1   **Effect of antenatal tetramethylpyrazine on lung development and YAP**

2   **expression in a rat model of experimental congenital diaphragmatic hernia**

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Abstract

Tetramethylpyrazine (TMP) is a chemical compound found in extracts derived from the Chinese

medicinal plant. Due to its remarkable therapeutic effects, availability, and low cost and toxicity,

TMP has been used to treat cardiovascular diseases and pulmonary hypertension in China. The

aim of this study was to investigate the therapeutic effects and underlying mechanism of TMP on

lung development using a rat model of nitrofen-induced congenital diaphragmatic hernia (CDH).

Pregnant rats were divided into three groups: control, CDH, and CDH+TMP. Nitrofen was

used to induce CDH. In the CDH and CDH+TMP, Fetuses only with left diaphragmatic

hernias were chosen for analysis. Lung and body weight were recorded and lung histologic

evaluations, image analysis, and western blot analysis of YAP , p-YAP and LATS1 were

performed after lung processing. A marked abnormal structure was observed, as evidenced by

pulmonary hypoplasia and vascular remodeling, in the CDH. These abnormalities were

improved in the CDH+TMP. There were significant differences between the CDH and

CDH+TMP in percentage of medial wall thickness, arteriole muscularization, radial alveolar

counts, AA%, and alveolar septal thickness. YAP expression was markedly increased in the

CDH compared to the control, which was not affected by antenatal TMP administration.

However, prenatal TMP intervention significantly increased expression of LATS1 and

39 phosphorylation of YAP in the CDH fetuses. Our results demonstrate that **a**ntenatal TMP

40 administration improved vascular remodeling and promoted lung development in a rat model

41 of CDH, potentially through increasing expression of LATS1 and phosphorylation of YAP.

42 **Key words:** congenital diaphragmatic hernia; tetramethylpyrazine (TMP); YAP

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Introduction

45   Congenital diaphragmatic hernia (CDH) is an uncommon congenital malformation,

46 occurring in 1 to 4 of every 10,000 pregnancies[1]. Although medical and surgical

47 management of CDH has improved, CDH is still associated with a high mortality rate of 60 -

48 70% [2][2][4][5]. The disease name comes from the original abnormality involving a hole in

49 the diaphragm, but over the last few decades clinicians have observed that the defect in the

50 diaphragm is not a determinant of survival. The primary causes of mortality in CDH include

51 pulmonary hypoplasia (PH) and severe persistent pulmonary hypertension of the newborn

52 (PPHN) [6]. It was traditionally thought that PH was caused solely by herniation of the

53 abdominal organs into the thorax through the pleuroperitoneal canals, which compresses the

54 developing ipsilateral lung and limits the expansion of the contralateral lung. However,

55 animal studies and evaluation of early human embryos have convincingly shown that

56 abnormal pulmonary development in the embryonic phase is the primary defect [7].

57   Current postpartum care, surgery, or medical treatment strategies have not proven to be

58 viable approaches for managing PH and improving the associated abnormal remodeling of the

59 pulmonary vasculature. However, with the development of modern imaging technologies,

60   CDH can now be accurately diagnosed in mid-gestation [8][9]. Therefore, it seems feasible to

61   consider prenatal intervention in cases with poor prognosis. Fetal surgical interventions, such

62   as fetoscopic temporary tracheal occlusion, are invasive, technically demanding, and limited

63   by maternal and fetal risks [10]. Evidence has demonstrated that tracheal occlusion does not

64   increase survival compared with standard postnatal care [11]. Therefore, less invasive

65   approaches, such as antenatal pharmacologic treatment to stimulate lung growth and

66   maturation, have been proposed and investigated in the laboratory [13][14][14]. Although

67   some drugs have been found to improve pulmonary maturity and abnormal pulmonary

68   vascular remodeling in animal models, the potential side effects of antenatal treatments, such

69   as glucocorticoids [15] and sildenafil [16], limit their use. Therefore, it is necessary to explore

70   other pharmacologic antenatal interventions to determine which have fewer side effects.

