PIBB 生物化学与生物物理进展
Progress in Biochemistry and Biophysics
2007, 34(11): 1182~1189
www.pibb.ac.cn

Modulation of Retinoic Receptor Alpha and Beta and Its Links With Beta-catenin and Caspase-3 After Maternal Exposure to All-trans-retinoic Acid in KM Mouse Fetuses*

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Abstract Epidemiologic studies suggest that intake of excess all-trans-retinoic acid (RA) during embryogenesis induces various developmental defects and the central nervous system (CNS) represents a major site of the teratogenic action of RA. It is therefore important to understand which parameters are affected early by excessive RA in order to devise and improve protective nutritional strategies. The modulations of beta-catenin and caspase-3 levels were investigated in the KM mouse embryo following maternal treatment with a single oral dose of 30mg/kg body weight of RA during the neurula period. In addition, retinoic receptors (RARs) are key transcription factors regulating gene expression in response to RA-activated signals. So the experiment was designed to evaluate whether the alterations in protein expression of RAR alpha and beta during the time of neural tube closure were induced by excessive RA. Maternal intake of excess RA induced early downregulation of RAR alpha and beta, beta-catenin and caspase-3 expression, which was followed by an increase in their expressive levels in the neural tube tissue of mouse embryos. This finding suggests that the alterations in the expression profile of RAR alpha and beta, beta-catenin and caspase-3 may be implicated in the teratogenesis induced by excess RA in KM mouse embryo.

Key words retinoic acid receptor, beta-catenin, caspase-3, developmental defects, RA, mouse

Vitamin A is an essential nutrient during embryonic development and has a profound effect on the morphogenesia and embryonic patterning of a wide range of species [1]. However, excessive intake of vitamin A or its derivatives during embryonic development causes a variety of congenital defects, depending on the dose and the stage at which it is administered [2~5]. All-trans-retinoic acid (RA) is an active metabolin of vitamin A and the maternal administration of RA at a dose of 30 mg/kg body weight on 7.75 day post coitum (d.p.c.) causes a series of developmental malformations in mouse embryo and neural tube defects (NTDs) are the most common phenotype [6]. NTD is one of the most common developmental defects in humans and it occurs at the rate of approximately 1 out of 1 000 births [7]. The etiology of this defect appears to be multifactorial and includes genetic and environmental factors. However, the exactly causes of NTDs is still unknown [8].

It is likely that vitamin A or its derivatives such as RA induces NTDs at the level of the cell signaling pathways rather than at the level of mutations. RA usually plays its physiological or teratogenic role by binding to the retinoic receptors (RARs) and as a result, modulating the transcription of downstream genes [9]. Thus, growing evidence points to the involvement of RARs in NTDs induced by RA. RARs belonging to the class Ⅱ superfamily heterodimerize with the retinoid X receptors (RXRs) and function as ligand-activated transcription factors to regulate a wide range of target genes that affect almost all biologic processes. Many studies reported the possible role that RARs might play in the NTDs induced by RA. However, the exactly range of RARs activity remains poorly understood.

* This work was supported by a grant from The Natural Science Foundation of Shandong Province (Y2005C63).
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Received: March 15, 2007 Accepted: May 31, 2007

In addition to the direct regulation of target genes, RA may affect two signaling pathways related to the development of nervous system: Wnt signaling and apoptosis signaling. Beta-catenin represents a central and nonredundant signaling component in the canonical Wnt pathway [10,11] and mediates its developmental effects by interaction with and activation of members of the LEF/TCF family of transcription factors [12,13]. During nervous system development, beta-catenin plays an important role in cell proliferation [14 ~17], apoptosis [14,18,19], cell fate determination [20] and morphogenesis of the neural tube [21,22], and its misregulation can result in dramatic brain malformation, failure of craniofacial development and optic cup and facial patterning defects[23,24]. It has been reported that RXRs can lead to the degradation of beta-catenin in an Adenomatous polyposis coli (APC)-independent way [25] and the beta-catenin can potentiate the activity of RA on RAR-responsive promoters[26].

