ORIGINAL ARTICLE

Cajal cells in the bladder of rats with anorectal malformations

Células de Cajal na bexiga urinária de ratos portadores de anomalia anorretal

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ABSTRACT

Objective: To evaluate the number of Cajal cells in the bladder of rats with anorectal anomalies induced by ethylenethiourea. **Methods:** Thirty-one fetuses of gravid Wistar rats were used. On the 11th day of gestation, 125 mg/kg ethylenethiourea at 1% diluted in water was given by gastrogavage. Fetuses were removed by C-section on gestation Day 21 and then divided into three groups: G1: urinary bladder of normal fetuses (mothers did not receive ethylenethiourea), G2: urinary bladder of fetuses with anorectal anomalies and no structural urological alterations, G3: urinary bladder of fetuses with anorectal anomalies associated with structural urological alterations. These urinary bladders were processed by the streptavidin-biotin-peroxidase method for Cajal cell investigation, evaluated at two different locations: suburothelium and detrusor muscle. **Results:** Statistically significant differences were found between groups G1, G2, and G3 as to average number of interstitial Cajal cells in the detrusor ($p < 0.001$) and suburothelium ($p < 0.001$). In both sites, the average numbers of cells in the groups were G3 > G2 > G1. **Conclusion:** The number of Cajal cells is increased in fetuses with anorectal anomalies, especially when associated with urologic malformations. The appreciation of abnormalities in morphology and distribution of Cajal cells can lead to an understanding of motility changes in several congenital and acquired urological diseases.

Keywords: Urinary bladder; Imperforate anus; Stromal cells; Rectum/abnormalities; Anal canal/abnormalities; Rats

RESUMO

Objetivo: Avaliar o número de células de Cajal na bexiga de ratos portadores de anomalia anorretal induzida pela etilenotiouréia. **Métodos:** Foram utilizados 31 fetos de ratas Wistar grávidas. No 11º dia de gestação foi administrada etilenotiouréia na dose de 125 mg/kg, diluída em água na concentração de 1%, por gavagem gástrica. Os fetos foram retirados por cesariana no 21º dia de gestação. Os fetos foram divididos em três grupos: G1: bexiga urinária de fetos normais (mães não receberam etilenotiouréia), G2: bexiga urinária de fetos que apresentavam anomalia anorretal sem manifestar alteração urológica estrutural, G3: bexiga urinária de fetos que apresentavam anomalia anorretal associada à alteração urológica estrutural. Essas bexigas urinárias foram processadas pela técnica da estreptavidina-biotina-peroxidase para pesquisa de células de Cajal avaliadas em duas localizações diferentes: suburotélio e detrussor. **Resultados:** Foram encontradas diferenças estatisticamente significativas entre os grupos G1, G2 e G3 quanto à média de número de células intersticiais de Cajal nas localizações detrussor ($p < 0,001$) e suburotélio ($p < 0,001$). Em ambas as localizações, a média do número de células no grupo G3 > G2 > G1. **Conclusão:** As células de Cajal estão presentes em número aumentado nos fetos portadores de anomalia anorretal, em especial quando há associação com malformações urológicas. O reconhecimento de anormalidades na morfologia e distribuição das células de Cajal pode nos levar ao entendimento das alterações de motilidade em várias doenças urológicas congênitas e adquiridas.

Descritores: Bexiga urinária; Ânus imperfurado; Células estromais; Reto/anormalidades; Canal anal/anormalidades; Ratos

INTRODUCTION

It is known that children with anorectal malformations (ARM) have a high incidence of associated abnormalities such as urologic and lumbosacral anomalies[1-7]. Many of these children with ARM have urinary tract dysfunctions that cause significant urologic problems with a morbidity that frequently exceeds that of isolated ARM[2].

Study carried out at Curso de Pós-Graduação em Cirurgia e Experimentação do Departamento de Cirurgia da Universidade Federal de São Paulo – UNIFESP – Escola Paulista de Medicina (EPM), São Paulo (SP), Brazil.

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Structural urologic anomalies associated with anorectal anomalies are well documented[1-3], but functional alterations of the lower urinary tract have not been well studied[4]. The neurogenic bladder of these patients is generally caused by lesions in the spinal cord (myelodysplasia), a very common association in patients with ARM[5]. Rarely, the cause of the neurogenic bladder is damage to pelvic nerves during anorectoplasty[5-7].

