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Apoptotic cell death and fertility in three unilateral cryptorchid rat models

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Abstract Three rat strains have been studied, using a sensitive apoptotic detection method for germ-cell degeneration, to resolve the controversy regarding the effect of cryptorchidism on the contralateral descended testis (CDT). Sprague Dawley and Buffalo rats were made cryptorchid by operation at 20–22 days of age, while trans-scrotal (T-S) rats were a congenitally unilateral cryptorchid strain. Sham operated rats or normal T-S littermates were used as controls. Experiments were performed over a period ranging from 2 weeks to 18 months. Testis weight was assayed, as was the detection of apoptosis by agarose gel laddering and immunohistochemistry by using the TUNEL method. Labeled cells in 150 cross-sectioned testis tubules were counted on the TUNEL stained slides and the mean number of labeled cells per tubule was calculated. Paternity studies on Sprague Dawley and T-S rats were carried out at 12 and 24 weeks of age to assess fertility by the resultant number of pregnancies and litter sizes. Both Sprague Dawley and T-S rat models showed a biphasic distribution of apoptosis levels. This biphasic distribution was not observed in Buffalo rats as they were only studied at later time points (12–20 weeks). A significant effect on either testis weight or apoptosis in the CDT compared with the control descended testis ($P \geq 0.1$) has not been found in these three cryptorchid models, and the present results are discussed with reference to observations of other researchers in rodents and humans. While the cryptorchid testis showed a high level of labeled apoptotic cells per tubule in all rat strains, fertility was not affected and remained the same as controls at 12 and 24 weeks. There was, however, a marked strain difference in fertility in T-S as compared with Sprague Dawley rats. After 24 weeks of cryptorchidism, both control and cryptorchid T-S rats had a 44% pregnancy incidence compared with a 90% pregnancy incidence in Sprague Dawley rats. In addition, litter size in T-S control and cryptorchid rats were small compared with those of Sprague Dawley rats at 12 and 24 weeks.

Key words Cryptorchidism · Contralateral descended testis · Apoptosis · Fertility · Rat model

Introduction

Cryptorchidism is one of the most commonly occurring congenital abnormalities, affecting 1–5% of boys. Histologic changes in the cryptorchid testis can be detected by 2 years of age, and if bilateral, a detrimental effect on fertility could result. The effect of unilateral cryptorchidism on fertility has been more controversial as the undescended testis is proposed to have a compounding effect on the contralateral descended testis (CDT). Studies in human unilateral cryptorchid patients have shown a decrease in fertility [6, 12, 13, 16] and spermatogenic parameters, while others have found normal paternity rates [2, 4, 5, 11].

Cryptorchid rat models have been used to analyze cryptorchidism and associated changes in fertility and to determine whether an effect is mediated on the CDT by the undescended testis. Some studies show no reduction in fertility in unilateral undescended testis (UL-UDT) rat models for up to 80 days, although a significant reduction was observed by others at 120–180 days of age [15, 19, 24]. However, the number of offspring was lower in rats with unilateral incarcerated inguinal hernia [21].

Both spontaneous and induced pathologic germ-cell death is known to occur via an apoptotic mechanism [3, 8, 14, 18]. In this study we analyzed the level of apoptosis in three unilateral cryprorchid rat models to

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determine whether an effect on the contralateral testis can be detected and we correlated this with paternity studies to assess fertility. We found that apoptosis was not significantly higher in the CDT as compared with control testes and this was correlated with normal fertility up to 168 days of cryptorchidism.

Material and methods

Rats

Sprague Dawley and trans-scrotal (T-S) rats were bred and maintained in the Royal Children's Hospital animal facility, as were the Buffalo rats, which were obtained from the University of Melbourne Biochemistry Department animal laboratory. Sprague Dawley and Buffalo rats were made unilaterally cryptorchid at 20–22 days of age, which was before descent of the testis was complete. An incision was made in the left-lower quadrant of the abdomen and the processus vaginalis freed from the surrounding scrotum and the testis pushed into the abdomen. The processus vaginalis was tucked back into itself and sutured at the level of the internal inguinal ring. Sham operated rats were used as controls. Congenital unilaterally cryptorchid T-S rats were examined using normal littermates as controls. Groups of 2–4 experimental and 2–4 control Sprague Dawley and T-S rats were used for each time point. Four Buffalo rats were used per group.

