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The application of a novel biomaterial based on the secreted products of human mesenchymal stem cells and collagen for spermatogenesis restoration in the model of experimental cryptorchidism.

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ABSTRACT

Male infertility is a widespread problem, and there is lack of effective methods for its treatment. The authors have developed a novel biomaterial for the stimulation of regenerative processes based on the secreted products of human adipose-derived mesenchymal stem/stromal cells (MSC). MSC conditioned medium (MSC-CM), containing bioactive factors produced by the cells, has a great regenerative potential, stimulates vascularization and innervation recovery of the tissue after injury, contributes to the activation of endogenous repair processes due to the additional attraction of stem and progenitor cells to the damaged area. This study investigated the ability of a novel biomaterial, consisted of MSC-CM combined with type I collagen, to stimulate spermatogenesis restoration. Experimental cryptorchidism (2 weeks) was used as a model of infertility. The results of this study clearly show that the administration of MSC suspension or MSC-CM combined with a collagen gel under the testis tunica albuginea effectively stimulates the recovery of spermatogenesis. It was found that biomaterial, containing concentrated MSC-CM, attenuated the hypotrophy of cryptorchid testes and stimulated the recovery of spermatogenesis by enhancing both the total number of spermatozoa and their motile fraction. Using this type of biomaterial, the authors observed 3-times more prominent effect on spermatogenesis compared to non-concentrated MSC conditioned medium. Importantly, this effect was comparable with the results obtained for local administration of MSCs themselves. Use of MSC-CM, containing MSC secretions, including specific growth factors to maintain vitality of spermatogonial stem cells (GDNF, FGF-2, etc.), in combination with collagen gel may be a therapeutic advantage to stimulate spermatogenesis and treat male infertility. This approach is considered to be even more preferable than administration of the cells themselves, due to the various clinical and ethical problems associated with transplantation of cellular material.

Keywords: infertility, mesenchymal stromal cells, conditioned medium, growth factors, spermatogenesis.

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INTRODUCTION

Couple's infertility is a serious social and medical issue nowadays. According to statistics, every sixths couple are infertile and every fifths couple have trouble conceiving, while most cases relate to male infertility. Due to multi-factor nature of this issue and lack of effective drugs make relative search for new means to stimulate spermatogenesis restoration.

Publications describe cases, where an experimental therapy for disturbed spermatogenesis was used and it involved introduction of cell cultures, which were enriched with stem and progenitor cells (1, 2, 3). Most research gave positive outcomes in animal models, and research dedicated to individual cases of clinical approbation is less frequent (3). Despite the fact that various kinds of therapy (xenogeneic, allogenic or autologous therapy) for spermatogenesis abnormalities produce a stable clinical effect, and researchers described marker control systems (markers of stemness, pre-differentiation, differentiation, proliferation etc.) for key phases of the physiological cycle of spermatogonial stem cells (SSCs), this attitude associates with a variety of risks, including possible neoplasia. At the same time, bioactive components proved to be a primary mechanism of actual therapeutic effects for stem and progenitor cells of many types. Therefore, a promising approach is development of new drugs and biomaterials that use stem cell secretions, in particular growth factors, cytokines, chemokines and other components, and it can also help with male infertility (4, 5).

Relevance is also given to search for optimum bio-compatible carrier to transfer and gradually release the above factors, which facilitates attraction of the necessary cells into the key target organ (6). Collagen is one of the most promising bio-carriers of that type (7, 8). Advantages of collagen include freedom from toxic and carcinogenic properties, low antigenicity, resistance to tissue enzymes, adjustable rate of lysis in the body, capability of complexing with biologically active substances and prolonging activity of such substances at the place of application, and stimulating regeneration of body's own tissues (9).

Increased production of any growth factor is most often not enough to restore the damaged tissue efficiently: the task requires a combination of several factors. Mesenchymal stem/stromal cells (MSCs), which play the crucial role in regulating body reparation and regeneration processes, appear to be a promising source of a balanced combination of bioactive factors to stimulate incarnation (3; 10). A conditioned medium was discovered, which contains MSC secretions derived from the adipose tissue, to exhibit a great regenerative potential, stimulate recovered blood flow and tissue innervation, following any damage, and facilitate activation of endogenous reparation processes, by additionally attracting stem and progenitor cells to the damaged area, and speed up healing of burning or mechanical wounds - the fact was proved *in vitro* and *in vivo*. Here, possible use of the combined conditioned medium was studied that contained human MSC secretions and type I collagen to stimulate spermatogenesis.

MATERIALS AND METHODS

Subjects/Materials. This research used 40 sexually mature male Wistar rates at the age of 3.5 to 4.0 months and of standard weight. All the experiments were carried out in accordance with the Declaration of Helsinki given by World Medical Association.