71   Tetramethylpyrazine (TMP, also called ligustrazine) is a chemical compound found in

72   extracts derived from the Chinese medicinal plant Ligusticum wallichii (*family Apiaceae*).

73   TMP possesses typical characteristics as a calcium antagonist. Due to its remarkable

74   therapeutic effects, availability, and low cost and toxicity, TMP has been considered an

75   effective therapy for various diseases, such as cerebral ischemia, cardiovascular diseases, and

76   pulmonary hypertension in China [17][18]. Moreover, in recent years, TMP has been found to

77   be an effective and safe treatment for fetal growth restriction (FGR) [19].

78   Most of our current understanding about the structural and molecular changes in CDH

79   originated from experimental animal models[20]. Administration of the herbicide nitrofen

80   (2,4-dichloro-phenyl-pnitrophenyl ether) to pregnant rats on embryonic day 9.5 (E 9.5) has

81   been shown to result in PH and diaphragmatic defects in the offspring, both remarkably

82   similar to human CDH[21][22].

83   The purpose of this study was to evaluate the effect of TMP on improving CDH-induced

84   abnormal pulmonary vascular remodeling and PH in the nitrofen-induced CDH model. We

85   also explored the possible underlying mechanism of TMP's effects by measuring expression

86   and activation of Yes-associated protein (YAP), which is an important protein in pulmonary

87   development and vascular reconstruction.

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Materials and methods

Experimental design and animal model

91   All animals were provided by the Institute of Laboratory Animais of Sichuan Academy

92   of Medical Sciences and Sichuan Provincial People's Hospital (Chengdu, China). The

93   protocol was approved by the Committee on the Ethics of Animal Experiments of Sichuan

94   Academy of Medical Sciences and Sichuan Provincial People's Hospital (Protocol Number:

95   2018-198). All surgery was performed under sodium pentobarbital anesthesia, and all efforts

96   were made to minimize suffering. This experiment was supported by Lilai Biotechnology

97   (Chengdu, China). Twenty adult female Sprague-Dawley rats weighing 240 - 305 g (average,

98   283 g) were used. All rats were bred after a night of controlled mating. A sperm-positive

99   vaginal smear confirmed mating and represented embryonic day (E) 0.5. CDH was induced in

100   pregnant rats at E9.5 via intragastric administration of a single oral dose of nitrofen (125 mg;

101   99% purity; Zhejiang Chemicals, Ningbo, Zhejiang, China) that was dissolved in 2 ml of

102   olive oil. Control rats received an equal amount of olive oil only. On E11.5, nitrofen-fed

103   pregnant rats were randomly divided into two groups: CDH or CDH+TMP. TMP (80 mg/kg,

104   Livzon Pharmaceutical Group Inc., China) was intragastrically administered to pregnant rats

105   on E11.5 once a day, for 10 days. In total, our study included three groups of pregnant rats as

106   follows: control (n = 5), CDH (nitrofen-induced CDH, n = 5), and CDH+TMP

107   (nitrofen-induced CDH with antenatal TMP treatment, n = 5). Rat fetuses were delivered via

108   Cesarean on E21.5 (prior to full term, E22) and immediately beheaded after being weighed.

109   Under an anatomic stereoscopic microscope, fetal lungs were removed and the bilateral

110   diaphragms were carefully examined for CDH. Lung tissue weight (LW) and body weight

111   (BW) of each fetus were recorded. The left lungs of the fetuses with left CDH only were

112   removed and processed for further analysis.

Lung preparation

114   Lungs were placed into 4% paraformaldehyde, fixed at 4℃ for 48 hours, and then

115   embedded in paraffin for histological analysis. Paraffin-embedded fetal lungs were

116   transversely cut into 5 μm sections with a microtome. Sections were stained with hematoxylin

117   and eosin (H&E) and an elastin histochemical stain. Lung samples to be used for western blot

118   analysis were stored at -80℃.

