INDUCTION OF MALE GERM CELL APOPTOSIS BY TESTOSTERONE WITHDRAWAL AFTER ETHANE DIMETHANESULFONATE TREATMENT IN ADULT RATS

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Objective. To carry out a detailed quantitative analysis of male germ cell apoptosis in seminiferous epithelium in a long period after EDS administration.

Methods. The apoptosis in adult rat testes was induced by a single i.p. injection of ethane dimethanesulphonate (EDS) in a dose of 75 mg/kg body weight. The TUNEL assay for in situ detection of apoptosis and quantitation of apoptotic germ were performed in testicular sections days 1, 3, 7, 14, 21 and 35 after EDS treatment. Plasma levels of testosterone (T) and luteinizing hormone (LH) were measured by RIA.

Results. First signs of seminiferous epithelium regression were manifested by a marked increase in the number of apoptotic cells on 3rd day after EDS treatment. The maximal value of germ cell apoptosis was established on 7th day post EDS that coincided with lowest T levels. Later, until the end of investigated period, the elevated values of all investigated parameters for quantification of germ cell apoptosis decreased, but remained still higher as compared to control and, in addition, also T concentrations returned to normal range and their mean values were lower than these in controls. The pachytene spermatocytes and spermatids were the predominant cell types that underwent apoptosis after EDS treatment.

Conclusions. Quantitative patterns of germ cell death after testosterone deprivation reveal in advance the kinetic of germ cell depletion and regeneration in a long period after EDS. These new findings bring additional support to the concept that germ cell apoptosis is a hormonally regulated process. Induction of germ cell apoptosis by EDS could be considered as a result of differential alterations occurring in the main testicular cell types, more than one pathway being probably involved in that physiological cell death in the testis.

Key words: EDS - Apoptosis - Spermatogenesis - Rat

Spermatogenesis in mammals is a dynamic and highly regulated process that encompasses numerous differentiating steps, leading to the production of the male gametes. Apoptosis is a process of programmed physiological cell death characterized by certain morphological and biochemical signs including a reduction in cell volume, blebbing of cell membrane, DNA fragmentation, chromatin condensation, migration and formation of apoptotic bodies (WYLLIE 1987; KERR 1992). The availability of a in situ method (TUNEL) to visualise and localise DNA fragmentation in male germ cells undergoing apoptosis, either spontaneously or in response to a variety of death triggering signals, opens new avenues in the understanding of the significance of germ cell apoptosis during normal and abnormal states of spermatogenesis (SINHA HIKIM and SWERDLOFF 1999). In adult mammals, including humans, germ cell death is conspicuous during normal spermatogenesis and plays a pivotal role in sperm output. Spontaneous germ cell apoptosis occurs in the testis during foetal

and postnatal development in mammals and apoptotic germ cells can be found at all stages of spermatogenic cycle (Boulogne et al. 1999). A growing body of evidence demonstrates both the spontaneous and induced germ cell death after various treatments, such as hormonal withdrawal, ionizing radiation, administration of cytotoxic drugs, hyperthermia, ischaemia and vitamin A deficiency (Blanco-Rodriguez 1998). Apoptosis has been proposed as a mechanism by which the testicular germ cells are removed during normal and various pathological conditions (Henriksen and Parvinen 1998). The survival of male germ cells in the immature and adult testes depends on FSH as well as on LH dependent intratesticular androgen production (Sharpe 1994).

Manipulation of spermatogenesis by deprivation of survival factors provides a basis for detailed study on the regulatory mechanisms of germ cell death. Treatment with ethane dimethanesulfonate (EDS) that selectively and temporary eliminates Leydig cells in the testis is a valuable tool for investigating male germ apoptosis in response to androgen withdrawal. (Henriksen and Parvinen 1998; Woolveridge et al. 1999). Male germ cell apoptosis in response to hormonal deprivation was investigated in experimental treatments with potent GnRH-antagonists (Billig et al. 1995; Sinha Hikim et al. 1995, 1997) or EDS and germ cell death was studied in details in a short time-window post EDS when LCs were completely missing from the testis (Sharpe 1994; Henriksen et al. 1995; Woolveridge et al. 1999). Kinetics of apoptotic events was not examined in a long period after EDS treatment when new LC population developed in the testis. In this respect the current study aimed to carry out a detailed quantitative analysis of the temporal changes of male germ cell apoptosis in seminiferous epithelium following EDS administration in order to develop our previous results and understanding on kinetics of germ cell death in the testis. (Bakalska et al. 2001).

