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Original Article

Expression of the P2Y2 receptor in the terminal rectum of fetal rats with anorectal malformation

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Abstract: Objective: The expression and distribution of a subtype of purine receptors (P2Y2) in the terminal rectum of fetal rats with anorectal malformations (ARM) were examined to investigate their possible impact on the development of the enteric nervous system (ENS). Methods: Pregnant Sprague-Dawley rats were randomly divided into a control group (5 rats) and an experimental group (20 rats). The experimental group was treated with ethylene thiourea (ETU). On gestational day 20, the intrauterine fetal rats were collected from both groups of pregnant rats. Sagittal sections of the pelvic perinea were stained with HE. P2Y2 protein and mRNA expression in the terminal recta of the fetal rats in the control group, the ARM group, and the ETU-treated group that exhibited no malformations (the ETU group) were detected by immunohistochemistry, western blot, and qRT-PCR. Results: The fetal rats in the control group showed normal position of the anal opening, with no malformation. The incidence of ARM was 89.2% for the fetal rats in the experimental group. The immunohistochemistry results showed that P2Y2 was expressed in the cytoplasm of the cells in the terminal rectum submucosa and myenteric plexus of the fetus rats in the control group, the ETU group, and the ARM group. The average integrated optical density (IOD) value for the ARM group was significantly lower than the IOD value for the control and ETU groups (186.48 ± 23.03 vs. 493.18 ± 19.70; 186.48 ± 23.03 vs. 479.48 ± 41.71, $P<0.01$), while the IOD value for the ETU group was comparable to the control group IOD (493.18 ± 19.70 vs. 479.48 ± 41.71, $P = 0.360$). The western blot and qRT-PCR results showed that the P2Y2 protein and mRNA expressions were significantly lower in the terminal rectum of the fetal rats in the ARM group than in the control and ETU groups (0.28 ± 0.08 vs. 0.51 ± 0.10, 0.28 ± 0.08 vs. 0.48 ± 0.12; 48.91 ± 12.17 vs. 98.03 ± 15.68, 48.91 ± 12.17 vs. 92.53 ± 10.43; $P<0.01$), while the P2Y2 protein and mRNA levels in the control group were comparable to the ETU group (0.51 ± 0.10 vs. 0.48 ± 0.12, $P = 0.494$; 98.03 ± 15.68 vs. 92.53 ± 10.43, $P = 0.058$). Conclusion: P2Y2 may participate in and affect the development of ENS in the terminal rectum of fetal rats with ARM.

Keywords: Anorectal malformation, fetal rats, enteric nervous system, P2Y2

Introduction

Congenital anorectal malformation (ARM) is one of the most common gastrointestinal malformations in children [1]. Although the cure rate for this condition has improved significantly, some pediatric ARM patients still suffer from difficulties with defecation and bowel control after surgery, which seriously affects their quality of life [2, 3]. Postoperative bowel dysfunction in children with ARM is closely related to abnormal development of the enteric nervous system (ENS) [4]; however, the mechanism by which abnormal ENS development affects bowel function is unclear. The ENS originates in the neural crest cells. After entering the intestine, the neural crest cells eventually differentiate into ganglion cells and glial cells through gradual migration, proliferation, and slow accumulation [5]. Disorders at any stage of this process can affect the development of ganglion cells, which leads to ENS dysplasia. The neurotransmitter adenosine triphosphate (ATP) is widely involved in neuron differentiation, migration, regeneration, and repair, and abnormal ATP expression affects ENS development. ATP must bind to the P2Y2 receptor for proper function [6, 7]. Therefore, the level of P2Y2 receptor expression may indirectly reflect the function of ATP as a neurotransmitter. In this study, we established an ARM animal model using ethylene thiourea (ETU) [8] to investigate the distri-

Table 1. The primers of β-actin and P2Y2

Gene	Sequences of Primers (5′–3′)	TM (°C)	Production
β-actin	F: GGAGATTACTGCCCTGGCT CCTA R: GACTCATCGTACTCCTGCTTGCTG	60	150 bp
P2Y2	F: CTCATTTGGCAGGGACTCAGG R: AATGGCAGCTGTTTGCATGG	60	141 bp

bution and expression of the P2Y2 receptor in terminal rectum of fetal rats with ARM and to explore the role of P2Y2 in ENS development.

