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Testosterone Productivity and Histostructural Changes of Autotransplanted Rat Leydig Cells

Moo Sang Lee, Sung Joon Hong, Hak Ryong Choi and Koon Ho Rha

To investigate the possibility of in vivo transplantation of Leydig cells as a new biologic androgen replacement therapy, the Leydig cells procured from 6 week-old male Sprague-Dawley rats were autotransplanted, and the level of testosterone secretion and histostructural changes were observed. The renal subcapsular and intraperitoneal transplant showed higher levels of testosterone compared to subcutaneous or scrotal counterparts, and the number of transplanted cells was correlated with the level of measured testosterone. Furthermore, if the Leydig cells were transplanted intraperitoneally after the uptake on synthetic collagen, testosterone levels were higher than the ones simply transplanted without synthetic collagen uptake, resulting in 27 fold increase at 3 months. The activity of ^{125}I-hCG decreased 20 to 40% at each month after transplantation compared to the normal levels, but no statistical significance was noted among different periods. The histologic examination revealed neovascularized capillaries and well demarcated sheet-like group of eosinophilic Leydig cells were observed at 4 weeks. But the evidence of destructive changes such as a focal inflammation with central dystropic ossification could be noted after 3 month. On electron microscopy, the marked indentation of nucleus and presence of lipochrome pigment were seen, and the number and size of smooth endoplasmic reticulum and mitochondria were reduced after 3 month. In conclusion, testosterone output could be increased to the physiologic range by increasing the number of transplant cells or utilizing collagen uptake but further effort is necessary on delaying or preventing the structural and functional decrement of Leydig cells.

Key Words: Transplantation, Leydig cell, androgen replacement

The Leydig cells of testes are the major source of testosterone in male. In testicular dysfunction or hypogonadism, androgen replacement is necessary in the somatic development in prepubertal period and to maintain the erectile function in men. Conventional treatment for hypogonadism in men consisted of periodic intramuscular injections of chemically modified testosterone. However long-term testosterone therapy may cause fluid and nitrogen retention, erythropoiesis, acne, cardiomegaly, hypertension, and bone density change (Santen and Swerdloff, 1990). Recently, Fox *et al.* (1973) and Boyle *et al.* (1976) reported the results of successful testosterone production by autotransplantation of gonadal tissue fragment in mice. Furthermore Tai *et al.* (1989) reported the similar results with the transplantation of collagenase-treated Leydig cells in castrated inbred rats. These results suggests the possibility of Leydig cell transplantation in men as a viable biologic androgen replacement therapy. But in these studies

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Department of Urology, Yonsei University College of Medicine, Seoul, Korea
Address reprints requests to Dr. M.S. Lee, Department of Urology, Yonsei University College of Medicine, C.P. O. Box 8044, Seoul 120-752, Korea
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transplanted gonadal tissues or cells have mainly been characterized by histological techniques on the light microscopic changes of secondary sex organs or functionally, by measuring the testosterone in blood plasma. In this study we investigated the testosterone productivity as well as the biochemical and histostructural changes of transplanted Leydig cells under various conditions which can be the foundation for clinical application.

MATERIALS AND METHODS

Male Sprague-Dawley rats weighing approximately from 170 g to 200 g (about 6 weeks old) were used in all experiments. The gelatin sponge (Gelfoam^{R}) which was extracted and synthesized from the pig skin collagen was obtained from Upjohn company (U.S.A.). Intraperitoneal injection of 25 mg/kg of pyrimidinetrione (Entobar^{R}, Hanrim Pharma, Seoul, Korea) was used for general anesthesia.

Isolation and characterization of Leydig cells

Bilateral orchiectomy was carried out through a scrotal incision under anesthesia. Testes were decapsulated and rinsed repeatedly with Hanks Balanced Salt Solution (HBSS: Flow Lab. McLean VA, USA) with 20 mM HEPES, 0.1% BSA (bovine serum albumin), 100 U/ml penicillin and 100 ug/ml streptomysin. The tissue were dissected into 1~1.5 mm and incubated in 10 ml HBSS containing 0.25 mg/ml type IV collagenase (Sigma Chemical Co., St. Louis, USA) at 34℃ for 30 minutes. The solution was repeatedly filtered through nylon mesh and washed with HBSS. The filtrate was centrifuged for 5 minutes at 120 g, and the cells were washed twice more and resuspended in adequate amount of HBSS. Discontinuous gradients of 20~50% percoll (Pharmacia Fine Chemicals, Uppsala, Sweden) were prepared as described by Kerr *et al.* (1985) in 45 ml polycarbonate tube. The crude cell preparation, mixed with 10 ml of 15% percoll, was applied to the gradient and then centrifuged at 800 g and 4℃ for 20 minutes. The isolated Leydig cells were characterized by 3β-hydroxysteroid dehydrogenase (3β-HSD) reaction.

