria such as Staphylococcus aureus were the major causative factors of FHN (Riddell and Springer 1985; Thorp *et al.* 1993; McNamee *et al.* 1998). However, subsequent studies have revealed that non-infectious factors can also directly induce the development of FHN in broilers, including femoral ischemia, mechanical stress due to rapid growth, disruption of lipid metabolism, and immunosuppression due to elevated corticosterone levels (Erken *et al.* 2012; Julian 1998; McNamee and Smyth 2000). Therefore, the glucocorticoid (GC)-induced animal model has been established to study FHN (Cui *et al.* 1997; Packialakshmi *et al.* 2015a). As a bone disorder, bone quality is intricately linked to the emergence and progression of FHN. Our previous studies have also demonstrated that GC-induced FHN broilers experienced significant impact on bone quality, accompanied by severe effects on skeletal growth (Zhang *et al.* 2017). Computed tomography (CT) scan results of the legs in broilers with severe spontaneous FHN also demonstrated a significant reduction in bone mineral matrix, with fractures of the proximal femur occurring in 10–15% of the broilers (Olkowski *et al.* 2011). Moreover, the effect of cartilage damage in FHN broilers cannot be ignored, given that cartilage is the first tissue to be stressed in the femoral head. In addition, prior studies have indicated that there was visible damage in the cartilage of FHN-affected broilers, and apoptosis was recognized as one of the primary reasons for cartilage damage (Zhang *et al.* 2019). Meanwhile, the extracellular matrix (ECM) homeostasis in cartilage was disturbed in FHN broilers (Yu *et al.* 2020). Hence, bone and cartilage play an important role in FHN-affected broilers.

Apoptosis is a well-established contributor to the development of FHN (Zhang *et al.* 2019; Liu *et al.* 2021; Yang *et al.* 2021). The signals that induce apoptosis can be mainly divided into 2 types: extrinsic and intrinsic pathways. Endoplasmic reticulum stress (ERS) is one of the intrinsic pathways, which plays a dominant role in FHN (Li *et al.* 2020). When the regulation of protein processing is disrupted, misfolded proteins accumulate in the endoplasmic reticulum (ER), which triggers the unfolded protein response (UPR) when the accumulation exceeds the ER loading capacity (Schroder and Kaufman 2005; Namgaladze *et al.* 2019; Li *et al.* 2020). Immunoglobulin heavy chain binding protein (BIP) dissociates from UPR sensors, releasing activating transcription factor 6 (*ATF6*), inositol-requiring enzyme 1 (IRE1), and PKR-like ER kinase (*PERK*), which can then activate subsequent downstream signaling pathways (Hetz *et al.* 2020). Although transient or acute ERS generally restores the protein homeostasis through the UPR, chronic or severe ERS could induce substantial cell dysfunction and death (Bartoszewska and Collawn 2020; Hetz *et al.* 2020). Moreover, hypoxia could induce ERS and trigger the activation of hypoxia inducible factor-1α (*HIF-1α*) in femoral head cartilage (Charlier *et al.* 2016; Yu *et al.* 2020). Some reports have also demonstrated that specific physiological processes within the ER undergo changes under hypoxia, and *HIF-1α* can regulate both the expression and activity of ERS sensor (Chevet *et al.* 2015; Akman *et al.* 2021). Thus, it appears that ERS has the potential to trigger apoptosis in FHN broilers, showing a potential link to *HIF-1α*.

A number of studies have demonstrated the potential role of bone remodeling and cartilage damage in FHN broilers, but limited research has been conducted to compare these alterations. In addition, *HIF-1α* plays a critical role in hypoxia and cartilage, whereas the relationship between FHN and ERS-related apoptosis has been well established. Our findings further suggest that cartilage could serve as an appropriate model to investigate the mechanism of FHN, while *HIF-1α* could potentially induce chondrocyte apoptosis through overactivation of ERS, ultimately leading to FHN in broilers.

2. Materials and methods

2.1. Animal experiment

Sample collection Six-wk-old Ross 308 broilers were obtained from a farm located in Jiangsu Province, China. All the birds were reared according to a standardized procedure and provided a basal diet (Appendix A).

After evaluation by the FHN score (Okazaki *et al.* 2009), the broilers were divided into 3 groups: normal, femoral head separation (FHS) and femoral head separation with growth plate lacerations (FHSL). In addition, cartilage damage was graded using the criteria established by the International Cartilage Repair Society (ICRS) score. Each group consisted of 6 broilers that were utilized for analysis.

Sampling After the broilers were restrained, blood was collected from the wing vein and subjected to centrifugation to obtain the serum. The broilers were then scarified and various organs including bone, femoral head, heart, liver, spleen, thymus, and bursa were collected.

Cortisol detection ELISA Kit (ANG-E32176C) obtained from Nanjing Angle Gene Biotechnology Co., Ltd., was used to determine the concentration of cortisol in the serum.

Histopathological analysis The femoral head was initially fixed with 4% paraformaldehyde and decalcified in EDTA decalcifying solution (G1105, Services, Wuhan, China). The decalcified cartilage tissues were then embedded in the paraffin, sectioned and then stained with hematoxylin and eosin (H&E), Alcian blue (G1560, Solarbio, Beijing, China), Toluidine blue (G2542, Solarbio), and Safranine O-solid green (G1053, Services, Wuhan, China) for histological observation.

Biomedical and bone indicator analysis ELISA kits purchased from Nanjing Angle Gene Biotechnology Co., Ltd., were used to detect the concentration of 4 different chicken specific bone indicators (osteocalcin (OT, ANG-E32164C), bone alkaline phosphatase (BALP, ANG-E32226C), cross-linked carboxy-terminal telopeptide of type I collagen (CTX-I, ANG-E32233C), and tartrate acid phosphatase 5b (TRACP-5b, ANG-E32229C) in the serum. In addition, the concentrations of creatinine, alkaline phosphatase (ALP), Ca, P were detected by an automatic biochemical analyzer (Hitachi Ltd., Tokyo, Japan).

Bone biomechanical tests The bone mineral density (BMD) of the femoral head and bone mechanical parameters were measured. The detailed procedures and analytical methods could be found in a previously reported study (Yu *et al.* 2022).

TUNEL staining of articular cartilage To examine chondrocyte apoptosis in FHN broilers, fluorescein (FITC) TUNEL Cell apoptosis Detection Kit (1501, Service, Wuhan, China) was used. The fluorescence density was analyzed using Image J software to determine the extent of chondrocyte apoptosis in the femoral head.

RNA extraction and real-time quantitative PCR The ABI PRISM 7300 HT Sequence Detection System (Applied Biosystems, CA, USA) was used to detect the mRNA level of related genes (such as *HIF-1α*, *ATF6*, *BIP*, *C/Ebp-homologous protein* (*CHOP*), etc.) in the cartilage. *GAPDH* was used as an internal control. The information of all the primers used has been depicted in Appendix B. The gene expression analyses were calculated using the $2^{-\Delta\Delta CT}$ value method.

Immunohistochemical analysis The 4-μm tissue sections of cartilage were processed for staining according to standard protocols. The details could be found in a previous study (Yu *et al.* 2022). The cells were considered positive when the nucleus was identified in combination with appropriate staining. The number of positively stained cells in each region was counted and the percentage of positive cells was finally calculated.

2.2. *In vitro* experiments

Primary chondrocytes isolation, culture, and identification The primary chondrocytes were isolated from broiler proximal tibial cartilages. The methods of isolation, culture, and identification and the reagents used in the experiment have been described in detail in a previous article (Yu *et al.* 2022).

In the present study, the second passage of chondrocytes was used for the subsequent experiments. The treated concentration of methylprednisolone (MP) was determined based on a previous study (Yu *et al.* 2022) and was set at 6 mg mL^{-1} for 15 min.

Detection of apoptosis The apoptosis rate of chondrocytes was detected by flow cytometry. The Annexin V-FITC/PI Double Staining Kit (A211-02, Vazyme, Nanjing, China) was used according to the manufacturer's instructions.

ERS activator or inhibitor treatment The chondrocytes were seeded and cultured to 70% confluence. The cells were then subsequently treated with different treatments: MP, ERS inhibitor (Salubrinal, SA, HY-15486, MCE, Shanghai, China) or ERS activator (Tunicamycin, TM, Solarbio, IT2670, Beijing, China) for 24 h to analyze the regulation of the ERS pathway. The protein and mRNA samples were collected after the transfection.

Transfection of HIF-1α-overexpressing protocadherin (PCDH) plasmid To generate PCDH-flag-HIF-1α, sequences encoding chicken full-length *HIF-1α* genes were cloned into PCDH flag vectors. HIF-1α-overexpressing PCDH plasmid (oeHIF-1α) and empty PCDH plasmid (oeNC) were obtained from Tsingke Biotech (Beijing, China). When the cells reached to 70% confluence, they were transfected with oeHIF-1α or oeNC using Lipofectamine 3000 Transfection Reagent (L3000015, ThermoFisher, Waltham, MA, USA) for 72 h according to the manufacturer's protocols.