Morphological analyses

Pulmonary vascular morphometric analysis

121   Lung slices were deparaffinized and hydrated using conventional methods[23]. The

122   sections were stained in Verhoeff's solution for 1 hour until the tissue sections were

123   completely stained black. Then, the sections were washed in running tap water three times

124   and soaked in 2% ferric chloride for 1 - 2 minutes, rinsed briefly with tap water, and checked

125   for black elastic fiber staining and gray background under a light microscope. The slides were

126   treated with 3% sodium thiosulfate for 5 minutes and rinsed in running tap water for 5

127   minutes. Slides were then counterstained in Van Gieson's solution for 5 minutes, dehydrated,

128   and rinsed in graded alcohols and xylene, cover slipped, and observed under a light

129   microscope.

130   External diameter (ED) and medial wall thickness (MT) of small pulmonary arteries with

131   a diameter of 20 ~ 60 μm that were associated with terminal bronchioles and distal airspaces

132   were quantified using Image-Pro Plus 6.0 (Media Cybernetics, Inc., Chengdu, China). ED

133   was defined as the distance between the external elastic laminae, and MT was defined as the

134   distance between the internal and external elastic laminae. Percentage of medial wall

135   thickness (%MT) was calculated using the following formula: $2 \times MT/ED \times 100$ [24][25].

136   Vessels with a measurable medial wall were considered muscularized; vessels without a

137   medial wall were considered non-muscularized; and vessels with an incomplete medial wall

138   were considered partially muscularized[15].

Lung maturation measurement

140   Lung growth was determined using the LW/BW ratio. The following morphological

141   parameters were measured: (1) Radial alveolar count (RAC) was used as an index of alveolar

142   proliferation and architectural maturity. RAC has been used to measure development of the

143   terminal respiratory unit, as originally described by Emery and Mithal [25]. RAC was

144   determined by counting the number of airspaces along a line drawn perpendicularly from the

145 center of a terminal or respiratory bronchiole to the closest edge of the acinus (pleural or

146 lobular connective tissue septum). (2) Percentage of lung alveolar area per unit area (%AA)

147 was measured by image analysis using Image Pro Plus version 6.0 (Media Cybernetics, Inc.,

148 Chengdu, China). (3) Alveolar septal thickness was also measured.

Western blot analysis

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150 Lung tissues were homogenized in RIPA buffer supplemented with Complete Protease

151 Inhibitor Cocktail tablets (Roche) and phosSTOP Phosphatase Inhibitor Cocktail tablets

152 (Roche). Protein concentrations were determined using the Pierce BCA assay (Rockford, IL).

153 Total protein (50 μg) was linearized in Laemmli sample buffer (Bio-Rad, USA) and then

154 separated by gel electrophoresis using prefabricated 10% SDS polyacrylamide gels

155 (Invitrogen). Proteins were then transferred to PVDF membranes (Hybond, USA).

156 Immediately after transfer, the membranes were blocked with 5% bovine serum albumin

157 (BSA) for 2 hours before antibody detection. Primary antibodies against YAP (1:500, CST),

158 large tumor suppressor kinase 1 (LATS1) (1:300, Proteintech), phosphorylated (p)-YAP

159 (1:1,000, CST), and β-actin (1:5000, Abcam) were incubated overnight at 4℃. The

160 membranes were further incubated with a goat anti-rabbit secondary antibody (1:5,000,

161 Abcam) at room temperature for 2~3 hours followed by extensive washing. An enhanced

162 chemiluminescence (ECL) kit (Thermo, USA) was used for antibody detection. All antibodies

163 used in this study were diluted in phosphate buffered saline (PBS). The gel image analysis

164 system (Tanon, China) was used for scanning analysis and the results are presented as relative

165 expression of the target protein calculated as: target protein expression = integrated optical

166 density value of target protein / internal reference integrated optical density value.

