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MORPHOLOGY AND PATHOMORPHOLOGY

Ectopic Organogenesis after Allotransplantation of Freshly Removed or Cryopreserved Neonatal Testicle under the Renal Capsule in Rats

V. I. Kirpatovskii[1], G. D. Efremov[1], and E. V. Frolova[2]

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In experiments on white outbred male rats, a freshly removed (20 experiments) or cryopreserved (10 experiments) testicle from newborn rats (1-2 days after birth) was transplanted under the renal capsule after bilateral orchiectomy. In all experiments with transplantation of freshly removed testicle, it was engrafted. In 3 months, histological examination revealed the formation of mature seminiferous tubules, but spermatogenesis was blocked at the stage of spermatogonia; groups of proliferating Leydig cells in the loose connective tissue between the tubules were also seen. In 6 and 12 months, the status of the seminiferous tubules remained unchanged, but structures typical of the epididymis and developing vas deferens were revealed. The number of proliferating Leydig cells increased. The initially low testosterone concentration in the blood of castrated males increased significantly as soon as in 1 month after transplantation and grew up to 3 months, remaining at a level ~50% of normal. Engraftment of cryopreserved neonatal testicular tissue was observed in 60% cases, however, engrafted tissue, similar to the fresh one, retained the ability for organogenesis with the formation of mature seminiferous tubules, epididymis, and groups of proliferating Leydig cells. The dynamics of blood testosterone concentration in rats with cryopreserved and fresh transplantation was similar. Subcapsular transplantation did not adversely affect the kidneys, which was seen from normal histological structure of the kidneys and creatinine and urea concentrations in the blood.

Key Words: *regenerative medicine; testis; organogenesis; cryopreservation; androgen deficiency*

The problem of protection of men's health, in particular sexual function, is becoming more urgent due to increasing incidence of erectile dysfunction and male infertility. This condition is often associated with androgen deficiency caused by age, occupational, or medical factors [5]. Spermatogenesis disturbances is the cause of infertility in 15-20% couples [11]. Androgen deficiency is also a common cause of spermatogenesis disruption.

Hormonal therapy with various testosterone preparations is the most common way to treat erectile dysfunction and impaired spermatogenesis [4,12]. However, the effectiveness of this therapy decreases with time and side effects often develop in the form of

[1]N. A. Lopatkin Research Institute of Urology and Interventional Radiology, Affiliated Branch of National Medical Research Radiological Center, Ministry of Health of the Russian Federation; [2]All-Russian Institute of Scientific and Technical Information, Russian Academy of Sciences, Moscow, Russia. ***Address for correspondence:*** vladkirp@yandex.ru. V. I. Kirpatovskii

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metabolic disturbances and allergic reactions requiring withdrawal [13].

The development of cell technologies with transplantation of stem cell or progenitor cells opens up new possibilities in the treatment of these conditions. The effectiveness of intratesticular transplantation of cultured allogenic and xenogenic bone marrow or adipose tissue mesenchymal cells, cultured fetal testicular cells [2,6], Leydig cells, or their precursors [3,9,15], or the complex of testicular, pituitary, and hypothalamus cells [1] is now intensively studied. However, cell culturing for obtaining the required cell mass is a very costly procedure. At the same time, fetal or neonatal tissue containing a large number of low-differentiated cells with high proliferative potential can be used in tissue therapy. Preserved cell—cell interactions in the transplanted tissue (stem cell “niche”) can support the formation of competent organ structures. The possibility of using cryopreserved testicular tissue transplants for restoring fertility clinical practice are now discussed [8,10].

We studied engraftment, growth, and hormonal activity of testicular tissue fragments taken from newborn rats and transplanted under the renal capsule. This transplantation variant was chosen because subcapsular space of the kidney is an immunoprivileged zone, which considerably reduces the risk of rejection of allo- or xenografts. We also studied the influence of cryopreservation on graft take and functional activity.