Transfection with small interfering RNA (siRNA) The siRNA sequences of chicken *HIF-1α* and scrambled (NC) were purchased from Genepharma Co., Ltd. (Shanghai, China). The primary chondrocytes were transfected with siRNA and Lipofectamime 3000 (L3000015, ThermoFisher, Waltham, MA, USA) for 48 h. *HIF-1α* siRNA sequences used were as following: sense: 5′-UUCUUUCUCAGAAUGAAGUTT-3′, antisense: 5′-UUCAUUCUGAGAAAGAAGCUG-3′.

Western blotting The detailed procedures and analytical methods could be found in a previously reported study (Yu *et al.* 2022). The information of antibodies was shown in Appendix C. Anti-GAPDH antibody was used as a control.

2.3. Statistical analysis

The statistical analysis was performed using IBM SPSS Statistics 19. All the data has been presented as mean±SE, and differences between the groups were determined by one-way ANOVA with Tukey's post hoc analysis (Dunnet's T3). ImageJ software was used to analyze the density of western blot bands and the positive cells obtained after immunohistochemical staining. The correlation analysis was performed by Pearson's or Spearman's test. $P<0.05$ was considered as statistically significant.

3. Results

3.1. Changes of immune in spontaneous FHN

Leg diseases are a persistent issue in poultry farming that lead to significant financial losses in contemporary intensive farming practices. Spontaneous FHN is one of the most common leg diseases in broilers (McNamee and Smyth 2000). As illustrated in Fig. 1, FHN can be classified into FHS and FHSL, depending on the level of severity. FHS is distinguished by the separation of the femoral head cartilage from the growth plate, without any visible lesions in the growth plate, whereas FHSL is identified by growth plate damage and epiphyseal fracture. Studies have indicated that FHN could cause limping and even starvation of broilers due to lack of access to water and food (Packialakshmi *et al.* 2015b). The result in Fig. 1 suggested that FHN-affected broilers exhibited lower BW. In addition, as depicted in Fig.1, there was slightly reduction in the indices of immune organs (spleen, bursa, and thymus). Cortisol is a type of GCs that can effectively inhibit the activities of the immune system. The findings revealed that the concentration of cortisol was significantly increased in broilers affected with FHSL. Hence, the suppression of the immune system could be occurring at an initial phase in FHS broilers, whereas a distinct alteration was evident in FHSL broilers.

3.2. Changes of bone remodeling in spontaneous FHN

The bone-related items were also analyzed in this study. As shown in Fig. 2-A, BMD of femoral head was significantly decreased only in FHSL broilers, thus suggesting that bone quality would be severely affected only when the epiphysis was broken. On the other hand, eleven mechanical parameters of femora were measured and have been presented in Table 1.

Similar to the BMD results, only FHSL broilers exhibited significant changes in the measured mechanical parameters. However, the biochemical results indicated that Ca/P was higher in both FHS and FHSL broilers, implying dissolution of bone calcium and increased bone absorption. Analyses of bone remodeling indicators further verified that bone formation (OT and BALP) was inhibited and bone absorption (CTX-I and TRACP-5b) was promoted in all FHN-affected broilers. In short, although bone-related serum indicators were altered in both FHS and FHSL broilers, only FHSL broilers showed alterations in bone biomechanics.

3.3. Spontaneous FHN showed different degrees of cartilage damage

Several methods were used to access cartilage, considering its crucial role within the femoral head. The ICRS score has emerged as a common criterion used to evaluate cartilage damage, and the results indicated that the degree of cartilage damage was closely related to the severity of FHN (Fig. 3-A). A higher percentage of cartilage from FHSL broilers exhibited an ICRS score of 3 or 4, in contrast to FHS broilers where the majority of cartilage was evaluated as grade 2 or 1. In addition, Fig. 3-B displayed varying staining outcomes of the femoral head, indicating that the lamina splendens of the superficial zone in cartilage was apparently thinner in FHS broilers and damaged in FHSL broilers. Additionally, both FHS and FHSL broilers exhibited an increased presence of intracytoplasmic vacuoles and pyknosis in the articular cartilage of the femoral head. Hence, our findings indicated a strong correlation between the degree of cartilage damage and the severity of FHN, and cartilage appeared to be

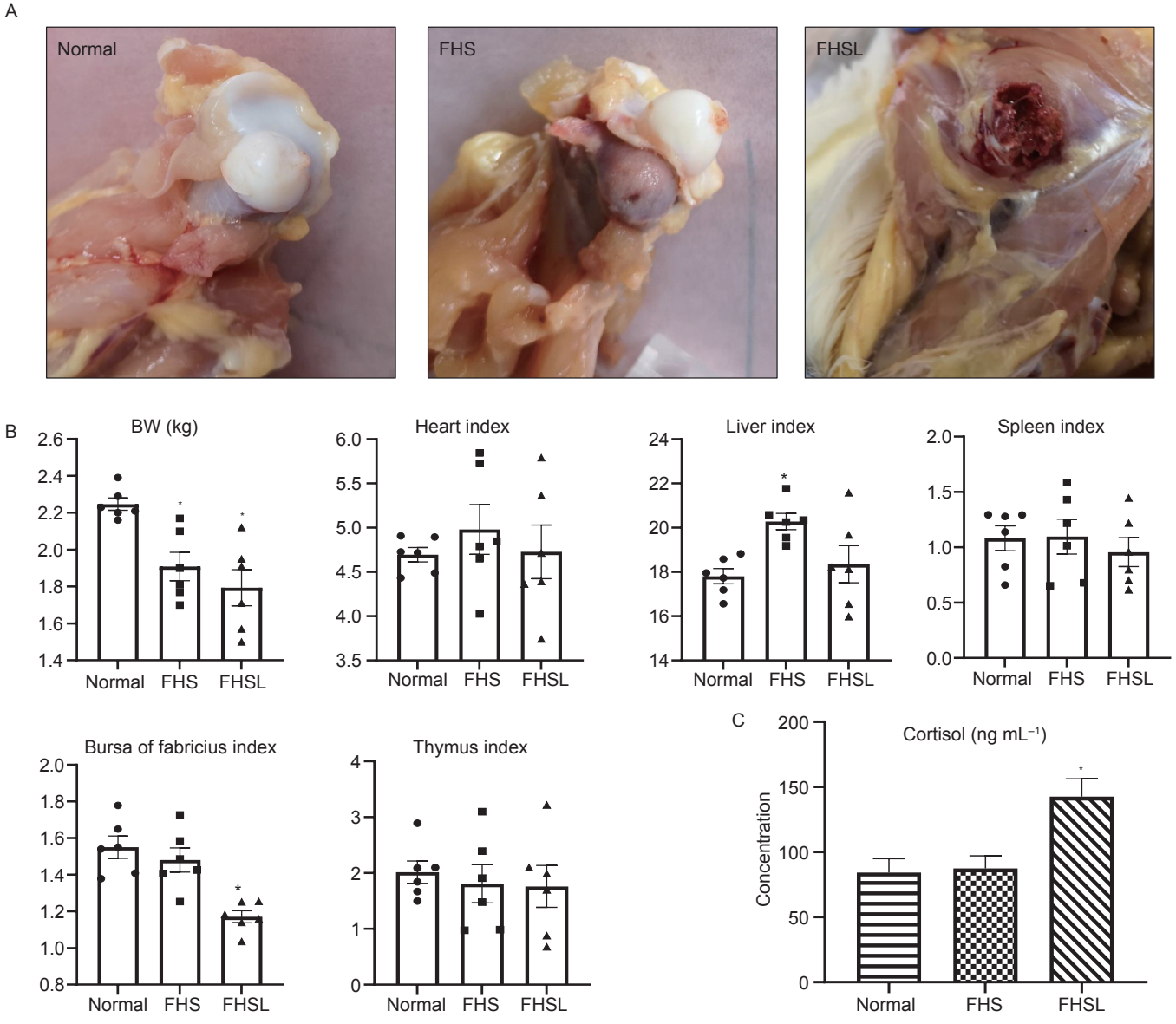


Fig. 1 The changes of fat metabolism in spontaneous FHN broilers. A, the femoral head in normal, FHS and FHSL broilers. B, the changes of BW and organ indexes. C, concentration of serum cortisol in normal, FHS and FHSL broilers. FHN, femoral head necrosis; FHS, femoral head separation; FHSL, femoral head separation with growth plate lacerations; BW, body weight. Data are mean±SE (n=6). *, P<0.05 *vs.* normal broilers.

superior to bone in studying the progression of FHN.

3.4. ERS was activated in spontaneous FHN

Since ERS-related apoptosis is closely associated to cartilage damage, we detected the expression of ERS-related genes. The results in Fig. 4-A revealed that that besides the 3 sensors of ERS (*IRE1*, *PERK*, and *ATF6*) being significantly activated, there was also a substantial increase in *CHOP*, which indicated overactivation of ERS, in broilers affected by FHN. These findings suggested that the ERS pathway was overactivated in both FHS and FHSL broilers, with a higher level in the latter. Moreover, analysis of apoptosis-related genes also revealed that the expression of pro-apoptotic gene *BID* was significantly increased in FHN broilers; while the mRNA level of the anti-apoptotic gene *BCL2* was also increased, but the increase was less pronounced in FHSL broilers. *Caspase3*, a functional performer of apoptosis, also displayed high expression (Fig. 4). Moreover, TUNEL staining showed increased apoptosis in both lamina splendens and articular cartilage, and the apoptosis rate increased with the severity of FHN (Fig. 4-D and E).

