HIF-1α upregulation exerts the antagonistic effect against angiogenesis inhibition in manganese deficiency-induced tibial dyschondroplasia of broiler chicks

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Abstract
Manganese (Mn) is an essential microelement for broiler breeding and its deficiency causes tibial dyschondroplasia (TD). Tibial growth plate (TGP) development and metaphyseal vascularization are crucial for tibia growth in fast-growing broiler chickens, but their roles in Mn deficiency-induced TD in chicks remain unclear. This study was designed to clarify this issue. A total of 36 one-day-old broilers were divided into the control group and Mn-deficiency (Mn-D) group, which were fed with a standard diet (60 mg Mn/kg) and Mn deficiency diet (22 mg Mn/kg) for 42 days, respectively. TGP and proximal tibial metaphysis were collected to perform the related assays. This study found that Mn deficiency decreased the tibia length and TGP thickness in the TD model. Also, Mn deficiency increased the irregular and white tibial dyschondroplasia lesions (TDL) region under the TGP, and reduced the expression levels of vascular endothelial growth factor (VEGF) and macrophage migration inhibitory factor (MIF). Combined with histological assessment, it was suggested that Manganese deficiency inhibited angiogenesis in the proximal tibial metaphysis. Meanwhile, Mn deficiency enhanced the expression levels of hypoxia-inducible factor-1 α (HIF-1α), autophagy-related protein 5 (ATG5), and microtubule-associated protein 1 light chain 3 β (LC3-II) in TGP, but decreased the expression level of SQSTM1 (P62), which suggested that autophagy was activated during this process. Collectively, these data indicate that HIF-1α up-regulation and concurrent autophagy activation exert a protective effect against Mn deficiency-induced angiogenesis inhibition, which may provide useful guidance to prevent TD in broilers.

Keywords Broiler · Manganese · Tibial dyschondroplasia · Angiogenesis · HIF-1α · Autophagy

Introduction
Manganese (Mn) is an essential trace element to maintain the normal biological process of livestock and poultry (Wang et al. 2021b), particularly in the growth and development of broiler tibia. It has been demonstrated that Mn deficiency diet increases the incidence of osteoporosis and tibial dyschondroplasia (TD) in fast-growing broilers (Zhang et al. 2020). TD, characterized by extensive non-mineralized and avascular cartilage near the tibial growth plate (TGP), is an intractable tibiotarsal bone disorder in fast-growing poultry, which results in the death of tibial chondrocytes due to insufficient or untimely blood supply (Jahejo and Tian 2021; Niu et al. 2021). TD seriously affects broilers’ movement and poultry welfare, which causes large economic losses to breeding farms. A previous study has confirmed that Mn deficiency affected the proliferation and differentiation of
chondrocytes in TGP via inhibiting related regulatory factors, leading to TD in broilers (Wang et al. 2021b). However, it has not been clarified whether Mn deficiency leads to TGP developmental disorder by affecting the angiogenesis of proximal tibial metaphysis.

Angiogenesis is closely associated with osteogenesis (Fu et al. 2020). During the elongation of the tibia, chondrocytes in the TGP resting zone complete longitudinal bone development by proliferation, differentiation, matrix mineralization and apoptosis (Yan et al. 2016). This process is inseparable from the metaphyseal blood vessels, which play a pivotal role in TGP chondrocyte development, such as mediating the transportation of oxygen, nutrients, and wastes, as well as providing vascular secretion signals to control TGP metabolism (Fong et al. 2009). Any defect in the development of metaphyseal vessels may affect the normal differentiation of TGP chondrocytes into bone, subsequently causing TD in broilers (Asmussen et al. 2021). As above mentioned, a well-functioning vascular system is critical for maintaining the normal metabolism and homeostasis of TGP and ensuring leg health of livestock and poultry, but whether Mn deficiency-induced TGP development disorder is related to the abnormal development of metaphyseal vessels remains to be further clarified.

