

The inhibitor of calcium activated neutral proteinase is an anti-meiotic agent. The spermicidal and anti-viral action

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Summary. In situ cytogenetic morphology and analysis showed that Epstein-Barr Virus (EBV)-infected Raji and EBV-producing P3HR-1 cells divide by meiosis and follow the life cycle of malignant cells in vitro. Meiosis was documented by the presence of condensed chromosomes, «o» chromosome, nuclear vlimata (NVs), NV invasion, extrusion of chromosomes, chromosomal transfer, metaphase fusion and aneuploidy. EBV-Raji, EBV-producing P3HR, HIV1-infected MOLT-4 cells (dividing by meiosis) and human spermatozoa (cellular products of meiosis) were highly sensitive to the endogenous inhibitor (CANP-I) of calcium-activated neutral proteinase (CANP). CANP-I-treated virally infected and virus producing cells showed necrosis and disappearance of immunofluorescent viral antigens, documenting the anti-viral action of CANP-I. CANP-I-treated spermatozoa exhibited arrested motility with subsequent necrosis documenting the in vitro spermicidal action of CANP-I. The testis of treated Wistar rats with 0.25 U CANP-I/gr body weight for six days, were devoid of spermatozoa, with pronounced toxicity and exfoliation of spermatocytes and spermatids, indicating the contraceptive action of CANP-I via spermatogenetic arrest. It is concluded that CANP-I is an anti-meiotic agent inhibiting CANP associated with meiosis. Hence CANP-I is a promising agent against various diseases involving meiosis.

Key words: Calcium-activated neutral proteinase, Calcium activated neutral proteinase inhibitor, Meiosis, Anti-meiotic agent, Antiviral agents, Spermicidal agents, Aneuploidy

Introduction

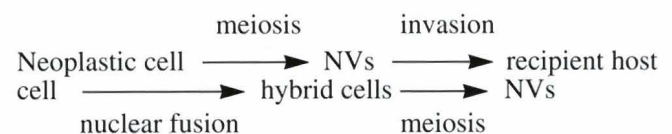
It has recently been reported that activated human lymphocytes and neoplastic cells produce spermatozoa-

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like cells (head with tail morphology) called nuclear vlimata (NVs). NVs were observed invading the cytoplasm and/or the nucleus of other cells by a process identical to fertilization and cell viral infection. Hence NVs are functional parasitic cellular elements (Logothetou-Rella, 1993a,b, 1994a).

It has further been documented, by in situ cytogenetic analysis, that NVs of activated lymphocytes and neoplastic cells are produced by meiosis identical to gametogenesis (Logothetou-Rella, 1994a,b).

In addition, in situ cytogenetic analysis showed that neoplastic NVs fused with host cell metaphases giving rise to hybrid cells. All observations led to the life cycle of neoplastic cells as follows (Logothetou-Rella, 1994a,b):



The cytotoxicity of the inhibitor of calcium-activated neutral proteinase (CANP-I) on lymphocytic, neoplastic NVs and generally aneuploid cells has documented that calcium-activated neutral proteinase (CANP) is associated with the process of meiosis and its cellular NV products (Logothetou-Rella, 1994a,b,c).

In this study, virally-infected cells and virus producing cells were examined for meiotic division. Furthermore, the anti-meiotic action of CANP-I was tested on spermatozoa, spermatogenesis, virally-infected and virus-producing cells.

Materials and methods

Cell lines

Burkitt tumor lymphoblasts (strain Raji) infected with Epstein-Barr virus (EBV) in vitro (Kottaridis et al., 1977) and P3HR-1 Burkitt lymphoma, EBV-producing cells were kindly donated by Dr. S. Kottaridis at Hellenic Anticancer Institute. The cell line MOLT-4

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(ATCC CRL 1582) infected with HIV-1 (Cann et al., 1990) was kindly donated by Dr. P. Markoulatos, at Hellenic Pasteur Institute. All cell lines were grown in RPMI-1640 (Gibco) supplemented with 10% foetal bovine serum (Seromed), penicillin (100 U/ml), and streptomycin (100 µg/ml) incubated at 37 °C in a CO₂-humidified incubator.

Cytogenetic analysis and morphology

Cytogenetic analysis and morphology was studied in situ and by the chromosomal spreading technique. Cytogenetic morphology defines the cell morphology exhibited after treatment of cells with hypotonic solutions (0.075M KCl). For the in situ study, EBV-infected Raji and EBV-producing P3HR-1 cells in complete medium, were plated in glass petri dishes, and incubated for cell attachment at 37 °C for 15 min. The medium was decanted and prewarmed KCl (0.075M) was added and incubated at 37 °C for 20 min. KCl was then removed and (3:1) alcohol-acetic acid was added and incubated at room temperature for 45 min. The cells were dried and stained with 6% Giemsa for 15 min.

Detailed chromosomal analysis was performed by the chromosomal spreading technique of Dutrillaux and Lejeune (1971), standard Giemsa and RhG banding. Two hundred consecutive metaphases were studied in each cell line.

Sperm processing

Semen samples from five fertile men were donated by Dr. F. Dimitriadou, Athens «Euromedica» IVF Center. Semen samples were processed with the swim-up technique in complete Earle's balanced salt solution (EBSS, Gibco), supplemented with pyruvate, antibiotics and 10% foetal bovine serum, of osmolarity 282-284 mOsm and pH 7.2-7.3. Motile spermatozoa in the top EBSS layer were removed (after 1 1/2 hour swim up), washed twice in complete EBSS by centrifugation at 400 g for 5 min and tested for sensitivity to CANP-I. CANP-I-treated and untreated spermatozoa were smeared on slides, fixed and stained with Papanicolaou, PAS and PAS-diacetate.

The spermicidal and antiviral action of CANP-I in vitro

The endogenous CANP-I (Sigma P-0787) was dissolved in RPMI-1640 with 25 mM Hepes (Gibco), filtered through 0.22 µm Sartorius filters, dispensed in aliquots and frozen at -20 °C. Fresh or thawed CANP-I was used.

Cytotoxicity of CANP-I was tested on human spermatozoa, EBV-infected Raji EBV-producing P3HR-1, and HIV-infected MOLT-4 cells in accordance to the method of Chang et al. (1989). Duplicate samples of cells were tested, each containing 200,000 cells. Cells were treated with various concentrations of CANP-I for 1 hour and cytotoxicity assessment was done at two days

post-treatment for spermatozoa and four days for the other cells, using the dye exclusion method of 0.4% trypan blue and eosin Y for spermatozoa. The degree of cytotoxicity was measured according to the formula:

$$\text{Cytotoxicity \%} = \left(1 - \frac{\text{Number of viable cells in the treated samples}}{\text{Number of viable cells in the untreated samples}} \right) \times 100$$

The anti-meiotic action of CANP-I in vivo

Five male, seven-week-old, Wistar rats were treated each with 0.25 U CANP-I/gr body weight i.p. daily for six days. Control group of five Wistar rats, received only RPMI-1640 with 25 mM Hepes. On the seventh day all rats were sacrificed and all organs removed were fixed in formalin, embedded in paraffin, sectioned, and stained with haematoxylin-eosin (HE) for histological study. The testis were fixed in Bouin acetic acid.

