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DEVELOPMENTAL BIOLOGY

Peculiarities of Development of Mouse Male Germ Cells after Intra-Testicular Injection of Dipin

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Abstract—It has been shown that a single intratesticular injection of the chemical mutagen dipin (experiment) or saline (control) into mice resulted in significant but reversible morphohistological damage of the spermatogenic epithelium. However, unlike the controls, in mutagenized tests these damages were more pronounced. Thus, the process of restoring a normal pattern of spermatogenesis was slower. In addition, on day 35 of fixation, mature gametes were almost completely absent in the cauda epididymis and a large number of sperm cells with abnormal head shape (58.5 versus 1.7% in the controls) appeared in the testes. Using spermatogonial and meiotic micronucleus assay, we found that dipin did not induce a rise in the number of gross chromosomal mutations in the spermatogonial stem cells (SSCs): on days 35, 56, and 100 postinjection, the incidence of aberrant spermatogonia and round spermatids was not significantly different statistically from the saline control. The degree of gametic chromatin decondensation was evaluated after treatment of the cauda epididymal sperm with sodium dodecyl sulfate (SDS) and dithiothreitol (DTT). Judging by the results of the in vitro sperm chromatin decondensation on days 7, 14, 35, 56, and 100 after the injection of dipin or saline, the number of decondensed nuclei decreased sharply in the studied samples as compared with the sperm from intact animals where sperm cells with fully decondensed chromatin prevailed.

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INTRODUCTION

Experimental studies of spermatogenesis in higher organisms occupy an important place in reproductive biology. Studies of the consequences of the impact of genetically and biologically active compounds on the differentiation and hereditary apparatus of male sex cells have been of special interest in recent years.

In our previous reports, data were presented that characterize the dynamics of destructive and restorative processes, which occur in the testicles of animals that have undergone the influence of the chemical mutagens dipin and nitrosomethyl urea (Zakhidov et al., 1994a–c, 1995; Zakhidov, Kulibin, 2006; Kulibin et al., 2008).

The aim of this work consisted in ontogenetic study of the dynamics of spermatogenesis in mice after intratesticular injection of the model mutagen dipin. This experiment imitates the conditions in vitro, in which the distance mutagen–cell is minimized and the chemical agent performs a direct and hardly modified intervention into the organization of the cell system. It is also considered that, in a limited space, the unfolding effect is more strict and accurate. As for the experiments in vivo, in the animal organism, mutagen molecules are absorbed and chaotically distributed in various organs; they are also are actively involved in complicated chains of synthetic metabolic processes, thus causing the formation of a large number of intermediate products, which possess cyto- and genotoxic activity. The total effect of the latter can intensify the pressure on spermatogenesis.

EXPERIMENTAL

Male mice of F_1-hybrids CBA × C57BL/6 2–3 months old were used in the study. The animals, divided into control and experimental groups, were kept in standard conditions of a vivarium and received food and water *ad libitum*.

The experimental animals received a single injection of the chemical mutagen dipin (15 μl) in a concentration of 0.45 mg/ml (dissolved in a saline solution) in the area of the interstitial tissue, while the control individuals underwent a single injection of saline (15 μl). The injections were performed using a glass capillary; for a uniform distribution of the solutions along the gonads, the latter were punctured in four places. The narcotization of the mice involved 400 mg of chloral hydrate per 1 kg of weight of the animal. The control and experimental mice were slaughtered by dislocation of the jugular vertebrae on days 7, 14, 35, 56, and 100 after the start of the experiment (4–6 males from each group per fixation spot). After the dissection, the testicles and epididymides were extracted.

For a morphohistological study of spermatogenesis, one of the pair of testicles was fixed in Bouin solution or in Davidson's modified fixative for four days. After dehydration in alcohol of ascending concentrations and embedding into paraffin blocks, sections were cut 7 μm thick and stained in Caracci hematoxylin and eosin. The sections were examined on a Leica DM RXA2 microscope (Germany).

Imprints of spermatogenic cells were prepared for cytogenetic analysis. The preparations were fixed in 10% neutral formalin for 10 min and stained after Felgen after washing in running water.

The frequency of formation of genetically abnormal spermatogenic cells was determined using the methods of counting spermatogonial and meiotic micronuclei, as well as the test on abnormalities of the form of the sperm cell heads (AFSH). From 300 to 500 spermatogonia (both undifferentiated and differentiating cells were taken into account), no less than 1000 early post-meiotic cells (round spermatids), and no less than 500 testicular sperm cells were counted from each animal. The number of aberrant spermatogonia and round spermatids was expressed per mil, while AFSH was expressed in percent.

The obtained digital data were processed using the SPSS statistical packet. To determine the reliability of differences, the Wilcoxon nonparametric test for the standard 5% level of significance was used.

To estimate the effect of the intratesticular injections of dipin or saline on the formation of the structure of the DNP-complex of sperm cells, which at the moment of influence were at various stages of spermatogenesis, decondensation of chromatin of mature sperm cells in vitro was used. Mice that were not subjected to any influences were used as the intact control. The caudal sections of the epididymides of the experimental, control, and intact mice were placed into a saline solution and reduced to fine fragments, after which a suspension of mature sperm cells was prepared. After centrifugalization (1000 g, 15 min), the supernatant liquid was drained off and the sperm cell sediments were dissolved in 1 ml of a 1% solution of sodium dodecyl sulfate (SDS, Sigma, United States), which is a detergent that destroys the plasmatic membrane. The obtained suspension was incubated at room temperature for 30 min. Then, 0.3 ml of 0.01M of the solution of dithiothreitol (DTT, Sigma) prepared on a *tris*-HCl buffer (pH8) was added, after which incubation was continued at room temperature. After completion of incubation of the sperm cells in the SDS/DTT solution, smears were prepared, which were fixed in 96% ethyl alcohol for 10 min after drying and stained with 0.1% solution of toluidine blue (Fluka, Switzerland). The smears of the stained sperm cells with varying degrees of nuclei decondensation were analyzed using an Opton microscope (Germany) with the general magnification 1000, examining no less than 100 randomly selected fields of vision.

