Morphological Changes in Paraurethral Area after Introduction of Tissue Engineering Construct on the Basis of Adipose Tissue Stromal Cells

A. V. Makarov[1,2], I. V. Arutyunyan[1,3], G. B.Bol'shakova[2], A. V. Volkov[1,2], and D. V. Gol'dshtein[1,2,3]

Translated from *Kletochnye Tehnologii v Biologii i Medicine*, No. 4, pp. 229-235, December, 2009
Original article submitted August 20, 2009.

We studied morphological changes in the paraurethral area of Wistar rats after introduction of tissue engineering constructs on the basis of multipotent mesenchymal stem cells and gelatin sponge. The tissue engineering construct containing autologous culture of the stromal fraction of the adipose tissue was most effective. After introduction of this construct we observed more rapid degradation of the construct matrix and more intensive formation of collagen fibers.

Key Words: *multipotent stromal cells; connective tissue; tissue engineering construct; transplantation*

Methods of introduction of preparations replenishing local connective tissue deficit are widely used in medical practice (in degenerative diseases of the skin and connective tissue, in congenital urinary tract pathologies, in stress-induced urinary incontinence, *etc*.). Urethral sling surgery, the least invasive surgical approach [3], is the method of choice for the correction of stress-induced urinary incontinence [2]. However, their efficiency is not always sufficient (treatment can be associated with complications). The methods based on the use of preparation creating additional volume in the site of their introduction and aimed at attaining pressure equilibrium between the urinary bladder and urethra have some advantages (biomaterials forming additional artificial uncontrollable sphincter increasing partial pressure in the urethra are introduced para- or periurethrally [15]).

These preparations should not contain toxic substances, rapidly degrading and eliminated substances, and substances migrating into surrounding tissues.

Certain positive effect and temporal remission of stress urinary incontinence can be attained after introduction of cell suspensions (multipotent mesenchymal stromal cells, MMSC) [4,13]. However, the effect is short lasting, because introduction of unbound cells is associated with their diffuse migration into surrounding tissues or massive death due to the absence of proper microenvironment.

Our aim was to create a tissue-engineering construct (TEC) including cell culture capable of replenishing the volume of connective tissue and fixing the cells in the site of injection and a carrier matrix ensuring cell integrity in low-invasive route of administration (injection).

When choosing the optimal carrier for TEC we excluded many synthetic preparations [5,6,15], preparations on the basis of organic acid polyesters [14], and gels on the basis of collagen and hyaluronic acid [6]. Gelatin sponge was used as a carrier for TEC [10,18].

Stromal fraction of the adipose tissue (SFAT) was chosen as the cell component of TEC because this culture corresponds to certain parameters: low-traumatic procedure of obtaining primary material, the possibility of isolation of autologous cell culture, high prolife-

[1]ReMeTeks Company; [2]Institute of Human Morphology, Russian Academy of Medical Sciences; [3]Medical Genetic Research Center, Russian Academy of Medical Sciences, Moscow, Russia. ***Address for correspondence:*** anvitmak@yandex.ru. A. V. Makarov

0007-4888/09/1484-0719

rative activity, and active synthesis of extracellular matrix [20]. MMSC isolated from the adipose tissue more intensively synthesize collagen fibers than cell cultures isolated from other sources [17,19].

Here we studied morphological changes in paraurethral area of female rats after introduction of TEC on the basis of adipose tissue MMSC and gelatin sponge.

MATERIALS AND METHODS

Isolation of adipose tissue stromal cell culture. SFAT cell cultures were isolated as described previously [12]. The material (subcutaneous fat) was transported into the laboratory in sterile tubes with transport medium (F12 with amikacin, 0.5 g/liter) within 1 h. All manipulations with the material were performed in a sterile laminar box (safety class II).

Tissue fragments were transferred into a Petri dish, thoroughly cleansed from blood vessels, and washed 3-4 times with Hanks saline with cephazolin (1 g/liter), then transferred into another Petri dish with Hanks saline with cephazolin and minced with scissors. The homogenized tissue was transferred with a pipette into a 15-ml centrifuge tube, Hanks saline was carefully discarded. Versen and 0.25% trypsin (1:1) were added to the tube so that the material was completely covered with the enzyme solution. The tubes were incubated for 1.5-2 h in a CO_2 incubator (37°C, 5% CO_2) and vortexed every 15 min.