Viral antigen detection

Viral capsid antigen (VCA) were detected on EBV-Raji and EBV-P3HR-1 cell smears by immunofluorescence, using known VCA antibody-positive human serum. HIV-1-MOLT-4 cell smears were also tested for HIV-1 antigens by immunofluorescence using known HIV-1 antibody-positive human serum. For negative controls antibody-negative human control serum was used. All treated and untreated cells were examined for viral antigens.

Results

Cytogenetic analysis and morphology of EBV-infected Raji and EBV-producing P3HR-1 cells

Both cell lines exhibited aneuploid meiotic metaphases characterized by condensed chromosomes, and presence of «o» chromosomes (Fig. 1). Aneuploid NVs were observed consisting of condensed chromosomes or condensed chromosomes with fine chromatin or pyknotic nuclear head (Fig. 2). NVs carrying one or probably fragments of chromosomes were identified free or invading the nucleus of other cells (Fig. 3). Extrusion of chromosomes and metaphase fusion was abundant (Fig. 4). Invasion of pyknotic head NVs or chromatin or chromosomes was identified in host-metaphases (Fig. 5) or nucleus. Cytoplasmic metaphases in host cells with simultaneous extrusion (meiosis) of chromosomes by the host nucleus were frequent (Fig. 5b).

Detailed chromosomal analysis is shown in Table 1. The two cell lines exhibited aneuploidy with structural chromosomal aberrations. There were no diploid metaphases and no metaphases of the same karyotype. Almost half of the metaphases were hyperdiploid and polyploid and the other half hypodiploid and

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hypohaploid.

The spermicidal and antiviral action of CANP-I in vitro

Treatment of spermatozoa with 4 U/ml CANP-I for one hour reduced the motility of viable spermatozoa to 3%, at two hours post treatment. At twenty-four hours post-treatment all spermatozoa were immotile and 80% necrotic. At forty-eight hours spermatotoxicity was 100%.

Cytology of treated spermatozoa with 4 U/ml CANP-I, showed spermatozoa with tail ends coiled into loops (Fig. 6a). Treatment with 10 U/ml CANP-I, showed

spermatozoa with severe coiled tail around the nucleus (Fig. 6b) and nuclear vacuolation (Fig. 6c).

Control spermatozoa samples showed translucent fibrillar and granular green basophilic glycosaminoglycan extracellular matrix (GSG-ECM) by Papanicolaou stain (Fig. 6d); PAS and PAS-D positive (Fig. 7a). Treated spermatozoa (10 U/ml CANP-I) showed haematoxylinophilic vacuolated, fibrillar to granular ECM by Papanicolaou stain; PAS and PAS-D negative (Figs. 6, 7). CANP-I cytotoxicity on virally-infected and virus-producing cells is shown in Table 2. EBV-producing cells were more sensitive to CANP-I than virally-infected cells. The concentration

Table 1. Numerical chromosomal analysis.

CELL LINE	%DIPLOIDY	%HYPERDIPLOIDY	%POLYPLOIDY	%HYPODIPLOIDY	HYPODIPLOID AND HYPOHAPLOID SETS OF			
					1-10	11-20	21-30	31-45
EBV-infected Raji	0	14	42	44	35	4	2	3
EBV-producing P3HR-1	0	9	40	51	33	9	3	6

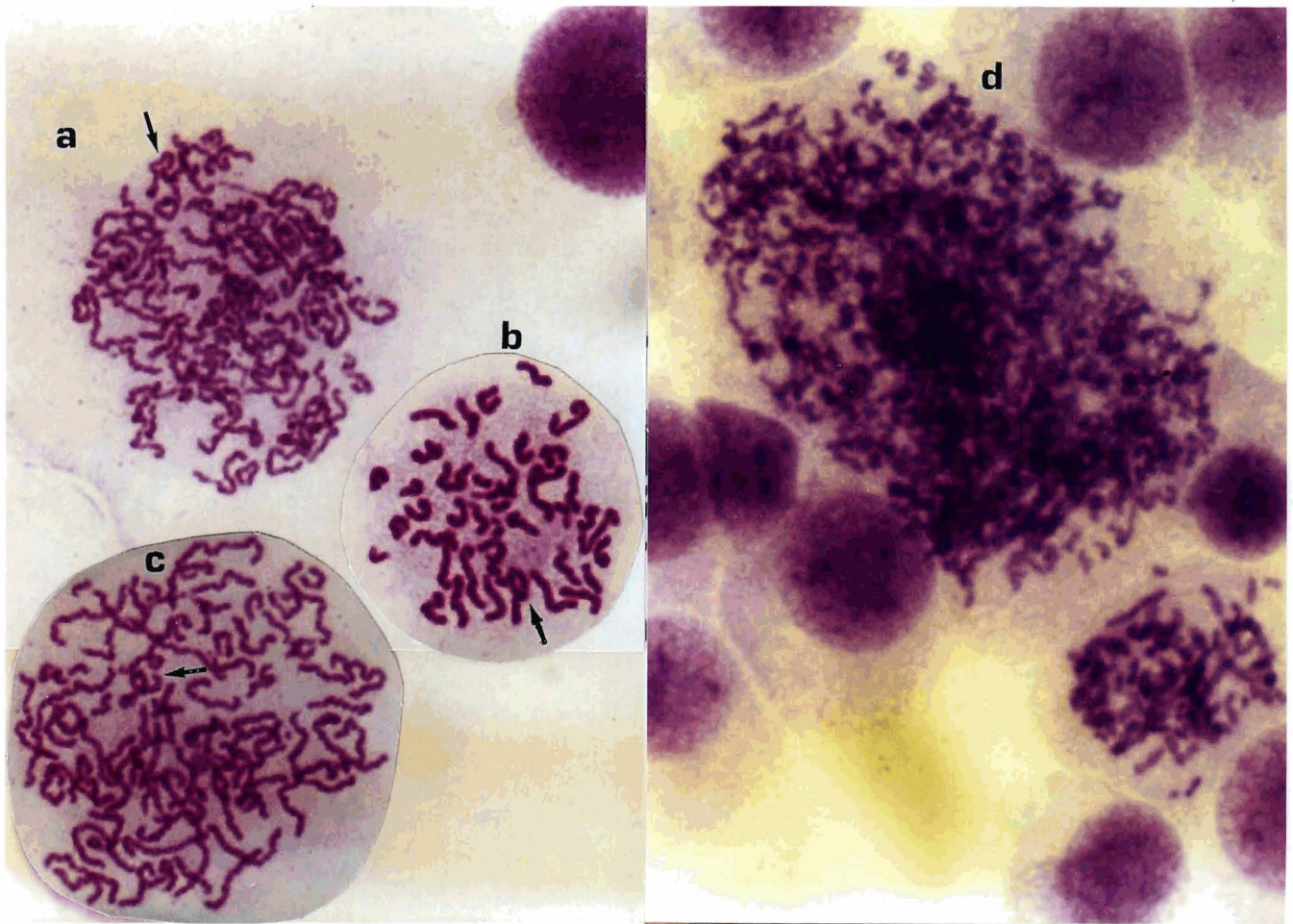


Fig. 1. Aneuploid metaphases with «o» (arrows) and condensed chromosomes. **Insets a,b:** metaphases of EBV-producing P3HR-1. **Insets c, d:** polypliod metaphases of EBV-infected Raji cells. Giemsa, x 1,000

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of 5 U/ml caused 100% cytotoxicity to all tested cell lines.

