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ANTHROPOMETRIC CHARACTERIZATION OF OS MORPHOFUNCTIONAL ASPECTS OF ADULT RAT TESTIS AFTER ETHANE DIMETHANESULPHONATE (EDS) TREATMENT

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ABSTRACT. EDS (ethane-dimethanesulphonate) selectively and temporary destroys Leydig cells (LCs) in the adult rat testis. We utilized EDS model to evaluate the interrelationships between the main testicular cell types following testosterone (T) withdrawal. We examined apoptosis in Leydig and germ cells, we also determined the timing for the acquisition of the new population of LCs, its development and differentiation, we analyzed dynamics of disturbance and restoration of steroidogenesis and spernatogenesis in relation to renewal of LCs cell population. We concluded that: restoration of new LC population after EDS passed through the same intermediate stages occurred during normal postnatal development. The kinetics of degeneration and regeneration of spermatogenesis broadly follows the changes in Leydig cell population. The quantitative pattern of germ cell death after testosterone deprivation by EDS revealed in advance the kinetics of germ cell depletion and regeneration in a long period after EDS. The intensity of androgen receptor (AR) immunostaining restored in tandem with development and differentiation of new LC population and as well as with the changes in the germ cells.

KEY WORDS. EDS, testis, Leydig cells, apoptosis, steroidogenesis, spermatogenesis,

INTRODUCTION

The methane sulphonic ester of ethylene glycol, ethane-1,2-dimethanesulphonate (EDS) is a unique testicular toxicant which selectively and temporary destroyed testicular Leydig cells (the main source of testosterone). Later a new population of

Leydig cells (LCs) regenerates, apparently from mesenchymal fibroblast-like precursors (Teerds, 1996). For this reason EDS has been used extensively to investigate the physiological role of LCs and their products. Experiments were conducted to determine the mechanism by which EDS eliminates totally LCs. Now it has been known that LCs undergo apoptosis (programmed cell death) in response to EDS (Morris et al., 1997). The apoptotic signal triggered by death factors is irreversible, i.e. a protease cascade is activated by the death signal and the proteases cleave various cellular components, which leads to biochemical and morphological changes of the cells and nuclei that are typical for apoptosis (Nagata, 1997). Apoptosis occur during normal postnatal development of testis as well as in some pathological conditions (Jacobson et al., 1997). In general, two morphologically and functionally distinct populations of LCs (i.e.„„fetal" LCs and „adult" LCs) are described in the mammalian testis (Ge et al., 1996; Habert et al., 2001; Davidoff et al., 2004). Fetal LCs differentiates from their mesenchimal precursors around day 14 of gestation in the rat testis and continues to be present at birth (Mendis-Handagama et al., 1998) and up to sexual maturity (Ariyaratne et al., 2000). However, most LCs in the sexually mature rat testis (the adult population of LCs) differentiates postnatal from spindle-shaped cells in the testis interstitium as early as postnatal day 10 (Hardy et al., 1989; Mendis-Handagama et al., 1998).

Androgens are very important for the initiation of the germ cell development during puberty and maintenance of spermatogenesis in adulthood. Androgens are primarily produced by the LCs in mammalian testis and androgen action is mediated via androgen-receptor localized in LCs, peritubular cells, Sertoli cells, but not in the germ cells (Sharpe, 1994). Experimental withdrawal of androgens by EDS produced degenerative changes in semniferous epithelium and hence EDS model is a valuable contemporary tool for investigation of cell-cell interaction and paracrine regulation in the testis. This paper collected and summarized our detailed studies from the last few years utilizing EDS model to evaluate importance of the main testicular cell types and their interrelationships. We examined apoptosis in the Leydig cells and in germ cells following androgen withdrawal. We also determined the timing for the acquisition of the new population of LCs, its consecutive changes in the differentiation and functional maturation (ultrastructural characteristics, the enzyme activity and immunoexpressin of 3ß-hydroxysteroid dehydrogenase (3ß-HSD) and androgen receptor (AR). Changes in Sertoli cell functional properties (androgen receptor immuno -expression) were studied in tandem with recovery of testosterone production. Based on these investigations we analyzed dynamics of disturbance and restoration of steroidogenesis and spermatogenesis in relation to renewal of Leydig cell population following EDS administration.