Hypoxia is a representative feature of the TGP microenvironment, which affects angiogenesis in the metaphysis and tibia development (Stegen et al. 2019; Zhang et al. 2015). Previous study has shown that hypoxia-inducible factor-1 alpha (HIF-1α) is a major modulator of hypoxia response and neovascularization (Im and Kim 2017). Hypoxia induces generation of almost all important angiogenic factors (Dong et al. 2020), such as vascular endothelial growth factor (VEGF) and macrophage migration inhibitory factor (MIF) (Alonso et al. 2019; Zhang et al. 2018). Numerous studies have reported that VEGF is a pro-angiogenic cytokine that sustains angiogenesis (Pulkkinen et al. 2020). MIF is involved in both angiogenesis and cell migration, as well as regulation of the angiogenic signaling (Alonso et al. 2019). Besides the direct regulation of angiogenesis, HIF-1α also regulates angiogenesis by promoting autophagy activation of chondrocytes (Lu et al. 2018). Autophagy is a lysosomal degradation pathway essential for cell survival (Yu et al. 2021), which plays an irreplaceable role in protecting chondrocytes from hypoxia (Singh et al. 2020). Many studies have shown that HIF-1α mediated autophagy defends the organism from pathological damage by converting damaged organelles and proteins into energy materials during hypoxia (Hu et al. 2020; Zhang et al. 2019). It has also been reported that autophagy activation provides essential amino acids and glucose for TGP angiogenesis under stress conditions to ensure the survival of chondrocytes (Jahejo and Tian 2021). At the same time, enhancing chondrocyte autophagy can also delay the progress of TD by affecting intracellular metabolic activity via regulating cell aging and death (Luo et al. 2019). It is known that the occurrence of TD is attributed to the cellular death of chondrocytes caused by insufficient blood supply, but the mechanisms by which Mn deficiency affects angiogenesis remain to be elucidated.

Therefore, this study employed Mn deficiency-induced broiler TD model to investigate the above-unresolved issues. Our results demonstrated that Mn deficiency inhibits metaphysis angiogenesis to prevent the development of the tibia in broiler chicks and HIF-1α may become a novel molecular therapeutic target for improving proximal tibial metaphysis vascularization in TD chicken.

**Materials and methods**

**Antibodies and reagents**

The following primary antibodies were used: VEGF (A5708), MIF (A1391), and HIF-1α (A11945) were bought from ABelonal Technology Co., Ltd. (Wuhan, China). ATG5 (A0432) was obtained from Abcam (Cambridge Science Park, Cambridge, UK). p62 (PM045) and LC3 (M186-3) were obtained from MBL Co., Ltd. (Nagoya, Japan). β-actin (GB12001) was purchased from Servicebio Technology Co., Ltd. (Wuhan, China). β-tubulin (66031-1-Ig) and GAPDH (10494-1-AP) were obtained from Proteintech Group, Inc. (Wuhan, China). Secondary antibodies used HRP-labeled Goat Anti-Mouse IgG(H+L) (CW1013A) and HRP-labeled Goat Anti-Rabbit IgG(H+L) (CW0102A) from CWBIO Biotechnology Co., Ltd. (Beijing, China). RNAiso Plus, Transcriptor cDNA Synth. Kit and SYBR Green I Master was purchased from Roche Co., Ltd (Shanghai, China).

**Experimental design**

One-day-old healthy commercial Arbor Acres (AA) broilers (n = 36) were purchased from New Hope Liuhe Group in Tai’an City, Shandong Province. All chickens were housed in standard wire cages (size, 80 × 60 × 50 cm³), using a recommended standard breeding temperature, humidity and daily lighting. Brooding temperature was maintained at 33°C to 35°C during the first week and steadily reduced to 29°C at the end of second week. During the experiments, the humidity was controlled at around 70%, and daily lighting was fixed with 23 h of light and 1 h of dark. Additionally, feed and water were provided *ad libitum*. After 3 days, chickens were randomly divided into two groups (n = 18), and each group was given the same basal diet with a different Mn supplement, i.e., control group (60 mg Mn/kg feed)
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and Mn-deficiency (Mn-D) group (22 mg Mn/kg.feed) (Table 1). Each group had 3 independent replicates (n = 6 per replicate). After 42 d of feeding, all chickens were sacrificed, and proximal TGP and metaphysis of tibial tissues (approximately 5 g) were collected. And then proximal tibial epiphysis was cut longitudinally to expose the tibial growth plate. The length of tibia and the thickness of growth plate were measured by a digital caliper (500–196, Mitutoyo, Japan). In addition, metaphysis of 3 broilers in each replicate was put into 4% formaldehyde fixation solution for histological assessment. TGP of 3 broilers in each replicate was immediately stored in liquid nitrogen and then transferred to -80 ℃ refrigerator for western blot and qRT-PCR analysis. All designing schemes in this study conform to animal welfare and have been approved by the Ethic Animal Care Committee of Shandong Agricultural University (NO: SDAUA-2019-057).

