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# ASTRAGALOSIDE-IV INHIBITS TIBIAL DYSCHONDROPLASIA VIA PI3K/AKT /HIF-1α SIGNALING IN CHICKENS

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## ABSTRACT

Tibial dyschondroplasia (TD) is a common leg-pathology of meat type broiler birds which is characterized by defective tibial growth plate (GP) cartilage failing to become bone. This study deals with an attempt to treat TD using Astragaloside IV (Astr IV) from *Astragalus membranaceus* which have been reported to promote osteogenesis but its protective affects against TD have not been studied yet. For this purpose, we selected 180-day old chickens and distributed them in three groups alike (n= 60); control group, TD group and Astr IV group (orally @50mg/ kg body weight from 8-18 days). The chickens were slaughtered on 7, 10, 14 and 18 days. The chicken performance indicators parameters, tibial bone parameters, and expression of proteins were studied by using immunohistochemistry. The results demonstrated that in TD group production parameters including weight improvement and feed consumption were decreased compared to control group during the study period. Tibial bone parameters were lowered non-significantly ($P$>0.05) during the experiment except tibial GP, which was increased significantly ($P$<0.05) in TD chickens compared with control group on day 7, 10 and 14. While, Astr IV group indicated that performance indicators and tibia bone parameters were near to control group compared to TD group. On 14 and 18 days both TD incidence and TD score significantly ($P$<0.05) amplified in TD group compared to Astr IV group. The results of immunohistochemistry analysis regarding the proteins expression showed P-AKT ($P$<0.01), HIF-1alpha ($P$>0.05), and VEGF ($P$<0.01) expressions were increased in TD group. While the expression of AKT, PI3K and VEGFR1 were decreased non-significantly ($P$>0.05) in TD group chickens. However, the expression of Akt, PI3K and VEGFR1 proteins was up-regulated, while P-AKT, HIF-1alpha, and VEGF were down-regulated in Astr IV treatment group. Altogether, Astragaloside IV diminishes TD through regulating PI3K/Akt/HIF-1α signaling in chickens.

**Keywords:** Tibial dyschondroplasia, Chickens, Astragaloside-IV, Growth plate, Chondrocytes.



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## INTRODUCTION

The broiler industry is touching to highest peak of economic benefits due to rapid growth of broiler chicks, but on other hand incidence of various skeletal diseases like tibial dyschondroplasia (TD) is increasing in chicks. TD is a leg-pathology of broiler because of high growth rate, which mostly occurs in fast growing birds and characterized by deformed bones leading to the lameness (Jiang *et al.,* 2020; Liu *et al.,* 2021). It is a common leg problem which cause about 30% cases in chickens and commercial broilers (Huang *et al.,* 2018; Li *et al.,* 2020). TD is a serious threat to growing poultry industry in USA China and worldwide. It has affected and reduced the production performance and immunity of broiler chicks, which can lead to thoracic cyst,

osteomyelitis and more intensify the TD in broilers (Iqbal *et al.,* 2018a, b).

The exact cause and mechanism of disease is still unknown; however, scientists have proposed that interruptions and delay in growth plate cartilage undergoing endochondral ossification often result in development of TD lesions (Huang *et al.,* 2017a, b; Iqbal *et al.,* 2018c). The cellular hypertrophy of chondrocytes is a key factor for the longitudinal growth in avian species, so variations in proliferative and hypertrophic zones during endochondral ossification in different species of birds are strongly correlated with alteration in growth rate (Kulyar *et al.,* 2022). Researchers believe that disturbance of vascularization and angiogenesis to bone cells including osteoblasts and osteoclasts and mesenchymal stem cells are the probable reasons for impedance in endochondral ossification. In this way growth plate cartilage cannot become complete bone and ultimately white opaque mass is deposited in GP (Mehmood *et al.,* 2019b). It is assumed that any failure in growth plate cartilage during ossification leads to retention of avascular cartilage and could be the probable cause of lameness in birds (Luo *et al.,* 2018; Jiang *et al.,* 2019).

Many ectoparasiticides including thiamethoxam have been known for their potent role in development of musculoskeletal disorders in chicken (Zahoor *et al.,* 2022). In particular, the thiram can induce interruptions in endochondral ossification process as occur in TD. It has been reported that Thiram interacts with chondrocytes in GP of chickens and ultimately cause TD in birds with clinical signs and symptoms similar to TD (Iqbal *et al.,* 2022). Furthermore, it has been reported that TD may be related to genetic selection. But, the exact model of inheritance for TD occurrence cannot be proposed till yet (Kulyar *et al.,* 2021).