Apoptosis is as much a part of embryonal development as is cell proliferation and differentiation. This cell suicide is controlled by cell genes involved in induction or prevention of programmed cell death. During embryogenesis apoptosis implicates cell elimination, which is necessary for fashioning of the body and moulding of the tissues. An exaggerated or a defective apoptosis can perturb the normal development and morphogenesis of individual organisms and organs and cause various developmental abnormalities [27]. Caspase-3 is an active cell death protease involved in the execution phase of apoptosis. Its null mutant mice exhibits a remarkablely NTD that has been attributed in part to a failure of apoptosis of neurons [28~31]. The present study was undertaken to investigate the modulation of RAR alpha and beta and its links with beta-catenin and caspase-3 after maternal exposure to excess RA in KM mouse fetuses.

1 Materials and methods

1.1 Animal housing and retinoic acid treatment

Time-pregnant KM mice were purchased from the Laboratory Animal Center of Shandong University. Noon of the day of vaginal plug was considered 0.5 d.p.c. The dams were housed at 23℃ and 50% relative humidity with a 12 h light/dark cycle and allowed pelleted food and fresh water ad libitum. The animal-use protocols complied with the Public Health Service Policy on Humane Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee. To investigate the effects of RA on RAR alpha and beta, beta-catenin and caspase-3 genes during organogenesis, 7.75 d.p.c. dams were given a single oral dose of 30 mg/kg body weight of RA (Sigma, Saint Louis, MO, USA) dissolved in sesame oil. The control dams were treated only with an equal volume of sesame oil (vehicle). RA was formulated and dispensed under yellow light to prevent photoisomerization. The females were killed by cervical dislocation at 4, 18, 42, 66 and 90 h after RA or vehicle treatment (five per group). The fetuses from RA and vehicle-treated dams were collected surgically by abdominal incision, freed from extraembryonic membranes, and quickly frozen in liquid nitrogen and stored at –80℃ for future analysis.

1.2 Semiquantitative assessment of beta-catenin gene expression by reverse transcriptase polymerase chain reaction (RT-PCR)

The RNA was isolated from the fetuses and converted to cDNA by RT-PCR using a similar protocol described earlier [32]. For semiquantitative measurement, the number of cycles within an exponential phase was determined by initial trials to ensure gene amplification in the linear exponential phase. Samples were amplified for 25 cycles for beta-actin and 30 cycles for beta-catenin in GeneAmp 2 400 thermal cycler. (Perkin Elmer). The annealing temperature was optimized using a gradient. An annealing temperature of 50℃ was used for beta-actin and beta-catenin. The primers for beta-actin (housekeeping) and beta-catenin, chosen by the Primer 5 program, were (sense primer first): beta-actin, 5′ CG CGGGCGACGATGCTC 3′ and 5′ TTCACGGTTG-GCCTTGGGGTTCAG 3′ (289 bp); beta-catenin, 5′ CACGCAAGAGCAAGTAGCTG 3′ and 5′ GGGAT-GCCACCAGACTTAAA 3′ (492 bp). Negative controls with the same primers but no cDNA in the PCR reactions were used. After amplification, 10 μl of PCR products were mixed with DNA dye and ran on 2.0% agarose gel stained with ethidium bromide for electrophoretic separation. The gels were photographed in a backlighted UV transilluminator and quantified with Image-Pro Plus 5.0. Total pixel counts for beta-catenin were normalized by the beta-actin.