Materials and methods

Establishing the ARM animal model

A total of 40 adult Sprague-Dawley rats (SPF grade, provided by the Experimental Animal Center of the Third Military Medical University, China) weighing 200-250 g were selected for mating. Thirty female rats and 10 male rats were placed in cages with a female to male ratio of 3:1 at 10 pm. At 8:00 the next morning, a vaginal smear test was performed. If sperm or a vaginal plug were observed, the rats were recorded as being at gestational day 0. Twenty-five female rats with clear conception were randomly divided into two groups: the experimental group containing 20 rats and the control group containing 5 rats. The rats in the experimental group were administered 125 mg/kg of 1% ETU by intragastric infusion on gestational day 10 [9], while the rats in the control group were infused with saline on gestational day 10. On gestational day 20, all fetal rats were collected from the two groups of pregnant rats. Malformations were observed visually or with a dissecting microscope. The experimental group was then divided into rats with malformations (the ARM group) and rats that received the treatment but exhibited no malformation (the ETU group). The terminal recta were collected aseptically from the three groups of fetal rats and stored at -80°C for later use in qRT-PCR experiments. The remaining specimens were fixed with 4% formaldehyde for 12-36 h, followed by conventional dehydration and paraffin embedding.

Equipment and reagents

The primary equipment used in this study included a real-time quantitative PCR system (Bio-Rad, UAS), a Leica QWin image analysis system (Forma Scientific, Germany), a dual band infrared laser imaging system (Odyssey, USA), and an optical microscope (Leica, Germany). The primary reagents used in this study included ETU (Sigma, 1504-I00G), diethyl phosphorocyanidate (DEPC) stock solution (Sigma St. Louis, USA), an immunohistochemical staining kit (Beijing Zhongshan Biological Technology Co., Ltd., China), an RT-PCR kit (Takara Bio-Engineering (Dalian) Co., Ltd., China) and a rabbit anti-mouse P2Y2 monoclonal antibody (Beijing Biosynthesis Biotechnology Co., Ltd., China).

Experimental methods

HE staining

The paraffin-embedded pelvic perineum and terminal rectum tissues were serially sectioned to a thickness of approximately 4 mm and observed under an optical microscope after conventional HE staining.

Immunohistochemical detection

Ten paraffin sections each were selected from the ARM group, the ETU group, and the control group. The sections were incubated with a rabbit anti-mouse P2Y2 primary antibody (1:200) at 4°C for 12 h, and the S-P kit from Fuzhou Maixin Co. was used as the secondary antibody. After DAB staining, hematoxylin restaining, dehydration and destaining, the sections were mounted on slides. The cells that stained brownish yellow in the cytoplasm were scored as positive cells. Negative and positive controls were included, and the image results were analyzed using the Leica QWin and Image-pro plus 6.0 software.

Western blot analysis

Total protein was extracted from ten specimens each from the ARM group, the ETU group, and the control group using a total protein extraction kit from Beyotime Institute of Biotechnology (Jiangsu, China). After the protein concentration was determined by BCA, the samples were stored at -20°C for later testing. For western blotting, 60 μl of total protein was run on a 10% SDS-PAGE gel and transferred to a nitrocellulose membrane. The membrane was then blocked with 5% skim milk and incubated with a rabbit anti-mouse P2Y2 antibody (1:200) and a β-actin antibody (1:1000) at 4°C for 12 h. The

