

Original Article

Leydig cell transplantation restores androgen production in surgically castrated prepubertal rats

Jie Sun¹, Ye-Bin Xi², Zhong-De Zhang³, Ping Shen³, Huai-Yuan Li⁴, Min-Zhi Yin³, Wei-Yi Li², Cheng-Ren Shi⁵

¹Department of Urology, Shanghai Children's Medical Center, Shanghai 200127, China

²Department of Immunology, Shanghai Jiao Tong University, School of Medicine, Shanghai 200025, China

³Department of Pathology, Shanghai Children's Medical Center, Shanghai 200127, China

⁴Department of Clinical Laboratory, Shanghai Children's Medical Center, Shanghai 200127, China

⁵Department of Pediatric Surgery, Xin Hua Hospital, Shanghai Jiao Tong University, Shanghai 200092, China

Abstract

Prepubertal testicular dysfunction and the subsequent development of hypogonadism affects an estimated one in 200 children worldwide. As the testosterone levels are dynamic during development and puberty, traditional hormone treatment regimens are often inadequate, thereby leaving associated physiological conditions unresolved. Therefore, we have investigated the potential therapeutic effect of mature Leydig cell transplantation for the treatment of prepubertal primary hypogonadism through the use of a surgically induced hypogonadistic rat model system. In the experiment, Leydig cells were surgically isolated from mature Sprague–Dawley rats and transplanted into prepubertal recipients. Serum testosterone levels and microscopic analysis of the stained testicular interstitium were compared with sham-treated controls, as well as with castrated and intact rats during sexual development. At 4 weeks post-implantation, serum testosterone was detectable in Leydig cell recipients, but not in surgical controls, and progressively increased as a function of time until reaching levels comparable with sexually mature males at 12 weeks post-implantation. Histological analysis revealed a high rate of Leydig cell survival as well as steroidogenic secretory activity. Therefore, we conclude that mature Leydig cell transplantation in prepubertal hypogonadism recipients has therapeutic potential in rats and merits further investigation for clinical application.

Asian Journal of Andrology (2009) 11: 405–409. doi: 10.1038/aja.2009.22; published online 18 May 2009.

Keywords: androgens, Leydig cells, male hypogonadism, testis

1 Introduction

An estimated one in 200 children suffers from androgen deficiency worldwide [1], causes of which include congenital abnormalities, trauma, infections and tumour growth [2]. Further, prepubertal testicular

dysfunction, if improperly treated or left untreated, can lead to primary hypogonadism and altered sexual development.

Although hormonal treatment has become common practice in the clinical treatment of androgen-deficient adults, prepubertal and pubertal cases pose a greater level of complexity, as levels of testosterone and its metabolites are dynamic during development and puberty. Thus, traditional hormone treatment regimens are often inadequate for paediatric patients, and fail to prevent associated physiological retardation and psychological problems [3].

As Leydig cells are the primary source of intrat-

Correspondence to: Professor Cheng-Ren Shi, Department of Pediatric Surgery, Xin Hua Hospital, Shanghai Jiao Tong University, 1665 Kong Jiang Road, Shanghai 200092, China.

Fax: +86-21-3501-0514

E-mail: shicr629@163.com

Received: 17 December 2008

Revised: 9 March 2009

Accepted: 13 March 2009

Published online: 18 May 2009

esticular and circulating androgens and are permanent residents of the mature testicular interstitium, we investigated the potential therapeutic benefits of Leydig cell transplantation using a rat model system, in which mature donor Leydig cells were transplanted into orchietomized prepubertal recipients. Prepubertal males were chosen as recipients to minimize the influence of preoperative androgen levels and to examine the effects of Leydig cell transplantation during sexual development.

2 Materials and methods

A total of 70 male Sprague–Dawley rats (purchased from the Chinese Academy of Sciences) were used in this study. In total, 30 of these were used for the surgical procedures, which comprised 10 sexually mature male rats (12 weeks old; 300.0 ± 9.1 g) that served as Leydig cell donors and 20 prepubertal male rats (4 weeks old; 120.2 ± 8.3 g) that underwent orchietomy and served as either recipients of mature donor Leydig cells or surgical controls.