The bilateral abdominal cryptorchism was simulated.

Methods. Abdominal cryptorchism simulation technique.

The testes were pulled out from the scrotum into the abdominal cavity through the inguinal canal (which was easy thanks to the wide canal lumen in rats) and fixed to the abdominal wall in the area of lateral canals with an interrupted stitch, using an atraumatic Prolene 4/0 suture and passing it through the distal testis pole to prevent any possible block of communication between the seminal duct and epididymis. At the same time, the authors were careful to avoid stitching the different duct and blood vessels. In 2 weeks, testes were brought down to the scrotum by removing the fixating ligature and flushing testes into the inguinal canal. Apparent, however potentially reversible spermatogenesis abnormalities were demonstrated for this period of keeping testes in the abdominal cavity (11). At the same time, no treatment was provided in the control set, while the perididymis was punctured and a collagen gel was administered that contained biological

preparations to stimulate spermatogenesis or components of such preparations, using an insulin syringe, in the experimental set, before bringing the testes down.

Sets of experiments.

In total 6 sets of experiments were performed:

In set 1, testes were brought down to the scrotum, after keeping them in the abdominal cavity for 2 weeks, and administering no treatment.

In set 2, sub-perididymal administration of 0.1 ml of collagen gel with 50% conditioned medium was performed, and this medium contained human adipose tissue MSC (AT MSC) secretions, before bringing the testes down (CM dose 1).

In set 3, the same amount of gel was administered that contained concentrated conditioned medium of AT MSCs, before bringing the testes down to discover any possible therapeutic effect boost due to higher concentrations of regeneration stimulations factors that are secreted by MSCs (CM dose 2).

In the set 4, 0.1 ml of a mixture of collagen gel and standard low-glucose DMEM (DMEM-LG) were administered into the testes, which served as the basis for conditioning and suspending the AT MSCs, before mixing them with the collagen gel. This set was the control set.

In set 5, collagen gel that contained AT MSC culture (250k cl.) was administered before bringing the testes down. This set of experiments aimed at comparing the extent of biologic effect of AT MSCs alone and their secretions.

In set 6, exclusive isolated AT MSC culture (no collagen base) (250k cl.) was administered to determine how the collagen matrix affects the biologic effect of the cell therapy.

Isolation and culture of human adipose-derived MSC.

MSCs were isolated from the subcutaneous fat depots of healthy donors of both sexes, which were obtained via a minor surgery under a local anesthesia or during scheduled surgeries. Their biomaterial was chopped with vessel scissors in a sterile laminar flow unit to obtain a suspension of fine particles (not more than 2 mm³ in size) and mixed with enzyme solutions of type 1 collagenase (200 IU/mL, Worthington Biochemical, USA) and dispase (40 IU/mL, Sigma, Germany) with tissue volume (mL) and enzyme solution volume (mL) in the ratio of 1:2. The sample was incubated at 37°C for 30-45 minutes with continuous stirring. Following the incubation, an equal amount of the MSC growth medium was added and centrifuged at 200 g for 8 minutes. A white superficial layer of mature adipocytes and bits of tissue that did not get treated with the enzymes by means of a vacuum pump were removed and the residual matter was suspended, which contained adipose stromal cells and vessel wall and blood cells, in a sterile deionized water to lyse RBCs. To restore the osmotic pressure, a certain amount of 10-fold phosphate buffer was added and filtered through nylon filters with pore size of 100 µm (BD FalconCellStrainer, USA) and centrifuged at 200 g for 5 minutes. The supernatant and re-suspended the remaining matter in a medium that supports growth of undifferentiated human mesenchymal progenitor cells (Advance Stem Cell Basal Medium, further – AdvanceSM, HyClone, USA) was removed, which contained 10% growth factors mixture (Advance Stem Cell Growth Supplement, HyClone) and 100 IU/mL of penicillin/streptomycin (HyClone). The isolated cells were put in Petri dishes (Corning, USA) in the concentration of 5x10⁴/cm³ and incubated in a CO₂ – incubator (5% CO₂; 95% air) at 37°C. The medium was changed next day to remove any unattached cells. The medium was changed every 2-3 days; after reaching 70-80% confluent, the cells were transplanted in the ratio of 1:3, using QTase (HyClone) solution. The cell vitality was evaluated by staining the cells with a trypan blue solution and counting live and dead cells, using a cell counter (Cell Counter, Invitrogen, USA).

Some AT MSC passage 4 was detached from the plastic surface, using the QTase (HyClone) solution, to be further administered in rats, then counted and dissolved in the DMEM-LG medium (HyClone, USA) in the concentration of 250k cl. per 100 mL of the medium and mixed with 2.5% bovine collagen gel (Viscoll, Imtek,

Russia) in the ratio of 1:1 by volume. Their cell suspension or mixture of cells and 0.5% collagen gel (1:1) were administered in corresponding experimental groups.

