



RESEARCH ARTICLE

Effect of Grape Seed Extract on Tibial Dyschondroplasia Incidence, Liver Weight, and Tibial Angiogenesis in Chickens

Hammad Qamar¹, Aoyun Li¹, Zhibo Zeng¹, Yaping Wang¹, Khalid Mehmood^{1,3}, Muhammad Waqas^{1,4}, Mudassar Iqbal^{1,3}, Jialu Zhang¹ and Jiakui Li^{1,2*}

¹College of Veterinary Medicine, Huazhong Agricultural University, Wuhan 430070, PR China; ²College of Animals Husbandry and Veterinary Medicine, Tibet Agricultural and Animal Husbandry University, Linzhi, Tibet 860000, PR China; ³University College of Veterinary & Animal Sciences, Islamia University of Bahawalpur-63100, Punjab, Pakistan; ⁴Faculty of Veterinary & Animal Sciences, University of the Poonch, Rawalakot, District Poonch 12350, Azad Jammu & Kashmir, Pakistan

*Corresponding author: lijk210@sina.com

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ABSTRACT

Among avian metabolic disorders, Tibial dyschondroplasia (TD) has a wide prevalence worldwide in chickens that are identified by non-mineralization and avascularization of the growth plate (GP). Grape seed extract (GSE) reduces the inflammation and oxidative stress by scavenging free oxygen specie and has some anti-cancer and angiogenic activity. This study was carried out to evaluate the role of GSE for the therapy of the chickens affected with TD. Arbor Acres broiler chicks (n=240) weighing 47 \pm 6g were fed starter feed till first 3 days and then portioned equally into three groups (Control, TD and GSE group; n=80 each). Thiram was incorporated in the feed of TD and GSE chicks until day 6 for inducing TD (50 mg/kg of feed), whereas usual feed was offered to control group. All the birds in TD and GSE group showed the symptoms of TD on day 7 and GSE group was given GSE (50 mg/kg/day) intraperitoneally till the termination of the experiment. Chickens fed with thiram-feed exhibited high TD score and GP measurement. After the GSE treatment, the TD score dropped remarkably and, inflammation and TD lesions reduced. Moreover, body weight gain and liver weight were increased in comparison with TD chicks after application of GSE. GSE supplementation alleviated the adverse effects of thiram and increase in angiogenesis was observed. In conclusion, GSE recovered the TD affected broilers through downregulating the HIF-1 α , Hsp90 and CA2 expressions.

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INTRODUCTION

Tibial dyschondroplasia (TD) is widely prevalent metabolic disorder especially in fast growing birds like turkey and chicken (Nabi *et al.*, 2016a, 2016b). It is characterized by non-mineralization and avascularization of the growth plate (GP) which generally leads to lameness and uncharacteristic growth of chondrocytes (Nabi *et al.*, 2016a). Normally, chondrocytes are involved in bone remodeling, cartilage vascularization and ossification. However, TD lesion is formed as a result of endochondral ossification halt, the proliferation of cartilage cells and development of avascular cartilage mass in the tibia metaphysis region (Tian *et al.*, 2013).

Recently, it is reported that TD accounts for 30% of all bone disorders in poultry with approximately 10% morbidity rate in China leading to substantial reduction in the farmer's profit margin (Zhang *et al.*, 2018). Clinical and subclinical effects of TD include; reduced immunity, osteomyelitis, compromised growth rate and breast cyst formation, that ultimately reduce carcass and meat quality besides raising ethical and welfare issues (Tian *et al.* 2013; Shahzad *et al.*, 2014a; 2015). Thiram induces TD in chicks, affects liver health (Iqbal *et al.*, 2016), checks the angiogenesis and disrupts the normal arrangement of chondrocytes (Zhang *et al.*, 2018). These problems are well documented formerly but fundamental etiology is still not clear (Iqbal *et al.*, 2016). Globally broiler health

and production is negatively affected by TD leading to massive economic loss to the poultry industry. Therefore, research focusing on etiology and pathogenesis of TD is required to address current knowledge gap and subsequent development of preventive and therapeutic strategies for TD.

