The Investigation of the Relationship Between HSP-27 Release and Oxidative DNA Damage in Broiler Chickens with Tibial Dyschondroplasia by Using Histopathological and Immunohistochemical Methods

ABSTRACT

Tibial dyschondroplasia (TD) is a skeletal disorder that occurs in the proximal metaphyses of tibiotarsus and sometimes tarsometatarsus, resulting in the development of avascularized and non-mineralized abnormal cartilage and causing significant economic loss. In this study, we aimed to show the histopathological changes and the relationship between the release of Heat-Shock Protein 27 (HSP-27) and oxidative DNA damage in broiler chickens with tibial dyschondroplasia, using histopathologic and immunohistochemical methods. Our study material consisted of totally 20 animals out of 42 days old 205 Ross 308 broiler chickens, 10 with TD lesions and 10 healthy control subjects. Tissue samples taken from animals performed necropsy was exposed to routine tissue follow-up. Macroscopically, unilateral and bilateral thickening and swelling were observed in the growth plates of tibiotarsal joints of the broiler chickens diagnosed with tibial dyscondroplasia. Histopathologic examination of the tibiotarsal joints of broiler chickens affected by TD revealed an increase in the number of immature chondrocytes, as well as deficiencies in vascularization and calcification. In the immunohistochemical study; HSP-27 and 8-OHDG release was positive in the chondrocytes located on the Proliferative Zone, Maturation Zone and Hypertrophic Zone. However, the positivity was the most profound in the PZ and MZ, while less in the HZ chondrocytes. As a result; we demonstrated by immunohistochemical methods that the increase in the HSP-27 release is parallel to the increase in 8-OHDG release in TD lesioned areas and this may be related to oxidative stress.

INTRODUCTION

Tibial dyschondroplasia (TD) is a skeletal disorder that occurs in the form of avascularized and non-mineralized abnormal cartilage growth in the proximal metaphyses of the tibiotarsus and sometimes tarsometatarsus of poultry (Dinev, 2012; Genin et al., 2012; Tian et al., 2013; Velada et al., 2011). The disease is attributed to abnormal differentiation of chondrocytes leading to bone growth, cartilage vascularization, and mineralization (Nabi et al., 2016a; Shahzad et al., 2014; Tian et al., 2013). TD, most frequently seen more in rapidly growing poultry, is a growth plate disease, which is more common in broiler chickens and less common in turkeys and ducks. The most important cause of abnormal cartilage development has been reported to be the failure of the cartilage tissue in adapting itself to such a rate of growth due to rapid growth (Angel, 2007; Dinev, 2012; Shim et al., 2013). TD is attributed to various factors such as genetics, gender, nutritional content or nutritional deficiencies, toxins, growth rate and environmental factors, although its etiology is not clearly understood (Gay et al., 2007; Houshmand et al., 2011).
Heat shock proteins (HSPs), also known as stress proteins, form a highly conserved family of proteins released by all prokaryotic and eukaryotic cells, protecting the organism and the cells from injury and increasing resistance. The release of these proteins is increased by the effects of various stress factors such as toxins, free radicals, temperature, infections, and nutritional inadequacy (Haslbeck & Vierling, 2015; Dökümancıoğlu et al., 2018; Meher et al., 2018).

Heat shock proteins are composed of six main families, such as HSP-100, HSP-90, HSP-70, HSP-60, HSP40 and small HSPs (sHsps) according to their molecular weights and functions (Meher et al., 2018). Heat shock protein-27 (Hsp 27) is a member of the small HSPs, playing important roles such as cytoprotective, antiapoptotic, anti-aging, and embryogenesis (Leonardi et al., 2004; Rogalla et al., 1999; Thompson et al., 2001; Hasbeck & Vierling, 2015, Dökümancıoğlu et al., 2018).

In this study, we aimed to show the histopathological changes and the relationship between the release of Heat-Shock Protein 27 (HSP-27) and oxidative DNA damage in broiler chickens with tibial dyschondroplasia, using histopathologic and immunohistochemical methods.