MATERIAL AND METHODS

Mature Wistar rats received a single intraperitoneal injection of EDS at dose of 75 mg/kg body weight dissolved in dimethylsulphoxide and water (1:3, v/v). The animals were killed on days 1, 7, 14, 21 and 35 after initial treatment (N=4 per group). Plasma samples were stored at -20° C until used for ***hormonal analysis*** of

testosterone and LH ***by RIA***. One testis was fixed in Bouen's solution, embedded in paraffin, and after ***hematoxylin-eozin coloration***, examined by light microscope. Some paraffin sections were incubated 10 min with 0.3 μg/ml ***acridine orange*** diluted in PBS and 0.1μg/ml ***propidium iodide*** (Bakalska et al., 2000), observed in Zeiss epifluorescence microscope and examined the different stage of apoptotic process of the LCs. ***In situ assay of apoptosis*****:** Apoptotic cells were also detected by using terminal deoxynucleotidyl transferase (TdT)-mediated digoxigenin-11-dUTP nick end labeling (***TUNEL***) ***method*** that resulted in a high degree of specificity and low background staining (Sharpe et al., 1998). For assessment of apoptosis, the percentage of seminiferous tubules with apoptotic germ cells was determined, as well as the ***apoptotic index*** (Woolveridge et al., 1999). E***lectron microscopy:*** testicular fragments were fixed in 2,5% glutaraldehyde in 0.1 M cacodylate buffer, 1% osmium tetraoxide and embedded in durcupan. Electronograms were made on an Opton EM 109. The functional activity of the new LCs was detected by ***immunohistohemistry*** for steroidogenic enzymes 3β-HSD (Atanassova et al., 1999) using rabbit poly anti-body (Prof. I.Mason, Edinburgh University). Immuno- histochemistry for AR visualization was performed according to McKinnell et al. (2001).

RESULTS

Staining with acridine orange and propidium iodide shows that 24h after EDS treatment the interstitium of rat testis contains Leydig cells in the different stage of apoptotic process, identified according to the criteria previously described by Desjardins & MacManus (1995) in the cell cultures (HT29). The fluorescent microscopic observation indicated that some LCs might be determined as „preapoptotic" cells. Some LCs begin to increase in volume and were designated as „swelling" cells, others cells had started to round – „rounding" cells. Some LCs exhibited fragmented nuclei – „apoptotic body/bodies" – a hallmark of apoptosis. The „ghost-like" cells described by Desjardins & MacManus (1995) were also observed (fig.1). The different stages of LC apoptosis are presented on schema (fig.2).

Detailed light- and electronmicroscopical descriptions of the degeneration and disappearance of LCs after EDS have been previously reported (Bakalska et al., 2000, 2002). Briefly, at day 7 after EDS treatment any LCs were not observed in the testicular interstitium (fig.3). Two weeks after EDS in some intertubular areas, elongated spindle-shaped cells, so-called Leydig cell-progenitors**,** were noted. They had little cytoplasm, poor presence of endoplasmic reticulum and other organelles. By three week post-EDS the progenitors cells are transformed into immature LCs contained numerous lipid inclusions, mitochondria and appearance of smooth endoplasmic reticulum (SER). On day 30 some of immature cells during further differentiation obtained morphological characteristics of mature adult-type LCs (abundant SER and tubular mitochondria). Their quantity increased markedly and by 6 weeks was similar to the control group. The absence and regeneration of LCs and their steroidogenic activity after EDS was proved by immunohistochemistry with 3β-HSD – key enzyme in testicular steroidogenesis, used as a specific marker for

visualization and quantification of LCs (Atanassova et al., 1999). First 3β–HSD positive cells appeared in the testicular interstitium two weeks post-EDS and a marked increase in the of 3β-HSD activity occurred between 21st and 30th after EDS, when the immunostaining intensity was comparable to control (fig.4). LC immunoreactivity for 3β-HSD correlated with values of serum testosterone levels measured by RIA. The severe drop in T concentration (below 0.1 ng/ml) on day 7th (in comparison with control 2.14 ± 0.39) was followed by gradual increase in T levels after two weeks post-EDS (0.51± 0.25). Hormonal profile after EDS correlated with changes of the immunoexpression of androgen receptors. Seven day post-EDS there was total loss of AR immunostaining in Sertoli, Leydig and peritubular cells. Two weeks after EDS treatment AR expression was recovered in these cell types, but there was no evidence for stage-specific pattern of AR immunoexpression in Sertoli cells, that is characteristic for adult control testis.

First signs of seminiferous epithelium regression were manifested by marked increase in frequency of apoptotic germ cells in comparison with control testis. Germ cells apoptosis was accompanied with total loss of elongated spermatids in late stages of spermatogenic cycle by 7 day post-EDS and later from of all the stages by day 14. Apoptotic index was dramatic increased by 7 day after treatment when its value was 10 fold higher than control. Afterwards it decreased, but remained still significantly higher compared to the control. The germ cells undergoing apoptosis were mostly 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 that corresponded to the lowest plasma level of T. First sign of recovering of spermatogenesis was seen by day 21 and was manifested by appearance of elongated spermatids in late stages of spermatogenic cycle. Degenerative alterations in seminiferous epithelium were still present and some Sertoli-cell only tubules with germ cells depletion were observed.