Materials and Methods

Animals and treatments. Adult Wistar rats bred in our animal house were maintained under standard conditions. Food and water were provided ad libitum. Testosterone withdrawal in adult rats was induced by single i.p. injection of EDS in a dose of 75 mg/kg body weight dissolved in dimethylsulfoxide and water (1:3, v/v). Animals were sacrificed at 1, 3, 7, 14, 21 and 35 days after EDS. Plasma samples were stored at –20° C until used for hormonal analysis of testosterone and LH by RIA. Testes were fixed in Bouin's solution, dehydrated and embedded in paraffin.

In situ assay of apoptosis. Paraffin cross sections (5 μm) were mounted on coated slides, deparaffinized and rehydrated. Apoptotic cells were detected in situ by using terminal deoxynucleotidyl transferase (TdT)-mediated digoxigenin-11-dUTP nick end labelling (TUNEL) method that resulted in a high degree of specificity and low background staining (Sharpe et al. 1998). Endogenous peroxidase activity was blocked in 3 % (v:v) H_2O_2 in methanol for 30 min. After two washes (5 min each) in PBS (0.01 M, pH 7.4), slides were placed on an ice-cold plate, and 5 nM digoxigenin-11-dUTP and 50 U/ml terminal deoxynucleotidyl transferase (TdT) (both from Roche Diagnostics GmbH, Mannheim, Germany) were added in enzyme buffer comprising 30 mM Tris-HCl, pH 7.2, 140 mM sodium cacodylate and 1.5 mM $CoCl_2$ (both from Sigma Chemical Co., St. Lous, MO). For negative control slides, only the enzyme buffer lacking TdT was added. The slides were immediately cover slipped using GelBond (FMC Bio-Products, Rockland, ME), sealed with a rubber solution (Cowgum; Cow Proofing Ltd., Slough, UK) in hexane, and incubated at 37 °C for 30 min. The cover slips were then removed and the slides washed twice in PBS, followed by blocking for 10 min at room temperature (RT) with normal rabbit serum (NRS) in PBS (1:4). Sections were then incubated for 90 min at RT in a humidified chamber with sheep anti-digoxigenin antibody (Roche Diagnostics GmbH, Mannheim, Germany) diluted 1: 100 in the blocking solution. After a further two washes in PBS the slides were incubated for 30 min with biotin-conjugated rabbit anti-sheep immunoglobulins (Vector Laboratories, Peterborough, UK) diluted 1:500 in TBS containing NRS (1 part NRS: 4 parts TBS) at RT in a humid chamber. Two washes in TBS (5 min each) were followed by incubation for 30 min at RT with horse-radish peroxidase-avidin biotin complex (Dako, Glostrup, Denmark) diluted in 0.05 M Tris-HCl, pH 7.4, according to the manufacturer's instructions. The sections were again washed twice in TBS, then visualised with liquid DAB+Substrate-Chromogen System (Dako, Glostrup, Denmark) and reaction was stopped in water. Finally, sections were lightly counter stained with hematoxylin, dehydrated, cleared in xylene, and cover slipped using Pertex mounting medium (CellPath plc).

Quantitation of apoptotic cells. For assessment of apoptosis, the percentage of seminiferous tubules with apoptotic germ cells was determined by scoring 75 ran-

domly selected tubules per section on four sections from different animals at each time point after EDS administration (WOOLVERIDGE et al. 1999). The number of apoptotic cells per tubule was assessed on four sections. The apoptotic index was calculated by multiplying the percentage of tubules containing apoptotic germ cells by the number of apoptotic germ cells per tubule at each time point after EDS. The data obtained were statistically analyzed by Student's t-test.

Hormone measurements. Serum luteinizing hormone (LH) and testosterone (T) levels were measured by radioimmunoassay (RIA) as described by FRASER and SHANDOW (1977) and SHARPE and BARTLETT (1985), respectively.