As cartilage is typically anoxic under physiological conditions, the role of *HIF-1α* in cartilage cannot be ignored (Zhang *et al.* 2015; Okada *et al.* 2020). Furthermore, studies have reported a close relationship between *HIF-1α* and ERS (Stegen *et al.* 2019; Akman *et al.* 2021). In this study, both the ERS pathway and *HIF-1α* were found to be overactivated in FHN broilers (Fig. 4-C and G). Thereafter, we performed the correlation analysis of cartilage in normal, FHS and FHSL broilers. The correlation analysis further revealed a significant positive relationship between the level of *HIF-1α* and the severity of the spontaneous FHN (Fig. 4-H). A significant positive correlation was also observed between *HIF-1α* and *caspase3* or the fluorescence intensity of TUNEL

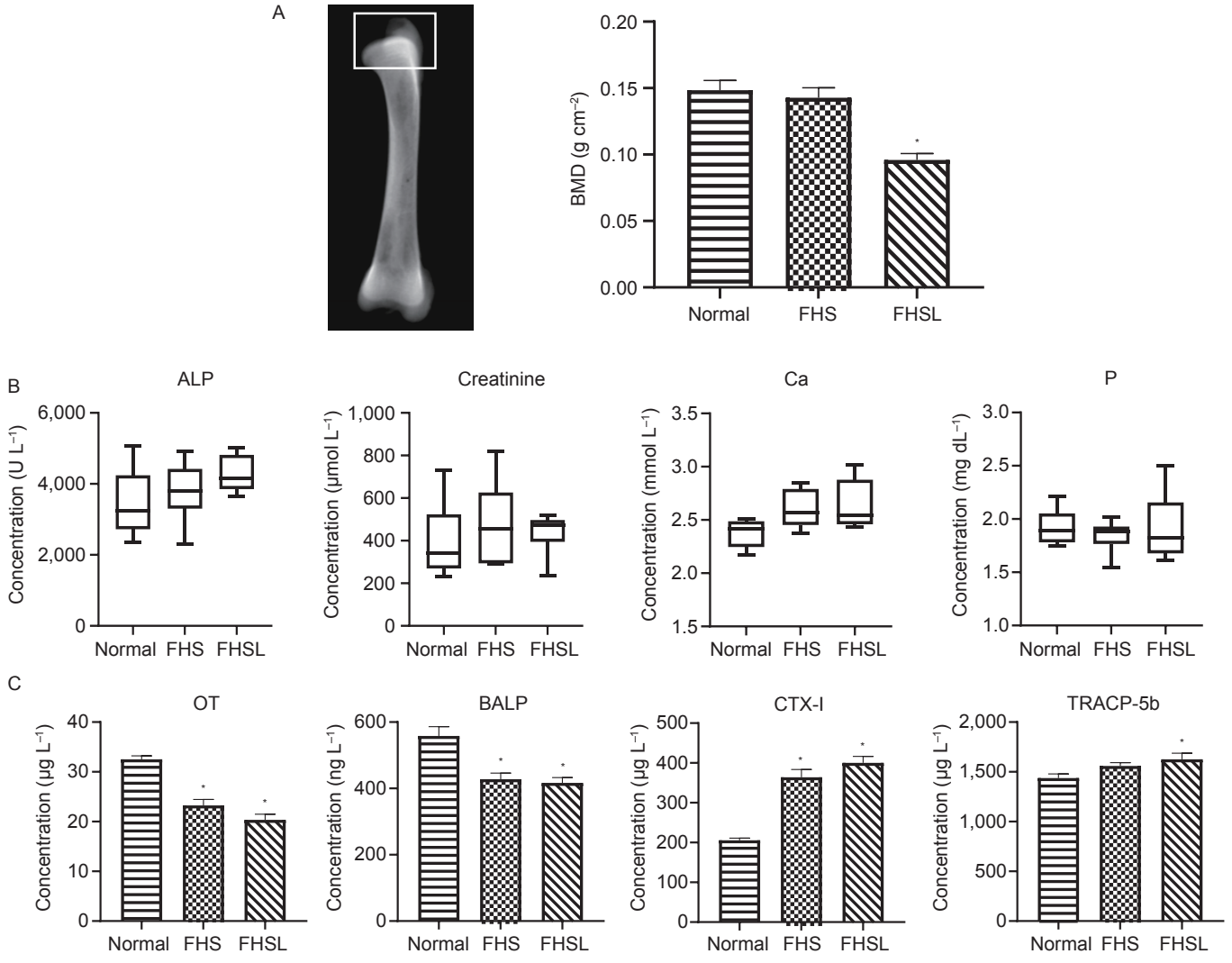


Fig. 2 The bone parameters of femur in spontaneous FHN broilers. A, the BMD of femoral head in normal, FHS and FHSL broilers. B, the changes of serum indicants related to bone metabolism. C, changes in indicators related with lipid metabolism. FHN, femoral head necrosis; FHS, femoral head separation; FHSL, femoral head separation with growth plate lacerations; BMD, bone mineral density; ALP, alkaline phosphatase; OT, osteocalcin; BALP, bone alkaline phosphatase; CTX-I, cross-linked carboxy-terminal telopeptide of type I collagen; TRACP-5b, tartrate acid phosphatase 5b. Data are mean±SE (n=6). *, P<0.05 *vs.* normal broilers.

Table 1 Mechanical parameters in femora of three animal groups[1)]

Parameter	Unit	Normal (mean±SE)	FHS (mean±SE)	*P vs.* normal	FHSL (mean±SE)	*P vs.* normal	*P vs.* FHS
Stiffness	N m^{-1}	145.07±11.82	152.64±13.95	0.902	93.94±11.04	0.026	0.011
Elastic modulus	MPa	1933.31±203.59	1882.78±184.11	0.979	1273.73±162.30	0.050	0.081
Bending rigidity	N m^2	0.34±0.02	0.36±0.03	0.717	0.24±0.01	0.014	0.003
Yield force	N	213.39±16.60	219.80±16.49	0.961	104.20±17.45	0.001	0.001
Ultimate force	N	183.59±17.99	200.03±18.95	0.755	127.83±9.84	0.066	0.017
Work to yield	J	0.35±0.05	0.39±0.08	0.863	0.19±0.05	0.191	0.078
Total work	J	0.54±0.05	0.46±0.04	0.391	0.35±0.03	0.021	0.240
Yield stress	MPa	52.02±8.48	46.56±6.87	0.834	29.65±4.01	0.078	0.210
Ultimate stress	MPa	54.44±6.51	46.76±6.18	0.581	19.27±2.38	0.001	0.007
Strain to yield	μstrain	0.06±0.00	0.06±0.01	0.935	0.05±0.01	0.841	0.641
Total strain	μstrain	0.10±0.03	0.08±0.01	0.858	0.10±0.01	0.969	0.728

[1)] Among them, 3 other parameters (stiffness, elastic modulus and bending rigidity), 2 parameters related to force (yield force and ultimate force), 3 parameters related to energy (work to yield, and total work), 2 parameters related to stress (yield stress, and ultimate stress), and 2 parameters related to strain (strain to yield, and total strain). FHS, femoral head separation; FHSL, femoral head separation with growth plate lacerations. Data are mean±SE (n=6).

(Fig. 4-I). These results indicated that high-level *HIF-1α* may induce ERS, resulting in an elevated apoptosis rate in chondrocytes, ultimately leading to FHN.

3.5. MP induced apoptosis in chondrocytes

Chondrocytes were chosen for cellular experiments due to their exclusive presence in cartilage. Therefore, in the present study, MP was directly delivered to chondrocytes instead of broilers to further verify the potential roles of *HIF-1α* and ERS in FHN-affected broilers. From the result of Fig. 5-A, it was evident that MP was responsible for severe cell death and the number of chondrocytes did not increase significantly in the following 48 h. Moreover, the findings from the Annexin V-FITC/PI double staining demonstrated that the chondrocytes treated with MP exhibited an approximately 15% increase in apoptosis rate (Fig. 5-B). The results demonstrated that MP not only induced apoptosis in chondrocytes, but also impaired the ability of proliferation.

In addition, similar to the results observed in FHN broilers, *HIF-1α* was activated in MP-treated chondrocytes (Fig. 5-C).

3.6. MP activated ERS pathway to induce apoptosis in chondrocytes

To verify that apoptosis was induced by the ERS pathway, ERS inhibitor SA and activator TM were used in this study. As shown in Fig. 6-A, SA was found to inhibit the ERS pathway, whereas the expression levels of 3 ERS sensors were significantly increased in chondrocytes treated with TM or MP (Fig. 6-B). It has been reported that activated *CHOP* serves as a prominent indicator of ERS-induced apoptosis, and it is usually activated through the *PERK-ATF4-CHOP* and *ATF6-CHOP* pathways (Delbrel *et al.* 2018; Akman *et al.* 2021). The finding of *CHOP* further confirmed that both MP and TM could excessively activate ERS, while SA exhibited an opposite effect by suppressing it.