DISCUSSION

During the last decade experimental hormonal manipulation by EDS become unique appropriate model, which enable to answer many questions related to morpho-functional status of the adult mammalian testis. This experimental approach played essential role in understanding of LC postnatal development and evaluation of the importance of androgens for spermatogenesis. Although it has been known for a number of years that EDS can induce LC degeneration (Jackson et al., 1986) it is only recently that the mode of cell death has been established as apoptosis (Morris et al., 1997). The molecular events involved in EDS-induced LC apoptosis are still unclear. However in vitro experiments suggest that the covalent binding of EDS to glutathione may be essential for cytotoxicity (Kelce & Zirkin, 1993) and that the regulation of apoptosis is dependent upon specific gene products Bax, Bad, Bcl-xl, Bcl-2 and Fas-L (Woolveridge et al., 1999). The morphological feature we followed for the identification of apoptotic cells by staining with DNA-specific fluorescent dyes enable us to show the different stages of LCs apoptosis 24 hours after EDS treatment

and our findings were in agreement with studies of (Morris et al. 1997; Taylor et al., 1998;Yokoi et al.,1998).

The establishment of new LC population after EDS was suggested to begin from progenitor LCs that underwent active cell divisions giving rise of immature LCs (Ge et al., 1996; Teerds, 1996). In our study we observed the progenitor LCs two weeks post EDS, as well as the immature LCs, which are transformed into mature LCs by 3-4 weeks after treatment. The differentiation of new LCs after EDS passed through the same intermediate stages, occurred during normal postnatal development.

Germ cell apoptosis is very common phenomenon during various stages of mammalian testicular development and can be triggered by various pathological conditions (Koji & Hishikawa, 2003). Testosterone is important factor for maintenance of normal spermatogenesis and fertility of adult males and drop of T levels results in apoptosis of differentiating germ cell types (Kim et al., 2001). The highest values of germ cell apoptosis we established by day 7th after EDS coincided with the lowest T plasma levels and complete absence of LCs. The loss of immunoexpression of 3β-HSD in the testis after EDS confirmed that LCs had been totally eliminated (Morris et al., 1997). Appearance of single newly formed LCs by 14 day post-EDS we found concedes with stage dependant loss of germ cells due to lack of appropriated LC population. At 21 day after EDS when the new LC population was relatively well presented we observed the first signs of seminiferous epithelium regeneration with appearance of elongating spermatids. Therefore the changes in Leydig and germ cell populations after EDS occurred hand by hand indicative for close interrelationship between interstitium and seminiferous epithelium.

Our data indicated that the decrease in germ cell apoptosis two weeks post-EDS and onwards coincided with gradual recovery of T production. The great importance of T support for germ cell survival and its adequate signaling via androgen receptors 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) (De Gendt et al., 2004). The changes in LC and germ cell populations after EDS treatment are temporary and reversible which has been reported by some authors (Teerds, 1996; Taylor et al., 1998) and in our studies as well.

Summarizing the analysis of our data we could conclude that:

- The restoration of new LC population after EDS repeats the normal dynamics of LC development within similar time range.
- The kinetics of degeneration and regeneration of spermatogenesis broadly follows the changes in Leydig cell population after EDS treatment.
- The quantitative pattern of germ cell death after testosterone deprivation by EDS reveals in advance the kinetics of germ cell depletion and regeneration in a long period after EDS. These new finding brings additional support to the concept that germ cell apoptosis is a hormonally regulated process.

- The intensity of AR immunostaining restored in tandem with development and differentiation of new LC population and as well as with the changes in the germ cells.

