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Morphological Changes in Rat Uterine Tissues and Possibility of Spontaneous Labor as a Result of Injection of Multipotent Mesenchymal Stromal Cells against the Background of Hydrometra

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The possibility of pregnancy and labor was evaluated and tissue changes after injection of autologous bone marrow multipotent mesenchymal stromal cells with transfected GFP gene were studied in rats with experimental hydrometra. Injection of stromal cells to the uterine cicatrix increased the number of vessels (vascular walls or their elements) formed *de novo* with participation of injected cells. The animals produced progeny 2 estrous cycles earlier, the percentage of "puerperal" rats in this group was higher, their progeny was more numerous and they had the maximum numbers of little rats. The maternal mortality was lower after injection of stromal cells. Injection of stromal cells led to development of a trend to more rapid reparative processes in the uterus in animals with cicatricial stenosis of its lumen.

Key Words: *uterine cicatrix; hydrometra; autologous bone marrow multipotent mesenchymal stromal cells; angiogenesis*

In a previous study we detected changes in the uterine cicatrix, developing in rats after ligature of the uterine horns (UH) and injection of autologous bone marrow multipotent mesenchymal stromal cells (ASC) with transfected GFP gene. Large groups of blood vessels with blood cells inside were detected after injection of ASC into the cicatrix (formed 2 months after ligature) on the right, but not on the contralateral side. Examination of unstained sections in reflected UV light showed sufficiently bright fluorescence in the vascular endothelium and outer tunic in the UH cicatrix only on the side of ASC injection. Expression of GFP gene in the vascular endothelium and outer tunics indicated that ASC could differentiate in the endothelial and pericytic directions. Hence, injected into the cicatrix, ASC formed blood vessels by differentiating into endotheliocytes and pericytes [2]. This study was carried out on just few observations (6 rats per period of the study); the probability of pregnancy and spontaneous labor in rats with experimental hydrometra after removal of the ligature from UH and injection of ASC in atrophic foci in all layers of the uterus were not studied.

Here we studied the changes in the rat cicatricial and uterine tissues and evaluated the probability of spontaneous labor after UH ligation and subsequent injection of ASC in these sites.

MATERIALS AND METHODS

Inbred WAG female rats (n=180; 180-200 g; 6 months) served as the model animals. All manipulations on animals were carried out under total inhalation ether narcosis in a clean operation room in accordance with Regulations for Studies with the Use of Experimental Animals.

Inferior median laparotomy was carried out under aseptic conditions. UH was mobilized on sterile gauze.

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A catgut ligature was brought under each UH close to the body of the uterus and the horn was ligated. The abdominal cavity was then sutured [2].

Autologous stromal cells were isolated by washing out the bone marrow from the femoral epiphyses of male WAG rats. The resultant cell suspension was explanted in plastic flasks (Nunc); free cells were discarded 48 h after bone marrow explantation. Adherent cells were cultured in α-MEM with 10% fetal calf serum (FCS, BioloT) at 37°C in a CO_2 incubator with 5% CO_2 under conditions of saturated humidity. The medium was replaced with a fresh portion every 3 days. The monolayer culture was inoculated at a density of 1000-5000 cell/cm^2 (depending on the growth characteristics of the FCS used) and subculturing was carried out in standard versene and trypsin solutions.

Passage 2 ASC were transfected with plasmid pEGFP-N1 DNA (Clontech Laboratories Inc.) containing GFP gene. The transfection protocol and methods for evaluating the expression of GFP gene inserted in ASC were described in detail previously [2].

Four hours after transfection the cells were diluted with nontransfected cells in 1:2.5 proportion. Relaparotomy was carried out, fragments of nonlysed suture material were removed, and 100 μl of mixed ASC suspension were injected into the cicatrix which formed 2 months after ligation of both UH. Rats with ligated UH without ASC injection and intact animals served as controls. Twelve rats were examined per period.

Fragments of UH with cicatrices, removed 4 days, 1, 2, and 3 weeks after injection of ASC, were fixed in 4% paraformaldehyde solution in phosphate buffer (pH 7.4) during at least 24 h, dehydrated in ascending ethanols, clarified in xylol, and embedded in histoplast. Unstained sections (5-7 μ) were examined in an AxioImager M1 microscope at magnification of up to 1500 in the fluorescent mode at an Alexa 488 filter.

Three weeks after the ligature was removed from UH and ASC were injected (26 rats) or not (46 rats), the males were placed in cages with the remaining females. The day of delivery and number of newborn rats were recorded. As the duration of pregnancy in these animals was 22 days, they were observed over 10 days after the males were put into cages (3 periods of pregnancy onset and delivery).

