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RESEARCH ARTICLE

Recovery of Chickens Affected with Tibial Dyschondroplasia by Application of Grape Seed Extract through Downregulating CA2 Gene and Enhancing Liver Functions

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A B S T R A C T

Globally Tibial Dyschondroplasia (TD) is a serious metabolic syndrome in broilers that is identified by the accretion of the growth plate (GP) into the tibialmetaphyseal portion. Grape seed extract (GSE) is a well-known significant antioxidant since it possesses multifaceted anti-inflammatory, anti-cancer and reducing reactive oxygen ability. The experiment was intended to evaluate the proficiency of GSE to recover the chicks affected with TD. For this purpose, 210 chicks were bought and fed normal starter feed for the first 3 days. After that two groups were made having 70 (Control group; normal feed) and 140 chickens (Thiram group; normal feed+ thiram @ 50 mg/kg). Further two equal divisions were made in Thiram group on 7th day viz TD group and GSE group (@ 50 mg/kg/day). GSE significantly ($P<0.05$) reverted the blood serum profile and liver antioxidants metabolites and, reduced the histopathological abrasion of the liver as compared to the TD group. Furthermore, GSE chicks exhibited a significant decrease ($P<0.05$) in CA2 gene expression and less mortality, TD score and GP size. In inference, GSE is a natural plant-based extract which owns solid scavenging and bone healing property, and strong antioxidant capacity. GSE provided recovery to chicks affected with TD chicks via dropping TD score, whereas stabilized the GP width through the CA2 mRNA downregulation.

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INTRODUCTION

Broiler chickens are challenged with various leg problems however, Tibial Dyschondroplasia (TD) is a serious metabolic disease affecting broiler production globally. In this disease, white cartilage is accumulated in the tibial metaphyseal portion, which causes the non-mineralization and avascularization in the tibial growth plate (GP) of the chicken (Tian *et al*., 2013; Mehmood *et al*., 2018a). Normally, chondrocytes in the GP have a key role in cartilage vascularization, minerals deposition and bone formation but in TD there is abnormal development of chondrocytes (Shahzad *et al*., 2015).

Numerous reasons involved in the induction of TD in chickens e.g. daily management, commercial variety, feed nutrient content, genetic growth rate and food toxins (Rekaya *et al*., 2013). Leg infections, bone fractures and lameness are the secondary infections that are also noticed in TD and it leads to severe financial loss and disturbs animal health and welfare. The onset of TD disease mainly involved the pattern of osteoblasts and osteoclasts differentiation and their growth (Ruff *et al*., 2013). Thiram has been regarded as a susceptible element to bring TD in broilers (Zhang *et al*., 2019) and the signs are analogous to the chickens affected with TD in a natural way (Rath *et al*., 2007). Recently scientists confirmed the effectiveness of Thiram as a strong TD inducing agent along with the reduction in production parameters and liver index (Iqbal *et al*., 2016), and reduction in angiogenesis in tibia GP and destruction of the normal

columns of chondrocytes (Zhang *et al*., 2018), however the ultimate pathway involved in TD induction in chickens remains unclear.

Proanthocyanidins are the natural compounds found in plant-based foods (like grape seeds). Grape seed proanthocyanidin extract (GSE) is extracted from grape seeds which contained favorable amounts of polymerized oligomers, polyphenolic flavonoids and oligomeric proanthocyanidins. Chinese used traditional medicines and GSE for the betterment of their fitness and animal health (Zhen *et al*., 2014). GSE played an important role in promoting anti-mutagenic (Sharma and Katiyar 2010), myocardial protecting (Demirkaya *et al*., 2009) and neurons protecting (Ahn *et al*., 2011) ability in various experimental trials. It is a well-known significant antioxidant since it possesses versatile anti-inflammatory, anti-cancer and absorbing reactive oxygen ability (Sayin *et al*., 2014). As compared to vitamin A, C and E GSE showed significantly increased protection against DNA damage, free radicals and lipid peroxidation (Xu *et al*., 2015). Moreover, GSE improved the immunity, body weight gain and spleen oxidative status in mice (Long *et al*., 2016). It could also help in the prevention of liver and kidney damage induced by the toxic effects of drugs (Engelbrecht *et al*., 2007). Broilers challenged with aflatoxicosis showed significantly increased activities of serum antioxidants and decreased contents of malondialdehyde (MDA) after the administration of GSE (Rajput *et al*., 2018). However, until now no one has reported the beneficial effects of GSE against TD.