Viral antigen detection

Control cell smears showed 15-20% immunofluorescent EBV-infected Raji cells, 25-30% EBV-producing P3HR-1 cells and 60% HIV-1-infected MOLT-4 cells. Smears of all cell lines were found free of detectable immunofluorescent viral antigens after treatment of cells with higher than 4 U/ml CANP-I (Figs. 8, 9).

The anti-meiotic action of CANP-I in vivo

The testis of all CANP-I-treated rats were smaller in size than the placebo ones and showed spermatogenic arrest at the spermatocytic level with occasional bionecrotic and necrotic seminiferous tubules. Primary and secondary spermatocytes showed a different degree of degeneration and exfoliation in the lumen of seminiferous tubules within the same testis and among rats (Fig. 10). Exfoliated syncytia of intact and degenerated spermatids were pronounced in the lumen (Fig. 11). Degenerated spermatids were

Table 2. Effect of CANP-I on virally-infected and virus-producing cells.

CANP-I (U/ml)	CYTOTOXICITY %		
	EBV-INFECTED RAJI CELLS	EBV-PRODUCING P3HR-1 CELLS	HIV-1-INFECTED MOLT-4 CELLS
2	22	95	
4	97	100	97
5	100	100	100
10	100	100	100

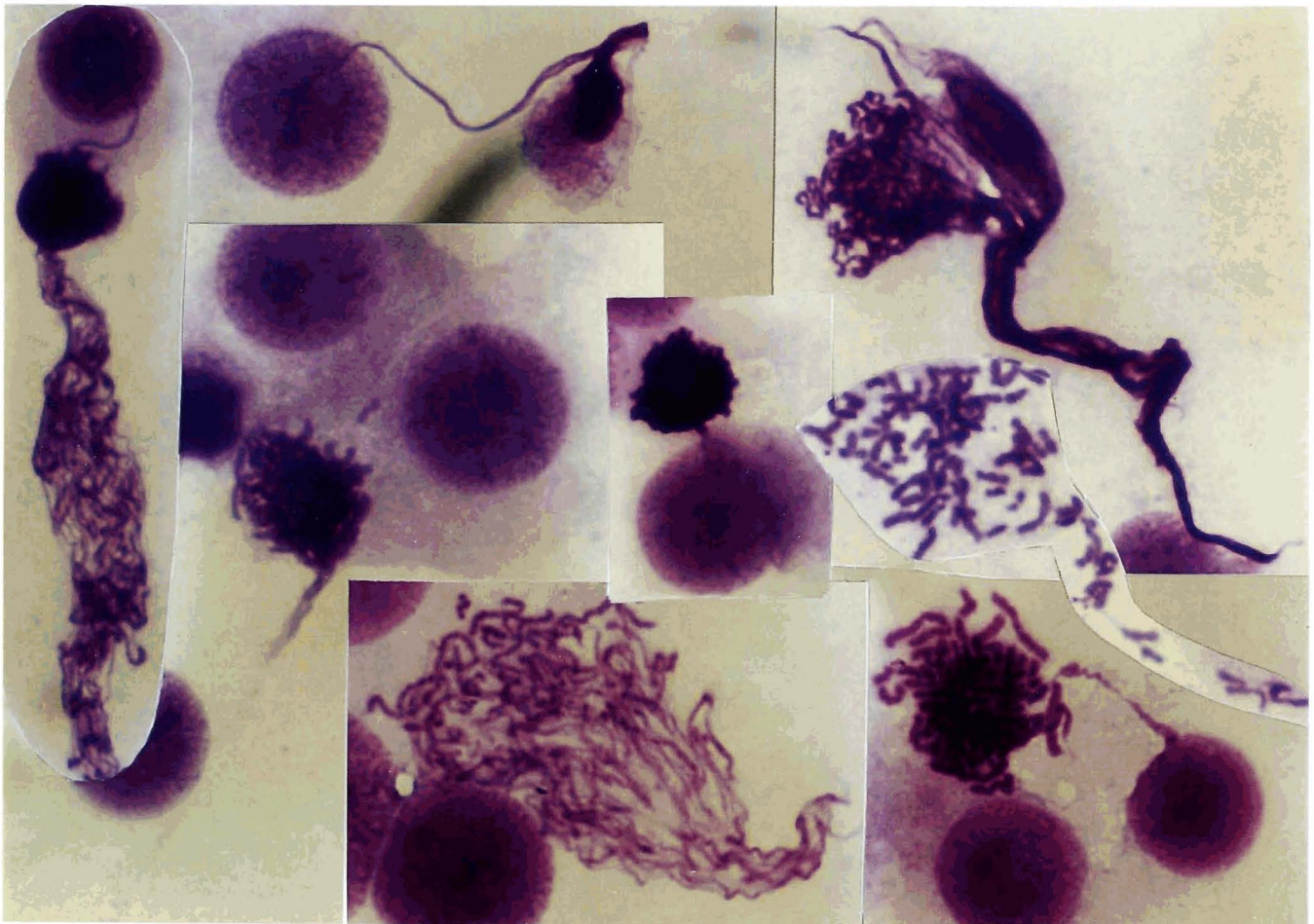


Fig. 2. NVs of different morphology in EBV-Raji and EBV-P3HR-1 cells. Giemsa, x 1,000

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characterized by nuclear vacuolation and nuclear ring formation (Fig. 12). Most of the seminiferous tubules were devoid of spermatozoa and a few contained only degenerated ones (Fig. 13). The ducti epididymidis showed lumen containing exfoliated

degenerating spermatoocytes, without spermatozoa (Fig. 14).

Control rats exhibited full spermatogenesis in all seminiferous tubules and ducti epididymidis full of intact spermatozoa (Figs. 15, 16).

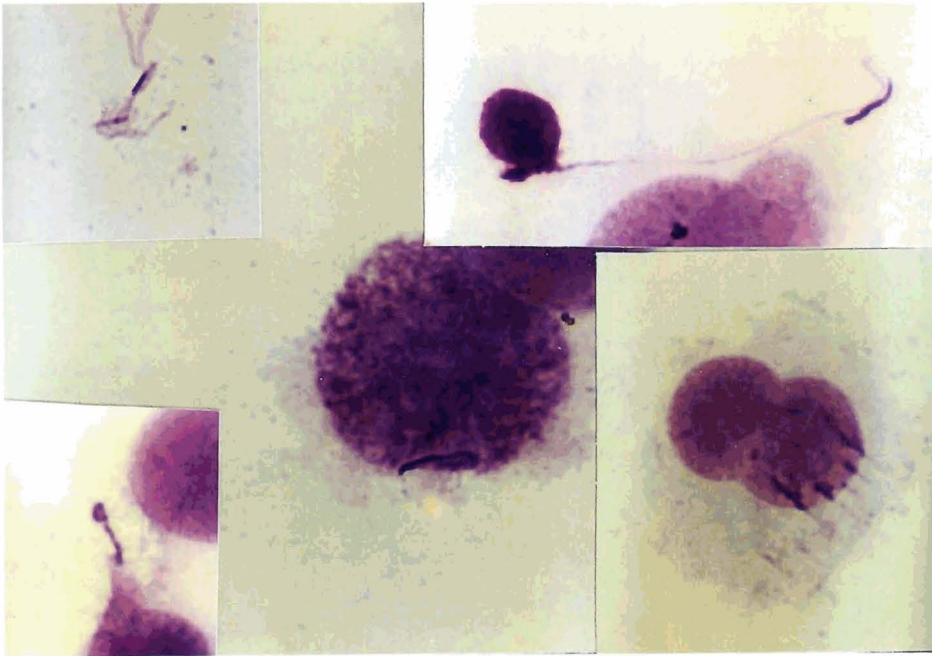


Fig. 3. NVs, of one chromosome or probably chromosomal fragments, free or invading other nuclei in EBV-P3HR-1 cells. Giemsa, x 1,000

