Placental and Cleft Palate: Preliminary Insights from Integrated Metabolomic and Transcriptomic Analyses

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Article

Keywords: Cleft palate, Placenta, Dexamethasone, Metabolomics, Transcriptomics

Posted Date: February 1st, 2024

DOI: https://doi.org/10.21203/rs.3.rs-3855331/v1

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Additional Declarations: No competing interests reported.
Abstract

The correlation between glucocorticoids and cleft palate, a prevalent congenital abnormality, remains controversial, particularly concerning the uncertain status of placenta-palate formation. Utilizing a dexamethasone-induced cleft palate model in New Zealand rabbits, an integrated analysis of untargeted metabolomics and transcriptomics was conducted to explore the correlation between placental pathology and cleft palate. After dexamethasone treatment, approximately 60% of rabbit embryos developed cleft palates. Obvious pathologic change were observed on placenta including fibrosis, calcification, and necrosis. Transcriptomic analysis identified 4,744 differentially expressed genes in the placenta, involving pathways related to hormonal responses, vascular development, and inflammatory reactions. Metabolomic data revealed significant metabolic differences in both the placenta and amniotic fluid, with notable increases in urea levels in the placenta, while urea and arginine levels were markedly reduced in the amniotic fluid. Furthermore, metabolic disruptions in urea cycle, particularly an increase in arginase activity, may related to placental pathological changes. Overall, there is a correlation between placental pathology and cleft palate. Disruption of the urea cycle may contribute to placental lesions associated with the development of cleft palate. This offers a novel direction for understanding the mechanism of cleft palate formation, suggesting a potential significant role of placental metabolic disorders.

Introduction

Cleft palate is one of the most common congenital craniomaxillofacial birth defects in humans, resulting from abnormal development of the jaw and palate during embryonic development [1–2]. It imposes a significant financial and psychological burden on patients and their families due to the high costs and extensive medical assistance required [3]. Glucocorticoids (GCs), steroid hormones commonly used in treating inflammation, autoimmune diseases, and cancer, have been associated with orofacial clefts in offspring when used by pregnant women, as suggested by reports from the National Birth Defect Prevention Study (NBDPS) [4–5]. However, the causative link between GCs and human cleft palate formation remains controversial [6].

Dexamethasone, a widely used GC, has been shown to affect the proliferative capacity, apoptosis level, and intercellular adhesion of palatal mesenchymal cells, potentially leading to cleft palate formation in animal models [7–8]. Among various glucocorticoid-induced animal models of cleft palate, the dexamethasone-induced rabbit model is particularly valuable for exploring the mechanisms of cleft palate formation and craniofacial development. Our previous studies have established a New Zealand rabbit model for congenital cleft palate induced by dexamethasone. This model's significance stems from the similarities in bone accretion and muscle fiber composition between rabbits and humans, along with the animal's larger size compared to rodents, offering easier surgical access and greater tolerance for procedures [9]. Notably, there is still a lack of comprehensive research on the specific mechanisms, particularly the phenotypic variability in offspring following maternal exposure to dexamethasone. Thus, our study aims to explore the potential sources of this phenotypic variability in the model's offspring.
Recent research has increasingly illuminated the critical role of placental function and pathology in the development of congenital conditions, notably congenital heart defects (CHD), where abnormalities in placental form and function, such as altered blood flow and oxygen saturation, are now understood to significantly impact fetal cardiovascular development, potentially leading to CHD, and thus underscore the importance of placental health in predicting and managing these complex congenital conditions[10]. However, the correlation between the placenta and cleft palate formation has not yet been reported. Studies have shown that alterations in placental morphology and function can profoundly impact fetal development, potentially leading to a range of developmental disorders [11–12]. The placenta serves as a physical and nutritional link between mother and fetus and as a crucial mediator of hormonal and environmental influences, with its health and integrity being central to fetal development and wellbeing [13]. These insights underscore the importance of further investigating placental characteristics in the context of cleft palate, particularly how alterations in placental structure and function might contribute to this condition.

Recent multi-omics approaches have enhanced our understanding of disease pathogenesis from genetic, environmental, or developmental origins to their functional consequences and interactions [14–15]. In this study, we examined placental lesions in dexamethasone-treated New Zealand rabbits and analyzed phenotypic differences in offspring at both the transcriptional and metabolic levels. This exploration aims to provide insights for this model's future application and a new direction in understanding the mechanisms of cleft palate.

MATERIALS AND METHODS

2.1. Experimental design

The experiment design, including establishment of a dexamethasone-induced congenital cleft palate model in New Zealand rabbits, metabolomics, transcriptomics, statistical analysis, and biological validation, is summarized in a flow chart (Fig. 1).