Proanthocyanidins are naturally occurring compounds found in plant-derived foods like berries and grape seeds. Studies have reported that grape seed extract (GSE) possesses a wealth of proanthocyanidins oligomers, oligomeric polymers and flavonoids polyphenols. In China, GSE has been widely used as health and immunity promoter in humans and animals (Zhen *et al.*, 2014). In a couple of researches, GSE provided protection against cardiac arrest (Demirkaya *et al.*, 2009), mutagenic compounds (Sharma *et al.*, 2010) and nervous breakdown (Ahn *et al.*, 2011). GSE reduces inflammation and oxidative stress by scavenging free oxygen species and also possesses anti-cancer activity (Sayin *et al.*, 2014). GSE has shown more potent antioxidant capability than other natural antioxidants (vitamins A, C and E) to mediate protection of cells from free oxides and hydroxides, fat peroxidation (induced by free radicals), and RNA/DNA damage (Xu *et al.*, 2015). Moreover, GSE improved the immune functions, restored the antioxidant status of the spleen and enhanced the growth rate and weight gain in mice (Long *et al.*, 2016). GSE remarkably improved the antioxidant status and growth rate and decreased the malondialdehyde contents in chickens challenged with aflatoxins (Rajput *et al.*, 2018).

The aim of the current study is to evaluate the GSE effects on incidence of TD, liver weight, tibial angiogenesis, tibia parameters and HIF-1 α , Hsp90 and CA2 expressions in broilers exposed to TD.

MATERIALS AND METHODS

Animal welfare and ethics: All the experimental procedures were submitted to an Animal Welfare and Ethical Committee of Huazhong Agricultural University, China and relevant proclamation and guidelines were followed strictly (Approval code no.: 31273519).

Birds raising plan: Arbor Acres broiler chicks (n=240) weighing 47 \pm 6g were acquired from an established hatchery and controlled environment with 93 °F temperature and 60% humidity was provided. For first 3 days, chicks were fed starter feed and then portioned equally into three groups (n=80 each) (Control, TD and GSE group). Thiram (50 mg/ kg of feed) was added in the feed of TD and GSE group until day 6 for inducing tibial dyschondroplasia (Shahzad *et al.*, 2015; Iqbal *et al.*, 2016), whereas usual feed was offered to control group. All the birds in TD and GSE group showed the symptoms of TD on day 7 and GSE group was given Grape seed extract (50 mg/kg/day) intraperitoneally (Long *et al.*, 2016) till the end of the experiment.

Morphological inspection, tibia parameters and liver weight: Chickens were raised for 18 days and physical inspection and weighing was done on daily basis and dead and lame birds were recorded. Chickens, 20 in numbers, were selected randomly and slaughtered humanely from

each group on days 7, 10, 14 and 18. Tibia bone parameters i.e. bone width, length and weight, tibia GP size and liver weight were noted. The calculation of the TD score was done according to the method explained by Pines *et al.* (2007). Some of the bones were stored in 4% paraformaldehyde for hematoxylin and eosin staining and some at -80°C for future use for RT-qPCR and western blotting.

Hematoxylin & Eosin (H&E) staining and Immunohistochemistry (IHC): The individual tibiotarsal bones (n=12) were preserved simultaneously in phosphate buffered saline (PBS) and 4% paraformaldehyde at 4°C. Decalcification was done with 10% ethylenediamine tetra acetic acid, then dehydrated in ethanol and washed out in xylene, and covered with paraffin wax. Four μ m thick incisions were made to prepare the slides. These paraffin embedded segments were dewaxed in xylene and stained with H&E stain. The light microscope was used to observe the pathological changes in the tibial bones.

For IHC analysis, the PBS and peroxidase solution were used for the washing of slides and then primary antibody treatment was done at 4°C overnight. The PBS was used again for washing and secondary antibody treatment was done in the dark room for 2h at 25°C.

Real-time quantitative PCR (RT-qPCR): The RNA extraction from tibial GP (n=12) was done by using Trizol extraction method and then cDNA was constructed using a commercial kit for RT-qPCR analysis according to manufacturer specifications. Gene specific primers were used. For HIF-1 α F: TGAGAGAAATGCTTACACACAG R: TGATGGGTGAGGAATTGGTTTAC (Mehmood *et al.*, 2018), for Hsp90 F: CCTCCTCCATACGTGATGTG TCA R: GCCTGGGCATTGATGAAGATG (Mehmood *et al.*, 2017; Nabi *et al.*, 2018) and for GAPDH: CCTTCAT TGACCTTCACTACATGGTCTA R: TGGAAGATGGT GATGGCCTTTCCATTG (Mehmood *et al.*, 2018) primers were used. Reference gene used during the PCR was GAPDH.