After incubation, Hanks saline or culture medium were added to the tube for reducing enzyme concentration. The cells were precipitated by centrifugation (1100 rpm, 10 min). The supernatant was removed and the pellet was resuspended in culture medium (DMEM/F12 1:1 supplemented with 10% ECS, 584 mg/liter L-glutamine, and antibiotic (500 mg/liter amikacin) and transferred to ciltural flasks. The cells attached to the substrate within 12-24 h.

After removal of floating cells, the culture looked homogenous and was presented primarily by spindle-shaped fibroblast-like cells (15-17-μ long) with few processes, centrally positioned nucleus, and low number of granules in the cytoplasm.

Cell transfection with marker gene Green Fluorescent Protein (GFP). For cell transfection with marker gene GFP we used a construct on the basis of adenovirus (AD-5 EGFP; kindly provided by B. S. Naroditskii, Doct. Biol. Nauk, Head of Laboratory of Molecular Biotechnology, N. F. Gamaleya Institute of Epidemiology and Microbiology)

For evaluation of the working dilution of the virus, the cells were seeded in a 6-well and incubated in a medium with different content of the transfecting agent. The optimum dilution was determined by the efficiency of transfection and the absence of the cytotoxic effect.

Two days before transplantation, the confluent cell monolayer was washed from serum and incubated in serum-free medium with chosen virus concentration for 2.5-3 h at 37°C. After incubation, the cells were repeatedly washed and low-serum medium (1%) was added.

The efficiency of transfection was evaluated visually. Green fluorescence appeared 24 h after transfection.

Devitalization of cell culture. The required amount of cells were suspended in physiological saline, transferred into cryotubes, and placed into liquid nitrogen (without cryoprotectors and staged freezing). After freezing, the tube was placed into hot water (90°C) for complete defrosting. The procedure was repeated 3 times. Cell viability was excluded by staining with 0.4% trypan blue; cell death was also confirmed by their inability to attach to the plastic over 48 h under standard culturing conditions.

Experimental stage of the study. Experiments were carried out on female Wistar rats weighing 250-300 g (*n*=96, Stolbowaya nursery). Animal experiments were performed with Order No. 755 of Ministry of Health of USSR (August 12, 1977). The experiment was approved by Bioethical Committee of Institute of Human Morphology, Russian Academy of Medical Sciences.

The animals were divided into 4 groups: main, reference, and 2 control groups. The construct containing minced gelatin sponge and autologous culture of SFAT cells was injected to animals of the main group. Animals of the reference group received minced gelatin sponge and allogenic cell culture. Control group 1 received only TEC matrix and control group 2 received minced gelatin sponge and devitalized culture of SFAT cells. Two animals in the main and control groups received TEC with cell culture transfected with GFP in each point of the study.

Before the experiment, subcutaneous fat was obtained from the withers of rats of the main group for isolation of autogenous and allogenic cultures of SFAT cells. The hairs on the withers were removed, the skin was cut longitudinally, and the subcutaneous fat was exposed by the blunt and sharp methods. A fragment of the adipose tissue (0.5×0.5 cm) was cut and after hemostasis the wound was sutured. The obtained samples in the transport medium were transported to the cultural laboratory; the temperature of the medium was maintained at 4°C. For ensuring autogenous transplantation, the animals were marked by auricle cuts.

The transplant (0.1 ml volume, cell concentration 5 mln) was paraurethrally injected using an insulin syringe with a standard needle (0.6 mm diameter) to animals anesthesized with ether.

In each group (n=6 in each group), the animals were sacrificed on days 7, 14, 30, and 60 of the experiment with ether overdose. After sacrifice, the skin around the clitoris including the upper part of the vagina was cut. The clitoris was mobilized by the blunt and sharp technique up to the pubic bone together with the portion of the vagina and cut at a depth of 1 cm.