1.3 Determination of RAR alpha and beta, beta-catenin and caspase-3 protein in tissue extracts by Western blot

About 5～18 embryos (n=5) were used for total protein extraction and the subsequent steps were carried out on ice and centrifuged at 4℃. Briefly, the powdered embryos were mixed with 1 ml of 5× stop buffer (20% glycerol, 10% SDS, 250 mmol/L Tris pH 6.7) and homogenized in a 2-ml Dounce tissue homogenizer with pestle B. After five strokes, the homogenate was transferred to a 1.5-ml tube and centrifuged at 2 000 r/min for 30 s to get rid of the unbroken tissues. The supernatant was incubated on the ice for 5min and centrifuged for an additional 5 min at 5 000 r/min. The supernatant was stored at –80 ℃. Protein concentration from the neural tube tissue extracts was quantified by external absorbent method and corrected by the formula of ($1.45\times A_{280}-0.74\times A_{260}$). An equal amount of protein was loaded in each well. An aliquot of 15 μg of the supernatant protein from each sample was heated with 2×sodium dodecyl sulfate (SDS) sample buffer (0.1 mol/L Tris-HC1 pH 6.8, 20% glycerol, 4% SDS, 0.01% bromphenol; 10% β-mercaptoethanol (ME)) at 95℃ for 5 min, and separated electrophoretically on 10% SDS-polyacyramide gel for beta-catenin and 12% SDS-polyacyramide gel for RAR alpha, RAR beta and caspase-3. The proteins were transferred onto 0.45 μm pore-size polyvinylidene difluorede (PVDF) membranes and blocked overnight with 5% milk Tween-Tris buffer saline. Membranes were exposed to anti-RAR alpha (developed in rabbit, Santa Cruz) or anti-RAR beta (developed in rabbit, Santa Cruz) or anti-beta-catenin (developed in mouse, Zymed) or anti-caspase-3 (developed in rabbit, Boster) or anti-beta-actin antibodies (developed in rabbit, Zymed) in the blocking buffer and later incubated with horseradish peroxide-conjugated antirabbit or antimouse IgG (Zymed). Proteins were visualized with diaminobenzidine at room temperature, photographed in a backlighted UV transilluminator and digitized with Image-Pro Plus 5.0. Total pixel counts for RAR alpha,RAR beta, beta-catenin and caspase-3 were normalized by the beta-actin as described for PCR above.

2 Statistical analysis

For analysis of the gene and protein expression at all the time-points between vehicle and RA treated groups, one-way analysis of variance (ANOVA) was used. The error bars represented standard error of the mean. A probability (P) of ≤0.05 was considered significant.

3 Results

3.1 Protein expression of RAR alpha and beta

5～18 fetuses from the independent dams (n=5) were used for protein analysis of RAR alpha and beta by Western blot at 18, 42, 66 and 90 h after the vehicle and RA treatment. Experimental data showed that the level of RAR alpha protein increased rapidly at 18 h after vehicle treatment and reached the peak value at 42 h, then decreased continuously at 66 and 90 h after maternal treatment. RA treatment resulted in a significant decrease (P≤0.05) at 18 and 42 h in RAR alpha protein level in fetuses after maternal treatment, and no significant difference was found at 66 and 90 h between the vehicle and RA-treated groups after maternal treatment (Figure 1).

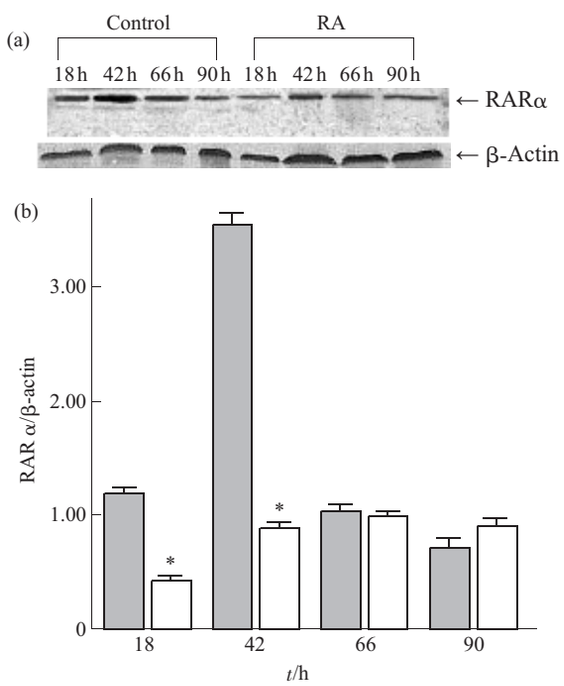

Fig. 1 RAR alpha protein expression in the neural tube of mouse embryos of vehicle and RA-treated groups

