A New Method of Culturing Leydig Cells and Prospects of Their Practical Use in Andrology

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We present a new method of obtaining a xenoculture enriched with Leydig cells from neonatal porcine testes providing 97-99% viability of cells in the culture. Genital extratesticular transplantation of the obtained xenoculture was applied, which minimized traumaticity of surgery and the number of postoperative complications and had undoubted advantages over the subcapsular cell culture depot.

Key Words: *Leydig cells; allotransplantation; xenotransplantation; testis transplantation; testis*

The development of transplantation methods in andrology was associated with the creation and clinical approbation of different variants of testis transplantation. Several variants of testis allotransplantation are known: free transplantation of the organ carried out as a subcutaneous injection of testis suspension or transplantation of testis fragments of different size [4]; transplantation of the whole testis [3,5], transplantation of testis in Millipore chambers [8]; whole-organ transplantation on the arterial [3] or arteriovenous pedicle [6,7].

Free transplantation of the testis was considered to be an ineffective method for the treatment of androgen deficiency. Transplantation of the testis on the vascular pedicle sometimes led to pronounced, but short-lasting clinical effect. Allotransplantation of the testis has not been widely used in clinical practice, because this operation is complex and traumatic, requires special surgical skills (technique of vascular suture, microsurgical technique, *etc*.), is associated with many postoperation complication (vascular thrombosis in the graft, rejection reaction, *etc.*), and produces insufficient clinical effect. Moreover, a series of synthetic androgens appeared, which cast doubt on advisability of organ transplantation of the testis, because the aim of this transplantation is functional activity of Leydig cells comprising a negligible percent of the testis mass.

A new stage in the development of transplantation andrology was the development of methods for culturing embryonic Leydig cells. Allotransplantation of cultured neonatal endocrine cells from the testis was proposed for the treatment of fertile and copulative disturbances in male patients: a clinical effect was observed after transplantation of hormone-producing cells under the tunica albuginea.

However, fetal human testes are not used in experimental studies due to ethical and legal reasons.

In light of this we developed and patented a new method of obtaining primary culture enriched with Leydig cells from neonatal piglet testis (porcine tissues are very close to human tissue by antigenic composition).

MATERIALS AND METHODS

The testes were aseptically isolated from prenatal or neonatal piglets and placed into vials containing 20 ml RPMI-1640 medium supplemented with antibiotics (penicillin, streptomycin, kanamycin, and

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fungizone). After 5-min washout the testes were transferred into new vials with the same medium. The procedure was repeated 3 times. Then the testes were transferred into a sterile Petri dish containing 10 ml RPMI-1640 medium supplemented with antibiotics (penicillin, streptomycin, kanamycin, and fungizone).

After removal of the outer connective tissue layer and epididymides, the content of the tunica albuginea was squeezed with pincers, minced, and transferred to a vial containing 10 ml DMEM with 0.1-2% collagenase and 0.01-1% DNase (disaggregation medium). The cell yield from the tissue decreased when the concentrations of collagenase and DNase were below 0.1 and 0.01%, respectively. Increasing the concentration of collagenase and DNase above 2 and 1% was associated with decreased viability of cells.

The vial was placed in a CO_2 incubator at 37°C (6% CO_2) for 30-60 min and slightly rocked every 5 min. Cell viability was controlled every 10 min in a Goryaev chamber by trypan blue and acridine yellow staining. The incubation was stopped after the cell viability decreased to 95-97%. The cell suspension was filtered through a capron filter and washed free from enzymes by double centrifugation in sterile polypropylene tubes containing 50 ml RPMI-1640 medium supplemented with antibiotics (penicillin, streptomycin, kanamycin, and fungizone) and 5% FCS (10 min at 800-900 rpm in a refrigerator centrifuge).

The pellet was resuspended in 5 ml DMEM containing glutamine and 20% FCS. Cell viability and count were determined in a Goryaev chamber. Cultures with cell viability below 95% were not used in the study. The cells were seeded into plastic flasks (200-800 thousands cells per 1 ml) and cultured in a CO_2 incubator at 37°C and 6% CO_2. The presence of Leydig cells was determined by staining with Sudan dyes.