Four groups of 10 rats each served as non-surgical controls and were grouped according to age: 4, 8, 12 and 16 weeks old. Serum samples from these rats were, thus, collected in a cross-sectional manner, but later analysed as if originating from a longitudinal study.

All rats were housed individually in a temperature- and light-controlled environment (25°C, 12L: 12D cycle) and pellet food (purchased from the Chinese Academy of Sciences) and water were provided *ad libitum*.

2.1 Orchietomy

Prepubertal rats were anesthetized and both testes were surgically resected. Specifically, the right spermatic cord was ligated at the level of the upper part of the scrotum and the intact right testis was removed, although the left spermatic cord remained. The parenchymal tissue of the testis was removed through the albuginea incision, re-sutured and, in control animals, returned to the scrotum. In transplantation recipients, the albuginea served as the placement site for donor Leydig cells.

2.2 Isolation and characterization of mature Leydig cells

A total of 10 sexually mature male rats were killed by decapitation. The bilateral testes were collected individually, the albuginea were punctured and the testes

were digested in M199 media containing 0.5 mg mL^{-1} collagenase and 1% bovine serum albumin in a 34°C oscillating incubator for 15 min, as described earlier [4]. Digested cell suspensions were transferred to a stationary 50-mL conical tube and placed on ice for 2 min, allowing the tubules to settle. The resultant supernatant was filtered through a 70- μm nylon cell strainer (Falcon, BD Biosciences, Franklin Lakes, NJ, USA) and then centrifuged at 350 g for 20 min at 4°C. The pellet was resuspended in 10 mL of M199 media, loaded in a four-step Percoll gradient (5, 30, 58 and 70%, Sigma), and centrifuged at 800 g for 30 min at 4°C. Three cell layers were apparent and the bottom Leydig cell-enriched fraction was collected and washed twice using M199 media.

The presence of Leydig cells in the isolated cell fraction was confirmed by 3β -hydroxysteroid dehydrogenase (3β -HSD) staining, an indicator of steroidogenic activity. Cell viability was estimated by trypan blue exclusion, and data were represented as a percentage of the total number of Leydig cells observed by light microscopy.

2.3 Leydig cell implantation

Isolated mature Leydig cells were injected into the cyst of the left albuginea in 10 prepubertal transplant recipient rats immediately after surgical resection.

2.4 Testosterone production

Serum testosterone levels were measured in transplant recipients and surgical controls by enzyme-linked immunosorbent assay (ELISA) (Access Immunoassay System, Beckman) at 4-week intervals (4, 8, and 12 weeks post-surgery). Serum testosterone levels were also measured in normal, untreated rats undergoing sexual maturation (4, 8, 12 and 16 weeks of age at the onset of analysis). Results of testosterone production in transplant recipients were compared with surgical and untreated developmental controls.

2.5 Histology

Surgical controls and Leydig cell transplant recipients were killed by decapitation 12 weeks after the surgery. The cysts located on the left side of the scrotum were dissected, fixed in formalin, dehydrated using a graded ethanol series and embedded in paraffin. Tissue sections (4- μm) were stained with hematoxylin and eosin, and examined microscopically.

Secretory activity was assessed using the method

reported earlier by Threadgold [5]. In brief, samples were fixed in Helly's solution for 24 h, incubated in 3% potassium dichromate for 72 h, dehydrated using a graded series of ethanol and embedded in paraffin. Tissue sections (4- μm) were stained in a Sudan black B saturated solution for 30 min, washed with 70% alcohol, washed with 50% alcohol and distilled water, and mounted with glycolgelatin. Stained slides were then examined using light microscopy as described above, and the secretory activity of Leydig cells was quantified on the basis of staining intensity.

2.6 Statistical analysis

Serum testosterone data were tested for normality and equal variance before analysis by one-way ANOVA (analysis of variance) (SPSS Inc., Chicago, USA). Analysed data sets included surgical controls and Leydig cell transplant recipients at 4, 8 and 12 weeks after surgery, as well as 4-, 8-, 12- and 16-week-old untreated developmental controls. Statistical significance was set at $P < 0.001$.