Western blot analysis: The tibial bone GP (n=12) were used for the analysis of western blot. The western blot analysis was done according to the method explained by Mehmood *et al.* (2018). In short GP samples homogenization was done in PBS and subjected to 4°C cold environment for 2h. Then centrifugation was done at 14000 rpm for 10 min and SDS-PAGE was performed on 10% polyacrylamide gel to get the equivalent quantity of proteins. The incubation of membranes was done for 1.5h in 5% skimmed milk and subjected to the primary antibody at 4°C for overnight. Saline having 0.1% Tween 2.0 was used to wash the membranes for 5 min and then subjected to secondary antibody (anti-mouse goat) for 30 min at normal temperature. The washing of membranes with saline was done again and images were taken using a standard imaging device.

Statistical analysis: Two-way ANOVA was performed to evaluate the data obtained through this experiment using SPSS software (SPSS Version 19.0) and student t-test was done to compare the means. The whole data was presented as means \pm standard deviation (SD) and significant results were considered if P<0.05.

RESULTS

Weight gain and mortality: Average weight gain and mortality was recorded throughout the experiment. Results revealed that thiram affected the growth rate and weight gain of the chicken (Fig. 1A). The survival percentage graph has been shown in Fig. 1B. The stress of the TD reduced after day 10 however weight gain remained low as compared to GSE and Control chicks. Provision of GSE to chicken improved the growth rate and weight gain as compared to the TD group. After providing thiram containing feed, mortality was more in chicken of GSE and TD group. However, on day 8 and afterward, after the provision of normal feed, the mortality rate was declined in TD chicks but it was higher as compared to other groups. GSE administration reduced the TD stress in GSE chicken and mortality was dropped remarkably (Fig. 1C).

Effect of GSE on TD incidence and liver weight: Almost 90% of chicks were affected from TD after the provision of thiram in the feed from day 4 to 7 and lameness was observed clearly. Highest TD score was seen in TD chickens as compared to the other groups however relative TD score was less on day 18. Phenotypic evaluation of tibia of healthy chickens showed normal condition and no signs of lameness were observed. After the administration of GSE, TD score was declined, while diminishing lameness and decreasing TD incidence remarkably (Fig. 2B). Liver weight was adversely affected by thiram administration. The thiram decreased the liver weight on day 7 and 10 in the GSE and TD group. However, provision of GSE reduced TD stress and improved liver weight significantly on day 14 and 18 as compared to TD chicks (Fig. 2A).

The general tibial parameters examination: The tibia bone parameters i.e. bone length, weight and width and GP width was measured during the whole experiment.

Tibia length (Fig. 3B), weight (Fig. 3D) and width (Fig. 3C) were tended to be less on all days in TD chicken as compared to GSE and Control chicken, but results were not significant. However, GP measurements revealed that TD chicks had an enlarged size of the GP on day 7, 10 and 14 as compared to Control group. Application of GSE reduced the size of GP, and non-significant results were observed on day 18 when compared with healthy chicks and GSE chicks retained the capability to gait and move properly. Whereas TD chicks also showed some recovery on day 18 but GP of TD chicks was more adversely affected.

Clinical evaluation of the TD and GSE chickens: The symptoms of TD were seen on day 4 in chickens fed thiram containing feed and the complete onset of TD was noticed on day 7 and almost 90% of the chickens offered thiram feed showed lameness, weakness, lazy body gestures and difficulty in feeding and drinking. However, after treatment with GSE, chicken become healthy and lameness diminished and normal feeding and drinking habit was restored. A white opaque distended GP having avascular cartilage was observed in TD affected chicks however GSE supplementation restored the GP size and physical characters were normal (Fig. 4).

Histopathological investigation of the growth plates of the tibia: Histological investigation showed that the Control group exhibited well-established network of blood vessels and well-arranged columns. TD chicks exhibited mass of dead and degraded cells, and abnormal arrangement of bone cells. GSE supplementation alleviated the adverse effects of thiram and enhanced angiogenesis which restored the chondrocytes arrangement and network of blood vessels (Fig. 5A). On day 7 the length of trabecular bone was reduced in TD chicks as compared to healthy chicken. After GSE application, trabecular bone length and blood vessel numbers were restored and improved remarkably as compared to TD affected birds (Fig. 5B).

