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Quantification of Germ Cell Apoptosis and Survival in Relation to Renewal of Leydig Cell Population after EDS Treatment of Adult Rats

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The aim of the present study was detailed quantitative analysis of male germ cell apoptosis and survival in relation to renewal of Leydig cell (LC) popilation in a long period after EDS administration. The apoptosis in adult rat testes was induced by EDS and was assessed by TUNEL method. The first signs of seminiferous epithelium regression were manifested by marked increase in number of apoptotic cells on 3rd day post EDS. The maximal value of germ cell apoptosis was established on 7th day post EDS that coincided with lowest testosterone (T) levels and lack of 3β-HSD immunopositive cells. The regeneration of seminiferous epithelium two weeks post EDS and onwards correlated with decrease of elevated germ cell apoptosis and development of new LC population. Quantitative patterns of germ cell death and survival occurred in tandem with changes in T production and reveal in advance the kinetic of germ cell depletion and regeneration in a long period after EDS.

Key words: EDS, apoptosis, spermatogenesis, 3β-HSD.

Introduction

Mammalian spermatogenesis is a highly ordered process that depends on adequate levels of testosterone [12]. Despite decades of study, however, the mechanism(s) by which testosterone regulates spermatogenesis remains uncertain. It has been shown that germ cell loss by apoptosis occurs normally during spermatogenesis [16]. Several studies have shown that testosterone withdrawal from the rat testis results in increased germ cell apoptosis, suggesting that testosterone may function as a cell survival factor, in some way protecting germ cells from apoptotic death [16].

Manipulation of spermatogenesis by deprivation of survival factors provides a basis for detailed study on the regulatory mechanisms of germ cell death. Ethane dimethanesulfonate (EDS) is a unique cell-specific toxin with action confined exclusively

to the Leydig cells [10]. EDS is a valuable tool for investigating apoptosis in the testis in response to androgen withdrawal because it selectively eliminated both basal and LH-stimulated testosterone production, thus providing a model in which there is complete androgen ablation within the testis [18]. The predominant germ cell types undergoing apoptosis as a result of androgen withdrawal induced by EDS include pachytene spermatocytes and spermatids [7].

Male germ cell apoptosis in response to testosterone deprivation was investigated in a short time-window post EDS when LCs were completely missing from the testis [7, 18]. Kinetics of germ cell apoptosis and survival was not examined in a long period after EDS treatment when new LC population developed in the testis and restored testosterone production essential for regeneration of seminiferous epithelium. In this respect the current study aimed to carry out a detailed quantification of germ cell apoptosis and survival in tandem with recovery of steroid-producing function of LC in order to develop our previous understanding on germ cell degeneration and regeneration in the testis [3].

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 intraperitoneal injection of EDS at 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 and quantitation of apoptotic cells: 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 [14]. For assessment of apoptosis, the percentage of seminiferous tubules with apoptotic germ cells was determined by scoring 100 randomly selected tubules per section on four sections from different animals at each time point after EDS administration [18]. 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.

Imminohistochemistry for 3β-HSD: Leydig cells were immunostained with rabbit polyclonal antibody against 3β-HSD, kindly provided as a gift form Prof. I. Mason (Edinburgh University).

Hormone measurements: Serum LH and testosterone (T) levels were measured by radioimmunoassay (RIA) as described by Fraser and Shandow [6] and Sharpe and Bartlett [13], respectively.

Results

Our previous studies have shown that a single dose (75 mg/kg bw) of EDS administrated to adult male rats causes apoptotic death of Leydig cells resulting in complete loss of Leydig cell population within the next 24 hours [2]. First signs of seminiferous epithelium regression were manifested by marked increase in frequency of apoptotic germ cells in

Table 1. Summary of EDS–induced changes in germ cell apoptosis, testosterone levels and 3β-HSD immunoexpression in adult rats.
Data represent mean ± SD (n=4; *** p< 0.001, ** p< 0.01, * p< 0.05, ns – non significant, in comparison with control value).