On the other hand, overactivated ERS can disrupt the expression and structure of *BCL2* family members (McCullough *et al.* 2001; Hetz *et al.* 2006). In TM- or MP-treated chondrocytes, the pro-apoptotic gene *BID* was found to be activated whereas the expression of anti-apoptotic genes (*BCL2* and *BCL10*) was inhibited, indicating that TM or MP induced chondrocyte apoptosis (Fig. 6-C and D). The activation of apoptosis was further confirmed by increased *caspase3*. Conversely, SA-treated chondrocytes showed a lower apoptosis level. The findings in Fig. 6-F and G also indicated that SA could effectively inhibit apoptosis activated by MP. These results confirmed that MP induced apoptosis through activation of ERS.

Moreover, the result of HIF-1α was illustrated in Fig. 6-E, indicating the TM or MP treatment increased the expression of *HIF-1α*, while SA inhibited it. Therefore, the level of ERS could also affect *HIF-1α* in return, there was a strong correlation between them.

3.7. Activation of ERS or overexpression of HIF-1α can induce chondrocytes apoptosis

To investigate the relationship between apoptosis and ERS or *HIF-1α*, flow cytometry was performed. As shown in Fig. 7, TM obviously increased the apoptosis rate, while SA treatment alone did not affect the apoptosis rate in chondrocytes. Moreover, overexpression of *HIF-1α* in chondrocytes resulted in a similar effect as MP treatment alone, inducing significant apoptosis with a comparable rate in chondrocytes (oeHIF-1α=20.3%, MP=18.96%). Interestingly, co-treatment with oeHIF-1α and MP or TM further increased the apoptosis rate, but SA reduced the apoptosis rate induced by plasmid oeHIF-1α. Another intriguing finding shown in Fig. 7 was that chondrocytes treated with MP alone exhibited a significantly higher apoptosis rate (18.96%) compared to those treated with TM alone (8.3%). The results suggested that the high apoptosis rate in MP-treated chondrocytes could be primarily linked to the activation of *HIF-1α* rather than ERS. Furthermore, although the activation of the ERS pathway by *HIF-1α* overexpression led to apoptosis, inhibition of ERS did not completely reduce the apoptosis rate induced by HIF-1α.

3.8. *HIF-1α* affected the ERS pathway and apoptosis

To gain deeper insights into the role of *HIF-1α*, the plasmid oeHIF-1α and siRNA were designed to treat chondrocytes.

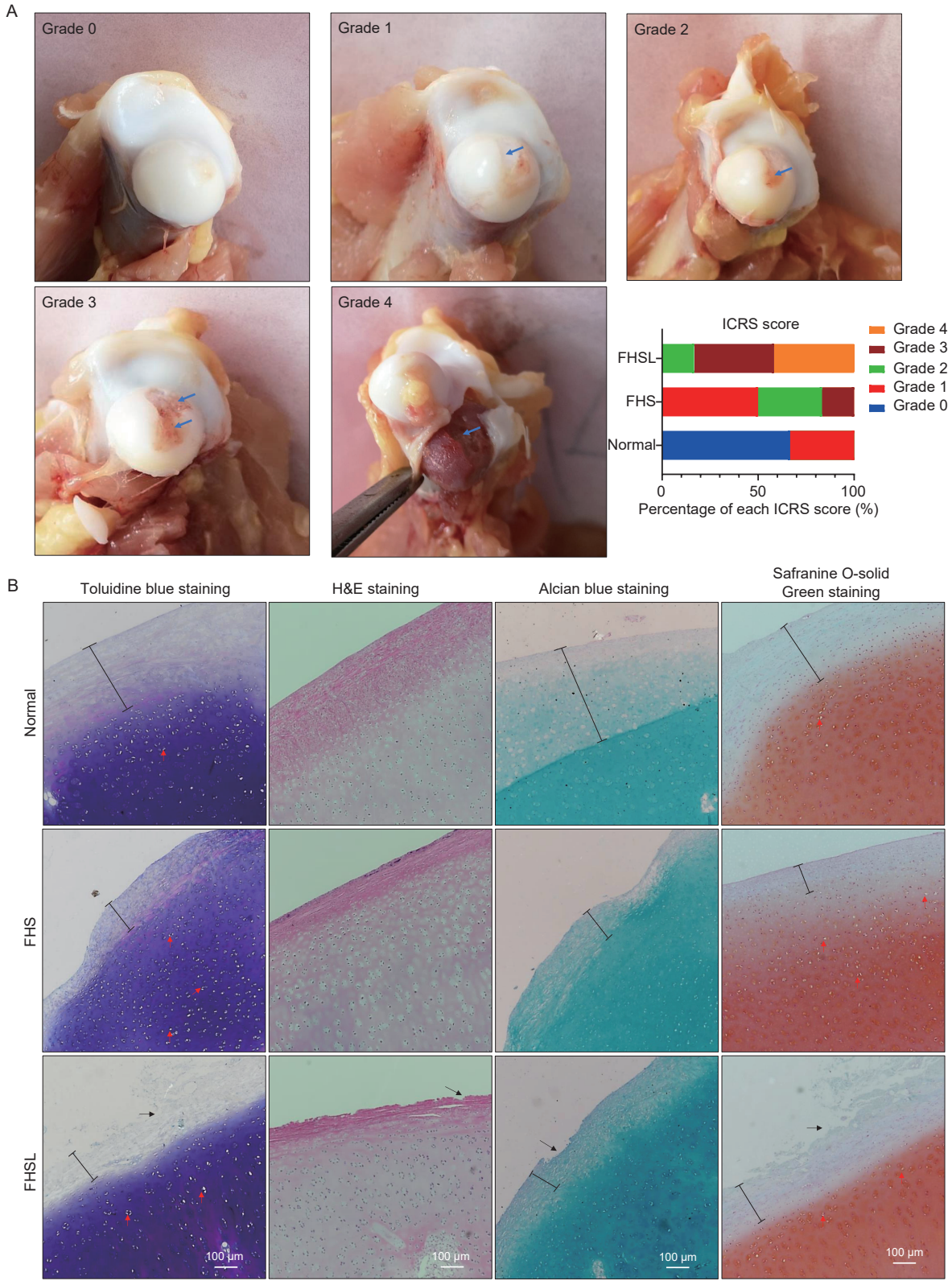


Fig. 3 The cartilage changes in spontaneous FHN broilers. A, femoral head cartilage with different ICRS score. Grade 0, normal cartilage; grade 1, nearly normal (superficial lesions, soft indentation and/or superficial fissures and cracks); grade 2, lesions extending down to <50% of cartilage depth; grade 3, cartilage defects extending down>50% of cartilage depth as well as down to calcified layer and down to but not through the subchondral bone; grade 4, cartilage defects extending down to the subchondral bone. The blue arrow pointed to the damage part of cartilage. B, cartilage tissue with toluidine blue, H&E, alcian blue and safranine O-solid green staining. The black arrow pointed to the damaged the lamina splendens of superficial zone, and the red arrow pointed to the vacuoles in the chondrocytes. ICRS, international cartilage repair society; FHS, femoral head separation; FHSL, femoral head separation with growth plate lacerations; H&E, hematoxylin and eosin.