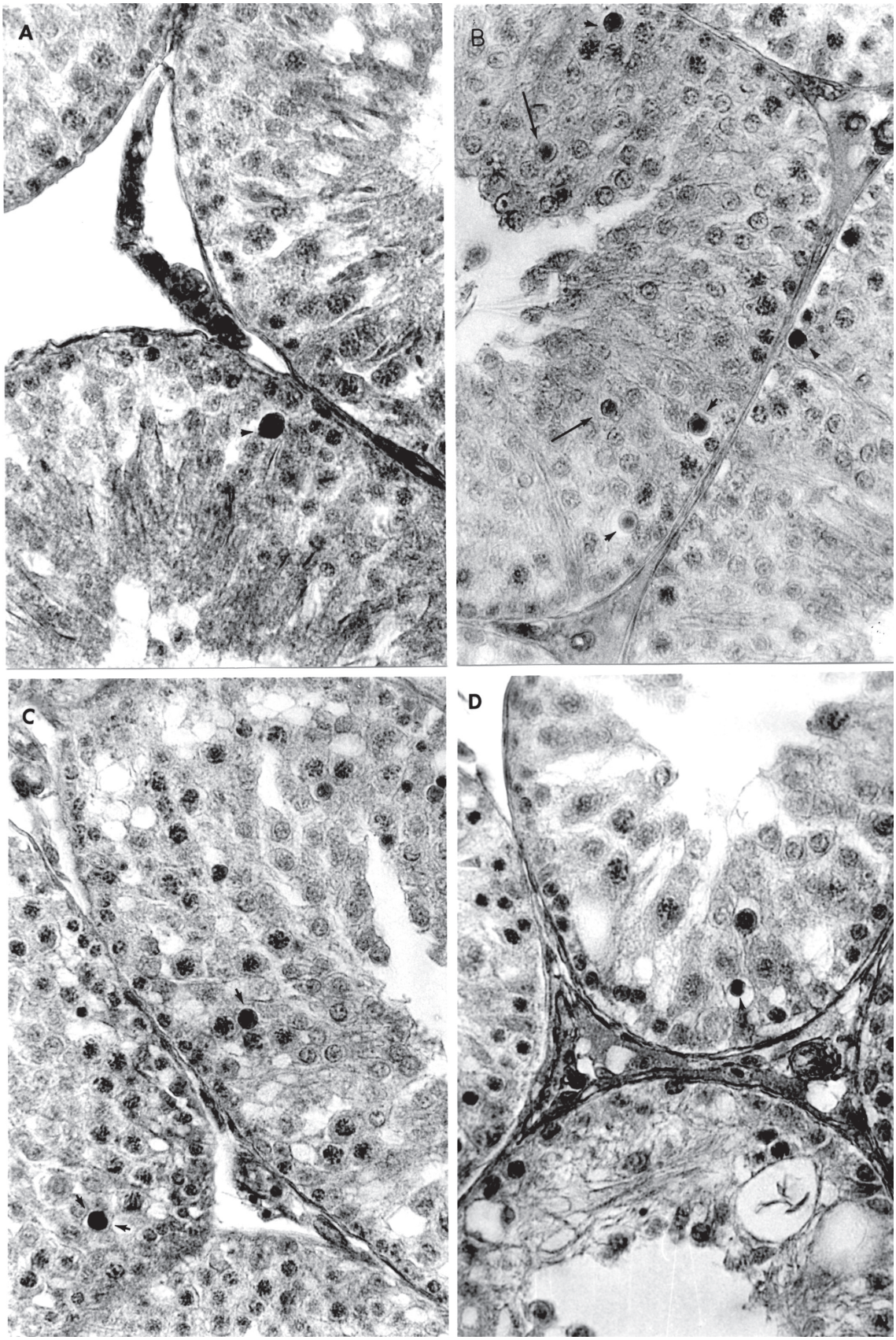
Results

As we previously have shown (BAKALSKA et al. 2000), a single dose (75 mg/kg bw) of EDS administered to adult male rats completely destroyed by apoptosis the mature Leydig cells population of the testis. One day after EDS-treatment most of the Leydig cells exhibited various degrees of nuclear and cytoplasmic disintegration followed by rapid and complete loss of Leydig cell within the next 24 hours. Three days post EDS germ cell complement of all the stages of spermatogenic cycle was intact. First signs of seminiferous epithelium regression were manifested by marked increase in frequency of apoptotic germ cells in comparison with control rat testis (Fig. 1A) where single apoptopic germ cells were observed in the seminiferous tubules. Apoptotic cells were identified by specific TUNEL reaction for DNA fragmentation and morphologically by cell shrinkage and compaction of nuclear chromatin into sharply defined dense masses. As a consequence of condensation, cell dying by apoptosis initially rounded up and retracted from their neighbors. Seven days after EDS treatment apoptotic germ cell were still frequently found in seminiferous tubules that were depleted from elongating spermatids at late stages of the cycle (Fig. 1B). Apoptotic cells were seen mainly in early (I-VI) and middle (VII-VIII) stages of the cycle. Two and three weeks post EDS germ cell apoptosis was less evident, although spermatogenesis looked more affected due to total loss of elongated spermatids in all fourteen stages of cycle and disappearance of some pachytene spermatocytes (Fig. 1C). Seminiferous tubules lacking germ cells (Sertoli cell only tubules; SCO) were also found in the testes at these time points (Fig. 1D). The germ cells undergoing apoptosis were mostly pachytene spermatocytes and round spermatids in stages I-VIII from spermatogenic cycle. Some leptotene and zygotene spermatocytes in late stages (IX-XIV) could be found in EDS treated animals. The validity of the TUNEL method was assessed using two controls. In negative control no staining was obtained when TdT was omitted. As a positive control we used testicular sections from adult rats that had been treated with methoxyacetic acid (MAA, 650 mg/kg) 24 h earlier to cause widespread apoptosis of pachytene primary spermatocytes.