Production of conditioned medium that contains human AT MSC secreted products.

In order to obtain a conditioned medium AT MSC passage 4-5 with 80% confluent were washed three times with the Hanks' solution (PanEco, Russia). The DMEM-LG medium was added to the dishes. The cells were cultivated for 7 days, and then the conditioned medium was collected, purified from the cell debris by centrifuging for 10 minutes at 300g, concentrated 25 times, if necessary, by means of ultrafiltration through membranes made of regenerated cellulose with the given cutoff of 10 kDa in centrifugal cartridges (Millipore, USA).

Preparation of conditioned medium (CM)-based biomaterial that contains human AT MSC secretions and collagen gel.

All procedures were conducted in a laminar flow unit, biological safety level 2, aseptically, while preserving all components at the temperature of 4°C. 50 mL of 2.5% bovine collagen gel (Viscoll, Imtek, Russia) were inserted into sterile 1.5 mL test tubes each. Then, 50 mL of non-concentrated AT MSC CM (for the group CM dose 1) or 30 mL of a fibronectin solution (1 mg/mL, Imtek, Russia) and 20 mL of 25 times concentrated AT MSC CM (for the group CM dose 2) were added to the gel. 50 mL of 2.5% bovine collagen gel were mixed with 30 mL of the fibronectin solution (1 mg/mL) and 20 mL of the DMEM-LG medium for negative control. After adding all necessary components, the test tubes were mixed quickly by vortexing, centrifuged for a minute and a sterile insulin syringe was filled with the mixture. The obtained biomaterial was stored at 4°C to prevent polymerization and used for administration within 30 minutes.

Evaluation methods

The extent of damages developed in the testes was evaluated, after they were kept for 2 weeks in the abdominal cavity, in a month and 3 months, following their bring-down. Then the testes and epididymis were removed and their weight was measured. Also samples of the testicular tissue were collected for histology, which was carried out according to the standard technique by staining histologic sections with hematoxylin and eosin. The extent of impaired spermatogenesis was evaluated by its blockage (the most mature cell forms were determined in seminiferous tubules) as well as a portion of desolated and sclerous seminiferous tubules.

The epididymis were homogenized in 5% glucose solution with the tissue and solution ratio of 1:10 and further the number of actively motile and immotile spermatozoa were determined in the supernatant fluid in the Gorjaev's chamber according to the standard semen analysis procedure.

RESULTS

Decreasing weight (hypotrophy) is a negative process that was observed in cryptorchid testes. According to the research, testis weight reduced from 1.98 ± 0.09 g to 0.82 ± 0.06 g on the average ($p < 0.001$), after keeping the testes in the abdominal cavity for 2 weeks (figure 1).



FIGURE1 A normal testis (on the left) and a cryptorchid testis (on the right).

The weight of these organs practically remained the same in a month, following the bring-down of the control set, and in experiments, where gel and non-concentrated CM (dose 1) and DMEM administered into the testis, while the weight increased up to 1.0-1.1 g on the average in the experiments, where the concentrated CM and MSCs were administered in combination with gel or no gel (figure 2). The weight did not demonstrate any significant growth in 3 months, following the bring-down, in the control set and in the experiments, which involved CM dose 1 and DMEM (the differences with data at the bring-down and a month after are statistically insignificant), while the weight continued its significant growth, following administration of the gel and concentrated CM and MSCs, and exceeded values of other experiments ($p < 0.05$). The unexpected results were obtained in the set, where the gel + MSCs were used and where a decrease in organ weight was observed in 3 months.

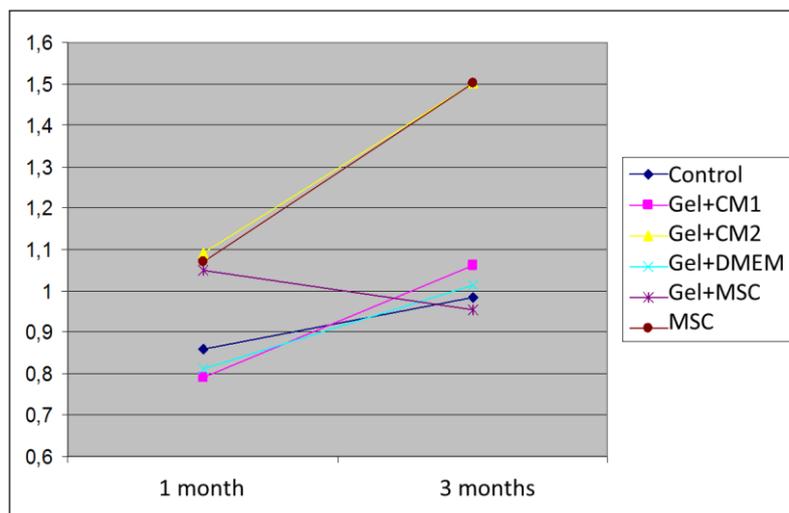


FIGURE2. Weight dynamics of cryptorchid testes in different sets after they were brought down into the scrotum.