Neural tube tissues were assayed for RAR alpha protein by Western blot 18 h, 42 h, 66 h and 90 h after maternal treatment. Representative gel (a) is shown over respective bar (b). ▩: Control; □: RA. Results are expressed as ($\bar{x}\pm s$), n=5. *Indicates significantly different from control group at P≤0.05.

Expression of RAR beta protein was upregulated at 18 h and reached the peak value at 42 h after

maternal treatment in the vehicle groups, while downregulation of RAR beta protein was noticed at 66 and 90 h. RA treatment resulted in a significant decrease ($P \leqslant 0.05$) at 18 and 42 h and a significant increase at 66 and 90 h in fetuses after maternal treatment (Figure 2).

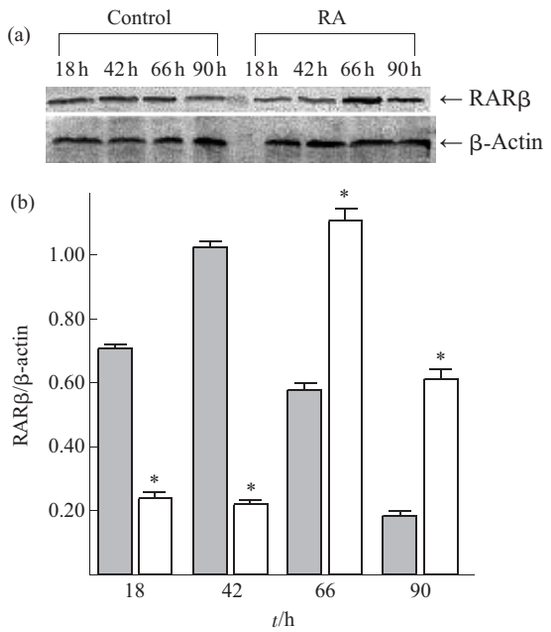

Fig. 2　RAR beta protein expression in the neural tube of mouse embryos of vehicle and RA-treated groups

Neural tube tissues were assayed for RAR beta protein by Western blot 18 h, 42 h, 66 h and 90 h after maternal treatment. Representative gel (a) is shown over respective bar (b). ▩: Control; □: RA. Results are expressed as ($\bar{x} \pm s$), n=5. *Indicates significantly different from control group at $P \leqslant 0.05$.

3.2　Expression of Beta-catenin and Caspase-3

5～18 fetuses from the independent dams (n=5) were used for mRNA analysis by RT-PCR at 4,18,42 and 66 h and protein analysis by Western blot at 18, 42, 66 and 90 h after the vehicle and RA treatment. In the vehicle-treated groups, the amount of beta-catenin mRNA transcript and protein displayed a continuously decreased pattern. RA treatment resulted in a marked decrease ($P \leqslant 0.05$) at 4 and 18 h and a marked increase at 66 h in beta-catenin mRNA level in fetuses after maternal treatment, and no significant difference was found at 42 h between the vehicle and RA-treated groups after maternal treatment (Figure 3). In addition, the level of beta-catenin protein was decreased distinctively at 18 and 42 h and increased distinctively at 90 h in fetuses after maternal treatment, and no significant difference was found at 66 h between the vehicle and RA-treated groups after maternal treatment (Figure 4). The similar expression patterns of beta-catenin mRNA and protein both in the vehicle