2.2. Animals and ethics statement

The rabbits were provided by, and housed in, the Animal Experiment Center of Sichuan University, China. All experimental procedures on animals were in accordance with the National Institute of Health Guidelines for the Care and Use of Laboratory Animals. The study was approved by the Ethics Committee of West China College of Stomatology, Sichuan University (Protocol number: WCHSIRB-D-2017-090). This study adheres to the ARRIVE guidelines to ensure transparency and reproducibility in reporting animal research.

2.3. Induced congenital cleft palate model in New Zealand rabbits
Twenty-four female New Zealand rabbits, aged 40 weeks and weighing 4.5-5.0 kg, were selected and randomly divided into the dexamethasone group (DEX group) (n = 12) and the control group (n = 12) and fed in separate cages. On the same day, the female rabbits were caged with New Zealand male rabbits at 8:00 a.m., and the next day was considered as the first day of pregnancy (Embryonic day 0, ED0). The successful pregnancy of all females was confirmed at ED 7. From ED 13 to 16 inclusive, the pregnant rabbits in the DEX group were injected intramuscularly, at 8.00am daily, with 1.0 mg of DEX (1.0 ml: 5.0 mg, dexamethasone sodium phosphate, TianJin China). The control group were injected intramuscularly with 1 ml of saline.

On ED21, the pregnant rabbits were humanely euthanized using intravenous injection of a euthanasia agent approved for rabbits. The GD19 fetuses were carefully extracted following ethical guidelines for animal handling. The uterus was exposed by dissection, and then the amniotic fluid was extracted by syringe, and the embryos and their placental tissues were collected. The palatal phenotype was observed and assigned to one of three groups: control, dexamethasone cleft palate (D-CP), or dexamethasone non-cleft palate (D-NCP). Placenta samples were placed in freezing tubes and stored in liquid nitrogen, amniotic fluid samples were centrifuged and transferred to cryovials, and all placenta and amniotic samples were transferred to a -80°C freezer. In each of the three groups, ten samples were collected for metabolomics analysis and three samples were collected for transcriptomics analysis.

2.4. Sample preparation

Placenta tissue and palate tissue: The samples were thawed at 4°C and homogenized in 200 µL reagents (MeOH: H₂O = 4:1). To mix the samples, 800 µL reagent (MeOH: H₂O = 4:1) was then added. Samples were vortexed for 30 s and sonicated for 10 min, and then centrifuged (14000 rpm, 4°C) for 30 min. To 600 µL of the supernatant was added 500 µL reagent (MeOH: H₂O = 4:1) into the residual precipitation. Next, the same steps were performed as before. Supernatants were combined, and dried using liquid nitrogen. The sample was re-dissolved in 100 µL reagent (ACN: H₂O = 6:4). The sample was vortexed for 15 min and centrifuged (14000 rpm, 4°C) for 10 min. Quality control (QC) samples were prepared by taking 30 µL from each sample and mixing these together.

Amniotic fluid: The samples were thawed at 4°C and vortexed for 30 s. Then 50 µL of amniotic fluid was taken, and added into 250 µL of spiked methanol. Next, the samples were vortexed (4°C, 1000 rpm) for 30 min, and then centrifuged (4°C, 13300 rpm) for 20 min. A 200 µL aliquot of supernatant was then taken and placed into another tube, and dried using liquid nitrogen. The sample was re-dissolved in 200 µL of mobile phase mixture (solvent A-solvent B, 20:80, v/v). Quality control (QC) samples of amniotic fluid were prepared as described above for the placenta.

2.5. HILIC-Q-Orbitrap-HRMS analysis and data processing

The measurement was performed using an Ultimate 3000 rapidseparation liquid chromatograph coupled with Q Exactive Plus Q-Orbitrap HRMS (Thermo Fisher Scientific, Waltham, MA, USA).
Chromatographic separation was performed on a BEH Amide column (2.1X100 mm, 1.7 m) (Waters, Milford, MA, USA) with a flow rate of 0.3 mL/min. The column was maintained at a temperature of 40°C. The mobile phase was composed of solvent A (water) and solvent B (acetonitrile-water, 90:10, v/v), both containing 10 mM ammonium formate and 0.15% formic acid. The gradient was linearly changed as follows: 0 – 2 min, 100% B; 2 – 9 min from 100% B to 85% B; 9 – 14 min from 85% B to 50% B; 14 – 17 min, 50% B for column cleaning; 17 – 25 min, 100% B for column re-equilibration. The injection volume was 2 L, and the solvent for needle wash was 0.15% formic acid in methanol-water (50:50, v/v).