Histological and morphological studies. Histological examination of tissue samples was performed immediately after animal sacrifice. The material was fixed in 10% neutral formalin on Lilly phosphate buffer for 72 h and then washed with running water for 24 h. After standard histological processing, the tissue samples were embedded into paraffin (Gistomix) using histological rings (Biovitrum). Sections (5 μ) were obtained on a Microm HM430. For survey examination of sections of the paraurethral area, the preparations were stained with hematoxylin and eosin and after Mallory; picrosirius red staining was used for evaluation of the total area of collagen fibers and Romanovskii–Giemsa staining was used evaluation of the morphology of cell infiltration. GFP was detected using immunohistochemical staining of histological sections using rabbit polyclonal antibodies to GFP (Abcam). Histological preparations were studied and photographed using a Zeiss Axioplan-2 microscope and Axiocam digital camera.

For evaluation of morphological changes in the transplantation site, morphometric study of the construct matrix, cells infiltrating the area of construct transplantation, collagen fibers, and blood vessels was performed.

Six digital photos at ×200 were taken in each section in the transplantation area. The fields of view were chosen using random number table. The calculation was performed by the methods of fields using a 100-point grid [1], and then the relative volume of each element was determined.

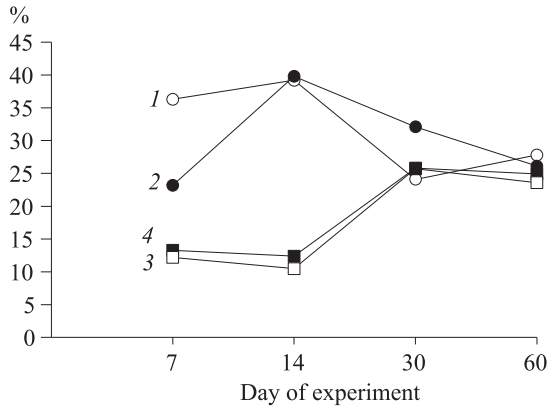

Fig. 1. Changes in volume density of cell infiltrate after injection of TEC containing autologous (*1*), allogenic (*2*), and devitalized (*3*) cell cultures and matrix alone (*4*) at different terms of the experiment.

The qualitative composition of the cell infiltrate was evaluated by counting 100 cells in the transplantation area in each animal. The following cell elements were counted: macrophages, fibroblasts, fibrocytes, and cells of the transplant.

Changes in the content of collagen fibers were studied on 4-μ sections stained with picrosirius red yielding most precise visualization of collagen [11]. On each section from the transplantation area, up to 4 photos were taken at ×200 and processed using Adobe Photoshop CS3 software. The fraction of collagen fibers was calculated by the ratio of all collagen pixels to the total number of pixels.

The data were processed statistically using standard software (Statistica 6.0, Sigma Stat 3.5). The differences were significant at $p \leq 0.05$.

RESULTS

On day 7 of the experiment, the maximum proportion of infiltrate in the transplantation area was observed in the main group (Fig. 1). Appreciable infiltration was noted in the reference group, while in both control groups this parameter was minimum. No significant differences between the control groups were revealed.

On day 14, the volume density of infiltrate in the main and reference groups did not differ; control groups were also similar by this parameter. However, the content of infiltrate in animals receiving TEC containing viable cells considerably surpassed the corresponding parameter in the control groups. On days 30 and 60, the volume density of infiltrate decreased in the main and reference groups and increased in both control groups. On day 60, the volume density of infiltrate became similar in all groups.

At early terms of the experiment, the infiltration in the main and reference groups was presented by transplanted cell culture, which disappeared or was transformed into connective tissue cells by day 30. The latter hypothesis was confirmed by detection of the label in analogous cells during histochemical study. In groups receiving cell cultures, transplanted cells constitute a considerable portion of infiltrate on days 7 and 14. The increase and leveling of the number of cells in control groups at late terms can be explained by the fact that on days 7 and 14 the matrix occupied practically total field of view and masked the cells.