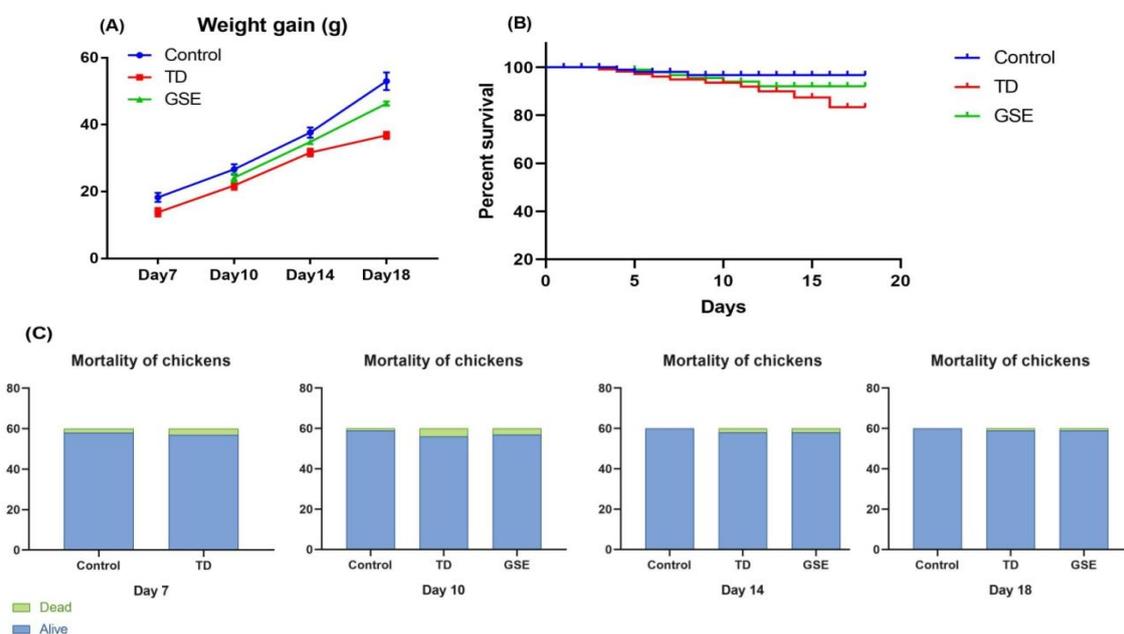


Fig. 1: Weight gain, percent survival and mortality. Average weight gain of the chicken (A), survival percentage (B) and number of dead chickens (C). Thiram provision reduced the weight gain and survival percentage and increased the mortality of chickens as compared to control group however Grape Seed Extract (GSE) administration improved weight gain and survival percentage and decreased mortality as compared to TD group. The data are presented as mean±SD. *P<0.05.

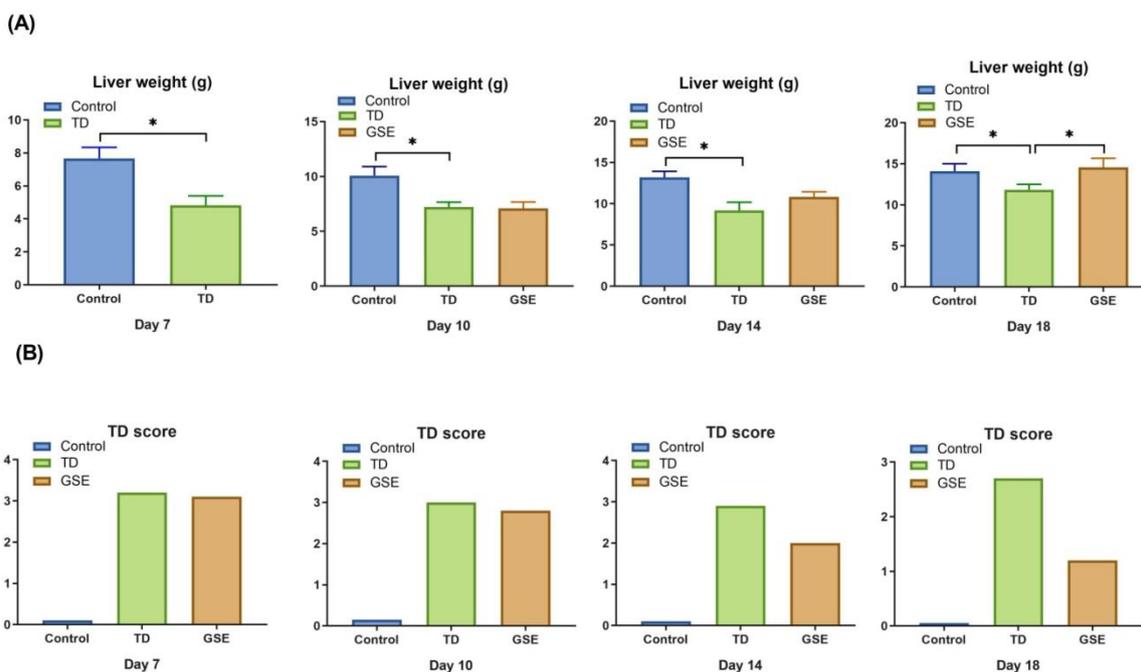


Fig. 2: Liver weight and TD score. Average liver weight (A) and average TD score (B). GSE supplementation increased the liver weight and decreased the TD score as compared to the TD group. The data are presented as mean \pm SD. * $P < 0.05$.