**MATERIALS AND METHODS**

**Experimental animals**

The experiments were performed according to the ethical conditions confirmed by the Ethics Committee of Experimental Animal Teaching and Researcher Center, Ataturk University, Erzurum, Turkey (Decision No: 25.03.2011/3/10). Our research consisted in a total of 205 42 days old Ross 308 broiler chickens, of which 20 were used for study material, being 10 with TD lesions and 10 healthy control subjects. The necropsy was performed on broiler chickens sacrificed using the method of cervical dislocation. Tissue specimens taken from the tibiotarsal joints of the animals that were sacrificed by cervical dislocation were subjected to routine histopathologic and immunohistochemical examinations.

**Histopathological examination**

The samples were fixed in 10% formalin for 24 h. Then, the samples were decalcified in 36.8% formic acid and 6.8% sodium formate. Finally, the samples were post-fixed and embedded in paraffin. After routine procedures, sections of 5-6 μ thickness were obtained and stained routinely with haematoxylin-eosin, and examined under a light microscope (Presnell & Schreibman, 1997).

**Immunohistochemical examinations**

After the deparaffinizing process, antigen retrieval (pH 6.0) was applied in the microwave for 15 min. Then the sections were incubated in 3% H₂O₂ for 10 min to prevent endogenous peroxidase activity. The sections washed with phosphate buffered saline (PBS) were incubated with polyclonal rabbit HSP-27 antibody (clone:ab78806, dilution 1/100; Abcam, UK) and rabbit 8-OHdG (clone:sc66036, Santa Cruz Biotecnology, dilution 1/200); at room temperature for 30 min. Sections rewashed with PBS were stained with expose mouse and rabbit specific horseradish peroxidase/diaminobenzidine (HRP/DAB) detection IHC kit (Catalog No: ab80436, Abcam, UK) as recommended by the manufacturer. 3,3-diaminobenzidine (Dako Cytomation) was used as the chromogen. Slices which were passed through alcohol xylol series following counterstaining with Mayer’s hematoxylin were examined under light microscope (Dökümancıoğlu et al., 2018).

**RESULTS**

**Macroscopic findings**

Macroscopically, difficulty in standing up, walking, specially lameness was observed in 10 of the 205 animals. Weight loss and hair loss were observed in these animals. Unilateral thickening in seven and bilateral thickening in eleven growth plates of the tibiotarsal joints of these animals were noticed as well as swelling (Figure 1). On the cross section, opaque cartilaginous structure was detected, varying in color from white to gray and extending from the distal end of the epiphysis plate into the metaphysis.
Histopathologic Findings

Histopathologically, no lesions were observed in the animals in the control group (Figure 2A). Histopathologic examination of the tibiotarsal joints of the broiler chickens affected by TD revealed an increase in the number of immature chondrocytes (Figures 3A, B), as well as deficiencies in vascularization and calcification. Degenerative changes were also observed in some chondrocytes and necrosis in some others.

Immunohistochemical Findings

HSP-27 release was positive in the chondrocytes located on the Proliferative Zone (PZ), Maturation Zone (MZ), and Hypertrophic Zone (HZ). However, the positivity was the most profound in the PZ and MZ, while less in the HZ chondrocytes (Figures 3C, D). The expression of HSP27 in articular cells was weak in the Control group (Figure 2B). The expression of 8-OHdG was present in the PZ, MZ, and HZ chondrocytes (Figures 3E, F). Its expression was moderate in the MZ and PZ, and weak expression was observed in the HZ chondrocytes. Similar to HSP27 expression, in 8-OHDG expression in articular cells for the TD group than for the control group (Figure 2C).