So, this experimental trial was carried out to evaluate the effects of GSE on production parameters, TD score, serum profile and liver functions in TD affected broilers.

MATERIALS AND METHODS

Ethical approval: At Huazhong Agricultural University, Wuhan, China, the Ethics Committee of Research and Animal Welfare Department are responsible for approval of ethics and experimental design. So, Approval was taken from this committee (approval code no. 31273519).

Experimental design: Day old chicks (Arbor Acres, n=210), having 47±5g weight, were bought from a commercial hatchery at Chengdu and reared for 18 days. The standardized environment with proper ventilation i.e. 60% humidity level and 93 °F temperature was provided. For the first 3 days, all the chicks had free access to fresh water and starter feed. After that two groups were made having 70 and 140 chickens in Control and Thiram group respectively. Control group received the normal diet whereas thiram (@ 50 mg/kg/day) was given in the Thiram group to induce TD (Iqbal *et al*., 2016; Mehmood *et al*., 2018a, 2018b). Further two equal divisions (n=70 each) were made in Thiram group on 7th day viz TD group and GSE group. TD group received the normal diet without the addition of thiram whereas grape seed extract was given @ 50 mg/kg daily via the intraperitoneal route to GSE group from day 7 till the end of the experiment (Wen *et al*., 2008; Long *et al*., 2016).

Analysis of production performance: Production performance i.e. body weight, feed consumption, FCR (feed conversion ratio) along with the no. of dead birds and lame birds were noticed. The cervical dislocation was done to slaughter the fifteen (n=15 from each treatment) randomly selected chicks on day 7, 10, 14 and 18. After slaughtering the GP width of the tibia was recorded and few bones were saved at −80°C for the polymerase chain reaction (RT-qPCR) analysis in the future.

Analysis of serum biochemicals: Collection of blood was done from all treatments (n=15 each) on day 7, 10, 14 and 18 by cardiac puncture. Centrifugation (3000×g for 20min) was done to obtain the blood serum from blood samples and stored at -20°C till the analyses of AST, ALT and ALP concentrations (stated in U/L; unit per litre).

Analysis of liver antioxidants and hematoxylin & Eosin (H&E) staining: On day 7, 10, 14 and 18 liver samples were collected from all treatments and were kept under freezing temperature (-70°C) for future use for the analyses of T-AOC, GSH-Px and SOD (shown in U/mg; Unit per milligram of protein) contents, and MDA proportion (shown in nmoles/g; nanomoles/gram). For histopathological inspection, liver tissues were fixed by using neutral buffered formalin (10%), then rooted and cleansed with paraffin and xylene correspondingly, small parts of 5 µm were cut down and H&E staining was done (Rajput *et al*., 2018).

Extraction of RNA and RT-qPCR analysis: Tibial GP (n=10) from all groups were used to extract the RNA by Trizol chemical (Invitrogen, United States). Then cDNA was translated from RNA by a commercially available kit. Following primers were made and used during the RT-qPCR. For CA2 R: 5′-CATTCATGCAGTGGTGGAGT AGTCA-3′ F: 5′-CCATTCAAACCAAGGGGAAAC AT-3′ and for GAPDH R: 5′-TGGAAGATGGTGATGGCCTTT CCATTG-5′ F: 5′-CCTTCATTGACCTTCACTACATG GTCTA-3′ primers were used. GAPDH was regarded as a housekeeping gene. All the samples were run in quadruple by means of Applied Biosystems PCR machine.

Statistical analysis: All Data were analyzed using two-way ANOVA design in SPSS 19.0 software. Significant results between two means were sorted out using student t-test. Whole data are shown as mean values ±SD (standard deviation) and a significant difference was declared if values having $P<0.05$.