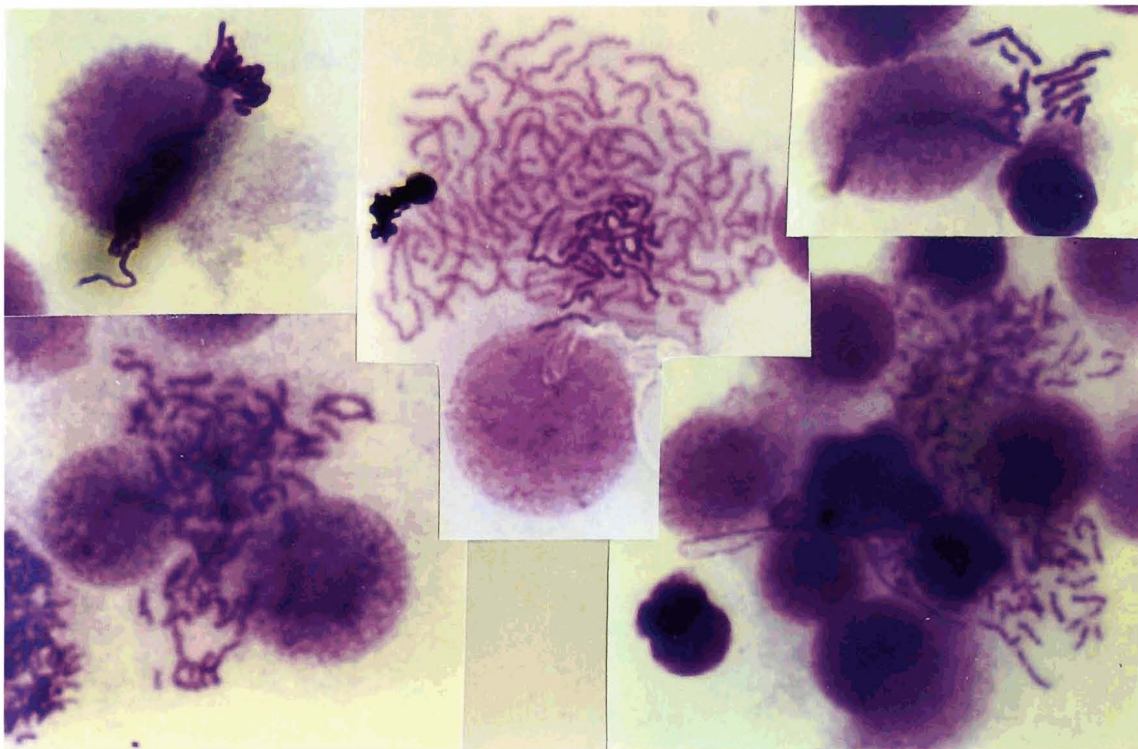


Fig. 4. Extrusion of chromosomes and metaphase fusion observed in both cell lines. Giemsa, x 1,000

Discussion

Spermatozoa are normal motile haploid cellular products of meiosis of in vitro short life-span, embedded in mucin, capable of invading and fusing with the oocyte, giving rise to hybrids (embryos) for propagation

and survival and can therefore be classified as parasites. Spermatozoa were used in this study as an appropriate in vitro system to study the effect of CANP-I on meiotic cellular products. CANP-I treated affected spermatozoa motility first and subsequently viability by inhibition of CANP. This documents that spermatozoa motility and

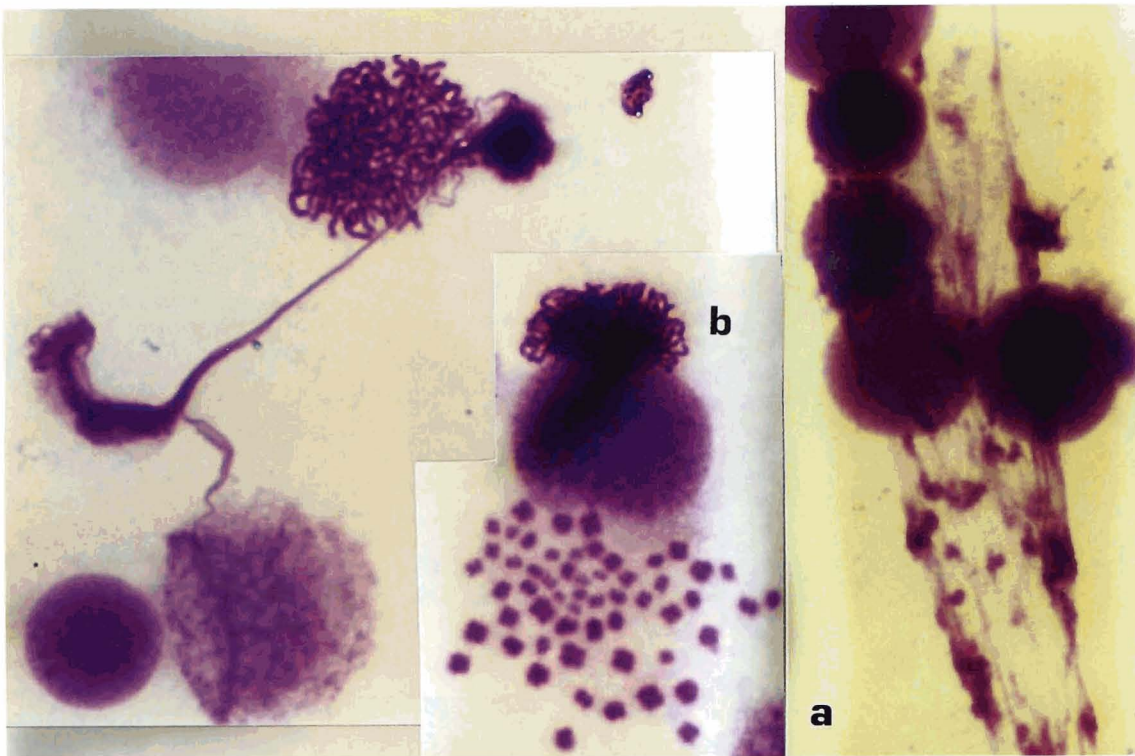


Fig. 5. Invasion of a pyknotic head NV into a metaphase and chromatin strand into a nucleus. **Inset a:** Transfer of chromosomes from one nucleus into another. **Inset b:** A parasitic cytoplasmic metaphase in a host cell the nucleus of which is undergoing meiosis. Giemsa, x 1,000