The quadrupole tandem orbitrap mass spectrometer Q Exactive Plus (Thermo Fisher Scientific) was equipped with a heated electrospray ionization (HESI) source, with the following source parameters: sheath gas flow at 35 arb, auxiliary gas flow at 10 arb, spray voltage at +3.2 kV in positive mode, capillary temperature at 320°C, auxiliary gas heater temperature at 350°C, s-lens RF level at 60. The data dependent MS2 parameters were: 1 microscans, mass resolution at 17,500 fwhm, 1e5 AGC target, 50 ms as maximum IT, TopN = 8 (fragmented the 8 most abundant ions of the previous full scan spectrum), isolation window within 1 m/z, and CE stepped at 20, 30, 40 eV.

Raw data were acquired by Xcalibur (Version 4.0, Thermo Fisher Scientific). Scheduled PRM data were integrated by TraceFinder (Version 4.1, Thermo Fisher Scientific) for predesigned transitions, based on a mass tolerance of 10 ppm centered around the theoretical m/z. For untargeted data processing, Compound Discoverer (Version 3.1, Thermo Fisher Scientific) was used for retention time alignment, peak detection, peak deconvolution, compound identification, KEGG pathway annotation, missing value imputation, and data normalization. The maximum shifts of mass accuracy and retention time for alignment were set as 5 ppm and 15 s, respectively. The parameters for peak detection were set as follows: mass tolerance, 10 ppm; S/N threshold, 10; minimum peak intensity, 100,000; maximum peak width, 30 s. The predicted adducts contained [M + H]+, [M + H–H2O]+, [M + Na]+, and [M + NH4]+ in positive mode.

The metabolic features were matched against the Human Metabolome Database (HMDB and KEGG database with a mass tolerance of 10 ppm. The structural similarity matching of metabolic features at the MS/MS level was based on three sources: our in-house database, the public database MassBank of North America (MoNA), and the commercial database mzCloud. After QC-based normalization, the reproducible metabolic features were selected from the dataset in which CV was less than 30% in pooled QC samples. Subsequently, the pre-processing dataset was submitted to R (version 4.0.2) for further statistical analysis.

Significant differential metabolites were determined by criteria as ANOVA P < 0.05 (FDR adjusted), absolute value of Log2 (fold change (FC)) > 1 and VIP (from partial least squares discriminant analysis (PLS-DA) and orthogonal partial least squares discriminant analysis (OPLS-DA)) > 1 was performed using the ropls R package.

2.6. RNA sequence analysis and data processing
Total RNA was extracted from tissues using the Trizol reagent (Invitrogen, USA) according to the manufacturer’s protocol. The RNA concentrations and purities were measured by a NanoDrop spectrophotometer, and RNA integrity was assessed by an Agilent bioanalyzer 2100. After passing the test, and following the instructions to build a library, the RNA from the samples was sequenced using the BGISEQ-500 platform to obtain transcriptome data. To get clean reads, the data was filtered using SOAPnuke software to remove low quality reads. After obtaining the clean reads, we use Hisat2 (version 2.0.4) to align the clean reads to GCF_000003625.3_OryCun2.0. Messenger RNA levels were quantified by the value of fragments per kilobase of exon per million mapped reads (FPKM). Differentially-expressed mRNAs were identified as those with q-values < 0.05 and | log2 (fold change) | >1.

### 2.7. Histopathological examination (H&E staining)

The placental tissues were fixed in 10% PBS neutral formalin, dehydrated, washed, and paraffin-embedded. The paraffin-embedded specimens were sectioned at xx µm, and stained with hematoxylin-eosin (H&E).

### 2.8. Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted from placenta tissue using TRIzol® (Life Technologies, Carlsbad, CA, USA), according to the manufacturer’s instructions. The RNA (1 µg) was reverse transcribed to cDNA templates using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA, USA). For semi-quantitative RT-PCR, the cDNA (25 ng) was amplified using SYBR® Green PCR Master Mix (Life Technologies) and oligonucleotide primers for specific target sequences from the Applied Biosystems 7500 Real-Time PCR system. The qRT-PCR parameters were as follows: denaturating at 95°C for 10 min, followed by 40 cycles of denaturing at 95°C for 15 s, and annealing/extension at 60°C for 60 s. The system software automatically calculated threshold cycles (Ct). The expression of GAPDH normalized the expression level of the target mRNA. The collected data were quantified using the $2^{-\Delta\Delta C_{t}}$ method. Primer sequences are listed in Table 1 (Supplementary material.).

