Original Article
Apoptotic genes expression in placenta of clubfoot-like fetus pregnant rats

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Abstract: Objective: To investigate the apoptotic gene expression of placenta in an all-trans-retinoic acid (ATRA) induced fetus congenital clubfoot pregnant rat model. Methods: Sprague-Dawley (SD) rats were divided randomly into ATRA-exposed group and control group. On day 10 of pregnancy, a dose of 120 mg/kg ATRA dissolved in mineral oil was given intragastrically to the rats in the ATRA-exposed group and equivalent volume of mineral oil was given intragastrically to the control rats. Fetuses were delivered on day 20 of pregnancy, the placenta was collected for the pathological and biochemical analysis. Results: Clubfoot-like deformity fetuses were observed in the ATRA-exposed group and none with deformity was found in the control group. The pro-apoptosis in placenta of ATRA-exposed group was measured by flow cytometry. Moreover, compared with the control group, lower expression of Bcl-2 and higher expression of BAX were found in the ATRA-exposed group in both mRNA and protein level. Immunohistochemical labeling of Bcl-2 in the control group was more intense while BAX labeling in the ATRA-exposed group was more intense. Additionally, the caspase-3 activity was also significantly increased in the ATRA-exposed group than control group. Conclusion: In our research, we found a pro-apoptosis in placenta in the ATRA-exposed pregnant rats, indicating a possible association between placental apoptosis and congenital clubfoot.

Keywords: Animal model, apoptosis, placenta, clubfoot

Introduction

Congenital talipes equinovarus (CTEV), also called isolated clubfoot, is one of the most common orthopedic birth defects with four clinical foot characteristics: forefoot adduction, hindfoot varus, midfoot cavus, and hindfoot equinus. CTEV occurs in 1 per 1,000 live births in the United States, but the birth prevalence varies substantially among different ethnic groups. Most cases of CTEV are bilateral, but the right side is affected more often than the left in unilateral cases [1, 2]. CTEV affects the physical and psychological health of children, however, the etiology of clubfoot remains unknown and controversial.

Many studies have shown that ATRA could induce clubfoot in animal mode, and this model has proven to be suitable for detecting some causative factors for CTEV [3, 4]. For example, researchers have focused on the influence directly on the fetus limb development in the animal model [5].

Apoptosis is a programmed cell death which a cell actively induces its own death. But an imbalance of this process in placenta leads to placental dysfunction [6]. Placental dysfunction causes the inadequate supply of nutrients and oxygen to support normal growth of the fetus, resulting in fetal blood flow redistribution and diminished amniotic fluid with subsequent reduction of the space around the fetus [7, 8], and the mechanisms above affect the physiological development and position of the lower limbs [9]. Thus, it is plausible that apoptosis in placenta may be related to CTEV. However, few researches concentrate on placenta apoptosis and congenital clubfoot.

In our study, we detected apoptosis in placenta, and tried to explore the relationships between placental factors and limb malformation in this animal model.

Table 1. Primers and sequences in real-time PCR

Target gene	Primer sequences	Tm (°C)
Bcl-2	forward: 5'-GGGATACTGGAGATGAAGACT-3' reverse: 5'-CCACCGAACTCAAAGAAGG-3'	55
BAX	forward: 5'-GCAAACTGGTGCTCAAGG-3' reverse: 5'-TCCCGAAGTAGGAAAGGAG-3'	55
GAPDH	forward: 5'-GGGGCTCTCTGCTCCTCCCTG-3' reverse: 5'-AGGCGTCCGATACGGCCAAA-3'	55

Materials and methods

Animals and models

Female SD rats weighing 250-300 g were kept in cages and maintained under standard conditions of temperature, humidity and light. The SD rats were fed with rat chow and water ad libitum. The rats were assigned randomly into two groups depending on random number table: one ATRA-exposed group (n=10) and one control group (n=10). The day 0 of pregnancy was considered when the vaginal plug was detected after overnight mating. On day 10 of pregnancy, the experimental rats received a single dose of ATRA (SIGMA-ALDRICH) (120 mg/kg dissolved in mineral oil 40 mg/ml) by gavage. On the same day, the control rats were given an equivalent dose of mineral oil (SIGMA-ALDRICH) [10]. Fetuses and placentas were delivered in aseptic conditions by cesarean section under ether anesthesia on day 20 of pregnancy. Fetuses with external malformations were examined and photo was obtained. Part of ATRA-exposed and control placentas were fixed by 10% neutral formalin and embedded in paraffin wax. The remaining placentas were used for biochemical analysis.