RESULTS

The technique of testes isolation from prenatal and neonatal piglets and the technique of obtaining xenoculture enriched with Leydig cells were worked out in 46 and 92 experiments. The results of all experiments were identical.

The minced testicular tissue from 1-day-old piglets was disaggregated with a mixture containing DMEM, 2% collagenase, and 0.1% DNase.

The cells in DMEM supplemented with glutamine and 20% FCS were transferred into flasks (seeding dose 500 thousands cells per 1 ml, 3.5 ml suspension per flask) and cultured at 37°C and 6% CO_2. After 6-8 h the cells adhered to the plastic and

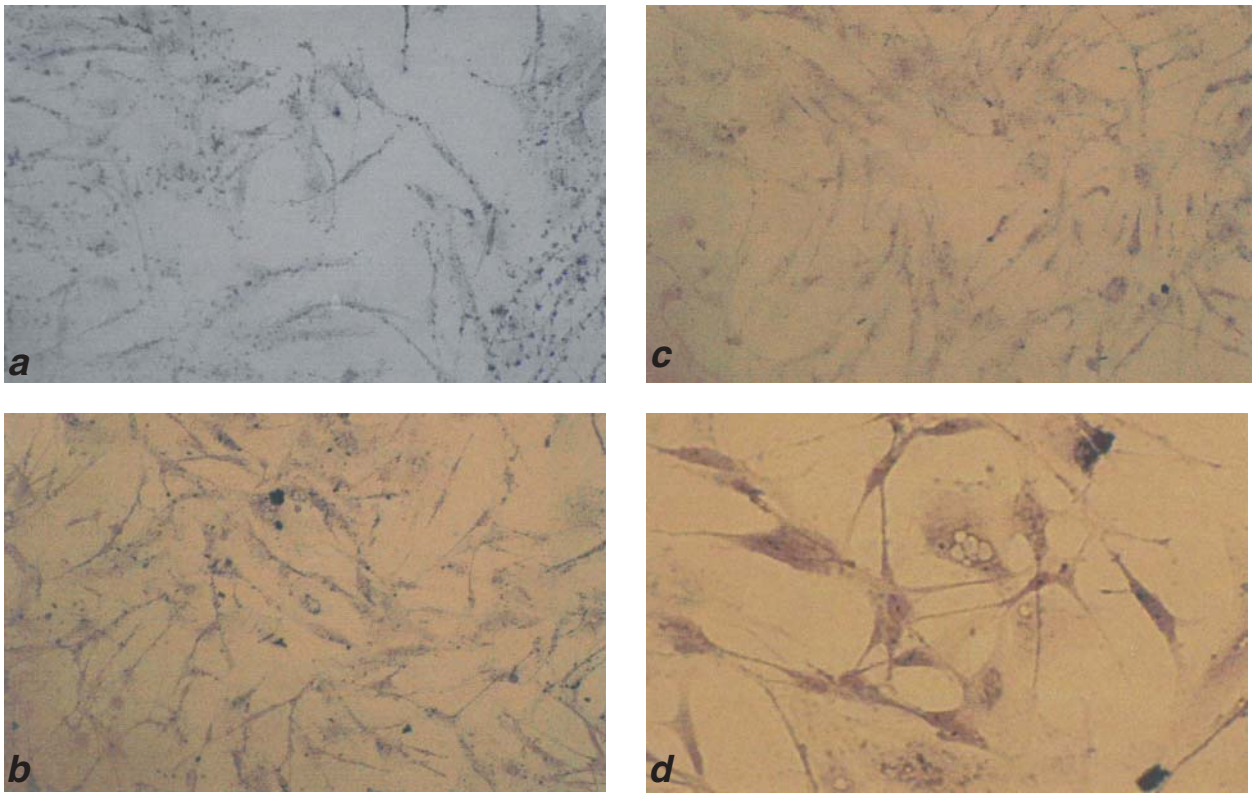

Fig. 1. Culture of testicular cells enriched with Leydig cells. *a*) sudan black staining, *b-d*) poststaining with neutral red.

after 10-16 h they flattened and start to grow. A monolayer formed after 36-48 h primarily consisted of polygonal cells enriched with Sudan-positive vacuole inclusions and fine-grained substance. Cell viability in culture was 97-99% (Fig. 1, 2).