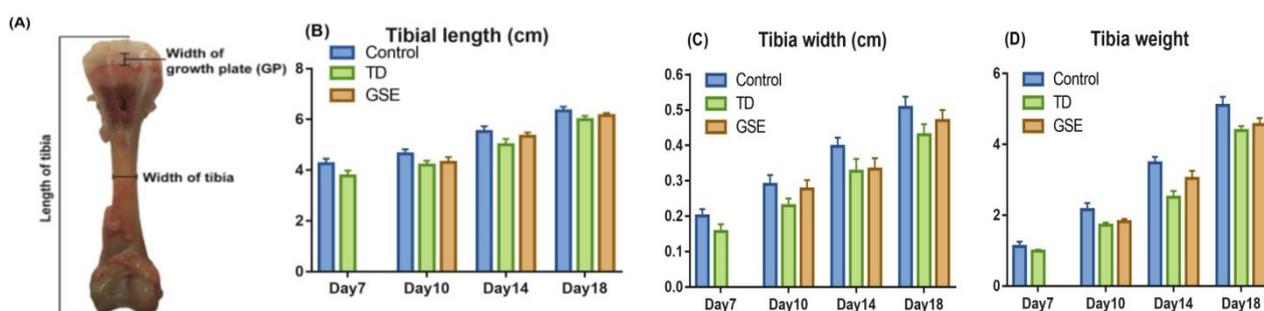


Fig. 3: Tibia Parameters. Tibia of the chicken (A), Tibia length (B), Tibia width (C) and Tibia weight. Non-significant results were obtained among three groups. Tibia length, width and weight were not affected by thiram provision. The data are presented as mean \pm SD. * $P < 0.05$. ** $P < 0.01$.

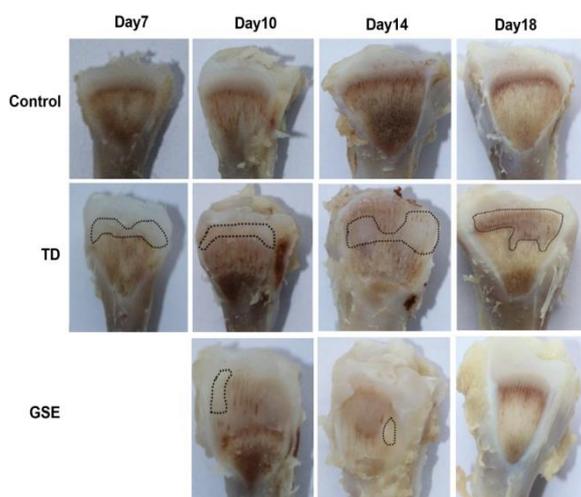


Fig. 4: Growth plate of proximal tibia at various days. Control group had normal growth plate whereas Tibia Dyschondroplasia (TD) chickens had extended growth plate. The dotted area represents the TD lesion. Grape Seed Extract supplementation decreased TD lesion remarkably.

Immunohistochemistry of Tibial growth plates: The immunohistochemistry was done to check the antibody expression of HIF-1 α , Hsp90 and CA2 in tibia GP

(Fig. 6A). Thiram group showed more cells in terms of HIF-1 α , Hsp90 and CA2 expression and localization as compared to healthy chicks. Supplementation of GSE reversed the adverse effects of TD by decreasing relative expression and localization of HIF-1 α , Hsp90 and CA2 as compared to TD affected chicks (Fig. 6B).

The HIF-1 α , Hsp90 and CA2 protein expression in growth plate: HIF-1 α gene expression was significantly high in TD chickens as compared to the control group on day 7 as shown in Fig. 7A. Similar trend was observed on other days. Administration of GSE tended to decrease the expression of target genes as compared to TD group on 10, 14 and 18 days but results were non-significant. On day 18 GSE administrations reduced the gene expression in TD affected chickens to the control level. Similar results were obtained in case of protein expression (Fig. 7A).