All experiments were approved by the institutional Research Committee, and were performed strictly according to the Nanjing Medical University (Nanjing, China) regulations for animal care.

Flow cytometric analysis

Placental apoptosis was measured using an Annexin V-FITC apoptosis detection kit (BD Pharmingen) [11]. Fresh cell suspensions collected from placental were rinsed five times with PBS and resuspended in 100 μl of binding buffer at a concentration of 1×10^6 cells/ml. The cells were then stained according to the manufacturer's recommended protocol, and an immediate analysis was per-formed using a fluorescence activated cell sorter (FACS) Calibur flow cytometer (BD).

RNA purification and real-time PCR

Total RNA was isolated from placentas using Trizol Reagent (Invitrogen), according to the manufacturer's instructions, and quantified by measuring the absorbency at 260 nm wavelength. Reverse transcription was performed from 1 μg of total RNA for each sample using the Transcriptor First Strand cDNA Synthesis Kit (Roche) according to the manufacturer's instructions. Quantitative real-time PCR amplification in a volume of 5 μl were performed on ABI Prism 7900 HT (Applied Biosystems) with the FastStart Universal SYBR Green Master (Roche) according to the manufacturer's instructions for quantification of gene expression. Cycling conditions were standard absolute quantification thermal cycling program and using the SDS 2.3 software to determine the cycle threshold (Ct). The thermocycler program included a step of denaturation at 95°C for 10 min followed by 40 repeats of 95°C for 15 s and 60°C for 1 min. The sequences of primer pairs are shown in **Table 1**. The housekeeping gene Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control. The relative expression ratio of a target gene was calculated using $2^{(-\Delta\Delta Ct)}$ method.

Western blot analysis

Placenta were solubilized in lysis buffer (10 mM EGTA, 5 mM EDTA, proteinase inhibitor cocktail, 20 mM PNPP, 1 mM Na_3VO_4, 30 Mm β-Glycerophosphate, 0.05 mM NaF and so on). The homogenates were centrifuged at 10,000 rpm for 20 min (4°C), the supernatants were harvested as cytosolic fractions for immunoblot. Protein content was measured by BCA protein assay kit (Biouniquer technology) according to the manufacturer's instructions. The protein samples (50 μg) were fractionated by 15% SDS-PAGE and transferred to nitrocellulose membrane (NC, Milipore Corp). Blots were blocked and immunostained with primary rabbit polyclonal antibody for Bcl-2 (Santa Cruz, 1:400 dilution), BAX (Santa Cruz, 1:400 dilution), followed by secondary antibody (Cell
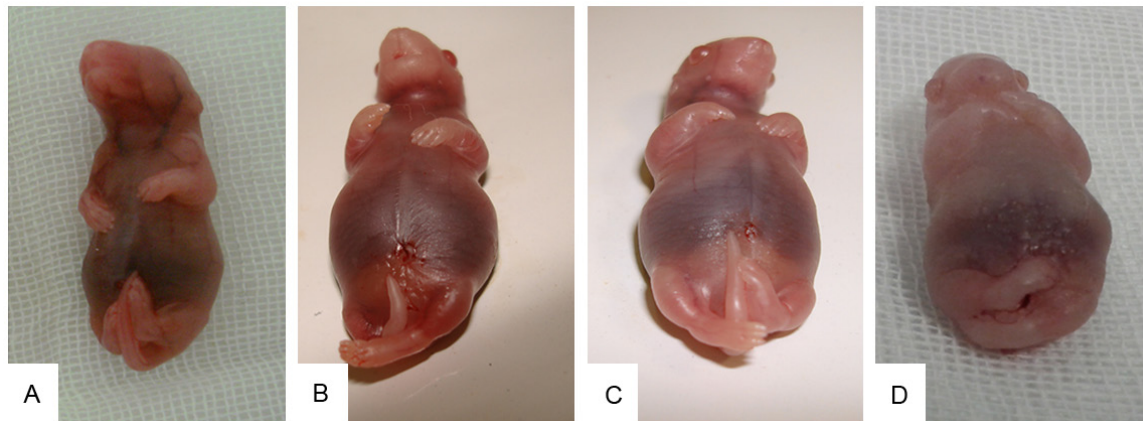
Figure 1. Twenty-day Embryos' hindlimb appearance in control and ATRA group. A: Normal fetus. B: Left side hindlimb clubfoot-like deformity. C: Right side hindlimb clubfoot-like deformity. D: Bilateral clubfoot-like deformity.