RESULTS

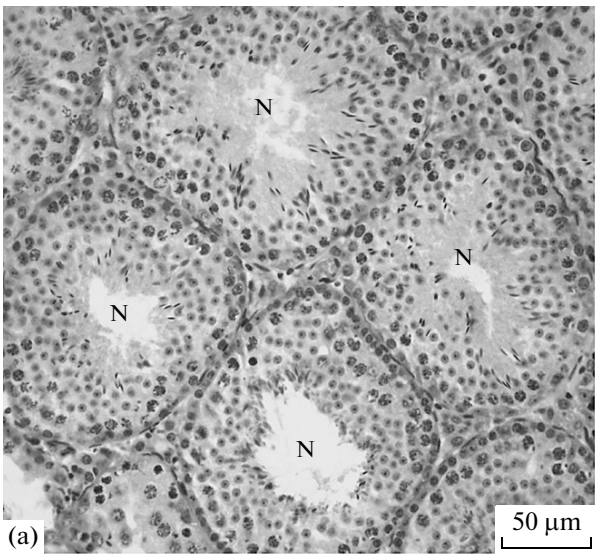
A morphological analysis showed that seven days after the start of the experiment, the seminiferous tubules in the testicles of the control mice, which were injected with saline, were heterogeneous. In most cases, the inner structure of the tubules looked quite normal: various generations of the developing male germ cells were situated in regular, orderly layers around the tubule lumina (Fig. 1a). In other tubules, the structure of the spermatogenic epithelium was noticeably damaged: the lumina in such tubules looked wider, cells with two nuclei appeared, as did spermatids with large nuclei (Figs. 1b, 1c). At the same point of fixation, in the experimental males, after a single injection of the chemical mutagen dipin into the gonads, the pathological changes of spermatogenesis were more pronounced, chaotic, and varying in the overwhelming majority of tubules (Figs. 1e, 1f). In one of the four studied animals, complete degeneration of the spermatogenic tissue was observed in almost all tubules.

Under the influence of the saline or dipin, the largest centers of degenerative changes could be seen distinctly directly in the places where the testes were punctured with the capillary (Fig. 1d).

On day 14 of fixation in the testicles, deep structural changes in the spermatogenic system were registered in three out of five studied control mice (Fig. 2a), in other males the developing sex cells and Sertoli cells looked normal (Fig. 2b). In two out of six experimental males, destructive changes in the structure of the spermatogenic epithelium were also observed, whereas in other mice, unlike the controls, damages of the spermatogenic process characteristic of the influence of dipin were distinctly pronounced: most often postmeiotic cells were found, while meiotic cells were either absent altogether or single (Fig. 2d). In addition, a noticeable increase in the intertubule spaces was noted (Fig. 2c).

On day 35 of fixation, the morphological structure of spermatogenesis in the testes of the control mice again became more complicated and obtained a regular, orderly aspect in the majority of tubules. In some tubules, however, morphologically formed sperm cells were still absent (Fig. 3a). Compared with the controls, in the mutagenized gonads of experimental ani-