On day 7, the composition of the infiltrate differed: fibroblasts and fibrocytes predominated in the main and reference groups and macrophages were more abundant in the control groups (Fig. 2, Table 1).

On day 30 of the experiment, fibroblasts and fibrocytes still predominated in the main and reference groups, their number being maximum in the main group. The number of fibroblasts and fibrocytes in the

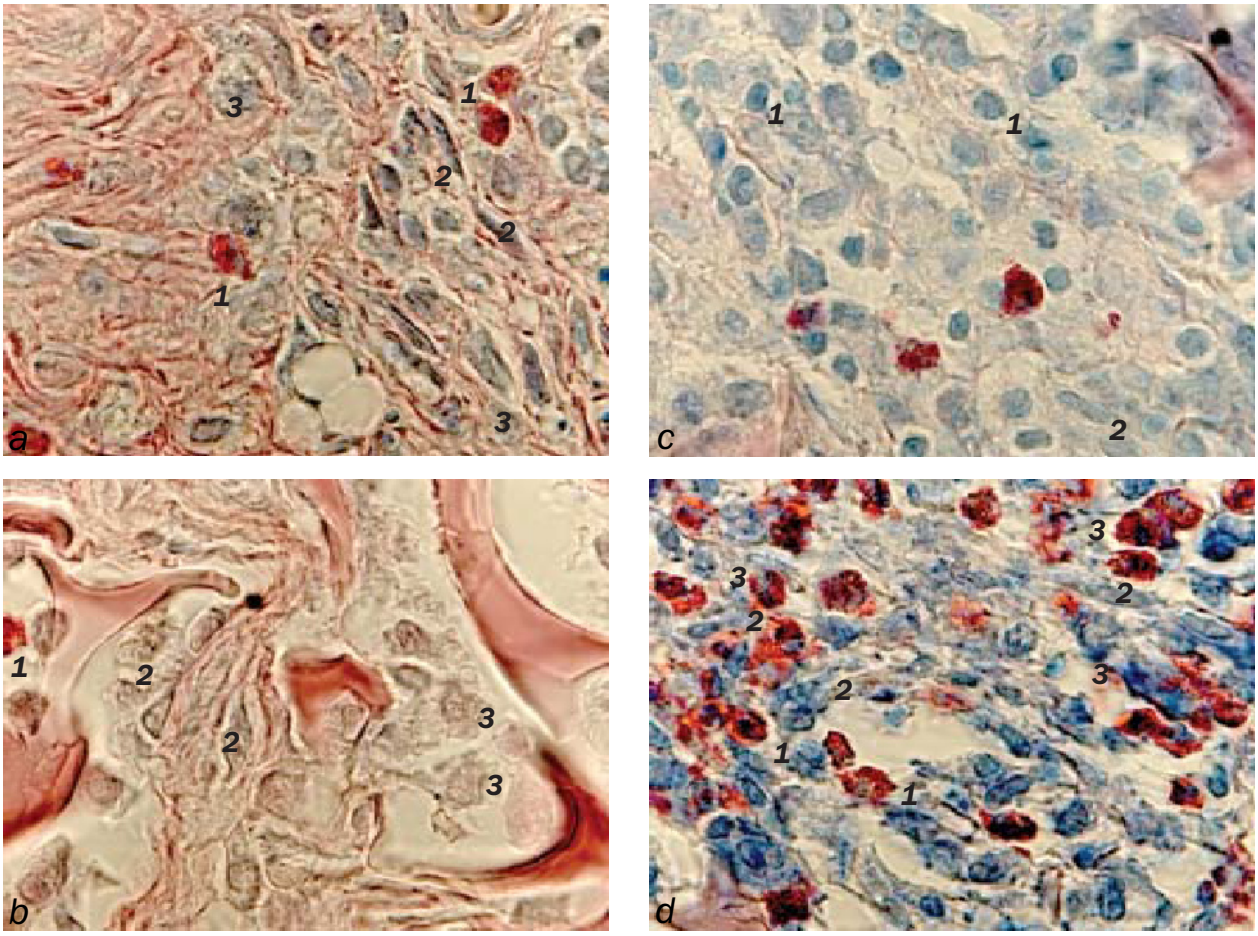

Fig. 2. Cell composition at the site of transplantation on day 7. *a*) groups with transplantation of autologous cell culture (main group), *b*) allogenic (reference group), c) devitalized (control 2), and matrix alone (control 1). *1*) macrophages, *2*) fibroblast-like cells, *3*) cells of the transplant (immunohistochemical staining detects GFP on serial sections). Romanovskii–Giemsa staining (×100).

two first observation groups corresponded to the area of formed collagen fibers (Fig. 3).