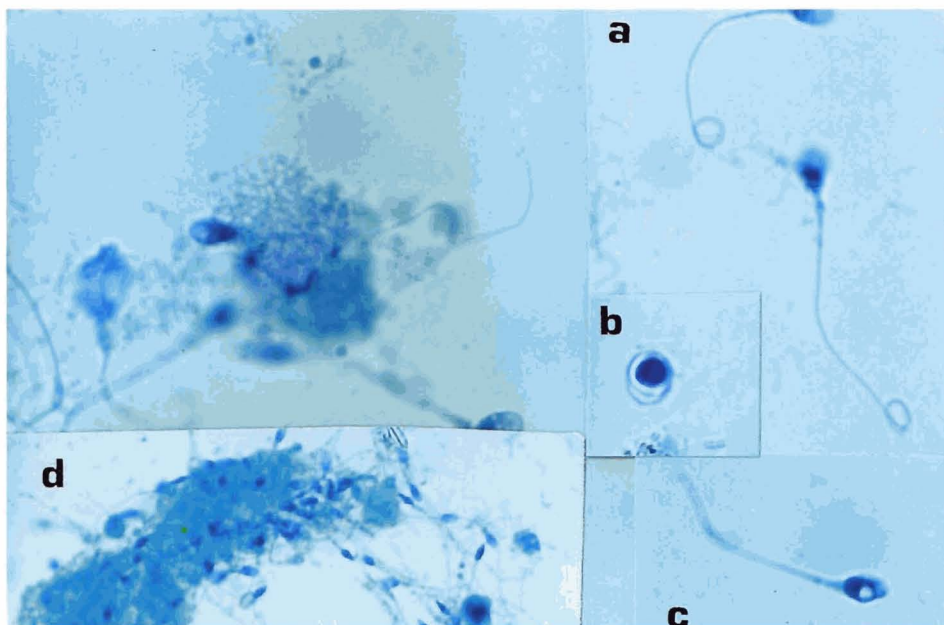


Fig. 6. CANP-I-treated spermatozoa showing basophilic green translucent, fibrillar to granular and haematoxylinophilic fibrillar to granular ECM. Papa, x 1,000. **Insets a,b:** coiled tail ends of CANP-I-treated spermatozoa. Papa, x 1,000. **Inset c:** nuclear vacuolation. Papa x 1,000. **Inset d:** Only basophilic green translucent fibrillar to granular ECM of untreated spermatozoa. Papa, x 400

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viability are dependent upon CANP. The increased spermatozoa motility observed with high Ca²⁺ concentration (Fakih et al., 1986) may be achieved via activation of spermatozoa endogenous CANP. This is the first report that spermatozoa contain CANP, opening new fields in the study of unexplained idiopathic male infertility and unsuccessful fertilization. The high Ca²⁺ release by the oocyte cortex upon polyspermy

prevention (Steinhardt et al., 1977) indicates involvement of the oocyte CANP-I and regulation of the CANP-CANP-I system in gamete interaction, for high Ca²⁺ activates CANP and CANP-I (Melloni et al., 1984). Vacuolation of semen GSG and the alteration of the basophilic ECM into haematoxylinophilic granules by CANP-I treatment, indicate that CANP is bound to GSG, a common substrate for CANP and CANP-I.

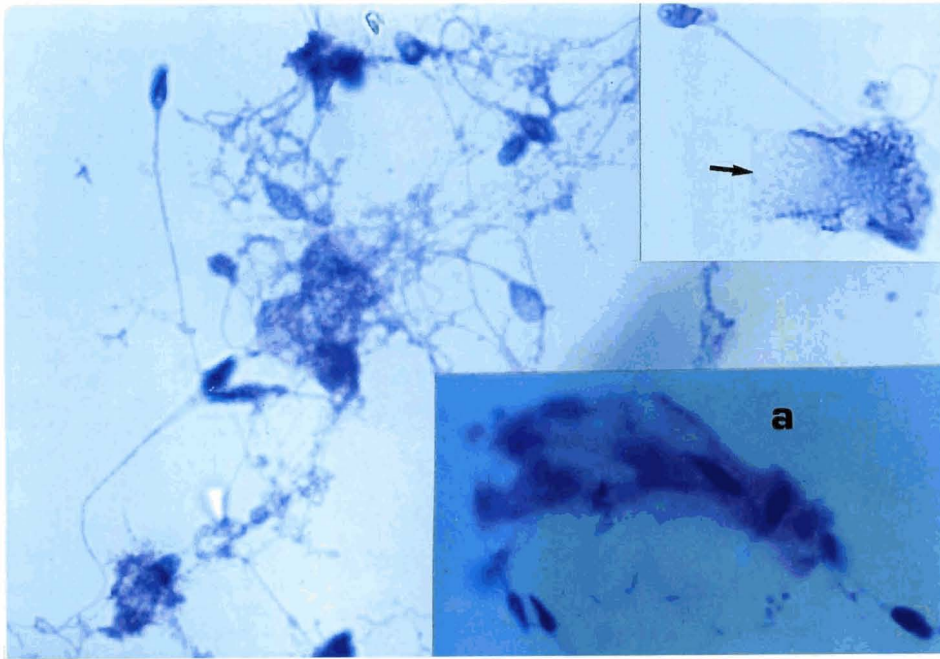


Fig. 7. CANP-I-treated spermatozoa and ECM, PAS-D negative. Arrow points at vacuolated ECM. PAS-D, x 1,000. **Inset a:** Untreated spermatozoa and ECM, PAS-D, positive. PAS-D x, 1000

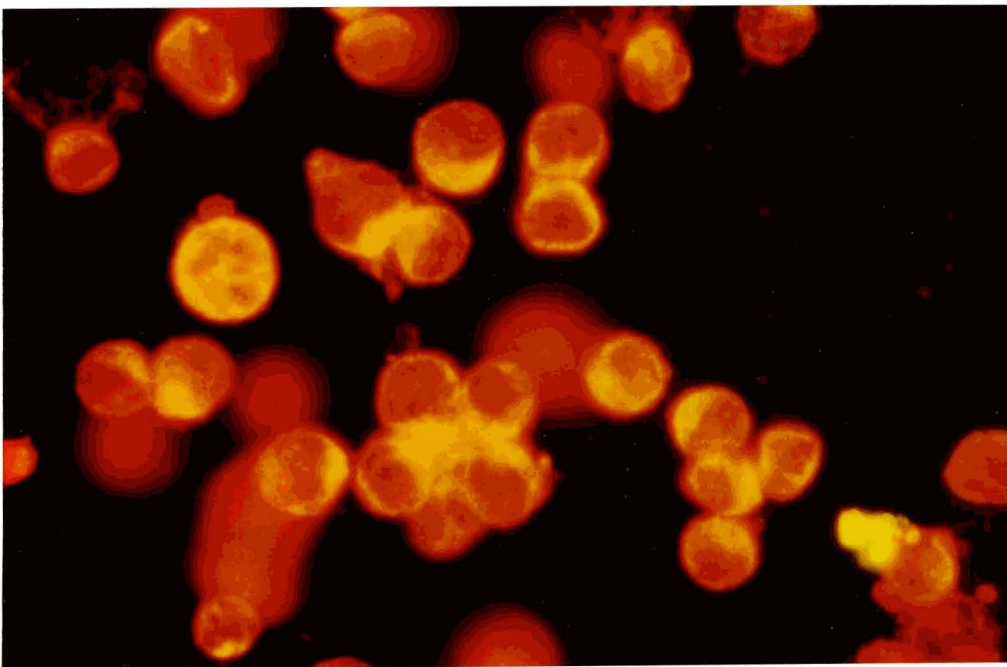


Fig. 8. Untreated EBV-Raji cells showing immunofluorescent VCA. Immunofluorescence, x 1,000

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Sperm nuclear vacuolation caused by CANP-I is identical to that of CANP-I on malignant cells and activated lymphocytes (Logothetou-Rella, 1994a,c) indicating that the mode of CANP-I cytotoxicity to different kinds of cells is the same. CANP is known to hydrolyze cytoskeletal proteins (Kawashima et al., 1988) and is therefore needed by spermatozoa, in order to penetrate the oocyte.

