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New discovery of cryptorchidism: Decreased retinoic acid in testicle

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Abstract This study focuses on investigation of cryptorchidism induced by flutamide (Flu) and its histopathological damage, and detects retinoic acid concentration in testicle tissue, in order to find a new method for clinical treatment to infertility caused by cryptorchidism. Twenty SD (Sprague Dawley) pregnant rats were randomly divided into Flu cryptorchidism group (n = 10) and normal control group (n = 10). HE stained for observing morphological difference. Transmission electron microscope (TEM) was used for observing the tight junction structure between Sertoli cells. Epididymal caudal sperms were counted and observed in morphology. The expression of stimulated by retinoic acid gene 8 (Stra8) was detected using immunohistochemistry, western blot, and Q-PCR. High performance liquid chromatography (HPLC) analysis was made on retinoic acid content. Sperm count and morphology observation confirmed cryptorchidism group was lower than normal group in sperm quantity and quality. The observation by TEM showed a loose structure of tight junctions between Sertoli cells. Immunohistochemistry, western blot, and Q-PCR showed that cryptorchidism group was significantly lower than normal group in the expression of Stra8. HPLC

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showed that retinoic acid content was significantly lower in cryptorchid testis than in normal testis. In the cryptorchidism model, retinoic acid content in testicular tissue has a significant reduction; testicles have significant pathological changes; damage exists in the structure of tight junctions between Sertoli cells; Stra8 expression has a significant reduction, perhaps mainly contributing to spermatogenesis disorder.

1. Introduction

Cryptorchidism is one of the most common congenital malformations in the genitourinary system in boys with an incidence up to 3–4% (Kolon et al., 2014). With social development as well as increased physical and chemical factors in environmental pollution, this incidence has a trend to increase year by year (Guerrero-Bosagna and Skinner, 2014). Studies show that 38% of patients with unilateral cryptorchidism suffer from oligospermia with an infertility rate of 60–100% (La Vignera et al., 2009). Cryptorchidism has become one of important reasons for infertility in males. Currently, the mechanisms causing cryptorchidism and testicular damage are not yet clear, but it is believed that this is a result from genetic and environmental factors (Kurahashi et al., 2005). Studies confirm that Flu can block the effect of androgens in embryonic stage, affect testicular development, and inhibit testicular descent (Okur et al., 2006). In this study, cryptorchidism rats model was used to detect retinoic acid content in testicular tissue and investigate pathology, thereby laying a foundation for the study of male infertility caused by cryptorchidism and providing new ideas for clinical research of treatment programs.

2. Materials and methods

2.1. Reagents

Reagents were analytically pure flutamide (Sigma, F9397, USA), corn oil (Aladdin, Shanghai), rabbit Stra8 antibody (Abcam, ab49602, USA), rabbit Scp3 primary antibody (Abcam, ab85621, USA), goat anti-rabbit secondary antibody detection kit (ZSGB-BIO, PV-6001, Beijing), RNA rapid extraction kit (BioTeke, Beijing), RNA reverse transcription kit (TaKaRa, DRR037S, Japan), total protein extraction kit (KeyGEN, Nanjing), SDS-PAGE gel preparation kit (Beyotime), ECL detection kit (Millpore, Germany), and Stra8 primer sequence (Sangon Biotech, Shanghai).

2.2. Animals and drug

SD rats (purchased from Animal Center of Chongqing Medical University; animal certificate number: SYXK (Chongqing) 2011–0001; weight 280–310 g) were reared in Animal Center, Children's Hospital of Chongqing Medical University (rearing temperature: 20–25 °C, relative humidity: 40–60%). Once adulthood was reached, males and females were caged together (1:1). Then, observations were made at 08:00 daily on whether the vaginal plug appeared. The female rat having such plug was separately reared (designated as 0 d of gestation, namely, GD0). 20 pregnant rats were randomly divided into 2 groups: (1) Flu group, 10 pregnant rats, GD 11–19 d, injected with anti-androgen drug Flu 25 mg/kg (prepared using corn oil) and (2) control group, 10 pregnant rats, regularly reared, not given any drugs or reagents. According to the criteria by van Haaster and de Rooij (1993), the total number of male offspring and the number of the offspring of cryptorchidism were observed with the incidence of cryptorchidism calculated on postnatal day 20 (PND20). Normal and cryptorchid testicle tissues were extracted from the offspring on PND60, embedded in paraffin, and sliced (4 μm), for HE, immunohistochemical staining. Some of testicular tissues on PND60 were extracted and stored in liquid nitrogen for Western blot and Q-PCR.

2.3. HE staining

The testicular tissue from each group was sliced and stained with HE for observing changes in morphology and histology.

2.4. TEM

The rat testis specimens were cut into small pieces (about $1 \times 1 \times 1\ mm^3$), fixed in 3% glutaraldehyde for 1 h, washed 3 times with PBS, fixed for 1 h in 1% OsO4, dehydrated with ethanol, embedded in epoxy resin, made into ultra-thin slices (50 nm), and stained for observing the tight junction structure in testicle under TEM (Philips TECNAI 10, USA).

2.5. Sperm count

Sperm collection: The bilateral caudal epididymis was placed in two preheated (37 °C) dishes, respectively, and cut longitudinally into pieces with ophthalmic scissors. 1 dish was added 10 mL PBS solution (preheated to 37 °C) where No. 1 sperm suspension was prepared for sperm counts. The other dish was added with 1 mL PBS solution where No. 2 sperm suspension was prepared for observing sperm morphology. Then it was placed in a water bath at 37 °C (constant temperature) and incubated for 10 min, so that sperms could be fully free. Sperm count: The hemocytometer was used to count caudally epididymal sperm density. (1) The hemocytometer pipette was used to transfer 10 μL No. 1 sperm suspension to fill the count pool of the count plate in the said meter; (2) the counting plate was stood still for 15 min on a box covered with a damp cloth, so that all the sperms could settle on the same focal plane of the count plate; (3) the low magnification (10×) microscope was used to find the square of the count plate center, and a count pool where sperms were distributed evenly was selected for counting under the high magnification (40×) microscope. Sperms in 4 small squares in the big square of the center were counted (namely, small squares at four corners). The calculation formula is as follows: total sperm count in four small