GENETICS

Destructive Effect of DNA Topoisomerase II Inhibitor Vepesid on Mouse Spermatogenesis

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Inhibition of DNA topoisomerase II with vepesid induced structural and functional reorganization of chromatin in meiotically dividing spermatocytes I, which later led to the block of their differentiation and long-lasting disorders in spermatogenesis. Vepesid induced decondensation of spermatocyte I chromatin, block of desynapsis, and elongation of lateral elements of spermatocyte autosome synaptonemal complexes during late pachytene and diplotene of meiosis. This confirms the involvement of type II DNA topoisomerase in chromatin condensation and homologous chromosome desynapsis at the stage of diplotene and the role of this enzyme in structural organization of the synaptonemal complex. Vepesid induced the formation of dichotomy and breaks of the pericentromer regions of subelements of lateral elements of the autosomal synaptonemal complexes; the number of cells with associations of axial elements of sex chromosomes with autosomal synaptonemal complexes increased, univalents of autosomes and sex chromosomes appeared. Mesna, a modifier of toxic effects of antitumor drugs, had no toxic effect on spermatogenic cells. Mesna reduced the lethal effect of vepesid during combined treatment, but did not ensure long-term protection of spermatogenesis.

Key Words: *DNA topoisomerase II; vepesid; Mesna; spermatogenesis; meiosis*

DNA topoisomerase II (topo II) is a structural and functional enzyme component of chromatin participating in structural transformations of chromatin during meiotic prophase I.

Active expression of topo II genes during meiotic prophase I was demonstrated [4,7]. However the role of topo II in the organization of chromatin and synaptonemal complex (SC) in mammals, a unique nucleoprotein structure forming between synapting homologous chromosomes during meiotic prophase I and participating in chromosome synapsis, recombination, and desynapsis remains unknown.

Electron immunocytochemistry showed that during diplotene topo II antigens are located on chromatin, axial (AE) and lateral (LE) elements of SC [12]. In addition, *top2/top2* meiotic mutants of *S. cerevisiae* yeast are arrested during transition from late pachytene to diplotene [15].

No topo II mutations are known for mammals, in contrast to yeast and drosophila, and therefore we selected another approach to investigation of the role of topo II in meiosis. We used antitumor drug vepesid (Vp-16, Bristol Mayers Squibb), a topo II inhibitor, for experimental modeling of meiotic mutation *in vivo*. It was previously shown that Vp-16 produced a mutagenic effect on spermatogonias and spermatocytes I (SPC-I) [2]. Exposure of late pachytene and diplotene cells to Vp-16 led to the formation of micronuclei [8].

In this study we analyzed structural changes caused by Vp-16 in chromatin and SC and on the course of spermatogenesis.

In addition, well-known side effects of Vp-16 prompted us to investigate the possibility of reducing the

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toxic effect of Vp-16 on spermatogenic cells with antitoxic modifier Mesna (Asta Medica). This drug is now widely used in clinical practice as uroprotector during oxazaphosphorine treatment [10]. Animal experiment showed that Mesna reduces the lethal effect and hematological toxicity of Vp-16 [1].

MATERIALS AND METHODS

Experiments were carried out on male CBA mice aged 2-4 months (22-28 g) from Stolbovaya Breeding Center, Russian Academy of Medical Sciences. The animals were divided into groups (6 males per group): 1) intact; 2) single intraperitoneal injection of Vp-16 in a dose of 30 mg/kg (LD_{50}); 3) Mesna (4 injections 25, 20, and 15 min after the first one, total dose 200 mg/kg); 4) Mesna in a dose of 200 mg/kg 30 and 5 min before Vp-16 in doses of 30 and 80 mg/kg and 15 and 30 min after Vp-16.

The animals were sacrificed by cervical dislocation at different terms after drug injections (3 h to 170 days).

For histological analysis the testis was fixed in Bouin fixative, paraffin sections were stained with hematoxylin and eosin.

For electron microscopy testicular tissue fragments were fixed in glutaraldehyde, postfixed in OsO_4, and contrasted with lead uranylacetate. Ultrathin sections were contrasted with lead citrate.

Total preparations of flattened SPC-I nuclei were prepared as described previously with some modifications [13]. The preparations were fixed in 4% paraformaldehyde (in 0.1 M sucrose, pH 8.4-8.6) and contrasted with 50% $AgNO_3$ (pH 3.5-5.5). Ultrathin sections and SC preparations were examined under a JEM 100B electron microscope.