Statistical analysis

Values are presented as mean ± standard deviation (SD). All data were statistically analyzed using SPSS, version 21 (SPSS, Inc, Chicago, Ⅲ). Statistical analysis was performed using one-way ANOVA and the Chi-square test. Values of $P < .05$ are considered statistically significant.

Results

Incidence of CDH and pulmonary vascular remodeling

We determined the incidence of CDH in the three groups: none of the 80 fetuses in the control group presented with CDH; 48 out of 70 fetuses (68.6%) presented with CDH in the CDH group; and 51 out of 76 (67.1%) fetuses presented with CDH in the CDH+TMP group. There were no significant differences in CDH incidence between the CDH and CDH+TMP groups ($P = .85$). In the experimental groups, fetuses with left CDH only were included in our analyses (Table 1). Accordingly, the fetuses were further divided into three groups: controls (n = 80), CDH (n = 35), and CDH+TMP (n = 41).

Table 1. Pulmonary vascular morphometry

Group	N	ED (μm)	%MT	Wall structure (%)		
				M	PM	NM

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Control	80	25.22±8.82[&]	17.86±4.18	49.56±9.76	4.27±1.85	46.17±8.97
CDH	35	30.19±11.35	34.84±5.91*	58.46±11.21	15.69±4.77	25.85±9.39[§]
CDH+TMP	41	27.27±9.87[&&]	18.54±4.11**	51.07±8.90	7.69±2.05	41.24±10.11[§§]

184 Values are expressed as $\bar{x} \pm s$. M: fully muscularized; PM: partially muscularized; NM:

185 non-muscularized. & P = .57, && P = .71, vs Control; * P < .01, vs Control; ** P < .01, vs

186 CDH; § P < 0.01, vs Control; §§ P < .01, vs CDH.

187 Comparison of pulmonary vascular morphometry showed that ED was not statistically

188 different between the CDH group and the other groups (P = .57, P=.71). Fetuses in the CDH

189 group had a significantly increased %MT and decreased non-muscularized vessels compared

190 to the controls (P < .01, P < .01), whereas the fetuses in the CDH+TMP group showed

191 significantly reduced %MT and increased non-muscularized vessels compared to the CDH

192 group (P < .01, P < .01) (Table 1, Fig 1).

193 **Fig 1.** Medial wall thickness of the pulmonary artery was significantly increased in the CDH

194 group (B) compared to the control group (A). Medial wall thickness was decreased in the

195 CDH+TMP group (C) compared to the CDH group (B) (VVG, original magnification ×400).

LW/BW ratio and lung morphometric analysis

197 Similar to our previous study, fetal lungs in the CDH group were markedly hypoplastic,

198 as evidenced by lower LW/BW ratios, decreased RAC and %AA, and thicker alveolar septum

199 compared to the CDH group. Treatment with TMP significantly promoted fetal lung

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200   development, but the development still lagged compared to controls. The results are shown in

201   Table 2 and Fig 2.

202   **Table 2.** LW/BW ratio and lung morphometric analysis

Group	N	%LW/BW	RAC	%AA	Alveolar septal thickness(μm)
Control	80	3.31±0.29	5.7±0.8	67.3±9.8	13.53±3.22
CDH	35	2.29±0.36$^{\&}$	2.3±0.3*	43.6±6.5$^{\Delta}$	22.02±5.06§
CDH+TMP	41	2.99±0.41$^{\&\&}$	3.1±0.4**	57.1±7.5$^{\Delta\Delta}$	14.70±3.87§§

203   Values are expressed as $\bar{x} \pm s$. & $P < .01$, vs Control; && $P = .02$, vs CDH; * $P < .01$, vs

204   Control; ** $P < .01$, vs CDH; Δ $P < .01$, vs Control; $\Delta\Delta$ $P < .01$, vs CDH; § $P < .01$, vs

205   Control; §§ $P < .01$, vs CDH.

206   **Fig 2.** Lungs from the CDH group (B) were characteristic of the fetal canalicular stage,

207   showing poorly formed saccules and thickened septal walls compared to lungs in the control

208   group (A), which showed well-differentiated saccules and thin septal walls. Striking changes,

209   including an increase in air saccule size, thin septal walls, and maturation of the pulmonary

210   interstitium, (H&E, original magnification ×100) were seen in the CDH+TMP (C) groups.