Determining Mn concentrations using microwave digestion-ICP-MS

The contents of Mn in diet, serum and tibia were measured using microwave digestion-ICP-MS. The sample was digested and treated with nitric acid. Then, the obtained samples were quantitatively transferred with polytetrafluoroethylene and diluted with ultrapure water. All conditions and operations follow the microwave digestion-ICP-MS requirements and Mn levels was determined by the standard mode (Dong et al. 2021).

Histological assessment

Metaphysis samples were fixed in 4% paraformaldehyde at 4 ℃ in PBS, and then decalcified in 10% EDTA. As referred to the previous method (Chi et al. 2021; Huang et al. 2017), the metaphysis samples were dehydrated, removed, and embedded with ethanol, xylene and paraffin, respectively. Paraffin sections of 5 μm thicknesses were cut, stained with hematoxylin and eosin and mounted on Superfrost plus-coated slides for microscopic examination. In addition, the number of blood vessels in metaphysis was determined by Image-Pro Plus 6.0 software.

Quantitative real-time polymerase chain reaction (qRT-PCR) analysis

In this study, the proximal tibial epiphysis was dissected longitudinally to expose the TGP. Then TGP was isolated from each group, ground to powder with liquid nitrogen, and total RNA was collected using the RNAiso Plus kit (Invitrogen, CA, USA) according to the manufacturer’s protocol. The concentration of extracted RNA was assessed by analyzing A260/A280 ratio using the MicroDrop® spectrophotometer (BIO-DL Corporation, Shanghai, China). The purified RNA (2 μg per sample) was reverse-transcribed into cDNA using Transcriptor cDNA Synth. Kit. qPCR reactions were carried out on a Light Cycler 96 (Roche, Basel, Switzerland) with SYBR Green I Master as previously described (Zhao et al. 2021). The house-keeping gene β-actin was used to normalize the Ct value and the 2−ΔΔCt method was employed to calculate relative mRNA levels. Primers used in this study are shown in Table 2.

Western blotting analysis

TGP was ground into powder with liquid nitrogen and then put at 4 ℃ for 30 min for lysin in buffer to prepare total protein. The concentration of protein was determined by the bicinchoninic acid assay (Thermo Fisher Scientific, USA). The detailed procedure was referred to the previous study (Liu et al. 2016). Approximately 20 μg of protein was separated by SDS-PAGE on 10% gel of polyacrylamide and then transferred to PVDF membranes (Millipore Corporation, USA). PVDF membrane was blocked in 5% skim milk for 1.5 h and then incubated with primary antibody at 4 ℃ overnight. The membranes were washed 3 times with TBST (containing 0.1% Tween 20) for 8 min each time and then incubated with appropriate secondary antibodies for 50 min at room temperature. Images were captured

Table 1: Ingredients and nutrient composition of diets to AA broilers

| Item         | Content  | Ingredient                  | 1-3week | 4-6week |
|--------------|----------|-----------------------------|---------|---------|
| Corn         |          |                             | 60.96   | 67.18   |
| Soybean meal |          |                             | 10.81   | 10.25   |
| Corn gluten meal |      |                             | 22.54   | 17.84   |
| Fish meal    |          |                             | 2.00    | 1.00    |
| CaHPO₃       |          |                             | 1.59    | 1.22    |
| Limestone    |          |                             | 1.36    | 1.78    |
| Salt         |          |                             | 0.3     | 0.3     |
| Lysine       |          |                             | 0.34    | 0.33    |
| Microconstituents |   |                             | 0.10    | 0.10    |
| Total        |          |                             | 100     | 100     |

Nutrient composition %

| Metabolizable energy (MJ/kg) | 13.39 | 13.39 |
| Crude protein %              | 23.00 | 20.00 |
| Non-phytate phosphorus %     | 1     | 1     |
| Lysine %                     | 0.45  | 0.35  |
| Methionine%                  | 1.1   | 1     |

Nutrient level of the diets was based on NRC recommendations.

Supplied per kilogram of diet: vitamin A, 12,500 IU; vitamin D, 33,500 IU; vitamin E, 18.75 mg; vitamin K, 32.65 mg; vitamin B, 26 mg; Biotin 0.0325 mg, folic acid 12 mg, pantethenic acid 50 mg; Cu 8 mg, Zn 40 mg, Fe 80 mg, I 0.35 mg, Se 0.15 mg.
with the Chemidoc XRS (Bio-Rad, Marnes-La-Coquette, France) and quantified by Image J analysis. The density of each band was normalized to its respective loading control. Three independent experiments were carried out for biological replicates.