Staining medium, consisting of 0.1 ml β-nicotinamide adenine dinucleotide (6 ml/ml), 0.1 ml 5β-androstan-3β-ol-17- one and 0.2 ml nitro-blue tetrazolium (1 mg/ml) were added to 0.2 ml of isolated cells then incubated at 34℃ for 30 minutes. A drop was placed on a slide and examined under a light microscope.

The percentage of blue stained cells was estimated. Prepared cells were transplanted either through the 26G needle or transplanted with collagen absorption.

Localization for effective transplantation

Six groups each composed of 10 animals were prepared for the selection of adequate transplant site. Group I was sham operated with scrotal incision. Group II was castrated only and Group III, IV, V and VI were castrated and Leydig cells injected group which were transplanted at peritoneal, dorsal subcutaneous, intrascrotal, and renal subcapsular spaces, respectively. In each group, 1×10^{6} cells in 200 ul solution were autotransplanted. Serum testosterone levels were checked at 1, 2 and 3 months.

Testosterone productivity in different number of cells

Different number of cells, 1×10^{6}, 3×10^{6} and 5×10^{6} in 200 ul of HBSS, respectively, were autotransplanted in the peritoneal cavity of castrated rats. Ten animals were used in each group and blood testosterone levels were checked after 1 month.

Synthetic collagen-absorbed transplantation

Direct injection and synthetic collagen-absorbed Leydig cell transplantation were compared for testosterone productivity. According to the modified method from Van dam *et al.* (1989), gelatin sponge was cut into small pieces (5×5×5 mm) with a scalpel. 3×10^{6} cells in 200 ul of media was absorbed for 1 minute to gelatin sponge in a 5cc plastic tube. Then the sponge was placed and fixed at the inner surface of peritoneum with absorbable suture. Subgroups were consisted as follows; Group I: sham-operated control, Group II: cas-

trated sham-operated control, Group III: intraperitoneal injection of 3×10^6 cells, Group IV: castrated and synthetic collagen implanted group.

Twenty animals were used in each group. Serum testosterone levels were measured at 1, 2 and 3 months. Serial light and electron microscopic study and ^{125}I-hCG binding assay were prosecuted monthly after transplantation with cells isolated from cell mass found in the peritoneal cavity of transplanted rats with collagen absorption.

Testosterone measurement

Testosterone concentrations were estimated by direct radioimmunoassay using testosterone kit (Testosterone, ICN Biomedicals Inc. CA, USA) and counted with gamma counter.

^{125}I-labelled hCG binding activity

Cell suspensions, containing 5×10^6 Leydig cells isolated from normal testis and transplanted cell masses were incubated for 2h at 34℃ with a constant
amount (2×10^4 cpm) of R5I-labelled hCG (Du Pont Co., Wilmington, USA) in HBSS at 1, 2 and 3 months after transplantation. After adding 1ml of cold HBSS/0.2% albumin, the mixture was centrifuged at 1500g and 4℃ for 10 minutes. The supernatants were discarded and the radioactivities were counted. Non-specific binding of ^{125}I-labelled hCG was determined by incubation in the presence of excess of unlabelled hormone.

Histologic examination

Animals transplanted Leydig cells with collagen absorption were sacrificed at 1, 2, 4 weeks, and 3 months. Seminal vesicles and cell masses obtained from transplant site were fixed at formalin. After dehydration and paraffin embedding, 4 um sections were prepared and stained with hematoxylin and eosin for light microscopic study.

For the electron microscopic study, cell masses were fixed in 2% glutaraldehyde, 2% paraformaldehyde solution diluted with 0.1M cacodylated buffer (pH 7.4) for 24 hours, and then fixed in 1.33% osmium tetroxide, dehydrated in graded ethanol, and embedded in Epon 812. Thin sections were cut and stained with uranyl acetate and viewed on a transmission electron microscope (Hitachi H-500).

Statistical analysis

Statistical significance of the difference between variables were analysed with the Mann-Whitney U test. The difference was regarded as significant when the p value was less than 0.05.

RESULTS

Isolation of Leydig cell and response to 3β-HSD

The average weight of ipsilateral testis of 6 week old rat was 1.09 ± 0.04 g (mean±S.D.) ranging from 1.03 to 1.14 g. The cells composing the middle band treated with 3β-HSD showed blue cytoplasmic granular staining(Fig. 1), and the mean composition of Leydig cell was 82.3 ± 7.4%. The mean isolated Leydig cell count of ipsilateral testis was $3.54(\pm0.48)\times10^6$.