Cell line

The 32Dcl23 (32D) cell line was cultured in Iscove's modified Dulbecco's medium (Gibco, Grand Island, N.Y.) with 10% fetal calf serum (Trace Laboratories, Melbourne, Australia) and 20% pokeweed mitogen spleen cell conditioned medium [7]. Cultures were incubated at 37 °C and 5% CO_2 in a humid atmosphere.

Specimen collection

Testes were removed from Sprague Dawley rats between 2 and 24 weeks post-operation; from Buffalo rats at 12, 16, and 20 weeks post-operation; and from T-S rats at 4–12 weeks and at 6, 12, and 18 months of age. The testes were weighed and half of each testis was fixed in formalin for TUNEL staining, while the remaining half was snap frozen for extraction of DNA to run the apoptosis gel ladder assay.

TUNEL method

Paraffin-embedded 5-μm sections were mounted on silane-coated slides (2% solution of 3 aminopropyltriethoxysilane (Sigma Chemical Co., St. Louis, Mo., USA). Sections were dewaxed, rehydrated, rinsed in phosphate-buffered saline (PBS), and then permeabilized with 20 mg/ml Proteinase K (Sigma) for 30 min to unmask fragmented DNA. Endogenous peroxidase was quenched using a 5 min incubation in a 0.6% solution of hydrogen peroxide in PBS. The slides were placed in terminal transferase equilibration buffer (a mixture of 0.2 M sodium cacodylate, 25 mM Tris HCl, 0.25 mg/ml bovine serum albumin at pH 6.6, 0.2 M sodium cacodylate, and 25 mM cobalt chloride), then incubated in a humid chamber with terminal transferase enzyme (TdT; 1.25 U/μl; Boehringer Mannheim, Melbourne, Australia) and 50 μM digoxygenin-11-UTP (Boehringer Mannheim) for 30 min at 37 °C. The reaction was stopped by placing the slides in PBS at 37 °C for 30 min with agitation every 10 min. The sections were then rinsed in PBS, covered with peroxidase conjugated antibody to digoxygenin (1/500 dilution; Boehringer Mannheim), and incubated for 30 min at room temperature. After washing in PBS, the immunoreaction was visualized by developing sections with diaminobenzidine (DAB; Sigma) at room temperature. The sections were counterstained with hematoxylin, dehydrated, cleared, and mounted in DPX (BDH, Poole, UK).

Apoptosis gel ladder assay

DNA was extracted from rat testes and the 32D cell line, after washing with PBS, using DNAzol (Life Technologies, Australia) followed by ethanol precipitation, and phenol–chloroform and chloroform extraction. After an additional ethanol precipitation, the pellet was resuspended in 50 μl of TE buffer (0.2 M Tris–HCl, 0.05 M EDTA, pH 8). Ethanol precipitations were carried out overnight at −20 °C and tubes were microfuged at 14,000 rpm for 30 min at 4 °C to ensure an optimal yield of low molecular weight DNA fragments. The DNA pellet was dissolved in TE buffer and made up to a concentration of 2 mg/29 μl with water. The DNA labeling method was adapted from Tilly and Hsueh [23] using 25 U TdT enzyme and 5 μl ^{32}P dCTP (10 mCi/ml with a specific activity of 3000 Ci/mmol; DuPont, Boston, Mass., USA) and incubated for 60 min at 37 °C. The labeled DNA products were purified and electrophoresed on a 2% agarose gel with lamda-HindIII DNA markers (Boehringer Mannheim). The gel was dried in a gel dryer without heat and exposed to X-omat autographic film (Kodak, Melbourne, Australia).

Paternity studies

Sprague Dawley and T-S rats were used at 12 and 24 weeks for paternity studies with up to ten unilaterally cryptorchid and ten normal littermates per group. Males were housed with two virgin 12-week-old females for 2 weeks, which is a standard protocol for determining male fertility [14, 18, 24]. Males were removed and the females were left to deliver naturally. The frequencies of resultant pregnancies and litter sizes were recorded.

Statistical analysis

Mean values and standard deviations (SD) were calculated for all experimental groups and Students *t*-test was performed to determine the level of significance.

Results

Testis weights

The CDT of the Sprague Dawley and T-S rats increased in weight up until 6 weeks (Sprague Dawley) and 12 weeks (T-S) post-operation, after which testis weight reached a plateau, as did the testes of the control rats (Fig. 1). The UL-UDT weight remained constant from the 4th week post-operation and from the 6th week in T-S rats (Fig. 1). In Buffalo rats, the UL-UDT weights were significantly lower than the CDT weights and controls that had similar weights at the three time points assayed.