MATERIALS AND METHODS

Experiments were carried out on outbred white male rats (n=30). Bilateral orchiectomy was performed under ether anesthesia. In 1 month, transplantation with freshly excised or cryopreserved testicle fragments of newborn rats (1-2 days after birth) under the renal capsule of recipients was performed (Fig. 1, *a*). To this end, the outer edge of the renal capsule was cut with microsurgical scalpel, a tunnel was formed in the subcapsular space, and a fragment of neonatal testicle was placed there. The abdominal cavity was sutured with atraumatic Vicryl 4/0 thread.

In series I (20 experiments), freshly removed neonatal testes were washed in physiological saline, cut in halves, and transplanted into both kidneys of castrated rats. In series II (10 experiments), the removed testes were washed in saline, placed at room temperature in a cryoprotective medium containing (g/liter): 3.8 K_2HPO_4, 1.02 KH_2PO_4, 0.57 KCl, 0.43 $NaHCO_3$, and 68.4 sucrose. After 5-min incubation, cryoprotectant DMSO was gradually with 10 min intervals added to the solution to concentrations of 5, 10, and 15%. After 10-min incubation, the fragments of neonatal testis were placed in a dry plastic tube and stored in a freezer at -90°C over 2-4 weeks until transplantation. Prior to transplantation, the tubes containing the tissue were removed from the freezer, placed in a water bath at 37°C, and after complete thawing they were transferred to a solution similar to the cryoprotecting one containing 10% DMSO for 10 min, then transferred to a solution with 5% DMSO for 10 min, and finally, in a solution without DMSO for 10 min. Transplantation was carried out as described above.

In series I, the animals were examined in 1, 3, 6 and 12 months; in series II — in 1, 3, and 6 months. The presence of the transplant under the renal capsule was visually assessed, the kidney with the transplant was removed for histological examination, and blood samples were taken to measure testosterone concentration and biochemical parameters. Histological examination was carried out according to a standard procedure, paraffin sections were stained with hematoxylin and eosin. Blood serum testosterone concentration was determined by immunochemiluminescence on an Access 2 immunochemical analyzer (Beckman Coulter).

To evaluate the functional state of the kidneys, serum creatinine and urea were measured using standard reagents on an ADVIA-2000 biochemical analyzer (Siemens).

Results were statistically processed using Student’s t test. The differences were significant at $p<0.05$.

RESULTS

After transplantation of freshly removed neonatal testicular tissue (series I), transplant engraftment was observed in all cases. In 1 month, it looked like a whitish formation under the renal capsule (Fig. 1, *b*). In 3, 6, and 12 months, graft dimensions significantly increased. It protruded above the kidney surface (Fig. 1, *c*) and grew the kidney parenchyma (Fig. 1, *d*). In some experiments, at 6 and 12 months, a clear heterogeneity of the newly formed tissue was observed, probably related to epididymis formation (Fig. 1, *c*).

Histological examination in 1 month after transplantation showed that approximately $^1/_3$ of the newly formed testicular tissue was presented by loose connective tissue with small aggregations of Leydig cells and few eosinophilic leukocytes. The rest two-third was presented by tubules of various maturity lined with spermatogenic epithelium (Fig. 2, *a*). The developing epididymis structures were located in a separate area. The kidney looked little affected. The structure of the glomeruli and tubules was preserved. At the boundary of the kidney and newly formed testicular tissue, diffuse lymphoid cell infiltration was observed. In 3 months after transplantation, the newly formed testicle tissue was presented mainly by mature tubular structures and epididymal elements. The basal mem-