The explanation to this fact was found when the histologic analysis of testes was performed in this set of experiments. Connective tissue granuloma was revealed in all specimens in the group that was watched for 3 months, and this granuloma took up a great portion of the organ and contained an aggregate of histiocytes that surrounded the central basophilic zone (Figure 3). A massive connective tissue capsule formed around the granuloma, and pronounced atrophy of the adjacent seminiferous tubules was observed. In other words, reduced organ weight was most likely associated with replacement of the parenchyma by the connective tissue and caused by the pressure exerted by the growing granuloma on the adjacent seminiferous tubules.

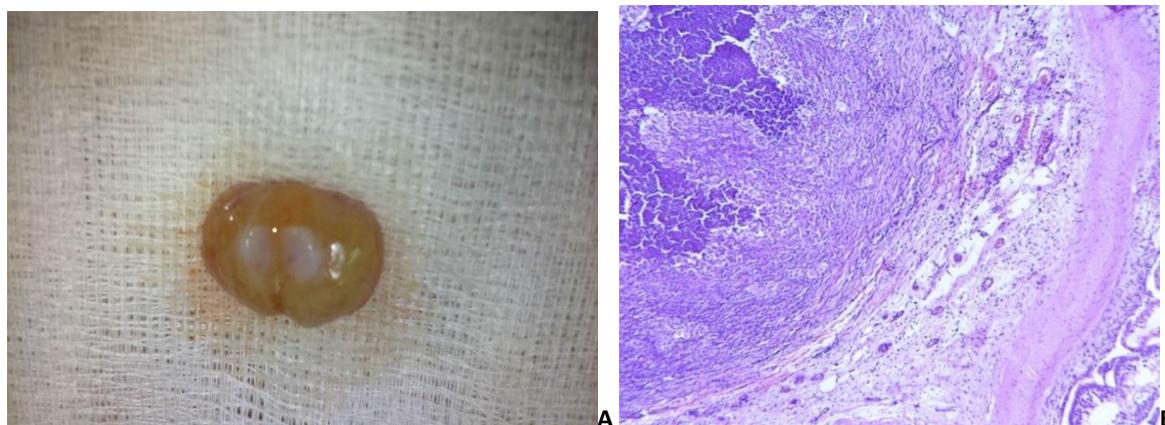


FIGURE3 Histiocytic granuloma in the tissue of a cryptorchid testis 3 months after administration of collagen gel in combination with AT MSCs and bringing the testis down into the scrotum. A – appearance, B – histologic pattern (haematoxylin and eosin, x200).

A month after eradication of the bilateral abdominal cryptorchism, strongly pronounced abnormalities were determined in the histologic analysis of the spermatogenesis state involving spermatozoa maturation blockage already at earlier stages in the control set (untreated animals). Thus, even dark spermatogonia A (primary progenitors of spermatozoa) were found in as much as 60% of the cases, and more mature spermatogonia B and primary spermatocytes just in 20% of the specimens. More mature forms (secondary spermatocytes, spermatozoa) were lacking in all cases (table 1). In other words, these animals experienced spermatogenesis blockage at the level of spermatogonia or primary spermatocytes. Slight improvement of spermatogenesis was observed in the sets of experiments, while administering gel that contained non-concentrated AT MSC CM, dark spermatogonia A were found in all specimens, whereas mature cells were also rare or were absent at all. The significant improvement of testis state was observed in the sets, when using collagen gel in combination with conditioned MSC CM or gel in combination with MSCs, and also when administering gel-free MSC culture into the testis. Complete spermatogenesis (up to the stage of spermatozoa) was found in these experiments or in some experiments (set 3), or in all experiments (set 5 and 6). Some spermatogenesis improvement was also observed in the sets, when administering DMEM medium (set 4), however, it was not that prominent. Spermatogenesis blockage happened at the level of primary or secondary spermatocytes in this set of experiments.

TABLE 1. Frequency of different spermatozoa maturation stages in cryptorchid testes a month after bringing them down (number of manifesting animals / number of animals in the group).

