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Cell apoptosis during the cloacal embryonic development in rats with anorectal malformations

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Abstract: Objective In the normal embryonic development of anorectum, apoptosis plays an important role. To explore the role of apoptosis in anorectal malformations (ARM), this study investigated cell apoptosis during the cloacal embryonic development in ARM embryos. **Methods** ARM embryos were induced by intragastric administration of ethylenethiourea (125 mg/kg) for pregnant rats on embryonic day 10 (E10). The distribution of apoptotic cells in the cloaca was ascertained by hematoxylin and eosin and TUNEL staining in the normal control embryos ($n = 102$) and ARM embryos ($n = 147$) on E13, E13.5, E14, E15 and E16. **Results** On E13, apoptotic cells were detected in the urorectal septum of rat embryos in the control group. With the development of embryos, the number of apoptotic cells in the mesenchyme of urorectal septum gradually increased and a large number of apoptotic cells were seen in the dorsal rectal mesenchyme. On E14, apoptotic cells appeared at the terminal rectum and the dorsal cloacal membrane. On E15, the urorectal septum fused with the cloacal membrane and apoptotic cells in the urorectal septum mesenchyme continuously extended down to the fusion region. Compared with the control group, apoptotic cells in the urorectal septum, the dorsal rectal mesenchyme and the cloacal membrane of the ARM rat embryos were significantly reduced during the embryonic development. The development of the urorectal septum was delayed and it did not fuse with the cloacal membrane in ARM embryos. **Conclusions** During the embryonic development of cloaca, abnormal apoptosis in the urorectal septum, the dorsal rectal mesenchyme and the cloacal membrane may be one of the reasons for anorectal malformations. The proper regulation of cell apoptosis may be one of the key mechanisms for normal development of anorectum in the embryonic stage.

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Key words: Anorectal malformation; Cloaca; Apoptosis; Embryo; Rats

肛门直肠畸形大鼠泄殖腔胚胎发育过程中细胞凋亡的研究

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[**摘 要**] **目的** 肛门直肠正常胚胎发育过程中,细胞凋亡发挥重要作用。该研究调查了肛门直肠畸形(anorectal malformation, ARM)胎鼠泄殖腔胚胎发育过程中细胞凋亡情况,以了解 ARM 胚胎发育过程中细胞凋亡的作用。**方法** 在胚胎发育的第 10 天,通过胃管注入乙烯硫脲(125 mg/kg)致畸孕鼠,诱导 ARM 胚胎。在胚胎发育的第 13,13.5,14,15 和 16 天,利用苏木素-伊红和 TUNEL 染色技术检测正常对照胚胎 ($n = 102$) 和 ARM 胚胎 ($n = 147$) 泄殖腔凋亡细胞的分布。**结果** 对照组胚胎第 13 天尿直肠隔可见凋亡细胞,随着胚胎发育,尿直肠隔间质内的凋亡细胞逐渐增多;直肠背侧间质可见大量凋亡细胞。胚胎第 14 天,直肠末端和未来肛门开口处的泄殖腔膜开始出现凋亡细胞。胚胎第 15 天,尿直肠隔与泄殖腔膜融合,尿直肠隔间质内的凋亡细胞一直向下延伸到融合部位。ARM 胎鼠与对照组胎鼠相比,在胚胎发育过程中尿直肠隔间质、直肠背侧间质和泄殖腔膜的凋亡细胞均明显减少。ARM 胎鼠尿直肠隔的发育明显延迟,未与泄殖腔膜融合。**结论** 在泄殖腔的胚胎发育过程中,尿直肠隔间质、直肠背侧间质和泄殖腔膜细胞凋亡的异常是导致 ARM 的原因之一。细胞凋亡的正常调控是保证肛门直肠胚胎期正常发育的关键机制之一。 [**中国当代儿科杂志,2009,11(9):709 – 713**]

[**关 键 词**] 肛门直肠畸形;泄殖腔;凋亡;胚胎;大鼠

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Anorectal malformation (ARM) is a common congenital malformation of the digestive tract seen in pediatric surgery departments. With an incidence rate of 1/5 000-1/1 500, it is one of the congenital malforma-

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tions routinely monitored by the World Health Organization. Particularly in intermediate-type and high-type ARM patients, bowel dysfunction such as incontinence and constipation often happens after surgery and seriously affects the life quality of patients[1,2]. Therefore, ARM has become of great concern to researchers throughout the world.