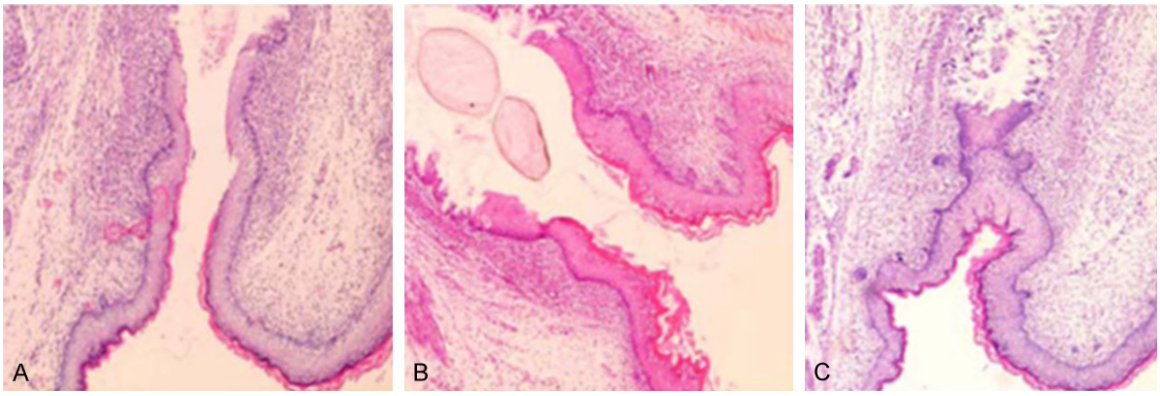

Figure 1. A. Sagittal plane from the control group: normal anal opening, with a clear outline; B. Sagittal plane of the ETU group: normal anal position and opening, with observable meconium in the rectum; C. Sagittal plane of the ARM group: the anal opening was a closed blind end.

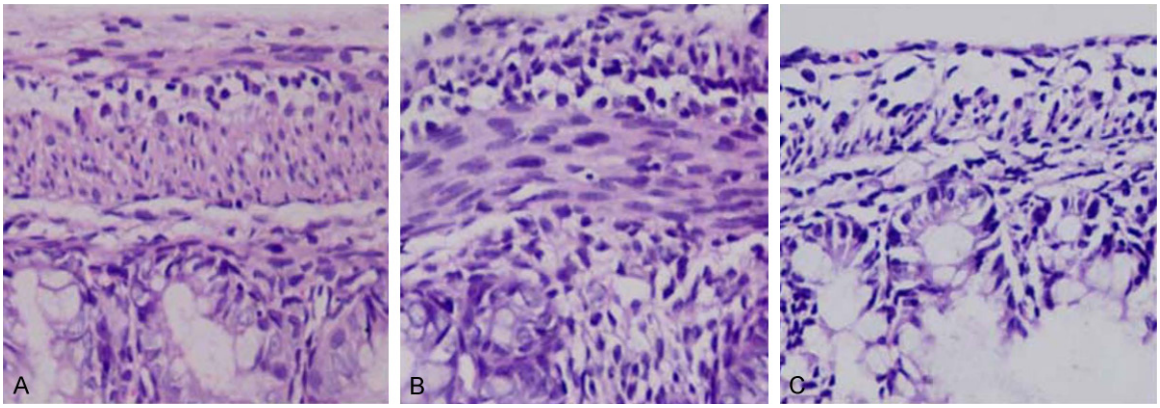

Figure 2. A. Cross-section from the normal group: the terminal rectum submucosa and myenteric plexus exhibited a large number of darkly staining spherical or ellipsoid neurons with a large nuclear volume, abundant cytoplasm and clear axons; B. Cross-section from the ETU group: a large number of darkly staining neurons with a large nuclear volume; C. Cross-section from the ARM group: a smaller number of neurons, with smaller nuclear size, shrinkage, light staining, and irregular shapes.

next day, the membrane was washed and then incubated with an 800 CW labeled goat anti-rabbit IgG (1:10000) secondary antibody for hybridization. The results were analyzed using a dual band infrared laser imaging system. The protein content was determined by density analysis, and the amount of protein expression was expressed as a relative gray value.

qRT-PCR

Primers were synthesized by Takara Bio-Engineering Co., Ltd. (Dalian, China) (**Table 1**). Total RNA was extracted from 10 specimens each randomly selected from the ARM group, the ETU group, and the control group, and cDNA synthesis by was performed using a reverse transcription kit (purchased from Takara). The Ct values for β-actin and P2Y2 amplification were measured for each sample, and duplicate tubes were measured for each sample to reduce operator error. The relative gene expression was calculated using the Ct value as the statistical parameter and the following equations: 1) Ctaverage = (Ct1+Ct2)/2 (duplicate tubes); 2) dCt = Ctaverage-median value; 3) gene expression = 2 (-dCt); 4) relative quantity = expression of the target gene/expression of the internal reference gene (β-actin) ×100.