RESULTS

Growth performance and mortality: The feed consumption (Fig. 1B) and body weight (Fig. 1A) were improved after the administration of GSE but when compared with the TD and Control group, there was no significant difference. Feed conversion ratio (FCR) was decreased significantly ($P<0.05$) in the GSE group when compared with the TD group on the 18th day (Fig 1C). Overall death ratio (Fig. 1D) was increased significantly ($P<0.05$) in broilers affected with TD in comparison with other groups. However, GSE administration reduced the stress in birds and mortality frequency was decreased significantly ($P<0.05$) when compared with TD chicks.

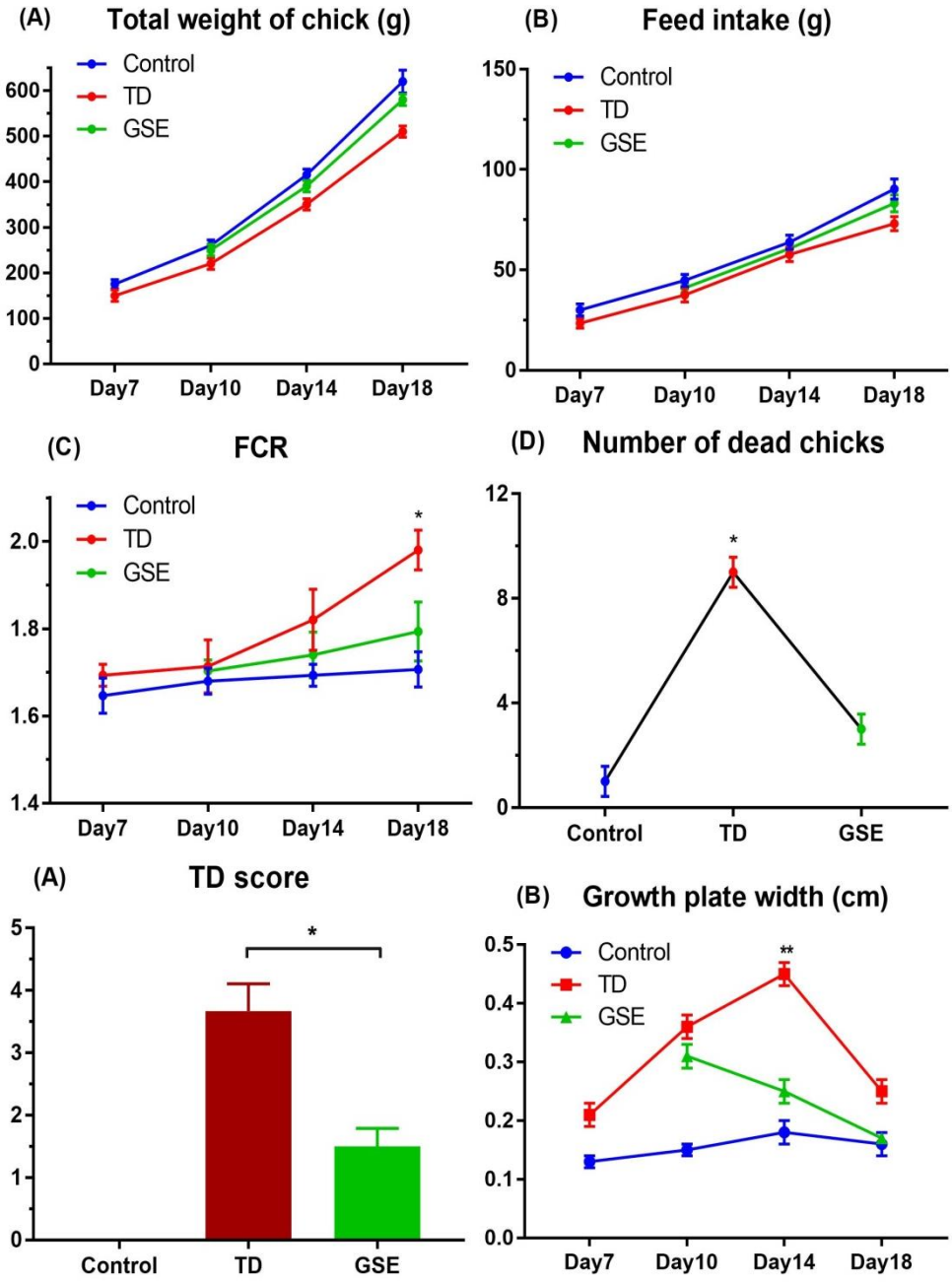

Fig. 1: Production performance evaluation. Mean chicks weight (A); Feed consumption (B); Feed conversion efficacy (C); Mortality of chicks between all treatments (D). GSE supplementation improved the body weight, FCR, feed consumption and survival rate as compared to the TD chicks. The mean results ±SD are shown in graphs. *P<0.05.