Western blot analysis of YAP, LATS1, and p-YAP

212   YAP expression was significantly increased in fetal lungs from the CDH group

213   compared to the control group ($P < .01$), while there was no significant difference in LATS1

214   between the two groups ($P = .65$). TMP prenatal intervention did not significantly affect YAP

215 expression ($P = .28$), but significantly increased LATS1 ($P < .01$) and p-YAP ($P < .01$)

216 expression in the CDH lung tissues. Equal loading of electrophoresis gels was confirmed by

217 β-actin staining of the stripped membranes (**Fig 3**).

218 **Fig 3.** (A) Western blot analysis of lysates derived from control, CDH, and CDH+TMP lung

219 tissues. (B) YAP expression was significantly increased in the lungs of the CDH group

220 compared to the control group ($P < .01$). TMP prenatal intervention did not significantly

221 affect YAP expression in CDH fetal lung tissue ($P = .28$), but significantly increased LATS1

222 ($P < .01$) and p-YAP ($P < .01$) expression.

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Discussion

225   TMP has been used in traditional Chinese medicine for many years to treat various

226 diseases, including pulmonary hypertension, cardiovascular and neurovascular disease, FGR,

227 and others. Therefore, we hypothesized that TMP could also be used to treat CDH with PH

228 and PPHN. In this study, we used a rat nitrofen-induced CDH model to evaluate the effects of

229 prenatal TMP administration on improving pulmonary vascularization. Our results indicate

230 that prenatal TMP therapy significantly reduced medial thickness of small arteries and

231 increased the number of non-muscularized arteries, while decreasing the number of fully or

232 partially muscularized arteries in CDH rats. These data indicate that TMP decreases vascular

233 remodeling, resulting in increased pulmonary blood flow, and further suggests that pulmonary

234 hypertension in CDH rats can be alleviated by prenatal TMP therapy. However, the

235 mechanism by which TMP inhibits pulmonary vascular remodeling in the CDH rat model

236 remains unclear. Emerging evidence supports that YAP plays an important role in vascular

237 remodeling and related cardiovascular diseases [27]. Therefore, we hypothesized that TMP

238 alters YAP expression and activation in CDH.

239   In mammals, YAP is the key functional effector of the hippo pathway, which mainly

240 comprises mammalian STE20-like protein kinase 1/2 (MST1/2), Salvador family WW

241 domain containing 1 (SAV1), large tumor suppressor 1/2 (LATS1/2), Mps one binder

242 (MOB1), YAP/transcriptional coactivator with PDZ-binding motif (TAZ), and transcriptional

243 enhancer associate domain family members 1-4 (TEAD1-4) [28][29]. When the Hippo

244 pathway is activated, the YAP/TAZ complex is phosphorylated by LATS1/2, which results in

245 its nuclear exclusion, ubiquitination, and subsequent proteolytic degradation [30]. Hippo/YAP

246 signaling plays an important role in cardiovascular development and vascular homeostasis

247 [31]. Moreover, Hippo/YAP signaling has been found to contribute to vascular remodeling

248 and related cardiovascular diseases, including pulmonary hypertension, atherosclerosis, aortic

249 aneurysms, restenosis, and angiogenesis [27]. New evidence suggests that YAP regulates

250 proliferation and survival of pulmonary arterial vascular smooth muscle cells (VSMCs) and

251 pulmonary vascular remodeling [32][33]. In addition, LATS1 was found to be inactivated in

252 small remodeled pulmonary arteries, as well as distal pulmonary arterial VSMCs in idiopathic

253 pulmonary hypertension [32]. In our study, we found that upregulated YAP expression in the

254 CDH rats was associated with increased pulmonary vascular resistance and altered pulmonary

255 arterial muscularization. We also found that TMP treatment increased LATS1 expression and

256 YAP phosphorylation. Therefore, we speculate that pulmonary vessel remodeling and

257 pulmonary hypertension in CDH is partly due to an increase in LATS1 and YAP expression

258   and activity.