As compared to healthy chicken the TD chicken showed an almost two-fold increase ($P < 0.01$) in Hsp90 gene expression on day 7. The same kind of expression tendency was noticed on days 10, 14 and 18 between TD and normal chicken. However, TD and GSE group showed the same expression level on day 10. Though, after GSE administration a significant reduction in gene expression was seen in chicks treated with GSE as

compared to TD chicks on day 14 and 18. On day 14 Hsp90 gene was highly expressed in the GSE group as compared to the control group but it declined later on reaching to the control level on day 18. Similar results were obtained in case of protein expression (Fig. 7B).

CA2 protein expression was determined to evaluate the effect of GSE on CA2 protein expression. Interesting results were obtained which are in line with our previous research results. TD chicks had a high protein expression of CA2 than normal chicken whereas GSE reduced the protein expression of CA2 and facilitated recovery in TD chicks.

DISCUSSION

Tibial Dyschondroplasia (TD) is a severe bone metabolic disorder that adversely affects the proximal growth plate

(GP) of the tibia and usually occurs in fast growing chicks (Nabi *et al.*, 2016a, 2016b). Present study revealed that chicks fed with thiram-feed exhibited higher TD score and GP measurement with tibial inflammation and inability to walk properly as compared to healthy chicks which leads to reduced growth rate, weight gain and liver weight and increased mortality as reported formerly (Zhang *et al.*, 2019). Treatment with GSE remarkably reduced the TD score and inflammation along with diminishing lesions from the GP region. The chicks recovered quickly and started to locomote properly leading to restoration of normal frequency of feeding and drinking due to which body weight gain and liver weight was increased and mortality decreased. Similar findings have been reported earlier (Iqbal *et al.*, 2018a; Zhang *et al.*, 2019).