Fig. 2: Mean TD score between three treatments (A); Growth plate width of the proximal tibia on various days (B). GSE chicks exhibited a significant decrease in score of TD and GP measurement. **P<0.01; *P<0.05.

Effect of GSE on TD score, growth-plate width, and lameness: The TD score (Fig. 2A) and GP width (Fig. 2B) was increased significantly (P<0.01) in chicks exposed to thiram when compared with healthy chickens through GSE supplementation significantly decreased (P<0.01) the TD score and GP width. Throughout the experiment, chicks were physically inspected and TD chicks (Fig. 3B) showed signs of stressful body state, lameness and improper gait whereas, healthy birds were active and walk normally (Fig. 3A). After GSE supplementation, the chickens' normal gait and the standing position were restored (Fig. 3C). Distended GP was observed in proximal tibia of TD chicks (Fig. 3G), but GSE supplementation leads to the development of a normal growth plate (Fig. 3H).

Analysis of blood serum profile and liver histopathological inspection: The AST (Fig. 4A) and ALT (Fig. 4B) concentrations were increased significantly (P<0.05) accompanied by the reduction in the ALP level (Fig. 4C) in thiram treated chickens as compared to the healthy chickens. The high levels of serum biochemicals indicate liver destruction and abnormal liver metabolism. From liver H&E staining results, karyopyknosis and irregular arrangement of hepatocytes were observed in TD affected birds whereas Control group possessed the normal hepatocytes with a tight arrangement. After GSE supplementation, liver histopathological lesions disappeared and, irregular cells lining and haemorrhages were decreased (Fig. 5). After GSE supplementation, a remarkable decrease (P<0.05) in ALT and AST contents while the increase in ALP level significantly (P<0.05) were noticed in comparison with TD chicks but non-significant results were attained after comparing with healthy chickens on day 18.