Hypoxia inducible factor 1-alpha (HIF-1α) is regulator of vascular endothelial growth factor (VEGF) production by hypertrophic chondrocytes. New blood vessels formulation and regulation of hypoxic responses that is necessary for chondrocyte development are controlled by HIF-1α (Mehmood *et al.,* 2018a). Most of the bones go through endochondral ossification in which a cartilage (avascular) is gradually replaced by a well vascularized bone. Bone cells including osteoclasts, osteoblasts and chondrocytes express VEGF (Mehmood *et al.,* 2017). This factor not only mediates fundamental physiologic and pathophysiologic capillary formation but also critically influence vascularization, degradation of matrix, maturation, differentiation and functional activity of bone cells. VEGFR1 and VEGFR2 are important receptors of VEGF, which are activated by VEGF (Mehmood *et al.,* 2019a, b), VEGFR1 has more affinity to VEGF, while during bone formation it play vital role for osteoblast activity and osteoblastogenesis (Mehmood *et al.,* 2019b).

Phosphoinositide 3-kinase (PI3k) has anti-apoptotic activities, when it becomes active then it stimulates phosphorylation and activation of Akt for cell survival (Nabi *et al.,* 2018a). It has been studied that PI3K/Akt signaling play important role during osteoarthritis pathogenesis (Chen *et al.,* 2017; Qamar *et al.,* 2020). Furthermore, PI3K and Akt have been concerned in numerous characteristics of cartilage and bone (Liang *et al.,* 2018; Qamar *et al.,* 2019a). The initiation of these signalings in the chondrocytes ultimately causes hypertrophy and proliferation of chondrocytes (Waqas *et al.,* 2019). The PI3K/Akt signaling support the differentiation of osteoblasts and any interruption in this process may lead to bone loss and attenuate bone formation (Lauzon *et al.,* 2016). Rao *et al.* (2017) stated that activation of PI3K and Akt is critical for chondrocytes protection during apoptosis and endoplasmic reticulum stress. Moreover, previous experiments reveal that decrease in the signaling of PI3K/Akt also decrease the process of osteogenesis and bone sclerosis in mouse (Lin *et al.,* 2018a). So, their results indicate that inhibition of PI3K/Akt attenuate hypertrophy of chondrocytes and degradation of cartilage (Lin *et al.,* 2018b).

Traditional Chinese medicines (TCM) are used from many years in China for the treatment of various diseases (Yang *et al.,* 2014). TCMs are formulated either from plant/ plants derivatives (Men *et al.,* 2022). Astragaloside IV is a traditional Chinese medicine which promotes osteogenesis and enhance the type I collagen synthesis via regulation of Col1a2 and ALP gene expression (Lin *et al.,* 2018a, b). So, the goal of our study is to inhibit tibial dyschondroplasia while improving hypoxic injury by using Astragaloside IV.

## MATERIALS AND METHODS

**Experiment Design:** The day old broiler chickens (n=180) were bought from market. All the birds were fed on normal feed for 3-days and after that, they were randomly divided; control group, TD group and Astr IV group. The Control-group was offered normal feed from 3-18 days. Thiram and Astr IV groups were given Thiram @50 mg (Jiang *et al.,* 2018; Zhang *et al.,* 2018a) per kilogram in normal feed for 3-7 days. Then, Astr IV group was offered Astragaloside IV @50 mg per kilogram (Zhou *et al.,* 2017; Mehmood *et al.,* 2019b) of body weight orally from 8-18 days, whereas TD-group was only offered normal feed without thiram from 8-18 days. All the animal's experiments were performed at Huazhong Agricultural University according to animal ethics committee (approval # 31272517), China.

**Samples Collection:** During the experiment, the lameness, severity and incidence of TD, tibia index changes, growth plate pathology, and expression of

proteins were studied. The chickens (n= 15) were randomly euthanized on days 7, 10, 14 and 18 from every group. The production parameters including feed intake and weight gain, while tibia parameters (tibia length, weight, width and GP width) were recorded before and after the slaughtering respectively. The tibia bones on each slaughtering day from each group were dissected out and freeze at –60 °C for Immunohistochemistry.

**Incidence and severity of TD:** The GP of tibial bones were cut with the help of blade in longitudinal patten to calculate TD incidence and TD score (severity of TD) as per previous studies (Kaukonen *et al.,* 2017; Zhang *et al.,* 2018b, c). TD incidence was calculated by calculating the birds showing the sign of lameness and chickens which have TD-score≥1 in respectively group. Whereas, TD severity was determined in percentage as No. of birds having TD-score≥3 divided by No. of slaughtered birds in the group.

**Immunohistochemistry Assay:** The immunohistochemistry assay on 18-days of tibiotarsal bones samples was done as per previous method (Mehmood *et al.,* 2019a). The histological tissues of 5µm thickness were prepared. After that, the slides were nurtured with the primary-antibodies against Akt, P-AKT, PI3K, VEGF, VEGFR1, and HIF-1alpha at 4°C for 12-hours. Then, it was washed with PBS and incubated with the secondary-antibodies of each protein (1:200) for 2-hours at 25°C temperature.