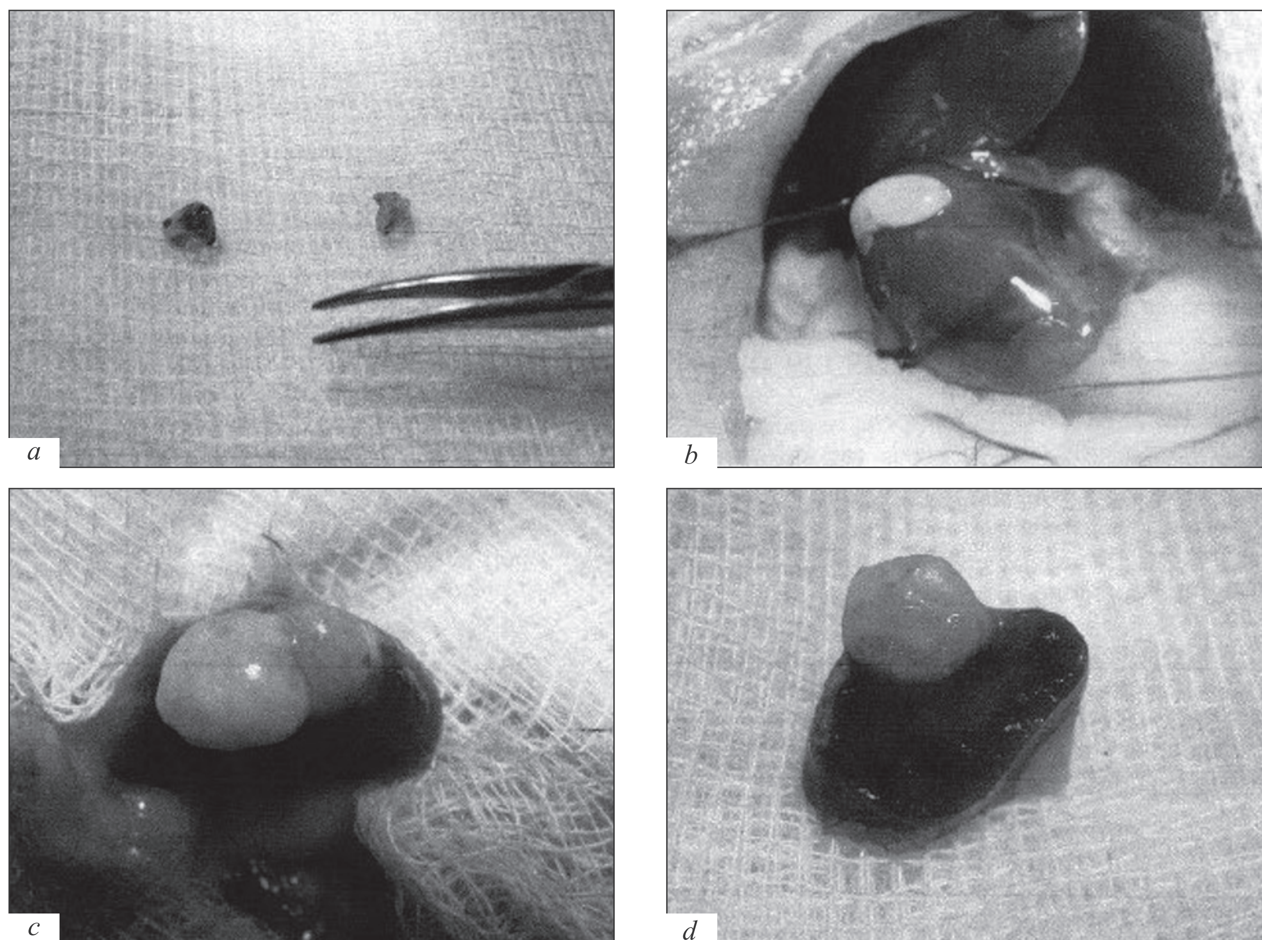

Fig. 1. Neonatal testis (*a*) and kidneys with the graft in 1 (*b*) and 6 (*c*) months; cross-section of the kidney in 6 months after transplantation (*d*).

brane of the tubules was lined with Sertoli cells; in the tubular epithelium, signs of active spermatogenesis up to the spermatogonia stage were revealed; complexes of proliferating Leydig cells were seen in the connective tissue between the tubules (Fig. 2, *b*). The kidney had normal structure with inflammatory plasma cell infiltration adjacent to the newly formed testicular tissue. In 6 and 12 months, the newly formed tissue was also presented predominantly by the seminiferous tubules, but in most of the tubules, spermatogenesis was arrested at the spermatogonia stage. In 6 months, structures typical for the epididymis (Fig. 2, *d*) were found in the preparations, and after 12 months, elements of the epididymis and the newly formed vas deference were seen (Fig. 2, *d*). Mild eosinophil infiltration was observed in the connective tissue between the tubules of the newly formed testicle. The adjacent kidney tissue looked unchanged.