Table 2. Characteristics of fetus and placenta

Characteristics	ATRA	NC	P
Fetus			
Weight of rats (g)	3.2392 ± 0.84917	3.3871 ± 0.71104	0.330[a]
Malformation	(63/122)	(0/131)	<0.001[b]
Placenta			
Weight of placenta (g)	0.4815 ± 0.10712	0.5110 ± 0.12414	0.187[a]
Diameter of placenta (cm)	1.3046 ± 0.13628	1.3438 ± 0.13512	0.133[a]

[a]by t-test, [b]by chi-square test.

Signaling, 1:2,000 dilution) conjugated with horseradish peroxidase (HRP) and the specific signals were visualized by the enhanced chemiluminescence (ECL Western blotting detection reagents, Amersham Life Science Limited). The amount of GAPDH (34 kDa) was used as a loading control to correct the expression of Bcl-2 (26 kDa) and BAX (23 kDa). Densitometric analysis was performed using Quantity One Software (Bio-Rad).

Immunohistochemistry

Immunostain were performed on 8-µm-thick paraffin-embedded tissue sections by streptavidin-avidin-biotin method (ZSGB-BIO). Standard protocol of immunohistochemistry reactions was according to the manufacturer's instructions. The primary antibodies were as follows: Bcl-2 (Santa Cruz, 1:50 dilution), BAX(Santa Cruz, 1:50 dilution). After incubation with primary antibodies, the sections were washed with PBS and incubated with the secondary antibody (ZSGB-BIO). Visualization was completed using diaminobenzidine (DAB) (ZSGB-BIO) and counterstained with hematoxylin solution. Experimental and control were taken into consideration to interpret stainings. Negative control was included and performed the same immunohistochemical method with omission of the primary antibodies.

Caspase-3 colorimetric assay

The activity of caspase-3-like proteases in the tissue lysate was detected by Caspase-3 Colorimetric Assay Kit (KeyGEN). The protocol and the data processing were according to the manufacturer's instructions.

Statistical test

The mean and standard deviations of each variable were calculated, ANOVA is not a common method in column analyses. Student's t-test is not suitable neither because of the non-normal distribution of the data when it was transferred. Then the Mann-Whitney U test (data with heteroscedasticity as well as non-normal distribution) could be conducted. All statistical analyses were carried out using Stata (Version 9.0), and P<0.05 was considered to be significant.

Results

Twenty-day embryos

Hypoplasia and malformation were found in the ATRA-exposed group, but none in control, the
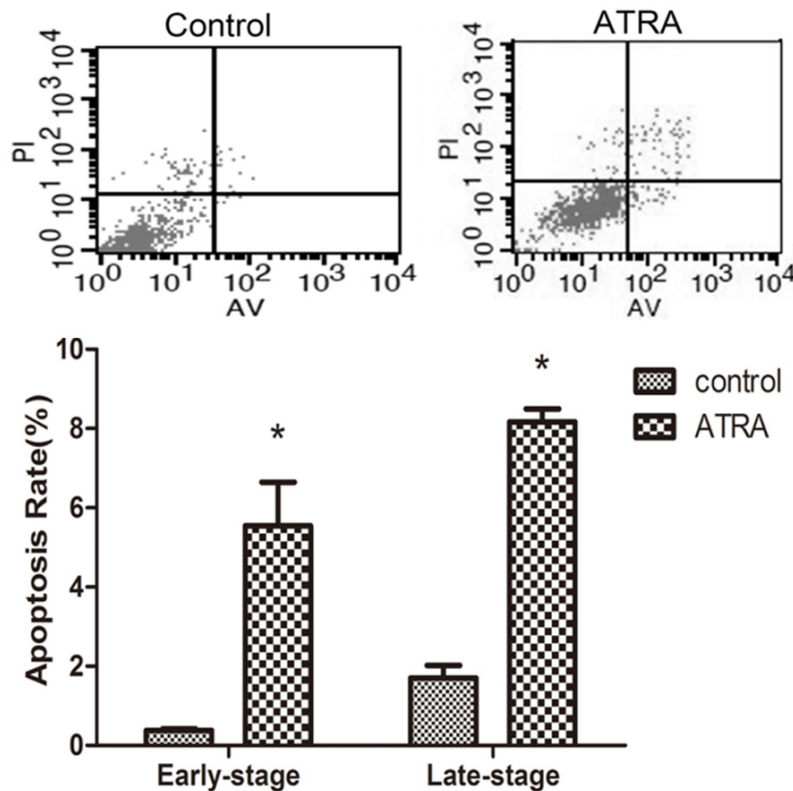
Figure 2. Flow cytometry for placenta apoptosis. The upper right quadrant contains late-stage apoptotic cells, and the lower right quadrant contains early-stage apoptotic cells. Placenta apoptosis was markedly caused in ATRA (n=8 for each group).