**Statistical Analysis:** Results of present study were analyzed through SPSS 19.0 and t-test. The graph and images were produced by using GraphPad Prism 7 software. The incidence and severity of TD were given as means ± SD.

## RESULTS

**Production Parameters:** The results presented that feed intake and wight gain was reduced ($P$>0.05) in TD group compared to control-group. While, feed intake and weight gain were decreased in TD-group during the experiment on 7, 10, 14, and 18 days ($P$>0.05). Whereas, feed intake and wight gain was increased in Astr IV group compared with TD-group as given in Figure 1. Administration of Astragaloside IV has positive effect on feed consumption and weight gain in chickens.

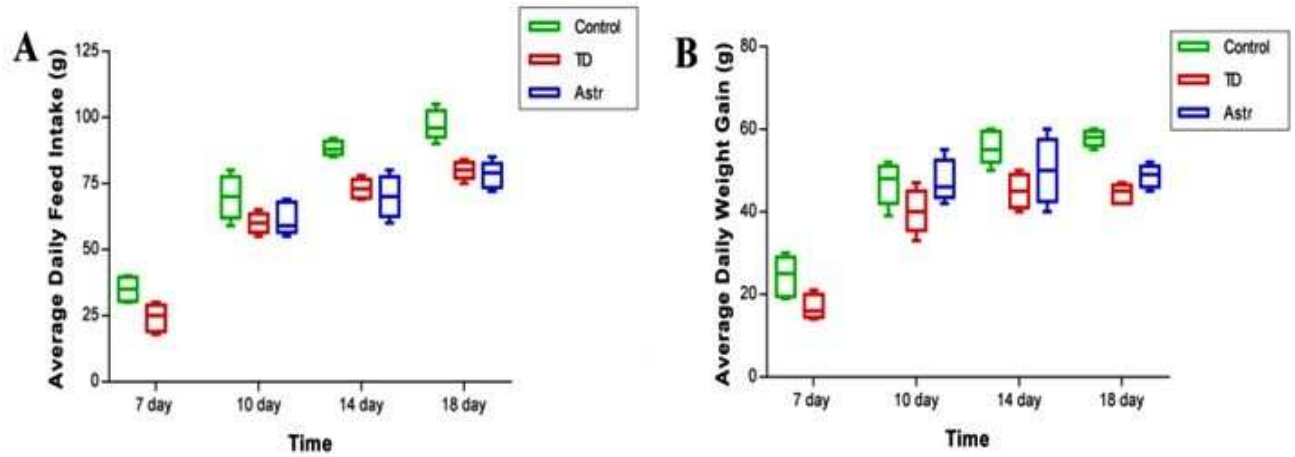


**Figure 1: The effect of Astragaloside IV on (A) feed intake (B) weight gain in TD chickens on various days. The values are mean ± SD.**

**Morphometery of Tibia:** The tibial parameters including length, width, and weight of tibia and width of GP were assessed in all the groups of chickens at various days as given in Figure 2. The length of tibia was increased in control group than TD but the difference was not significant. In Astr IV group, the length of tibia was higher than TD group but again the difference was non-significant. Similarly, the width of tibia was raised in control and Astr IV groups non-significantly compared to TD group on various days. The width of GP was increased significantly ($P$<0.05) in TD-group than control group chickens on various days. After the running of Astragaloside IV, the width of GP was reduced ($P$<0.05) in Astr-group up to normal level. The weight of tibia was more on various period in control group compared to TD-group.

**Incidence and severity of TD:** The result of present study indicated that TD incidence and TD severity was much higher in TD group compared with Astr IV group during the entire period of experiment. The lameness was much more in TD group chickens compared to Astr IV administered chickens. On day 7 and 10 TD incidence and TD severity was more in TD group as compared to Astr IV group but the difference was non-significant ($P$>0.05). Whereas, on day 14 and 18 both TD incidence

and TD score significantly ($P$<0.05) increased in TD group compared to Astr IV group. It indicated that Astr IV has beneficial effect towards TD score, TD severity, TD incidence and lameness reduction.