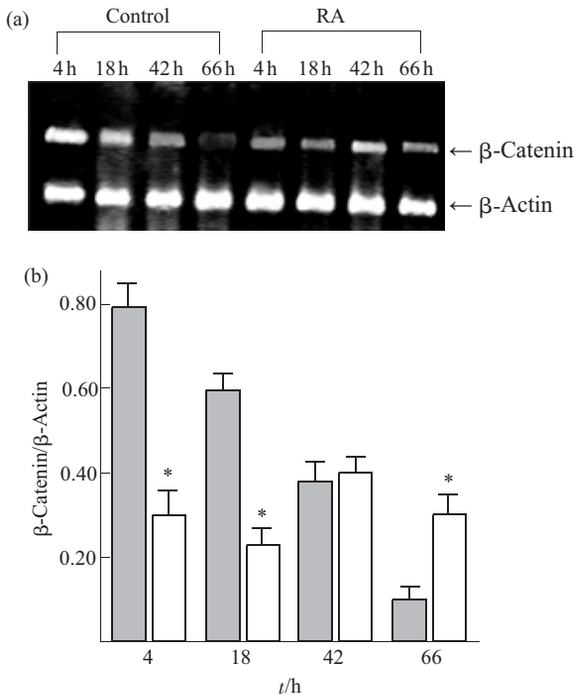

Fig. 3　Beta-catenin mRNA expression in the neural tube of mouse embryos of vehicle and RA-treated groups

Neural tube tissues were assayed for beta-catenin mRNA by RT-PCR 4 h, 18 h, 42 h and 66 h after maternal treatment. Representative gel (a) is shown over respective bar (b). ▩: Control; □: RA. Results are expressed as ($\bar{x} \pm s$), n=5. *Indicates significantly different from vehicle-treated group at $P \leqslant 0.05$.

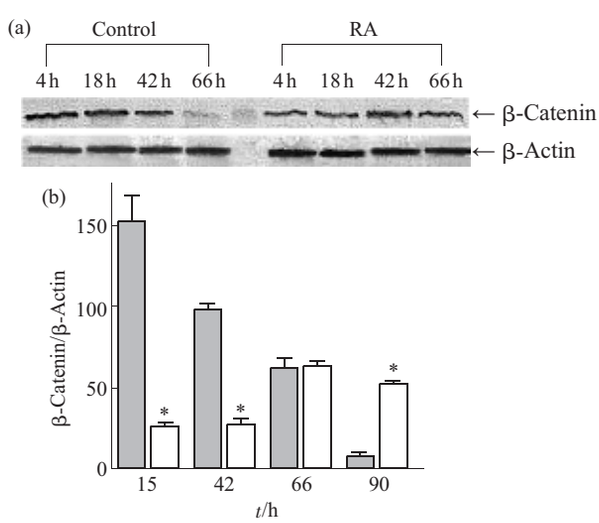

Fig. 4　Beta-catenin protein expression in the neural tube of mouse embryos of vehicle and RA-treated groups

Neural tube tissues were assayed for beta-catenin protein by Western blot 18 h, 42 h, 66 h and 90 h after maternal treatment. Representative gel (a) is shown over respective bar (b). ▩: Control; □: RA. Results are expressed as ($\bar{x} \pm s$), n=5. *Indicates significantly different from control group at $P \leqslant 0.05$.

and RA-treated groups hinted that the beta-catenin protein was the expressive consequence of the beta-catenin mRNA.Excess RA also affected the level of caspase-3 protein. In the vehicle treated groups, the level of caspase-3 protein increased rapidly at 18 h and reached the peak value at 42 and 66 h, then decreased to its virgin level at 90 h after maternal treatment. RA treatment resulted in a significant decrease ($P \leqslant 0.05$) at 42 h and a significant increase ($P \leqslant 0.05$) at 66 and 90 h in the level of caspase-3 protein in fetuses after maternal treatment, and no significant difference was found at 18 h between the vehicle and RA-treated groups (Figure 5).