Fig. 1. Sections of testicles of (a–d) control and (e and f) experimental mice on day 7 of the experiment. (HBV) Hypertrophied blood vessel, (GRS) gigantic round spermatids, (DC) double-nucleus cell, (DS) double-nucleus round spermatid, (DSE) disorganization of the spermatogenic epithelium, (MB) meiotic block, (N) tubules with a normal structure of the spermatogenic epithelium, (D) desquamation, (NV) nuclear vacuoles, and (*) tubule with reduced spermatogenesis. Staining: hematoxylin-eosin. For Figs. 1–3.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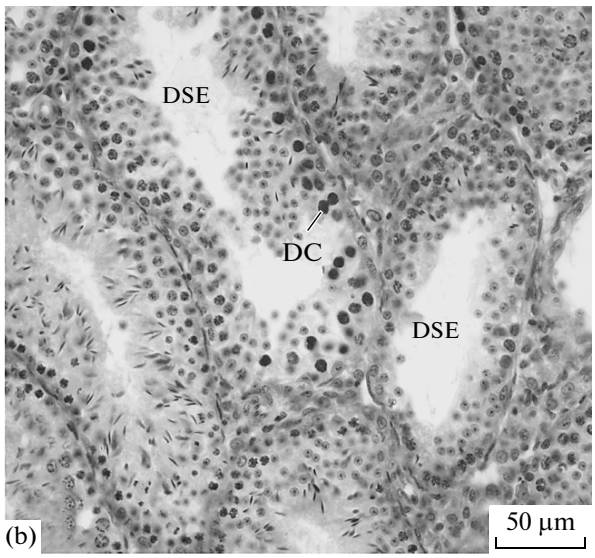
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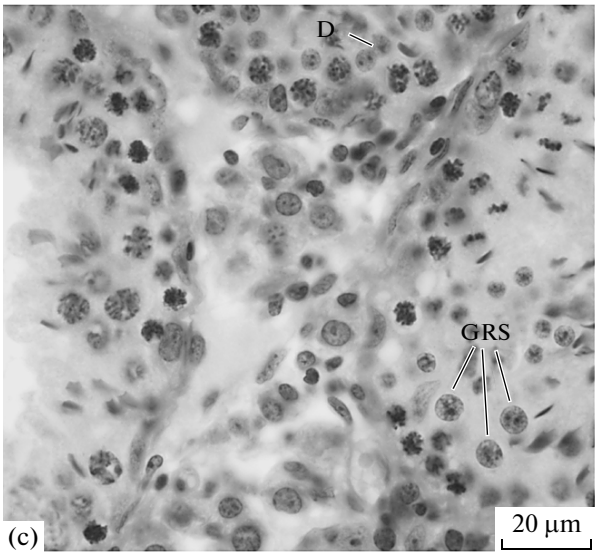
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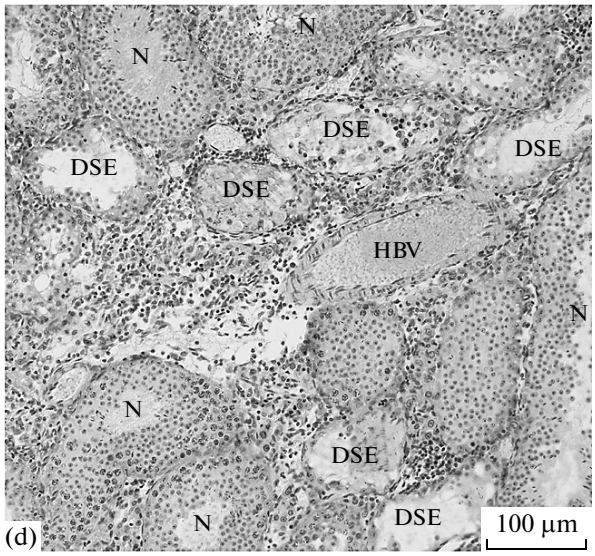
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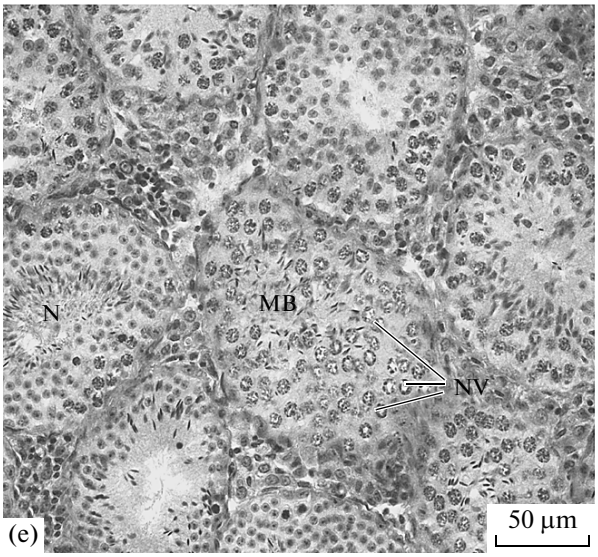
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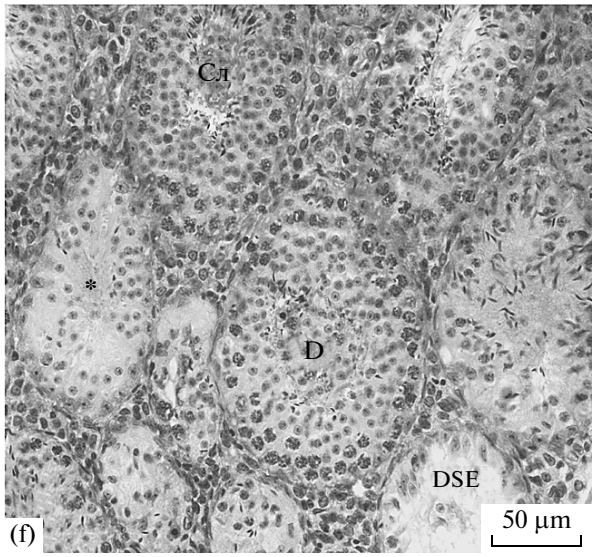
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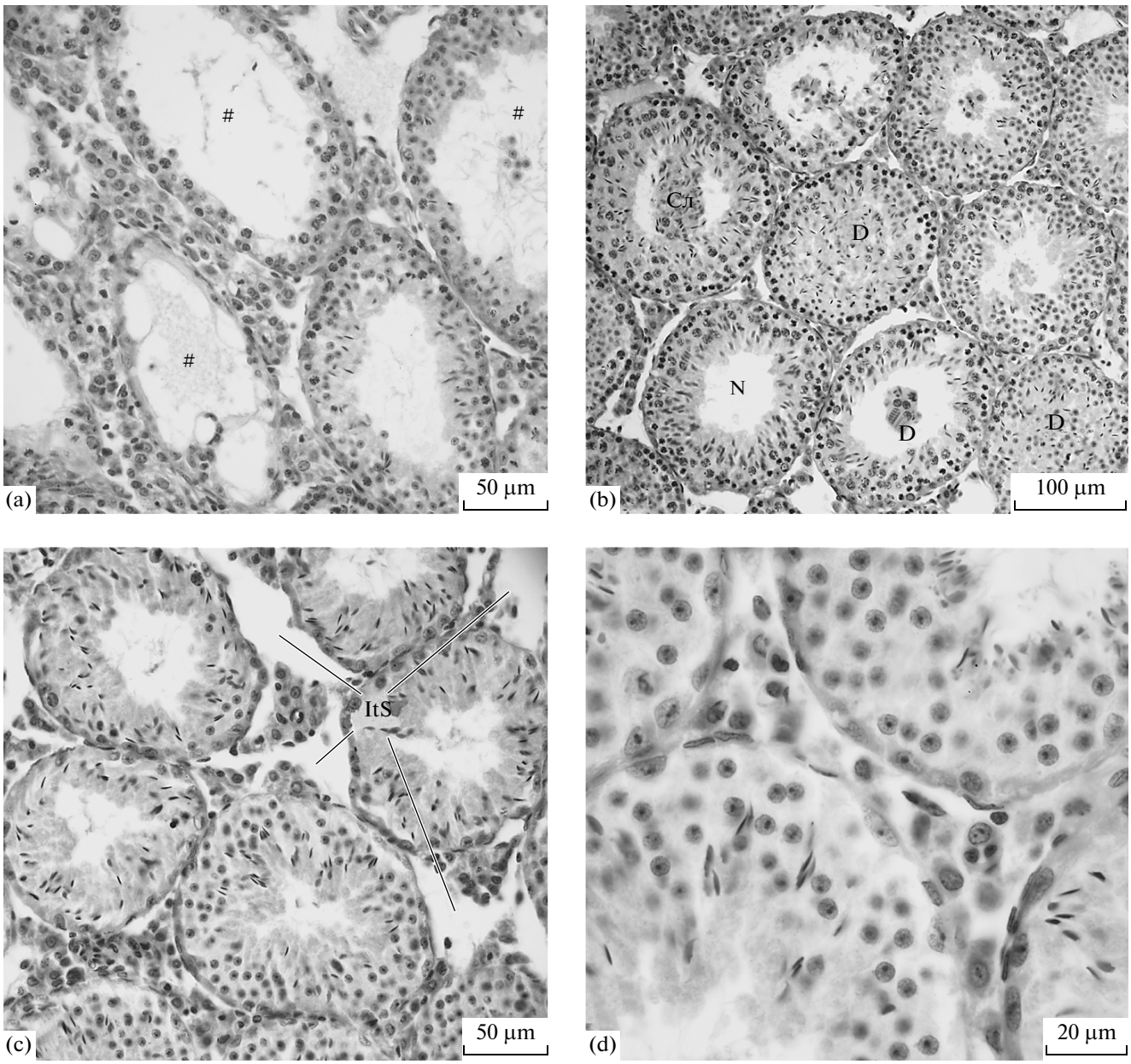