Three days after EDS the number of apoptotic cells per tubule increased nearly twice (2.88 ± 0.3) in comparison with control (1.61 ± 0.18; $p<0.001$) and 7 days after treatment the parameter remained still significantly higher ($p<0.001$) than in control. Two weeks post EDS and onwards the number of apoptotic cells showed values close to control (Fig. 2). Figure 3 shows the quantification of the temporal changes in percentage of tubules showing apoptosis. Three days post EDS this measure was 5-fold higher compared to the control ($p<0.001$). The maximum of the percentage of tubules with apoptosis was observed 7 days after treatment and the values remained higher until the end of the investigated period compared to control. The same tendency was found for the apoptotic index that dramatically increased at 3rd day after EDS administration (Fig. 4). By 7 days post EDS the parameter was more than10-fold higher than control, afterwards decreased but remained still significantly higher compared to control. The highest values of all investigated parameters for quantification of germ cell apoptosis were found by 7 day after EDS which corresponded to the lowest plasma level of testosterone measure by RIA (< 0.1 ng/ml in comparison to 2.14 ± 0.39 ng/ml in the control). Elevated germ cell apoptosis decreased at 14 day after treatment that coincided with considerable rise of T levels (0.51 ± 0.25 ng/ml) and even T concentration returned to normal range from 21 day post EDS (1.27 ± 0.3 mg/ml) their mean values remained lower than controls. Serum levels of LH became elevated 7 days after EDS administration (3.66 ± 1.76 ng/ml) compared to controls (0.86 ± 0.18) and they remained elevated up to 14 day post EDS (3.97 ± 1.18) followed by return towards normal range during 3-5 weeks after treatment.

Discussion

Several studies using spermatogenesis as a model system for studying the regulation of germ cell death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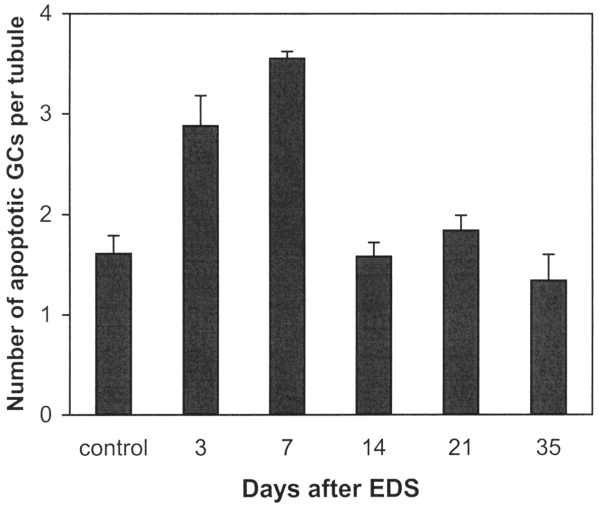

Fig. 2 Enumeration of germ cell number per seminiferous tubule showing apoptosis after EDS treatment. Each point represents the mean ± SD (n=4), p<0.001. GCs- germ cells.