Set	Spermatogonia A light	Spermatogonia B dark	Spermatogonia B	Spermatocytes primary	Spermatocytes secondary	Spermatozoa
Control (set 1)	5/5	3/5	1/5	1/5	0	0
CM dose 1 (set 2)	8/8	8/8	2/8	0	0	0
CM dose 2 (set 3)	6/6	6/6	6/6	6/6	5/6	2/6
DMEM-LG (set 4)	6/6	6/6	6/6	3/6	1/6	0
Gel + MSCs (set 5)	6/6	6/6	6/6	6/6	6/6	6/6
MSCs (set 6)	6/6	6/6	6/6	6/6	6/6	6/6

Quantitative analysis of distribution of spermatogenic epithelial cells of different types (table 2) revealed an extreme depletion of cells of all types in the control set, as well as in the experiments, where non-concentrated CM (dose 1) was administered (figure 4A). Cell population was restored essentially in all other sets of experiments, in terms of stem and progenitor cells (spermatogonia A), and more differentiated cell forms, up to mature spermatozoa (figure 4B).

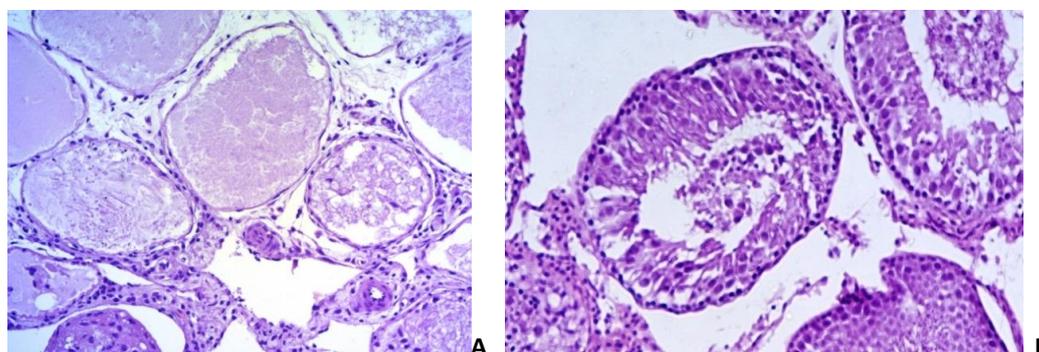


FIGURE 4. A - Atrophic tubules in cryptorchid testes a month after bringing them down (control set) B - restored spermatogenic epithelium a month after bringing the testis down, when AT MSC CM was administered into the testis Stained with haematoxylin and eosin, x200 and x400, accordingly.

TABLE 2. Quantitative description of spermatogenic epithelial cell sub-population a month after bringing cryptorchid testes down (cells per 1 μm^2).

Set	Spermatogonia A light	Spermatogonia B dark	Spermatogonia B	Spermatocytes primary	Spermatocytes secondary	Spermatozoa
Control (set 1)	1.62±0.08	1.04±0.06	0.34±0.4	0.37±0.4	0	0
CM dose 1 (set 2)	2.27±0.32	1.43±0.68	0.35±0.3	0	0	0
CM dose 2 (set 3)	23.72±1.12***	24.02±1.37***	20.59±0.81***	18.91±1.38***	10.52±1.04***	2.25±0.43**
DMEM-LG (set 4)	30.23±0.90***	22.92±1.24***	27.52±1.20***	15.17±1.26***	4.2±1.44	0
Gel + MSCs (set 5)	25.67±0.67***	21.65±1.60***	14.58±0.36***	19.77±0.39***	12.81±0.99***	5.53±1.61**
MSCs (set 6)	21.42±1.13***	19.08±1.58***	23.00±1.71***	18.06±2.04***	12.04±0.90***	6.42±0.64***

* - $p < 0,05$, ** - $p < 0,01$, *** - $p < 0,001$ (vs. the control group)

An essential number of tubules were found that lacked the epithelial lining in the control set. The number of empty (atrophic) tubules in this group was 63.24±5.16 tubules per unit of reference area of the specimen. The number of such tubules was approximately the same - 64.26±3.15 in the series of experiments, where DMEM was administered. At the same time, the number of atrophic tubules was significantly lower in the experiments, where gel was administered in combination of CM of varying concentrations, gel in combination with MSCs, or MSCs - 7.28±0.26, 3.18±0.24, 0 and 34.11±0.15 tubules per unit of reference area of the specimen, accordingly.

TABLE 3. Frequency of different spermatozoa maturation stages in cryptorchid testes 3 months after bringing them down (number of manifesting animals / number of animals in the group).