In 1893, Ramon and Cajal described small cells circumscribing intestinal myenteric ganglia, and these cells were called interstitial Cajal cells (ICC)[8]. Presently, with further study of this population of specialized cells of mesenchymal origin that express the tyrosine kinase kit (c-Kit) receptor on the surface, physiology of smooth muscle tissue is better understood. The ICC are located all along the digestive tract, adjacent to nerve fibers and myocytes, and are considered pacemakers in that they generate slow waves, facilitators of electrical stimuli propagation, and mediators of neurotransmission. The ICC can be recognized by electronic microscopy or by immunohistochemical demonstration of the c-kit receptor.

Knowledge as to their location, morphology, and physiological properties in the urinary tract is still limited[8], but it is known that they function as pacemakers or modulators of neurotransmission and neural conduction of electrical impulses, influencing contractile behavior[8-15]. In the bladder, these ICC are found below the lamina propria (suburothelium) where they interact with the urothelium and with sensitive nerves, and among smooth muscle bundles[8,12,14,15].

Since Cajal cells act as pacemakers signaling for regular contraction of smooth muscle and also regulate nerve impulse transmission to smooth muscles, appreciation of abnormalities in morphology and distribution of ICC can lead to an understanding of the functional modifications in several congenital and acquired urologic diseases[8-16].

OBJECTIVE

To evaluate the existence of changes in the number of ICC in the bladder of rats with anorectal anomalies induced by ethylenethiourea (ETU).

METHODS

Ethics

The experiment was approved by the Research Ethics Committee of the Universidade Federal de São Paulo; Protocol nº 0406/05.

The experiment received funding from the State of Sao Paulo Research Foundation - FAPESP; Process nº 05/56062-8.

Sample

Thirty-one fetuses were used from the litters of four OUT-B EPM-1 WISTAR rats from the animal facility at the Universidade Federal de São Paulo. The animals were maintained at this facility during the entire experiment.

Pregnant rats were distributed into 2 groups:

- Experimental group – Rats (E1, E2, E3) that received ETU on the 11th day of gestation, at a dose of 125 mg/kg in a 1% concentration in distilled water (12.5 ml/kg).
- Control group – Rats that received only distilled water at a volume of 12.5 ml/kg.

Pregnancy criterion

Vaginal smear with the presence of sperm after overnight mating. This was considered Day Zero (D0) of gestation. As of this moment, gravid rats were weighed and maintained in individual cages.

Induction of ARM

On the 11th day of gestation (D11), ethylenethiourea (ETU) was administered via gastrogavage of 125 mg/kg in a 1% concentration in water.

The pregnant rats were euthanized on the 21st day of gestation after having been weighed, and their uterine cavities were then opened for removal of the fetuses.

Fetuses were initially examined externally to determine sex, presence of ARM, and malformations of the spine and tail (Chart 1). Next, with the use of microscopy, fetuses were submitted to exploratory laparotomy to determine the type of ARM and investigate urologic malformations.

No fetus from the control group presented anorectal anomalies. The model used caused anorectal anomalies in 71% of fetuses. The types of anorectal anomaly found were: 14 of the cloaca, 4 ARM with perineal fistula, and 4 ARM with urethral fistula.

No fetus from the control group showed spinal malformations. These malformations were identified in 80% of experimental group fetuses, and were represented by agenesis of the tail or a short tail.

No fetus of the control group presented structural urologic alterations. Among the experimental group fetuses, 35% had alterations. Ureterohydronephrosis was found in 5 fetuses (Figure 1), unilateral renal agenesis in 4 fetuses, left kidney hypoplasia in one fetus, and persistence of the urachus in one fetus (Figure 2).

For ICC evaluation, bladders were removed from the fetuses and the animals were classified in three groups:

- Group 1 (n = 7): control: urinary bladders of normal fetuses from rats that did not receive ETU.
- Group 2 (n = 5): urinary bladders from fetuses with

ARM associated with spinal alterations, with no structural urologic alteration.

- Group 3 (n = 9): urinary bladders from fetuses with ARM associated with spinal alterations and structural urologic alterations.