### 2.9. TUNEL assay

TUNEL assay was performed using the One Step TUNEL Apoptosis Assay Kit (C1086; Beyotime Biotechnology) according to the experimental protocol.

### 2.10. Immunofluorescence staining

The placental tissue, fixed in 10% phosphate-buffered formalin, was dehydrated in a graded series of alcohol concentrations, embedded in paraffin, and cut into 5-µm sections. Next, the sections were placed in an appropriate amount of antigen repair solution (0.1M citric acid-sodium citrate buffer, pH 6.0) for antigen repair. Non-specific binding sites were blocked with PBS containing 5% goat serum for 1 hour at room temperature. For immunofluorescence staining, we incubated the specimens overnight with the first primary antibody (ARG1 (1:500) from Novus Biologicals (Abingdon, UK), GLB1(1:500) from Aviva
Systems Biology (San Diego, USA)) at 4°C. After washing with PBS, the corresponding secondary antibody was applied for 1 hour. The samples were washed with PBS, stained with DAPI (D8200; Solarbio) and mounted with cover clips.

2.11. Statistical analysis

All data, expressed as mean ± SD, were analyzed by GraphPad Prism 8.0 (GraphPad Software, CA, USA). The data analysis was conducted on a per-fetus basis, treating each fetus within a litter as an independent sample. Groups were compared using ANOVA, with appropriate tests for normality and homogeneity of variances. Statistically significant differences (*P < 0.05, **P < 0.01) were examined using the Student’s t-test.

RESULT

3.1 Placental Lesions were observed in Dex-induced Cleft Palate in Rabbits

After dexamethasone treatment, approximately 60% of rabbit embryos developed cleft palates, contrasting with the 40% embryos with intact palates (Fig. 2A). Generally, the degree of fetal and placental malformations was not consistent among three groups, and the D-CP had the highest degree of fetal and placental malformations. Histological examination using H&E staining revealed a significant increase in overall placental thickness for both D-CP and D-NCP groups, particularly in the decidua basalis layer (p < 0.01) (Fig. 2B). Pathological changes such as fibrosis, calcification, necrosis, and an increase in immature chromaffin cells were noted in the D-NCP and D-CP groups but absent in controls (Fig. 2C-D). Additionally, Masson staining indicated severe fibrosis in both D-NCP and D-CP groups, contrasting with controls (Fig. 2E). In terms of placental and embryonic weights, significant differences were observed. The placentas in the D-NCP group were notably heavier than those of the control group (p < 0.01), while the D-CP group’s placentas showed no significant weight difference from the controls. Conversely, control group embryos were heavier compared to both D-CP and D-NCP groups (p < 0.01), yet there was no discernible difference in embryo weight between these two Dex-treated groups. Placental efficiency, measured as the ratio of embryo weight to placenta weight, was higher in controls compared to the other groups (p < 0.05), but similar between the D-NCP and D-CP groups (Fig. 2E).

3.2 Transcriptomic profiling of placenta

In placenta, there were 4,744 differentially expressed genes (DEGs) (q-values < 0.05, | log2 (fold change) | >1) between the D-CP and control groups, of which 2,574 were up-regulated, and 2,170 were down-regulated in the D-CP group (Fig. 3A). There were 1118 DEGs (q-values < 0.05, | log2 (fold change) | >1) between the D-NCP and control groups, with 829 up-regulated and 289 down-regulated in the D-NCP group (Fig. 3B). There were 885 same genes between the above two DEGs sets (Fig. 3C). To discern the common pathological pathways in the placenta affected by dexamethasone, enrichment analyses were
performed on the 885 DEGs with the Metascape database. The results show hormonal responses, blood vascular development, wound responses, inflammatory reactions, and responses to decreased oxygen levels was significantly enriched (Fig. 3D). Furthermore, the key driving genes (KDG) analysis pinpointed 10 genes – CD44, SPP1, ITGB1, CCN2, MMP10, COL6A6, TNR, TNC, TNN, and PTGER4 – as playing pivotal roles in the observed genetic alterations (Fig. 3E).

To explore the source of the phenotypic varieties in fetus after DEX-treated, the transcriptomics differences of placenta between the D-CP and D-NCP groups were further analysed. There were 97 DEGs (q-values < 0.05, | log2 (fold change) | >1), of which 54 were up-regulated and 43 were down-regulated in the D-CP group compared to the D-NCP group (Fig. 3F). The Gene Ontology (GO) enrichment results of the 97 DEGs included regulation of angiogenesis, fatty acid metabolic process, regulation of cell junction assembly, response to glucocorticoids, regulation of vasculature development, response to nutrient levels, and regulation of excretion (Fig. 3G). The Disease Ontology (DO) enrichment results show hypercholesterolemia, carotid artery diseases, inflammation and maternal hypertension, showed were well enriched (Fig. 3H).