RESULTS

The walls of testicular tubules in intact mice contained Sertoli cells and sex cells at different stages of differentiation: spermatogonias, SPC, spermatides, and spermatozoa (Fig. 1, *a*). Electron microscopy of SPC-I showed typical structure of SC consisting of two LE and one AE with chromatin around it (Fig. 2, *a*). Examination of total preparations of flattened SPC-I pachytene nuclei showed 19 SC of autosomal bivalents and one sex bivalent (Fig. 3, *a*). Early, mid, and late pachytene were identified by changes in AE morphology of the sex chromosome and the degree of synapsis between them.

The histology and ultrastructure of the testes in males sacrificed 3 h and 1 day after injection of Vp-16 were virtually normal. However, electron microscopy of flattened SPC nuclei revealed increased number of late pachytene and prophase I diplotene cells with dichotomy of autosome LE in the pericentromer zone of one or both homologues (Fig. 3, *b*, Table 1). Breaks of the sublateral elements of SC in zones of LE dichotomy were seen in some cells (Fig. 3, *c*). Similar dichotomy of LE in autosomal SC and AE in sex chromosomes were described for total SC preparations of meiotic nuclei in different animals [11]. It was hypothesized that each LE in SC consists of at least two large sublateral elements connected to sister chromatides. VP-16-induced dichotomy and chromatid breaks of LE in the pericentromer zone of autosomal SC agree with the data on the presence of a specific topo II population in the pericentromer heterochromatin zone [5,14] and induction of breaks in the pericentromer chromosome area at the stages of late pachytene, diplotene, and diakinesis under the effect of Vp-16 [8,9]. It can be hypothesized that Vp-16 has a specific effect on the centromer population of topo II, which leads to chromatin restructuring in the chromosomal pericentromer area and, as a result, to the development of the above-described disorders.

The number of SPC with untimely desynapted LE of autosomal SC and AE of sex chromosomes increased during late pachytene and diplotene (Fig. 3, *d*), which was in line with the data on the formation of micronuclei by whole chromosome lagging in spermatides [8].

Associations between AE of sex chromosomes and LE of autosomal SC were often observed (Fig. 3,

TABLE 1. Number of SPC Nuclei with Aberrations of Autosomal SC and Sex Chromosome Structure at Late Pachytene and Diplotene Stages of Meiosis in Control Mice and Mice Injected with Vp-16 (30 mg/kg) and Mesna (200 mg/kg)

Group	Term	LE dichotomy in autosomal SC, %	Preterm desynapsis, %		Associations, %	
			LE of autosomal SC	AE of XY	X-autosome	Y-autosome
Control (*n*=26)	—	11.5	3.8	19.2	3.8	—
Vp-16 (*n*=82)	3 h, day 1	26.8	14.6	12.2	15.9	3.7
Mesna (*n*=60)	day 5	—	—	3.3	0.05	—
Vp-16+Mesna (*n*=68)	3 h, day 1	32.4	10.3	41.2	20.6	5.9

e), associations of Y chromosome AE with LE of autosomal SC were rarer compared to associations of X chromosome. This disturbed translocation of sex chromosomes to the nucleus periphery and sex body formation. According to some reports [6], this was a sign of the block of differentiation and subsequent degeneration of SPC-I at the pachytene stage.

One more type of disorders in structural elements of SC at the stage of late pachytene was detected as early as 3 h after injection of Vp-16; its most remarkable manifestation was more than 10-fold enlargement of flattened nuclei in comparison with the control. Electron microscopy of these giant nuclei showed 3-7-fold elongation of SC elements and, hence, chromosomes, narrowing of SC central space, thinning of LE, chromatin decondensation, and impaired desynapsis of homologues (Fig. 3, *f*).

No SPC with enlarged nuclei were detected on histological and ultrathin sections of the testes. This can be explained by specific reaction of topo II inhibitor-treated cells to their spreading on the surface of hypotonic solution.