These bladders were fixed in 10% buffered formaldehyde, dehydrated, and embedded in paraffin.

Chart 1. Associated malformations in fetuses of the experimental group

Group	Fetuses	ARM	Spine	Urologic
E1	F1	cloaca	short tail	normal
n = 10	F2	cloaca	tail agenesia	normal
	F3	normal	short tail	normal
	F4	perineal	tail agenesia	normal
	F5	urethral	tail agenesia	ureterohydronephrosis
	F6	cloaca	tail agenesia	normal
	F7	perineal	short tail	normal
	F8	cloaca	tail agenesia	normal
	F9	cloaca	tail agenesia	ureterohydronephrosis
	F10	cloaca	tail agenesia	ureterohydronephrosis
E2	F1	perineal	tail agenesia	normal
n = 11	F2	cloaca	tail agenesia	normal
	F3	cloaca	tail agenesia	hypoplasia left kidney
	F4	cloaca	tail agenesia	renal agenesia
	F5	urethral	tail agenesia	normal
	F6	cloaca	tail agenesia	normal
	F7	normal	short tail	renal agenesia
	F8	urethral	short tail	renal agenesia
	F9	urethral	tail agenesia	ureterohydronephrosis
	F10	cloaca	tail agenesia	ureterohydronephrosis
	F11	perineal	short tail	persistência de úraco
E3	F1	normal	normal	normal
n = 10	F2	normal	normal	normal
	F3	normal	tail agenesia	normal
	F4	cloaca	tail agenesia	normal
	F5	cloaca	tail agenesia	normal
	F6	normal	normal	renal agenesia
	F7	normal	normal	normal
	F8	normal	normal	normal
	F9	cloaca	tail agenesia	normal
	F10	normal	normal	normal

Association of ARM + spinal alteration + structural urologic alteration
Association of ARM + spinal alteration

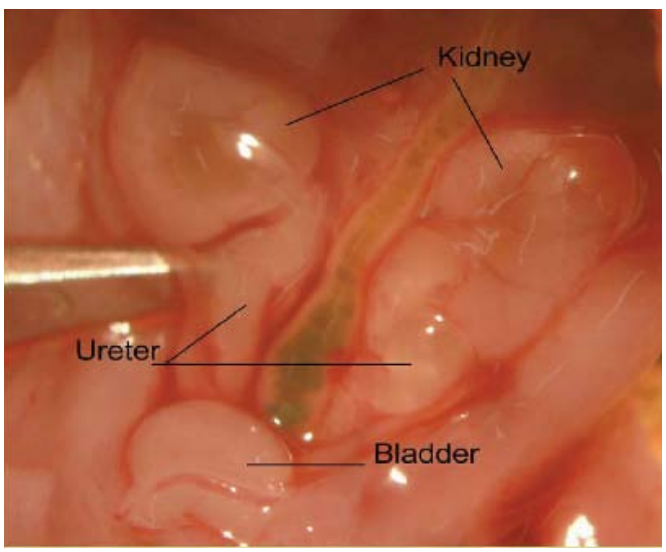

Figure 1. Ureterohydronephrosis in a rat with ARM

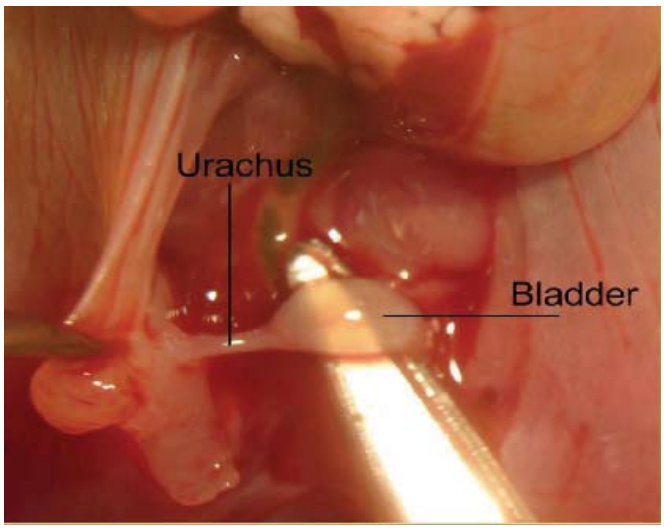

Figure 2. Persistence of the urachus in a rat with ARM

Immunohistochemical procedure

The previously paraffin-embedded material was once again sectioned into 3-micrometer slices and processed by the streptavidin-biotin-peroxidase technique for Cajal cell investigation:

