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Study of apoptotic DNA fragmentation in human spermatozoa

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The aim of our work was to define and better understand apoptosis in the spermatozoa of normal subjects, infertile patients and patients affected by specific tumoral diseases employing the method of the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling and confirming the results by electron microscopy. We studied 23 healthy, normozoospermic subjects (group A), 29 oligo-asthenoteratozoospermic patients, affected by various andrological pathologies (group B), 28 patients with Hodgkin's disease (C1) and 30 patients with testicular cancer (C2). Our data demonstrate that the percentage of apoptosis in normozoospermic subjects (group A) is significantly lower than in all the other groups (B, C1, C2) ($P < 0.001$). This confirms that high DNA fragmentation is one of the characteristics of spermatogenetic failure. The induction of apoptosis, which can also be a basic response to neoplastic disease, can even act right up to the mature male gamete. Our results suggest that apoptosis could be the final result of various pathologies and of a deregulation of spermatogenesis control systems.

Key words: apoptosis/electron microscopy/Hodgkin's disease/human spermatozoa/testicular cancer

Introduction

Apoptosis is a mode of cellular death based on a genetic mechanism that induces a series of cellular, morphological and biochemical alterations, leading the cell to suicide (Kerr *et al*., 1972; Majno and Joris, 1995; Nagata, 1997). This process takes place at specific moments in normal embryonic development to allow the definitive form of tissues and in adult life to discard cells which no longer have a function or which have an altered function (Vaux *et al*., 1999).

Apoptotic cells undergo characteristic modifications in form, which result in cell shrinkage. This is the most important morphological feature of apoptosis, which was originally defined as 'shrinkage necrosis' (Kerr, 1971). From a biochemical point of view, one of the most important characteristics of the apoptotic process is the activation of Ca/Mg-dependent endonucleases, that break DNA in a specific manner into nucleosomal units of about 185 bp. These can be identified as distinct bands of DNA using agarose gel electrophoresis and staining with ethidium bromide (Wyllie *et al*., 1980). The process is genetically controlled and can be triggered by an internal clock (Kerr *et al*., 1994; Wyllie, 1994) or by extracellular agents such as hormones, cytokines and numerous chemical, physical or viral agents.

Spermatogenesis is a process of cell differentiation characterized by mitotic and meiotic divisions, which transform the stem spermatogonia into final mature spermatozoa. This process leads to a clonal expansion that must be counteracted by mechanisms able to control the overproduction of male gametes. In recent years considerable evidence has accumulated to suggest that apoptosis is responsible for cellular proliferation control in the testis (Sakkas *et al*., 1999a; Sinha Hikim and Swerdloff, 1999); the classic apoptotic morphological and biochemical patterns have been demonstrated in the cells of the rat seminiferous tubules (Brinkworth *et al*., 1995; Blanco-Rodriguez and Martinez-Garcia, 1996; Yazawa *et al*., 1999). Further studies have demonstrated spontaneous germ cell apoptosis in spermatogonia, spermatocytes and spermatids in the testis of normal men and in patients with non-obstructive azoospermia (Sinha Hikim *et al*., 1998; Jurisicova *et al*., 1999). Unlike numerous studies carried out in intratesticular cells, data on apoptosis in human spermatozoa are scarce (Baccetti *et al*., 1996; Sun *et al*., 1997).

The aim of our work was to define and better understand apoptosis in the spermatozoa of normal subjects, of infertile patients and of patients affected by specific tumorous diseases employing the classic method of the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling (TUNEL). Results were confirmed by electron microscopy.

Materials and methods

Patients

We studied 110 subjects who underwent seminal fluid evaluation at the Laboratory of Seminology and Immunology of Reproduction of the Department of Medical Pathophysiology of the University of Rome 'La Sapienza'. They were subdivided into the following three groups. Group A (normal subjects who acted as the control group): 23 healthy, normozoospermic subjects, aged 23–39 years, not medically or surgically treated in the 3 months prior to the study. Each seminal sample was studied *in toto* and after swim-up: group A1:

raw seminal samples; group A2: spermatozoa after swim-up. Group B (infertile patients): 29 patients, suffering from primary infertility for at least 2 years, aged 22–41 years, affected by various andrological pathologies, not medically or surgically treated in the 3 months prior to the study; this group was made up of oligoasthenoteratozoospermic (OAT) patients selected on the basis of the following seminal inclusion criteria: $\leqslant 20\times10^6$ cells/ml, forward motility $\leqslant$25%, atypical forms >70%. Group C: patients affected by neoplastic disease: 58 patients, aged 17–39 years, selected from the subjects attending our sperm bank before the start of chemotherapy or radiotherapy, which are potentially capable of blocking spermatogenesis or inducing damage to the genome with subsequent risk of transmitting genetic alterations to the offspring (Howell and Shalet, 1998). We subdivided this group into two subgroups according to pathology: group C1, 28 patients with Hodgkin's disease; group C2, 30 patients, 20 of whom had seminoma and 10 testicular embryonic carcinoma. These patients had undergone unilateral orchidectomy about a month before the evaluation of seminal fluid.

Seminal samples

The samples were collected by masturbation into sterile plastic jars, after 3–5 days of sexual abstinence. They were allowed to liquefy for 30 min at room temperature (22°C) and were then evaluated according to the World Health Organization (1999). The variables taken into consideration were: volume of ejaculate (ml), sperm concentration ($\times10^6$/ml), forward motility (%), morphology (% of atypical forms).