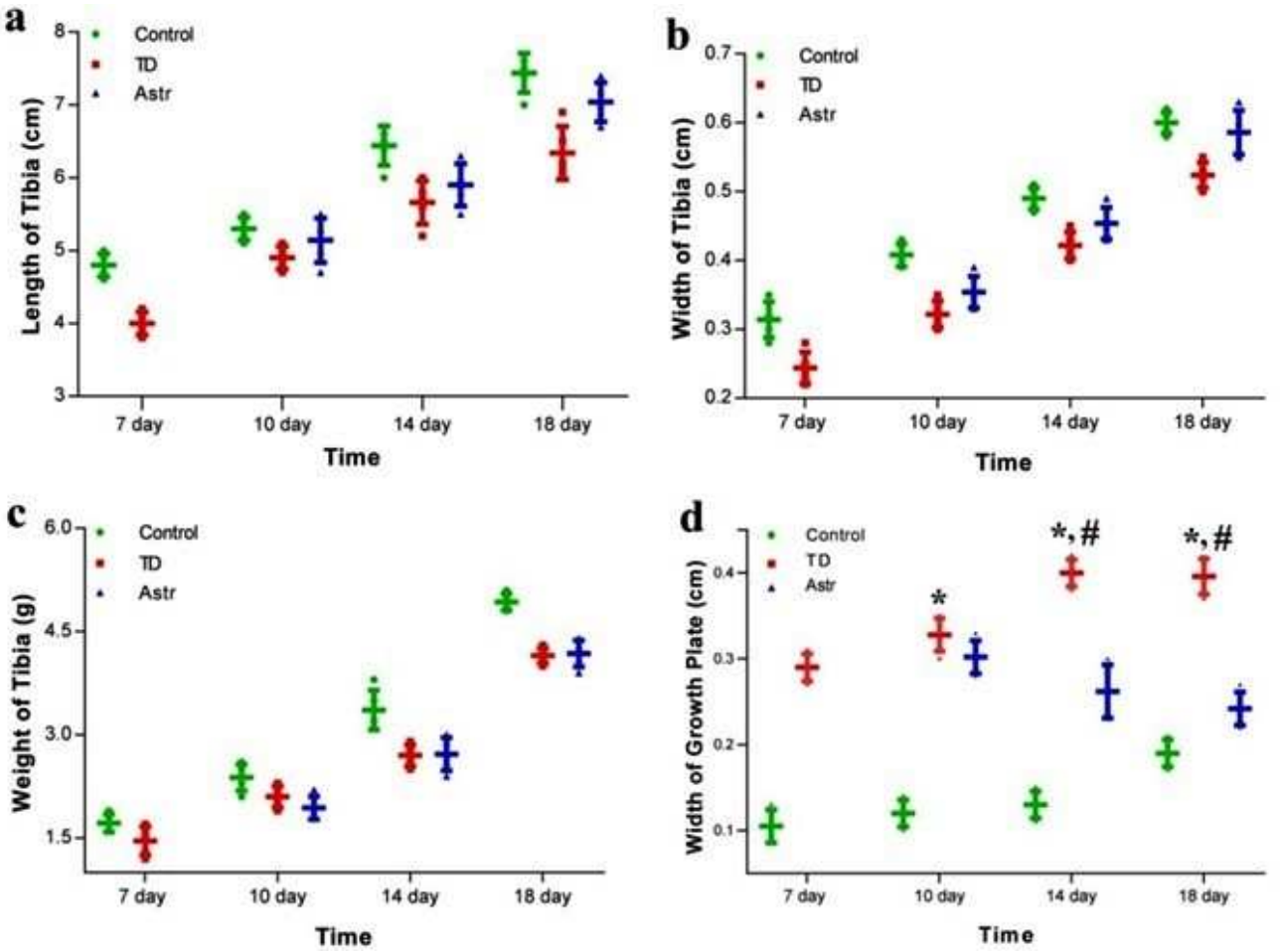


**Figure 2: Effect of Astragaloside IV on morphometric studies of tibia. * Indicates $P$<0.05 significant difference between TD and Control whereas # indicates significant ($P$<0.05) difference between TD and Astr groups.**

**Table 1: TD incidence and severity in chickens (n= 60 in each group).**

| Days | TD group | ASTR IV group |
|---|---|---|
| **TD Incidence (%)** | | |
| 7 | 95.30±1.5 | 94.80±1.5 |
| 10 | 92.39±1.5 | 59.90±1.0 |
| 14* | 89.33±1.0 | 36.35±0.5 |
| 18* | 78.20±0.5 | 19.22±0.5 |
| **TD Severity (%)** | | |
| 7 | 70.25±0.5 | 72.50±1.5 |
| 10 | 61.10±1.5 | 43.33±1.0 |
| 14* | 51.35±1.0 | 16.50±0.5 |
| 18* | 35.40±1.0 | 0.0±0.0 |

*$P$<0.05 significant difference

**Immunohistochemistry analysis:** The results of immunohistochemistry analysis about localization of Akt, P-AKT, PI3K, VEGF, VEGFR1 and HIF-1alpha proteins in the growth plates of tibial bones in chicken on day 18 showed that the localization of AKT and PI3K proteins was less and P-AKT was enhanced in TD-group compared with control. Conversely, Astr IV treatment normalized it by increasing the localization of AKT and PI3K and decreasing the localization of P-AKT protein localization in the cells compared to TD group. The immunohistochemistry analysis of AKT, P-AKT and PI3K proteins in GP of tibial bones in chicken on day 18 showed in Figure 3.