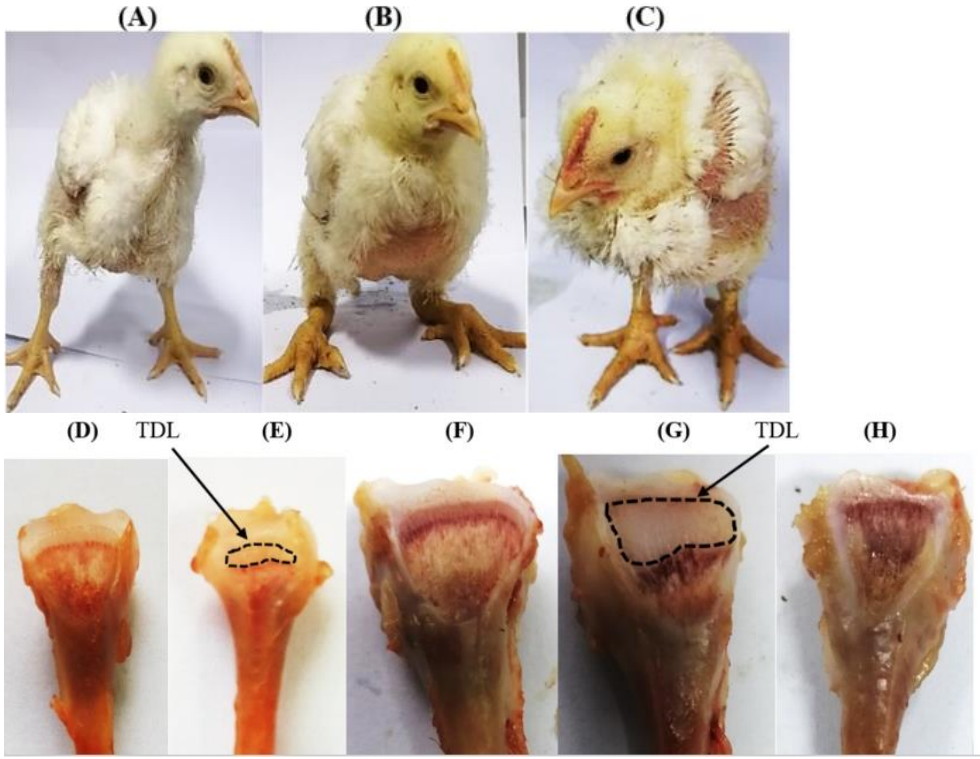

Fig. 3: Physical inspection of chickens. On day 18 Control (Chick looks active) (A), TD (Lameness can be observed) (B) and GSE group (Normal gait restored) (C). On day 7 tibia growth plate of Control (Normal GP) (D) and TD group (Chick had TD Lesion) (E). On day 18 growth plate of Control (Normal GP) (F), TD (Chick had TD Lesion) (G) and GSE group (Extended GP width) (H). Extended GP width and improper gait were observed in the TD group.

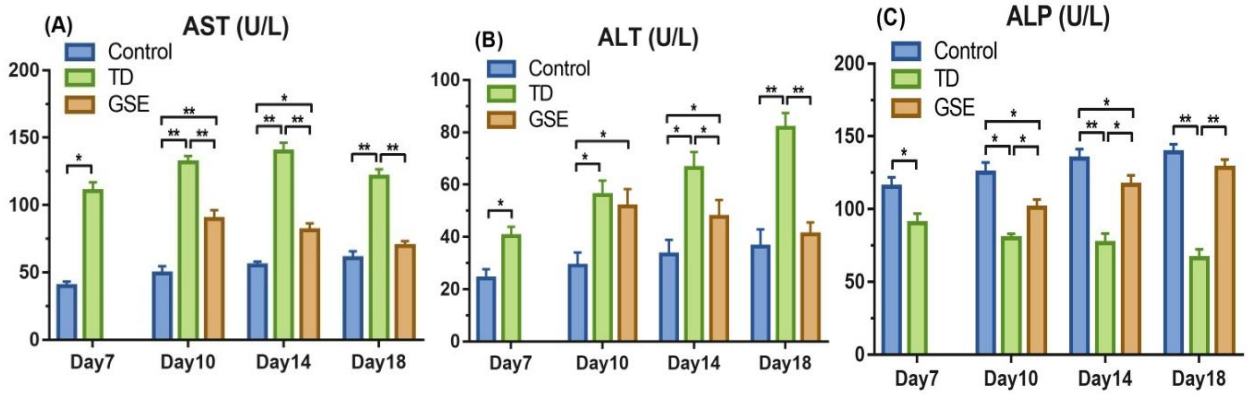

Fig. 4: Blood serum profile analysis. Aspartate aminotransferase (AST) (A), Alkaline phosphatase (ALP) (B) and Alkaline phosphatase (ALP) analysis (C). The mean results±SD are shown in graphs. TD chicks exhibited increased contents of AST and ALT and decreased amount of ALP in comparison with other chicks. *P<0.05; **P<0.01.