**Statistical analysis**

One-way analysis of independent sample T-test was performed to analyze the data using the software package SPSS Statistics 21.0. Values were expressed as the mean ± SD and statistical significance was set as p < 0.05 and p < 0.01.

**Results**

### Mn deficiency causes tibial dyschondroplasia in broilers

This study has established a TD model using fast-growing broilers by feeding with 22 mg Mn/kg diet for 42 d, and the actual Mn content in the control group and the Mn-D group was 60.3 ± 1.02 mg Mn/kg.feed and 21.8 ± 1.4 mg Mn/kg.feed, respectively. Broilers in the Mn-D group gradually displayed a series of typical clinical symptoms on the 30th day, such as dullness, depression, and finally lameness (Fig. 1 A). Meanwhile, we found skeletal malformations in Mn deficiency chickens showing proximal tibia distortion and distal tibia dilation. Irregular and slightly white areas of tibial chondrodysplasia (TDL) can be seen below the TGP, and the area below TDL showed significant blood insufficiency (Fig. 1B). In addition, the length of tibia (Fig. 1 C) and the thickness of TGP (Fig. 1D) in Mn-D group were shorter than those in control group. The serum Mn content (Fig. 1E) in control group and Mn-D group was 7.58 ± 1.18 µg/L and 5.58 ± 1.06 µg/L, respectively, and the tibial Mn content (Fig. 1F) in control group and TD group was 7.28 ± 1.07 µg/g and 6.09 ± 0.88 µg/g, respectively, which were significantly decreased in Mn-D group. Overall, these results suggest that manganese deficiency leads to TD in broilers, which has an adverse impact on the development and tibial growth of broilers.

### Mn deficiency inhibits angiogenesis of metaphysis in broilers

To investigate the difference in vascular distribution between Mn-D group and control group, the results of HE staining on the proximal metaphysis of tibial tissues were shown in Fig. 2. In the control group, the distribution of blood vessels was well-proportioned and well-organized, and the columnar structure was well-preserved, part vessels orderly encircling the TGP (Fig. 2A). However, in Mn-D group, the arrangement was irregular (Fig. 2A) and the area of blood vessels was extremely decreased in the proximal metaphysis region compared to control groups (Fig. 2B). Then, we analyzed the expression profiles of MIF and VEGF in Mn deficiency-induced TGP tissues. As shown in Fig. 3, qRT-PCR analysis showed that the mRNA expression levels of VEGF and MIF were significantly down-regulated by Mn deficiency (Fig. 3A and B). Also, Mn deficiency significantly decreased VEGF and MIF protein levels (Fig. 3C and D). These results indicate that Mn deficiency-inhibited mRNA and protein expression levels of VEGF and MIF in TGP tissues contribute to angiogenesis inhibition and subsequent disturbed endochondral ossification.

### Mn deficiency induces HIF-1α up-regulation in TGP of broilers

To examine the effect of Mn deficiency on the transcriptional levels of HIF-1α in TGP of broilers, we performed qRT-PCR to detect the relative mRNA levels of HIF-1α in two groups. As shown in Fig. 4A, there were significant increases in expression levels of HIF-1α in the Mn-D group as compared to the control group. In addition, immunoblot results showed that Mn deficiency remarkably increased the protein level of HIF-1α (Fig. 4B). These data demonstrate that Mn deficiency induces HIF-1α upregulation in proximal TGP.

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**Table 2** Primer sequences for quantitative real-time PCR

| Gene ID | Gene      | Forward primer (5′-3′) | Reverse primer (5′-3′) | Target size (bp) |
|---------|-----------|------------------------|------------------------|------------------|
| 39509   | VEGF      | CCTGTGTGCCTCTGATGAGATGTG | CGCTATGTCGCTACTGATGGG  | 128              |
| 10085237| MIF       | GGCAGCAGAAGACACTGACC   | CGTTGAGCGGTATATGGCAAG   | 111              |
| 374177  | HIF-1α    | CAGCCAGGTCGCCAGAAGC    | ATGCTAGCTACCAATGATGCG   | 118              |
| 421784  | ATG5      | TGGAGGACAATTGCACACTTGGG | TGTAGTGGTTGTCCAGGATTGGC | 132              |
| 416269  | p62       | TCCCTGTGGACCCAGCAAGAC  | TCCCTGAGCCCCACGACTGAC   | 124              |
| 427559  | LC3-II    | CGTGGTAGACCCAGCACAGA   | AAGCCGTCTCTGTCTTTCTCG   | 80               |
| 396526  | β-actin   | GCCCTGGAACCTAGCAATG    | CTTCCTGCTGCTGATCCACATCTG | 129              |
Mn deficiency induces the activation of autophagy in TGP of broilers