DISCUSSION

Obtaining rapidly growing breeds as a result of genetic selections conducted in the production of broiler chickens led to an increase in the incidence of leg problems resulted from developmental bone tissue disorders. Leg problems occur as a result of incomplete development of bone and cartilaginous tissues and primarily as a consequence of tibial dyschondroplasia. Bone formation is known to be influenced by many factors including genetic diseases, toxins, antinutritional feed, age, gender, diet, physical activity and endocrine system. Bones are composed of inorganic salts that accumulate in the organic matrix composed of collagen fibrils and glycoproteins (Angel, 2007; Gay et al., 2007; Hasky-Negev et al., 2008; Dinev, 2012; Shim et al., 2012).

Factors such as genetics, age, gender, nutrition and flock management were reported to play an important role in the pathogenesis of TD, although its etiology is not fully known (Rath et al., 2007; Houshmand et al., 2011). There are studies reporting morphological, histopathological, biochemical and molecular changes observed in the tissues and cells of the animals affected by TD (Velada et al., 2011; Genin et al., 2008; Genin et al., 2012; Imık et al., 2012).

In the proximal growth plates of tibiotarsal bone in TD, lesions are usually bilateral, but occasionally they can be unilateral. Clinically, while swelling, deformity and lameness are observed in these areas at the beginning and bone fractures are formed at a later stage. For this reason, TD leads to significant economic loss worldwide, besides affecting the animal welfare negatively (Rath et al., 2005; Imık et al., 2012; Pelicia et al., 2012).

In previous studies, thickening of the epiphyseal plate and the formation of an abnormal opaque cartilage mass extending from the distal end of the epiphyseal plate into the metaphysis was detected macroscopically (Rath et al., 2005; Imık et al., 2012; Pelicia et al., 2012; Nabi et al, 2016b). In this study, clinically and macroscopically similar lesions were observed in 10 of the 205 broiler chickens.

This non-mineralized abnormal cartilage mass is devoid of blood vessels and has a soft consistency. In such cases, the most prominent histopathological lesions are the presence of hypertrophic and immature
The Investigation of the Relationship Between HSP-27 Release and Oxidative DNA Damage in Broiler Chickens with Tibial Dyschondroplasia by Using Histopathological and Immunohistochemical Methods

Kapakin KAT, Kapakin S, Imik H, Gumus R, Eser G

The Investigation of the Relationship Between HSP-27 Release and Oxidative DNA Damage in Broiler Chickens with Tibial Dyschondroplasia by Using Histopathological and Immunohistochemical Methods

Chondrocytes in the epiphyseal plate (Rath et al., 2005; Imik et al., 2012). In addition, degeneration and necrosis of chondrocytes may also be seen. While extreme hypertrophy of chondrocytes increase in the number of immature chondrocytes, degeneration and necrosis of chondrocytes were observed in the tibiotarsal joint. This was accompanied by a vascularization (Rath et al., 2007; 2005; Imik et al., 2012; Nabi et al., 2016b; Zhang et al., 2018). The histopathological findings observed in the broiler chickens with TD lesions in the present study were determined to be compatible with the findings of previous studies. Since the etiology of TD could not be fully clarified yet, many new studies are conducted on this issue. For this reason, the studies investigating the factors that are likely to play a role in the pathogenesis of the disease are becoming prominent recently. In these studies, Hypoxia inducible factor-1 (HIF-1α), (Genin et al., 2008; Mehmood et al., 2017) vascular endothelial growth factor (VEGF) (Zhang et al., 2013; Mehmood et al., 2018; Zhang et al., 2018) and HSPs (Genin et al., 2012; 2018) release were shown by several genes (Tian et al., 2013, 2014), biochemical and immunohistochemical methods in poultry with TD lesions (Genin et al., 2012; Rath et al., 2005; Nabi et al., 2016a, b; Shahzad et al., 2014, 2015; Iqbal et al., 2016).

Stress proteins, known to be produced by all living creatures, play important roles in all cell metabolism, including cell growth and death. The release of these proteins is increased when exposed to environmental factors such as hypoxia, reactive oxygen metabolites, heavy metals, heat, and in all conditions where cells are damaged, such as infection or tumor (Haslbeck & Vierling, 2015, Dökümancıoğlu et al., 2018; Meher et al., 2018).