Swim-up

One millilitre of each seminal sample of group A was diluted 1:2 with Earle's solution and centrifuged for 10 min at 300 *g*. After centrifugation the supernatant was discarded and an aliquot of 0.5 ml of Earle's solution was layered on the pellet. The spermatozoa were allowed to migrate for 30 min at 37°C, in 5% CO_2. After migration, the supernatant was gently aspirated and examined with a light microscope and processed for the study of apoptosis.

TUNEL

DNA fragmentation was evaluated using TUNEL (Boehringer Mannheim, Cat. No 1684795, Mannheim, Germany) which can be summarized as follows: seminal fluid was washed twice in phosphate-buffered saline (PBS) and cytocentrifuged (Shandon Inc., Pittsburgh, PA, USA; cytospin 3) for 5 min at 200 *g* on polylysine-coated slides that were fixed in methanol for 30 min at room temperature. They were then incubated with a solution of 0.1% Triton X-100 (Sigma, St Louis, MO, USA; T-8787) and 0.1% sodium citrate (Sigma, C-8532) for 2 min on ice. After washing the slides twice and letting them air dry, a 30 µl TUNEL mixture, composed of terminal deoxynucleotidyl transferase (TdT) and fluorescein-dUTP, was added to each sample. They were covered with 22×22 mm coverslips. The samples were incubated 60 min at 37°C in a moist chamber in the dark, washed three times with PBS and then analysed using a fluorescence microscope (Leitz Dialux 22, Leica, Wetzlar, Germany), counting at least 500 cells.

In each experiment we used the following controls: positive control, fixed and permeabilized spermatozoa incubated with DNase I (Sigma, D-4263) (1 µg/ml) for 10 min at room temperature, in order to induce DNA fragmentation; negative control, fixed and permeabilized spermatozoa incubated in a moist chamber in the dark for 60 min at 37°C with 30 µl of a solution containing fluoresceinated nucleotides without TdT.

Head morphology in apoptotic spermatozoa

In order to evaluate the relationship between DNA fragmentation and the morphology of single spermatozoa, we have reported the percentages of the various types of sperm head atypical forms showing apoptosis, according to the following classification: amorphous, small, tapered, round, double. For this purpose, we carried out a double evaluation of spermatozoa showing the characteristic fluorescence pattern by reading simultaneously each microscopical field using both transmitted (bright field) and reflected (dark field) light (×500). We did this for each group (A1, A2, B, C1, C2).

Electron microscopy

Fixation of human spermatozoa was performed using the Karnovsky mixture (Karnovsky, 1965) for 2 h at 4°C. The fixed spermatozoa were then centrifuged at 3000 *g* for 15 min. The pellet was washed in 0.1 mol/l cacodylate buffer (Agar Scientific Ltd, Stansted, Essex, UK) at pH 7.2 overnight and then post-fixed for 1 h at 4°C in 1% buffered osmium tetroxide (Electron Microscopy Sciences, Fort Washington, PA, USA), dehydrated in ethanol, embedded in Epon resin (Electron Microscopy Sciences) and semithin and ultrathin sections were cut with a Reichert (Reichert-Leica, Nussloch, Germany) ultramicrotome. Ultrathin sections were collected on copper grids, contrasted with uranyl acetate and lead citrate and observed with a Zeiss (Jena, Germany) EM109 electron microscope.

Statistical analysis

Comparison between percentage of apoptosis and variables of groups A1 and A2 was carried out by employing the analysis of variance (ANOVA) test for pairwise data and by confirming the significance using the Mann–Whitney *U*-test. Mean values of group A1 (control group) were compared using Student's *t*-test with mean values of groups B, C1 and C2. Finally, mean values of group B were compared using Student's *t*-test, with those of groups C1 and C2.

Correlation index (*r*) was calculated to compare the age of the subjects/patients and the sperm parameters (sperm concentration, motility, morphology) with the percentage of DNA fragmentation in the various groups studied.

Results

Detection of DNA fragmentation using TUNEL

The mean and SD of age, sperm parameters, and percentage of apoptosis of the five groups are reported in Table I. The results of A1 were taken as controls for the other groups.

Comparison of the values of the percentage of apoptosis found in the spermatozoa of A2 (carried out using ANOVA test for paired data, confirmed by Mann–Whitney *U*-test) showed a statistically significant reduction ($P < 0.001$) compared with A1. The percentage values of apoptosis found in the spermatozoa of groups B, C1 and C2, compared with A1, showed a statistically significant increase ($P < 0.001$). The percentages of apoptosis found in the spermatozoa of groups C1 and C2, compared with B, were not statistically significant.

Regarding the correlation calculated between the percentage of apoptosis and the age of the patients and the sperm parameters, the results can be summarized as follows (Figure 1): age of the patients (not reported in the Figure): there was no correlation in all the groups studied; sperm concentration: there was a significant negative correlation in group C2; forward motility: there was a significant negative correlation in group A1, C1 and C2; atypical forms: there was a significant positive correlation in groups B, C1 and C2.