259   YAP transcriptional targets often include positive regulators of cell proliferation and

260   negative regulators of cell death. Thus, inactivation of Hippo signaling leads to organ

261   enlargement, which is a signature phenotype of Hippo pathway activation [34][35]. However,

262   in this study, we found that increased YAP expression in CDH lung tissues did not lead to

263   increased lung size; rather, upregulation of YAP led to a decrease in lung size and an apparent

264   cessation in development. A recent study suggested that early inactivation of the Hippo

265   pathway during early stages of lung development resulted in a sharp decrease, rather than the

266   expected increase, in lung size [36]. Furthermore, researchers found that, despite nuclear YAP

267   localization in the epithelium, Shhcre;Lats mutant rats had smaller lungs with halted

268   development after primary branch formation, one of the more severe lung developmental

269   phenotypes. This developmental defect is likely attributed to disrupted localization of

270   apical-basal polarity determinants, cell adhesion molecules, extracellular matrix components,

271   and spindle misorientation in dividing cells. Instead of a single cell layer of epithelium that is

272   critical for effective extension and growth of the branches, Shhcre;Lats mutant rats showed a

273   multilayered epithelium with cells protruding into the lumen. Similar phenotypes were also

274   described in the kidneys and salivary glands of transgenic animals over-expressing YAP or

275   mutants with a LATS1/2 deletion [37][38]. These findings suggest that in branching organs,

276   such as the lung, kidney, and salivary gland, there is a primary role for Hippo signaling to

277   maintain an organized epithelium, which is critical for degerming organ size [36]. Moreover,

278   increased Yap activity could lead to impaired differentiation and maturation of lung epithelial

279   cells and decreased surfactant proteins [39][40], all of which are in accordance with many

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280   disease manifestations in CDH lung tissues.

281   Interestingly, we found that antenatal administration of TMP was beneficial for

282   improving PH, as evidenced by the LW/BW ratio, alveolar septal thickness, RAC, and %AA

283   in the CDH+TMP group compared to the CDH group. We speculate that these results are

284   related to increased LATS1 expression and inhibition of YAP activity.

285   This study is the first to report the effects of prenatal TMP administration on lung

286   development in a rat model of CDH. We revealed a significant role of Hippo signaling in

287   CDH-associated PH and pulmonary hypertension. We also found that antenatal nitrofen

288   exposure increased YAP expression and structural abnormalities in the lung, including

289   abnormal vascular remodeling and impaired alveolarization. We also noted that antenatal

290   TMP treatment promoted lung development and improved vascular remodeling. Although

291   further studies are needed to determine the exact mechanisms of CDH-induced PH and

292   antenatal TMP administration in improving lung structure, the current findings suggest that

293   increased YAP activity is associated with delayed pulmonary development and abnormal

294   vascular remodeling, and antenatal TMP therapy improves lung structure and function via

295   increasing LATS1 expression and phosphorylation of YAP.

296   There are several limitations to note in our study. There was a lack of data regarding the

297   whole process of fetal lung development, and therefore we could dynamically reflect the

298   changes of the Hippo signaling pathway in lung development. Furthermore, there was a lack

299   of direct evidence supporting the relationship between the Hippo signaling pathway,

300   CDH-induced PH, and TMP prenatal intervention. These limitations need to be addressed in

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301   future studies.