Day after treatment	Number of apoptotic cells / ST	% of ST with apoptotic cells	Apoptotic Index	Testosterone levels, ng/ml	Intensity of 3β –HSD IHC
Control	1.61 ± 0.18	6.81 ± 2.03	11.00 ± 3.57	2.14 ± 0.39	+ + +
3	2.88 ± 0.30 ***	35.22 ± 7.07 ***	100.74 ± 18.79***	not measured	–
7	3.55 ± 0.07 ***	40.80 ± 8.91 ***	144.14 ± 21.40***	< 0.1 ***	–
14	1.58 ± 0.14 ns	18.43 ± 1.59	29.27 ± 4.90***	0.51 ± 0.25 *	+ + +
21	1.84 ± 0.15 ns	15.61 ± 6.02	29.20 ± 13.32 *	1.27 ± 0.3 *	+ + +
35	1.34 ± 0.26 ns	18.22 ± 1.12	24.46 ± 5.88 **	not measured	+ + +

ST – seminiferous tubule
IHC – immunohistochemistry

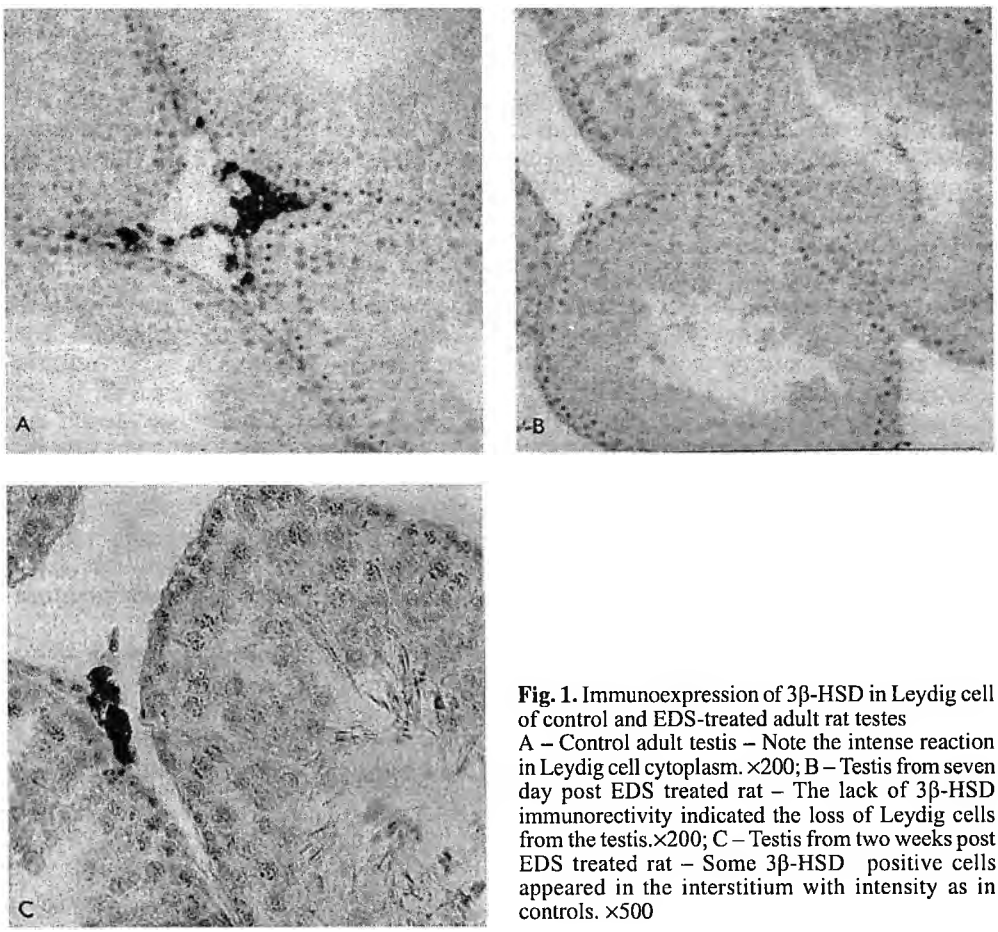

Fig. 1. Immunoexpression of 3β-HSD in Leydig cell of control and EDS-treated adult rat testes
A – Control adult testis – Note the intense reaction in Leydig cell cytoplasm. ×200; B – Testis from seven day post EDS treated rat – The lack of 3β-HSD immunorectivity indicated the loss of Leydig cells from the testis.×200; C – Testis from two weeks post EDS treated rat – Some 3β-HSD positive cells appeared in the interstitium with intensity as in controls. ×500

comparison with control rat testis 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.

Quantification of germ cell death using three parameters (number of apoptotic cell per seminiferous tubule, percentage of tubules with apoptotic cells, apoptotic index) demonstrate significant increase in apoptosis on day 3 after EDS treatment with maximal values on day seven (Table 1). Two weeks post EDS and onwards both the elevated percentage of tubules with apoptosis and the germ cell apoptotic index decreased but remained still higher compared with the controls, whereas the number of apoptotic germ cells per seminiferous tubule tended to reach the control values. Our data showed that predominant germ cell types undergoing apoptosis as a result of androgen ablation include pachytene spermatocytes and round spermatids.