Mammalian testis consisting of spermatocytes

dividing by meiosis, spermatids and spermatozoa, provide an excellent system for studying anti-meiotic agents in vivo. CANP-I was spermicidal and cytotoxic to spermatocytes and spermatids in vivo, without affecting other tissues, documenting that CANP-I is an antimeiotic agent and CANP is associated with meiosis in vivo. The selective action of CANP-I (Logothetou-Rella, 1994c) is once more documented, since all tissues, consisting of cells dividing by mitosis, were free of cytotoxicity and

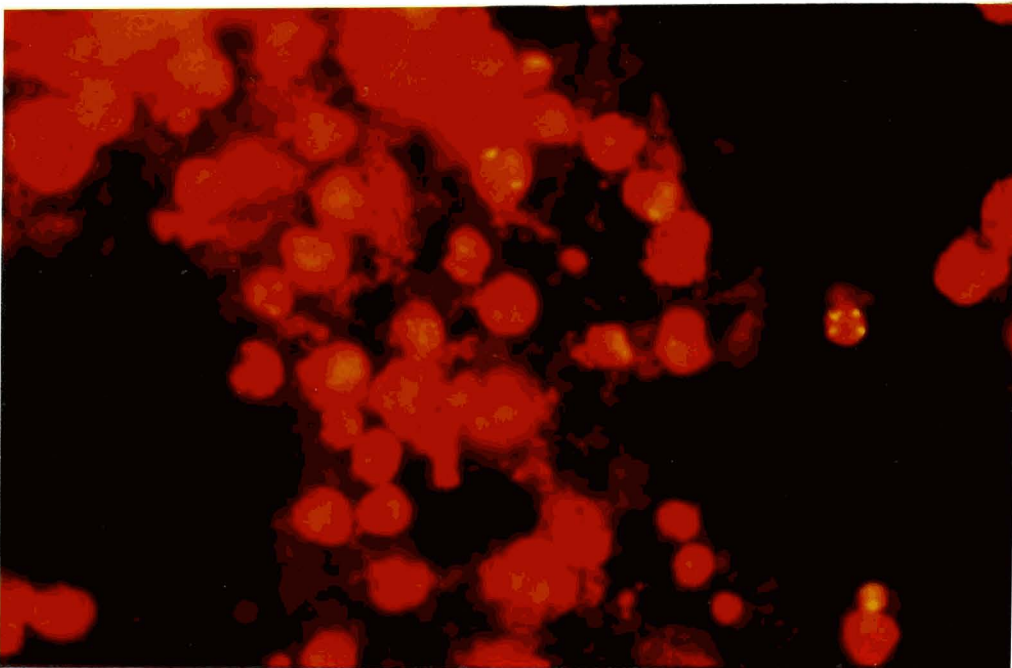


Fig. 9. CANP-I-treated EBV-Raji cells devoid of immunofluorescent VCA. Cells are small due to degeneration. Immunofluorescence, x 1,000

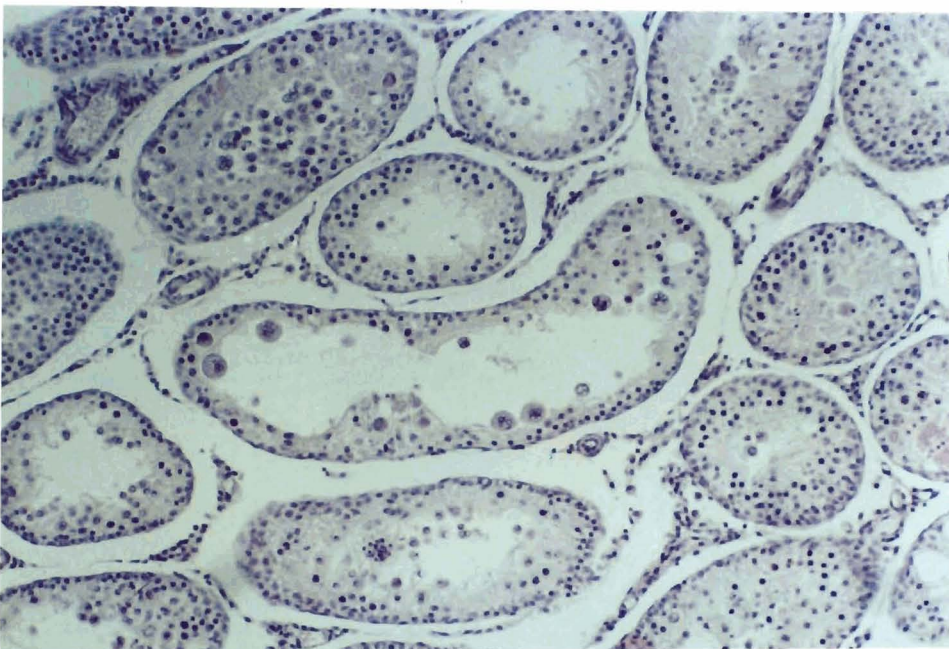


Fig. 10. Seminiferous tubules of CANP-I-treated rats devoid of spermatozoa, with exfoliated spermatozoa. H&E, x 100

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only testicular tissue, of high meiotic activity, was affected.

This study further documents that virally-infected and virus-producing malignant cells divide by meiosis; meiosis of these cells was demonstrated by the presence of NVs, NV invasion, «o» and condensed chromosomes, metaphase fusion, transfer of chromosomes and aneuploidy (Logothetou-Rella, 1994b). Although

aneuploidy is well known to be caused by meiosis (Goodenough and Levine, 1974) it has mostly been ignored in autoimmune diseases. Aneuploidy caused by random meiosis has recently been shown in malignant cells and activated lymphocytes (Logothetou-Rella, 1994a,b). Virally-infected and virus-producing cells also divide by random meiosis giving rise to aneuploidy showing only numerical chromosomal similarity.