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Availability of data and materials

304   The authors declare that all data supporting the findings of this study are available within the

305   article or from the corresponding authors on reasonable request.

306

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310

Authors' contributions

312   JZ Liao is first author. WY Liu obtained funding. JZ Liao, Q Li, LB Zhang, and WY Liu

313   designed the study. JZ Liao, Q Li, and LB Zhang collected the data. JZ Liao and Q Li were

314   involved in data cleaning and verification. JZ Liao and Q Li analyzed the data. JZ Liao

315   drafted the manuscript. WY Liu, JZ Liao, and F Hou contributed to the interpretation of the

316   results and critical revision of the manuscript for important intellectual content and approved

317   the final version of the manuscript. All authors have read and approved the final manuscript.

318   JZ Liao and WY Liu are the study guarantors.

Competing interests

320   The authors declare no competing interests.

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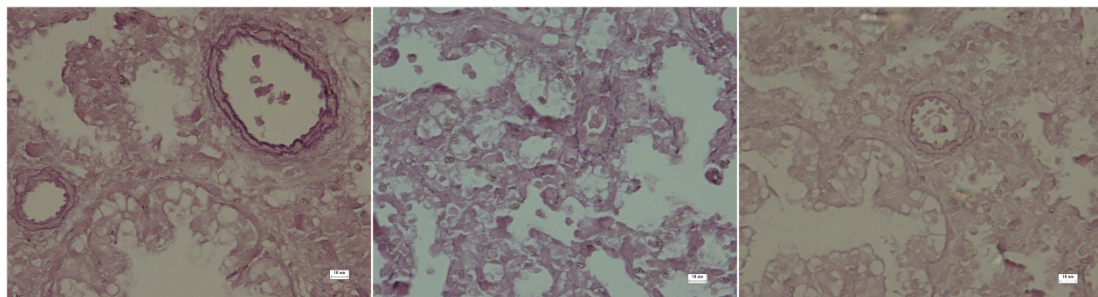
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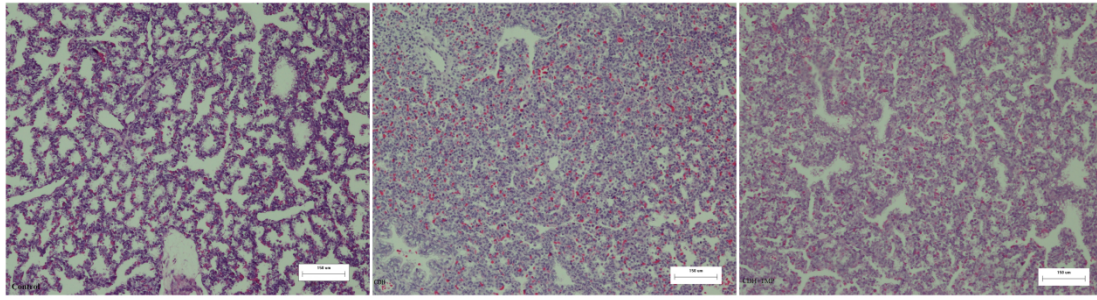
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431   **Figure 1**