Fig. 2. Sections of testicles of the (a, b) control and (c, d) experimental mice on day 14 of the experiment. (ItS) Inter-tubular spaces, (#) tubules with destructive spermatogenesis.

mals, tubules were often found with various damages and reduced spermatogenesis along with those where the spatial organization of the spermatogenic epithelium was more or less normal (Fig. 3b).

On day 56 of fixation, the structure of the spermatogenic system in the testicles of the control mice was restored in full measure in almost all tubules (Fig. 3c). At the same time, in the experimental animals, within one gonad, besides the tubules with an integral picture of spermatogenesis (Fig. 3D), tubules with a random distribution of differentiating sex cells were found (Fig. 3e).

On day 100 of fixation, tubules with externally normal, orderly, and active spermatogenesis prevailed completely in the testes of both control and experimental mice.

In the cytogenetic experiment, no reliable differences were found between the control and experiment in the frequency of incidence of spermatogonia with micronuclei (Fig. 4a) at any points of fixation (days 35, 56, and 100 of aftereffect). A similar picture was observed in the population of round spermatids as well: the difference in the estimation of frequencies of chromosome mutations during the entire experiment did not exceed the level of significance (Fig. 4b).

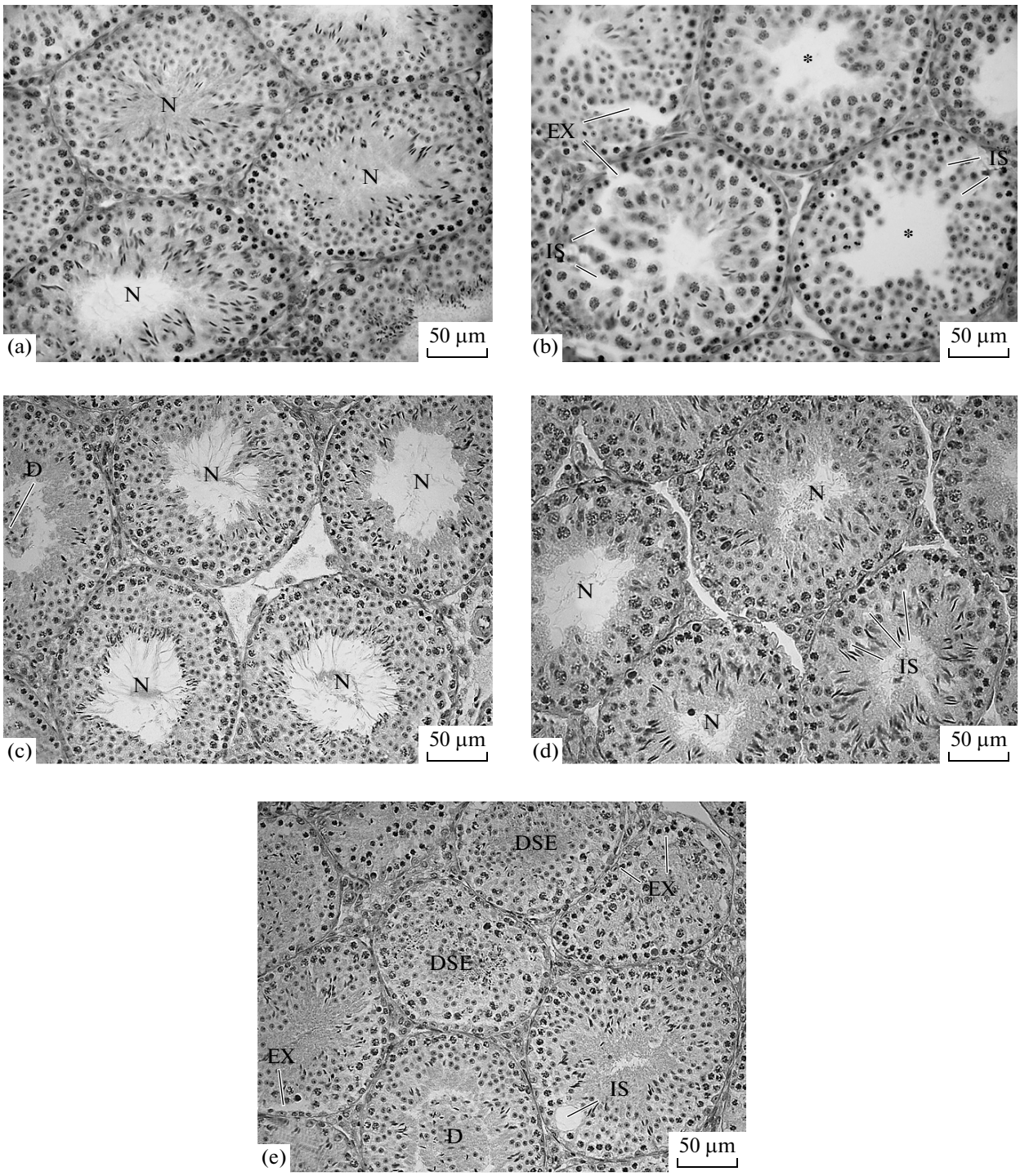

Fig. 3. Sections of testicles of the (a, c) control and (b, d, and e) experimental mice on days 35 (a, b) and 56 (c, d, and e) of the experiment. (IS) Inter-cellular spaces and (EX) exfoliation.

Meanwhile, the incidence of round spermatids with micronuclei in mice that were injected with the saline solution or dipin exceeded the level of micronuclei aberrations two- and three-fold, which is usually observed during spontaneous mutagenesis (Lahdetie, 1983; Zakhidov et al., 1994a). Importantly, both in the controls and in the experiment, on day 100 of the aftereffect, the output of genetically abnormal spermatogonia and round spermatids diminished significantly compared with the previous days of fixation.

The test on the abnormalities of the forms of sperm cell heads showed (Fig. 4c) that, on day 35 of fixation,

the incidence of aberrant forms increased sharply compared with the control. In the testicles of two out of six studied males, the number of atypical gametes was 100%. The low percent of sperm cells with morphologically abnormal heads was noted on days 56 and 100 of the experiment in both the controls and the experiment.

Light-optical observations revealed varying sensitivity of the nuclear material of the de-membranized epididymial sperm cells to the influence of the carbothiolic DTT reagent. By the degree of unpacking of chromatin and swelling of the nuclei, mature gametes were conditionally divided into three main groups: under-condensed (which, on the whole, did not differ from the normal, native nuclei), partially, and completely decondensed. Cells with abnormal nuclei, some of which contained vacuoles, were also found in the population of mature sperm cells treated with SDS/DTT (Figs. 5a, 5b). Figure 5c shows that on day 7 of fixation, in both the controls and the experiment, the percent ratio of the under-condensed, partially, and completely decondensed nuclei was approximately identical. On day 14 of the after-effects, no differences between the sperm cells of the control and experimental animals were found; in both cases, the number of under-condensed nuclei increased considerably, while the number of nuclei with completely decondensed chromatin decreased as significantly. On day 35 after the start of the experiment, the ratio of cells with varying degrees of nucleus decondensation in the control samples was almost the same as on the previous days of fixation. No analysis of the experimental material was performed, since only single sperm cells with under-condensed or abnormally decondensed nuclei were found in the epididymis on day 35 of the experiment. On days 56 and 100 of fixation, the controls and the experiment differed little: in both cases, sperm cells with under-condensed and partially decondensed nuclei predominated in the studied samples.