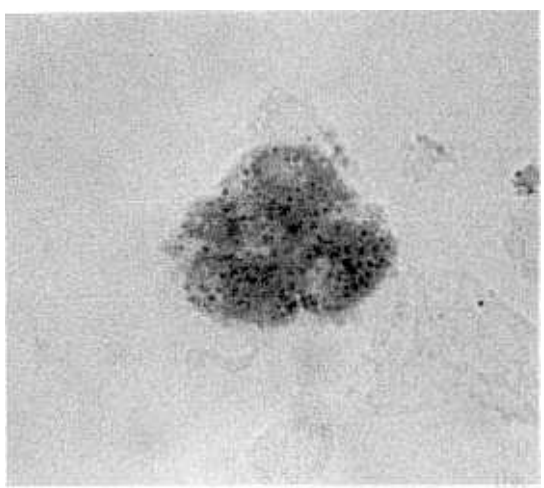

Fig. 1. The 3β-hydroxydehydrogenase reaction to isolated Leydig cells. Typical blue dots were seen in in the cytoplasm of Leydig cells (×400).

Effect of transplant site on testosterone production

The level of testosterone one month after implantation of 1×10^6 Leydig cells were highest in subcapsular implant group with mean of 0.51 ng/ml, followed by intraperitoneal implant group 0.42 ng/ml, which were significantly increased compared to the castrated group 0.15 ng/ml ($p<0.01$), and the elevated levels were maintained until two month. But at the 3 month after transplant, the level of testosterone in subcapsular group was 0.33 ng/ml, intraperitoneal group was 0.23 ng/ml, which were significantly decreased compared to the results of second month ($p<0.05$). The subcutaneous and intrascrotal transplantation group did not show significant difference compared to the castrated group. The testosterone level of control group at 1, 2, and 3 months were 1. 87, 1.36, and 1.65 ng/ml, respectively. The relative increment of testosterone secretion after substracting the control testosterone level was highest after two months in intraperitoneal transplant group with 28.6%, which was similar to the subcapsular group (27.9%) (Fig. 2).

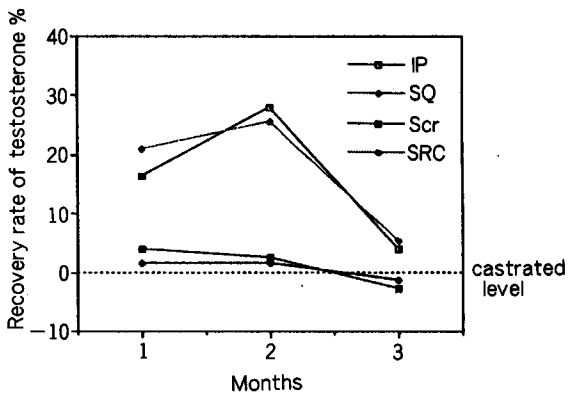

Fig. 2. *The actuarial recovery rates of testosterone secretion according to implant site at each month compared with castrated level. IP, intraperitoneal; SQ, subcutaneous; Scr, scrotal; SRC, svbcapsular renal transplantation.*