3 Results

3.1 Microscopic findings of isolated Leydig cells

The mean number of Leydig cells isolated from a single mature male rat donor was $(4.87 \pm 0.96) \times 10^6$. Cellular staining for 3β -HSD was granular and localized to the cytoplasm. Viability was assessed by the trypan blue exclusion method, and cell survival was determined to be 100%.

3.2 Serum testosterone levels

Serum testosterone levels in the untreated developmental controls were age-dependent and positively correlated with sexual maturity. In brief, serum testosterone levels in untreated prepubertal rats (< 4 weeks old) were low, sharply increased with the onset of puberty at 4–6 weeks of age, and peaked after sexual maturity at 12 weeks of age. Serum testosterone levels in surgical orchiectomized controls were non-detectable 4 weeks post-surgery using the ELISA method.

Mean serum testosterone levels in mature Leydig cell transplant recipients were much greater (0.712 ng mL^{-1}) than in surgical controls (0.000 ng mL^{-1}) 4 weeks post-implantation ($P < 0.001$) and gradually, yet obviously, increased as a function of time ($P < 0.001$). Although mean serum testosterone levels were predominantly reduced in mature Leydig cell recipients 4 weeks post-

transplantation in comparison with untreated 8-week-old rats ($0.712 \text{ vs. } 2.080 \text{ ng mL}^{-1}$; $P < 0.001$), the testosterone levels 12 weeks post-transplantation were comparable with untreated 16-week-old rats ($3.978 \text{ vs. } 4.200 \text{ ng mL}^{-1}$) (Figure 1).

3.3 Histological appearance of implanted Leydig cells

In surgical orchiectomy controls, no structures were observed within the albuginea on the left side of the scrotum compared with untreated developmental controls. However, in mature Leydig cell transplant recipients, a large cluster of cells was observed 12 weeks post-transplantation. These cells displayed characteristics similar to mature Leydig cells; they were large, slightly eosinophilic and possessed round nuclei.

3.4 Histochemical staining in transplanted Leydig cells

Large dark granules were visible within the transplanted Leydig cells, indicating positive Threadgold staining [5]. In tissue sections of untreated controls, darkly stained granules were localized to the mesenchyma of the testis. No positive staining or granules were observed in the scrotal cysts, or more specifically, with-

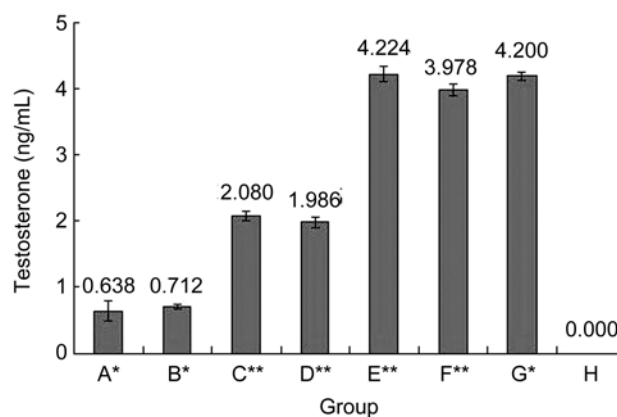


Figure 1. Serum testosterone levels in experimental treatment groups: (A) untreated 4-week-old rats ($n = 10$); (B) prepubescent Leydig cell recipients 4 weeks post-implantation ($n = 10$); (C) untreated 8-week-old rats ($n = 10$); (D) prepubescent Leydig cell recipients 8 weeks post-implantation ($n = 10$), (E) untreated 12-week-old rats ($n = 10$); (F) prepubescent Leydig cell recipients 12 weeks post-implantation ($n = 10$); (G) untreated 16-week-old rats ($n = 10$); (H) orchiectomized controls 4 weeks after surgery ($n = 10$). Bars represent mean serum testosterone concentration \pm standard deviation (s.d.). * $P < 0.001$, compared with orchiectomized controls, ** $P < 0.001$, compared with the earlier age group.

in the filament structure of surgically orchiectomized controls. In mature Leydig cell transplant recipients, a large number of darkly stained granules were observed 12 weeks post-implantation, indicating the secretory capability of transplanted Leydig cells.