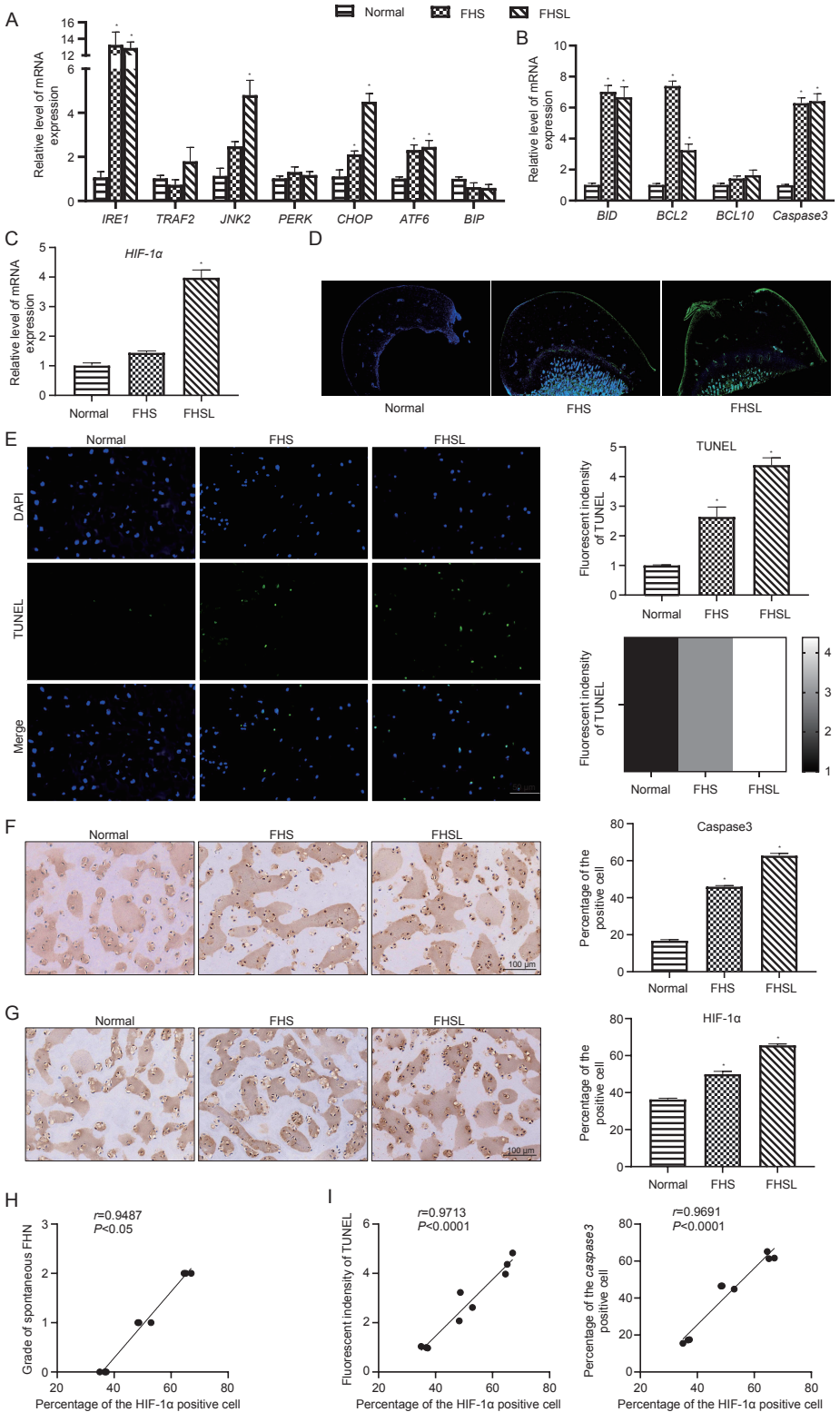


Fig. 4 The changes of ERS pathway and apoptosis in spontaneous FHN broilers. A and B, mRNA expressions of ERS and apoptosis related genes in cartilage. C, mRNA expressions of *HIF-1α* in cartilage. D, TUNEL scan of whole cartilage in spontaneous FHN broilers. E, TUNEL results of cartilage in spontaneous FHN broilers. F and G, immunohistochemical results of Caspase3 and HIF-1α in cartilage. H, correlation analysis for the percentages of *HIF-1α* positive cell and grade of spontaneous FHN in broilers, performed by Spearman's correlation. I, correlation analysis for the percentages of *HIF-1α* positive cell and fluorescent intensity of TUNEL/percentage of *caspase3* positive cell in samples from the broilers, performed by Pearson's correlation. ERS, endoplasmic reticulum stress; FHS, femoral head separation; FHSL, femoral head separation with growth plate lacerations; IRE1, inositol-requiring enzyme 1; TRAF2, TNF receptor associated factor 2; JNK, c-Jun N-terminal kinase; PERK, PKR-like ER kinase; CHOP, C/Ebp-homologous protein; ATF6, activating transcription factor 6; BIP, immunoglobulin heavy chain binding protein; HIF-1α, hypoxia-inducible factor-1α; BID, BH3 interacting domain death agonist. Data are mean±SE (n=3). *, P<0.05 *vs.* normal broilers.

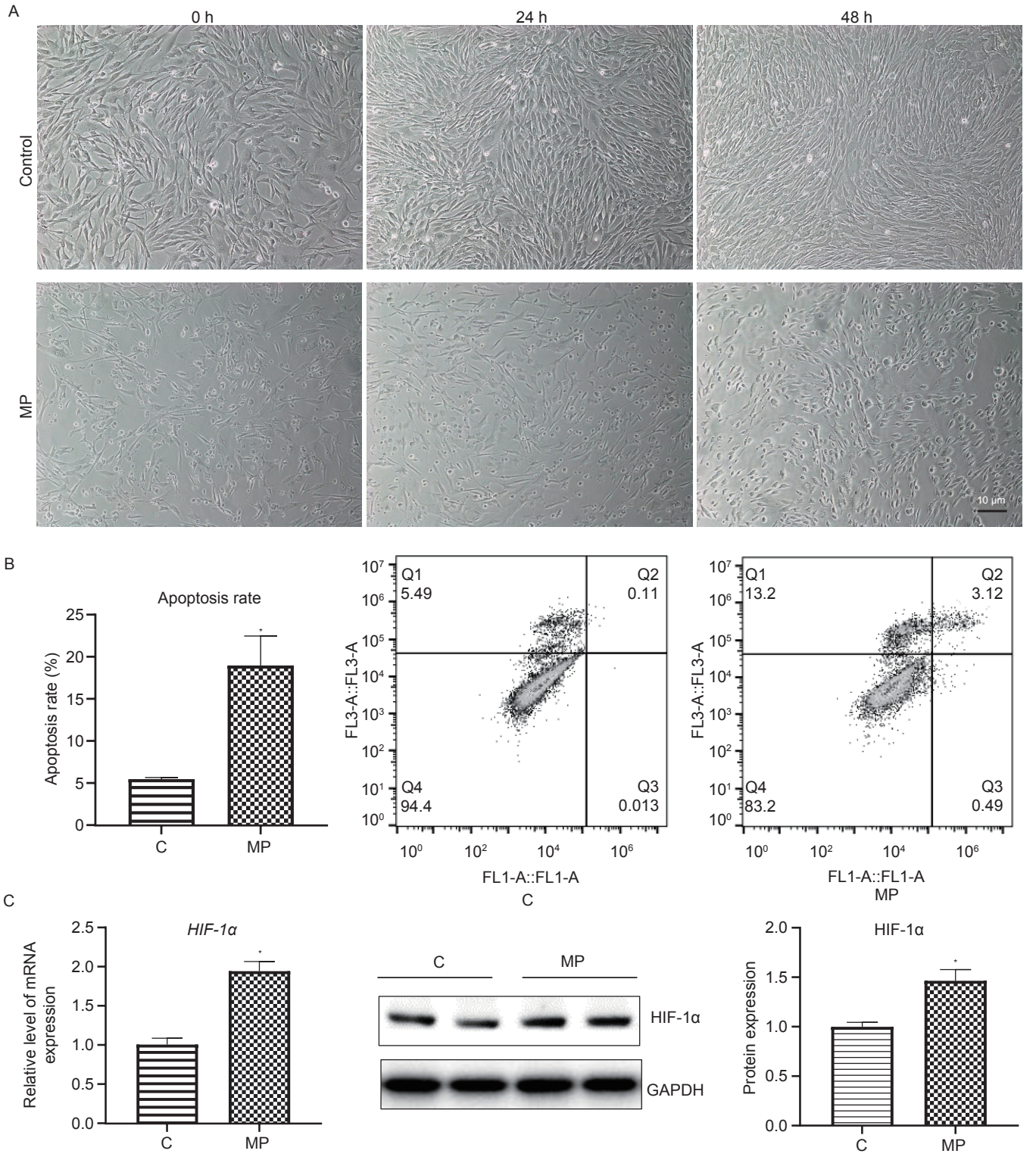


Fig. 5 The results in MP-treated chondrocytes. A, the result of chondrocytes with MP treatments. B, the results of apoptosis in MP-treated chondrocytes by flow cytometry determination. C, the results of HIF-1α in chondrocytes with MP treatment. MP, methylprednisolone; HIF-1α, hypoxia-inducible factor-1α; GAPDH, glyceraldehyde-3-phosphate dehydrogenase. Data are mean±SE (n=3). *, P<0.05 *vs.* normal broilers.