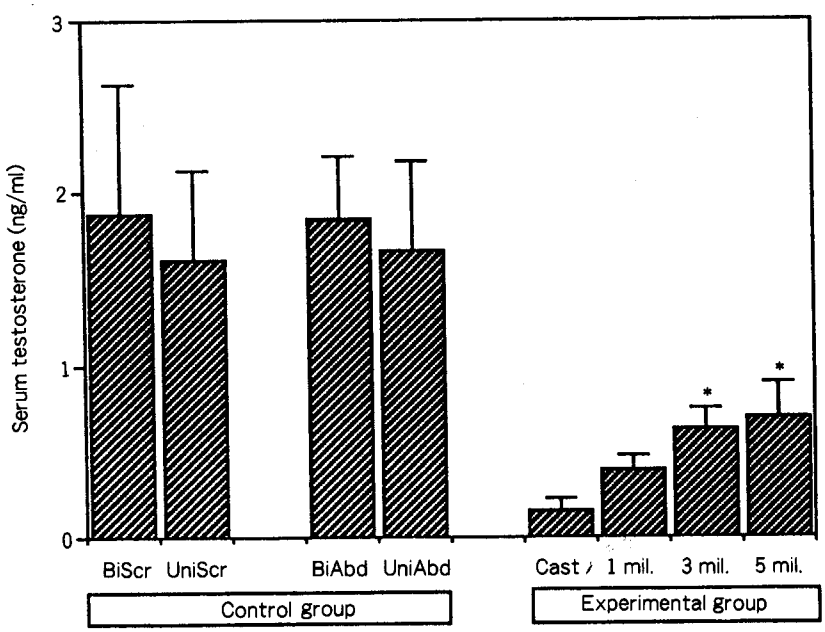

Fig. 3. *Changes in testosterone secretion according to the number of Leydig cells one month after transplatation. BiScr, bilateral scrotal testis; UniScr, unilateral scrotal testis; BiAbd, bilateral abdominal testis; UniAbd, unilateral abdominal testis. Cast, castrated group; 1, 3, 5 mil, each transplant 1, 3, 5 million.*
**$p<0.05$, compared with 1 million cell transplanted group.*

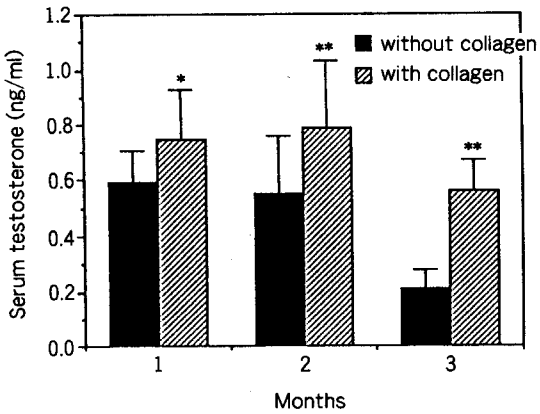

Fig. 4. *Comparison of serial testosterone concentrations according to the transplanted method.*
**p<0.05, **p<0.01, compared with group which transplanted without collagen absorption at each month.*

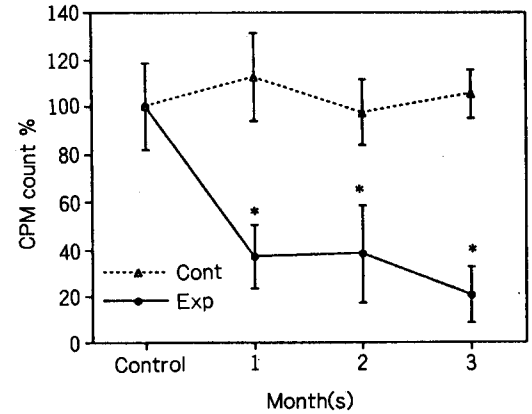

Fig. 5. *Relative changes in monthly ^{125}I-hCG binding activities after synthetic collagen-absorbed Leydig cell transplantation. Vallues were represented as average±standard deviation compared*
**p<0.01, compared with control group at each month.*

Effect of number of transplanted cells on testosterone production

In the control group, the number and the location of testis did not show any significant difference ($p>0.05$) in the testosterone production. Testosterone level one month after transplant of 1, 3, 5×10^6 Leydig cells was 0.39, 0.63, 0.81 ng/ml, which were significantly increased compared to the 1×10^6 cell group ($p<0.05$)(Fig. 3). The correlation coefficiency between the number of cells and the secretion of testosterone was 0.967 ($p=0.024$). The actual amount of increased secretion after subtraction of testosterone level of castrated group was 16.4%, 32.9%, 43.4%, respectively for 1, 3, 5 $\times10^6$ cells.

Effect of transplant method on testosterone production

The increases of testosterone in injected group, after one month and two months were 27% and 43.6%, but the level was decreased up to 0.21 ng/ml, which was 38% level compared to second month. However in the collagen uptake group, the level was 0.56 ng/ml, which was 70% of the second month level (Fig. 4).

The changes of hCG receptor activity

Eighty percent of cells procured from the normal testis with 3β-HSD staining were functioning Leydig cells, but the ratio of functioning Leydig cells 1, 2, and 3 month after uptake on the synthetic collagen is 55%, 69%, and 27%, respectively. The binding capacity of ^{125}I-hCG was 37.7%, 38.0%, and 21.0% compared to the controls at respective periods decrernervs which were significant ($p<0.01$) (Fig. 5).

Light microscopic findings

In comparison of light microscopic findings of seminal vesicle after 3 month with intact control, the secretory function and the volume of epithelial cells were reduced in transplanted group than in non-castrated control, but epithelial bridge was still maintained and the degree of atrophy in glandular component was less than that of castration group, suggesting the minimal testosterone production of transplanted Leydig cells (Fig. 6).