The results were evaluated by Student's *t* test. The differences were considered significant at a confidence interval of 95% and higher. The distribution of the studied signs was close to the normal.

RESULTS

Atrophy, sclerosis, and many plethoric vessels were found at the site of ligature 4 days after its removal and ASC injection (Fig. 1, *a*).

Studies of unstained histological sections in reflected UV light showed solitary small vessels, consisting completely from fluorescent cells, in the myometrial cicatrix and adjacent tissues and in the myometrium. Fluorescent cells sometimes formed just part of the vascular wall (Fig. 2, *a*).

Sometimes the fluorescent cells formed annular structures, just slightly resembling blood vessels. The fluorescent cells were oval – while the vascular wall cells are typically elongated. These cells were rather large, up to 7 μ in size; a dark unstained large oval nucleus was clearly seen against the background of bright fluorescent cytoplasm. Fluorescent cells in these structures formed a row or even several layers – a sort of a multilamellar vascular wall consisting of several tunics (Fig. 2, *a*).

Hence, just by day 4 after injection, ASC formed groups in the myometrial cicatrix (presumably, these groups of fluorescent cells formed as a result of proliferation of one cell) and annular structures similar to young vessels: thin single-walled vessels with wide virtually round lumen.

The absence of blood cells in these young vessels could indicate that they were not involved in microcirculation because of their functional and structural deficiency (the walls were not completely formed in places and there was a lumen) or because they were not connected to the existing vessels and capillaries. These vessels were most likely formed in tissues *de novo* and were included in the microcirculatory bed only later.

The uterine tissues remained atrophic 1 week after ASC injection, but manifest signs of edema and numerous small plethoric blood vessels, resembling granulations, emerged (Fig. 1, *b*). Numerous small blood vessels with all tunics consisting of fluorescent cells were seen in the cicatrix and adjacent tissues. The darker nucleus was seen against the background of brightly fluorescing cytoplasm in these cells. Erythrocytes were seen in vascular lumens, that is, the vessels worked. The erythrocyte fluorescence was significantly less intense than the vascular wall fluorescence (Fig. 2, *b*). Importantly that the erythrocytes are characterized by significant autofluorescence [3-5].

The number of vessels in tissues adjacent to the cicatrix increased 2 weeks after injection of ASC, but only at the expense of large and medium-sized vessels. The walls of many vessels were thick and sclerosed. The number of small vessels, looking like granulations, decreased significantly (Fig. 1, *c*). Study of unstained sections in reflected UV light revealed numerous vessels of different diameters with fluorescent cells and structures in the vascular walls. Vascular walls or their individual elements consisted completely from fluorescent cells. The majority of vessels contained different volumes of blood cells, which indicated their