Statistical analysis

All data were analyzed using SPSS 18.0 statistical software. The data are presented as Mean ± SD based on a univariate analysis of variance (ANOVA). The groups were compared using an

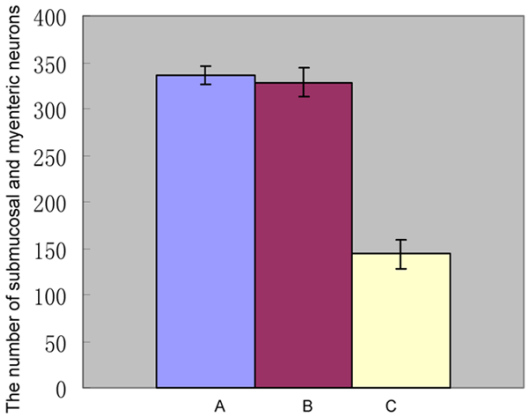

Figure 3. Comparison of the number of submucosal and myenteric neurons from the fetal rats in the normal group (A), the ETU group (B), and the ARM group (C).

LSD-t test and P<0.05 was considered statistically significant.

Results

General observations

The 63 fetal rats in the control group had a survival rate of 100%, and no malformations were observed. The 204 fetal rats in the experimental group had a survival rate of 93.6%, and the incidence of ARM was 89.2%. Twenty-two of the rats in the experimental group (10.8%) exhibited no malformations.

HE staining results

For the fetal rats in the control and ETU groups, the position and opening of the rectum and anus were normal, with a clear outline of perianal muscle and well-differentiated rectal wall layers (**Figure 1A**, **1B**). For the fetal rats in the ARM group, the terminal rectum was a blind end, showing no normal anal structure (**Figure 1C**). For the fetal rats in the control and ETU groups, the terminal rectum submucosa and myenteric plexus showed a large number of darkly stained spherical or ellipsoid neurons with a large nuclear volume, abundant cytoplasm and clear axons (**Figure 2A**, **2B**). For the fetal rats in the ARM group, the stratified structure of the tissue was unclear. We observed many cavities and a smaller number of neurons, with small nuclear size, shrinkage, light staining, irregular shape, sparse distribution, fewer axons, and less cytoplasm (**Figure 2C**). The number of neurons was significantly lower in the ARM group than in the control and ETU groups (143.60 ± 14.93 vs. 335.90 ± 10.46, 143.60 ± 14.93 vs. 328.60 ± 15.75; $P = 0.00$), and the difference was statistically significant. However, the difference between the control and ETU groups (335.90 ± 10.46 vs. 328.60 ± 15.75; $P = 0.238$) was not statistically significant (**Figure 3**).

Immunohistochemistry results

In the control and ETU groups, P2Y2 was highly expressed mainly in the cytoplasm of cells from the terminal rectum submucosa and myenteric plexus in fetal rats (brownish yellow staining in **Figure 4A**, **4B**). In the ARM group, P2Y2 was expressed at low levels in the cytoplasm of cells from the terminal rectum submucosa and myenteric plexus (pale yellow staining in **Figure 4C**). The integrated optical density (IOD) values were significantly higher in the control and ETU groups than in the ARM group (493.18 ± 19.70 vs. 186.48 ± 23.03, 479.48 ± 41.71 vs. 186.48 ± 23.03; $P = 0.00$), and the difference was statistically significant. However, the difference between the control and ETU groups (493.18 ± 19.70 vs. 479.48 ± 41.71, $P = 0.360$) was not statistically significant (**Figure 5**).