To assess the effect of Mn deficiency on autophagy status in proximal TGP of broilers, the mRNA and protein expression profiles of three autophagy marker proteins were analyzed. Compared with the control group, the mRNA and protein expression levels of ATG5 in the Mn-D group were significantly increased (Fig. 5A and D), while mRNA and protein expression levels of p62 in Mn-D group were significantly decreased (Fig. 5B and E). Moreover, Mn deficiency increased the LC3-II mRNA expression level and LC3-II protein level (Fig. 5C and F). These data showed that Mn deficiency induces the activation of autophagy in TGP of broilers.

Discussion

TD is one of the most common leg diseases in broilers, accounting for nearly 30% of leg diseases in fast-growing broilers (Mehmood et al. 2019; Waqas et al. 2019). Mn is an essential element for bone growth (Defu Li et al. 2020; Gajula et al. 2011), and its deficiency leads to the failure of endochondral osteogenesis in tibia of fast-growing broiler chickens (Liao et al. 2019). The National Research Council
During this process, MIF and VEGF are secreted by chondrocytes in hypertrophic chondrocyte zone of TGP to induce vascular invasion and osteogenesis, which play a crucial role in regulating angiogenesis and enhance endothelial cell adhesion, migration, proliferation and microtubule formation (Zhang et al. 2018). In this study, we found that, in Mn-D group, the number of blood vessels was sharply reduced and the expression levels of VEGF and MIF in TGP were down-regulated, which led to the transportation hurdles of nutrition to chondrocytes. Ultimately, cartilage in the zone of mineralization and calcium deposition fails to complete the calcification. Our research group previously found that Mn deficiency induces an increase in apoptotic death of hypertrophic chondrocytes in TGP (Wang et al. 2015), while the present data further revealed that insufficient blood supply in TGP is another factor contributing to the death of chondrocytes.

During the elongation of the tibia, chondrocytes are transformed into TGP cartilage and bone tissue adjacent to the metaphysis through proliferation and differentiation to increase tibia length. Metaphyseal vascularization is closely associated with this process, and the failure of angiogenesis is liable to increase the incidence of TD (Sivaraj and Adams 2016). During this process, MIF and VEGF are secreted by chondrocytes in hypertrophic chondrocyte zone of TGP to induce vascular invasion and osteogenesis, which play a crucial role in regulating angiogenesis and enhance endothelial cell adhesion, migration, proliferation and microtubule formation (Zhang et al. 2018). In this study, we found that, in Mn-D group, the number of blood vessels was sharply reduced and the expression levels of VEGF and MIF in TGP were down-regulated, which led to the transportation hurdles of nutrition to chondrocytes. Ultimately, cartilage in the zone of mineralization and calcium deposition fails to complete the calcification. Our research group previously found that Mn deficiency induces an increase in apoptotic death of hypertrophic chondrocytes in TGP (Wang et al. 2015), while the present data further revealed that insufficient blood supply in TGP is another factor contributing to the death of chondrocytes.
vascularization, resulting in inhibition of TGP development, but it needs to be further investigated.

Fig. 4 Mn deficiency enhances HIF-1α mRNA and protein expression levels in TGP of broilers. (A) mRNA expression levels of HIF-1α were determined by qRT-PCR assay. (B) Protein levels of HIF-1α were determined by western blotting analysis and the results were quantified and analyzed using Image-Pro Plus 6.0. Data were expressed as mean ± SD (n = 3 of each independent replicate). Compared with the control, * p < 0.05; ** p < 0.01

TGP resides in a low oxygen environment and it is an avascular tissue that obtains nutrition and oxygen by