Concerning the relationship between DNA fragmentation

Table I. Patient age, sperm parameters and percentage of apoptosis in the various groups (mean ± SD; range in parentheses)

Subjects	Age (years)	No. of spermatozoa/ml ($\times 10^6$)	Forward motility (%)	Atypical forms (%)	Apoptosis (%)
Group A1	32.0 ± 4.7 (23–39)	96.5 ± 37.1 (50–180)	57.6 ± 5.8 (50–65)	47.1 ± 4.7 (42–55)	2.5 ± 1.2 (0.9–4.4)
Group A2	32.0 ± 4.7 (23–39)	28.6 ± 12.0 (10–55)	87.4 ± 4.5 (80–90)	25.7 ± 2.8 (22–32)	1.2 ± 0.7[a] (0–2.5)
Group B	32.7 ± 4.8 (22–41)	13.7 ± 5.8 (1.5–20)	14.7 ± 8.3 (0–25)	82.1 ± 8.8 (72–100)	11.0 ± 4.3[a] (6.5–24)
Group C1	26.9 ± 5.5 (17–38)	62.7 ± 39.3 (4–140)	44.3 ± 13.4 (10–65)	51.9 ± 10.8 (42–85)	11.3 ± 4.9[a,b] (5–22)
Group C2	28.5 ± 4.7 (21–39)	42.7 ± 31.8 (8–120)	40.5 ± 12.8 (15–65)	56.7 ± 10.6 (44–80)	11.3 ± 4.4[a,b] (4.9–20.4)

[a]$P < 0.001$ versus group A1.
[b]Not significant versus group B.
For definition of groups, see text.

and the sperm head morphology of all the cells showing apoptosis, the results can be summarized as follows: 32.5% amorphous, 43.0% small, 2.2% tapered, 9.5% round, 11.5% double: 1.3% normal (Figure 2). The percentages of the various types of head morphology found in each group are shown in Table II. Figure 3 shows morphological pictures of atypical spermatozoa in apoptosis evaluated using fluorescence microscopy at reflected and transmitted light.

Evaluation of the apoptotic patterns using electron microscopy

Electron microscopy showed characteristic apoptotic patterns in a greater number of spermatozoa in groups B, C1 and C2 compared with control group A. This is in agreement with the results obtained by TUNEL.

Ultrastructural apoptotic features in the nucleus and cytoplasm of the spermatozoa in the ejaculates showed different patterns related to the various phases of the apoptotic process. They can be summarized as follows (Figures 4 and 5): the nuclei showed an irregular (Figure 4a,b), enlarged (Figures 4c and 5a,b), or fragmented shape (Figures 4d,e and 5e). Initially chromatin was partially disrupted by the presence of one or more nuclear vacuoles in which a fine fibrillar material was observed (Figures 4a,c and 5a,b). In some cases, the chromatin appeared condensed near the periphery, close to the nuclear envelope in one or several large and homogeneous masses (Figure 4b,d,e). In other cases, chromatin showed an incomplete condensation pattern, displaying also a loose fibrillar-microgranular network (Figure 5a–d). The nuclear envelope, even though mainly continuous (Figures 4a,b and 5a,b), sometimes appeared irregular, enlarged (Figure 4c–e), or disrupted (Figure 5a,c,e). Excessive production of membrane in the post-acrosomal region was observed (Figure 4a). The acrosome appeared normal; sometimes it was partially empty, undulated (Figure 4a), enlarged (Figure 5f) or completely absent (Figures 4c,d,e and 5a–d).

The cytoplasmic droplet around the neck and middle piece was mainly condensed and occupied by lysosomes, autophagic vacuoles as well as autophagolysosomes containing membrane structures (Figure 4b–e). Mitochondria were scarce (Figure 4a,c). The plasma membrane was generally normal. In some cells, the plasma membrane had an irregular profile or formed large evaginations (Figures 4b and 5c). The cell membrane was also mainly endocytosed, forming numerous clear, large vesicles in the cytoplasm with transparent contents (Figures 4b,c and 5a,c,e).

Numerous large spheroidal elements were found in the samples from groups B and C. They were sometimes devoid of the nucleus, and contained some organelles such as mitochondria, vacuoles, and abundant microgranular cytoplasmic material (Figure 5f). Other large spheroidal elements had residual parts of the axoneme and residual fragments of nuclear chromatin (Figure 5e). These spheroidal elements can be considered the final stage of the apoptotic process called apoptotic bodies.

Discussion

Programmed cellular death is an extremely interesting biological phenomenon and is involved in the regulation of the growth, differentiation and homeostasis of many multicellular organisms (Raff, 1992). In response to various stimuli, cells are able to trigger or to hinder the expression of genes responsible for cell suicide (Kerr *et al*., 1972). The physiological demise of cells due to apoptosis has received a great deal of attention in the last few years. As far as the testis is concerned, studies have shown that germ cell apoptosis is increasingly involved in male gonadal pathophysiology (Billig *et al*., 1996). In fact, intratesticular programmed cell death was suggested as a possible mechanism for the control of the clonal expansion of spermatogenesis. The most relevant biochemical characteristic of apoptotic death is the activation of endogenous endonucleases, which induce numerous breaks in the double strand following degradation of DNA and chromatin condensation. This breakage can be detected using exogenous DNA polymerase (nick-translation assay) or terminal deoxynucleotidyl transferase (TdT). Indeed, the reaction based on the TdT is specific for apoptotic cells and allows us