Western blot results

The relative expression of P2Y2 protein in the terminal rectum tissues of fetal rats was significantly lower in the ARM group than in the control and ETU groups (0.28 ± 0.08 vs. 0.51 ± 0.10, 0.28 ± 0.08 vs. 0.48 ± 0.12; $P = 0.00$), and the difference was statistically significant. However, the difference between the control and ETU groups (0.51 ± 0.10 vs. 0.48 ± 0.12) was not statistically significant ($P = 0.494$) (**Figures 6** and **7**).

qRT-PCR results

P2Y2 mRNA expression in the terminal rectum tissues of fetal rats was significantly higher in the control and ETU groups than in the ARM group (98.03 ± 15.68 vs. 48.91 ± 12.17, 92.53 ± 10.43 vs. 48.91 ± 12.17; $P = 0.00$), and the difference was statistically significant. However, the difference between the control and ETU groups (98.03 ± 15.68 vs. 92.53 ± 10.43) was not statistically significant ($P = 0.058$) (**Figure 8**).

Discussion

Congenital ARM is one of the most common gastrointestinal malformations in children. This

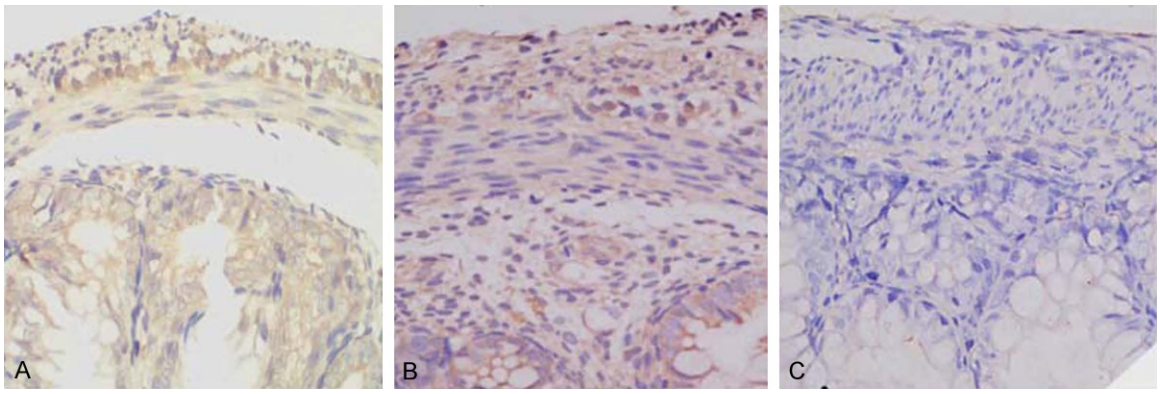

Figure 4. A. High expression of P2Y2 in the control group; B. High expression of P2Y2 in the ETU group; C. Low expression of P2Y2 in the ARM group.

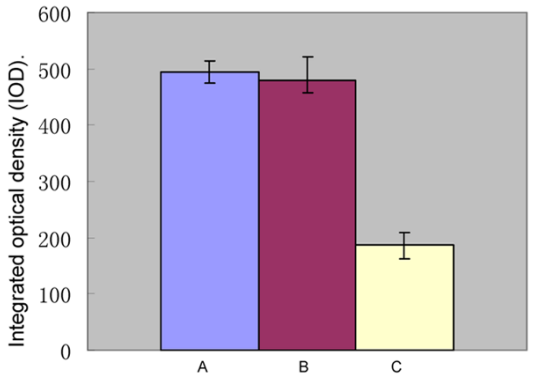

Figure 5. Comparison of the IOD values for P2Y2 in the terminal rectum of fetal rats in the control group (A), the ETU group (B), and the ARM group (C).

congenital malformation that is routinely monitored by the World Health Organization (WHO). It was previously thought that postoperative bowel dysfunction in children with ARM was related to postoperative Hirschsprung's enterocolitis, megarectum or rectum weakness [10]. Recent studies have shown that abnormal neurodevelopment in the terminal rectum is an important cause of postoperative bowel dysfunction in children with ARM [11, 12], but the mechanism of the abnormal ENS development remains unclear. The ENS is comprised of neurons, neurotransmitters, and proteins to transmit signals between neurons. The neurons in the ENS regulate intestinal function by secreting excitatory neurotransmitters (e.g., SP, Ach and 5-HT) and inhibitory neurotransmitters (e.g., NO, VIP, and ATP). A deficiency or excess of neurotransmitters can lead to abnormal ENS development, which can cause many motility disorders [13].