DISCUSSION

The data obtained in the study showed clearly that single intratesticular injections of the chemical mutagen dipin or the saline solution into mice led to significant destructive changes in the spatial organization of the spermatogenic epithelium. However, in both cases, the injuries were of reversible nature. The restorative process in the mutagenized testicles was slower than in the gonads of the control mice. This similarity in the reaction of the spermatogenic system in the control and experimental animals allows us to conclude that the appearance of morphohistological disturbances of spermatogenesis can be associated with the mechanical damage (injury) of testicles during micromanipulations. As a matter of fact, according to some observations (Weinbauer et al., 1985; Sprando et al., 1996; Baran et al., 2011), it is the intratesticular injections into mammals and not the injected chemical substances (including the saline solution) that cause damage in the structure of the seminiferous tubules and spermatogenic epithelium. Interesting observations were made by Andreeva and Serova (1992). According to their data, such micromanipulations with the mouse zygotes as the perforation of the male pronucleus with a glass microneedle, injection of buffer solution or genetic construction, later on, after the transplantation of the zygotes into the oviducts of the recipient females led to disturbances in the development of the embryos, their death, or the appearance of offspring with lowered vitality. As the authors emphasize, the most traumatic stage in the experiment is puncturing of the zygote's pronucleus. These observations were confirmed to some degree by other studies that showed that micro-injections of the buffer solution or the so-called piercing of the pronucleus had a strong damaging impact that influenced the preimplantation stages of development of the rodents negatively (Popova et al., 2002; Popova et al., 2003).

The specific nature of the action of dipin as a highly active mutagen directly on the mouse testicles was reflected in the absence of any sharp increase in the frequencies of spermatogonial and meiotic micronucleic aberrations at remote terms after the start of the experiment (days 35, 56, and 100 of fixation), compared with the control. Based on the data of the kinetics of spermatogenesis in mice (Meistrich, 1986), this means that the intratesticular injections of dipin (as well as the saline solution) did not induce any gross changes in the structure of the chromosome apparatus of the spermatogonial stem cells. It is difficult to understand why the clustogenic effect of dipin on SSCs in the mouse testicles is not manifested to the same degree and as irreversibly as was established by us earlier in the experiments with intraperitoneal injections into animals (Zakhidov et al., 1994a; Kulibin et al., 2008). It is likely that in intraperitoneal injections, dipin becomes involved in various metabolic and biochemical processes and forms complexes with multiple enzymes, activating them or, on the contrary, inhibiting them. As a result, a multitude of intermediate metabolites may form, which possess a stronger mutagenic origin. They are capable of overcoming powerful protective barriers, such as the basal membrane and hematotesticular barrier, as well as specific, extremely effective systems of detoxication and reparation, which are characteristic of mammalian testicles. Apparently, in pure form, dipin molecules do not possess such an ability. In other words, the clustogenic potential of dipin injected intratesticularly proved insufficient to cause deep changes in the hereditary structures of SSCs.