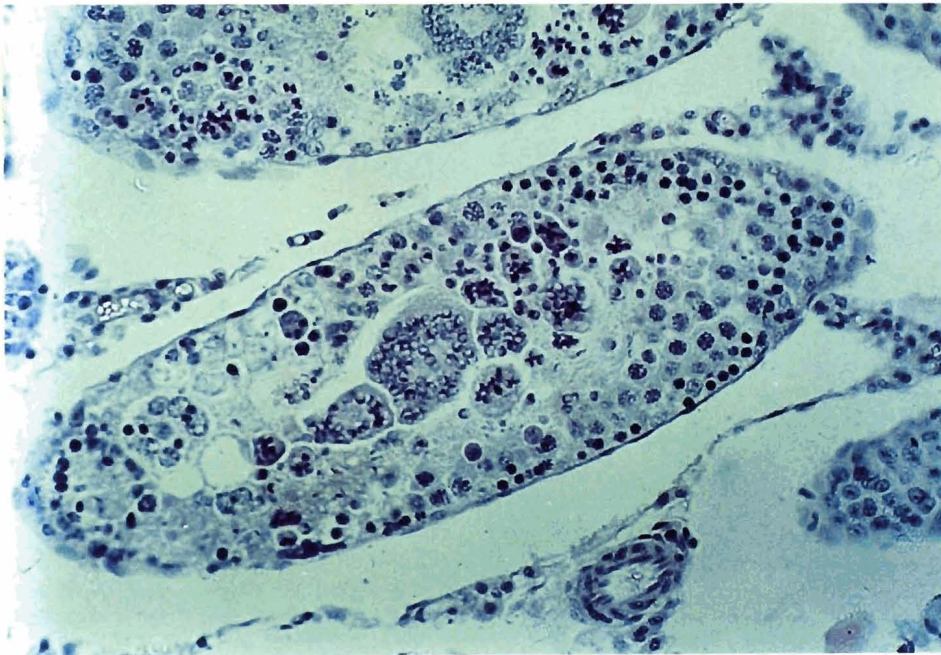


Fig. 11. Seminiferous tubules of CANP-I-treated rats showing degenerating spermatocytes and exfoliated syncytia of degenerating spermatids in the lumen. H&E, x 200

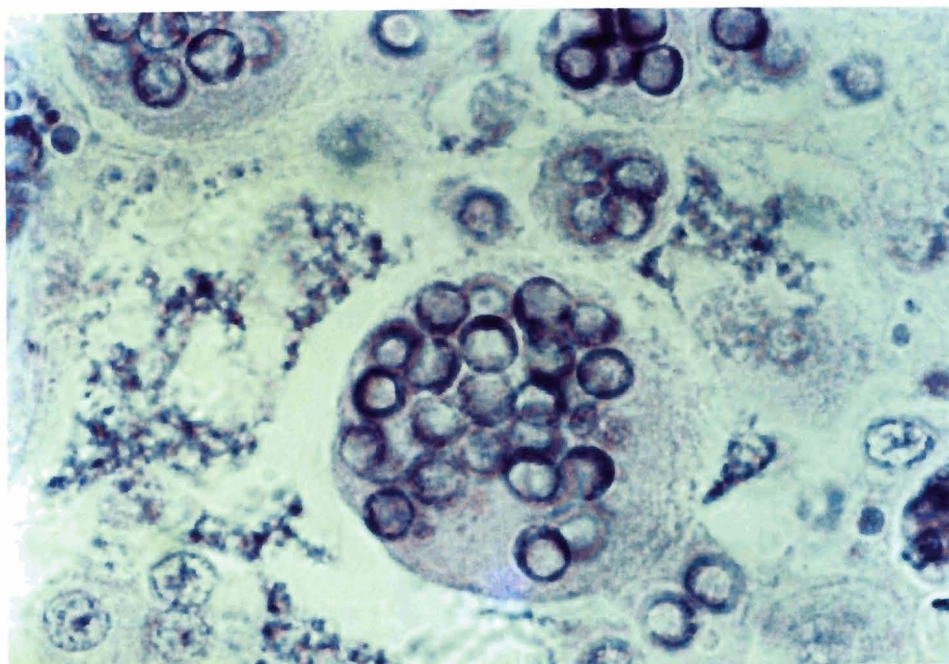


Fig. 12. Syncytia of degenerating spermatids characterised by nuclear vacuolation and nuclear ring formation in the lumen of seminiferous tubules of CANP-I-treated rats. H&E, x 1,000

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Virally-infected and virus-producing cells follow the life cycle of malignant cells (Logothetou-Rella, 1994b).

In this study virus-producing cells were more sensitive to CANP-I than virally-infected cells. This is probably attributed to the different CANP content of these cell lines. CANP-I was cytotoxic to all aneuploid cells, inhibiting all the events of the malignant cell life cycle such as meiosis, NV formation, NV invasion, cell,

nuclear and metaphase fusion. Although the three cell lines are sensitive to CANP-I because they are malignant, the disappearance of immunofluorescent viral antigens after treatment documents the antiviral action of the CANP-I and indirectly the CANP content of viruses. Viruses just like spermatozoa, malignant and lymphocytic NVs need CANP in order to invade host cells for propagation and survival. It is suggested that

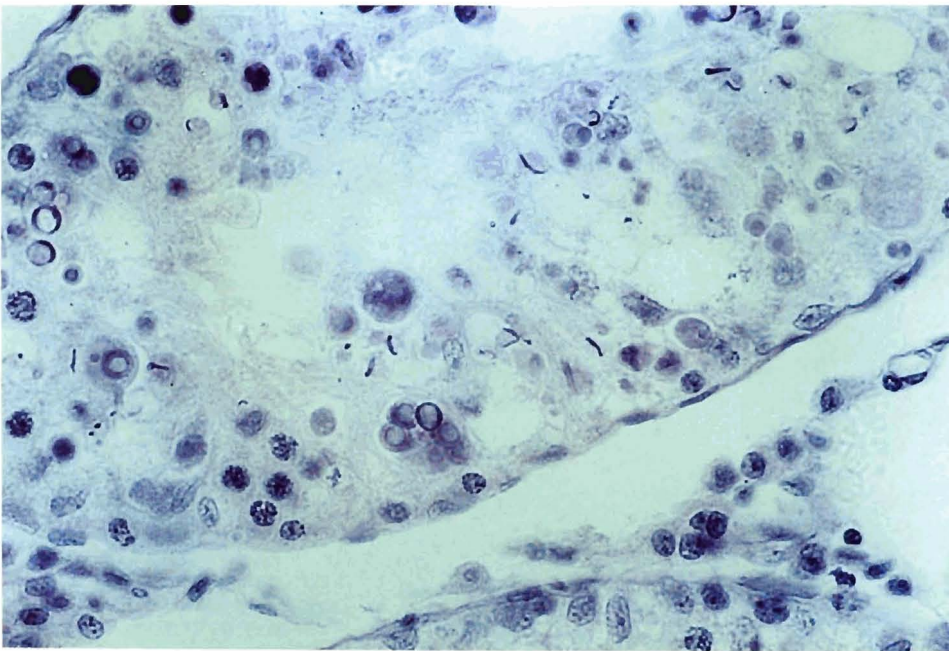


Fig. 13. Seminiferous tubule with remnants of degenerated spermatozoa of CANP-I-treated rats. H&E, x 400