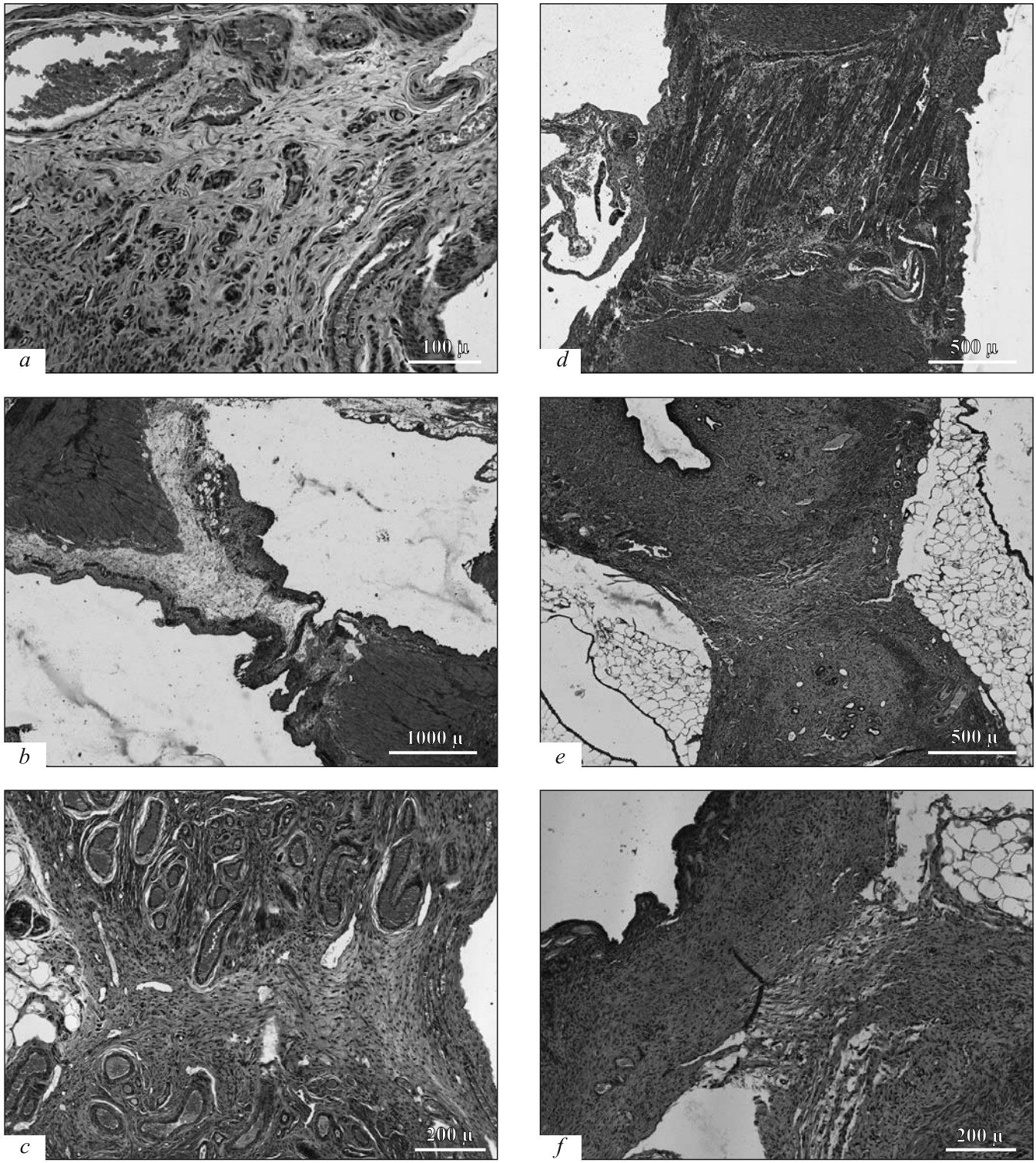

Fig. 1. Morphological changes in the rat UH cicatrix and tissue 4 days (*a*), 1 (*b*), 2 (*c*), and 3 weeks (*d*) after injection of ASC. Hematoxylin and eosin staining. *a*) Numerous thin-walled large and small vessels; *b*) drastic stenosis and degeneration of UH tissues at the site of ligature; *c*) stenosis and degeneration of tissues at the site of ligature, numerous wide plethoric vessels nearby; *d*) sites with myometrial fibrils in UH cicatrix alternate with connective tissue sites; numerous thin-walled plethoric vessels in the same area, many of them twisted; *e*) UH cicatrix and tissues in a control rat after removal of ligature; *f*) intact rat UH lined with endometrium throughout the entire study, the myometrium is even, with few vessels.