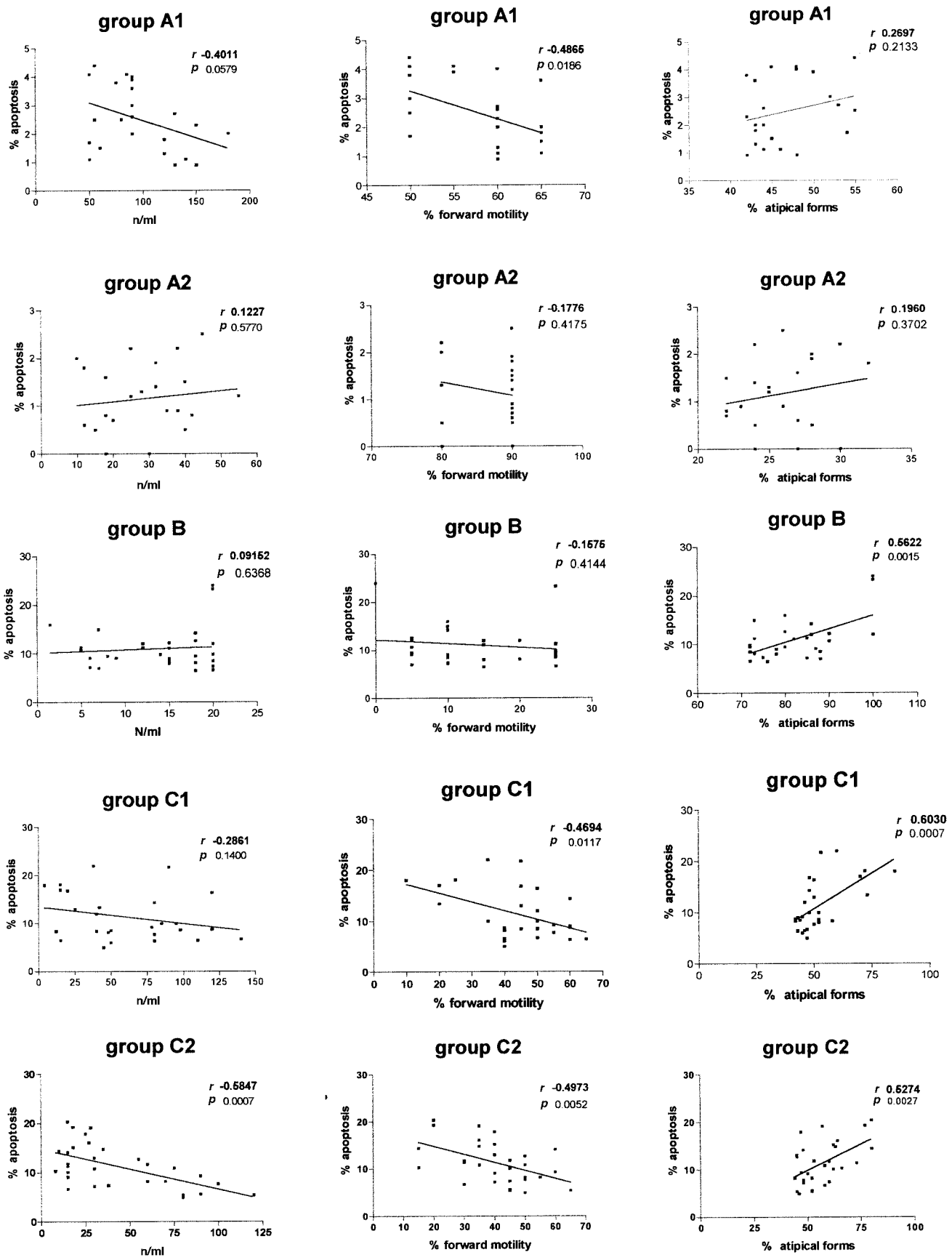

Figure 1. Relationship between the percentage of apoptosis and sperm concentration (no./ml), percentage of forward motility, percentage of atypical forms, in the three goups A, B, C (normal subjects, infertile patients and patients with neoplastic disease respectively; A1 = raw semen; A2 = spermatozoa after swim-up; C1 = Hodgkin's; C2 = seminoma or testicular embryonic carcinoma).

to distinguish DNA strand breakage in apoptotic cells from that of necrotic cells (Gorczyca *et al.*, 1993).

The aim of our paper was to evaluate the presence of apoptosis in human spermatozoa, employing terminal deoxynucleotidyl transferase, and to quantify this process, correlating it with sperm parameters in selected groups: normal subjects, infertile patients and patients affected by neoplastic disease. We selected a group of healthy, fertile subjects with normal semen parameters as a control in order to verify whether there was an apoptotic sperm cell population in the seminal fluid of subjects without pathologies and free of current or previous medical treatment. Infertile patients were chosen on the basis of a severe OAT in order to verify whether such spermatogenetic damage was correlated with apoptosis. Neoplastic patients (seminoma, testicular carcinoma, Hodgkin's disease) were chosen on the premise that in the pathogenesis of neoplastic diseases there is an alteration of apoptosis (Macleod and Jacks, 1999). In fact, cellular growth can be consequent not only to an increased proliferation but also to a reduction of apoptosis after appropriate stimulus.

In all the groups, we evaluated sperm apoptosis in raw semen; in the normal subjects we also studied spermatozoa selected after swim-up. This latter control was carried out because swim-up allows the recovery of the best spermatozoa from a kinetic and morphological point of view. These spermatozoa should be the least involved in apoptosis since their role is to fertilize the female gamete, the final act of the reproductive process. In fact, they represent the cellular pool which is similar to that selected during the transit in the female genital tract and is also employed in some assisted reproduction techniques.

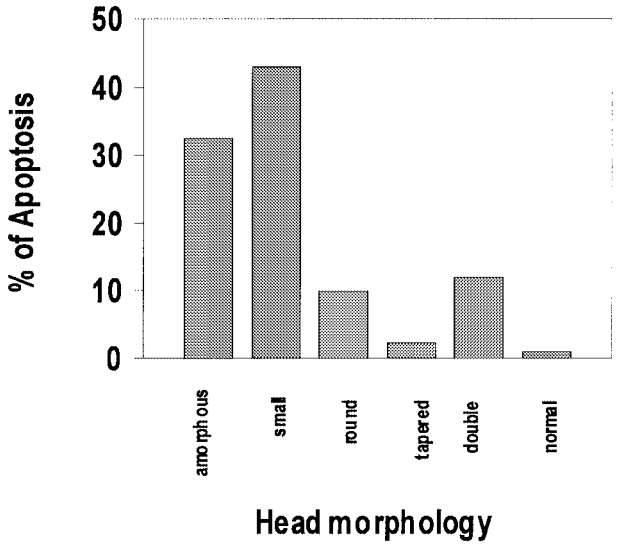

Figure 2. Relationship between DNA fragmentation and the sperm head morphology of all the cells showing apoptosis.