Previous experimental transplantations of Leydig cell culture under rat testicular tunica albuginea and clinical trials showed that these operations are associated with serious complications. They often damage the testicular parenchyma (due to sharp

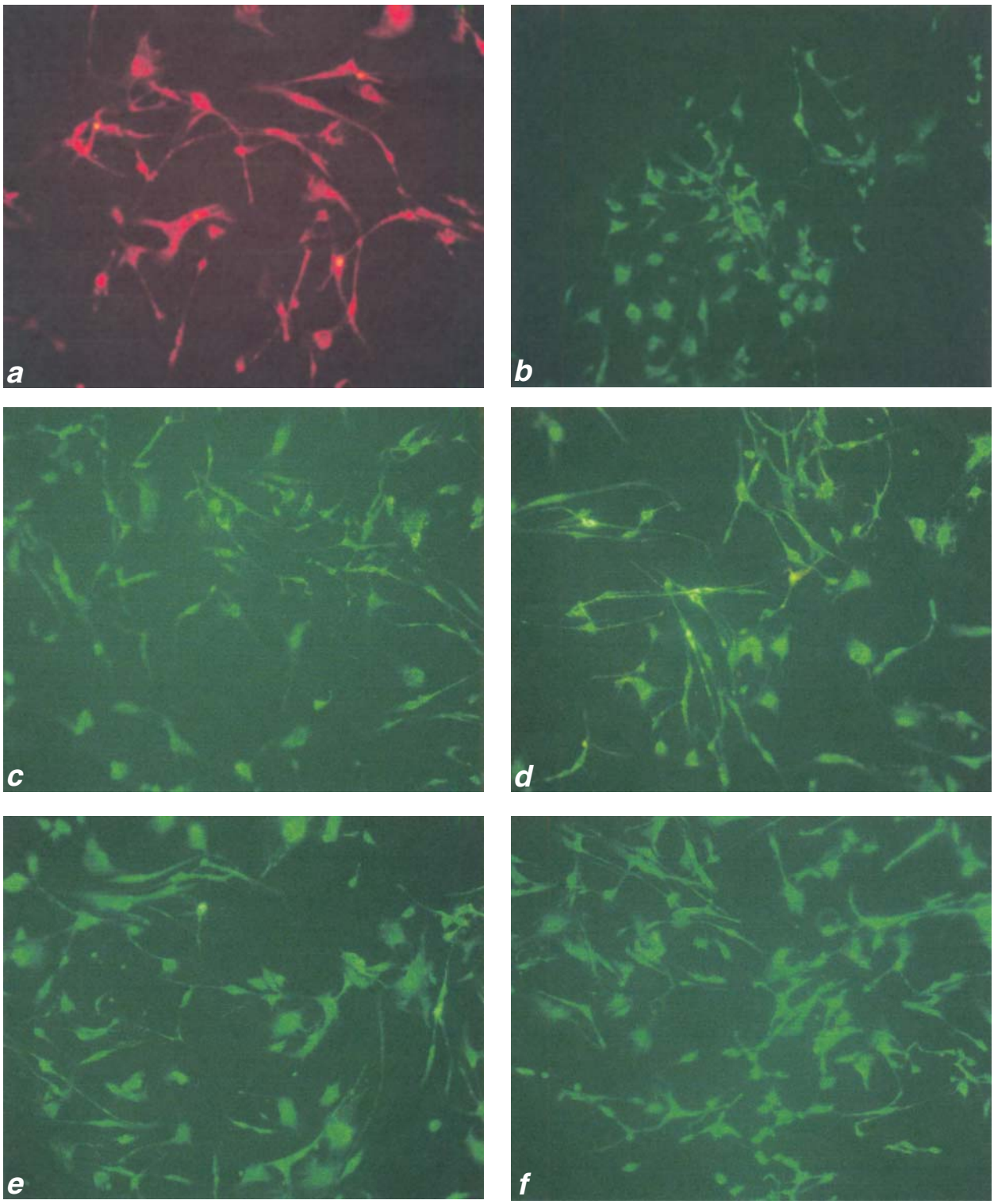

Fig. 2. Culture of testicular cells enriched with viable Leydig cells. Staining with acridine yellow and ethidium bromide.