HSPs are known to be released at a certain level in growth plates during normal bone development. HSP-27, similar to other HSPs in the group, has an important role in bone development (Leonardi et al., 2004), as well as protecting and strengthening...
the cell against various stress factors (Rogalla et al., 1999; Thompson et al., 2001; Hasbeck & Vierling, 2015, Dökumacıoglu et al., 2018). In previous biochemical and immunohistochemical studies, HSP-90 and HSP-70 release were reported to be higher in TD-lesioned poultry than in healthy poultry. They noted that the release of HSP-90 and HSP-70 was higher in chondrocytes located on the hypertrophic zone (HZ) and the maturation zone (MZ) as compared to other zones in healthy broiler chickens. However, in broiler chickens with TD lesions, the release of these proteins was found to be higher in the proliferative zone (PZ) when compared to other zones (Genin et al., 2012; Shahzad et al., 2014; Iqbal et al., 2016; Mehmood et al., 2018). However, there was no immunohistochemical study showing HSP-27 release in broiler chickens with TD lesions in the literature reviewed. In our study, HSP-27 release was positive in the chondrocytes located on the Proliferative Zone, Maturation Zone and Hypertrophic Zone. However, the positivity was the most profound in the PZ and MZ, while less in the HZ chondrocytes.

This increase of HSP-70 and HSP-90 release in the areas with TD lesions was associated with an increase in HIF-1α in previous studies (Genin et al., 2008). HIF-1α protein is an important regulator for maintaining the viability of the cells and tissues in adapting themselves to low oxygen pressure (Cheng et al., 2016). Under oxidative stress conditions developed as a result of hypoxia, HIF-1α protein causes an increased HSP synthesis, by binding to the promoter regions of the DNA, and thus providing transcription of the HSP gene (Huang et al., 2018).

The increase in the release of HIF-1α and the release of HSP-90 and HSP-70 along with hypoxia and oxidative stress as a result of a vascularization in lesioned areas in TD in broiler chickens were shown by immunohistochemical methods in previous studies (Mehmood et al., 2018).

The 8-Hydroxy-2'-deoxyguanosine (8-OHdG) is a highly sensitive biological marker that is used to detect DNA damage due to oxidative stress. Previous studies revealed a positive correlation between HIF-1α release and oxidative DNA damage in oxidative stress (Schipani et al., 2001; Huang et al., 2018). To the best of our knowledge, there is no immunohistochemical study showing the relationship between HSP-27 release and oxidative DNA damage in broiler chickens with TD lesions.

In this study, an increase in 8-OHdG along with an increase in HSP-27 release was remarkable in the immunohistochemical staining of TD lesions. Therefore the relationship between HSP-27 release and 8-OHdG in TD lesioned broiler chickens was demonstrated by immunohistochemical methods for the first time in the literature, in this study.

In recent years, increasing the level of HSP in order to stimulate the defense mechanisms was considered as a method in the treatment of diseases of the nervous system (Kampinga & Bergink, 2016), cardiovascular system (Hu et al., 2017) or cancer (Chatterjee & Burns, 2017), and many studies were conducted on this topic. At the same time, there are studies currently suggesting the idea that increasing the release of HSPs to provide revascularization and reduce oxidative stress in the treatment of TD lesions would be beneficial (Huang et al., 2017; Huang et al., 2018, Mehmood et al., 2017; Mehmood et al., 2018). In accordance with the results of the present study, we agree with other researchers that HSPs proteins should not be overlooked in the development of new methods for the prevention or treatment of TD, which leads to significant economic loss regarding poultry.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENT

This study was supported by the Coordinator of Scientific Research Projects (2012/56) at Atatürk University.

REFERENCES


The Investigation of the Relationship Between HSP-27 Release and Oxidative DNA Damage in Broiler Chickens with Tibial Dyschondroplasia by Using Histopathological and Immunohistochemical Methods