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 (Table 1). Elevated germ cell apoptosis decreased at 14 day after treatment that coincided with considerable rise of T levels and even T concentration returned to normal range from 21 day post EDS their mean values remained lower than controls.

Immunohistochemistry of 3β-HSD was performed for identification and visualization of Leydig cells in the testis [1]. In controls intense reaction was observed in the Leydig cell cytoplasm (Fig.1A; Table 1). The complete loss of Leydig cells on day 7^{th} was confirmed by lack of 3β-HSD immunopositive cells in the testis (Fig.1B). On 14^{th} day the first positive cells can be found in the interstitium with intensity of 3β-HSD immunostating as in controls (Fig.1C). The frequency of 3β-HSD-positive cells increased after 21th day post EDS.

Discussion

Apoptosis has been considered as a mechanism by which the testicular germ cell are removed during normal and various pathological conditions [8]. The survival of male germ cells in the immature and adult testes depends on FSH as well as on LH dependent intratesticular androgen production [12]. Testosterone withdrawal from the testis has been shown to increase germ cell apoptosis [7, 15, 17] suggesting that testosterone functions as a germ cell survival factor.

Our observation and the data from the literature showed that germ cell death caused by testosterone withdrawal in adult EDS treated rats is mediated by apoptosis [2,10]. We have demonstrated by detailed quantitative analysis profound time-dependent increase in germ cell apoptosis in seminiferous epithelium after testosterone deprivation by EDS administration. The highest values of germ cell apoptosis we established by day 7th after EDS coincided with the lowest testosterone plasma levels and complete absence of Leydig cells. The loss of imminoexpresion of 3β-HSD in the testis after EDS confirmed that Leydig cells had been eliminated, resulting in undetectable testosterone levels during first week post EDS. The induced germ cell death, we found, could be interpreted as an "echo" of preceding Leydig cell apoptosis latter documented by M o r r i s et al. 1997 [10].

The time dependent increase in germ cell apoptosis we found after EDS treatment is consistent with previous results [7, 15, 17]. It has been established that apoptosis occurs in the normal testis and is associated predominantly with spermatogonia [4]. On

the other hand, in response to androgen withdrawal as a result of hypophysectomy [11], administration of GnRH antagonist [15], or anti-LH antibodies [9], the cell types that have been shown to undergo apoptosis are pachytene spermatocytes and round spermatids. Our data showed that predominant germ cell types undergoing apoptosis as a result of androgen ablation include pachytene spermatocytes and round spermatids. Quantitative analysis of time and cell specificity of germ cell apoptosis in the present study develops our previous data [3] that testosterone withdrawal caused stage-dependent loss of haploid germ cells (spermatids) due to differential sensitiveness 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 spermatocytes from the seminiferous epithelium.

The kinetics of germ cell apoptosis we found correlated with dramatic change in T levels which were irrespective 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 [16]. Our data indicated that the decrease in elevated germ cell apoptosis after two weeks EDS and onwards coincided with gradual recovery of testosterone production and regeneration of depleted germ cell types suggesting that testosterone may play a role in germ cell survival. The great importance of testosterone support for germ cell survival and its adequate signaling via androgen receptor (AR) was recently demonstrated by comparative studies on transgenic mice with total knockout of the AR in the testis (ARKO mice) and Sertoli cell-selective knockout of AR (SCARKO mice) [5]. On the other hand, it is interesting that all the values of quantitative parameters for germ cell apoptosis we established remained significantly higher than controls until the end of the investigated period even 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.

In conclusion our results indicate that quantitative patterns of germ cell death and survival occurred in tandem with dramatic changes in testostetone production, respectively the presence of Leydig cells and they revealed 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 and survival is a hormonally regulated process.