extent of these deformities ranged from mild to severe (**Figure 1A-D**). Fetuses of clubfoot-like deformity including unilateral (left or right side) and bilateral clubfoot were 51.6% (63/122) among all fetuses in ATRA-exposed group. The general information of fetus and placenta was listed in **Table 2**.

Flow cytometry

Placenta apoptosis was analyzed using Annexin V-FITC and PI cell staining with a flow cytometer. The results indicated that the rate of apoptosis in the ATRA-exposed group was increased compared to that of the control group (*P*<0.05; **Figure 2**).

Apoptotic gene expression

The expression of Bcl-2 gene and BAX gene in each groups were statistically significant. In the ATRA-exposed group, the Bcl-2 gene was low expressed (*P*<0.05) while BAX gene was high expressed (*P*<0.05) compared to the control group in both mRNA level (**Figure 3A**, **3B**) and

protein level (**Figure 3C**, **3D**).

Immunohistochemical labeling of Bcl-2 labeling (**Figure 4B**, **4C**) in control group was more intense than ATRA-exposed group, while BAX (**Figure 4E**, **4F**) in ATRA-exposed group was more intense than control group in cytoplasm.

The level of caspase-3 activity were increased statistically significant (*P*< 0.05) in ATRA-exposed group than control group (**Figure 5**).

Discussion

The certain mechanisms for CTEV remain uncertain, and theories on causative factors include: nerve lesion [12]; muscular abnormality [13]; vascular defect [14]; intra-uterine restriction [15]; a genetic component, either alone idiopathic congenital clubfoot or with a gene-environment interaction [16]. In this study we demonstrated the relation between abnormal placental apoptosis and CTEV.

Apoptosis occurs during normal development and is essential for the regulation of the balance between the loss of non-functional or old cells and the formation of new ones in organs. Researchers have suggested that apoptosis is important for the normal development, remodeling, and aging of placenta [17], however, the dysfunction of the apoptotic mechanism is associated with the dysfunction of placenta. In our research, we utilize the flow cytometric analysis to reveal an increased rate of apoptosis in placenta. Thus, it is plausible that a pro-apoptosis in placenta is related to CTEV.