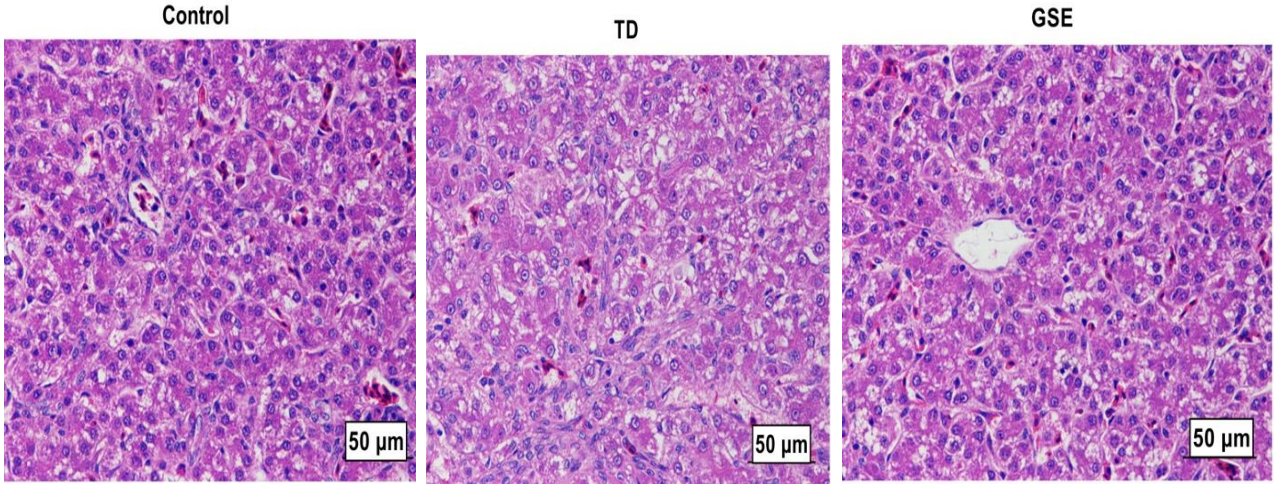

Fig. 5: H&E staining. From liver H&E staining results, karyopyknosis and irregular arrangement of hepatocytes were observed in TD affected birds whereas Control group possessed the normal hepatocytes with a tight arrangement. After GSE supplementation, liver histopathological lesion disappeared and, irregular cells lining and haemorrhages were decreased.