These results are in good correlation with description of SC structure of *S. cerevisiae* yeast homozygotic

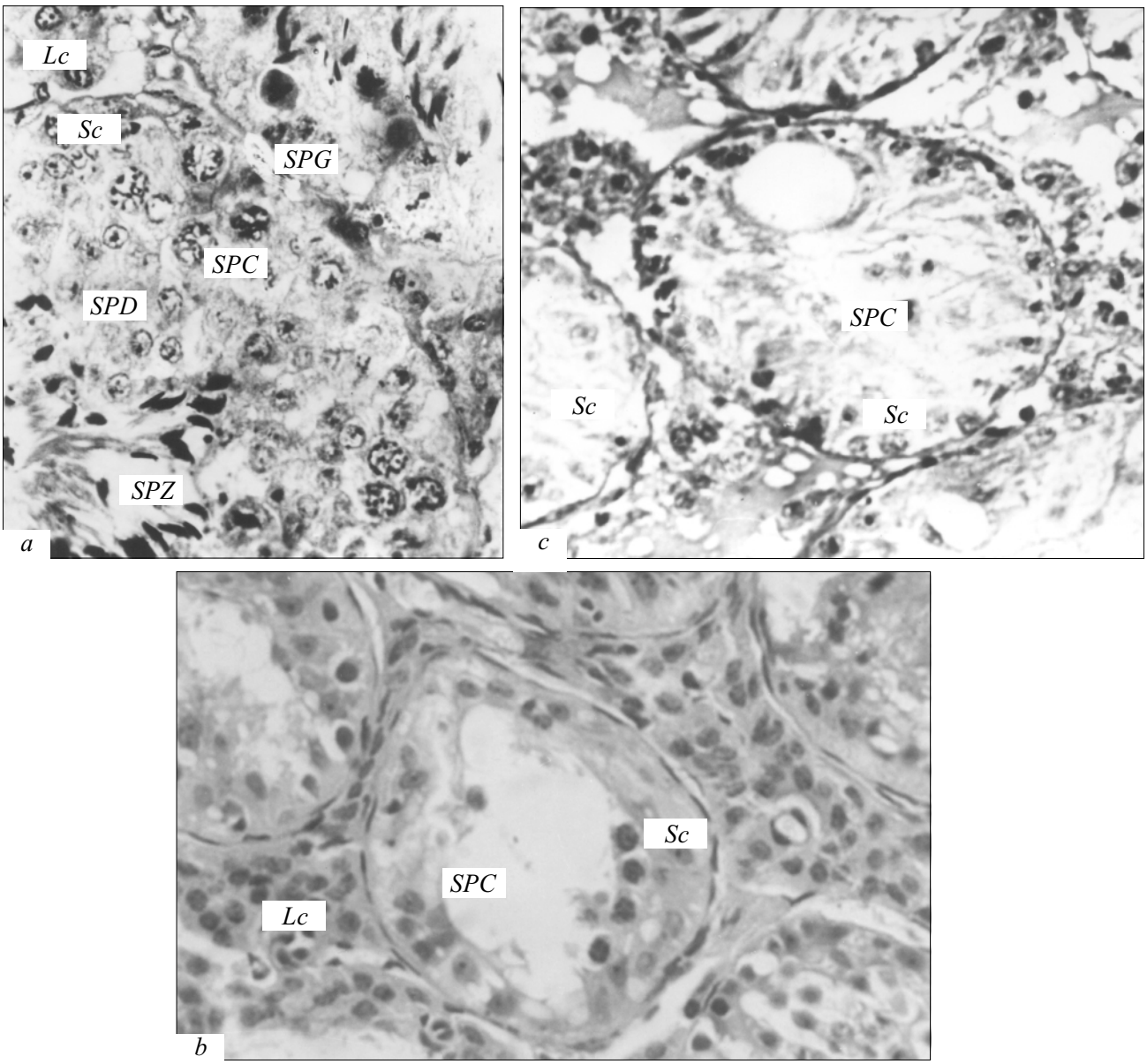

Fig. 1. Histological sections of mouse testis. *a*) control. Testicular tubule wall contains Sertoli cells (*Sc*), spermatogonias (*SPG*), spermatocytes (*SPC*), spermatides (*SPD*), and spermatozoa (*SPZ*). Leydig cells (*Lc*) in the interstitium, ×650; *b*) degenerative changes in some tubules at the periphery of the testis on day 22 after injection of vepesid (30 mg/kg). Reduction of spermatogenic epithelium. Cell hyperplasia in the interstitial space, ×470; *c*) atrophy of testicular tubules on day 170 after combined treatment with vepesid (80 mg/kg) and Mesna (200 mg/kg). Hyalinization of the interstitium, ×470.