Typical cluster of Leydig cells was seen in the cell mass from collagen uptake group but

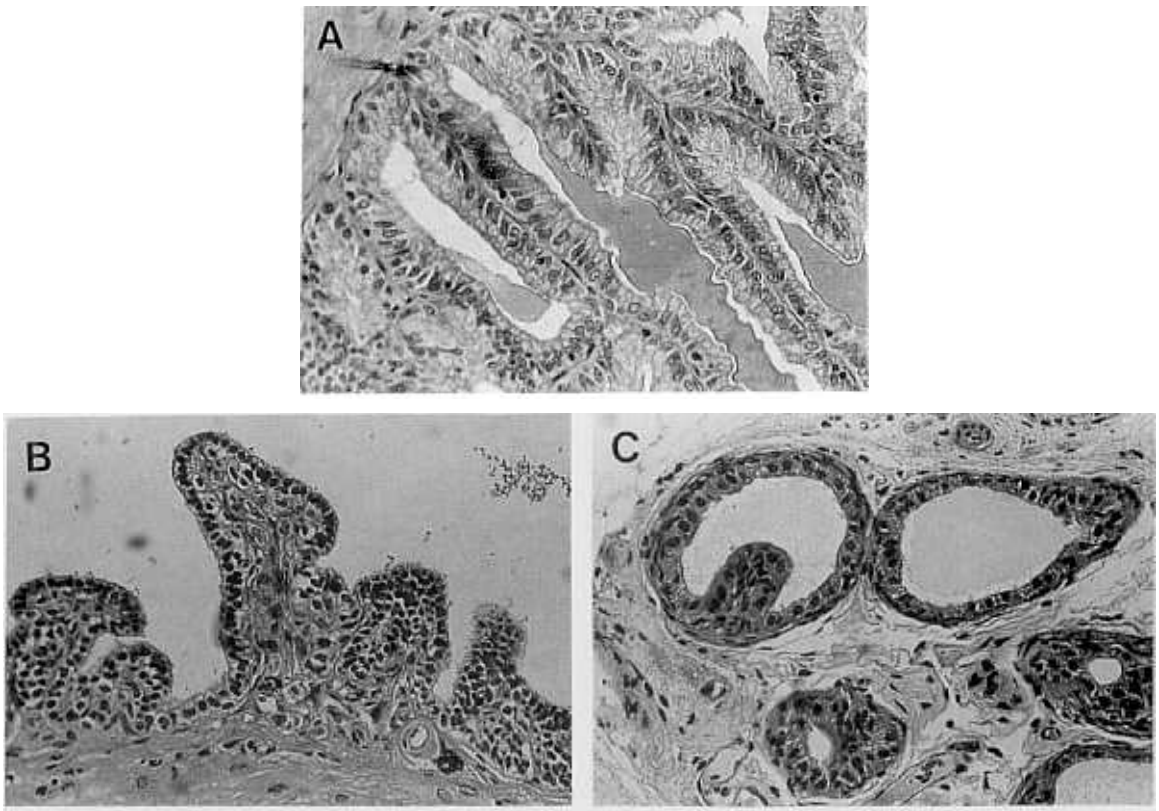

Fig. 6. Histological changes of seminal vesicle three months after treatment. A.sham-operated, non-castrated nornal control group; B.synthetic collagen-absorbed intraperitioneal Leydig cell transplant group (3×10^6 cells) after castration; C.castrated, non-transplanted group. The secretory function and the volume of epithelial cells are reduced in transplanted group than in non-castrated control, but epithelial bridge is still maintained and the degree of atrophy in glandular component is less than that of castration group suggesting the minimal production of testosterone from transplanted Leydig cells (H-E stain, ×200).

not in the locally injected group. The collagen uptake group showed undissolved synthetic collagen at 1 week and the transplanted Leydig cells mixed with infiltrated inflammatory cells were observed between collagen. At 2 weeks the findings were similar excent the periphery began to show normal cell pattern with formation of cell clusters. Erythrocytes began to appear without visible microvasculature which can be an evidence of neovascularization from peritoneal attachments. At 4 weeks the neo-vascularized vessels and existence of aggregated sheet-like pattern of Leydig cell clump were clearly visible. At 3 month, destructive changes such as the focal inflammation with central dystrophic ossification was noted (Fig. 7).