Apoptosis

The three rat models showed very low numbers of labeled apoptotic cells per tubule in the CDT and controls throughout the experimental time frame. In the

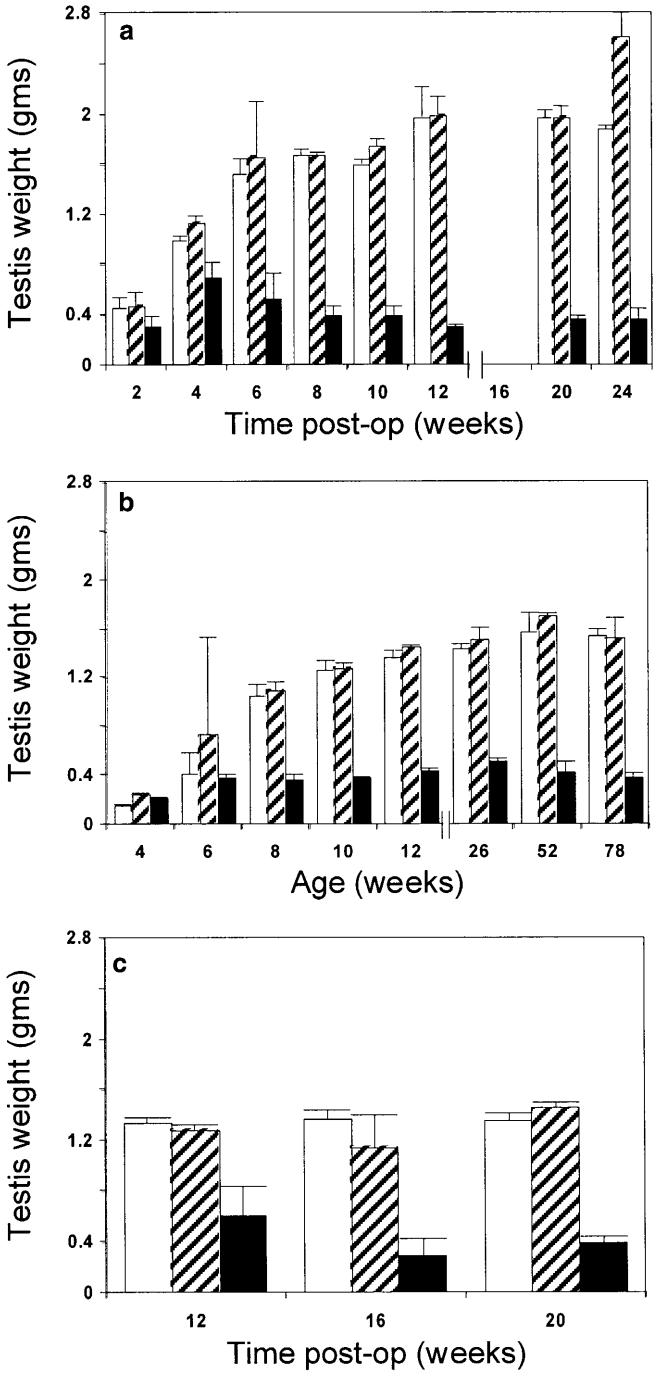

Fig. 1 Measurements of testis weights are shown for **A** Sprague Dawley, **B** T-S, and **C** Buffalo rats. Sprague Dawley and Buffalo rats were made cryptorchid at 3 weeks of age, whereas T-S rats were naturally cryptorchid and their age is shown accordingly. *Bars* indicate control descended testis (*white*), CDT (*hatched*), and UL-UDT (*black*). Two to four UL-UDT and CDT were assayed per rat group and four to eight testes were weighed in the control group. Mean values ± SD are shown for each group