1. deparaffinization in an incubator/oven;
2. deparaffinization in xylol;
3. rehydration in 70% absolute alcohol, running water and distilled water;
4. antigen retrieval with 0.01M citrate buffer, pH 6.0, in a microwave oven at maximum potency for three 5-minute cycles, and kept in incubation for a further 20 minutes at room temperature, followed by washes in running water and distilled water;
5. blocking of endogenous peroxidase with a solution of hydrogen peroxide (H_2O_2), 10 volumes, for two 10-minute cycles, followed by washes in running water and distilled water;
6. blocking of nonspecific reaction by incubation in phosphate buffer, pH 7.2, with the addition of 1% bovine serum albumin for 15 minutes, discarding excess after this time;
7. incubation in the primary antibody (Polyclonal Rabbit Anti-Human CD117, c-kit- DakoCytomation) at a dilution of 1:150, overnight, at a temperature of approximately 8°C;
8. wash in buffer;
9. incubation in biotinylated secondary antibody at room temperature for 30 minutes;
10. wash in buffer;
11. incubation in the streptavidin-biotin-peroxidase complex at room temperature for 30 minutes;
12. wash in buffer;
13. incubation with the diaminobenzidine enzymatic substrate;

14. wash in running water and distilled water;
15. Harris' counter-staining with hematoxylin.

The slides were analyzed by computed image analysis system, comprised by a Sony® CCD-IRIS video camera coupled with an optic Olympus (BX 50) microscope, using panchromatic objectives to transmit images to a Pentium 4 microcomputer, with 502 megabytes of RAM, Windows 98® and equipped with a digitalizing board and Image-Pro-Plus® (version 4.1) software. Images of the suburothelium and detrusor corresponding to six fields with a 400X magnification were captured. Cajal cells were counted with the help of the Image Tool imaging analysis program.

Statistical study

The number of ICC in six high magnification (400X) fields in the suburothelium and detrusor was summarized per group and represented by mean, standard deviation (SD), median, and minimum and maximum values.

Normal distribution of the variable within the groups was tested by the Kolmogorov-Smirnov test.

A variance analysis (ANOVA) technique was applied and the differences were located by Tukey's multiple comparison test.

A 0.05 ($\alpha = 5\%$) significance level was adopted.

RESULTS

Detrusor (Table 1 and Figure 3)

Statistically significant differences were found among the groups as to the average number of ICC in the detrusor – Figure 4 ($p < 0.001$), in which:

- G1 is less than G2 ($p = 0.028$);
- G1 is less than G3 ($p < 0.001$);
- G2 is less than G3 ($p = 0.002$).

Suburothelium (Table 1 and Figure 5)

Statistically significant differences were found among the groups as to the average number of ICC in the suburothelium – Figure 6 ($p < 0.001$), in which:

- G1 is less than G2 ($p = 0.001$);
- G1 is less than G3 ($p < 0.001$);
- G2 is less than G3 ($p = 0.039$).

Table 1. Number of Cajal cells in six 400X fields in the suburothelium and detrusor muscle in three study groups

Site	Group G1 N = 7	Group G2 n = 5	Group G3 n = 9
Detrusor			
mean ± SD	24.9 ± 5.7*	40.6 ± 5.0*	62.3 ± 12.8*
median	27	42	63
minimum – maximum	15 – 31	32 – 45	46 – 81
Suburothelium			
mean ± SD	22.7 ± 6.5*	47.4 ± 14.1*	61.2 ± 7.9*
median	24	48	58
minimum – maximum	11 – 29	34 – 70	53 – 78