Our data demonstrate that the percentage of apoptosis in the fertile subjects (group A1) was significantly lower than all the other groups (B, C1, C2). A lower percentage of apoptosis was detected only in group A2. The significant difference in the percentage of apoptosis between groups A1 and A2 demonstrates that the simple selection of motile spermatozoa really can eliminate most of the apoptotic spermatozoa, excluding them from a possible fertilizing process. However, when certain assisted reproduction techniques are employed in which the natural selection of the male gametes does not take place (e.g. intracytoplasmic sperm injection), the higher percentage of apoptosis could lead to the risk of microinjecting a spermatozoon with fragmented DNA. In physiological conditions, such spermatozoa would be excluded from fertilization, as demonstrated by the reduced percentage of apoptosis in post swim-up spermatozoa (group A2).

In the group of patients with OAT (group B) we demonstrated a percentage of apoptosis $>10\%$ confirming that high DNA fragmentation is one of the characteristics of spermatogenetic failure. One of the possible hypotheses on the aetiology of OAT is represented by the action of oxidative stress. It has been demonstrated that reactive oxygen species (ROS) act at various levels on spermatozoa. In fact, they can act on the polyunsaturated fatty acids (PUFA) in the sperm membrane. The alteration of the PUFA pattern of the membrane produces significant modifications in most mammalian cells including changes in the activity of different lipid-dependent enzymes and in the resistance to physical or chemical stress (Lenzi *et al.*, 1996). Moreover, ROS are capable of inducing damage to sperm chromatin via single and double DNA strand breaks (Hughes *et al.*, 1996). In a recent work, it was demonstrated that ROS can cause a dramatic increase in DNA fragmentation when incubated with spermatozoa and that this time-dependent damage is significantly reduced when antioxidants are added (Lopes *et al.*, 1998). Moreover, it has been demonstrated that low concentrations of hydrogen peroxide can induce apoptosis, while necrosis takes place only at higher concentrations (Sentman *et al.*, 1991). The effect of oxidant agents could be modulated by the same genes which regulate the equilibrium of cell life and death. In fact, some authors have proposed that Bcl-2, the negative regulator gene of programmed cellular death, protects the cells from apoptosis owing to its capacity to reduce ROS production (Kane *et al.*, 1993); this allows the

Table II. Percentages of the various types of head morphology showing apoptosis found in each group

Groups	Amorphous	Small	Tapered	Round	Double	Normal
A1	51.9	42.9	1.3	0	2.6	1.3
A2	80.0	6.7	0	0	13.3	0
B	20.6	33.0	3.7	21.4	20.2	1.0
C1	36.9	54.0	1.0	0.3	6.0	1.7
C2	40.8	50.7	1.1	1.5	4.4	1.5

For definition of groups, see text.