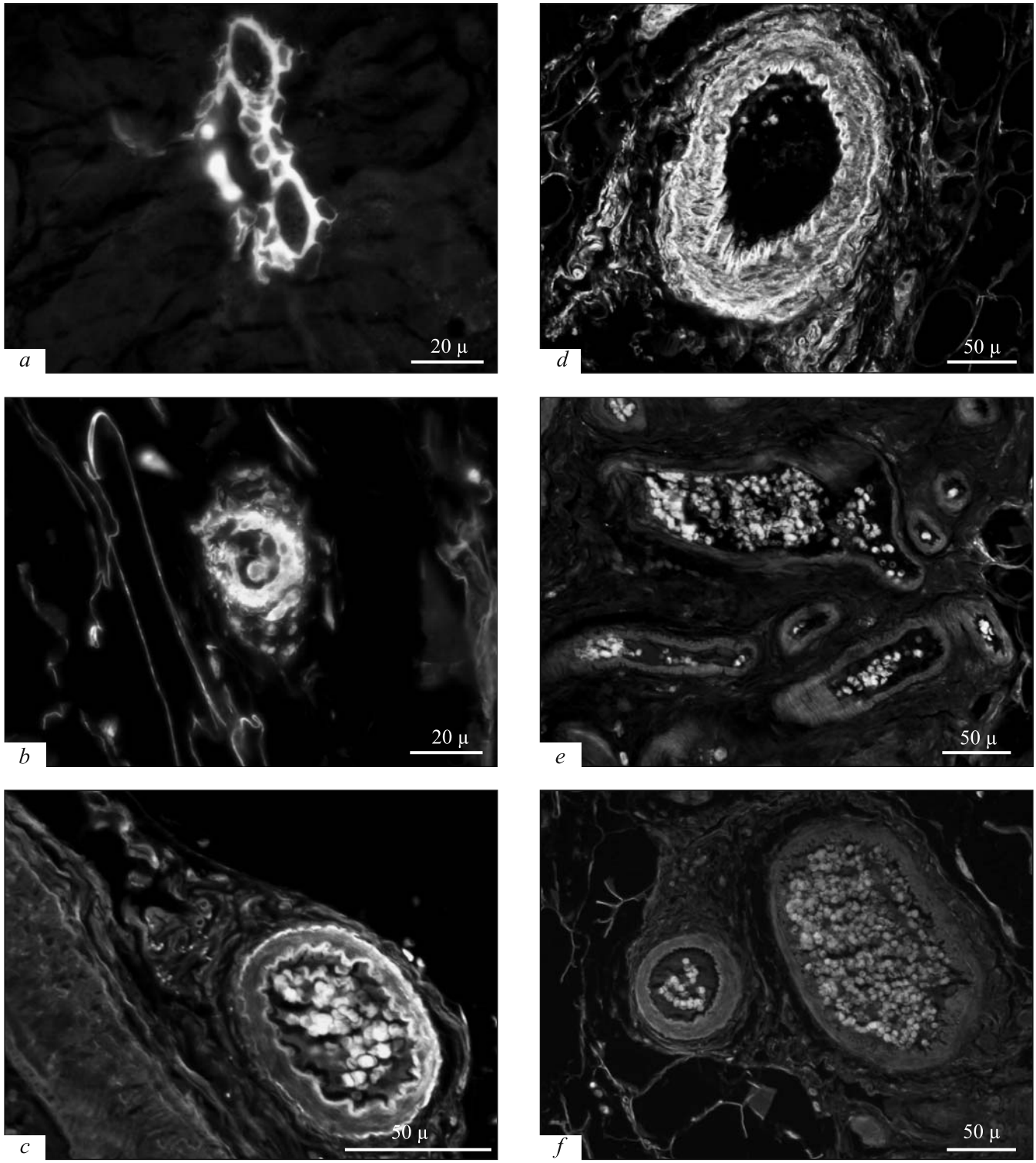

Fig. 2. Angiogenesis in rat UH cicatrices 4 days (*a*), 1 (*b*), 2 (*c*), and 3 weeks (*d*) after ASC injection. Unstained sections in reflected UV light with Alexa 488 filter. *a*) Annular structures resembling vessels with walls from large cells, with dark nuclei against the background of brightly fluorescing cytoplasms; *b*) numerous small blood vessels, all their tunics consisting from fluorescent cells; *c*) large arterial vessels with brightly fluorescing endothelial and adventitial tunics; *d*) fluorescence of all tunics of sclerosed vessels, other structures fluorescing less intensely against the background of brightly fluorescing internal and external tunics; *e*) control rat UH cicatrix after removal of ligature: no specifically fluorescing objects in vascular walls, only erythrocytes fluorescing; *f*) no fluorescent objects except erythrocytes in intact rat UH.

TABLE 1. Deliveries in Rats after ASC Injection and Hydrometra Resolution

Parameter	After ASC injection (*n*=26)	Without ASC (*n*=46)
Number of females with newborns	8 (30.8%)	13 (28.3%)
Date of labor	09.01.2013	16.01.2013
Maximum number of newborns	7	4
Mean number of newborns (*M*±*m*)	3.13±2.23	1.92±1.12
Maternal mortality	2 (7.7%)	4 (8.7%)

full value and active functioning (Fig. 2, *c*). Rather large arterial vessels with fluorescent endothelium and adventitial tunic in sclerosed wall contained brightly fluorescing erythrocytes (Fig. 2, *c*).

The number of vessels in the uterine cicatrix decreased by week 3 after ASC injection. On the other hand, twisted vessels emerged, this presumably indicating their varicose dilatation because of bloodflow resistance (Fig. 1, *d*). The number of vessels with fluorescent walls was the maximum. These vessels were present in the cicatrix, adjacent fat, and in uterine tissues, but the fluorescence intensity decreased significantly, which was best of all seen in large vessels (Fig. 2, *d*).