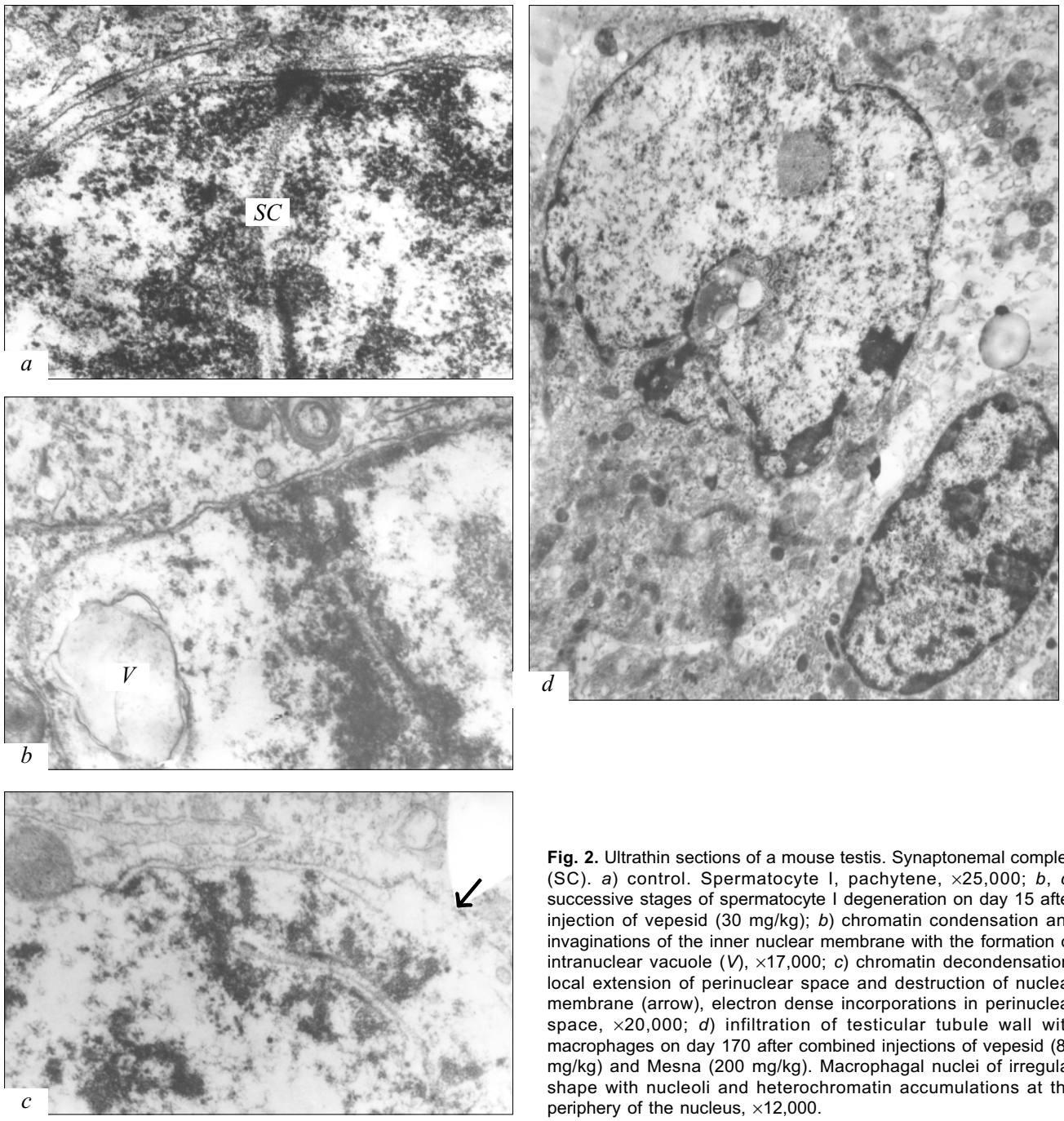

Fig. 2. Ultrathin sections of a mouse testis. Synaptonemal complex (SC). *a*) control. Spermatocyte I, pachytene, ×25,000; *b*, *c*) successive stages of spermatocyte I degeneration on day 15 after injection of vepesid (30 mg/kg); *b*) chromatin condensation and invaginations of the inner nuclear membrane with the formation of intranuclear vacuole (*V*), ×17,000; *c*) chromatin decondensation, local extension of perinuclear space and destruction of nuclear membrane (arrow), electron dense incorporations in perinuclear space, ×20,000; *d*) infiltration of testicular tubule wall with macrophages on day 170 after combined injections of vepesid (80 mg/kg) and Mesna (200 mg/kg). Macrophagal nuclei of irregular shape with nucleoli and heterochromatin accumulations at the periphery of the nucleus, ×12,000.

by the *top2/top2* meiotic mutation (block of desynapsis, chromatin decompactization in meiotic chromosomes, and arrest of meiosis during transition from late pachytene to diplotene) [15]. Therefore, the proposed experimental scheme can be regarded as the experimental model of meiotic *top2/top2* mutation in mammals.