The bcl-2 family members play a central role in the regulation of apoptosis [18, 19]. Apoptosis is activated via the extrinsic or intrinsic pathway. Activation of the intrinsic pathway leads to alteration in mitochondrial membrane permea-
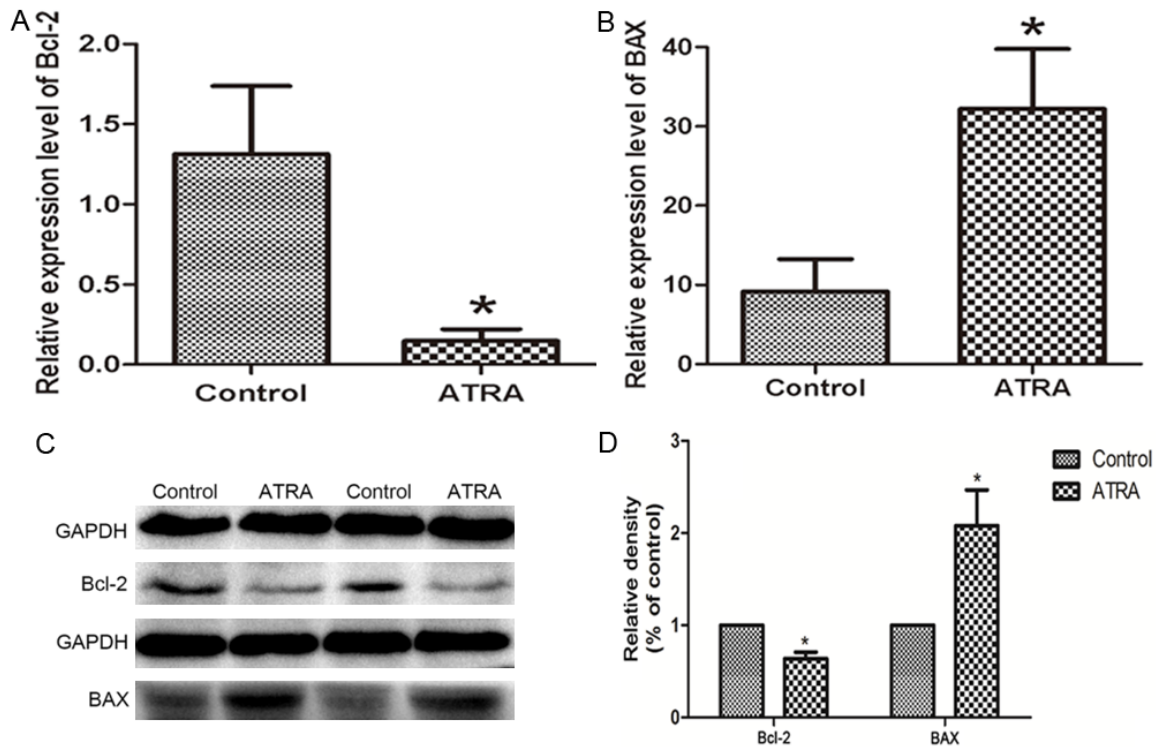
Figure 3. Apoptotic Gene Expression in placenta. A: The mRNA expression pattern of Bcl-2 in control and ATRA (n=10 for each group). B: The mRNA expression pattern of BAX in control and ATRA (n=10 for each group). C: The panel shows a representative Western blot. D: Relative density pattern of Western blot in control and ATRA.
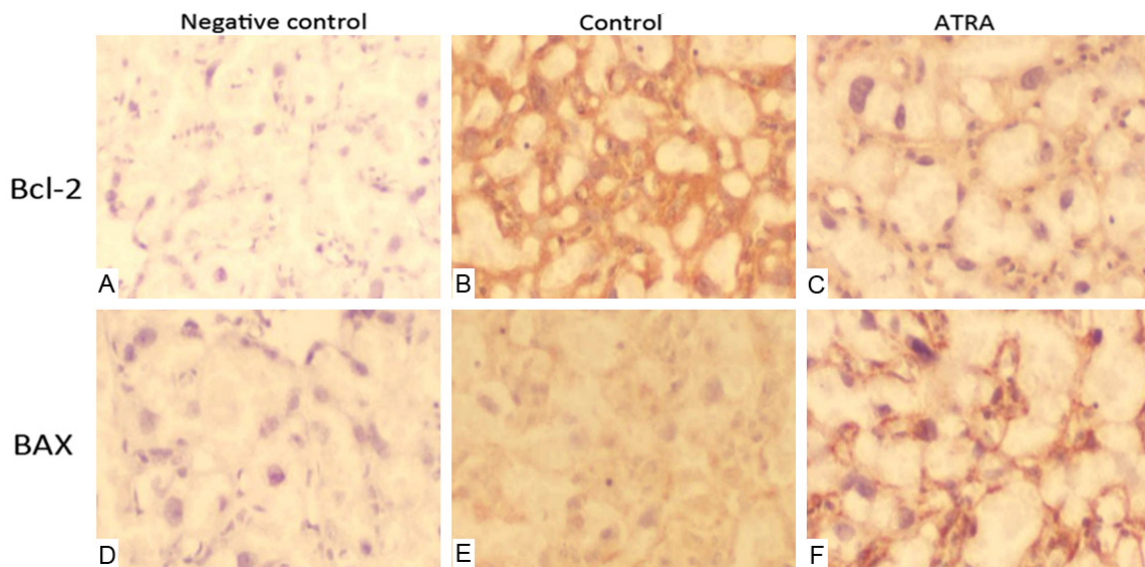