Electron microscopic findings

Leydig cells seen both in normal testis and freshly isolated tissue from testicular interstitium have the typical polyhedral shape with centrally placed nucleus. Small amount of heterochromatin was located in the periphery of nucleus and numerous smooth endoplasmic reticulum and mitochondria were seen in the cytoplasm. After 3 months, among the various shapes of Leydig cells observed, generally marked disfigurement of nucleus and presence of lipochrome pigment were seen in

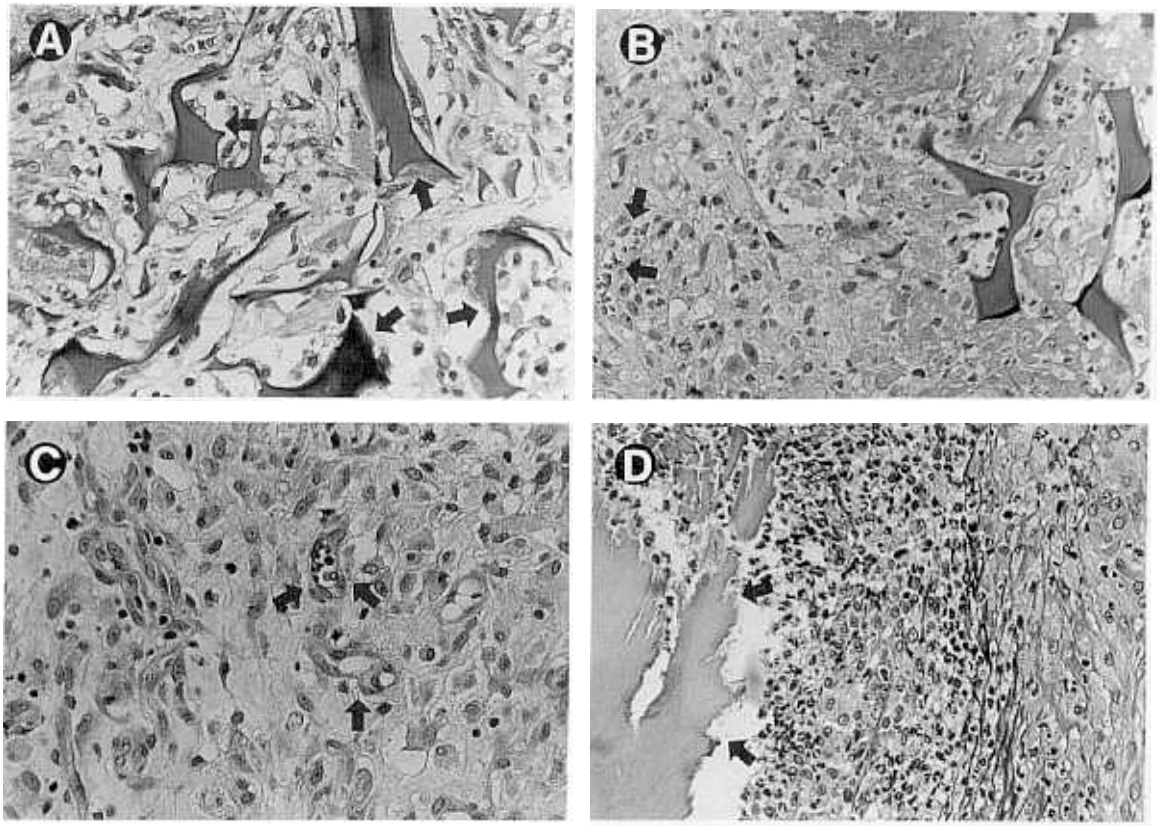

Fig. 7. The changes in light microscopic findings of Leydig cells transplanted with synthetic collagen uptake.
A. One week after transplantation. Arrows indicate the irregular shaped undissolved synthetic collagen. The mixture of transplanted Leydig cells and infiltrated inflammatory cells is observed (×100).
B. Two weeks after transplantation. Red blood cells (arrows) begin to apprear at the periphery of the cell mass. Still the undigested synthetic collagens are partly seen in the center (×100).
C. Four weeks after transplantation. Neovascularization (arrows) and aggregated sheet like pattern of Leydig cells are evident (×200).
D. Three months after transplantation. The synthetic collagen disappeared entirely. Peripherally located remaining Leydig cells are found. Dystrophic ossification (arrows) is seen in the center. The inflammatory cells are seen between the Leydig cell clusters and dystrophic ossification (×100).

most cells and the number and size of smooth endoplasmic reticulum and mitochondria were reduced. Intracytoplasmic lipochrome pigments were increased in proportion to the cellular deformity (Fig. 8).