Similar data were obtained with topo I inhibitor [3]. It seems that inhibition of the structural function of both topo I and topo II led to chromatin decondensation, chromosome elongation, and the resultant elongation of SC elements.

Numerous nuclei containing homogeneously stained chromatin and degenerating elements of autosomal SC and sex chromosomes, but virtually no normal SPC nuclei at the stage of prophase I were observed on total SC preparations on days 11, 15, and 22 after injection of Vp-16. Examination of histological sections at this term revealed some narrowed tubules containing vacuolated Sertoli cells and solitary spermatogonias and SPC at the periphery of the testis (Fig. 1, *b*). Analysis of ultrathin sections at these terms revealed progressing degenerative changes in SPC I nu-

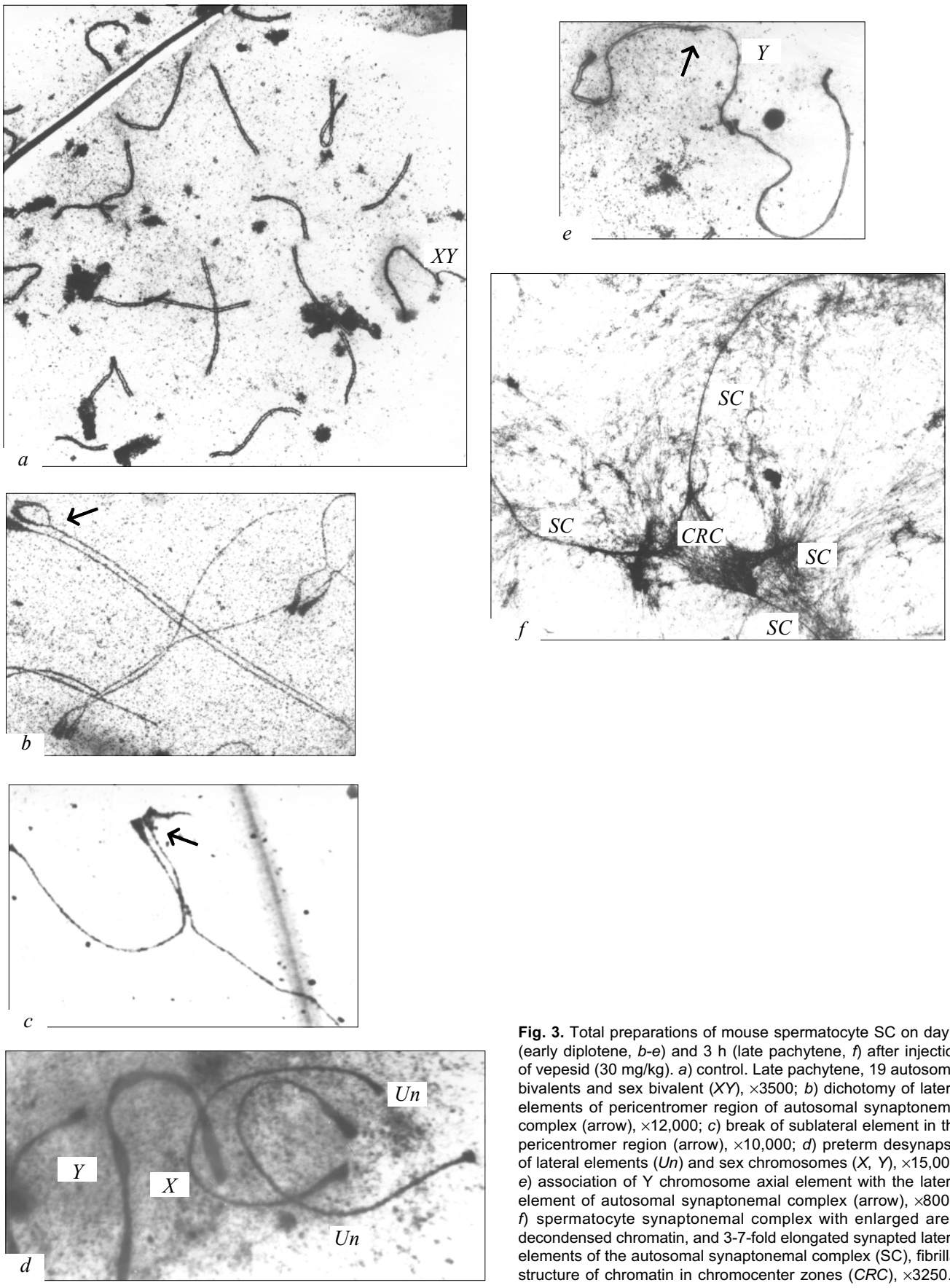

Fig. 3. Total preparations of mouse spermatocyte SC on day 1 (early diplotene, *b*-*e*) and 3 h (late pachytene, *f*) after injection of vepesid (30 mg/kg). *a*) control. Late pachytene, 19 autosomal bivalents and sex bivalent (*XY*), ×3500; *b*) dichotomy of lateral elements of pericentromer region of autosomal synaptonemal complex (arrow), ×12,000; *c*) break of sublateral element in the pericentromer region (arrow), ×10,000; *d*) preterm desynapsis of lateral elements (*Un*) and sex chromosomes (*X*, *Y*), ×15,000; *e*) association of Y chromosome axial element with the lateral element of autosomal synaptonemal complex (arrow), ×8000; *f*) spermatocyte synaptonemal complex with enlarged area, decondensed chromatin, and 3-7-fold elongated synapted lateral elements of the autosomal synaptonemal complex (SC), fibrillar structure of chromatin in chromocenter zones (*CRC*), ×3250.

clei: sharp chromatin condensation, invagination of internal nuclear membrane with formation of intranuclear vacuoles, local dilatation of the perinuclear space containing electron dense inclusions of unknown nature, and destruction of nuclear and cells membranes leading to the release of chromatin with SC elements into the cytoplasm of Sertoli cells (Fig. 2, *b*, *c*).

Hence, topo II inhibitor Vp-16 damaged spermatogenic cells at the level of stem spermatogonia and SPC I, which led to massive SPC I degeneration and long-lasting spermatogenesis disorders. Vp-16 induced decondensation of SPC chromatin, elongation of SPC autosomal SC LE and block of their desynapsis during late pachytene and diplotene. The specificity of Vp-16 effect and the key role of topo II in chromatin condensation, structural organization of SC, and desynapsis of homologous chromosomes during late pachytene and diplotene were confirmed in our study of spermatogenesis and SC after treatment with taxol (not inhibiting topo II).