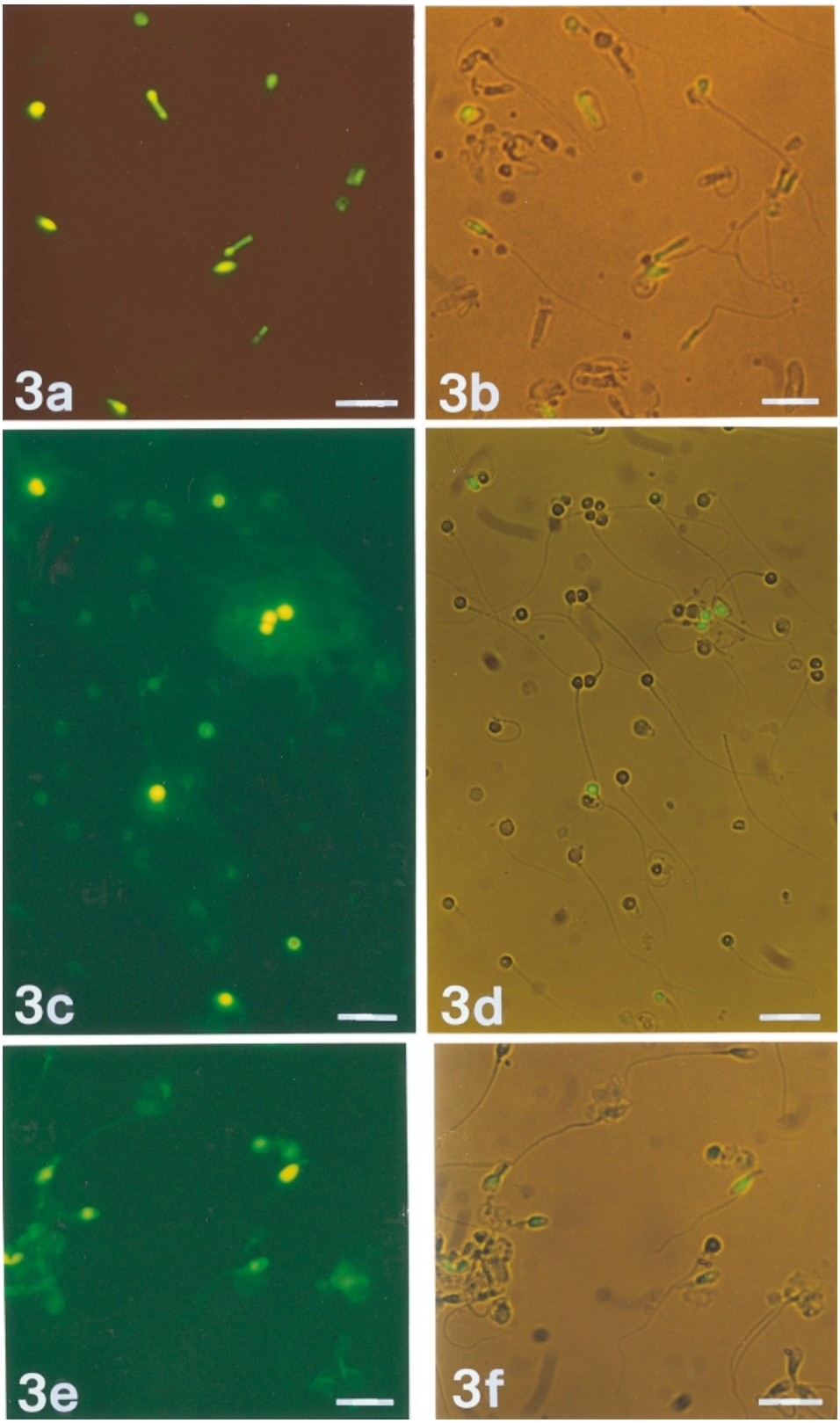

Figure 3. Apoptotic spermatozoa detected by fluorescence microscopy (500×): (**a**,**b**,**c**,**d**). Patients infertile: tapered and round heads evaluated at reflected light on dark field (**a**,**c**) and at transmitted light on light field (**b**,**d**). (**e**,**f**) Patient affected by seminoma: spermatozoa evaluated at reflected light on dark field (**e**) and transmitted light on light field (**f**).

cell to avoid the accumulation of these substances and to maintain a level of oxidation in keeping with cell survival. One could postulate that ROS, acting negatively on sperm function, may also be responsible for triggering DNA apoptotic fragmentation.