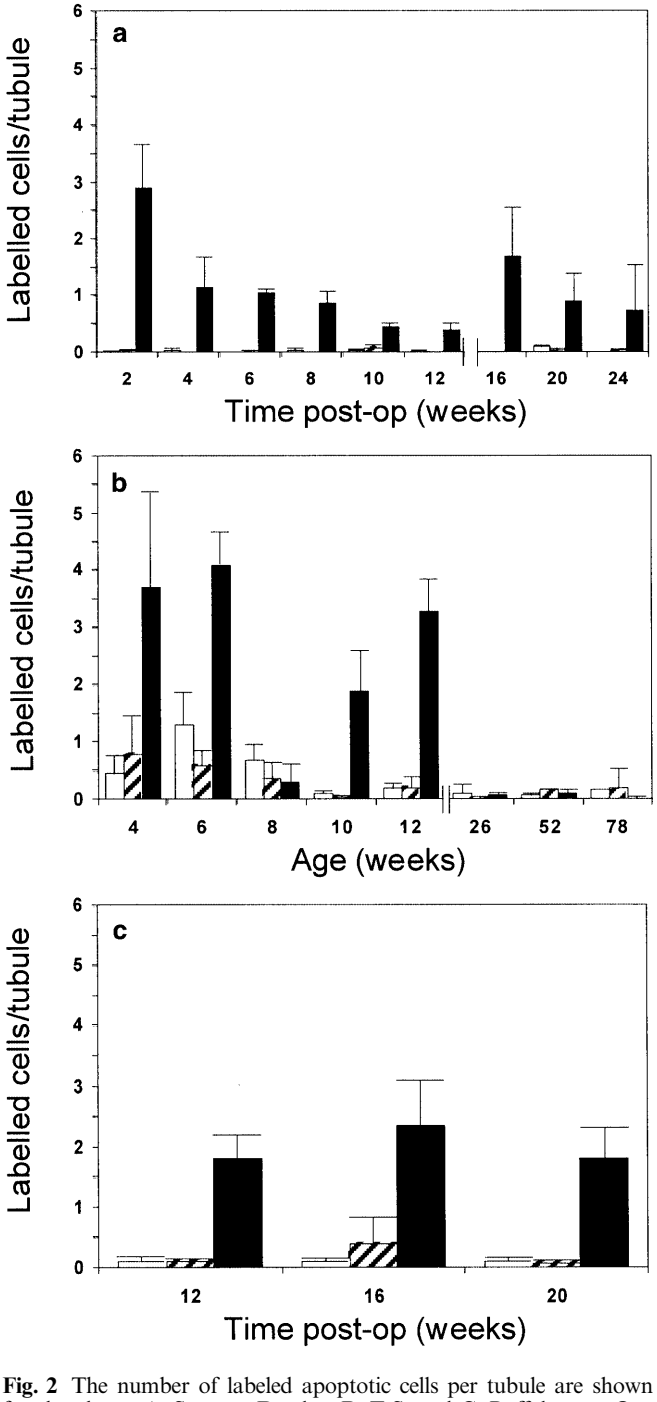

Fig. 2 The number of labeled apoptotic cells per tubule are shown for the above: **A**, Sprague Dawley; **B**, T-S; and **C**, Buffalo rats. One hundred and fifty tubule cross-sections were counted for each testis and two to four testes were sampled per group. *Bars* indicate control descended testis (*white*), CDT (*hatched*), and UL-UDT (*black*). Mean values ± SD values are shown for each group

UL-UDT, significant levels of labeling were observed ($P < 0.0003$; Fig. 2) and the number of labeled apoptotic cells per tubule exhibited a biphasic distribution in both Sprague Dawley and T-S rats, with a second peak at 16 and 12 weeks, respectively. By comparison, the UL-UDT Buffalo rat group had a consistently significant level of labeled apoptotic cells from 12–20 weeks post-operation. Overall, the number of labeled apoptotic cells per tubule was comparatively higher in T-S rats in all three testis groups compared with the Sprague Dawley and Buffalo strains. The gel ladder assay confirmed the findings of the TUNEL staining in the T-S rat testes