increase in the intratesticualr pressure), cause mechanical damage to the blood-testis barrier, and are associated with high risk of orchitis and orchiepididymitis. This leads to the development of active antitesticular and antisperm autoimmunity (pronounced accumulation of complement-fixing antibodies against testicular tissue and humoral antisperm immunity), which sharply impairs functional state of the testes (spermatogenesis and hormone-producing function).

We developed and applied a new method of Leydig cell culture transplantation: depositing of no less than 2 mln. cells into membranes of the scrotal part of the spermatic cord. The cell culture was introduced into a preliminary created hydrocortisone infiltrate (injection of 1.0 ml 2.5% hydrocortisone acetate suspension).

A course of general ozone therapy was carried out for 5 days before surgery. To this end, 400 ml of freshly prepared ozonized isotonic NaCl solution (ozone concentration 1.8-2.8 mg/liter) was infused daily. After surgery the intravenous infusions were continued according to the same scheme.

General state of the patients after Leydig cell transplantation was satisfactory. Minor pain in the operated area was observed during 3-4 days after surgery. The immune status (cell and humoral immunity) was unchanged on day 10 after surgery; the parameters corresponded to the preoperation level, including the level of complement-fixing antibodies against the testicular tissue, spermagglutinins and spermimmobilizins.

Within 2-6 weeks after surgery the patients noted an increase in libido, spontaneous morning erections became more frequent and potent, general working capacity also increased. Significant increase in plasma testosterone concentration (to 14-21 mmol/liter) was found in all patients. Parameters of spermatogenesis improved in the majority of patients 3 months after surgery.

The patients were followed up for 1-1.5 years. The positive clinical and laboratory dynamics was observed in the majority of cases.

Thus, transplantation of the culture enriched with Leydig cells can be considered as an effective method of stimulation of spermatogenesis. The advantage of this technology is obvious: sparring puncture operation in an outpatient department provides stable therapeutic effect and does not require long-term drug therapy or injection of synthetic androgens. Compared to subcapsular cell depot, this new method of cell culture transplantation excludes traumatization of the testicular parenchyma, reduces the risk of autoimmune aggression against testicular tissue and spermatozoa, inflammatory diseases of the scrotal organs (orchitis and orchiepididymitis), and improves survival of the allogeneic cell culture in the glucocorticoids infiltrate. Preventive and postoperation courses of intravenous ozone therapy create favorable general (hemocirculatory, immunological, *etc.*) and local (improvement of regional hemodynamics, *etc.*) conditions for grafting, adaptation, and further functioning of the transplanted cell culture.

TABLE 1. Comparative Characteristics of Spermiological Parameters of Patient K.

Parameter	Before surgery	After surgery	
		3 months	6 months
Density, mln/ml	6	12	18
Parameter, %			
actively moving	0	Solitary	20
abnormal	100	81	62

TABLE 2. Comparative Characteristics of Spermiological Parameters of Patient I.

Parameter	Before surgery	After surgery	
		3 months	6 months
Density, mln/ml	5	16	28
Parameter, %			
actively moving	0	15	48
abnormal	100	70	45

The efficiency of the proposed method was confirmed by clinical observations.

Patient K., 36 years (clinical diagnosis: moderate androgen deficiency; infertility; grade III oligospermia) considered himself ill for 8 years and had pronounced changes in spermatogenesis. The patient was observed by an urologist and received vitamins B_{12}, A, E, plant preparations (Speman Forte, Mustong, *etc.*), chorionic gonadotropin, and testosterone preparations). The treatment produced a transient positive effect, but reproductive function was not restored. The last 2 years the patient noted decreased libido and weak erections. Examination revealed no pathology of internal organs.