433   **A**   **B**   **C**

434   **Figure 2**

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436   **A**   **B**   **C**
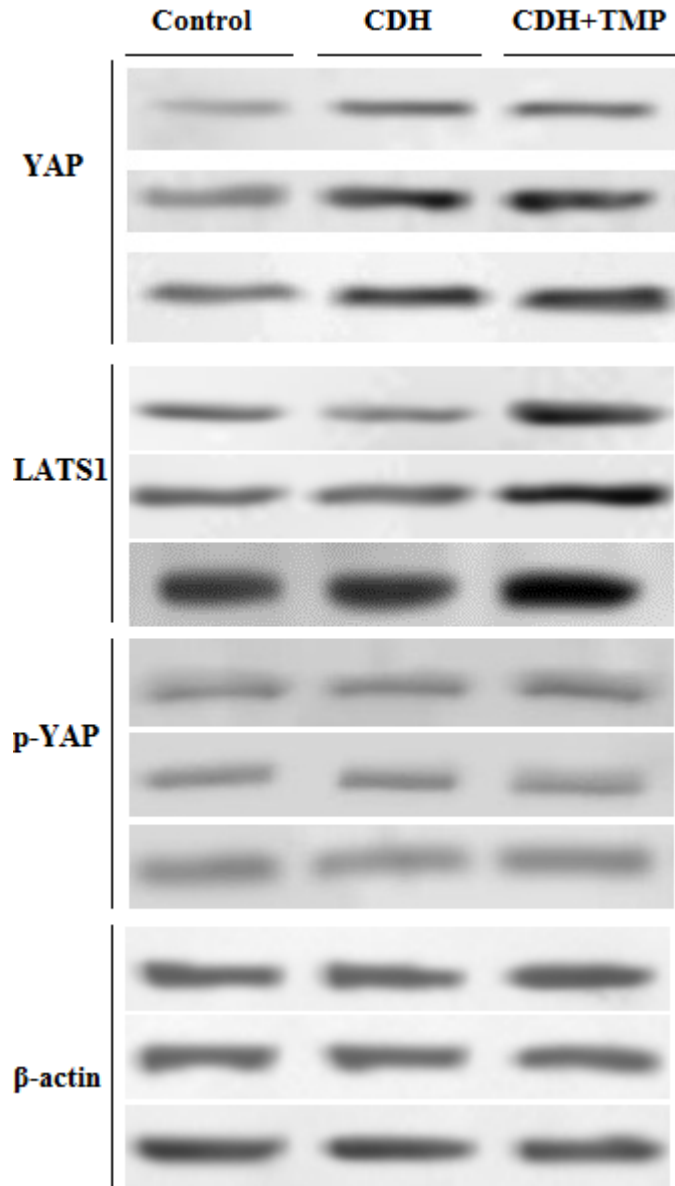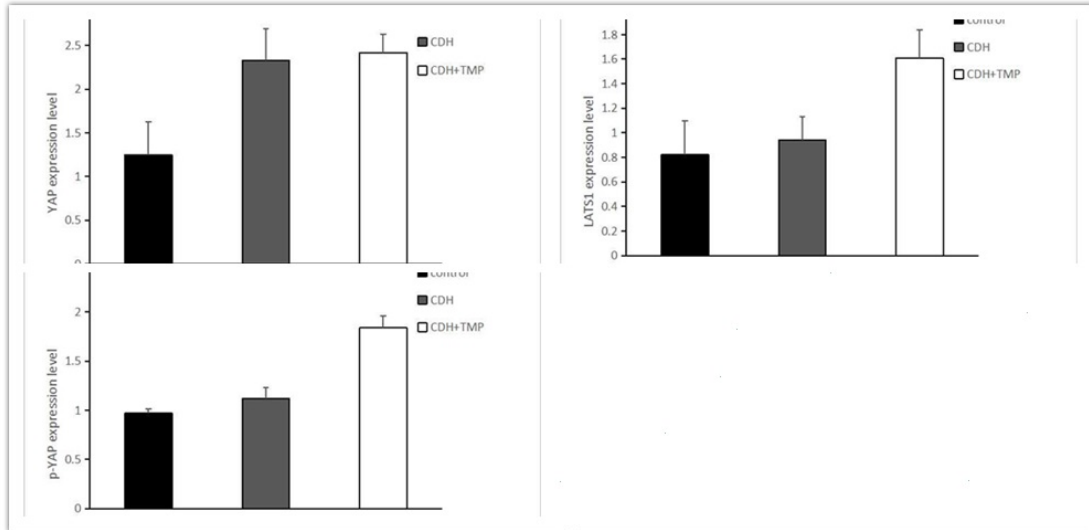
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447   **Figure 3**

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449   A

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451 B

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