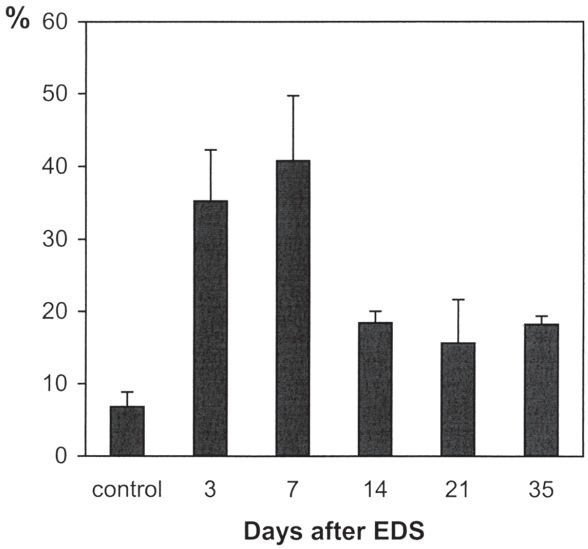

Fig. 3 Quantification of the temporal changes in percentage of tubules showing apoptosis after EDS administration. Each point represents the mean ± SD (n=4), p<0.001.

have provided evidence that both spontaneous and induced germ cell death after removal of hormone support in adult rats occurs almost exclusively via apoptosis (Sinha Hikim and Swerdloff 1999). Testosterone appears an essential factor for the maintenance of normal spermatogenesis and fertility in the adult males and the drop of T levels results in apoptosis of differentiating germ cells types (Henriksen et al. 1995; Kim et al. 2001). The presence of internucleosomal chromatin degradation in apoptotic cells resulted in the use of DNA fragmentation as a reliable diagnostic tool for the occurrence of apoptosis. The technique of TdT-mediated dUTP nick-end labelling (TUNEL) to 3'-end of fragmented DNA is being used routinely to detect in situ apoptotic cells in various tissues.

Our observation in the present study showed that germ cell death caused by testosterone withdrawal in adult EDS treated rats is mediated by apoptosis. By detailed quantitative analysis we demonstrated profound time-dependent increase in germ cell apoptosis in seminiferous epithelium after testosterone deprivation by

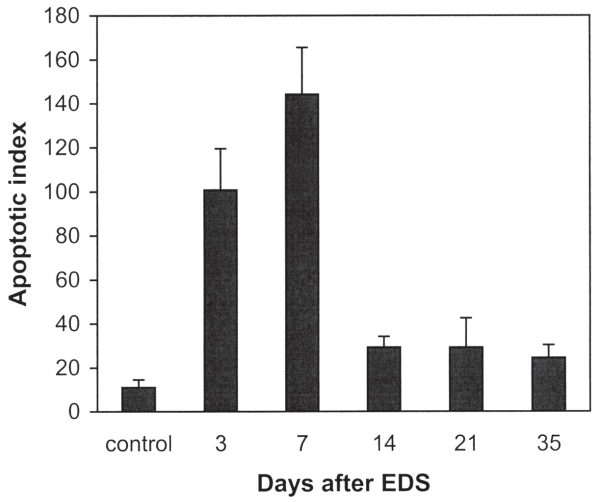

Fig. 4 Temporal changes in apoptotic index following EDS administration. The values of apoptotic index were calculated by multiplying the percentage of tubules containing apoptotic cells by the number of apoptotic germ cells per tubule. Each point represents the mean ± SD (n=4), p<0.001.