### 3.3 Metabolomic profiling of placenta

We employed Partial Least Squares Discriminant Analysis (PLS-DA) to distinguish the metabolite features in our study. In the placenta, there were 113 differentially expressed metabolites (DEMs) between the Dexamethasone-Treated Cleft Palate (D-CP) group and the control group (q-value < 0.05, | log2(fold change) | > 1), with 42 being upregulated and 71 downregulated in the D-CP group (Fig. 4A). Between the Dexamethasone-Treated Non-Cleft Palate (D-NCP) group and the control group, there were 92 differentially expressed metabolites (q-value < 0.05, | log2(fold change) | > 1), with 54 upregulated and 38 downregulated in the D-NCP group. And further differentiation of the metabolic features between the D-NCP and D-CP groups was achieved using Orthogonal Partial Least Squares Discriminant Analysis (OPLS-DA). In the placenta, there were 101 significant differential metabolites (VIP > 1, p < 0.05) identified between these two groups. Notable among these were lower levels of alanine, glutamine, L-aspartic acid, and taurine in the D-CP group, while cortisol, xanthosine, and lysoPC levels were higher (Fig. 4C). The pathways enriched in differences between the D-CP and control groups included Purine Metabolism and Arachidonic Acid Metabolism, as well as Galactose Metabolism and Aspartate Metabolism. The pathways that were differentially enriched between the D-CP and D-NCP groups included the Urea Cycle. Commonly highly enriched metabolic pathways between these groups were the Urea Cycle, Aspartate Metabolism, Nicotinate and Nicotinamide Metabolism(Fig. 4B).

### 3.4 Metabolomic profiling of amniotic fluid

In the amniotic fluid, there were 231 differentially expressed metabolites (DEMs) between the D-CP group and the control group (q-value < 0.05, | log2(fold change) | > 1), with 127 being upregulated and 104 downregulated in the D-CP group. Between the D-NCP group and the control group, there were 248
differentially expressed metabolites (q-value < 0.05, | log2(fold change) | > 1), with 118 upregulated and 130 downregulated. And we identified 34 differential metabolites (VIP > 1, p < 0.05), including D-glucose, taurine, and arginine, which decreased in normalized abundance among the control, D-NCP, and D-CP groups. Additionally, 59 differential metabolites (VIP > 1, p < 0.05), including xanthosine, leukotriene C4, and norepinephrine, were found to increase in normalized abundance among these groups. A comparison in the amniotic fluid revealed 28 differential metabolites (VIP > 1, p < 0.05) between the D-CP and D-NCP groups (Fig. 4D), with significant decreases in pyridoxal 5’-phosphate and PC levels in the D-CP group. The pathways enriched in differences between the D-CP and control groups included Glycine and Serine Metabolism and Spermidine and Spermine Biosynthesis. The differentially enriched pathways between the D-CP and D-NCP groups included Spermidine and Spermine Biosynthesis and Beta-Alanine Metabolism. Commonly highly enriched metabolic pathways in these groups were Glycine and Serine Metabolism, Spermidine and Spermine Biosynthesis, and Methionine Metabolism (Fig. 4B).

3.5 Increased arginase activity in urea cycle is involved in placental pathology

Metabolomics and transcriptomics data were combined analysed to identify differential metabolites and genes sharing a pathway. The urea cycle emerged as a potential target pathway implicated in dexamethasone-induced placental lesions. Metabolomic data for the placenta showed that several relevant metabolites in the urea cycle were altered after dexamethasone treatment, in particular urea was significantly elevated in the D-CP and D-NCP groups compared to controls (p < 0.05) (Fig. 5A). Interestingly, in the amniotic fluid, the levels of urea and arginine were significantly decreased in the D-NCP and D-CP groups compared to the controls (p < 0.01) (Fig. 5A). The expression of ARG1 and GLB1 in transcriptomics was also significantly increased in D-CP group compared to control group (p < 0.05) (Fig. 5A). The qRT-PCR results showed that the ARG1 and GLB1 mRNA were significantly increased in the D-NCP and D-CP groups compared to the control group (p < 0.05) (Fig. 5A). Immunofluorescence results showed that arginase-1 and β-galactosidase expression was increased after dexamethasone treatment in the decidua basali of the placenta (p < 0.05) (Fig. 5B-G). TUNEL staining showed that there was a significant increase in the numbers of apoptotic cells in the placenta of the D-CP and D-NCP groups compared to the control group (p < 0.05) (Fig. 5J-L).