Studies show that in the normal embryonic development of anorectum, apoptosis plays an important role[3-5]. However, the role of apoptosis in the ARM embryonic development is still unclear. While pregnant rats are treated with ethylenethiourea (ETU), about 40%-80% of the offspring are ARM embryos, in which the deformations range from simple atresia ani to urorectal fistula, similar to the human ARM. Therefore, ETU was used in this study as a teratogenic reagent for Wistar rats to produce ARM embryos, in order to study the role of apoptosis in the embryonic development of cloaca in ARM rats.

Materials and methods

Sample preparation

This study was approved by the Ethics Committee of the China Medical University. ETU (2-imidazolidinethione; $C_3H_6N_2S$) was provided by the Sigma-Aldrich Chemical Company, Seelze, Germany. Twenty-five female Wistar rats, with body weights ranging from 250 to 300 g, were time-mated. When sperms were found in female vaginal smears in the morning, rat embryos were designated as embryonic day 0 (E0). They were randomly assigned to two groups: control group (n = 10) and experimental group (n = 15). Pregnant rats in the experimental group were gavage fed with 125 mg/kg ETU on E10, while pregnant rats in the control group received the same volume of normal saline. On E13, E13.5, E14, E15, and E16, two pregnant rats from the control group and three pregnant rats from the experimental group were sacrificed respectively. The embryos were fixed in 4% paraformaldehyde solution, routinely dehydrated, paraffin-embedded and continuously cut into 4 μm-thick sections at sagittal planes. Sections were preserved alternately. Parts of sections were used for hematoxylin and eosin staining and the others were used for TUNEL staining.

Laboratory methods

The distribution of apoptotic cells in the cloaca was ascertained by hematoxylin and eosin and TUNEL staining. The TUNEL staining kit and protease K used were provided by Roche Diagnostics, Germany.

The sections used for TUNEL staining were routinely dehydrated and dewaxed, incubated in 20 μg/mL protease K solution (pH 7.6) at room temperature for 20 minutes, then rinsed by PBS, and incubated with 50 μL TUNEL reaction mixture at 37℃ for 60 minutes. After PBS washing, the sections were incubated in 50 μL converter-POD solution at 37℃ for 30 minutes. Diaminobenzidine tetrahydrochloride color developed under the light microscope and then the sections were counterstained and mounted. For negative control, PBS was applied on specimens instead of TUNEL reaction mixtures. Other sections were stained with hematoxylin and eosin. All sections were photographed using a digitized microscope camera (Nikon Corporation, Japan).

Results

There were a total of 102 rat embryos in the control group and no malformations were observed in any of those. A total of 153 rat embryos were produced in the experimental group, among which 6 were stillborn. In addition to ARM, other deformities could be seen in the experimental group, including spina bifida, meningocele, tailless, short-tail, omphalocele and gastroschisis. The distribution of rat embryos in the experimental group is shown in Table 1.