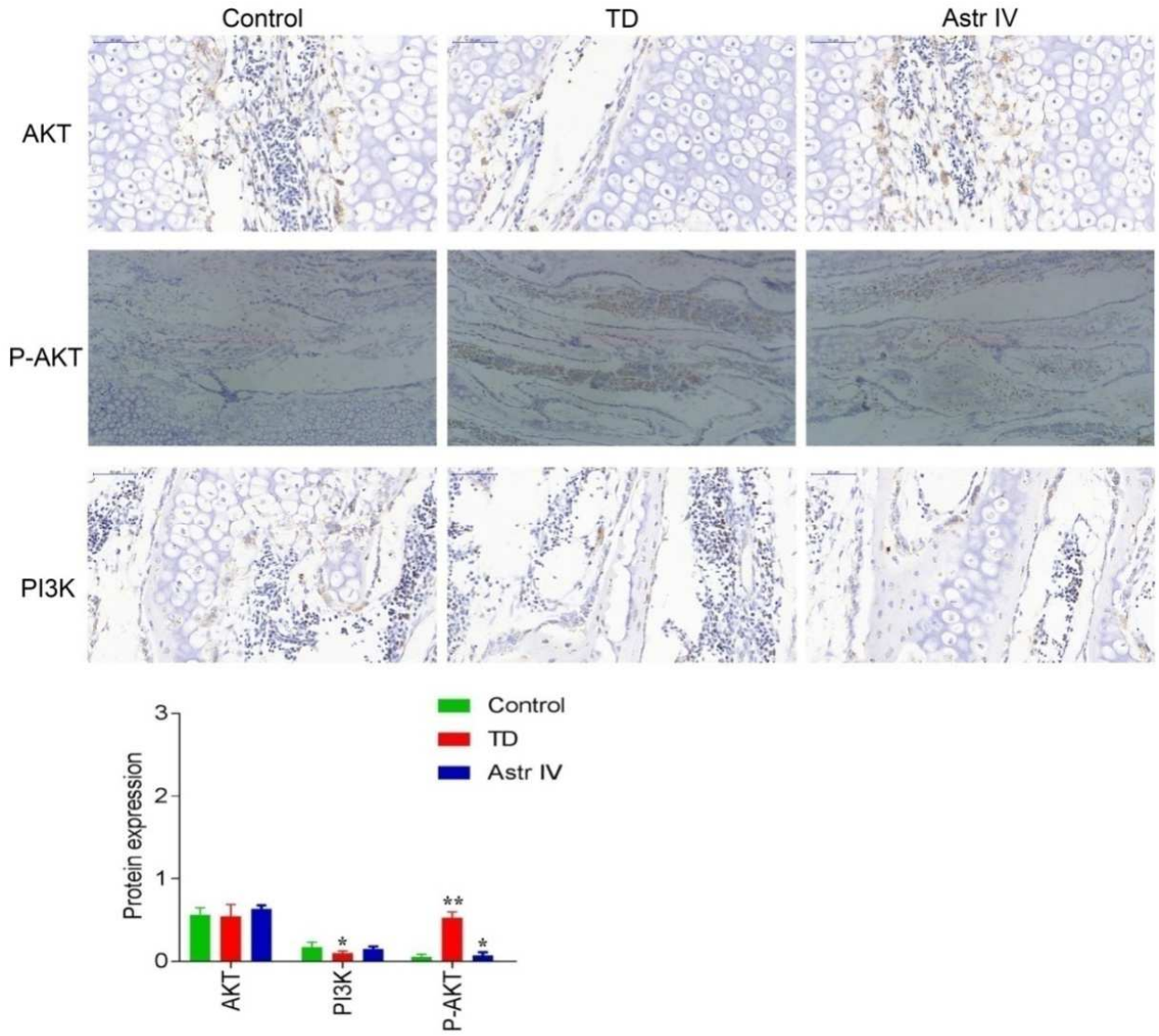


**Figure 3. Immunohistochemistry analysis of Akt, P-AKT and PI3K proteins localization in GP cells of various groups on 18$^{th}$ day. The values are mean±SD. **P*<0.05; ***P*<0.01.**

Similarly, the expression of VEGF and HIF-1alpha proteins was more in TD-group as compared to control. While, the localization of VEGFR1 was less in TD group compared with control chickens. However, localization of VEGF, VEGFR1 and HIF-1alpha proteins were regularized near to control group in Astr IV treated chickens compared with TD-group (Figure 4).