It is impossible to identify in a discrete experiment the vessels in Figure 2 (fragments *c*, *d*) – whether the vessels are new or existed previously. Hence, in such cases we stated the presence of sufficiently large vessels with fluorescent objects in their tunics, which was confirmed by studies of sections stained with hematoxylin and eosin (Fig. 1, *c*, *d*). Two weeks after ASC injection only the endothelium and adventitium clearly fluoresced in these vessels with sclerosed walls, while by week 3 the whole wall fluoresced, though less intensely. The endothelial lining and adventitial tunic fluoresced brighter than other structures in the vascular wall, but this fluorescence was often indiscernible against the background of other layers' fluorescence (Fig. 2, *d*). This indicated a reduction of the endothelium and adventitial fluorescence.

It seems that because of mobilization of their own pool of bone marrow pluripotent stromal cells, the injected ASC carrying foreign DNA of transfected GFP started to be gradually removed by week 3 from the structures formed with their participation. These ASC were replaced by autologous cells and were destroyed and utilized in some other organs and tissues. However, the vessels initially consisting from injected ASC and then from host's own cells remained in uterine tissues. This promoted improvement of blood supply to atrophic and sclerosed cicatricial tissues and created conditions for the cicatrix reorganization.

Improvement of microcirculation in the cicatrix could promote regeneration of collagen and elastin fibrils with emergence of finer structures and their orderly location [1].

Injection of ASC leads to rapid formation of vessels in the UH cicatrix in the site of injection, while normally the process starts with activation and mobilization of one's own mesenchymal stromal cells, their division and migration to the target body regions, and only then the formation of target structures.

Use of ASC leads to mobilization of stem cells, stimulation of their proliferation and migration; these cells migrate to already formed structures (vessels) and incorporate in their walls with elimination of ASC with foreign DNA and foreign GFP. This saves time needed for activation, production, and migration of a sufficient amount of autologous mesenchymal stromal cells and their differentiation in the endothelial and pericytic directions. Angiogenesis starts much sooner and the number of vessels is higher in response to ASC injection.

The number of vessels in the uterine and cicatricial tissues of controls (animals with hydrometra without ASC injection) and intact rats was significantly less (Fig. 1, *e*, *f*); only erythrocyte autofluorescence [3-5] was detected (Fig. 2, *e*, *f*).

TABLE 2. Date of Delivery, Number of Rats with Newborns, and Number of Newborns after ASC Injection and Hydrometra Resolution

Without ASC		After ASC injection	
date of labor	number of newborns	date of labor	number of newborns
16.01.2013	1	09.01.2013	1
16.01.2013	1	11.01.2013	4
17.01.2013	1	14.01.2013	1
17.01.2013	4	16.01.2013	4
18.01.2013	1	20.01.2013	7
18.01.2013	1	20.01.2013	2
19.01.2013	3	06.02.2013	1
19.01.2013	3	10.02.2013	5
20.01.2013	3		
23.01.2013	1		
23.01.2013	1		
24.01.2013	3		
10.02.2013	2		

Eight (30.8%) of 26 animals had deliveries after ASC injections *vs.* 13 (28.3%) of 46 in the control group. Labor started 7 days earlier after ASC injection than in the control group (Tables 1, 2). The duration of the estrous cycle in rats (4 days) suggested that pregnancy started 7 days or 2 estrous cycles earlier in the group injected with ASC.

Presumably, injection of ASC and more rapid regeneration of UH structures more often led to recovery of patency in one or both UH. As a result, pregnancy involved all UH, and the number of newborn rats was higher (Tables 1, 2). It seems that the cicatrix area shrank as a result of ASC effects on the reparative processes and hence, the area for embryo implantation in each UH increased, which led to delivery of more numerous progeny.

The structure of UH regenerated gradually after ASC injection and without it. A small orifice emerged first, through which the spermatozoa penetrated, and oocyte fertilization became possible even without their migration proximally from the horn. The UH lumen was gradually growing wider and wider, this allowing normal delivery.

Failure of UH to restore for fetus passage as a result of intense myometrial contractions could lead to hysterorrhexis at the site of the cicatrix, by its edge, or in site of the fetus location. This complication (hysterorrhexis in labor) leads to animal death from hemorrhage, peritonitis, or both without specialized care.

Long ineffective contractile activity of the uterus can lead to amniorrhea and intrauterine fetal death because of hypoxia, the infected fetus then becomes the source of infection in the abdominal cavity and in the body, which leads to maternal death.

A lesser maternal mortality – even by just 1% – after ASC injection (Table 1) is a good result indicating more rapid recovery of UH lumen and better strength of the remaining cicatricial tissues capable of enduring full-value labor activity.

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