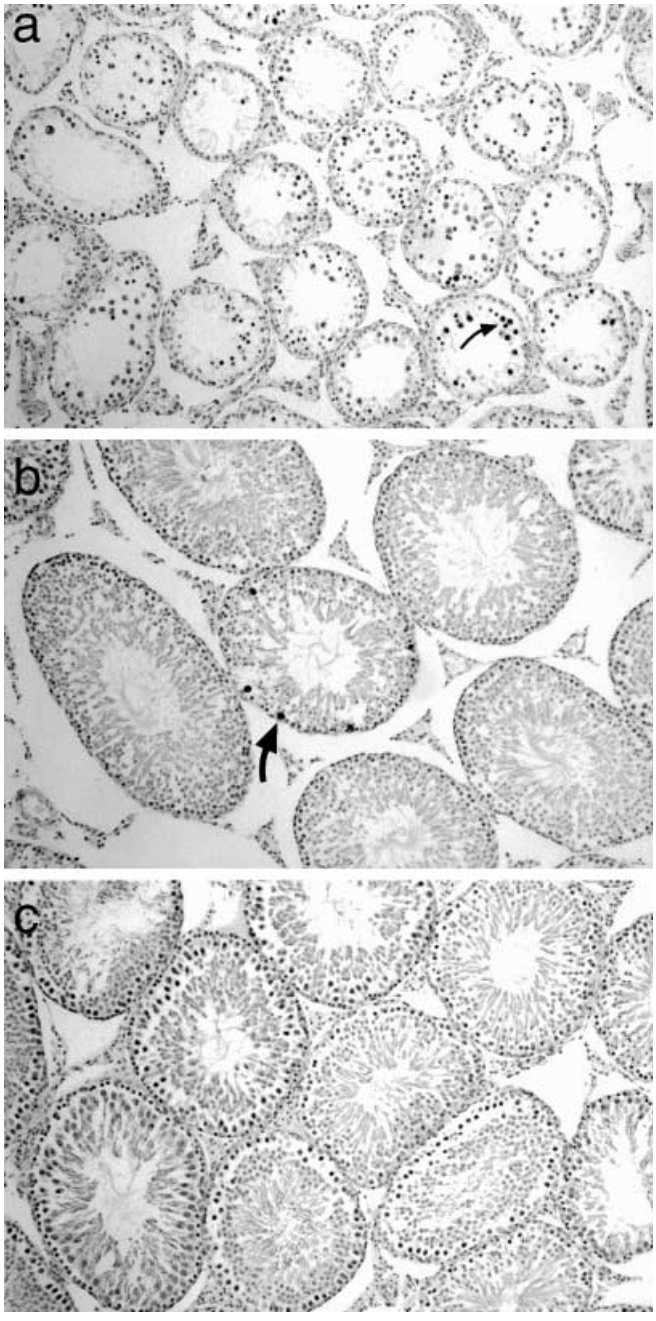

Fig. 3 Photomicrographs of testes stained by the TUNEL method are shown for **A** undescended testis, **B** CDT and **C** normal control testis in T-S rats at 10 weeks. Brown staining of the nucleus of apoptotic cells is indicated (*arrows*). Magnification ×400

(Figs. 3, 4). While there was a high level of apoptosis and very few germ-cells in the seminiferous tubules of UL-UDT (Fig. 3), the CDT or control testis had a sufficient number of germ-cells to maintain fertility.

Paternity studies

No significant difference was found in the number of pregnancies and litter sizes ($P \geq 0.26$) in Sprague Dawley control and cryptorchid groups or T-S rat groups at comparable ages, which were 12 and 24 weeks of age (Table 1). Contralateral effects were not observed in any of the strains and differences between fertility in cryptorchid and controls were not detected in T-S and Sprague Dawley rats. For this reason we did not perform paternity studies in Buffalo rats. T-S rats exhibited a lower fertility and this decreased with age from 67% to 65% at 12 weeks and to 44% at 24 weeks. Litter sizes were also considerably smaller in T-S rats as compared with Sprague Dawley rats.

Discussion

Only in the UDT group was there significant labeling of apoptotic cells per tubule in the three rat strains studied. In Sprague Dawley rats there was a high degree of apoptosis seen after the second week post-operation but the effect on testis weight was not evident until the 4th week post-operation. This delay is even more evident in the T-S rats, where there was significant apoptosis at 4 weeks but the effect on testis weight was not evident until 8 weeks. Over the entire assay period, apoptosis appeared to have a biphasic distribution in the UDT group in both the Sprague Dawley and T-S rats; however, this was not reflected in the testis weight, which remained constant. Sprague Dawley rats showed a rise in apoptotic cell number from 16 weeks, while the T-S rats showed an increase from 10 weeks, after which levels fell. By comparison, the control and CDT have a persistent low level of apoptosis supporting normal regulation of germ-cell death in the mature descended testis. The level of apoptosis indicated by the number of apoptotic cells per tubule was generally higher in T-S compared with Sprague Dawley and Buffalo rats.