The predominance of macrophages in the control groups at early terms of the experiments is determined by the development of the inflammatory process [7,21]. However, MMSC exhibit pronounced immunosuppressor properties, which also can affect the number of macrophages in the main and reference groups.

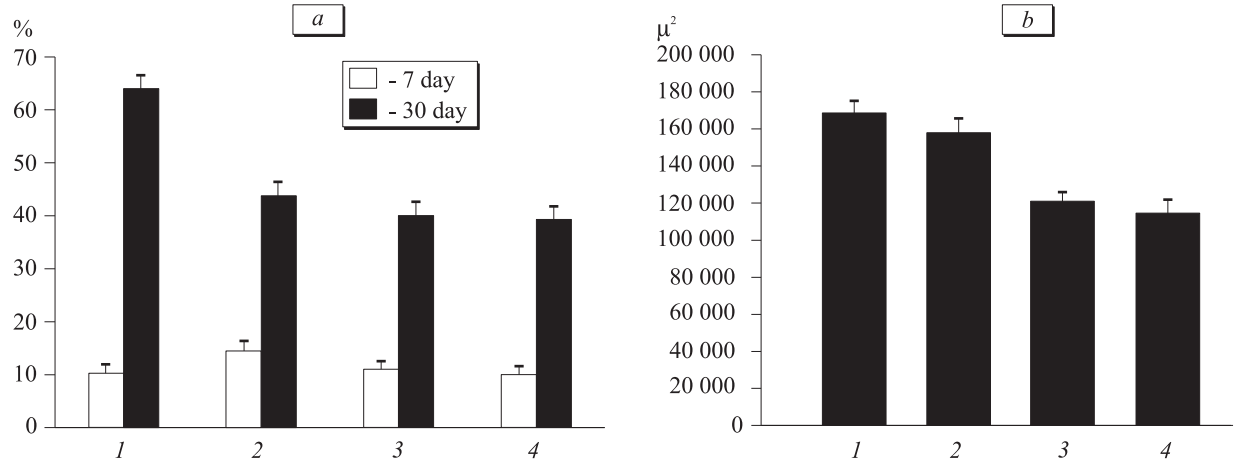

Fig. 3. Volume density of fibroblasts (*a*) and collagen fibers (*b*) after injection of TEC containing cell cultures and matrix alone. *1*) main group; *2*) reference group; *3*) control 1; *4*) control 2.

TABLE 1. Morphological Parameters of Volume Density of Infiltration Cells after Injection of TEC Containing Cell Cultures and Matrix on Day 7 of the Experiment (%)

Parameter	Groups			
	main	reference	control	
			1	2
Fibroblasts and fibrocytes	10.25	14.50	11.00	10.00
Macrophages	21.75	17.75	82.25	80.25

On the whole, the number of fibroblasts and fibrocytes corresponded to the area of the formed collagen fibers: on day 30, the amount of collagen fibers was maximum in the main group (injection of autologous cells culture), intermediate in the reference group (injection of allogenic cell culture), and minimum in both control groups. In control groups we found the matrix and a considerable number of macrophages, some cells were in the state of incomplete phagocytosis. In the control groups, the volume density of all macrophages was similar and on days 7 and 30 after surgery significantly surpassed that in groups with transplantation of cell cultures.

Thus, the intensity of cell infiltration on day 7 was similar in two control groups and was higher in the main and reference groups at the expense of transplanted cells.