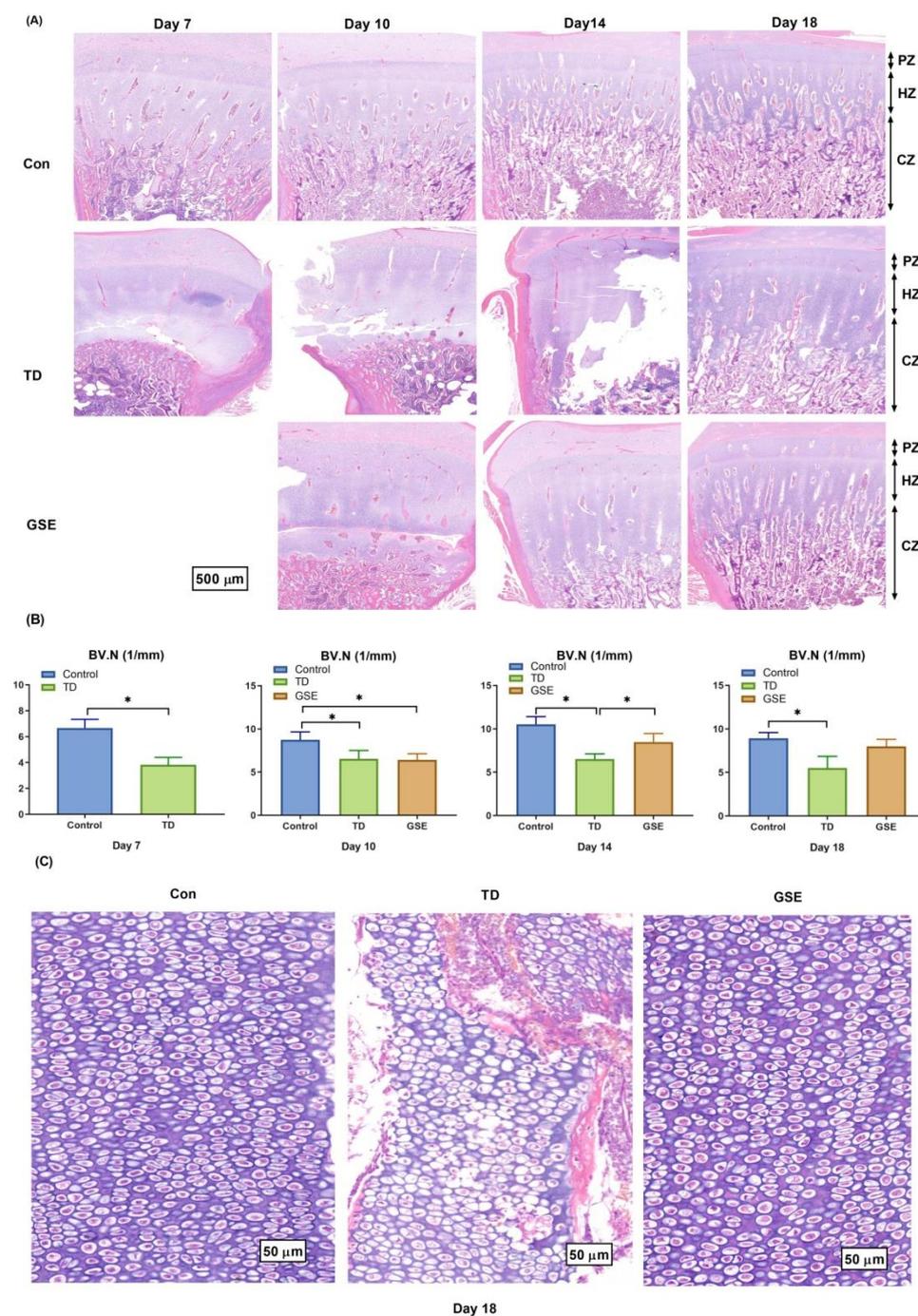


Fig. 5: Histopathological investigation of growth plates of tibia (A). Hypertrophic zone of growth plate magnified at 50um on day 18 (C). Control group had normal arrangement of cells surrounded by blood vessels whereas in TD group cells without nucleus and abnormal arrangement of cells with reduced number of blood vessels can be seen. However, GSE supplementation restored angiogenesis and the normal status of bone cells in hypertrophic zone (HZ) and proliferative zone (PZ). (CZ) is calcified zone. Measurement of blood version numbers analysis (BV.N) in various groups (B). The BV.N was evaluated with Image-Pro Plus 6.0. *P<0.01.

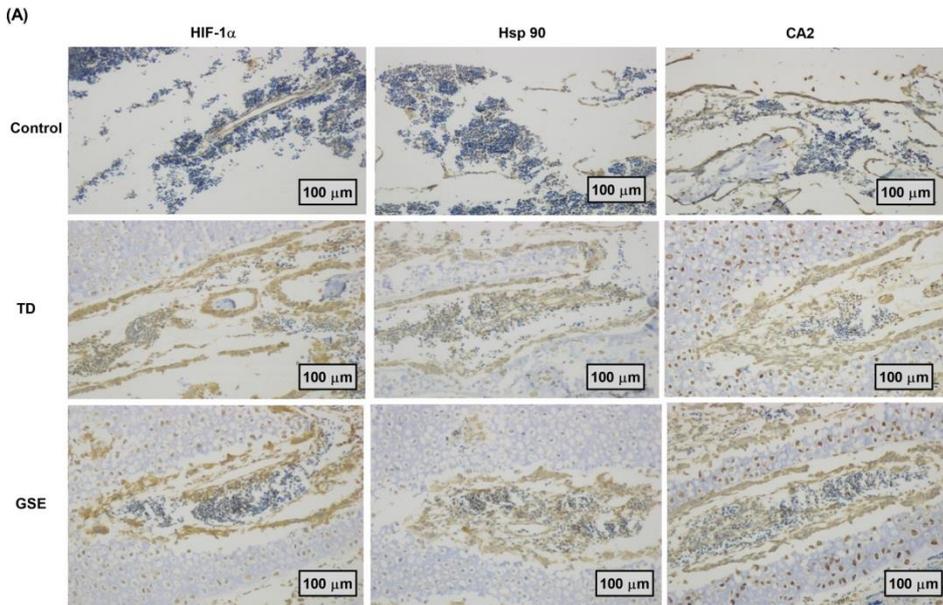


Fig. 6: Immunohistochemistry (IHC) of HIF-1 α , Hsp90 and CA2 in growth plate (A). In TD group HIF-1 α , Hsp90 and CA2 expressions were increased as compared to Control group while Grape Seed Extract supplementation reverse the results and HIF-1 α and Hsp 90 expression and CA2 localization was decreased. HIF-1 α , Hsp90 and CA2 relative expression during IHC (B). The results were obtained with Image-Pro Plus 6.0. *P<0.01.

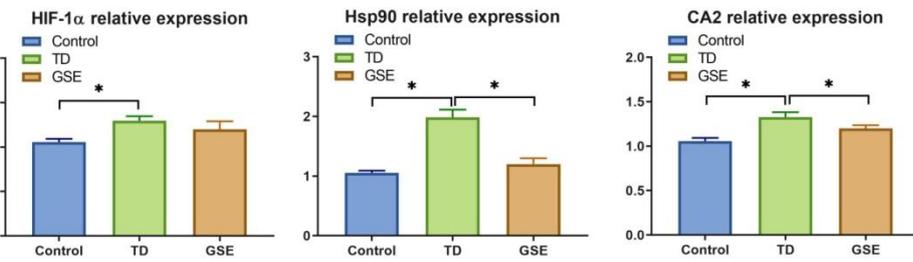


Fig. 7: Fold change in HIF-1 α (A), Hsp90 (B) and CA2 (C) expressions. The HIF-1 α , Hsp90 and CA2 expressions were increased in TD group as compared to control group but Grape Seed Extract administration decreased the HIF-1 α , Hsp90 and CA2 expressions as compared to TD group. The data are presented at various days of trial among three separate groups. The data are presented in mean \pm SD. *P<0.05, **P<0.01.