Regarding the patients affected by Hodgkin's disease or

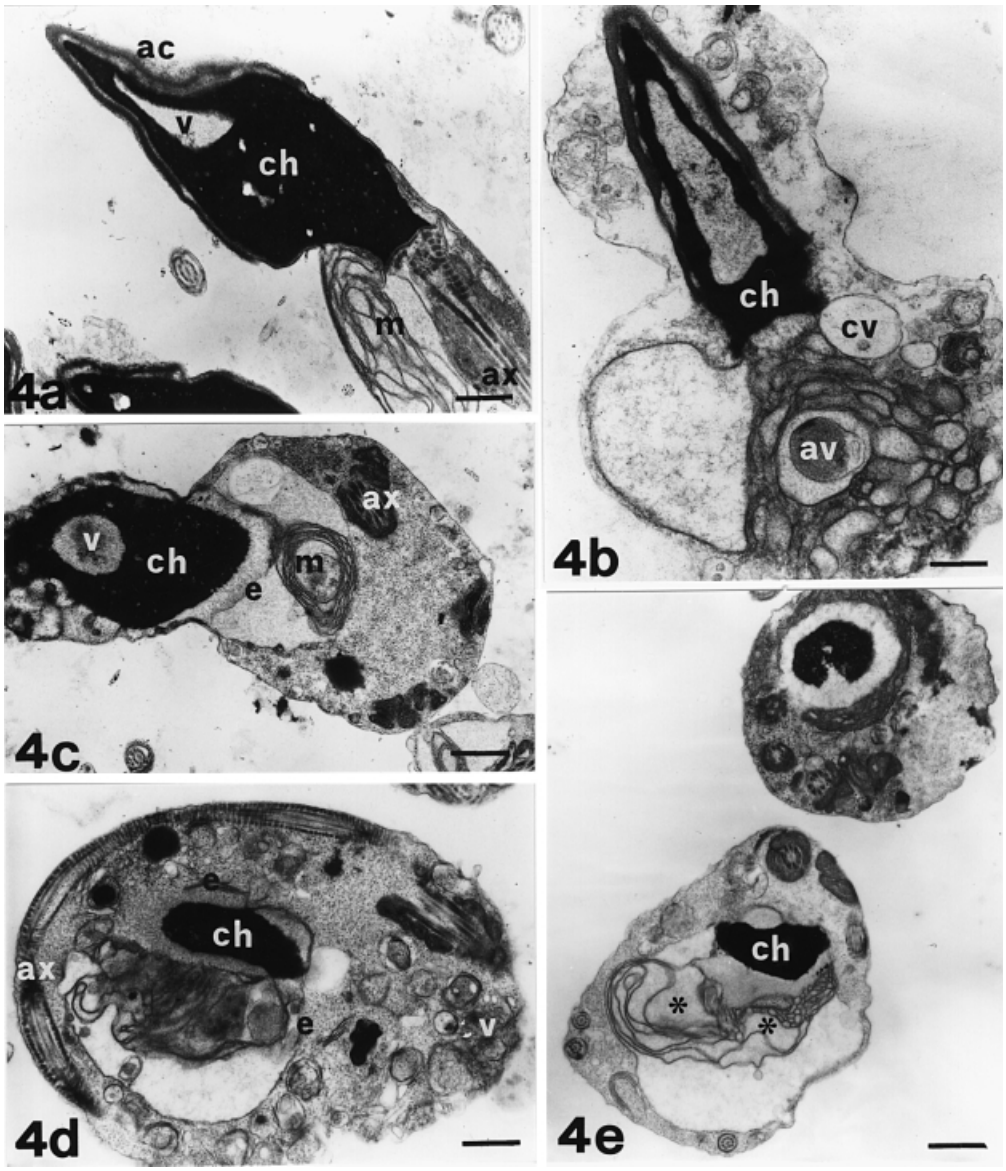

Figure 4. Electron microscopy: longitudinal/cross-sections of apoptotic spermatozoa. (**a**, **b**, **c**) Patient affected by seminoma. Vacuoles were observed in the nuclear chromatin (**a** and **c**). Chromatin was condensed near the envelope (**b**). The acrosome was partially empty (**a**) or absent (**c**). Excessive production of nuclear membrane in the post-acrosomal region was observed (**a** and **c**). **a**: bar = 0.8 µm; **b**: bar = 0.55 µm; **c**: bar = 1.15 µm. (**d**, **e**) Infertile patient. Nuclear chromatin was condensed and fragmented (**d**). The nuclear envelope (**e**) appeared enlarged, interrupted (**d**), and showed a very complicated network (asterisks) (**e**). The axoneme was rolled-up in the cytoplasm. **d**: bar = 1.05 µm; **e**: bar = 1.3 µm. v = vacuoles; ch.= nuclear chromatin; ac = acrosome; m = membrane; cv = cytoplasmic vacuoles; e = nuclear envelope; ax = axoneme; av = autophagic vacuoles.

testicular cancer, it is interesting to point out that the percentages of apoptosis are significantly higher compared with the control group. This demonstrates that in these patients the induction of apoptosis, which can also be a basic response to neoplastic disease, can act even up to the mature male gamete stage. Our results regarding the average of the sperm parameters of the patients with Hodgkin's disease (group C1) showed apparently normal spermatogenesis from a qualitative and quantitative point of view; this is in disagreement with the data of other groups, which have reported alterations of spermatogenesis (for a review see: Meirow and Schenker, 1995). However, the analysis with TdT and electron microscopy demonstrated a high DNA fragmentation with considerable damage of the sperm chromatin. It should be emphasized that our patients affected by Hodgkin's disease were studied very precociously, immediately after diagnosis, and that the average age of the patients was low (26.9 years). Furthermore, all the patients were studied before the start of the chemotherapy; in this way we excluded the cytotoxic effects of drugs on the apoptotic process in spermatozoa, and this could mean that a deregulation of cell death control in Hodgkin's disease is responsible for parallel sperm damage. This is also true for patients affected by testicular cancer. These patients present a similar high incidence of apoptosis; an interesting finding given that they were studied after the surgical removal of the neoplastic testis. Therefore, the spermatozoa we studied came from an apparently healthy testis, that is, not involved in the neoplastic pathology. This led us to postulate that in these