Fig. 5 The expression profiles of ATG5, LC3-II, and p62 in TGP of broilers. (A-C) Real-time quantitative PCR analysis of ATG5, p62 and LC3-II in Control and Mn-D groups. (D-F) Protein levels of ATG5, p62 and LC3-II were assessed by immunoblot assay and the results were quantified and analyzed using Image-Pro Plus 6.0. Data were expressed as mean ± SD (n = 3 of each independent replicate). Compared with the control, * p < 0.05; ** p < 0.01
diffusion from the metaphysis vascular (Alonso et al. 2019; Sivaraj and Adams 2016). Previous studies have shown that HIF-1α, a master regulator of the cellular response to hypoxia, is essential for growth and survival of TGP chondrocytes in vivo (Jahejo and Tian 2021). HIF-1α is expressed in a hypoxia-independent manner under physiological conditions, mainly in hypertrophic zone of TGP, where it is necessary for chondrocyte maturation and differentiation (Hu et al. 2020) (Lee et al. 2012). It has been shown that inadequate blood supply to chondrocytes leads to a decrease in oxygen concentration and an up-regulation of HIF-1α expression in TGP of chicks with TD (Huang et al. 2018). Moreover, chondrocytes lacking functional HIF-1α undergo massive cell death in the TGP, which leads to the tibia narrow and exhibits less vascularization in metaphysis (Oda et al. 2008). Another hypoxia experiment using AA chicks has shown that tibial angiogenesis is enhanced by upregulation of pro-angiogenic factors, such as MIF and VEGF, contributing to oxygen and nutrition delivery to TGP (Huang et al. 2017). These experiments indicate that hypoxia can induce angiogenesis. Data in this study showed that Mn deficiency induces the HIF-1α up-regulation, which indicates hypoxia in the TGP. At the same time, Mn deficiency induces down-regulation of the MIF and VEGF expression in TGP. This distinction may be explained that Mn deficiency suppresses hypertrophy of chondrocytes in TGP, resulting in decreased production of MIF and VEGF and subsequent inhibition of metaphyseal vascularization. Given the above mentioned, we speculated that HIF-1α up-regulation may mitigate the chondrocytes damage in TGP caused by Mn deficiency via antagonizing the decrease in angiogenesis.

Autophagy is an evolutionarily conserved process that plays an important role in maintaining intracellular homeostasis and degrading cytoplasmic components, such as damaged organelles and misfolded proteins, to provide energy for the body (Song et al. 2017). Recent study has shown that hypoxia can activate autophagy and promote lysosomal degradation of proteins and organelles (Muz et al. 2015). Hypoxia-induced autophagy is initiated by HIF-1α in a variety of cell lines (Zhang et al. 2008). Previous studies have shown that inhibition of autophagy genes results in the death of chondrocytes, suggesting that autophagy functions as a cytoprotective effect in tibial growth (Wang et al. 2021a) (Yang et al. 2020). It is reported that HIF-1α up-regulation can induce autophagy activation under stress conditions to ensure the survival of TGP chondrocytes (Hu et al. 2020; Singh et al. 2020). Data in this study showed that Mn deficiency induces the autophagy activation of TGP in chicks. It may be ascribed to the decreased angiogenesis in metaphyseal regions, which results in a hypoxia environment in TGP and subsequent HIF-1α up-regulation. We speculate that autophagy activation may provide essential energy for chondrocytes and metaphyseal vessels to ensure normal secretion of MIF and VEGF and normal angiogenesis in metaphysis, further indicating that HIF-1α up-regulation may protect against Mn deficiency-induced abnormal metaphysis angiogenesis in broilers. However, the role of autophagy in abnormal TGP development and anomalous metaphyseal angiogenesis induced by Mn deficiency has not been fully elucidated, which needs to be further investigated.

In summary, our findings demonstrate that HIF-1α up-regulation plays a protective role in Mn deficiency-induced TGP developmental disorder by antagonizing the angiogenesis inhibition and inducing autophagy activation, which provides new insight into the pathogenesis of Mn deficiency-induced TD.

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Contributions Lu Lu, Cong Jin, and Peng-Fei Dong: Conceptualization, Data curation, Investigation, Validation, Methodology, Writing–original draft preparation, Writing–review and editing; Zhen-Yong Wang: Funding acquisition.

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Data Availability The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval All animal experimental protocols were in accordance with the animal welfare and approved by the Ethic Animal Care Committee of Shandong Agricultural University (NO: SDAUA-2019-057).

Competing Interests The authors declare no competing interests.

Consent for publication We consent to the publication of the submitted manuscript.

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