We found no differences in the CDT compared with the control testis in relation to testis weight and the number of apoptotic cells per tubule in the three rat models, which were either naturally cryptorchid or made cryptorchid by surgical means. This agrees with the results of Quinn et al. [17], who found no effect on the testis weight, the Johnsen score, or on the tubular size in Wistar rats up to 120 days.

Fertility was not demonstrated to be affected as paternity rate and litter size were comparable with controls in all of the models. This is in agreement with the findings of Argawala and Mitra [1] and Kogan et al. [10], while Srinivas [19] found a decrease in paternity rate after 120 days. Stewart and Brown [20] found a normal sperm count at 100 days but a decreased sperm count at 150 days, and normal fertility at 130 days. Normal fertility but with a decreased number of offspring was found by Zakaria et al. [24]. Patkowski et al. [14] found a dramatic effect on paternity rates in the naturally cryptorchid Buffalo rat by 180 days but not at 90 days. The Buffalo rat strain we used is presumably genetically different to that used by Patkowski et al., as the strain we used was not congenitally cryptorchid. It is possible

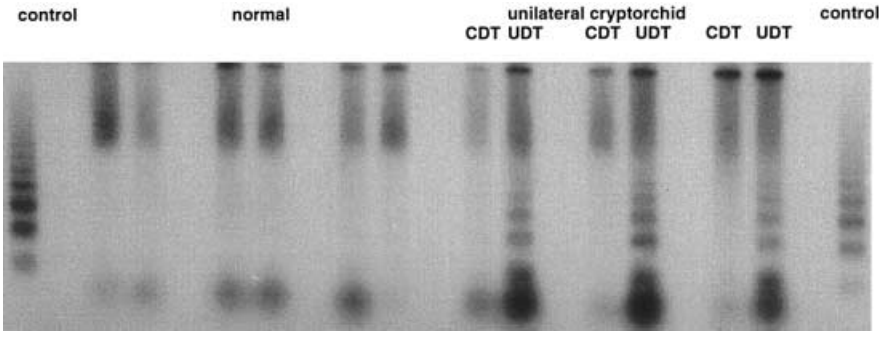

Fig. 4 The apoptosis gel ladder assay is shown for three normal T-S rats at 10 weeks and three unilateral cryptorchid rats at 10 weeks of age. The *control lane* for this experiment is an interleukin 3-dependent cell line, 32D, which had been grown for 24 h without growth factor to induce apoptosis. The size of the apoptotic bands in the tracks were calculated from the DNA standards and occurred at 180–200 bp intervals (band sizes increase in ascending order by this interval)

Table 1 Paternity studies using cryptorchid and normal Sprague Dawley and T-S rats

Rat strain	Experimental groups	Age (weeks)	
		12	24
Number of pregnancies			
Sprague Dawley	Control	100% (10/10)	90% (18/20)
	Cryptorchid	95% (19/20)	90% (18/20)
T-S	Control	67% (12/18)	44% (8/18)
	Cryptorchid	65% (13/20)	44% (8/18)
Litter size (mean ± SD)			
Sprague Dawley	Control	10.7 ± 2.7	12.3 ± 2.9
	Cryptorchid	11.0 ± 4.0	12.9 ± 4.4
T-S	Control	7.0 ± 2.8	6.3 ± 2.8
	Cryptorchid	6.0 ± 2.8	8.5 ± 3.7

that the low fertility of the naturally cryptorchid rats of this strain has allowed the relevant genes, both for cryptorchidism and those mediating an effect on the CDT, to be lost or suppressed in our colony over time.

In previous studies of the contralateral effect of cryptorchidism, other researchers have used various rat strains, time periods, and surgical procedures. It is, therefore, not surprising that results have remained divergent, with the effect on the CDT remaining contraversial. We have, therefore, studied three rat strains and have used a more sensitive apoptotic detection method for germ-cell degeneration to try and resolve this question. We have consistently found no effect on the CDT. There may still be a genetic susceptibility for contralateral effects on the testis, such as the congenitally cryptorchid Buffalo rat strain used by Patkowski et al. [14]. However, the congenitally cryptorchid T-S rat model did not show a contralateral effect, suggesting that congenital cryptorchidism is not sufficient for this effect to occur. Genetic susceptibility may be a key factor for a contralateral effect in the testis and could explain the disparate results in both rats and humans. Studies of the incidence of cryptorchidism and the correlation with genetic data are needed to see if the effect of cryptorchidism on fertility can be resolved.