References

1. Atanassova, N., C. McKinnell, M. Walker, K.J. Turner, J.S. Fisher, M. Morley, M.R. Millar, N.P. Groome, R.M. Sharpe. Permanent effect of neonatal estrogen exposure in rats on reproductive hormone levels, Sertoli cell number and the efficiency of spermatogenesis in adulthood. – Endocrinology, **140**, 1999, 5364-5373.
2. Bakalska, M., P. Angelova, A. Russinova, L. Kancheva, N. Atanassova, B. Nikolov. Morphological characterization of apoptotic Leydig cells after EDS-treatment in rats. – Macedonian J. Reprod., **6**, 2000, 55-60.
3. Bakalska, M., N. Atanassova, P. Angelova, Y. Koeva, B. Nikolov, M. Davidoff. Degeneration and restoration of spermatogenesis in relation to the changes in Leydig cell population following ethane dimethanesulfonate treatment in adult rats. – Endocrine Regul., **35**, 2001, 211-215.
4. Blanco-Rodriguez, J. A matter of death and life: the significance of germ cell death during spermatogenesis. – Int. J. Androl., **21**, 1998, 236-248.

5. De Gendt, K., J.V. Swinnen, P.T.K. Saunders, L. Schoonjans, M. Dewerchin, A. Devos, K. Tan, N. Atanassova, F. Claessens, Ch. Lecureuil, W. Heyns, P. Carmeliet, F. Guillou, R.M. Sharpe, G. Verhoeven. A Sertoli cell-selective knockout of the androgen receptor causes spermatogenic arrest in meiosis. – Proc. Natl. Acad. Sci., **101**, 2004, 1327-1332.
6. Fraser, H.M., J. Sandow. Gonadotrophin release by a highly active analog of luteinizing hormone releasing hormone in rats immunized against luteinizing hormone releasing hormone. – J. Endocrinology, **74**, 1977, 291-296.
7. Henriksen, K., H. Hakovirta, M. Parvinen. Testosterone inhibits and induces apoptosis in rat seminiferous tubules in a stage-specific manner: in situ quantification in squash preparations after administration of ethane dimethanesulfonate. – Endocrinology, **136**, 1995, 3285-3291.
8. Henriksen, K., M. Parvinen. Stage-specific apoptosis of male germ cells in the rat: mechanisms of cell death studied by supravital squash preparations. – Cell & Tiss. Res., **30**, 1998, 692-701.
9. Marathe, G. K., J. Shetty, R.R. Dighe. Selective immunoneutralization of luteinizing hormone results in the apoptotic cell death of pachytene spermatocytes and spermatids in the rat testis.- Endocrine, **3**, 1995, 705-709.
10. Moris, A. J., M. F. Taylor, I. D. Moris. Leydig cell apoptosis in response to ethane dimethane sulfonate after both in vivo and in vitro treatment. – J. Androl., **18**, 1997, 274-280.
11. Russel, L. D., Y. Clermont. Degeneration of germ cells in normal, hypophysectomized and hormone treated hypophysectomized rats. – Anat. Rec., **25**, 1992, 241-250.
12. Sharpe, R.M. Regulation of spermatogenesis. – In: The Physiology of Reproduction (Eds. E. Knobil, J. D. Neill). New York, Raven Press, 1994, 1394-1434.
13. Sharpe, R. M, J. M. S. Bartlett. Intratesticular distribution of testosterone in rats and the relationship to the concentrations of a peptide that stimulates testosterone secretion. – J. Reprod. Fertil., **73**, 1985, 223-236.
14. Sharpe, R. M., N. Atanassova, C. McKinnell, P. Parte, K. J. Turner, J. S. Fisher, J.B. Kerr, N.P. Groome, S. Macpherson, M.R. Millar, P. T. K. Saunders. Abnormalities in functional development of the Sertoli cells in rats treated neonatally with diethylstilbestrol: A possible role for estrogens in Sertoli cell development. – Biol. Reprod., **59**, 1998, 1084-94.
15. Sinha Hikim, A. P., C. Wang, A. Leung, R. S. Swerdloff. Involvement of apoptosis in the induction of germ cell degeneration in adult rats after gonadotropin-releasing hormone autagonist treatment. – Endocrinology, **136**, 1995, 2770-2775.
16. Sinha Hikim, A. P., R. S. Swerdloff. Hormonal and genetic control of germ cell apoptosis in the testis. – Reviews of Reproduction, **4**, 1999, 38-47.
17. Tapanainen, J. S., J. L. Tilly, K. K. Vihko, A. J. W. Hsueh. Hormonal control of apoptotic cell death on the testis: gonadotrophins and androgens as testicular survival factors. – Mol. Endocrinol., **7**, 1993, 643-650.
18. Woolveridge, I., M. De Boer-Brouwer, M.F. Taylor, K. J. Teerds, F. C.W. Wu, I. D. Morris. Apoptosis in the rat spermatogenic epithelium following androgen withdrawal: Changes in apoptosis-related genes. – Biol. Reprod., **60**, 1999, 461-470.