Fig. 8-A showed that the plasmid oeHIF-1α was able to increase the mRNA and protein levels of *HIF-1α*. Regarding the factors related to the ERS pathway, they were found to be obviously increased in MP- or oeHIF-1α treated chondrocytes, especially at the mRNA level. The *IRE1/TNF receptor associated factor 2* (*TRAF2*)*/c-Jun N-terminal kinase* (*JNK*) axis, *PERK/ATF4/CHOP* axis and *ATF6/X-box binding protein 1* (*XBP1*)*/autophagy related gene* (*ATG*) axis are typical pathways of ERS to induce apoptosis and autography (Urano *et al.* 2000; Hetz *et al.* 2006; Rellmann *et al.* 2021),

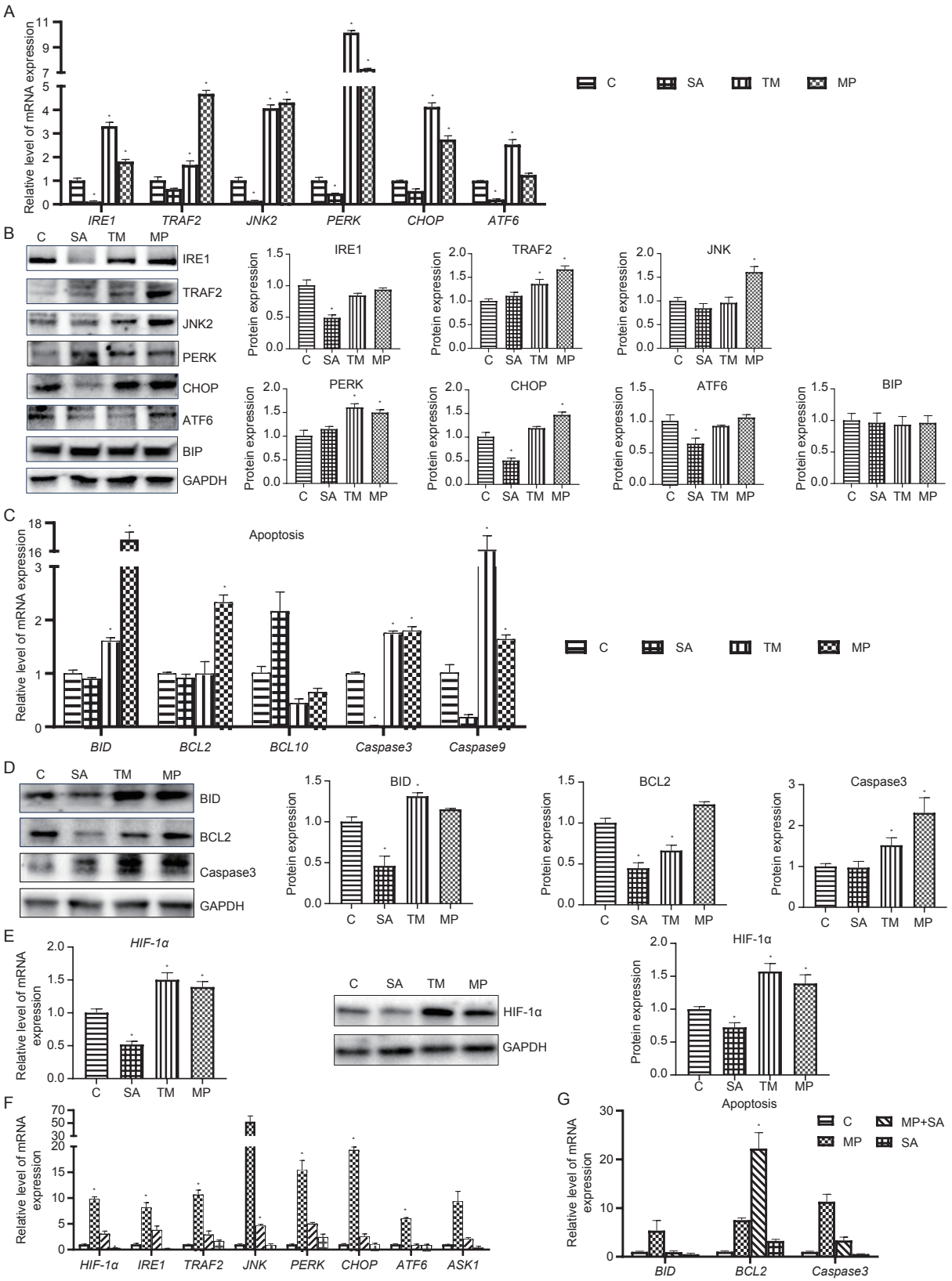


Fig. 6 The result of chondrocytes with SA, TM and MP treatment. A, mRNA expressions of ERS related genes in chondrocytes with different treatments. B, protein expressions of ERS related genes. C and D, mRNA and protein expressions of apoptosis related genes in chondrocytes. E, expressions of HIF-1α in chondrocytes. F, mRNA expression of genes related to the ERS in chondrocytes treated with MP and SA. G, mRNA expressions of apoptosis related genes in chondrocytes treated with MP and SA. SA, salubrinal; TM, tunicamycin; MP, methylprednisolone; IRE1, inositol-requiring enzyme 1; TRAF2, TNF receptor associated factor 2; JNK, c-Jun N-terminal kinase; PERK, PKR-like ER kinase; CHOP, C/Ebp-homologous protein; ATF6, activating transcription factor 6; BIP, immunoglobulin heavy chain binding protein; HIF-1α, hypoxia-inducible factor-1α; BID, BH3 interacting domain death agonist; GAPDH, glyceraldehyde-3-phosphate dehydrogenase. Data are mean±SE (n=3). *, P<0.05 *vs.* normal broilers.

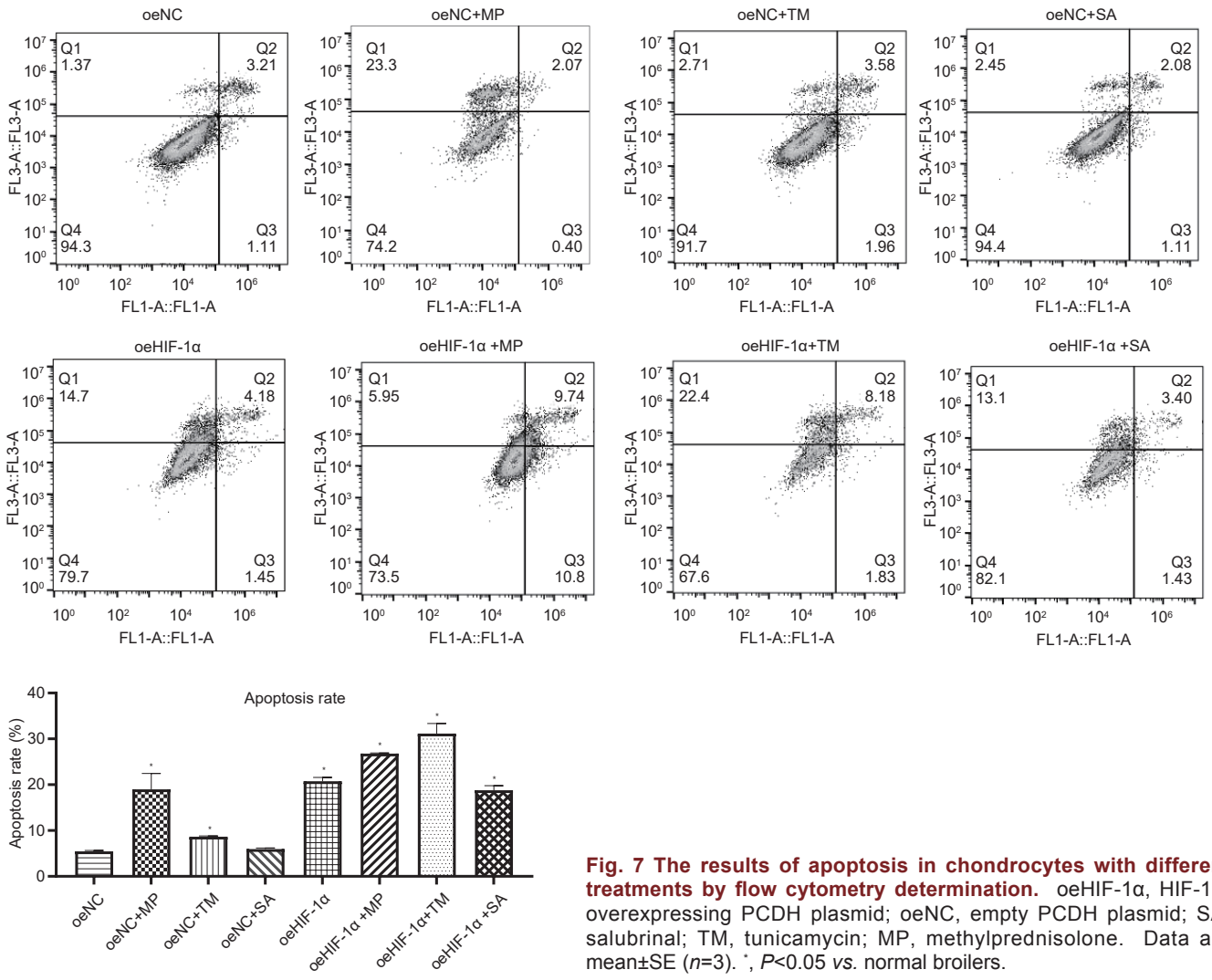


Fig. 7 The results of apoptosis in chondrocytes with different treatments by flow cytometry determination. oeHIF-1α, HIF-1α-overexpressing PCDH plasmid; oeNC, empty PCDH plasmid; SA, salubrinal; TM, tunicamycin; MP, methylprednisolone. Data are mean±SE (n=3). *, P<0.05 *vs.* normal broilers.

hence high levels of these axes can indicate the activation of ERS (Fig. 8-B and C). The result of *CHOP* expression revealed an excessive activation of the ERS pathway in chondrocytes treated with oeHIF-1α. Furthermore, we found that the pro-apoptotic gene *BID* was activated in 3 treated groups, whereas MP or oeHIF-1α treatment significantly increased the expression of *caspase-3* in chondrocytes (Fig. 8-D and E). The findings were further confirmed by the siRNA treatment, which showed that a decrease in *HIF-1α* levels effectively reduced chondrocyte apoptosis by inhibiting the overactivation of the ERS pathway (Fig. 9). Therefore, overexpression of *HIF-1α* could activate the ERS pathway and induce apoptosis in chondrocytes.