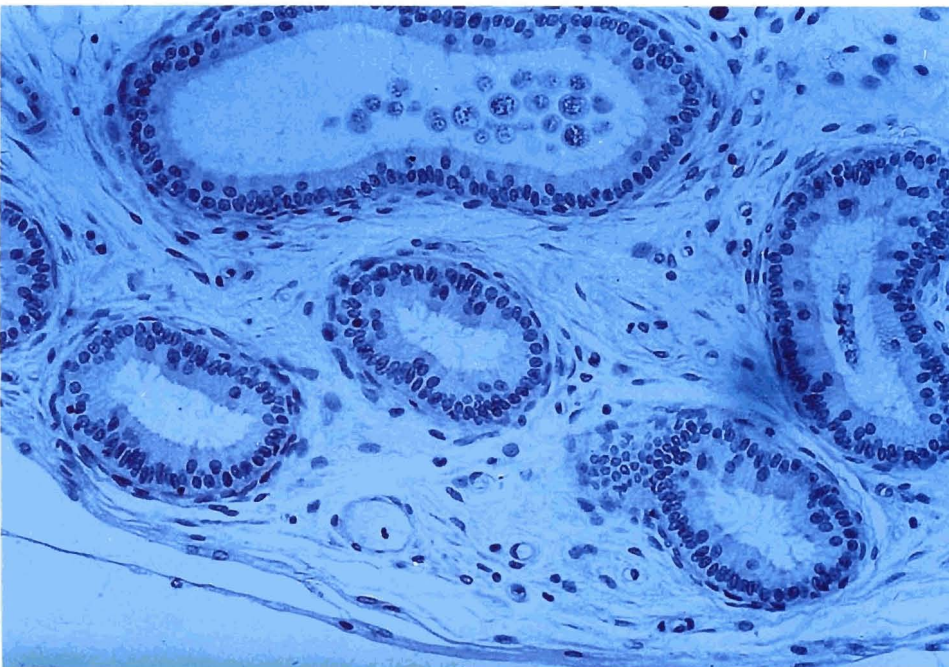


Fig. 14. Ductus epididymidis with exfoliated degenerating spermatocytes, devoid of spermatozoa. H&E, x 200

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viruses obtain CANP from the host cell intracellular CANP pools, during viral replication. The malignant cell lines P3HR-1, Raji and MOLT-4, easily infected by viruses in vitro as well as lymphocytes infected by HIV-1 in vivo, document that cells undergoing meiosis are vulnerable and susceptible to viral infections. This is probably attributed to the property of meiotic cells to act as donors and recipients of genetic material identical to

sperm-oocyte fertilization (Logothetou-Rella, 1994b). It cannot be excluded that viral, malignant and lymphocytic NV invasion into somatic host cells may switch on meiotic instead of mitotic division of somatic host cells and trigger initiation and promotion of neoplasia.

The antiviral action of CANP-I in vivo has not been documented in this study due to the lack of safe animal laboratory conditions.

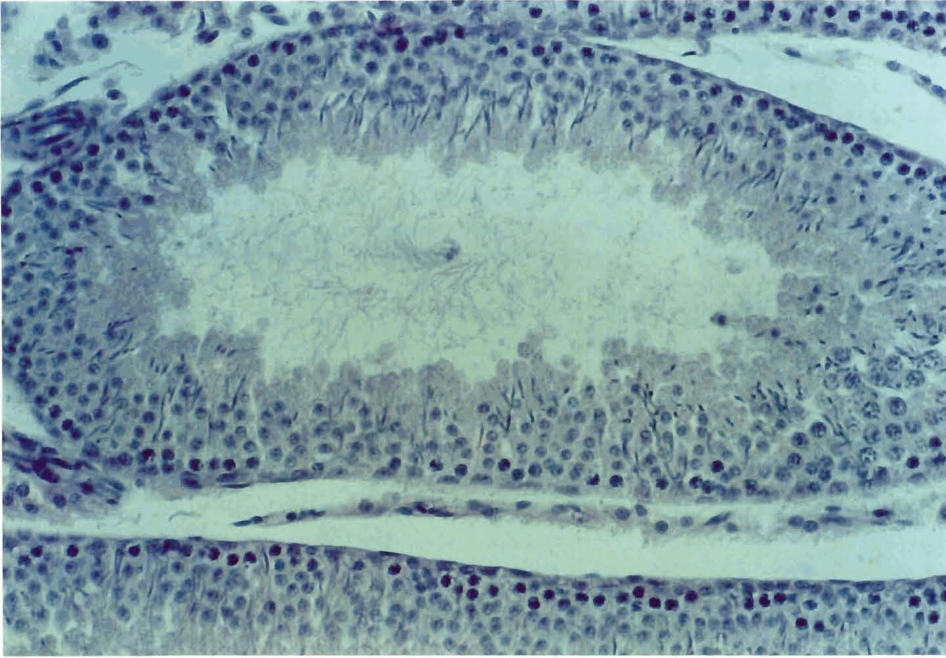


Fig. 15. Seminiferous tubule of untreated rat. H&E, x 200

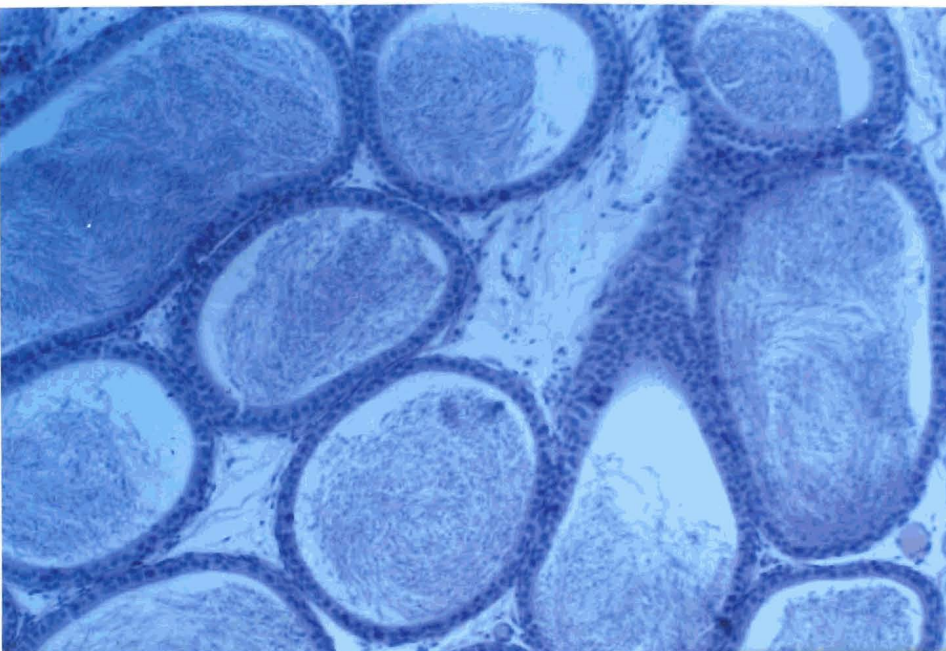


Fig. 16. Ductus epididymidis of untreated rat full of spermatozoa. H&E, x 200

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Meiotic aneuploidy, NVs, CANP content and GSG ECM are common properties of parasitic cells such as activated lymphocytes, malignant, virally-infected, virus-producing cells and gametocytes (Logothetou-Rella, 1993a, 1994a,b,d). CANP has also been associated with other parasites such as *Shistosoma mansoni* (Karcz et al., 1991), and with the invasion of human erythrocytes by the *Plasmodium falciparum* (Olaya and Wasserman, 1991). In addition, CANP has been detected in human arthritic synovial joints (Yamamoto et al., 1992) where lymphocytic NVs are involved.

In conclusion, CANP-I is an inhibitor of meiosis and can be used as a male contraceptive, antiviral agent and generally against autoimmune diseases involving meiotic cell division and interaction.

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