References

1. Argawala S, Mitra DK (1996) Fertility and unilateral undescended testis in the rat model. Pediat Surg Int 11: 266
2. Atkinson PM (1975) A follow-up study of surgically treated cryptorchid patients. J Pediat Surg 10: 115
3. Billig H, Furuta I, Rivier C, Tapanainen J, Hseuh AJW (1995) Apoptosis in testis germ cells: Developmental changes in gonadotrophin dependence and localization to selective tubule stages. Endocrinology 136: 5
4. Fallon B, Kennedy TJ (1985) Long-term follow-up of fertility in cryptorchid patients. Urology 25: 502
5. Gilhooly PE, Meyers F, Lattimer JK (1984) Fertility prospects for children with cryptorchidism. Am J Dis Child 138: 940
6. Hadziselimovic F (1985) Fertility and cryptorchidism. Am J Dis Child 139: 963
7. Hasthorpe S (1980) A hemopoietic cell line dependent upon a factor in pokeweed-mitogen stimulated spleen cell conditioned medium. J Cell Physiol 105: 379
8. Henriksen K, Harkovirta H, Parvinen M (1995) In-situ quantification of stage-specific apoptosis in the rat seminiferous epithelium: effects of short-term experimental cryptorchidism. Int J Androl 18: 256
9. Kirsten YY, Hawkins L, Dewolf WC, Morgentaler A (1997) Heat stress causes testicular germ cell apoptosis in adult mice. J Androl 18: 159
10. Kogan BA, Gupta R, Juenemann KP (1987) Fertility in cryptorchidism: further development of an experimental model. J Urol 137: 128
11. Lee PA, O'Leary LA, Songer NJ, Bellinger MF, Laporte RE (1995) Paternity after cryptorchidism: lack of correlation with age at orchidopexy. Br J Urol 75: 704
12. Lee PA, O'Leary LA, Songer NJ, Coughlin MT, Bellinger MF, LaPorte RE (1996) Paternity after unilateral cryptorchidism: a controlled study. Pediatrics 98: 676
13. Okuyama A, Nonomura N, Nakamura M, Namiki M, Fujioka H, Kiyohara H, Matsumoto K, Sonoda T (1989) Surgical management of undescended testis: retrospective study of potential fertility in 274 cases. J Urol 142: 749
14. Patkowski D, Czernik J, Jelen M (1992) The natural course of cryptorchidism in rats and the efficacy of orchidopexy or orchidectomy in treatment before and after puberty. J Pediat Surg 27: 870
15. Patkowski D, Czernik, Jelen M (1994) Division of the genitofemoral nerve in unilateral cryptorchid rats. J Pediat Surg 29: 832
16. Ponchietti R, Grechi G (1986) Fertility in unilateral cryptorchidism: review of 104 cases. Acta Europ Fertil 17: 277
17. Quinn FMJ, Crockard AD, Brown S (1990) Secondary changes in the scrotal testis in experimental unilateral cryptorchidism. J Pediat Surg 25: 402

18. Shikone T, Billig H, Hseuh AJW (1994) Experimentally induced cryptorchidism increases apoptosis in rat testis. Biol Reprod 51: 865
19. Srinivas M, Agarwala S, Datta Gupta S, Das SN, Shaha C, Mitra DK (1998) Fertility and unilateral undescended testis in the rat model. Pediat Surg Int 13: 392
20. Stewart RJ, Brown S (1990) Fertility in unilateral cryptorchism. J Pediat Surg 25: 672
21. Tanyel FC, Ayhan A, Buyukpamukcu N, Hicsonmez A (1991) Subsequent testicular histology: fertility and fecundity of rats subjected to unilateral incarcerated hernia during the prepubertal period. J Pediat Surg 26: 204
22. Tapanainen JS, Tilly JL, Vihko KK, Hsueh AJW (1993) Hormonal control of apoptotic cell death in the testis: gonadotropins and androgens as testicular cell survival factors. Mol Endrocrinol 7: 643
23. Tilly JL, Hsueh AJW (1993) Microscale autoradiographic method for the qualitative and quantitative analysis of apoptotic DNA fragmentation. J Cell Physiol 154: 519
24. Zakaria O, Shono T, Imajima T, Suita S (1998) Fertility and histological studies in a unilateral cryptorchid rat model during early and late adulthood. Br J Urol 82: 404