* ANOVA $p < 0.001$

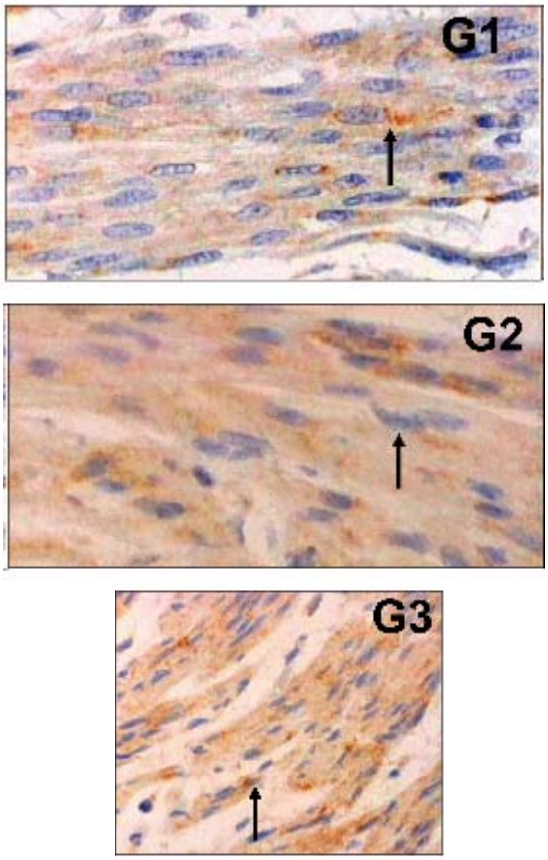

Figure 3. Photomicrography representing immunohistochemical staining with CD 117- c-kit in the detrusor muscle of animals: G1: normal fetuses; G2: fetuses with ARM; G3: fetuses with ARM and structural urologic alteration (black arrows show Cajal cells stained in brown) (400X)

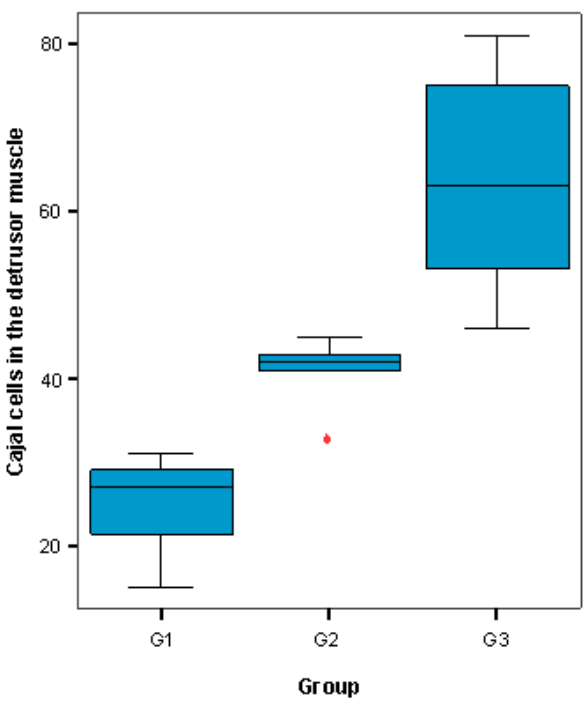

Figure 4. ICC in the detrusor

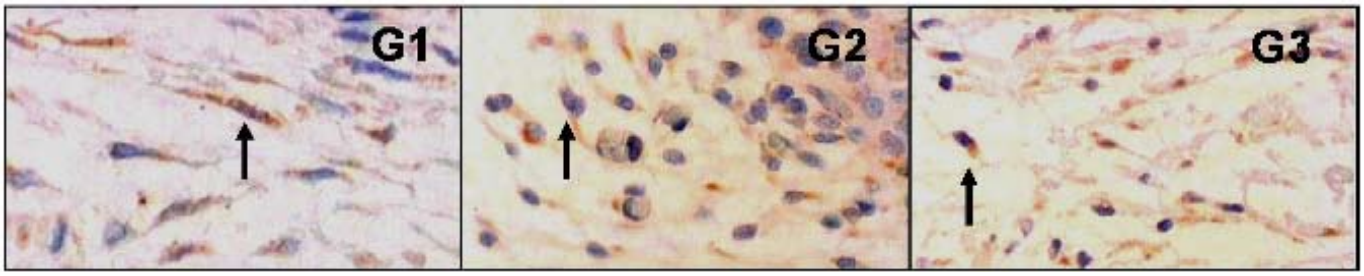

Figure 5. Photomicrography representing immunohistochemical staining with CD 117- c-kit in the suburothelium of animals: G1: normal fetuses; G2: fetuses with ARM; G3: fetuses with ARM and structural urologic alteration (black arrows show Cajal cells stained in brown) (400X)