Meanwhile, it is noteworthy that during the entire experiment in both cases (dipin or saline), the output of meiotic micronuclei was significantly higher than the incidence of spermatogonial micronuclei, despite the fact that both sperm cells and early postmeiotic

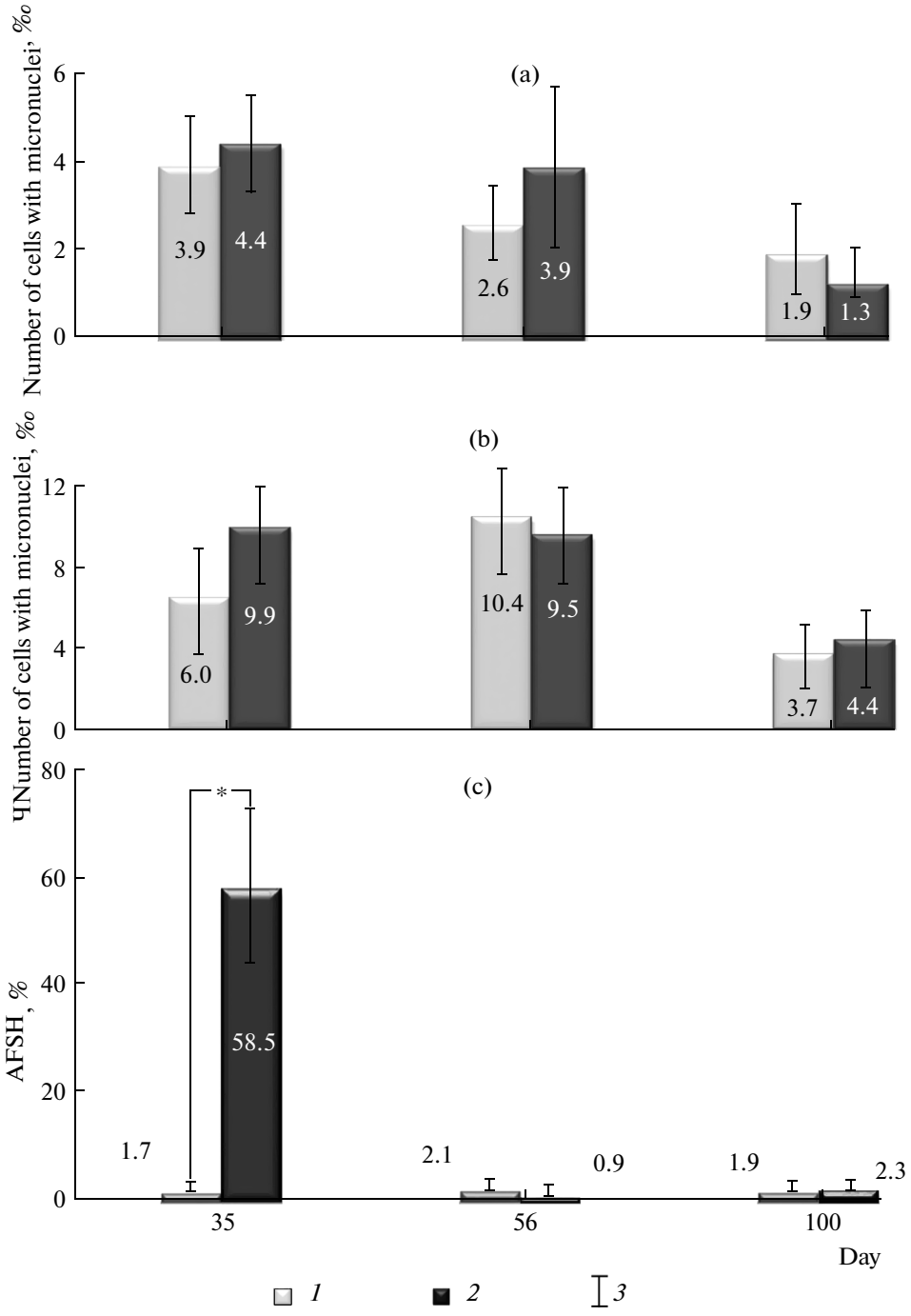

Fig. 4. Dynamics of change in the frequency of spermatogonia with (a) micronuclei, (b) round spermatids with micronuclei, and (c) testicular sperm cells with abnormal head forms in control and experimental mice. (1) Control, (2) experiment, (3) standard error of mean, $p \leq 0.05$.

cells take their origin from the cells that at the moment of the experimental manipulations were at the stage of SSCs. It can be assumed conditionally that some factor, most likely, of epigenetic character sharply weakens the functioning of reparation enzymes and cell selection in meiosis, as a result of which a certain percent of cells with potential latent ruptures of chromosome threads is revealed at the postmeiotic stages of development in the form of round spermatids with traces of chromosome damage.