In series II (transplantation of cryopreserved neonatal testicular tissue), the transplants were revealed in 12 of 20 kidneys and they looked similar to those in series I. In 3 and 6 months, dimensions of the newly formed cryopreserved testicular tissue were the same as in experiments with transplantation of fresh tissue. In 6 months, the heterogeneity of the newly formed testicle and appearance of an epididymis-like area were also noted (Fig. 3, *a*). Histological examination revealed a picture close to that in series I. The newly formed seminiferous tubules were lined with spermatogenic epithelium with spermatogenesis arrested at the stage of spermatogonia. Some tubules were lined only with Sertoli cells. In the interstitium, complexes of proliferating Leydig cells were detected. Along with seminiferous tubules, epididymis structures were also found in the preparations. The connective tissue capsule surrounding the transplant was clearly seen (Fig. 3, *b*).

The dynamics of blood testosterone showed that the hormone level decreased in 1 month after castration (Table 1). In both series of the experiments, a significant increase in testosterone concentration was detected in 1 month after transplantation; it continued to increase up to 3 months and then, remained at about the same level until the end of the observation period.

Blood creatinine and urea levels after testicular tissue transplantation did not differ from the normal at all observation terms, indicating that subcapsular

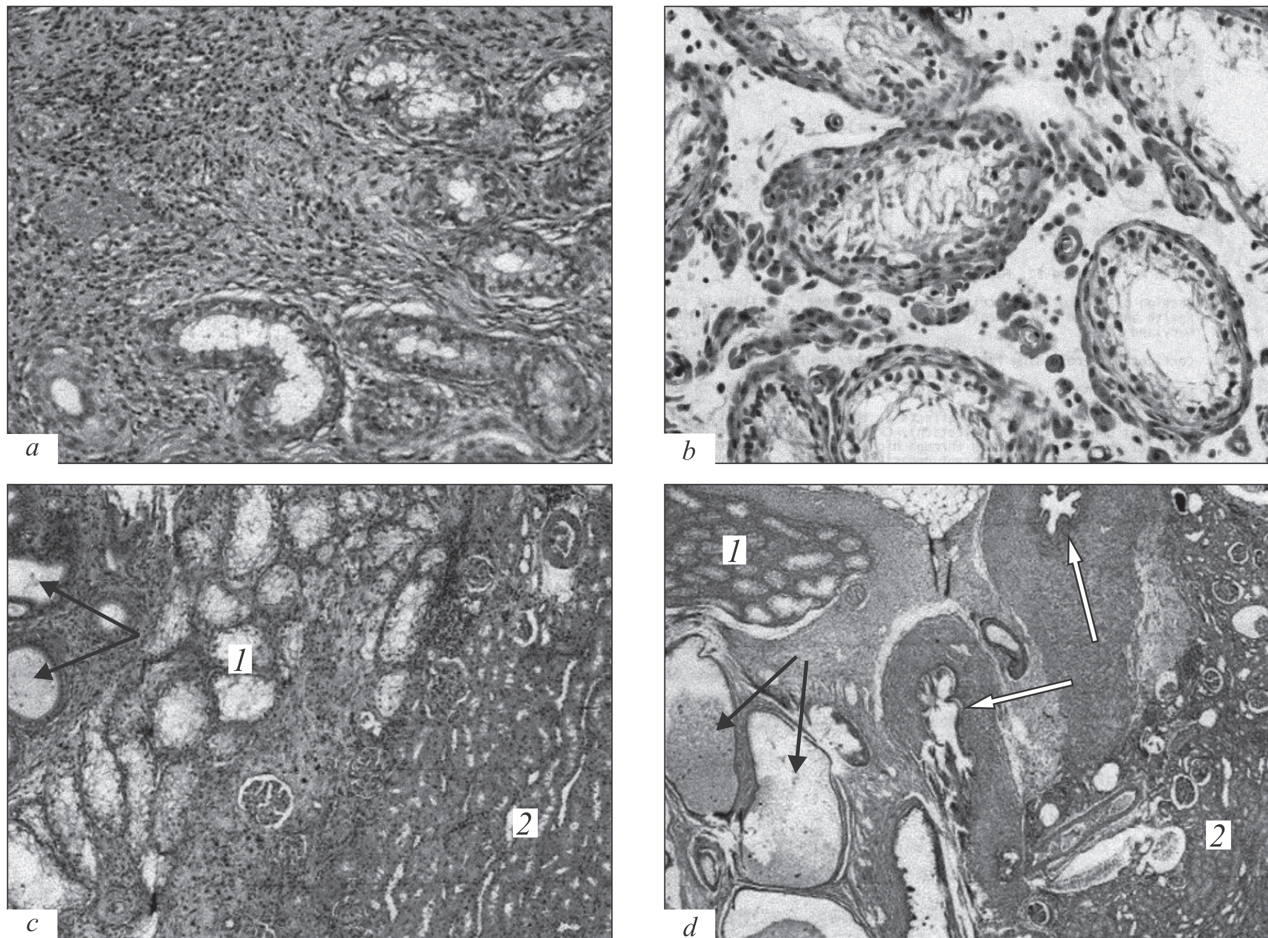

Fig. 2. Histological picture of the neonatal testicular tissue transplant in 1 (*a*), 3 (*b*), 6 (*d*), and 12 (*d*) months after transplantation. Staining with hematoxylin and eosin, ×200 (*a*, *c*, *d*), ×400 (*b*). *1*) Transplant, *2*) kidney tissue. Black arrows show the epididymis structures, white arrows show the vas deferens.

transplantation had no negative effects on kidney function.