Set	Spermatogonia A light	Spermatogonia B dark	Spermatogonia B	Spermatocytes Primary	Spermatocytes secondary	Spermatozoa
Control (set 1)	6/6	6/6	4/6	2/6	1/6	1/6
CM dose 1 (set 2)	8/8	8/8	7/8	4/8	4/8	0/8
CM dose 2 (set 3)	6/6	6/6	6/6	6/6	6/6	4/6
DMEM-LG (set 4)	6/6	6/6	6/6	4/6	1/6	0/6
Gel + MSCs (set 5)	-	-	-	-	-	-
MSCs (set 6)	6/6	6/6	6/6	6/6	6/6	6/6

The improved spermatogenesis was observed in all sets of experiments 3 months after bringing the cryptorchid testes down. Undifferentiated and poorly differentiated cell forms (light and dark spermatogonia A) were restored almost completely. The spermatogenesis was complete up to the stage of spermatozoa in some specimens in the control set; however, it was blocked at the level of primary and secondary spermatocytes in most cases (table 3). In the series of experiments, where gel was administered in combination with non-concentrated CM and DMEM, the situation did not differ much from the control series. At the same time, complete spermatogenesis was observed in most cases (CM dose 2) or in all cases (MSCs) in the sets of experiments, where concentrated CM (dose 2) and MSCs were administered. The set with administered gel +MSCs was not analyzed due to the fact that a histiocytic granuloma was observed in these experiments, which was another abnormal factor.

Quantitative estimation of different cell populations in seminiferous tubules confirmed restored population of undifferentiated and poorly differentiated cells (table 4). No significant differences were found in the number of light and dark spermatogonia in all sets of experiments. At the same time, the extent of restoration of spermatogonia B population in the control set was significantly less than in the experimental sets. The number of more mature forms was also overall lower in the control set vs. other sets, except for the experiments, where DMEM was administered. Administration of gel in combination with DMEM surely stimulated spermatogenesis vs. the control set to the level of spermatogonia B, while further cell maturation did not speed up with the same rate and did not pose the same statistical significance.

TABLE 4. Quantitative description of spermatogenic epithelial cell sub-population 3 months after bringing cryptorchid testes down (cells per 1 μm^2).

Set	Spermatogonia A light	Spermatogonia B dark	Spermatogonia B	Spermatocytes primary	Spermatocytes secondary	Spermatozoa
Control (set 1)	21.15±1.03	19.74±1.94	15.03±0.86	8.41±2.38	2.54±1.32	2.40±1.08
CM dose 1 (set 2)	19.81±1.80	15.65±1.05	20.59±0.69**	9.16±0.98	9.61±1.94*	0
CM dose 2 (set 3)	23.36±1.49	23.68±1.35	20.51±1.21*	14.81±0.98*	12.17±1.63**	5.14±1.21*
DMEM-LG (set 4)	28.24±1.39	27.95±1.58	27.65±1.32***	10.28±1.25	5.64±1.4	0
Gel + MSCs (set 5)	-	-	-	-	-	-
MSCs (set 6)	21.49±0.54	23.92±1.19	20.28±1.94*	16.17±2.24*	11.49±1.38**	6.98±1.17*

* - $p < 0,05$, ** - $p < 0,01$, *** - $p < 0,001$ (vs. the control group)

Statistical significance of differences is the same as in table 2.

Due to granulomas in specimens from the animals referred to set 5, individual local spermatogenesis foci observed with blockage at varying levels did not affect the clinical pattern, and interpretation of these set data proved difficult.

The number of atrophic tubules did not change essentially in the control set in 3 months vs. 1 month, and it was 75.26±6.17 tubules per unit of reference area of the specimen, the same as with the experiments, where DMEM - 52.12±2.19 was administered. The number of such tubules decreased continuously in the rest of the sets, and it was 0.03±0.01 tubules per unit of reference area of the specimen in the set of gel+CM dose 1, 0.78±0.13 in the set of CM dose 2, and 6.13±0.52 in the set of MSCs.

The effect of the biomaterial on the function of cryptorchid testis was also studied, and for this purpose the number of spermatozoa in the epididymis was determined as well as their motility fraction. According to these results, 61,375 (51,187; 71,562) thous. cells per epididymis were isolated on the average in intact rats, among which 693,750 (53,4375; 853,125) spermatozoa on the average retained their motility after the isolation.

A drastic decrease in total number of spermatozoa, as well as motile spermatozoa by 88-100% was observed a month after brining the testes down in all sets of experiments (figure 5). At the same time, a slight increase in total number of isolated spermatozoa vs. the control set was observed for the isolated spermatozoa in sets 2,3 and 5, while there was no difference in motile cells. The situation was different 3 months after brining the cryptorchid testes down. A significant increase in total number of spermatozoa and motile spermatozoa as well was observed at this time point in sets 2,3,6 with the highest growth obtained in the set, where concentrated CM was administered (set 3).