4. Discussion

With the development of the economy, the rising demand for chicken and other meat products has prompted the agricultural sector to focus on improving efficiency and increasing production (Gao *et al.* 2022). However, advancements in farming techniques and management have also resulted in a range of challenges and diseases, such as various leg diseases, which ultimately lead to animal welfare problems and economic losses (Cook 2000; Julian 2005). A number of studies have indicated that FHN is one of the most common leg diseases in in contemporary farms (McNamee and Smyth 2000). FHN hampers the mobility of broilers and restricts their access to sufficient food, resulting in weakened broilers that struggle to survive (Yu *et al.* 2022). Our findings also showed a progressive decline in broiler weight as the severity of FHN symptoms increased. Therefore, it is crucial to explore the mechanisms of FHN in broilers in order to provide new insights into the prevention of FHN.

In this study, we obtained broilers from a farm and divided them into 3 groups: normal, FHS and FHSL. During sampling, we observed that broilers with FHN tended to originate from cages located on the sides, closer to the heaters, and exposed to higher ambient temperatures compared to those in the middle. In addition, the outer cages were more susceptible to external factors such as human activity. This finding was consistent with some prior studies, which have indicated that the development of FHN could be influenced by both temperature variations and external stimuli (Shim and Pesti 2011; Wideman and Pevzner 2012). The development of the immune system is closely associated with stimulation, but prolonged exposure to stimulation leads to a suppression in immunity due to significant energy depletion (Singh 2000). In this study, FHN-affected broilers displayed

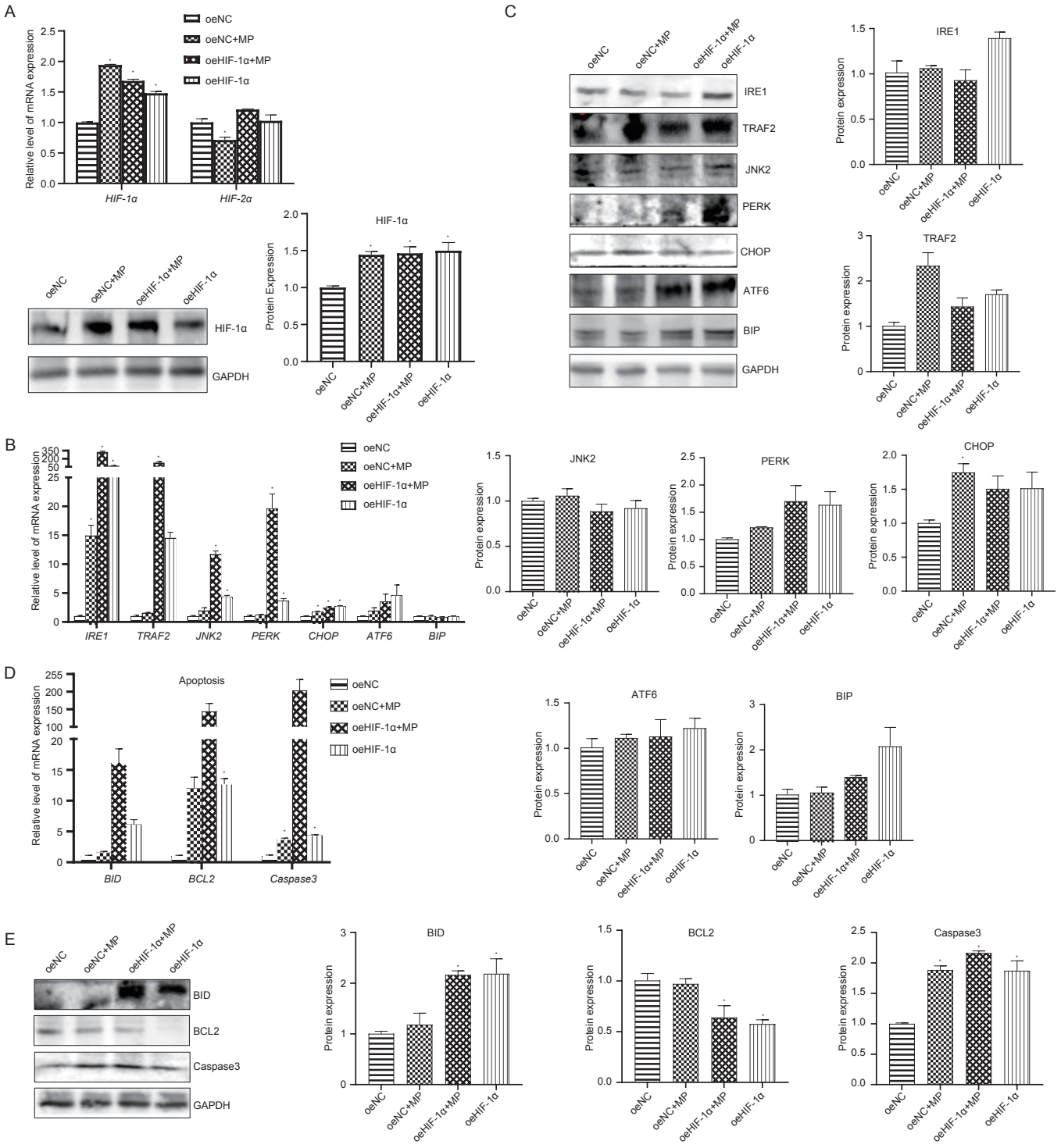


Fig. 8 The result of chondrocytes with plasma oeHIF-1α treatment. A, expressions of HIF-1α in chondrocytes. B, mRNA expressions of ERS related genes in oeHIF-1α-treated chondrocytes. C, protein expressions of ERS related genes. D, mRNA expressions of apoptosis related genes in chondrocytes. E, protein expressions of apoptosis related genes in chondrocytes. oeHIF-1α, HIF-1α-overexpressing PCDH plasmid; oeNC, empty PCDH plasmid; HIF-1α, hypoxia-inducible factor-1α; IRE1, inositol-requiring enzyme 1; TRAF2, TNF receptor associated factor 2; JNK, c-Jun N-terminal kinase; PERK, PKR-like ER kinase; CHOP, C/Ebp-homologous protein; ATF6, activating transcription factor 6; BID, BH3 interacting domain death agonist; GAPDH, glyceraldehyde-3-phosphate dehydrogenase. BIP, immunoglobulin heavy chain binding protein. Data are mean±SE (n=3). *, P<0.05 *vs.* normal broilers.

slight reductions in immune organ indexes and an elevated cortisol concentration. This finding provides further evidence that immunosuppression due to elevated corticosterone levels might be the causative factor of FHN (Julian 1998; Oppong and Cato 2015). In addition, we investigated the changes in bone, which showed an interesting result that although bone remodeling indicators were changed in the early stage of FHN, bone quality (including BMD, bone strength and so on) was significantly altered only in FHSL broilers. However, the results of ICRS scores, various cartilage staining and TUNEL