In the main group, the matrix was found in only few cases at all terms of the study (Fig. 4). On day 7 of the experiment, the content of matrix was considerable in the control groups (maximum in group 1) and somewhat lower in the reference group. On day 14, the matrix was found only in the control groups, while on days 30 and 60 its content was negligible in all groups. In the main and reference groups, the matrix of the construct was rapidly eliminated, while in the control groups it was sometimes detected even on day 60.

The percent of blood vessels in all groups did not exceed 4% at all terms of the study, but in the main and reference groups it significantly surpassed the corresponding parameter in the control groups.

The mean area of collagen fibers was higher in the main and reference groups at all terms of the study (Fig. 5); on days 7 and 14, this parameter in the main group was higher than in the reference group. In the control groups, the area of collagen fibers was similar and was lower than in the main and reference groups at all terms of the experiment.

Thus, the connective tissue formed more rapidly and contained more collagen fibers in groups receiving TEC with cell cultures.

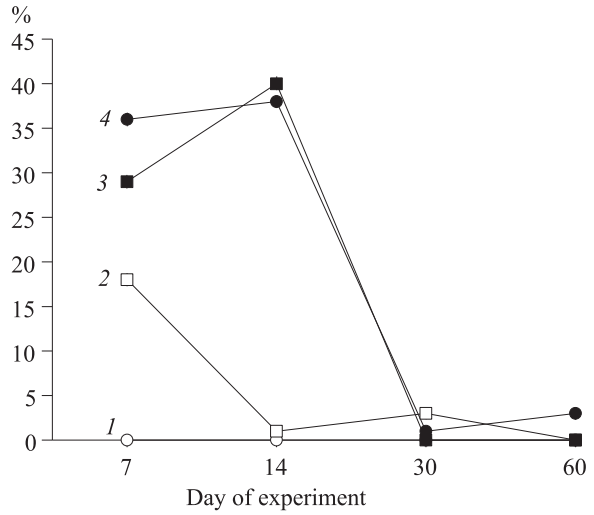

Fig. 4. Changes in volume density of the transplanted matrix after injection of TEC containing autologous (*1*, main group), allogenic (*2*, reference group), and devitalized (*3*, control 2) cell cultures and matrix alone (*4*, control 1) at different terms of the experiment.

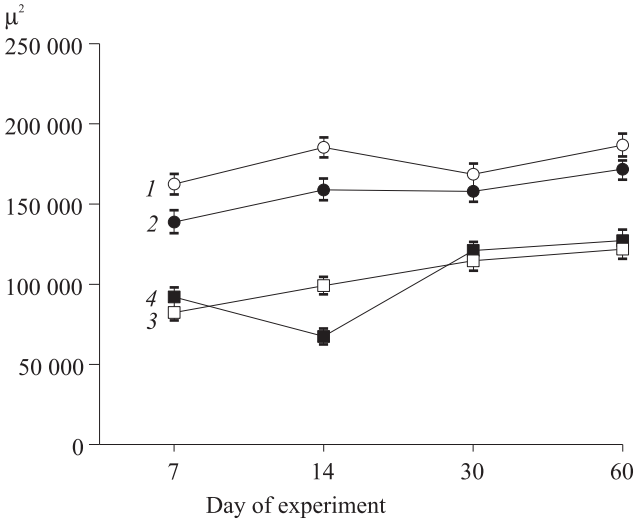

Fig. 5. Changes in the area of collagen fibers after injection of TEC containing autologous (*1*, main group), allogenic (*2*, reference group), and devitalized (*3*, control 2) cell cultures and matrix alone (*4*, control 1) at different terms of the experiment.

The formation of the connective tissue at the site of transplantation can attests to orthology of the regeneration processes and on the influence of microenvironment on differentiation of stromal cells.

TEC on the basis of gelatin sponge were previously used for creation of skin tissue equivalents; intensive formation of collagen fibers in the area of transplantation of cell cultures was demonstrated [20]. Some authors reported that MMSC isolated from the adipose tissue more intensively synthesize collagen fibers than cell cultures isolated from other sources [17,19].

We also observed more pronounced formation of collagen fibers by MSC, especially, by autologous MSC, and found a direct correlation between the presence of transplanted cells and the number of fibroblasts and fibrocytes at the site of transplantation and the area of formed collagen fibers.