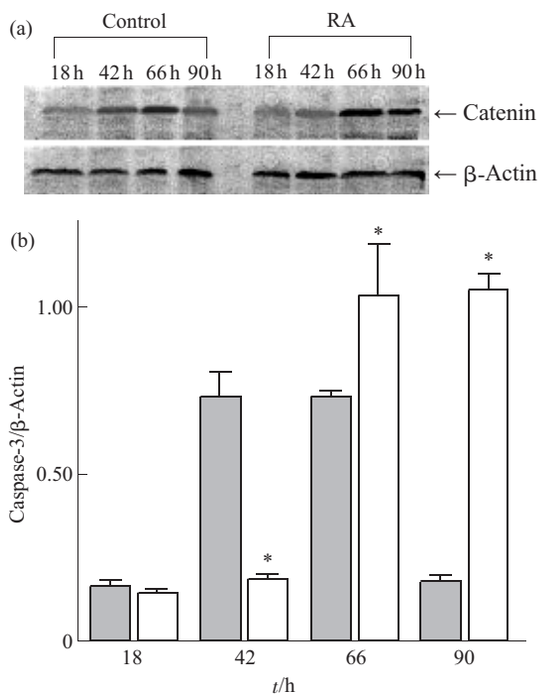

Fig. 5 Caspase-3 protein expression in the neural tube of mouse embryos of vehicle and RA-treated groups

Neural tube tissues were assayed for caspase-3 protein by Western blot 18 h, 42 h, 66 h and 90 h after maternal treatment. Representative gel (a) is shown over respective bar (b). ▩: Control; □: RA. Results are expressed as ($\bar{x} \pm s$), n=5. *Indicates significantly different from control group at $P \leqslant 0.05$.

4 Discussion

RA is a small (300 u), lipophilic signaling molecule that acts to mediate gene expression by binding to at least two classes of ligand-activated transcription factors: RARs and RXRs. The former category binds RA [33], while the latter binds 9-*cis*-RA [34]. At physiological dose, RA mediates multiple biological events during cellular growth, differentiation, apoptosis, vision, reproduction and embryonic patterning of a wide range of species. However, it is well known that both excess and deficiency of RA can cause a spectrum of dose and stage-specific birth defects during embryogenesis, and the central nervous system (CNS) represents a major site of the teratogenic action of RA which appears various NTDs. NTDs have a complicated and imperfectly understood etiology in which both genetic and environmental factors appear to be involved. The present study was conducted to analyze early molecular alterations associated with the teratogenesis induced by RA.

A number of studies on the teratogenic effects of RA have focused on RARs, firstly, because these receptors have a central role in RA-controlled gene regulation and, secondly, because of its involvement in neurogenesis. The majority of reports concerning RARs are loss/gain-of-function experiments, and the development of CNS responds to RARs agonists or antagonists by a failure of neural tube closure and various NTDs. Our results show that excess RA downregulates the level of RAR alpha and beta proteins at 42 and 66 h after maternal treatment, just a time around the neural tube closure. RA treatment also resulted in a significant increase at 66 and 90 h in fetuses after maternal treatment. It has been reported that compound null mutation of RAR genes lead to lethality in utero or shortly after birth and to numerous developmental abnormalities [35]. In addition, inositol can cure the folate-resistant NTDs in the *curly tail* mutant mouse by its up-regulation of RAR beta in the underlying hindgut endoderm and as a result the correction of a proliferation defect [36, 37]. The RARalpha-selective agonist (Am580, an arylcarboxamidobenzoic acid derivative) induces a list of developmental defects such as spina bifida aperta, micrognathia, exencephaly, tail malformations and so on in NMRI mice [38]. So we supposed that the RAR alpha and beta genes compromise the neural tube closure during embryogenesis and the disturbance of their proper expressive profile may take part in the teratogenesis induced by excess RA. However, the mechanism by which RA downregulates RARs mRNA remains unknown.