Thus, we demonstrated that allogeneic neonatal testicular tissue transplanted into the immunologically privileged zone (under the renal capsule) not only to survived, but also formed structures typical for the mature organ: seminiferous tubules containing Sertoli cells and spermatogenic epithelium, Leydig cell complexes in the interstitial tissue, epididymal structures, and vas deference. This suggests that stem and progenitor cells abundant in the neonatal tissue retain the capacity of directed differentiation after transplantation into a foreign region. It can be hypothesized that in case of tissue fragment transplantation an important role is played by preserved cell-cell cooperation (the so-called "niche") that is absent in the transplantation of cultured cells.

In this case, Leydig cells actively proliferating in the newly formed testicle can synthesize testosterone, which leads to elevation of its blood concentration. However, the increase in the hormone concentration ceased in 3 months and remained at a level about twice below the norm, probably due to limited space for the growth of newly formed organ under the renal capsule and, consequently, insufficient mass of Leydig cells.

At the initial stages after neonatal testicular tissue transplantation, signs of active spermatogenesis were histologically revealed, however at later terms, spermatogenesis was inhibited and arrested at the stage of spermatogonia. The spermatogenesis incompleteness in the newly formed seminiferous tubules can be determined by several factors: the absence of connections with other reproductive organs (prostate, seminal vesicles), low testosterone level, and the presence of simmering immune conflict as evidenced by connective tissue capsule formation around the graft and indistinct eosinophilic infiltration of the stroma observed in some experiments. In case of heterotopic porcine fetal testicle tissue transplantation to nude mice [7], mature seminiferous tubules were also formed with spermatogenesis arrest at the spermatogonia stage (1-year observation).