The testes were somewhat reduced (the volume of the right and left testes were 12-14 ml), the epididymides and vas deferens were unchanged, the internal male genital organs without pronounced pathology. Laboratory tests were carried out: infectious and immunological tests (PCR) were negative;

luteinizing hormone (LH), follicle-stimulating hormone (FSH), and prolactin were at the upper boundary of normal, testosterone 6.9 mmol/liter; prostate secretion analysis showed: leukocytes 4-5 per visual field, moderate number of lecithin grains, bacteria were absent; the state of cell and humoral immunity within normal ranges. The patient received intravenous ozone therapy before surgery (5 intravenous infusions of 500 ml ozonized isotonic NaCl solution; ozone concentration 1.8-2.8 μg/ml).

Transplantation of Leydig cell culture (not less than 2 mln cells) into the preliminary formed hydrocortisone infiltration (0.1 ml of 2.5% suspension of hydrocortisone acetate) in the scrotal part of the right spermatic cord was performed under local anesthesia (40 ml of 0.5% novocaine solution).

After surgery the patient was in a satisfactory state. Pain at the site of transplantation was minor. The course of intravenous ozone therapy was continued starting from day 2 after surgery (5 infusions).

There were no significant changes in the immune status, humoral and cell immunity (testosterone level after 1, 2, and 11 months was 12.0, 14.2, and 14.0 mmol/liter). Pronounced positive dynamics of spermiological parameters was observed (Table 1).

One month after transplantation, the patient noted an increase in libido, morning and spontaneous erections, and strengthening of erections.

Thus, our clinical study demonstrated high efficiency of Leydig cell transplantation into the wall of the spermatic cord for the treatment of fertile and copulative disturbances caused by moderate androgen insufficiency. The positive dynamics of clinical and laboratory parameters 11 months after the procedure attests to preserved functional activity of the transplanted culture.

Another observation also confirms high clinical efficiency of Leydig cell transplantation into a hydrocortisone infiltrate in the spermatic cord wall in the treatment of severe fertile disturbances. Patient I., 34 years (clinical diagnosis: idiopathic infertility; grade III oligospermia) was continuously observed by an urologist-andrologist during the last 6 years. Drug therapy (Sperman Forte, Tentex, tocopherol acetate, Proviron, *etc.*) produced no considerable positive dynamics of spermiological parameter. Physical examination revealed no pathologies of thoracic and abdominal organs as well as of internal and external gonadal organs.

Laboratory tests were carried out: infectious and immunological tests for sexually transmitted infections (PCR) were negative; LH, FSH, prolactin, and estradiol were within the normal range, testosterone 7.5 mmol/liter (moderate decrease); prostate secretion analysis showed: per visual field, lecithin number, bacteria were absent. Since the prescribed drug therapy was ineffective, transplantation of Leydig cell culture was recommended.

Transplantation of Leydig cell culture (not less than 2 mln cells) into the preliminary formed hydrocortisone infiltration (0.1 ml of 2.5% suspension of hydrocortisone acetate) in the scrotal part of the right spermatic cord was performed under local anesthesia (40 ml of 0.5% novocaine solution).

In the postoperation period the patient was in a satisfactory state, minor pain at the site of transplantation was observed for 3 days after the procedure. There were no significant changes in the immune status, humoral and cell immunity. The parameters corresponded to the preoperation levels (testosterone concentration after 1, 2 months, and 1 year was 18.2, 19.0, and 18.4 mmol/liter, respectively). Pronounced positive dynamics of spermiological parameters was observed (Table 2). The positive dynamics of plasma testosterone level 1 year after the procedure attests to preserved functional activity of transplanted Leydig cells.

Thus, xenotransplantation of Leydig cell-enriched culture performed according to the proposed genital extratesticular method (into the wall of the scrotal part of the spermatic cord) is an effective, low-traumatic, and technically simple method for the treatment of testicular forms of infertility and androgen deficiency. Further experimental and clinical studies will improve this technology.

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