Beta-catenin represents a central and nonredundant signaling component in the canonical Wnt pathway and plays important roles in cell proliferation, apoptosis, cell fate determination and morphogenesis during embryonic developmen. In the present study, RA treatment induced a decrease in the

level of beta-catenin mRNA and protein concomitant with underexpression of RAR alpha and beta in the time of neural tube closure. A significant increase was found in the level of beta-catenin mRNA and protein after the neural tube closure. Xiao *et al*. [39] have reported that RXRs can induce degradation of beta-catenin through an adenomatous polyposis coli (APC)-independent way and consiquently repress beta-catenin-mediated transcription. In addition, RARs repress the activity of the beta-catenin-LEF/TCF signaling pathway by interacting directly with beta-catenin in a retinoid-dependent manner [40]. We have known that conditional mutation of beta-catenin in the neural tube tissue leads to an elimination of the cells at the midhindbrain boundary [41]. Moreover, inhibition of beta-catenin expression is one of the mechanisms by which the RA induces the effects of anti-proliferation and neural differentiation [39, 42]. So we suppose that the downregulation of beta-catenin, which might be a consequence of RXRs-induced degradation, may disturb the balance of cell proliferation and difference and as a result participate in the teratogenesis in RA-treated mouse embryo. The increased expression of beta-catenin mRNA and protein after the time of neural tube closure may be a feedback effect of the organism in the RA treated embryos.

The decreased expression of caspase-3 protein is another event occurring earlier in NTDs induced by excess RA. Caspase-3 is an active cell death protease involved in the execution phase of apoptosis. Its null mutant mice exhibit a remarkably NTD that has been attributed in part to a failure of apoptosis of neurons[43]. It has been reported that RA can inhibit apoptosis triggered by hydrogen peroxide (H_2O_2) or pyrrolidine dithiocarbamate *via* downregulation of c-fos/c-jun by binding to RAR/RXR or suppression of c-Jun N-terminal kinase (JNK) by a nuclear receptor-independent mechanism [44 ~47]. RA also inhibits the apoptosis of medial edge epithelial cell by its upregulation of epidermal growth factor receptor (EGFR)[48]. So RA may dysregulated apoptosis through its modulation of RAR/RXR or a nuclear receptor-independent mechanism and consequently play its teratogenic effect in KM mouse embryo.

Embryonic growth and development entails differentiation, growth, apoptosis, and morphogenesis in a highly coordinated environment. Any alterations in the interaction between genes controlling these critical events during the embryogenesis can lead to an abnormal homeostasis and all kinds of developmental defects. In our study, maternal intake of excess RA inhibits RAR alpha and beta expression at an early stage, which may participate in the decreased expression of beta-catenin and caspase-3 in RA treated fetuses. So these molecular alterations might disturb some of the early events during neural tube closure and consequently induced teratogenesis in KM mouse embryo after maternal treatment.

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维甲酸致小鼠胚胎畸形发生过程中维甲酸受体 α/β 及 β-catenin 和 caspase-3 基因的表达变化 *

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摘要　流行病学研究显示，在胚胎发育过程中摄入过多维甲酸可致各种发育缺陷，其中神经管畸形最为常见．因此有必要探明维甲酸致各种发育缺陷的发生机制，以便为各种生长缺陷的预防和治疗提供实验依据．用 RT-PCR 及蛋白质印迹技术，探测了过量维甲酸对昆明小鼠胚胎神经管中维甲酸受体 α/β 及 β-catenin 和 caspase-3 基因表达的调整．结果显示，在神经管闭合期过量维甲酸显著降低了维甲酸受体 α/β 及 β-catenin 和 caspase-3 的基因表达，神经管闭合后，维甲酸受体 β、β-catenin 及 caspase-3 的基因表达又出现了一个明显的回升过程．提示，过量维甲酸改变了昆明小鼠胚胎神经管中维甲酸受体 α/β 及 β-catenin 和 caspase-3 基因的正常时间表达模式，这种异常的基因表达模式可能参与了维甲酸致昆明小鼠胚胎畸形的发生机制．

关键词　维甲酸受体，β-catenin，caspase-3，发育缺陷，维甲酸，小鼠

学科分类号　R363.1，R714.43

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* 山东省自然科学基金资助项目(Y2005C63).

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收稿日期：2007-03-15，接受日期：2007-05-31