**Astragaloside IV regulates PI3K/Akt/HIF-1α signalings:** Generally, in our experiment the results of immunohistochemistry analysis regarding the proteins expression showed P-AKT, HIF-1alpha and VEGF expression was more in TD group. While the appearance of AKT, PI3K and VEGR1 was less in TD group chickens. But, the localization of AKT, PI3K and VEGR1 proteins was up-regulated, while P-AKT, HIF-1alpha and VEGF was down-regulated by Astragaloside IV treatment in Astr IV group. The results show that Astr IV regulates the expression of various proteins. Most of the proteins belong to PI3K/AKT/HIF-1α signalings. The overall effect of the Astragaloside IV on regulating PI3K/Akt/HIF-1α signaling is shown in Figure 5.

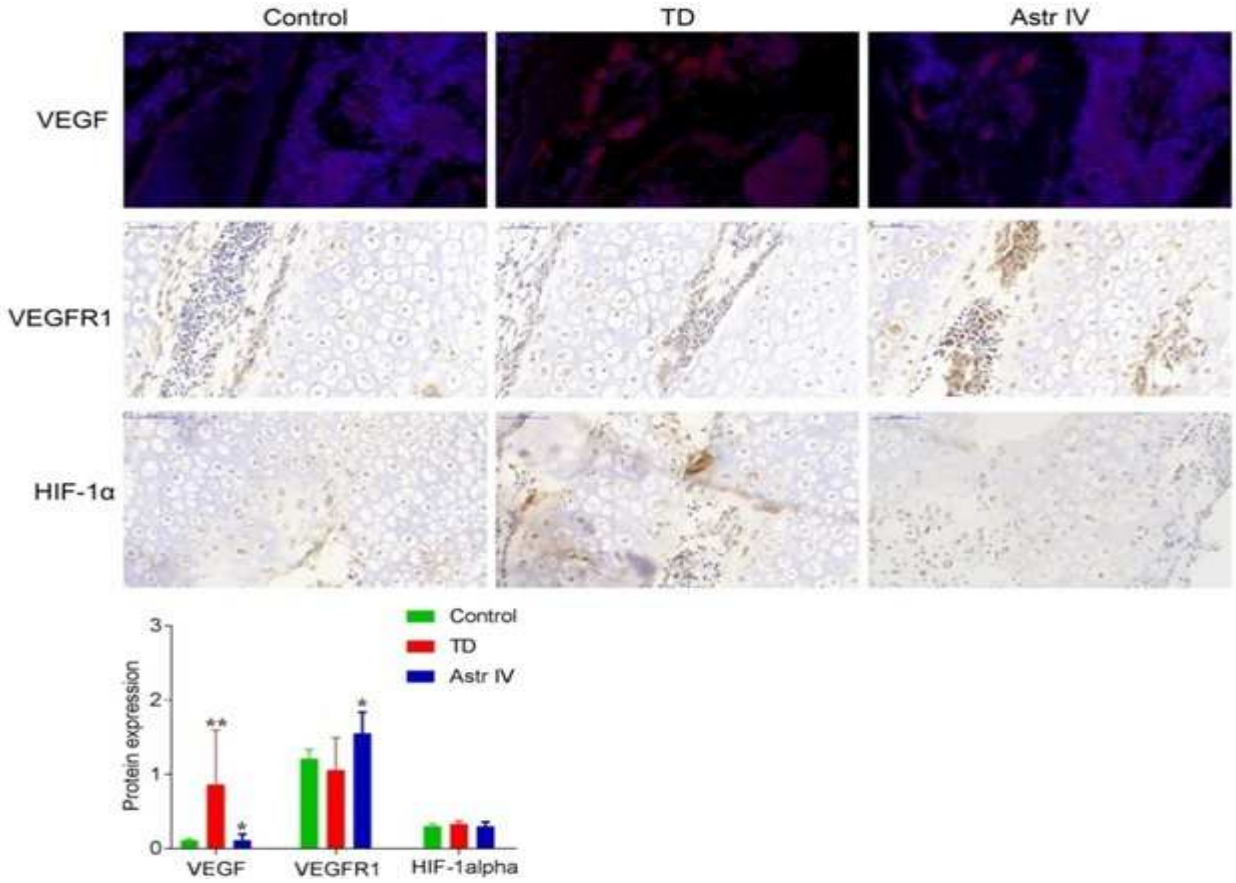


**Figure 4. Immunohistochemistry analysis of VEGF, VEGFR1 and HIF-1alpha proteins localization in growth plate cells of various groups on day 18. The values are mean±SD. *$P$<0.05; **$P$<0.01.**