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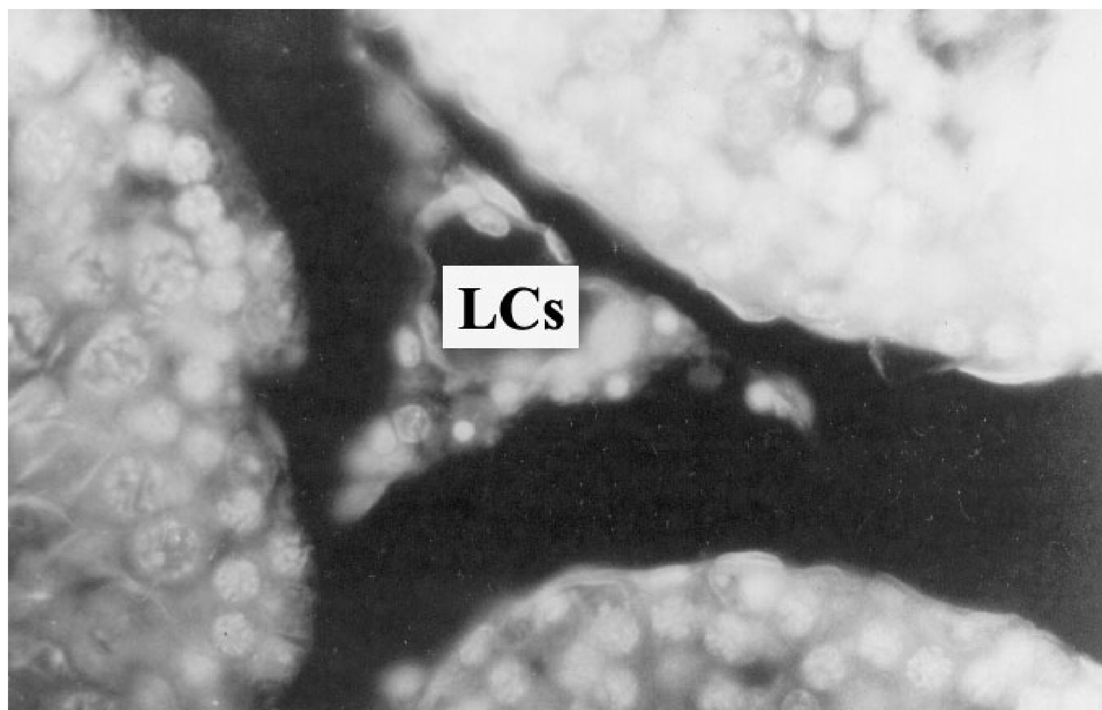

Fig.1. *Acridine orange and propidium iodide staining. Leydig cell degeneration 24h after EDS treatment. x 400*