An increase in total number of spermatozoa was observed in set 2 by 2.5 times on the average (from 2,126 thous. up to 5,259 thous.); at the same time, the motile fraction was 875 thous. spermatozoa on the average in this population, which is 16.7% of total fraction. The use of concentrated CM and growth factors with gel base (set 3) increased total spermatozoa population 3 months after the administration up to 19,745

thous. spermatozoa on the average, while the motile fraction was 36.7% on the average. A significant increase was observed in total number of spermatozoa in the animals that received a MSC culture shot under their perididymis vs. previous month, and it amounted to 9.156 thous. spermatozoa on the average. At the same time, 1,656 thous. spermatozoa referred to the motile fraction (18.1%), which is indicative of serious clinical performance of the MSC culture in restoring spermatogenesis.

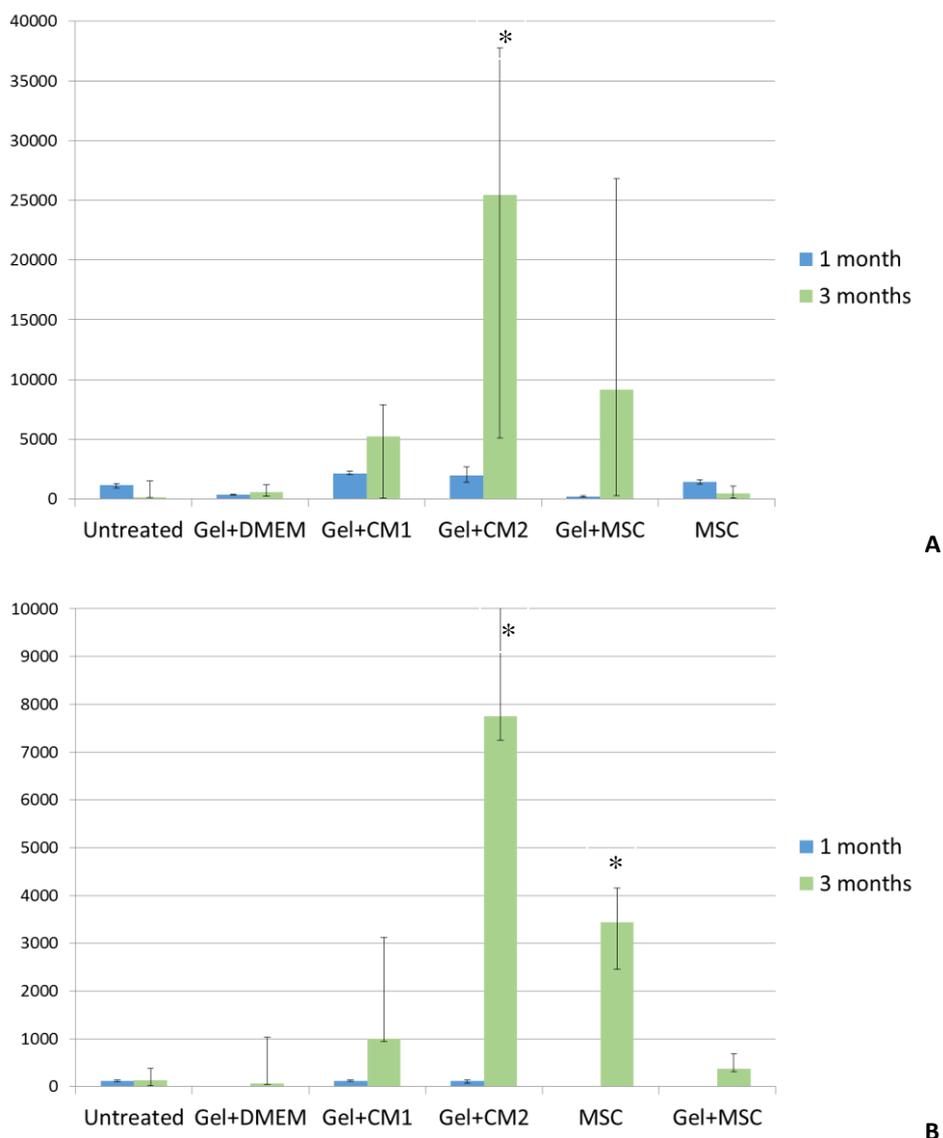


FIGURE 5 Total number of spermatozoa (A) and number of motile spermatozoa (B), which were isolated from epididymes in experimental groups (thous. per epididymis). These data are presented on a median line (25-, 75-percentiles). * - $p < 0,05$ (vs. the control group Gel + DMEM).