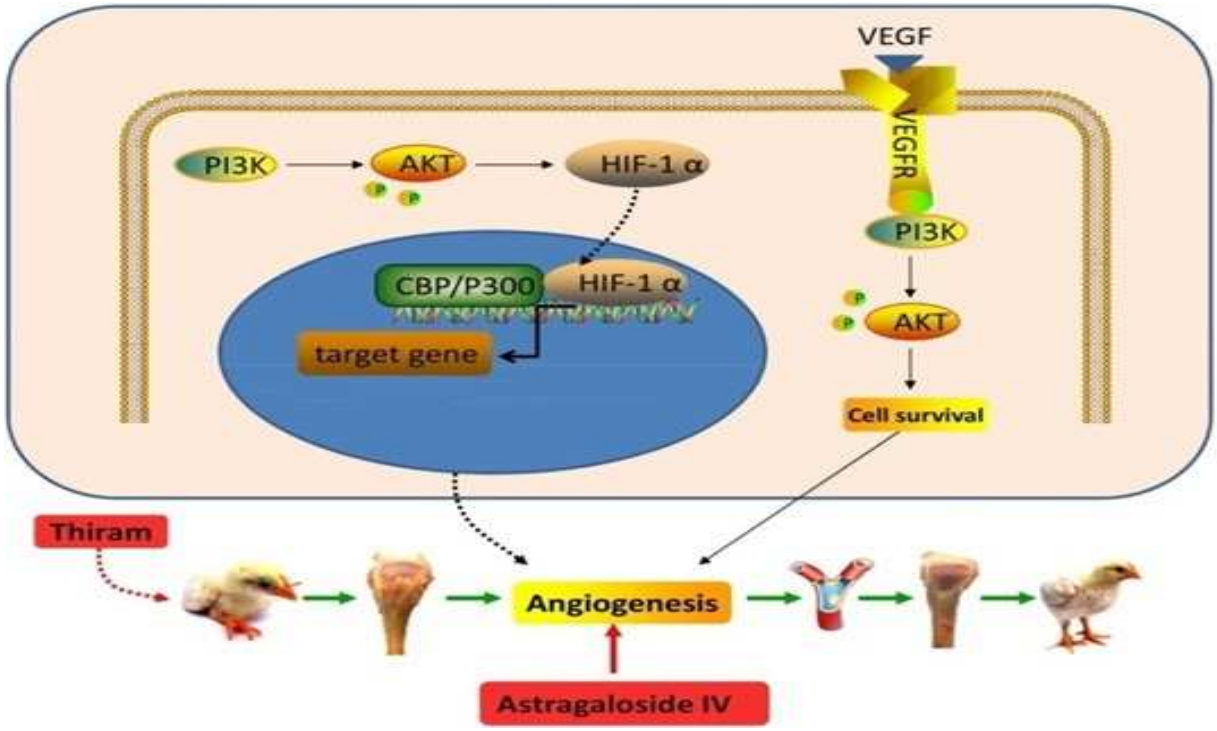


**Figure 5: Effect of the Astragaloside IV in regulating PI3K/AKT/HIF-1α signaling**

## DISCUSSION

The improved feed efficiency in broilers is often related to skeletal problems, leg weakness along with impaired walking ability that can affect the welfare of the birds. Much of the research work in broilers is addressing on methods using nanoparticles to improve the bone parameters in broilers alongside meat quality parameters (Bila and Tyasi, 2022; Khan *et al.,* 2022). TD is as also a leg abnormality of rapidly growing broiler birds, as it frequently occurs in meat type birds with high growth rate which results in bone deformity and lameness (Mehmood *et al.,* 2018b; Zhang *et al.,* 2018a). This skeletal disease of chickens leads to failure in growth plate cartilage to become bone, which results in avascular cartilage and severe lameness in chickens (Nabi *et al.,* 2018b). It is a frequently occurring leg abnormality and reported up to 30% in commercial broiler farms (Zhang *et al.,* 2018b; Qamar *et al.,* 2019b).

Actually, the specific reason and method of tibial dyschondroplasia is not known but many researchers addressed this issue by putting different reasons and logics. Most of them stated that the endochondral bone formation and ossification delay, which results in TD lesions, it prevented the differentiation of chondrocytes and bone formation (Yao *et al.,* 2018; Mehmood *et al.,* 2019a). Furthermore, it has been reported that TD may be related to genetic selection. But, the exact model of inheritance for TD occurrence cannot be proposed till yet (Zhang *et al.,* 2019a; Waqas *et al.,* 2020). The broiler industry is touching to highest peak of economic benefits due to rapid growth of broiler chicks, but on other hand various skeletal diseases like TD incidence is increasing in chicks (Zhang *et al.,* 2019b). This disease causes huge economic losses to broiler industry due to fracture during processing of carcass at slaughter houses.