Discussion

In this study, we adopted an innovative grouping approach (control, D-NCP, D-CP) to investigate the non-uniformity of palatal phenotypes in offspring following dexamethasone treatment. Beyond embryonic development, we explored the placental phenotype, an organ crucial for material exchange between the fetus and mother. It is well-established that placental dysgenesis is linked to fetal developmental diseases, such as hypoxia, malnutrition, and fetal distress [16–17]. Our findings indicate that, despite similar palatal phenotypes, the placentas in the D-NCP group exhibited significant pathological changes, including fibrosis and necrosis. These changes suggest a pronounced inflammatory response post
dexamethasone exposure, which could contribute to the development of cleft palate. Previous studies have reported dexamethasone-induced placental changes such as reduced placental weight, alterations in placental vascularization, and an increased incidence of placental inflammation [18–19]. These changes can impair the placenta’s capacity to support fetal development, potentially leading to various developmental disorders, including cleft palate. In our study, the presence of fibrosis and necrosis in the placental tissue, especially in the D-CP group, aligns with these reported effects of dexamethasone, highlighting the compound’s impact on placental health and its potential role in the pathogenesis of cleft palate.

Building on these insights, RNA-seq analysis revealed significant transcriptomic differences between the D-NCP and D-CP groups, particularly in pathways related to blood vessel development, inflammatory responses, and hypoxia. While both groups displayed alterations in these pathways, the magnitude of changes varied, indicating differential impacts of dexamethasone exposure. In the D-CP group, these changes were more pronounced, suggesting a stronger response to hypoxic stress and inflammation, which could contribute to the development of cleft palate. Conversely, the D-NCP group, despite showing similar pathway alterations, exhibited a less severe response, which might explain the absence of cleft palate formation in this group. In the D-CP group, the exacerbated response in inflammatory and hypoxic pathways might reflect a compromised placental function, leading to more severe developmental outcomes like Preeclampsia [20–22]. On the other hand, the D-NCP group, while still affected, may have retained a better adaptive response, preventing the full manifestation of developmental anomalies.

Interestingly, despite these differences, certain transcriptomic changes were consistent across both groups, suggesting some common mechanisms of dexamethasone action regardless of the phenotypic outcome. These shared changes might represent a fundamental response of the placenta to glucocorticoid exposure, affecting fetal development but not necessarily leading to cleft palate in every case. Furthermore, the complex role of glucocorticoids in fetal development is highlighted by these findings. While essential for normal development, their excessive levels, particularly under stress conditions, can lead to adverse outcomes. This dichotomy is evident in our findings, where dexamethasone exposure altered key developmental pathways, potentially through epigenomic mechanisms, resulting in varied outcomes in the D-NCP and D-CP groups [23–24].

Metabolomic profiles revealed significant metabolic variations in the placenta and amniotic fluid across the control, D-NCP, and D-CP groups. Specifically, the D-CP group exhibited elevated levels of metabolites such as xanthosine and cortisol, coupled with a reduction in essential amino acids like alanine and glutamine. These changes are indicative of a heightened inflammatory state and nutrient deficiency in the D-CP group, potentially impacting embryonic development [25–26]. Focusing on specific metabolites, elevated cortisol is a known indicator of increased stress and inflammation in the placenta. Elevated cortisol levels have been linked to altered fetal development and increased risk of developmental disorders, including cleft palate. Cortisol’s role in modulating placental function and its impact on fetal growth are well-documented, with recent studies suggesting that prolonged exposure to high cortisol levels during pregnancy can lead to epigenetic modifications affecting fetal gene expression [27–28]. Additionally, the decrease in amino acids such as glutamine and alanine is particularly concerning.
Glutamine plays a vital role in fetal growth and development, and its deficiency can lead to impaired placental function and fetal growth restriction. Recent research has highlighted the importance of adequate glutamine levels for healthy placental and fetal development, suggesting that a decrease in this amino acid could contribute to developmental anomalies like cleft palate [29–30]. The metabolomic changes observed in our study reflect the transcriptomic alterations, indicating an intricate interplay between genetic regulation and metabolic adaptation post-dexamethasone exposure. The pathway enrichment analysis of our metabolomic data further supports these observations, emphasizing pathways involved in inflammatory responses, nutrient transport, and hypoxia – all crucial for placental health and fetal development. These findings align with current research, enhancing our understanding of the complex molecular mechanisms underlying placental response to glucocorticoid exposure and its implications for fetal development and disease pathogenesis [30–31].