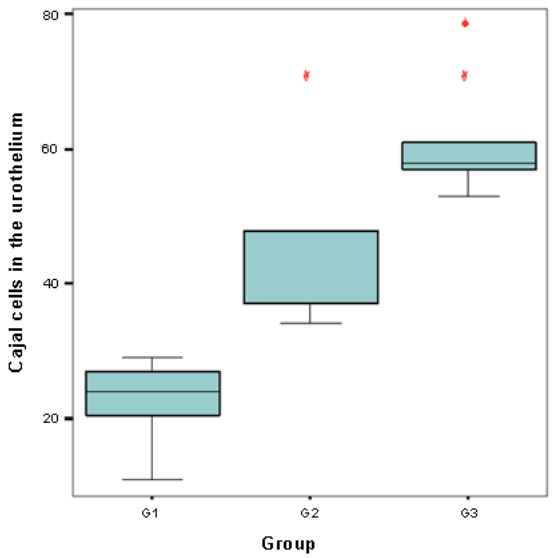

Figure 6. ICC in the suburothelium

DISCUSSION

Bladder dysfunction is the primary cause of morbidity and mortality in patients with ARM since it leads to deterioration of the upper urinary tract, repeat urinary infections, and urinary incontinence[2].

The reported incidence of neurogenic bladder varies from 7 to 29% in children with ARM[1,3-4]. Bladder dysfunction in these patients can be a result of a congenital neurologic deficit (myelodysplasia) or nerve lesion during surgical correction (transabdominal procedures and extensive retrovesical dissections). Ralph et al.[17], believe that atonic neurogenic bladder is a consequence of injury at the time of surgical correction, and that bladder hyperreflexia is due to a change in the upper motor neuron.

Myelodysplasia (anchored spinal cord, filum terminale lipoma, myelocele, myelomeningocele, diastematomyelia, syringomyelia, and associations of these lesions) are spinal cord lesions with an increased incidence in patients with ARM[7]. The presence of a spinal cord lesion is the main factor responsible for the bladder dysfunction experienced by these patients[4].

Some aspects of urinary bladder function are not well known, including spontaneous activity and complacency[8]. It is known that during the bladder filling phase, muscle cells relax and elongate maintaining intravesical pressure[8].

Recent studies demonstrated the presence of ICC in different parts of the urinary tract (ureteropyelo junction[9], urethra[10], vas deferens[11], bladder[8,12-14], and ureter[15]). These cells were first described by Cajal in the human intestine as primitive accessory components that modify smooth muscle contraction. The ICC function as pacemakers or modulators of neurotransmission and neuronal conduction of electric impulses[8-15].

The ICC have already been identified in the bladders of humans[13,15,18], rats[19], and pigs[8,12,14,20-21], and are located in the suburothelium where they interact with the urothelium and sensitive nerves (stellate morphology forming a cellular network), and around detrusor smooth muscle bundles (spindle-shaped).

In studying rats with anorectal anomalies, we noted increased numbers of ICC present in both the detrusor and suburothelium, especially when there is an associated urologic malformation.

Biers et al.[18] demonstrated in human bladders that Cajal cells occupy 13% of the detrusor muscle area in 'normal' bladders, 54% of the detrusor area in hyperactive bladders, and 91% of the detrusor area in neurogenic bladders. This project[18] leads to the suggestion that the greater the number of Cajal cells in the bladder, the greater the functional modification.

Recent studies[18,20-21], besides confirming that Cajal cells are responsible for generating action potentials and contracting detrusor smooth muscle cells, also demonstrated that drugs that inhibit the c-kit tyrosine kinase receptor (imatinib mesylate) reduce the pressure peak of the bladder in a dose-dependent manner and abolish spontaneous action potentials in the smooth

muscle, opening the way for a new treatment of hyperactive and neurogenic bladders.

CONCLUSION

The number of Cajal cells is increased in fetuses with ARM, especially when urologic malformations are associated.

This increased number of Cajal cells can indicate the presence of detrusor hyperreflexia or neurogenic bladder, functional abnormalities that can lead to renal failure, the primary cause of morbidity and mortality in patients with ARM .

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