Table 1 Distribution status of embryos in the experimental group (number)

	Embryonic day				
	13	13.5	14	15	16
Total embryos	30	29	30	31	27
Observed embryos	26	27	29	31	27
ARM embryos	12	15	17	20	18
Undetermined embryos	10	6	4	2	1

Normal embryonic development process of cloaca

In the control group, the genital tubercle appeared on E13, with a clear outline of the cloaca. An inverted V-shaped urorectal septum was seen between urogenital sinus and the rectum and a tail groove appeared. With the development of embryos, the genital tubercle gradually started to migrate towards the ventral and caudal sides and the urorectal septum extended into the cloaca. On E13.5, a wide cloacal canal was seen between the urogenital sinus and the rectum. On E14 the cloacal canal became significantly narrow. On E15 the median sagittal sections, the distalmost epithelium of urorectal septum fused with cloacal membrane epithelium, the cloacal canal closed and the rectum was completely separated from the bladder and the urethra; the anal

membrane was thin and the tail groove was about to connect with the rectum. In the sections aside of the median sagittal plane, the most distal epithelium of urorectal septum was closely adjacent to epithelium layers of cloacal member but still did not fuse since the fissure was still visible. On E16, the urorectal septum entered the perineal body and divided the rectum and urethra. The anal membrane ruptured, and the rectum communicated with the outside.

Embryonic development process of ARM cloaca

On E13, the genital tubercle was seen in ARM rats, with a clear outline of the cloaca. However, the cloacal membrane was short and had no obvious tail groove, and only a superficial depression appeared at the caudal part. An inverted V-shaped urorectal septum was seen between the urogenital sinus and the rectum. With the embryonic development, there were no obvious genital tubercle migration towards the ventral and caudal parts and no urorectal septum extension. The cloacal canal was still relatively wide on E14. On E15, the urorectal septum further descended and the genital tubercle development was delayed. However, the distance between the urorectal spetum and the cloacal membrane was still far and no fusion was observed. The cloacal canal was not closed and the rectum still communicated with the urogenital sinus. The development of the cloacal membrane was poor and the dorsocaudal rotation of cloacal membrane was not obvious. The terminal rectum was far away from the cloacal member. On E16, the urorectal septum was still not fused with the cloaca membrane. The distance between them was different depending on types of malformation, including rectal fistula and other anorectal malformations.

Distribution of apoptotic cells in the cloaca

On E13, apoptotic cells were detected in epithelial cell layers of the urorectal septum in the control group. Occasionally, apoptotic cells were seen in the mesenchyme of the urorectal septum. In the ARM group, apoptotic cells were seen in epithelial layers of the urorectal septum, but not in the mesenchyme region.

On E13.5, apoptotic cells were seen in the mesenchyme of the urorectal septum and more apoptosis was detected at the dorsal part of the rectum. In the ARM group, apoptotic cells were seen in the mesenchyme of the urorectal septum but significantly less apoptosis presented at the dorsal part of the rectum compared to the control group.

On E14, apoptotic cells were still found in the urorectal septum epithelium in the control group, and markedly increased apoptotic cells were seen in the urorectal septum mesenchyme, more in the ventral than the dorsal part, in the control group. Meanwhile, apoptosis started to appear at the lower rectum and the cloacal membrane at the location of future anus and urethra. A large number of apoptotic cells were seen at the dorsal rectum (Figure 1A). In contrast, in the ARM group no obvious apoptosis was seen in the urorectal septum mesenchyme and significantly less apoptotic cells were detected at the dorsal rectum than in the control group. Apoptosis still presented at the terminal rectum but was not obvious at the cloacal membrane (Figure 1B).

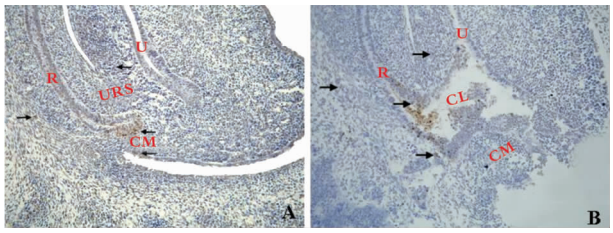

Figure 1 Distribution of apoptotic cells in the cloca on E14 (×200) **A**: control group. Apoptotic cells were found in the urorectal septum epithelium and mesenchyme. Apoptosis started to appear at lower rectum and the cloacal membrane; **B**: ARM group. Apoptotic cells were found in the urorectal septum epithelium but not in the urorectal septum mesenchyme and the cloacal membrane. The arrow indicates apoptotic cells.