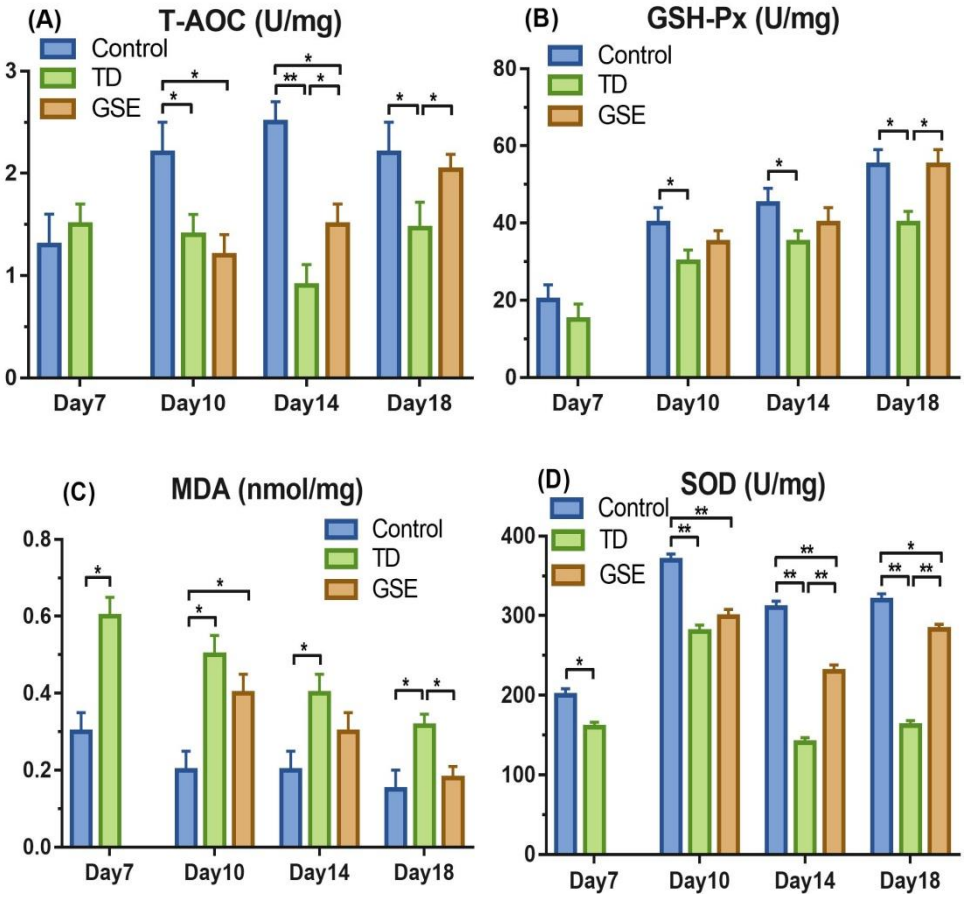

Fig. 6: Evaluation of liver antioxidant status. Measurement of T-AOC (A), GSH-Px (B), MDA (C) and SOD (D) were done using a spectrophotometer. The mean results ± SD are shown in graphs. Thiram provision raised the MDA amount and lessened the GSH-Px, SOD and T-AOC contents while GSE supplementation reverted the disturbed levels of enzymes and improved the bird's antioxidant status. *P<0.05; **P<0.01.

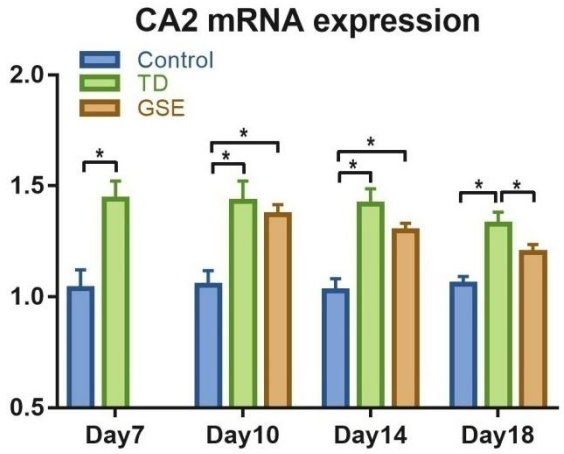

Fig. 7: Variation in CA2 gene expression. The mean results ±SD are shown in the graph. TD chicks exhibited higher expression of CA2 gene as compared to healthy chicks but GSE supplementation suppressed the mRNA expression of CA2 gene. *P<0.05.

Evaluation of liver antioxidant status: The liver antioxidant enzymes were measured to evaluate the GSE effect on oxidative stress and hepatic toxicity and results revealed that MDA percentage (Fig. 6C) was increased significantly (P<0.05) while significant (P<0.05) decrease in GSH-Px (Fig. 6B), SOD (Fig. 6D) and T-AOC (Fig. 6A) amount were observed in TD affected birds as compared to healthy chicks. However, supplementation of GSE significantly reverted (P<0.05) the disturbed levels of liver antioxidant enzymes when compared with the TD group but non-significant results were obtained when compared with healthy chickens.

CA2 mRNA expression in tibia growth plate: RT-qPCR results showed that on day 7 CA2 expression was up-regulated significantly (P<0.05) in chicks affected with TD in comparison with healthy chicks. The analogous trend was noticed on other days. GSE supplementation reduced the expression level of CA2 gene on day 10 and 14 but the non-significant change was detected when compared with the TD group. On day 18 GSE group exhibited significantly decreased (P<0.05) expression of CA2 gene as compared to the TD group, however, the non-significant change was noticed in comparison with healthy chicks (Fig. 7).

DISCUSSION

Globally, Tibial dyschondroplasia is a serious bone-related metabolic disorder in commercial broilers, which affects the metaphyseal part of the tibia and leads to the accretion of growth plate cartilage. Tibial dyschondroplasia like sudden death and ascites is one of the nutritional and metabolic syndromes which negatively affect the fast-growing broilers' production performance (Li *et al*., 2015). Poultry meat industry all over the world is challenged with a heavy financial loss because of TD prevalence (Li *et al*., 2008).

The normal structure of tibia growth plate in healthy chicks looks like an arc circular shape having a small, smooth and definite edge with equivalent width (Tian *et al*., 2013). However, in case of abnormal GP in TD chickens, avascularization, cell anomalous growth, non-mineralization and formation of a white milky plug can be observed (Shahzad *et al*., 2014b), which leads to abnormal

gait and complications in standing (Nabi *et al*., 2016). Recently scientists revealed that GP measurement was enlarged and TD score was higher in chicks treated with thiram because of which birds had more infection and difficulties in walking and standing (Mehmood *et al*., 2018a). Grape seed extract supplementation reduced the TD lesion in growth plate cartilage significantly and decreased the TD score and proper gait and normal standing ability was restored. These outcomes were also reported by former scholars (Iqbal *et al*., 2018; Mehmood *et al*., 2019b).