In addition to our findings in placental tissue, the metabolomic analysis of amniotic fluid revealed significant alterations, particularly in pathways like Glycine and Serine Metabolism, Spermidine and Spermine Biosynthesis, and Methionine Metabolism. These pathways play crucial roles in fetal development and placental health. For instance, alterations in Glycine and Serine Metabolism in the amniotic fluid can reflect changes in placental nutrient transport and fetal growth processes. Glycine and serine are essential for fetal cell proliferation and tissue growth, and disturbances in their metabolism could be associated with developmental anomalies [32–33]. Furthermore, the biosynthesis of spermidine and spermine, important for cellular growth and differentiation, was also found to be altered. These polyamines are vital for cell division and growth, and their dysregulation has been linked to various developmental disorders. Recent studies have indicated the importance of balanced polyamine levels for normal embryonic development, suggesting that deviations in their synthesis pathways could have profound effects on fetal outcomes [34]. Similarly, Methionine Metabolism plays a pivotal role in epigenetic regulation through methylation processes. Alterations in this pathway could affect DNA methylation patterns in the fetus, influencing gene expression and potentially leading to developmental irregularities. The importance of methionine and related metabolites in fetal development has been increasingly recognized, with studies showing that disruptions in methionine metabolism can have long-term consequences on fetal health and development [35–36]. These metabolomic insights from the amniotic fluid complement our findings in the placenta, offering a more comprehensive view of the biochemical environment experienced by the fetus. The changes observed in these specific metabolic pathways underscore the complex interplay between maternal treatments, such as dexamethasone, and fetal development. However, it is important to note that the functional implications of these alterations in amniotic fluid metabolites require further investigation to fully understand their impact on fetal development and long-term health outcomes.

Urea cycle pathway in both transcriptomic and metabolomic profiles of our study underscores its critical role in dexamethasone-induced placental changes. Arginine, a central amino acid in the urea cycle, is essential for maintaining placental blood supply and supporting embryonic development. Abnormalities in this pathway, particularly the increased activity of ARG1, are linked to vascular endothelial dysfunction, which can contribute to a variety of developmental and genetic disorders [37–38]. Our study’s findings of
elevated ARG1 activity suggest disruptions in the urea cycle, potentially impacting placental and fetal health. Furthermore, the increased levels of β-galactosidase (GLB1), identified in our study, serve as a marker of cellular senescence. This increase indicates a state of vascular endothelial cell senescence and apoptosis within the placenta, which can have significant implications for placental function and fetal development. Vascular endothelial dysfunction in the placenta is a critical factor that can lead to compromised fetal blood supply and nutrient delivery, potentially resulting in developmental abnormalities [39–40]. Recent research has highlighted the importance of the urea and arginine cycles in placental health. Arginine is not only crucial for the synthesis of nitric oxide, which regulates placental blood flow, but also plays a significant role in modulating immune responses and cellular metabolism within the placenta. Disruptions in arginine metabolism have been associated with conditions such as preeclampsia and intrauterine growth restriction, both of which are characterized by impaired placental function [41–42]. However, our study faces limitations in fully validating the functional implications of these findings. While we have identified key changes in the urea cycle and related metabolites, further research is necessary to elucidate the precise mechanisms by which these alterations impact placental function and fetal development. Such studies could involve more detailed functional assays and experimental models to directly assess the effects of altered urea and arginine cycle activities on placental health and fetal outcomes.

Declarations

Data availability

Data will be made available on request.

Acknowledgements

This study was supported by the Key Program of Science & Technology Department of Sichuan Province, China.(Grant No.2022ZDYF2641,2023ZDYF2883 and 2023ZDYF2596).

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Conflicts of interest

No conflict of interest exists.

Author Contribution

In this article, the authors contribute as follows: Authors Lin and Wei are jointly responsible for writing the whole article. They were involved in the design of the study, the analysis of the data and the interpretation
of the results, and together drafted the main part of the article. Authors Luo, Zhang, Jing, Wang, and Shi were responsible for producing Figures 1 through 5, collecting and collating data, and designing and drawing these charts based on the research results to ensure that the charts were accurate, easy to understand, and consistent with the content of the paper. Author Gong and Li made the design of the article, and revised and polished the whole article after the writing. They gave valuable advice on language, style and structure to make the essay more fluent and in line with academic norms. All authors participated in the discussion of the article, conducted in-depth discussion and analysis of the research methods and results, and jointly decided on the final content.