R: rectum; U: urethra; URS: urorectal septum; CL: cloaca; CM: cloacal membrane.

On E15, the apoptotic cells in the urorectal septum mesenchyme of the control group continuously extended to the fusion site of the urorectal septum epithelium and the cloaca membrane epithelium. Large quantities of apoptotic cells were seen at the terminal rectum, the future anal opening and at the dorsal of the rectum (Figure 2A). However, in the ARM group, no obvious apoptosis was detected at the urorectal septum mesenchyme and the cloacal membrane, while the number of apoptotic cells at the dorsal rectum was significantly reduced (Figure 2B).

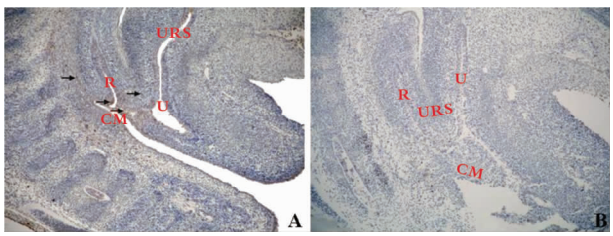

Figure 2 Distribution of apoptotic cells in the cloca on E15 (×100) **A**: control group. Apoptotic cells in the urorectal septum mesenchyme extended to the fusion site of urorectal septum epithelium and the cloaca membrane epithelium. Large quantities of apoptotic cells were seen at the terminal rectum and at the location of future anus; **B**: ARM group. No obvious apoptosis was detected at the urorectal septum mesenchyme and the cloacal membrane. The arrow indicates apoptotic cells.

R: rectum; U: urethra; URS: urorectal septum; CM: cloacal membrane

On E16, apoptotic cells were still seen at the ventral part of the urorectal septum in the control group. No obvious apoptosis was presented at the cloacal membrane and the urorectal septum in the ARM group.

Discussion

The pathological classification of ARM is very complicated. From simple anal atresia, various types of rectal and urinary fistula to serious cloacogenic deformities, it brings extreme pain to patients; therefore, ARM has become a cause of concern for researchers throughout the world. Yet the causes of ARM have still not been determined.

Regarding the studies on the embryonic development of the cloaca, classical theory believes that the existence of the urorectal septum and the fusion of the urorectal septum with the cloacal membrane are the key factors for the separation of the urogenital sinus and the rectum. However, this theory is opposed by some researchers[6-9], who think that in the embryonic development of the cloaca, the urorectal septum does not fuse with the cloaca membrane; instead they are just closely adjacent to each other. In this study, the fusion of the urorectal septum with the cloacal membrane was observed on the median sagittal sections in the control group on E15, and the cloaca was divided into the urogenital sinus and rectum; while on the sections besides the median sagittal plane, the urorectal septum epithelium was close to the cloacal member epithelium but did not fuse with it since the fissure was still present. While in the ARM group, the development of its urorectal septum was significantly delayed, without obvious head-tail extension and ventral-caudal rotation. The urogenital canal still existed and no fusion between the urorectal septum and the cloacal membrane was found on E15. The anal membrane did not rupture on E16 and different types of ARM appeared. This suggested that fusion indeed existed in the process of normal embryonic development, which is consistent with the classical theory. However, whether the descending and the fusion process of the urorectal septum are active or passive, has not yet been identified. Nevertheless, regardless of whether the descending and fusion process of the urorectal septum are active or passive, it is a key factor for separating the urogenital sinus from the rectum and its abnormal development can lead to ARM.

In the embryonic development, apoptosis is an active programmed process. It is a series of gene activity induced-changes in molecule, morphology and biochemistry that leads to cell death. Abnormal apoptosis in the process of embryonic development can cause developmental malformations affecting multiple tissues and organs such as finger/toe and esophagus/trachea. Whether there is an abnormal apoptosis in the embryonic development of ARM is currently not very clear.