ATP is an inhibitory neurotransmitter released by the intestinal non-adrenergic noncholinergic (NANC) nervous system that is responsible for synaptic purine transmission. Similar to VIP and NO, ATP is a NANC inhibitory co-neurotransmitter in the gastrointestinal tract. It is distributed in various tissues including the gastrointestinal tract. Because ATP regulates communication between different neuroglia and between the neuroglia and nerve trunk, it plays an important role in the proliferation, differentiation, migration, repair, and other activities of intestinal neuronal cells. Thus, ATP can serve as an important indicator of ENS development, but can only function by binding to the appropriate receptors. P2Y2 is an important ATP receptors that is widely distributed in the gastrointestinal tract [14]. Therefore, the level of P2Y2 receptor may indirectly reflect the function of ATP as a neurotransmitter in the gastrointestinal tract.

Studies in China and other countries have shown that ATP can promote the outward growth of axons by binding to the P2Y2 receptor. Arthur et al. [15] found that activation of the G protein-coupled receptor P2Y2 by ATP induced the colocalization of tyrosine kinase receptor A (TrkA) and P2Y2, which enhanced growth, differentiation, migration, and other processes in neurons. Thornton et al. [16] found that, after the slow excitatory synapses were induced in mice ilea, the potential was blocked by PPADS (a P2Y receptor antagonist). This only occurred in the downstream NOS-positive neurons, suggesting that P2Y receptors are involved in downstream conduction. Xiang et al. [17] found that guinea pig myenteric plexus and submucosal plexus cells that expressed P2Y2 also expressed NOS, whereas

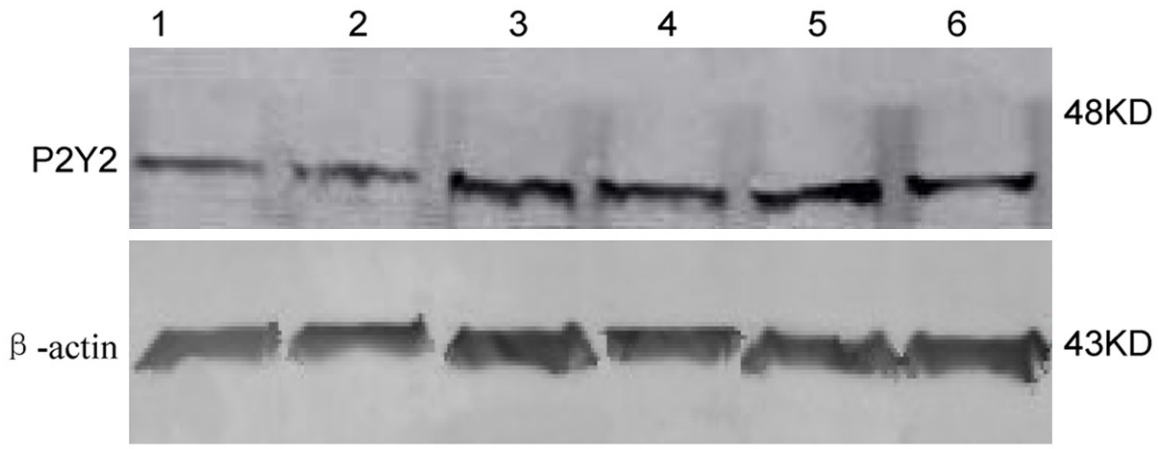

Figure 6. P2Y2 and β-actin expression levels in the three groups. 1 and 2: the ARM group; 3 and 4: the ETU group; 5 and 6: the control group.