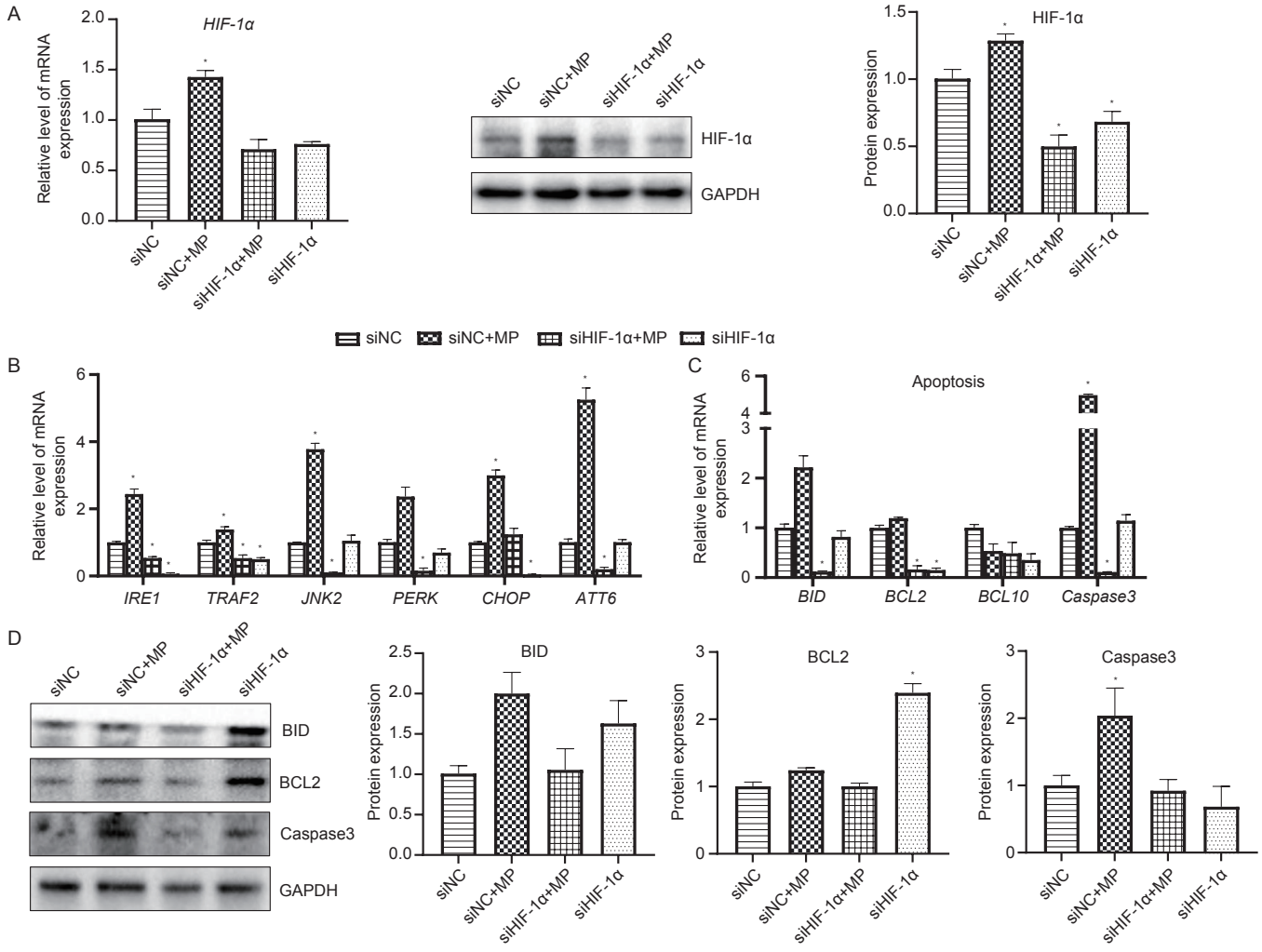


Fig. 9 The result of chondrocytes with HIF-1α siRNA (siHIF-1α) treatment. A, expressions of HIF-1α in chondrocytes. B, mRNA expressions of ERS related genes in siHIF-1α-treated chondrocytes. C, mRNA expressions of apoptosis related genes in chondrocytes. D, protein expressions of apoptosis related genes in chondrocytes. IRE1, inositol-requiring enzyme 1; TRAF2, TNF receptor associated factor 2; JNK, c-Jun N-terminal kinase; PERK, PKR-like ER kinase; CHOP, C/Ebp-homologous protein; ATF6, activating transcription factor 6; BIP, immunoglobulin heavy chain binding protein; BID, BH3 interacting domain death agonist; GAPDH, glyceraldehyde-3-phosphate dehydrogenase. HIF-1α, hypoxia-inducible factor-1α. Data are mean±SE (n=3). *, P<0.05 *vs.*normal broilers.

staining indicated a progressive increase in cartilage damage throughout the progression of FHN. Therefore, it is easier to access cartilage compared to bone for studies related to FHN.

MP is a type of GC that appears to cause immunosuppression to induce FHN in broilers, and it has been widely adopted in various studies to develop a FHN model in broilers (Boss and Misselevich 2003; Kerachian *et al.* 2009). Our previous study discovered a strong correlation between chondrocyte apoptosis and GC-induced FHN in broilers. (Zhang *et al.* 2019). Therefore, chondrocytes, being the sole cells present in the cartilage, were treated with MP to establish the FHN model *in vitro*. The mRNA results revealed that severe apoptosis was induced in FHN broilers and MP-treated chondrocytes, which was further supported by the results of caspase3, TUNEL staining and flow cytometry assays. The results of Zhang *et al.* (2019) also showed that there was a high level of genes associated with apoptosis in the cartilage of FHN broilers induced by GC. Consequently, the results suggested that apoptosis was increased in both FHN broilers and MP-treated chondrocytes.

Multiple studies have reported that the ERS pathway is the classical way to mediate apoptosis and has been implicated in various diseases (Delbrel *et al.* 2018; Rellmann *et al.* 2021). Normally, activation of mild or chronic ERS serves the purpose of restoring protein homeostasis and promoting survival. However, when stimulation persists, the persistent activation of ERS results in apoptosis and eventually culminates in cell death (Akman *et al.* 2021). The balance between pro-survival and pro-apoptotic responses is primarily regulated by two factors: *BIP* and *CHOP* (Schonthal 2013). When the ERS is in a pro-survival mode, *BIP* increases, whereas the expression of *CHOP* increases in a pro-apoptotic mode (Ma *et al.* 2002; Delbrel *et al.* 2018). In this study, we observed an increase in the expression of ERS-related genes in FHN broilers. Additionally, the severity of FHN was found to correlate with increased levels of ERS, particularly *CHOP*. We further investigated the potential role of ERS in MP-treated chondrocytes by activating or inhibiting the ERS pathway. The results showed that chondrocytes treated with

MP or TM (ERS activator) displayed comparable levels of genes associated with apoptosis in FHN broilers. Conversely, treatment with SA (an ERS inhibitor) led to a reduction in apoptosis levels, which was consistent with the findings of Huang *et al.* (2017). The apoptosis-inducing effects of MP and TM in chondrocytes were further corroborated by the flow cytometry findings. Furthermore, our previous results also demonstrated that ERS was overactivated in GC-induced FHN broilers (Zhang *et al.* 2019). The findings revealed that MP could activate ERS to induce apoptosis, and a high level of ERS might contribute to the occurrence of FHSL.

Cartilage is naturally hypoxic due to the lack of blood vessels (Sandell and Aigner 2001). Therefore, the vital role of *HIF-1α* in cartilage as a major factor mediating hypoxia response cannot be ignored (Stegen *et al.* 2019). Although the potential association between *HIF-1α* and ERS has been reported in some reports, there is a lack of emphasis on FHN in these studies (Huang *et al.* 2017; Delbrel *et al.* 2018). Our results indicated that *HIF-1α* was activated in MP-treated chondrocytes and broilers, and it was activated in spontaneous FHN-affected broilers, particularly in FHSL broilers, indicating that the severity of FHN was closely associated with the level of *HIF-1α*. This discovery was confirmed by the correlation analysis. In addition, prior studies have reported that stabilization of *HIF-1α* can effectively induce ERS in alveolar epithelial cells (Delbrel *et al.* 2018). Our findings further demonstrate a significant increase in mRNA and protein levels of genes associated with ERS and apoptosis in broilers affected by FHN and chondrocytes treated with MP. Furthermore, the opposite effect was observed in chondrocytes treated with *HIF-1α* siRNA, further corroborating our findings. The ERS pathway is influenced by the level of *HIF-1α*, and activation of the ERS pathway could effectively trigger apoptosis (Tang *et al.* 2017; Bartoszewska and Collawn 2020), which was confirmed by the flow cytometry results. Correlation analysis also revealed a strong association between the level of *HIF-1α* and the apoptosis rate in the cartilage of broilers affected by FHN. Therefore, *HIF-1α* might play a critical role in the development of FHN in broilers.

Our study showed that bone remodeling and cartilage homeostasis were affected in FHN broilers. However, there were evident alterations in bone quality only in FHSL broilers, while cartilage damage was observed to varying extents in both FHS and FHSL broilers. Therefore, cartilage might be more appropriate than bone to explore the mechanisms of FHN. In addition, there was an elevated level of *HIF-1α* observed in MP-treated chondrocytes and FHN-affected broilers, and a significant positive relationship between the level of *HIF-1α* and the FHN severity or apoptosis rate. Furthermore, overactivation of the ERS pathway induced chondrocyte apoptosis, while the *HIF-1α* played a key role in regulating both ERS and apoptosis processes. Therefore, *HIF-1α* might damage cartilage by inducing ERS-mediated apoptosis, resulting in the development of FHN in broilers. In addition, the elevated levels of *HIF-1α* can correspond to increased apoptosis rates in cartilage, potentially leading to more severe FHN (FHSL).

5. Conclusion

In conclusion, we found that FHN broilers have disrupted bone remodeling and imbalanced cartilage homeostasis, but only cartilage damage is exacerbated with FHN development. Furthermore, the development of FHN was found to be directly related to the levels of *HIF-1α*, a critical transcription factor responsible for cartilage. Specifically, it triggered chondrocyte apoptosis and thereby leading to cartilage damage, by activating the ERS pathway. Therefore, *HIF-1α* appears to be a key factor in the incidence and severity of FHN in broilers. Overall, these findings provided valuable insight into the underlying mechanisms of FHN.

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Declaration of competing interest

The authors declare that they have no conflict of interest.

Declaration of Generative AI and AI-assisted technologies in the writing process

During the preparation of this manuscript, the authors used ChatGPT 4.0 to assist in improving the clarity and readability of the language. All content generated was carefully reviewed and revised by the authors as necessary, and the authors take full responsibility for the final content of the article.

Ethical approval

This study was performed in accordance with the “Guidelines for Laboratory Animals” issued by the Ministry of Science and Technology of China (2006).

Appendices are associated with this study are available at https://doi.org/10.1016/j.jia.2024.03.084

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