Thiram is used in experimental birds for the induction of TD to explore the fundamental pathway of TD. However, liver metabolic activities are disturbed by the poisonous effects of thiram (Shahzad *et al*., 2014b; Mehmood *et al*., 2019a). Chicken tibial cartilage is developed by a series of processes and there is involvement of various key enzymes which plays a pivotal role in cartilage development. These enzymes are believed to have an important task in liver metabolism. Thiram negatively affects liver enzymes production like altering GSH and SOD concentration that can cause oxidative stress and halt liver functions. The MDA product is finally released as the consequences of disturbance in the lipid peroxidation of the plasma membrane caused by the oxidative stress (Perry *et al*., 2010; Shahzad *et al*., 2014b). Liver damages were observed after treating rats with thiram and AST contents were significantly higher as reported by a former scientist. Altering amounts of ALT and AST are regarded as imperative biomarkers of liver bad health. The ability of initial chondrocytes to differentiate into mature bone cells and the minerals precisely linked with cartilage restructuring can be attributed to the ALP activity (Phull *et al*., 2016). Our study revealed that AST contents were significantly increased after the provision of thiram to chickens. The high levels of serum biochemicals indicate liver destruction and abnormal liver metabolism. From liver H&E staining results, karyopyknosis and irregular arrangement of hepatocytes were observed in TD affected birds whereas Control group possessed the normal hepatocytes with a tight arrangement. After GSE supplementation, liver histopathological lesion disappeared and, irregular cells lining and hemorrhages were decreased. The liver antioxidant enzymes were measured to evaluate the GSE effect on oxidative stress and hepatic toxicity and results revealed that significant decrease in MDA percentage, while a significant increase in GSH-P, SOD and T-AOC amount were observed in TD affected birds as compared to healthy chicks. However, supplementation of GSE significantly revert the disturbed levels of liver antioxidant enzymes. Grape seed extract supplementation eases the oxidative stress and reduced the liver damages. Almost all the inflammatory disorders have similar underlying pathological mechanism because of which toxicity and the oxidative stress is increased (Li *et al*., 2015). Numerous factors are involved in the onset of the liver oxidative stress, however, the main detoxification and metabolism of the body are done by the liver so any disturbance in the liver directly affects the production parameters of the chicken. Supplementation of the antioxidants from an external source would be an appropriate and handy approach to cure and alleviate the after-effects of liver destruction and oxidative imbalance and stress (Li *et al*. 2015).

Carbonic anhydrase 2 (CA2) is present in many tissues but share an important and pivotal task in several types of pathological and physiological mechanisms. In osteoclast cells, bone resorption is controlled by CA2. Renal acidosis and genetic osteoporosis are the main metabolic disorders caused by the deficit of CA2 in humans (Borthwick *et al*., 2003). TD chickens at their early stage showed higher expressions of CA2 genes (Tian *et al*., 2009). Similar outcomes have been revealed in our experiment. The reason behind this is the initiation of bone resorption process by the CA2 gene to enhance the cartilage calcification (Tian *et al*., 2009). Supplementation of GSE remove the TD lesions, recovered the TD chicks and down-regulated the CA2 expression. Former scholars also reported the down-regulation of CA2 expression in rats after the supplementation of GSE (Kim *et al*., 2018).

Conclusions: The current experiment proved that grape seed extract is a natural plant-based extract which owns solid scavenging and bone healing property, and strong antioxidant capacity. GSE provided recovery to chicks affected with TD by dropping TD score, whereas stabilized the width of GP through the down-regulation of the CA2 mRNA expression. Farmers can use this inexpensive drug to recover the commercial broilers suffered from tibial dyschondroplasia.

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Authors contribution: JL and HQ devised and designed the research idea. HQ wrote the manuscript. KM and JL revised and reviewed the manuscript. AL, MW, MI, LZ and XL contributed to the reagents and materials.

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