In addition, Vp-16 treatment increased the number of cells with associations of sex chromosome AE with autosome SC, formation of univalents of autosomes and sex chromosomes. Vp-16 had a specific effect on pericentromer DNA causing dichotomy and breaks of LE elements in autosomal SC.

Studies of total SC preparations and analysis of histological and ultrathin sections on day 5 after injection of Mesna (group 3) showed no appreciable changes from the control (Table 1). This means that Mesna did not damage spermatogenic cells.

In group 4 dichotomy of LE and breaks of autosomal LE subelements were observed at the stage of late pachytene; preterm desynapsis of sex chromosomes and autosomes, associations of autosomes and sex bivalent were observed. The relative number of damaged cells increased in comparison with the results of the previous experiment (Table 1). Presumably, Mesna slightly potentiated the specific effect of Vp-16. The absence of SPC with elongated LE in SC and enlarged nucleus 3 h and 5 days after injections of Vp-16 and Mesna suggests that Mesna neutralized the effect of Vp-16 at these terms.

On day 35 after combined treatment with Vp-16 and Mesna solitary atrophic tubules were seen on histological sections of the testes. Changes in the ultrastructure of spermatogenic cell corresponded to those observed on days 11-22 after injection of the antitumor drug alone. In addition, numerous degenerating SPC nuclei were seen on total SC preparations.

On day 170 after combined treatment with Vp-16 and Mesna the animals were alive. Spermatogenic epithelium in the majority of tubules was presented by vacuolated Sertoli cells and solitary sex cells (Fig. 1, *c*). Analysis of ultrathin sections showed that in addition to solitary degenerating SPC and Sertoli cells, the walls of these tubules contained numerous macrophages, apparently migrating from the interstitium (Fig. 2, *d*). These data suggest that the effect of Vp-16 on spermatogenic cells can induce autoimmune process, which, in turn, can lead to irreversible disorders in spermatogenesis. We failed to obtain total SC preparations from these males.

Hence, injection of antitoxic modifier Mesna simultaneously with topo II inhibitor Vp-16 markedly reduced the lethal effect of Vp-16, but did not ensure long-lasting protection of spermatogenesis.

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