The advantage of TEC over unbound suspension cultures consists in the presence of optimum microenvironment promoting cell survival and preventing their migration into surrounding tissues. These effects and the presence of viable cell cultures in the construct provide long-lasting and local production of the connective tissue and collagen.

Morphological study showed that TEC containing viable culture of SFAT cells ensure considerable formation of collagen at the site of transplantation at all terms of the experiment. Transplantation of TEC containing autologous cell culture is most effective. Rapid degradation of the matrix, pronounced infiltration at the site of injection, and greater area of collagen fibers were noted in this case.

Immunohistochemical study revealed transplanted cells labeled with GFP at late terms of the experiment.

In our study, TEC containing devitalized cell culture was used as the additional control. However, comparative study revealed long persistence of the transplanted matrix and insufficient cell infiltration and collagen formation at the site of transplantation little differing from those in the group receiving matrix alone (without cell culture).

Thus, we demonstrated efficiency of TEC containing culture of autologous SFAT cells for local formation of the connective tissue. These advantages of the proposed TEC open prospects for successful treatment of connective tissue defects in some pathological states.

REFERENCES

1. G. G. Avtandilov, *Medical Morphometry* [in Russian] Moscow (1990).
2. V. I. Kulakov, L. V. Adamyan, and B. E. Sashin, *Endoscopy in Diagnostics, Treatment, and Monitoring of Women's Diseases* [in Russian], Moscow (2000), pp. 592-621.
3. L. K. Carr, D. Steele, S. Steele, *et al., Int. Urogynecol. J. Pelvic. Floor. Dysfunct.*, **19**, No. 6, 881-883 (2008).
4. V. Fano, M. Shatel, and M. L. Tanzi, *Acta Biomed.*, **78**, No. 3, 190-197 (2007).
5. M. Goldberg, *Clin. Oral. Investig.*, **12**, No. 1, 1-8 (2008).
6. C. D. Gregory and A. Devitt, *Immunology*, **113**, 1-14 (2004).
7. R. J. Hirsch and M. Stier *J. Drugs Dermatol.*, **7**, No. 9, 841-845 (2008).
8. L. Hong, I. A. Peptan, A. Colpan, and J. L. Daw, *Cells Tissues Organs.*, **183**, No. 3, 133-140 (2006).
9. S. H. Hsu and C. H. Lin, *Biorheology*, **44**, No. 1, 17-28 (2007).
10. L. C. U. Junqueira, G. Bignolas, and R. Bretani, *Histochem. J.*, **11**, 447-455 (1979).
11. J. B. Mitchell, K. McIntosh, S. Zvonic, *et al.*, *Stem Cells*, **24**, No. 2, 376-385 (2006).
12. M. Mitterberger, R. Marksteiner, E. Margreiter, *et al., BJU Int.*, **100**, No. 5, 1081-1085 (2007).
13. S. J. Peter, A. J. Domb, J. Kost, D. M. Wiseman, *Handbook of Biodegradable Polymers*, Harwood (1997), pp. 87-98.
14. R. Pickard, J. Reaper, L. Wyness, *et al., Cochrane Database Syst. Rev.*, **18**, No. 2 (2007).
15. C. Roques, E. Fattal, and Y. Fromes, *J. Gene Med.*, 11, No. 3, 240-249 (2009).
16. E. V. Shvetsova, O. S. Rogovaia, S. B. Tkachenko, *et al., Izv. Akad. Nauk. Ser. Biol.*, No 2, 169-173 (2008).
17. H. W. Sung, D. M. Huang, W. H. Chang, *et al.*, *J. Biomed. Mater. Res.,* 46, No. 4, 520-530 (1999).
18. W. Wagner, F. Wein, and A. Seckinger, *Exp. Hematol.*, 33, 1402-1416 (2005).
19. H. J. Wang, J. Pieper, R. Schotel, *et al.*, *Tissue Eng.*, 10, 1054-1064 (2004).
20. T. A. Wynn, *J. Clin. Invest.*, 117, No. 3, 524-529 (2007).