It was observed that in TD, proliferative and hypertrophic regions of GP had more dead cells, no nucleus, ruptured cell membrane, irregular arrangement of cells' columns and high rate of degradation of chondrocytes (Shahzad *et al.*, 2014a; Nabi *et al.*, 2016b; Mehmood *et al.*, 2019). Failure in calcification and vascularization, cartilage cells hypertrophy, and nuclear degradation led to the unfilled and opaque cartilage due to which interstitial cells increased leading to terminate the process of bone formation (Pines *et al.*, 2007). We also found the similar results and histological investigation showed that Control group exhibited well-established network of blood vessels and well-arranged columns. GSE supplementation alleviated the adverse effects of thiram and increased angiogenesis which restored chondrocytes arrangement, blood vessel numbers and network of blood vessels. Chicks with TD exhibited mass of dead and degraded cells, opaque cartilage and disorganized arrangement of bone cells. This is due to the fact that avascularization in tibia, non-calcification and increased extracellular matrix lead to the decline in angiogenesis that disturbs the nutrient transportation to the developing chondrocytes (Nabi *et al.*, 2016b). This subsequently leads to cell death and failure in completing the mineralization and calcification process by osteoblasts (Nabi *et al.*, 2016a).

HIF-1 α and HIF-1 β are the two transcription factors which are part of the heterodimeric transcription factor called HIF-1. Among these two the first one is more regulated. Whereas HIF-1 α plays a key role in the activation of VEGF genes via binding to the active site of VEGF specific region (Lu *et al.*, 2009). More prominently, in various kinds of diseases like cancer HIF-1 α is overexpressed and leads to the death of the organism. Our results showed that HIF-1 α was upregulated in TD chickens which is in agreement with the earlier reports regarding the upregulation of HIF-1 α in TD affected birds (Mehmood *et al.*, 2018). After administration of GSE HIF-1 α was downregulated in our study which has also been reported in earlier studies involving the application of GSE in diseased mice (Lu *et al.*, 2009).

Heat shock proteins (Hsp90) are well known to be greatly expressed in avian TD and cancerous cells (Nabi *et al.*, 2016a). The process of oncogenesis, angiogenesis, and metastasis are controlled by Hsp90 (Nabi *et al.*, 2016b). HIF-1 α and its receptors are considered to be pro-angiogenic substances and are mainly Hsp90 client proteins. Hsp90 have important role in angiogenesis and severely affected the TD birds due to hypoxic lesion in growth plate (Iqbal *et al.*, 2018b). Furthermore, lameness was noticed in TD chicks mainly due to the formation of avascular cartilage in proximal tibia (Nabi *et al.*, 2016b; Nabi *et al.*, 2018). A decline in the expression of Hsp90 was found in our trial after the use of GSE in TD chicken. GSE administration suppressed the Hsp90 expression and helped the chicks to get rid of TD lesions.

Carbonic anhydrase 2 (CA2) plays an important and pivotal role in various pathological pathways like bone resorption process in osteoblasts is initiated by CA2 (Borthwick *et al.*, 2003). Higher mRNA expression of CA2 in TD chicks has been reported by Qamar *et al.* (2019). Similar findings have been observed in our

experiment regarding protein expressions of CA2. This might be due to weakening of bones because of less deposition of minerals in TD chicks and CA2 initiated bone resorption to boost up the calcification process in cartilages (Qamar *et al.*, 2019).

Conclusions: From the above experimental trial, we concluded that GSE has effectively improved the tibial angiogenesis and reduce TD incidence in broilers. The current report confirmed the treatment mechanism of GSE in TD affected broilers through downregulating the HIF-1 α and Hsp90 gene and protein and CA2 protein expressions. GSE can be used as an effective plant-based drug for the control and therapy of TD.

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Authors contribution: JL and HQ devised and designed the research idea. HQ wrote the manuscript. KM revised and review the manuscript. AL, MW, MI, JZ, ZZ and YW contributed to the reagents and materials.

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