Fig. 1 Impairment of spermatogenesis and induction of germ cell apoptosis by EDS in adult rat visualized by TUNEL method:

Fig. 1A Testicular cross section of control rat. Note a single apoptotic spermatocyte (arrowhead) in seminiferous tubule in stage XIV-I of the spermatogenic cycle. x 400

Fig. 1B Testicular cross section of rat at 7th day after EDS administration. Apoptotic germ cells – spermatocytes (arrowhead) and round spermatids (arrows) are frequently seen in seminiferous tubules in early stages of the cycle. x 400

Fig. 1C Two weeks post EDS germ cell apoptosis (arrowhead) is accompanied with total loss of elongated spermatid in all the stages of the cycle. Note vacuolization at the periphery of the seminiferpus tubules. x 400

Fig. 1D Three weeks post EDS apoptotic germ cells (arrowhead) can be found in seminiferous tubules of germ cells. SCO tubules are seen in the testis. x 400

EDS administration. The highest values of all investigated parameters for quantification of germ cell apoptosis (the number of apoptotic cells per tubule, the percentage of tubules with apoptosis and apoptotic index) we established on day 7 after EDS which coincided with the lowest testosterone plasma levels and complete absence of Leydig cells. The induced germ cell death could be interpreted as an "echo" of preceding Leydig cell apoptosis documented by Morris et al. (1997), Taylor et al. (1998, 1999) and Kim et al. (2000). A growing body of evidence showed that the withdrawal of androgens in adult rats results in the acceleration of germ cell apoptosis at specific stages of the spermatogenic cycle (Henriksen et al. 1995; Sinha Hikim et al. 1997). Our data showed that predominant germ cell types undergoing apoptosis as a result of androgen ablation include pachytene spermatocytes and round spermatids in early (I-VI) and middle stages (VII-VIII) from the spermatogenic cycle. This differed from the results obtained from control animals in which apoptotic spermatids were rarely found and spermatogonia and spermatocytes during their meiotic division at stage XIV were the germ cell types undergoing apoptosis (Blanco-Rodriguez and Martinez-Garcia 1996). The preferential cell death was reported to occur in androgen dependent stages VII–VIII after testosterone withdrawal (Sharpe 1994). In the seminiferous epithelium testosterone is known to preferentially act in stages VII-VIII and it has been suggested to have an important key role in the conversion of round to elongated spermatids (O'Donnel et al. 1994). A possible explanation of our findings is that the competition for limiting amounts of survival signals after testosterone suppression may cause germ cell apoptosis first within the stage at which hormone levels need to be higher, but as entrance to apoptotic pathway is continuous, accumulating dying cells could be detected in other stages in the following days. The great importance of testosterone support for germ cell survival and its adequate signaling via androgen receptor (AR) was recently demonstrated by comparative studies in transgenic mice with total knockout of the AR in the testis (ARKO mice) and Sertoli cell-selective knockout of AR (SCARKO mice) (De Gendt et al. 2004)

Quantitative analysis of time and cell specificity of germ cell apoptosis in the present study supports our previous data (Bakalska et al. 2001) showing that testosterone withdrawal caused stage-dependent loss of haploid germ cells (spermatids) due to differential sensitivity of different germ cell populations. The time-dependent changes in germ cell apoptosis after EDS administration, we found in the present study, precedes the specific total loss of elongating spermatids and disappearance of pachytene spernatocytes from the seminiferous epithelium.

The kinetics of germ cell apoptosis we found correlated with dramatic change in T levels which were related to elevated LH concentrations. The similar relationship between germ cell apoptosis and T deprivation was reported in adult rats treated with GnRH-antagonist that were also deficient in gonadotropic hormones (Sinha Hikim and Swerdloff 1999). It is interesting that all the values of quantitative end points for germ cell apoptosis we established remained significantly higher than controls until the end of the investigated period and also testosterone levels tend to recover to normal range in tandem with restoration of spermatogenesis. Although most of the effects of EDS on spermatogenesis are attributed to the loss of testosterone in the testes, it can been suggested that EDS may have a direct affect on the function of Sertoli cells that produce factors required for meiotic division and subsequent germ cell maturation. Roberts et al. (1992) demonstrated that EDS inhibits the transferrin secretion from cultured Sertoli cells. Previously, we reported the profound ultrastructural alterations of Sertoli cells after EDS treatment (Bakalska et al. 2002).

In conclusion, our results indicate that quantitative pattern of germ cell death after testosterone deprivation reveal in advance the kinetic of germ cell depletion and regeneration in a long period after EDS. These new findings bring additional support to the concept that germ cell apoptosis is a hormonally regulated process. Induction of germ cell apoptosis by EDS could be considered a result from differential alterations occurred in the main testicular cell types and more than one pathway is probably involved in that physiological cell death in the testis.

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