On the other hand, the mutagenic influence of dipin was manifested in a significant increase in the incidence of sperm cells with abnormal head forms on day 35 of fixation. An increase in the percent of aber-

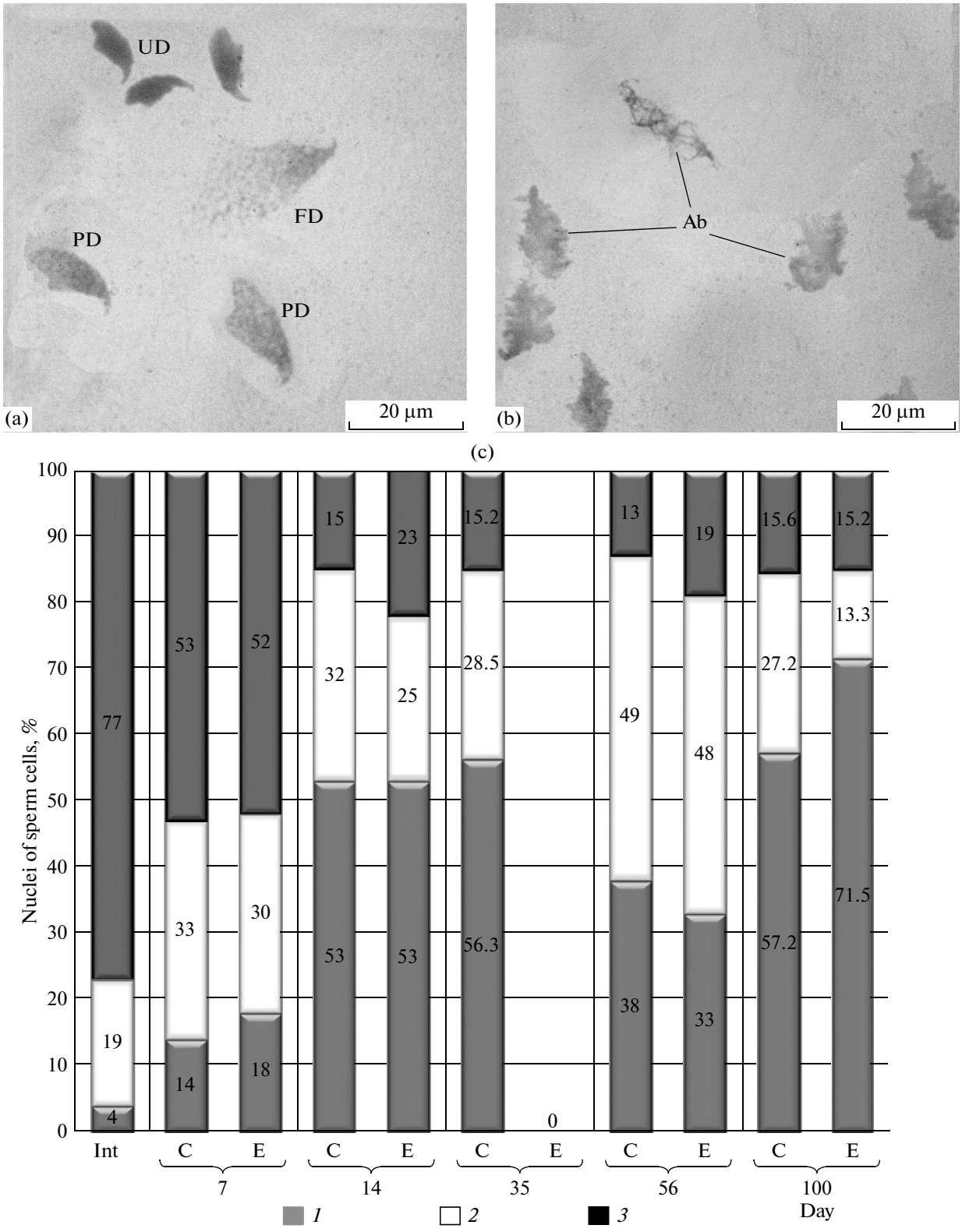

Fig. 5. Nuclei of epididymial spermatozoa of mice after combined treatment with SDS/DTT (a, b) and the ratio of the number of spermatozoa with varying degrees of chromatin decondensation after 40 min of incubation in DTT (c). (Ab) Abnormal; (UD) undecondensed; (FD) fully decondensed; and (PD) partially decondensed.

(C) Controls; (Int) intact controls; (E) experiment. (1) Undecondensed, (2) partially decondensed; (3) fully decondensed. Staining: toluidine blue.

rant forms in the population of testicular sperm cells is usually considered a morphological expression of point genetic mutations, microdeletions, or even large structural reorganizations of chromosomes caused by chemical and physical mutagens in premeiotic and/or early meiotic cells. Since we observed the high frequency of testicular sperm cells with an abnormal head form in the testicles only on day 35 of the aftereffect, we can conclude that, according to the data on the kinetics of spermatogenesis in mice, the high genetic sensitivity to the action of dipin was found in the nondifferentiated spermatogonia of type A1 but not SSCs, since on days 56 and 100 of fixation the number of sperm cells with abnormal head forms did not exceed the prescribed limits.