Figure 4. Photomicrograph for representative images of immunohistochemistry for Bcl-2 and BAX staining in placenta. (B, C) Placenta in ATRA with weak Bcl-2 staining, while with strong Bcl-2 staining in control. (E, F) Placenta in ATRA with strong BAX staining, while with weak BAX staining in control. (A, D) Negative control without the Bcl-2 and BAX primary antibodies. Magnification × 100 for (A-F).

bility because of an imbalance between pro- and anti-apoptotic Bcl-2 family members [20]. In our research, we found that ATRA-exposed group had low expression of Bcl-2 while BAX had high expression in both mRNA level and protein level compared to control group.
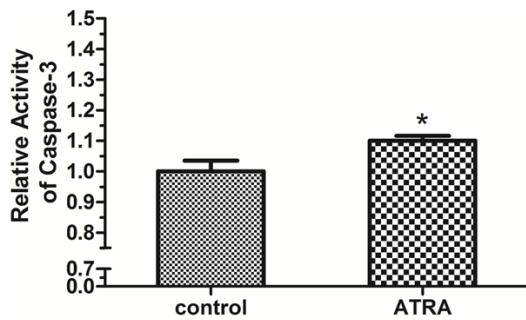
Figure 5. Caspase-3 activity assay. The results shown represent the mean caspase-3 activity. (n=9 for each group).

Immunohistochemistry in ATRA-exposed group proved the same tendency on Bcl-2 and BAX compared to control group in tissue section. It has been shown that the pro-survival Bcl-2 blocks the release of cytochrome C from the mitochondria, while the pro-apoptotic BAX leads to the release of cytochrome C [21]. The unbalance of BAX and Bcl-2 result in the increased permeability of mitochondrial membrane and cytochrome C are released into cytoplasm. In the cytosol, cytochrome C mediates the allosteric activation of apoptosis-protease activating factor 1, which is necessary for the proteolytic maturation of caspase-3 [22]. Caspases are the executors in the process of apoptosis. They are cysteine proteases with specificity for aspartic acid and are divided into two subgroups: the upstream or initiator caspases and the downstream or effector caspases. The effector caspases perform directly in the execution of apoptotic cells. Caspase-3 is one of the downstream caspases, once activated, it is thought to be one of these molecules which are responsible for the actual demolition during cell apoptosis [23]. Our research also showed a higher caspase-3 activity in ATRA-exposed group than control group. All the data we had detected supported that there existed a pro-apoptosis in placenta in ATRA-exposed group.

An imbalance of apoptotic pathways throughout pregnancy leads to dangerous pathological status with placental dysfunction [24]. When the normal trophoblastic invasion and modification of spiral arteries is disrupted, increased vascular resistance and decreased placental perfusion are consequently followed, which leads to insufficient supply for nutrients and oxygen for normal fetus growth [25]. In order to maintain the arterial oxygen concentration normal or minimally decreased until substrate delivery is severely reduced [26], chronic hypoxia is responsible for fetal blood flow redistribution favoring to the vital organs: brain, heart and adrenal gland, while lungs, digestive tract, kidneys and limbs are hypoperfused, which may induce the limitation of limb development. This phenomenon is the so-called "brain-sparing" effect, which may preserve the cerebral metabolism and reduce the amniotic fluid volume [27-29]. The normal amount of amniotic fluid is usually adequate to cushion the fetus and allows for normal growth and mobility. One of the major functions of amniotic fluid is to extend the uterus, thereby enabling the fetus to move freely and grow with equal pressure in all regions without excessive or localized constraint [30]. An overt or subclinical leakage of amniotic fluid decreases the intra-amniotic pressure may disrupt the normal physiological development and position of the lower limbs [9, 31]. We consider that it is placental pro-apoptosis which leads to placental dysfunction with deficiency of lower limb blood supply and development space that may induce clubfoot. Therefore, the imbalance of apoptosis in placenta may play a possible role in the development of congenital clubfoot.

In conclusion, we found a pro-apoptosis in placenta in the ATRA-exposed pregnant rats. These findings not only indicate an association between placental apoptosis and congenital clubfoot, but also reveal that anti-apoptotic therapy may reverse the progress of apoptosis in placenta and recovery the function of placenta, which may have some curative effect for CETV as an adjuvant therapy. Further studies which include the clinical placenta samples are still needed to confirm our findings.

Disclosure of conflict of interest

None.

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