In a special series, we examined the ability for ectopic organogenesis and hormonal activity of neo-

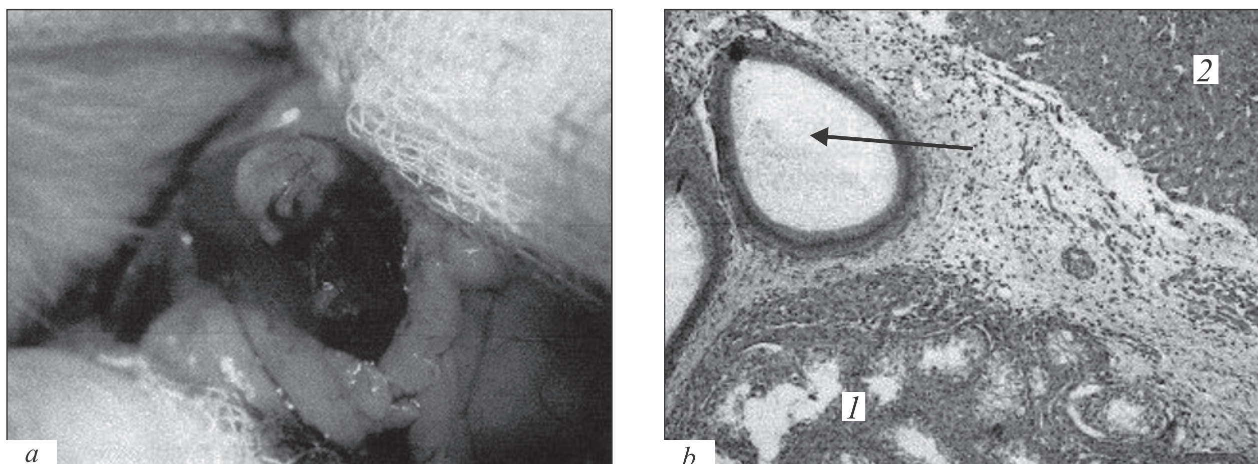

Fig. 3. External appearance (*a*) and histological picture of cryopreserved neonatal testicular tissue graft in 6 months after transplantation (*b*). Staining with hematoxylin and eosin, ×200. *1*) Transplant, *2*) kidney tissue. Arrow shows epididymis.

TABLE 1. Dynamics of Testosterone, Creatinine, and Urea Concentrations after Transplantation of Neonatal Testicular Tissue under the Renal Capsule (*M*±*m*)

Parameter	Norm	Orchiectomy	Months after transplantation			
			1	3	6	12
Testosterone (series I), ng/ml	2.39±0.12	0.61±0.04	0.73±0.02*	1.12±0.09**	0.94±0.07**	1.10±0.08**
Testosterone (series II), ng/ml	2.39±0.12	0.61±0.04	0.77±0.03*	1.51±0.11***	1.19±0.09**	—
Creatinine μmol/liter	59±1	61±2	57±2	62±1	58±2	63±2
Urea, mol/liter	6.7±0.1	6.9±0.2	6.6±0.1	7.1±0.1	6.8±0.1	6.4±0.1

Note. *$p<0.05$, ***$p<0.01$, ***$p<0.001$ in comparison with normal.

natal testes tissue after cryopreservation, which is important for assessing the possibility of neonatal tissue bank creation. It turned out that cryopreservation reduces the probability of transplant engraftment to 60%. However, the implanted cryopreserved grafts practically did not differed from freshly implanted. The histological pattern in both series was almost identical. The hormone-producing activity was also preserved: blood testosterone concentration increased to the same extent as in series I. Preservation of the functional usefulness of cryopreserved testicular tissue after the transplantation was confirmed previously [7,14].

Tissue transplantation under the renal capsule had no adverse effect on the functional state of the organ. Histologically, and according to the biochemical blood test, the kidney was not disturbed.

Thus, transplantation of neonatal testicle tissue fragments under the renal capsule demonstrated the possibility of formation of a new organ with a structure, typical for the native testis with its endocrine function and long-term functioning. Although in the newly formed tissue spermatogenesis was incomplete, the very ability of heterotopically transplanted neonatal tissue for organogenesis is indicative of the perspectivity of further research in this direction. We further continued the research with neonatal testicular tissue transplantation into the other immunoprivileged zone, under the tunica albuginea, to assess stimulation of impaired spermatogenesis and androgen deficiency correction.

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