In the general strategy of reproductive toxicology and auxiliary reproductive technologies, the analysis of the quality of semen occupies an important place. One of the main requirements in such analysis is study of the integrity of the nuclear material of mature spermatozoa. In other words, when assessing the fertilizing ability of sperm cells, an important parameter is the degree of condensation/decondensation of chromatin (gametic DNP-complex). In placental mammals and man, during the maturation of spermatids, the spatial reorganization of chromatin is accompanied by the synthesis and accumulation of spermio-specific protamine-like proteins, which are rich in arginine and cysteine. The newly synthesized high-base proteins, while replacing the fractions of histones of the somatic and meiotic types, participate in the specific setting of the DNA molecule. Condensation and stabilization of chromatin of the sperm cells occurs due to the formation of a large number of disulfide bridges, whose formation is finished almost completely in the head section of the epididymis. Transverse S-S-links, which are formed between the neighboring protamine-like proteins, provide high stability of the inactivated father genome against the action of various physical and chemical factors and preservation of its integrity during the passing of sperm cells along the complex routes of male and female reproductive systems. Damage in the process of change of the main nuclear proteins in spermiogenesis, defects in the interactions of DNA-proteins, hyperacetylation of histones, and ruptures of chromosome threads can lead to structural anomalies in the chromatin of the formed sperm cells. The immaturity of the gametic DNP-complex can reflect strongly on the process of chromatin decondensation in the future, which is a necessary condition for the formation of the male pronucleus, replication, condensation of chromosomes, and formation of a viable zygote (Dadoune, 1995, 2003; Chapman, Michael, 2003; Laberge, Boissonneault, 2005; D'Occhio et al., 2007). As our observations show, during the entire experiment, both in the experiment and in the control, the ratios of under-condensed (stable) and partially and completely decondensed nuclei in a population of mature mouse spermatozoa after the combined treatment with SDS/DTT were almost identical. However, in both cases, in the chosen condition of the experiment, these ratios differed sharply from the results of analysis of the process of decondensation of the nucleic chromatin in mature gametes of intact animals. The predomination of under-condensed nuclei in the control and experimental samples is explained, most likely, by the super-stabilization of chromatin due to the formation of a large number of additional disulfide bridges. Thus, a number of researchers (Johansson, Pellicciari, 1988; Hernandez-Ochoa et al., 2006) found that the long-term influence of inorganic lead on male mice intensified the processes of condensation and stabilization of nucleic material in the sperm cells, which was indicated by the high stability of gametic chromatin to decondensation in vitro. According to the data of Hernandez-Ochoa et al. (2006), in the controls, after the treatment of sperm cells with the SDS/DTT solution for 30 min, approximately 98% of the nuclei underwent full decondensation and only 2% of the nuclei showed resistance. At the same time, 41% of nuclei of mature gametes were not sensitive to the action of the decondensing agent: 40 and 18% of the nuclei underwent full and partial decondensation, respectively. The authors (Johansson, Pellicciari, 1988; Hernandez-Ochoa et al., 2006) are unanimous in believing that during the passage of sperm cells along the epididymis, Pb penetrates the nuclear material and interacts with the DNA-protein complex, forming S–Pb–S links in it, which may impede the normal process of chromatin decondensation. According to the observations of Sawyer and Brown (2000), after the chronic influence on rats for 6 weeks at a dose of 5.1 mg/kg, the antitumoral agent cyclo-phosphamide did not cause changes in the weight of testicles and epididymides nor in the number of the forming sperm cells. It also significantly suppressed the process of chromatin decondensation in the mature gametes in vitro.

The fact that at remote terms of influence (days 35, 56, and 100 of fixation) a large number of mature sperm cells remained insensitive to the action of a decondensing agent may be determined by prolonged and probably irreversible disturbances of the functional and topological integrity of Sertoli cells caused by the damaging action of intratesticular injections of dipin or saline. As is known, niche-forming Sertoli cells play a significant role in the development of spermatogenic cells, including those at postmeiotic stages of maturation. Hooley et al. (2011) showed that intratesticular injection of an adenovirus vector to mice, which carries the GFP transgene (green fluorescent protein) and which was expressed exclusively in the cytoplasm of Sertoli cells, led to disturbances in the function of these cells and, as a consequence, disintegration of the spermatogenic epithelium in some of the seminiferous tubules. Chapman and Michael (2003) put forward a hypothesis according to which the androgen-binding protein (ABP), which is secreted by

Sertoli cells and transported through rete testis into the epididymis, can participate indirectly in complex biochemical processes associated with the oxidation of thiolic (-SH) groups of cysteine and condensation/decondensation of chromatin in rat spermatozoa. In turn, a decrease in the ABP levels can cause qualitative defects in male gametes. On the other hand, we do not exclude the possibility that changes in the nature of decondensation of the gametic chromatin revealed by us may be based on some epigenetic changes in the system of development of male sex cells caused by mechanical damage (trauma) to the testicle. These so-called epimutations, which do not yield in effectiveness to true mutations, are capable of being preserved for several cell generations and even of being inherited and can provoke catastrophic changes in the processes of synthesis and accumulation of spermiospecific proteins and their interactions with the DNA molecule (Zamudio et al., 2008; Rajender, Agarwa, 2011).

Loss of the ability of decondensation of nuclei of mature spermatozoa, which at the moment of intratesticular injections were at intermediate stages of spermiogenesis, when the spermatid chromatin is open, active, and undergoes conformational changes (day 14 of fixation), or were at the point of transition from testicles into the epididymis (day 7 of fixation), could have been caused by mechanical damage to the hemato-testicular and hemato-epididymial barriers. Usually, damage to these barrier structures opens possibilities for the penetration of a large number of chemical substances, many of which possess genotoxic activity, into the spermatogenic system. In one of the early works by Qiu et al. (1995), it was shown that the chronic influence (6 weeks) of the antitumoral agent cyclophosphamide on male rats changed the nature of decondensation of the nuclei of epididymial gametes in the condition in vitro. According to the opinion of the authors, changes in the process of decompactization of chromatin could occur as a result of alkylation of the main nucleic proteins or DNA or as a result of formation of lacings between these macromolecules under the influence of metabolites of cyclophosphamide: mustard phosphoramide and acrolein.

Thus, based on the results obtained in this work, we can state that a single intratesticular injection of the chemical mutagen dipin (experiment) or saline (control) into mice causes significant destructive changes in the organization of the spermatogenic epithelium. These changes are of an irreversible nature, but they are manifested more abruptly in mutagenized testicles. This is evidenced by not only comparatively later restoration of the spermatogenic structure but also by the almost complete absence of gamete fixation in the cauda epididymis and the simultaneous appearance of a vast number of sperm cells with an abnormal head shape. If judged by the frequency of incidence of spermatogonial and meiotic micronuclei under the remote terms of the aftereffect, it becomes evident that neither dipin nor saline were inferior to each other in the cytogenetic effects on SSC. The fact that the main damaging effect of intratesticular injections of dipin or saline on the spermatogenesis of mice was manifested to the greatest degree in the decondensation of the nucleic chromatin of mature sperm cells in vitro deserves attention. Our observations confirm the point of view (Qui et al., 1995) that the quantitative estimation of chemically induced changes in the nature of swelling and decompactization of the nuclei of mature sperm cells can become a very useful tool in understanding the consequences of influence of reproductive toxicants on the spermatogenic functions.

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