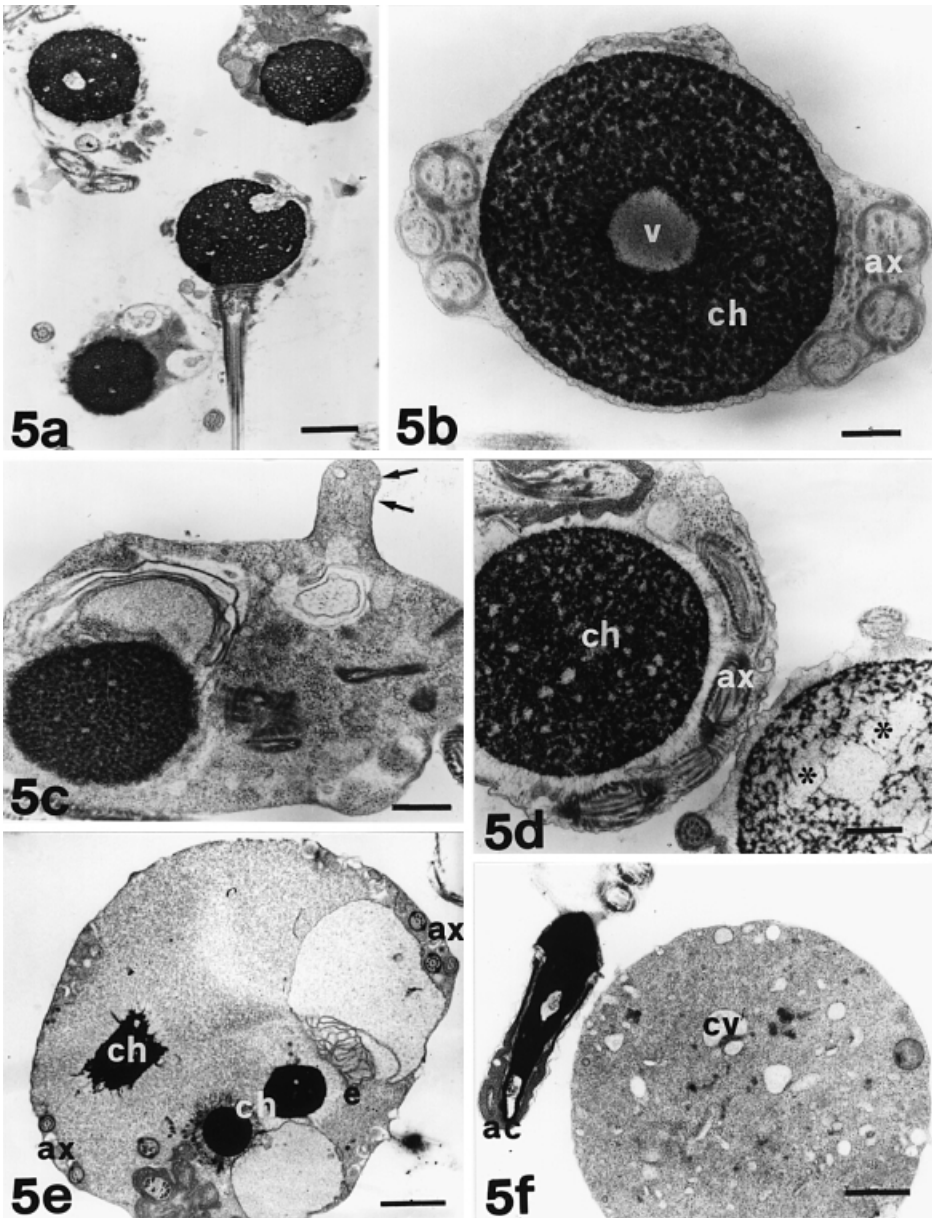

Figure 5. Electron microscopy: longitudinal/cross-sections of apoptotic spermatozoa. (**a**–**d**) Infertile patient affected by globozoospermia. Nuclear chromatin was decondensed. Nuclear vacuoles were present. Chromatin displayed loose fibrillar-microgranular pattern (asterisks) (**d**). Axonemes, rolled-up in the cytoplasm, showed structural abnormalities (**b**). Plasma membrane evagination (arrows) (**c**). **a**: bar = 1.3 μm; **b**: bar = 0.5 μm; **c**: bar = 0.75 μm; **d**: bar = 0.8 μm. (**e**, **f**) Patient affected by seminoma. Advanced stage of apoptotic degeneration (**e**). The nuclear chromatin was fragmented, the nuclear envelope was largely disrupted and abundant microgranular cytoplasmic material was evident. Apoptotic body containing clear vacuoles and abundant cytoplasmic material and a spermatozoon containing an enlarged acrosome were observed (**f**). **e**: bar = 1.5 μm; **f**: bar = 1.25 μm. v = vacuoles; ch = nuclear chromatin; ac = acrosome; cv = cytoplasmic vacuoles, e = nuclear envelope; ax = axoneme.

patients there was an intratesticular alteration in the system of apoptotic control as a reaction to the neoplastic cell proliferation. In fact, we can postulate a modification of the control system capable of expressing or overexpressing Fas on the spermatogenetic cells (Sakkas *et al*., 1999b) and/or of inducing an overexpression of Fas ligand by Sertoli cells in the testicular or seminal microenvironment (Nagata, 1995; Dondero *et al*., 1999; Lee *et al*., 1999). As is well-known, Fas is a membrane protein that contains a 'death domain', which is able to trigger apoptosis following bonding with its specific Fas ligand (Lee *et al*., 1999).

Regarding the possible correlation between the percentage of apoptosis and the age of the patients, we did not find any correlation in the various groups studied. This was as expected since the subjects under examination were young adults; only three were in their forties (40–41 years old). This is not an absolute finding and relates only to the patients in our study.

Concerning the relationship between apoptosis and seminal parameters, our study noted an increase of DNA fragmentation in line with the decrease of sperm concentration and motility. This correlation is even more evident with atypical forms, confirming that the results for morphology are strictly correl-

ated with sperm function. In this regard, our data indicate that most of the cells showing apoptosis also have atypical head forms. Indeed, examining the sperm morphological aspect of cells showing DNA fragmentation, we found that the most common atypical forms were small and amorphous heads. Amorphous heads seem to be the most characteristic defect in the spermatozoa of healthy subjects before and after swim-up selection. In groups B, C1 and C2 the most represented atypical forms are small heads, and then amorphous heads. The highest percentage of tapered, round and double heads were in group B. This can be explained by the fact that there are three patients in this group with monomorphic atypical heads: one 100% tapered, one 100% round (mostly double) and one 100% round. It is worth underlining that our data demonstrate that only rarely are spermatozoa with normal morphology affected by apoptosis. This shows that DNA fragmentation is strictly correlated with alterations of the mechanisms that allow normal spermatogenesis. This is confirmed by the finding that spermatozoa separated by swim-up not only show few signs of DNA fragmentation, but also a complete lack of correlation between apoptosis and seminal characteristics; this would be explained by the optimization of seminal parameters obtained by selection.

As shown in our ultrastructural observations, as well as described by others (Baccetti *et al*., 1996, 1997), several ultrastructural nuclear and cytoplasmic features in ejaculated spermatozoa were similar to the apoptotic features described in literature (Uchiyama, 1995). However, other specific ultrastructural degenerative features concerning the structure of the acrosome and the axoneme were observed in some apoptotic spermatozoa. These alterations may be considered as a consequence of the apoptotic sequence, and not strictly related to the apoptotic mechanism. Severe alterations in these organelles may be the consequence of a process triggered at a testicular level, whereas some alterations may be related to apoptosis starting at a post-testicular level. Other ultrastructural features such as an excessive production of nuclear membrane may be related to the apoptotic process. In addition, we observed an unusual chromatin pattern probably associated with the apoptotic process, especially in round-headed spermatozoa. In fact, we found decondensed chromatin associated with nuclear vacuoles, whereas in other round-headed spermatozoa a loose fibrillar-microgranular chromatin network was seen. This particular pattern is likely to be an advanced stage of the apoptotic process leading to the destruction of the nuclear material.

In conclusion, our results confirm that it is quite common to find sperm apoptotic DNA fragmentation in seminal fluid and demonstrate that apoptosis depends on the pathophysiological condition of the subject. It seems likely that apoptosis is the final result of various pathologies and of a deregulation of spermatogenesis control systems. The question to be answered is whether apoptotic spermatozoa are the result of a process triggered at the spermatogonial level or if nuclear fragmentation derives from a process during the last phases of spermatogenesis or even at the post-testicular level.

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