Hypoxia is very important for the regulation and expression of HIF-1alpha and accumulation of HIF-alpha is mostly happened by inhibiting the degradation of HIF-1α due to hypoxia. Because HIF-1alpha is subunit of HIF-1 and very sensitive to oxygen among other subunits (Masoud and Li, 2015). During tibial dyschondroplasia, the growth plate become hypoxic due to less vascularization compared with normal growth plate. In TD growth plates, HIF-1α up-regulated and belong the severity of TD (Yao *et al.,* 2020). Low oxygen level and hypoxia induce low cellular energy production due to lack of proper vascularization. They are also good regulators for the production of VEGF from hypertrophic chondrocytes. Synthesis of new blood vessels and regulation of hypoxic reactions that is essential for chondrocyte growth are controlled by HIF-1α (Zhang *et al.,* 2018c). In case of mammals, hypoxia is needful for endochondral bone formation, ossification and differentiation of cartilage (Zhang *et al.,* 2020). In hypoxia, HIF-1alpha signaling pathway is necessary for the regulation of heath shock proteins, so these proteins work as cellular chaperons for other proteins (Baird *et al.,* 2006).

In present study, VEGF expression was enhanced in TD-group compared to control. These findings are also previously reported by many researchers (Mehmood *et al.,* 2018a; Zhang *et al.,* 2018c). After Astr IV administration to TD affected chickens, VEGF was down-regulated significantly. These results are congruent with some other research report (Mehmood *et al.,* 2018a), they used various concentrations of Astr IV to reduce the expression of VEGF. Similarly, VEGFR1 expression was also increased in TD group chickens in our experiment. Previous researchers reported that VEGFR1 expression was increased on day 14 after thiram administration (Mehmood *et al.,* 2017). Astr IV treatment reduced VEGFR1 proteins expression in our experiment but again the difference was non-significant. Likewise, Mehmood *et al.* (2019b) reported that Astragaloside IV reduced the expression of proteins VEGFR1 and VEGFR2 receptors of VEGF.

Our results demonstrate an increase in the expression of HIF-1alpa gene and its protein. Further, Astragaloside treatment regulates it by reducing the expression of HIF-alpha. Our results are in line with other studies (Lin *et al.,* 2018a, b), they studied the effect of Astr IV with high and low doses, and their results suggested that HIF-1alpha gene and protein expression was more in model group compared with control but Astr IV treatment significantly lowered the expression of HIF-1alpha. Astr IV increased in P-AKT and decreased in AKT expressions in our experiment in TD affected chickens. The effect of Astr IV on HN4-induced oxidative stress and inflammation on epithelial cells is also assessed in previous reports (Chen *et al.,* 2017; Lin *et al.,* 2018a). They reported that Astragaloside IV has anti-inflammatory capability and furthermore it reduced the over expression of P-AKT in ammonia treated cells in bovine but the difference was non-significant. Astr IV can activate PI3K/AKT signals pathway by increasing the expression of P-AKT. It is studied that Astr IV has protective effect in mice against cardiac hypertrophy by down-regulating the appearance of P-AKT protein in heart tissues (Rao *et al.,* 2017; Liang *et al.,* 2018; Lin *et al.,* 2018a). The different concentrations of Astr IV were effective against gastric ulcer and significantly reduced the expression of P-AKT protein. Whereas, previous researcher reported that Astr IV can improve vascularization through vasodilatation and increase the expression of AKT in dose dependent manner (Lin *et al.,* 2018b). Astr IV increased the expression of AKT and PI3K in anemia model of mice (Lauzon *et al.,* 2016; Lin *et al.,* 2018b). Tibial dyschondroplasia affected growth plate is hypoxic in nature. So, the expression of HIF-

1alpha is increased in TD chickens. Astr IV increased the localization of AKT and PI3K, which can activate PI3K/AKT signals in TD chickens. Its mean expression of HIF-1alpha is regulated by PI3K/AKT signals. Altogether, Astr IV is important for the activation of PI3K/AKT signals, which controls HIF-1alpha in GP of TD chickens. On the basis of our results, it is seen that Astragaloside IV therapy has beneficial effect in thiram induced TD chickens through regulating PI3K/AKT/HIF-1α signalings.

**Conclusions:** Astragaloside IV diminishes TD through regulating the expression of various proteins PI3K, AKT, HIF-1α, VEGF, VEGFR1 and P- AKT in chickens. Our findings suggest that PI3K/AKT/HIF-1α is a potential new target in TD therapy, which provide new insights regarding the therapeutic potential of Astr IV for TD treatment. Furthermore, more studies for various genes and proteins need to be conducted to explore new insights regarding the therapeutic potential of Astr IV.

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