DISCUSSION

The primary function of testicular interstitium is the production and conduction of testosterone. Testosterone is essential in reproduction and in maintaining secondary sex characteristics, and is mainly produced from Leydig cells in testis. It is plausible to use Leydig cells in the androgen substitution therapy due to several reasons. First, each cell has independent capability to produce testosterone, which is the basis for cellular transplantation. Second, the spermatogenesis is greatly affected by intratesticular tempera-

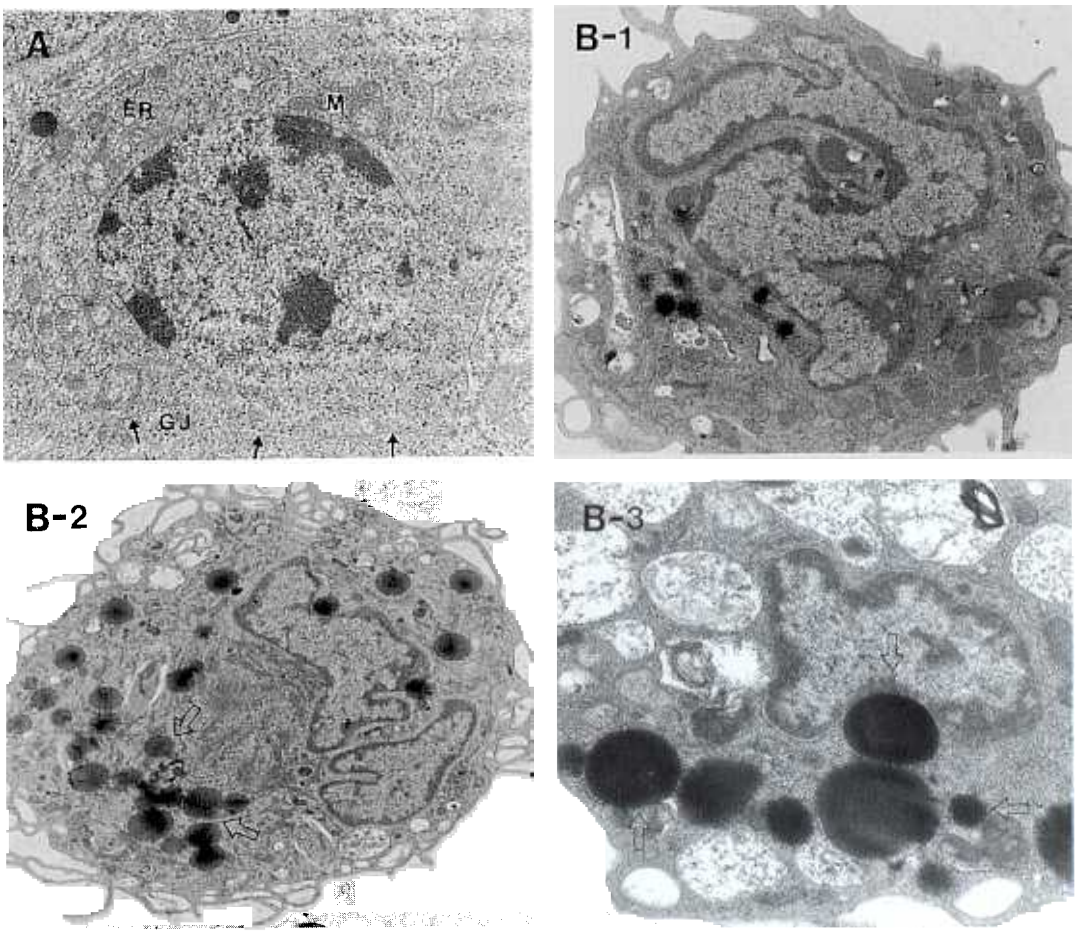

Fig. 8. *The changes in electron microscopic findings of transplanted rat Leydig cells compared with normal Leydig cell located in the testicular interstitium.*

A. Normal appearance of Leydig cell with typical polyhedral shape in the center of Leydig cell and the small amount of heterochromatin disposed at the periphery of nucleus. The large proportion of cytoplasmic vesicles believed to be an agranular form of endoplasmic reticulum (ER) is observed. Variable shape of mitochondria (M) with tubular cristae are present in the cytoplasm (×1,000)

B. Various Leydig cells isolated from synthetic collagen-absorbed Leydig cell transplants after 3 months.

B-1. Intracytoplasmic organelles are partially preserved. There are frequent invaginations of the cell surface suggesting pinocytosis (×14,000).

B-2. Intracytoplasmmic organelles were not well identified. Multiple nuclear indentations and small sized lipochrome pigments (arrows) are been (×14,000).