DISCUSSION

Experimental infertility simulation in animals leads to permanent impairments that develop for 2 weeks and manifest in a pronounced spermatogenesis blockage, total reduced Sertoli's cell count, infertility, hypodynamia and other phenomena, occurring against an apparent local immune inflammation, which is accompanied by focal and diffuse lymphocyte infiltration, lymphostastis, sclerosis and atrophy of the spermatogenic epithelium of seminiferous tubules. This simulation produces a stable and pronounced clinical effect, which is not easy to overcome. These data correlate with changes obtained by other authors (who reported pronounced spermatogenic disorders in cryptorchism patients with their spermatogenesis blocked in

seminiferous tubules at the level of primary spermatocytes, while most tubules contain exclusively Sertoli's cells) (12).

The use of growth factors to stimulate healing and tissue re-molding regulation is a flourishing field of regenerative medicine. Such factors, as a glial cells derived neurotrophic factor (GDNF), a primary fibroblast growth factor (FGF-2), a leukemia inhibitory factor (LIF) etc. are highly important to SSC vitality (13, 14). For example, SSC differentiation activation requires increased secretion of FGF-2 and GDNF, against inhibited KITLG. Publications on spermatogenesis morphogenesis show that administration of a vascular endothelial growth factor (VEGF165) does not stimulate genesis of seminiferous tubules or blood vessels, while it leads to an increased number of tubules that contain spermatogonia. This fact indicates a protective role of VEGF for SSCs (15).

The research brought into sharp focus that administration under the perididymis of an AT MSC suspension or conditioned medium that contains AT MSC secretions as combined with collagen gel stimulates effectively spermatogenesis restoration processes, using an experimental cryptorchism model. The maximum effect was noticed in month 3 of the observation. These results correlate with their earlier data that demonstrate pronounced spermatogenic cascade activation against experimental treatment of spermatogenic disorders, using a bilateral abdominal cryptorchism model and MSCs, however, fertility should be measured in these animals no sooner than on day 70 of the observation (16). A significant exceedance of secondary spermatocyte count in control groups (untreated animals and animals treated with gel+DMEM) seems interesting against administration of non-concentrated AT MSC CM, despite blockage at this level, which correlates with data for concentrated AT MSC CM and cells themselves. This may be indicative of non-concentrated AT MSC CM being potentially capable of further discovering final sperm forms, while reparation periods may be significantly prolonged. Therefore, AT MSC CM produces a pronounced and clinically relevant stimulating effect on spermatogenesis, with clinical response enhancing along with increasing concentration.

Human MSC secretome was studied in detail earlier, together with other researches: these cells secrete a variety of growth factors and cytokines, including key factors supporting SSCs, such as GDNF (17), VEGF, FGF-2 etc. (10; 18). Paracrine activity of MSCs likely determines obtained experimental therapy effects on testis function restoration and positive results of this histomorphologic study. Along with growth factors and other soluble molecules, MSCs also secrete different types of extracellular vesicles, that are also capable of penetrating seminiferous tubules and interstitium, when administered into a testis, and interacting with SSCs and supporting cells and transferring proteins and regulatory RNAs as well as other bioactive components to such cells, thus stimulating spermatogenesis restoration processes. However, the role of extracellular vesicles in spermatogenesis is underexplored, whereas their effect on spermatozoa maturation regulation was demonstrated (19, 20).

It should be mentioned that a specific granuloma was observed in 3 months in 50% of experimental animals, when administering AT MSCs within collagen gel, and that caused replacement of seminiferous tubules against pronounced local ischemia of the tissue (which is most likely associated with the collagen carrier serving as some kind of a cell niche for MSCs (21)) and reparation potential of MSCs focused on granulomatous tissue formation, following spatial orientation of the cells. Most seminiferous tubules were also observed in the rest of the animals of this set with no granuloma present to be characterized in 3 months of observation by a small number of Sertoli's cells and signs of atrophy and sclerosis, which may be indicative of stimulating effect blockage of the disoriented MSC. Therefore, administration of AT MSCs in combination with collagen gel did not lead to any positive changes in 3 months of observation, while the use of MSCs without collagen demonstrated a good and stable clinical effect.

CONCLUSION

The use of a combined biomaterial, based on human MSC secreted products and collagen gel as a depo has a pronounced stimulating prospermatogenic effect, which is manifested in total elevated spermatozoa count, and their restored motile fraction, and is also confirmed with histomorphologic methods, using an experimental cryptorchism model. Importantly, this effect is comparable with the results of local administration of MSCs themselves. The use of the conditioned medium that contains MSC secreted products, including specific growth factors to maintain vitality of SSCs (GDNF, FGF-2 etc.), combined with collagen gel may appear preferential to administration of the cells themselves, when considering a number of clinical and

ethical issues associated with cell material transplantation. A significant increase is highlighted not only in quantitative, but also qualitative composition of spermatogenesis against the experimental therapy, which deems this approach promising for male infertility treatment.

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