References


**Figures**
Figure 1

The experimental design. (Amniotic fluid was only metabolically analyzed.) NE—Normal Saline; DEX—Dexamethasone; AF—Amniotic Fluid.
Figure 2

Embryos, placenta, and palate alterations after dexamethasone treatment. A. Palatal morphology. (P— palate; *—cleft.) B. Appearance of the embryos and placentas. C. Placenta weight in every group. (n=16.) D. Embryo weight in every group. (n=16.) E. Placental efficiency in every group. (Placental efficiency is the ratio of the weight of the embryo to the weight of the placenta, n=16.) F-I. Placental thickness in every group. (LZT— Labyrinth Zone Thickness, DBT—Decidua Basali Thickness, JZ—Junctional Zone, n=3.). Scale bar, 1mm. J,K. HE staining of placental sections from three groups. (RBC—Red Blood Cell, Tb—
trophoblast, NV—Normal Vessel, ICC—Immature Chromaffin Cells. FV—Fibrotic Vessel, ▲—necrotic tissue, arrows in K indicate pathological characteristics existing in D-NCP group and D-CP group.) Scale bar, 50μm. L. Masson staining of placental tissue. (Collagen fibers are blue, cytoplasm, myofibrils, and glia are red, and nuclei are blue-black.). Scale bar, 100μm. Data in C, D, E, G, H, I were means and error bars represented SD. For all bar graphs, ***p < 0.001, **p < 0.01, *p < 0.05, n.s. ≥ 0.05.

**Figure 3**

Effects of dexamethasone exposure on the transcriptome of the placenta and Differences in transcriptomics of placenta between the D-CP and D-NCP groups. A. The volcano plot based on Control and D-CP samples. Eachpoint represents a specific gene; genes that were up-regulated, down-regulated, and not significantly differentially expressed (no-DEGS) were marked with different
colors. B. The volcano plot based on Control and D-NCP samples. C. There were 885 DEGs between the D-CP and Control groups were identical to DEGs between the D-NCP and Control groups, and the trends were the same. D. The top 20 enrichment results of the 885 DEGs. One per cluster, using a discrete color scale to represent statistical significance. E. The key driving genes in the 885 DEGs. KDG—Key Driving Genes; IG—Initial Genes; RG—Related Genes. G. Expression level of ADM, CD44, SPP1, ITGB1, CCN2, MMP10, COL6A6, TNR, TNC, TNN, PTGER4 in placenta tissue in three groups. F. The volcano plot based on D-CP and D-CP samples. G. The Gene Ontology (GO) enrichment results of the 97 DEGs. H. The Disease Ontology (DO) enrichment results of the 97 DEGs.

Figure 4

The differential metabolites among three groups in placenta and amniotic fluid and The differential metabolites between the D-CP and D-NCP groups. A. The diagram of the mechanism of GCs-induced cleft palate associated with placental dysfunction. (DEX—Dexamethasone; AF—Amniotic Fluid; CPE—Cleft palate embryo; UW—Uterine Wall; P—placenta). The red circle means that the substance in it are upregulated, and the green circle means that the substance in it are downregulated. B. Top 10 metabolic pathways with the greatest differential enrichment, with red indicating pathways commonly enriched in differential metabolic profiles (CP/CON displayed at the top, NCP/CP at the bottom). C. The volcano plot
based on the D-NCP and D-CP placenta samples; OPLS-DA scores plot based on the D-NCP and D-CP placenta samples; Clustering heat maps of differential metabolites in placenta in the D-CP and D-NCP group. D. The volcano plot based on the D-NCP and D-CP amniotic fluid samples; OPLS-DA scores plot based on the D-NCP and D-CP amniotic fluid samples; Clustering heat maps of differential metabolites in amniotic fluid in the D-CP and D-NCP group. E. Key differential metabolites level in D-CP and D-NCP group in placenta. F. Key differential metabolites level in D-CP and D-NCP group in amniotic fluid.
Figure 5

Increased arginase activity involved in placental pathological changes. A. Changes in metabolites involved in the urea cycle in the placental and amniotic fluid. B, C, D. Arginase-1 expression in placental tissue (n=3). Scale bar, 50μm. E, F, G. β-galactosidase expression in placental tissue. n=3. Scale bar, 50μm. H, I. ARG1 and GLB1 mRNA expression in placenta tissue in qRT-PCR. J, K, L. TUNEL staining of placental tissue. k was the labyrinth zone part, l was the decidua basali part (n=3). Scale bar, 100μm. Data in A, C, D, F, G, H, I, K, L were means and error bars represented SD. For all bar graphs, ***p < 0.001, **p < 0.01, *p < 0.05, n.s. ≥ 0.05.

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