Since the urorectal septum plays important role in the process of cloacal embryonic development [10-12], this study observed the distribution of apoptotic cells in the urorectal septum. In the control group, apoptotic cells existed in the epithelium and the mesenchyme of urorectal septum on E13, while apoptotic cells increased in the mesenchyme on E14 and could be seen both at the ventral and dorsal parts. With the embryonic development, apoptotic cells in the urorectal septum progressively descended to the fusion site of the urorectal septum and the cloacal membrane on E15. In the study on cloacal apoptosis in ARM mice, Kubota et al[13] found that apoptotic cells in the urorectal septum only exist on the apical part, while apoptosis in the urorectal septum region between normal embryos and ARM embryos were not significantly different. This result differs from this study and the studies done by Qi et al[4] and Sasaki et al[3]. Qi et al [4] found that apoptotic cells presented in the epithelium layer of rat urorectal septum but not in the mesenchyme region in the investigation on the normal embryonic development of cloaca. SaSaki et al[3] reported that apoptotic cells not only existed in the epithelium of mouse urorectal septum but also in the mesenchyme. However, these apoptotic cells in the mesenchyme only distributed at the ventral part. This study showed that in the process of rat cloacal embryonic development, apoptotic cells existed in both the epithelium and the mesenchyme of urorectal septum, while the apoptotic cells in the urorectal septum mesenchyme distributed more at the ventral than at the dorsal part. This distribution of apoptotic cells in the urorectal septum mesenchyme might relate to the ventral-caudal rotation of the urorectal septum. However in ARM embryos, apoptotic cells in the urorectal septum mesenchyme were not obvious even at E14-E15. E14-E15 is the critical period for development of the urorectal septum and abnormal cell apoptosis in this period may lead to developmental delay of the urorectal septum.

In the process of cloacal embryonic development, configuration changes of cloaca are the key mechanisms for its normal development and the abnormality in configuration changes may result in cloacal developmental malformations. Dorsal rectal mesenchyme is an important part of the cloaca and an important factor affecting

ventral-caudal migration of cloaca. In the control group, apoptotic cells appeared in the dorsal rectal mesenchyme on E13.5 and remained until the late embryonic stages. Compared with the control group, ARM embryos had significantly less apoptotic cells in the dorsal rectal mesenchyme. This indicates that the reduction of apoptotic cells in the dorsal rectal mesenchyme of ARM embryos may relate with the abnormal cloacal configuration.

In the control group on E14, large numbers of apoptotic cells were seen at the terminal rectum and the site of the future anal opening at the cloacal membrane. On E15, only a thin layer of anal membrane left between the rectum and the outside and most cells in the anal membrane were apoptosis-staining positive. On E16 the anal membrane ruptured and the rectum connected with the outside. However, in ARM embryos, no obvious apoptotic cells were observed in the future anal opening of the cloacal membrane, which hindered the thinning and rupture of the cloacal membrane and the communication of rectum to the outside, therefore leading to ARM deformity.

There are three critical processes in the embryonic development of cloaca: fusion and descending of urorectal septum, configuration changes of cloaca and rupture of anal membrane. Once the three critical processes show abnormal, different types of ARM occurred. Apoptosis plays important roles in the three processes of the cloacal embryonic development. In the cloacal development, abnormal distribution of apoptotic cells was found in the three key processes in the ARM group. This suggests that apoptosis abnormality is one of the reasons for the development of ARM. Similar to the development of finger/toe and esophagus/trachea, the normal regulation of cell apoptosis is one of the key mechanisms for normal embryonic development of anorectum. Specific genes involved in regulating the cell proliferation and apoptosis of the cloaca are numerous, including FGF10[14], BMP4, SHH[15] and GLI3[16] etc. However, the exact regulatory mechanisms remain unclear and need further investigations.

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