4 Discussion

Although the common clinical treatment for primary hypogonadism is androgen replacement [6], a number of adverse conditions induced by prolonged testosterone administration [7] highlight the need for alternative therapeutic options, especially for paediatric patients, who are in need of dynamic testosterone treatment regimens to mimic natural developmental fluctuations [8]. Therefore, we have investigated the therapeutic potential of Leydig cell transplantation, as Leydig cells are the primary source of intratesticular and circulating androgens [9]. Here, we report for the first time the successful restoration of endogenous testosterone production at levels comparable with untreated developmental controls in prepubertal castrated rats after mature Leydig cell transplantation.

Transplanted Leydig cells are a plausible substitute for traditional androgen therapy because of (1) the steroidogenic and secretory ability of individual Leydig cells [10]; (2) Leydig cells' resistance to moderate alterations in temperature, facilitating culture *in vivo* and *ex vivo*, and broadening potential transplantation sites [11]; and (3) the reported success of Leydig cell transplantation in mature male animal model systems [12]. Further, although Leydig cell steroid production lapses after only a week of culture, Leydig cell transplantation of 10% of the testicular volume has been shown to adequately maintain virility in males [13]. As Leydig cells normally comprise 2% and 5%–12% of the testicular cell population in mature rodents [14] and humans [15], respectively, the potential clinical application of Leydig cell transplantation should be further investigated.

Although it has been three decades since the first successful gonadal tissue transplantation was reported [16, 17], the method of cell isolation continues to reduce transplant efficacy, as transplanted cells often display evidence of necrosis or apoptosis [18]. Parameters associated with the transplantation procedure equally affect success, with transplantation delays resulting in altered Leydig cell function and structure, and an increased risk of infection.

In this study, we employed the Percoll technique of

cell isolation, which segregates cell types on the basis of their density gradient [19]. Isolated cellular fractions were screened for steroidogenic ability by 3 β -HSD staining, a method validated for 90% recovery of viable Leydig cells from the rat testis [20]. The viability of steroidogenically active cells was confirmed before transplantation by the trypan blue exclusion method [21]. Although a number of transplantation sites have been reported earlier to be successful [22–25], we placed donor cells within the albuginea to prevent accidental displacement. Histological analysis of testicular sections 12 weeks post-transplantation confirmed the presence of newly formed vessels that served to nourish the transplanted cells. Owing to the high degree of viability that we observed among transplanted cells, we believe that further investigation is warranted to assess clinical applicability.

The Threadgold staining method was employed in our study to evaluate the steroidogenic activity of transplanted Leydig cells. A high level of secretory activity was observed in transplanted cells, but not in surgical controls, providing further evidence that endocrine function was preserved for 3 months after allotransplantation.

Systemic testosterone levels were also measured in transplant recipients, untreated controls and in surgical controls to assess the steroidogenic robustness of the transplanted cells. To minimize the effects of androgen deficiency and to provide a direct link to the treatment of paediatric hypogonadism, prepubertal rats were selected as recipients in this study. Serum testosterone levels were comparable with untreated age-matched controls 12 weeks post-transplantation, whereas testosterone was undetectable in surgical controls. Importantly, although gonadotropins (luteotrophic hormone [LH] and follicle-stimulating hormone [FSH]) were not measured in this study, their fluctuating levels during pubertal onset and sexual maturation may have enhanced the implantation success [26]. Further investigation concerning the responsiveness of the hypothalamic–pituitary gonadal axis to Leydig cell transplantation is warranted. As Leydig cells are also participants in paracrine and endocrine regulatory feedback loops [27], Leydig cell transplantation may meet physiological and reproductive demands not achieved by simple hormone replacement therapy. The status of testicular immune privilege is now under investigation by other researchers. It has been shown that Leydig cells have nearly no immunogenicity, which makes the risk of

immunologic rejection after transplantation negligible and guarantees the long-term survival of transplanted cells [28, 29].

5 Conclusion

On the basis of the results of this study, we conclude that Leydig cell transplantation could restore androgen production in surgically castrated prepubertal rats. Its therapeutic potential for the clinical treatment of prepubertal primary hypogonadism, especially for patients with complete loss of endogenous Leydig cells, is worth investigating in the future.

Acknowledgment

We are grateful to the National Natural Science Foundation of China for financial support (Grant No. 30700878).

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