B-3. Increment of the volume of lipochrome pigments (arrows). Severe nuclear dysmorphism and myelin figure are observed in the cytoplasm (×24,800).

ture, which is lower than body temperature, but Leydig cells are relatively temperature resistant, which broadens possible sites of transplantation. Third, in vitro production of steroid by Leydig cells cannot last more than 1 week (Rommerts *et al.* 1982; Van Dam *et al.* 1989), but in vivo transplant of only 10% of testis in volume was shown to maintain virilization after castration(Boyle *et al.* 1976; Tai *et al.* 1989). Especially, the composition of

Leydig cell in adult testis is 5-12% (Kaler and Neaves, 1978), which is higher than that of adult rat (2.28%), makes technically feasible for isolation and utilization (Mendis-Handagama *et al.* 1987). With proper evaluation, a donor with two normally functioning testes can be selected, and sufficient amount of Leydig cells can be isolated from ipsilateral testis under topical anesthesia without any detrimental effect to the donor. The important aspect related to the biologic uptake is the methodology involved in isolation. It is essential to have proper method of isolation set up before the operation to have viable Leydig cells (Aldred and Cooke, 1982; Dehejia *et al.* 1982; Risbridger and de Kretser, 1982). Delay in isolation can bring the functional and structural changes of the Leydig cells itself, and the risks of contamination and infection are increased.

Among the various transplant site, both subcapsular and intraperitoneal implantation were appropriate which was concordant with the results of Bolye *et al.* (1976), who reported that intraperitoneal transplantation was acceptable. These findings suggest that the blood flow and the nutritional support are essential in early uptake in cell transplantation. Tai *et al.* (1980) reported transplanting 5×10^6 cells to renal subcapsular location, but it has probably technical limitation due to its spatial restrictions, thus we have utilized intraperitoneal transplantation with synthetic collagen absorption.

The secretion of testosterone was related to the number of cells implanted. In the control group, the testosterone levels of single intraperitoneal testis and both testes intact in the scrotum were not different, which can be explained by compensatory hypertrophy after orchiectomy and temperature resistance of Leydig cells. The efficiency of mechanical dissection and collagenase treatment is about 30 %, according to Zirkin and Ewing (1987), $10\sim13\times10^6$ Leydig cells with ipsilateral testis at 6 weeks. Although the transplant results also show 30% level of testosterone production compared with normal control group, it is probably due to combined effects of compensatory hypertrophy of remnant testis and decrease in ^{125}I-hCG binding capacity.

The use of synthetic collagen promotes formation of cellular clusters. Its advantages are mainly due to facile procurement of uptake cells, making both functional and structural evaluation of transplant cells possible. Progressive uptake with neovascularization was seen after 4 week beginning from periphery. The fact that the formation of clusters make possible the cell to cell transport of metabolic signal between Leydig cells (Eik-Nes, 1975), and gonadotropin hormone receptors are presented at gap junctions in Leydig cells (Samuels and Matsumoto, 1974), the use of synthetic collagen might be beneficial in early uptake of cells. We observed the 2.7 fold increase of testosterone level in collagen uptake group than the intraperitoneal injected group at 3 months. However the level of testosterone is not enough to maintain the secondary sex characteristics after 3 months, thus the maintenance of functional production after early successful uptake is a matter to be seen.

The presence of central dystrophic ossification and atrophy with florid peripheral vasculature was observed at 3 months, and the increase of lipochrome pigments and the decrease of endoplasmic reticulum and mitochondria are concordant with findings of Leydig cells devoid of luteinizing hormone stimulation (Dym and Madhwa Raj, 1975).

It is indirectly seen with decrease of hCG receptors in our study, but the other causes such as apoptosis should be considered (Cooke *et al.* 1981).

Two additional factors must be considered in the Leydig cell transplants. First is the loss of local testicular factors in transplantation.

The loss of local stimulatory factors can be explained with disruption of cell to cell interaction in testis. The steroidogenesis of Leydig cells are both under pituitary and non-pituitary controls. The possible non-pituitary factors are macrophages, inhibin and activin from Sertoli cells, growth factors, and various auto- or paracrine factors. (Bhasin *et al.* 1983)

The second is the cessation of physiologic replenishment form the Leydig precursor cells. The advent of mature Leydig cells at testicular interstitium in rats starts in week 2 (Aoki and Massa, 1972; Lording and de Kretser, 1972), and the origin of the precursor cells is

presumed to be fibroblasts (Mendis-Handagama *et al.* 1987; Keeney *et al.* 1988). Currently the maturation theory is gaining more acceptance but its full elucidation is not yet complete. Christensen and Peacock (1980) reported maturation of precursor cells by administering hCG, and Swerdloff and Heber (1981) suggested FSH as a possible stimulant. In 1985, Kerr *et al.* showed appearance of more Leydig cells in hypophysectomized rats by injecting LH and FSH simultaneously.

Thus the simultaneous implantation of Sertoli cells and immature form of fibroblasts which is believed to be precursor of Leydig cells, combined with proper stimulation can result in more testosterone of production. Further study is necessary in experimental elucidation of the mechanism in combined transplant and stimulation.

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