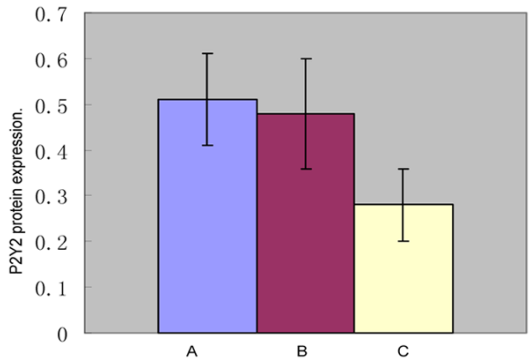

Figure 7. Comparison of the P2Y2 protein expression in the terminal rectum of fetal rats from the control group (A), the ETU group (B), and the ARM group (C).

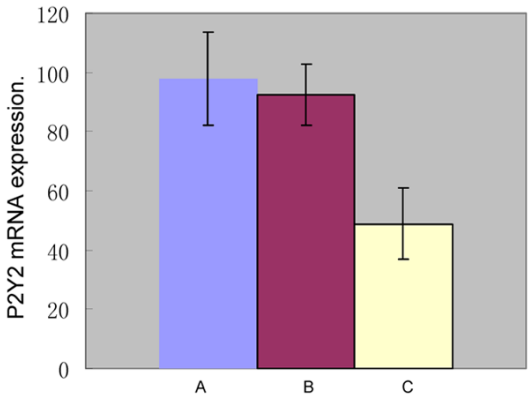

Figure 8. Comparison of the P2Y2 mRNA expression levels in the terminal rectum of fetal rats in the control group, the ETU group, and the ARM group.

cells that did not express P2Y2 receptor also did not express NOS. These studies demonstrated that ATP and NO may have similar functions as inhibitory neurotransmitters in the ENS.

In the ENS, P2Y receptors primarily regulate muscle relaxation. Flavia et al. [18] found that the P2Y receptor antagonist suramin inhibited ATP-induced muscle relaxation, confirming that P2Y receptors participate in muscle relaxation. Giaroni et al. [19] also found that, in the small intestine of mice, ATP exerts its inhibitory effect by binding to P2Y receptors on neurons. Another study showed that [20], in the stomach, small intestine, and large intestine of mice, ATP inhibited the nervous system by binding to its receptor P2Y. Donnell et al. [21] found that P2Y2 is expressed in the intestinal wall of patients with Hirschsprung disease (HD) and that P2Y2 expression in intestinal tissue with HD lesions is significantly lower than in normal intestine. This suggests that P2Y2 may ENS development, leading to HD. A P2Y receptor deficiency could lead to dysfunction in intestinal relaxation, resulting in intestinal spasticity.

This is the first report of the expression and function of ATP and its receptor P2Y2 in the terminal rectum of model organisms with ARM. In this study, we found that P2Y2 was expressed in the cytoplasm of the cells in the terminal rectum submucosa and myenteric plexus. We observed high levels of P2Y2 expression in the control and ETU groups and weak levels of P2Y2 expression in the ARM group. The number of the neurons in the terminal rectum sub-

mucosa and myenteric plexus was significantly lower in the ARM group than in the control group ($P<0.01$). Western blot and qRT-PCR results also showed that the levels of P2Y2 protein and mRNA expression were significantly lower in the ARM group than in the control group ($P<0.01$). There was no significant difference in the number of neurons in the terminal rectum submucosa and myenteric plexus or in P2Y2 between the control and ETU groups ($P>0.05$). This rules out the possibility that the downregulation of the P2Y2 receptor was due to a toxic effect caused by ETU treatment. Our results show that the P2Y2 receptor was downregulated in the terminal rectum of fetal rats with ARM. This result suggests that postoperative constipation in children with ARM may be due to the downregulation of P2Y2, which interferes with ATP function, leading to intestinal spasticity. We only investigated the expression of P2Y2 receptor, and did not assess ATP expression. Experiments are currently underway to determine whether ATP expression is also abnormal in these animals, and whether there is a relationship between P2Y2 and ATP expression levels.

Acknowledgements

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Disclosure of conflict of interest

None.

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