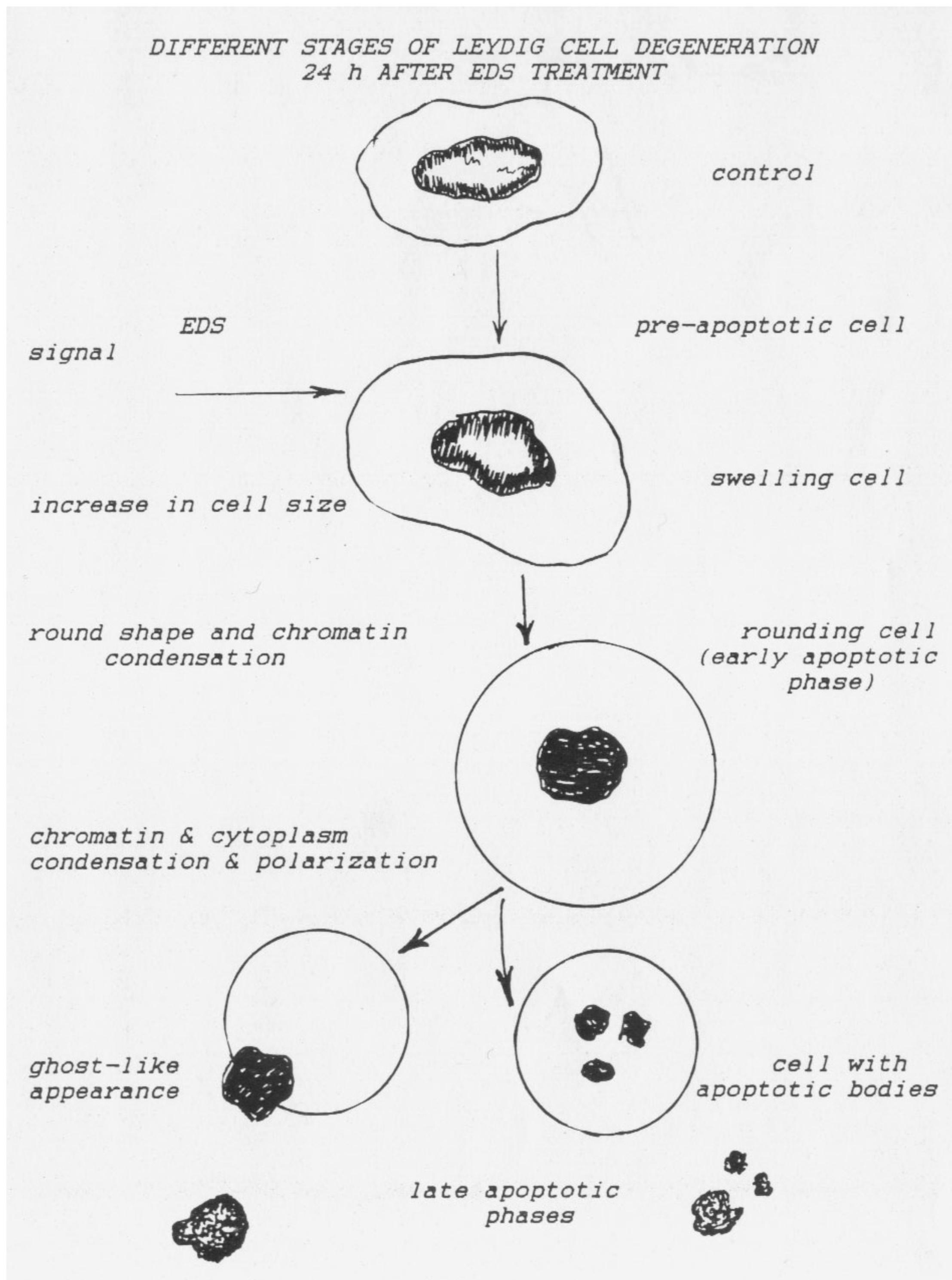

Fig.2. *Schema presented the different stages of LC apoptosis (according to Desjardins & MacManus, 1995)*

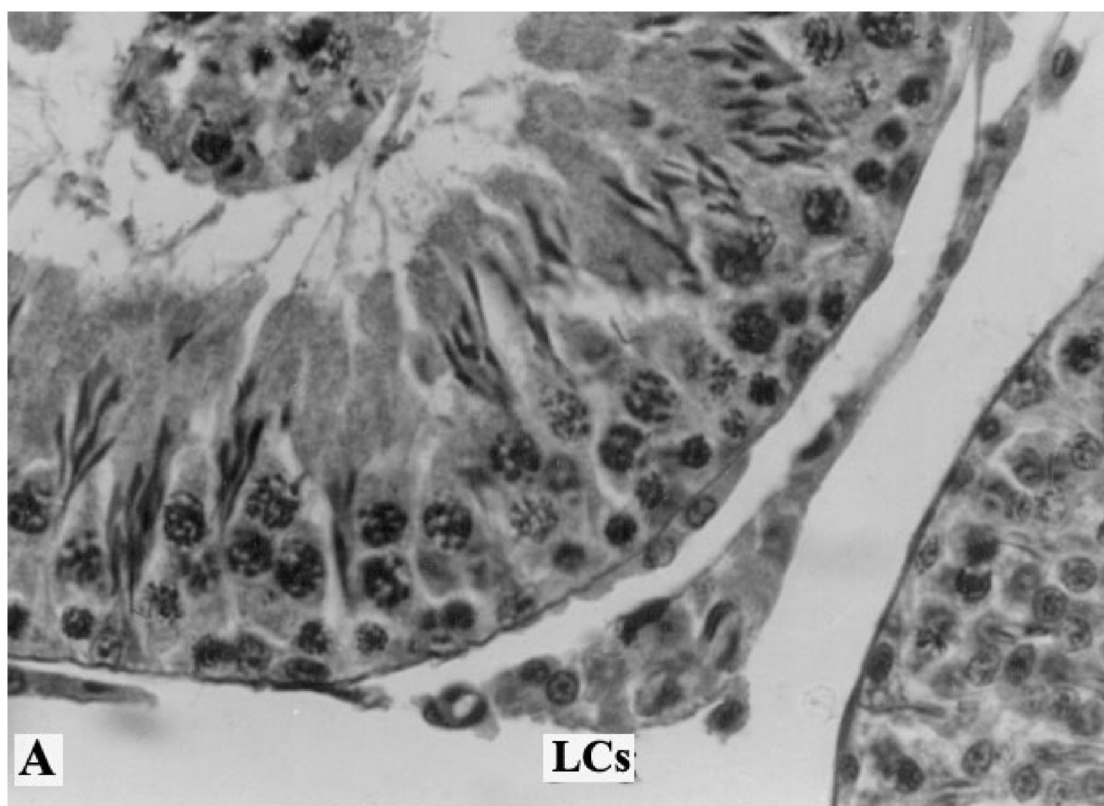

Fig. 3. Para*ffin testicular sections stained with hematoxylin-eosin:*
Fig.3 A – cont*rol testis. x 400*

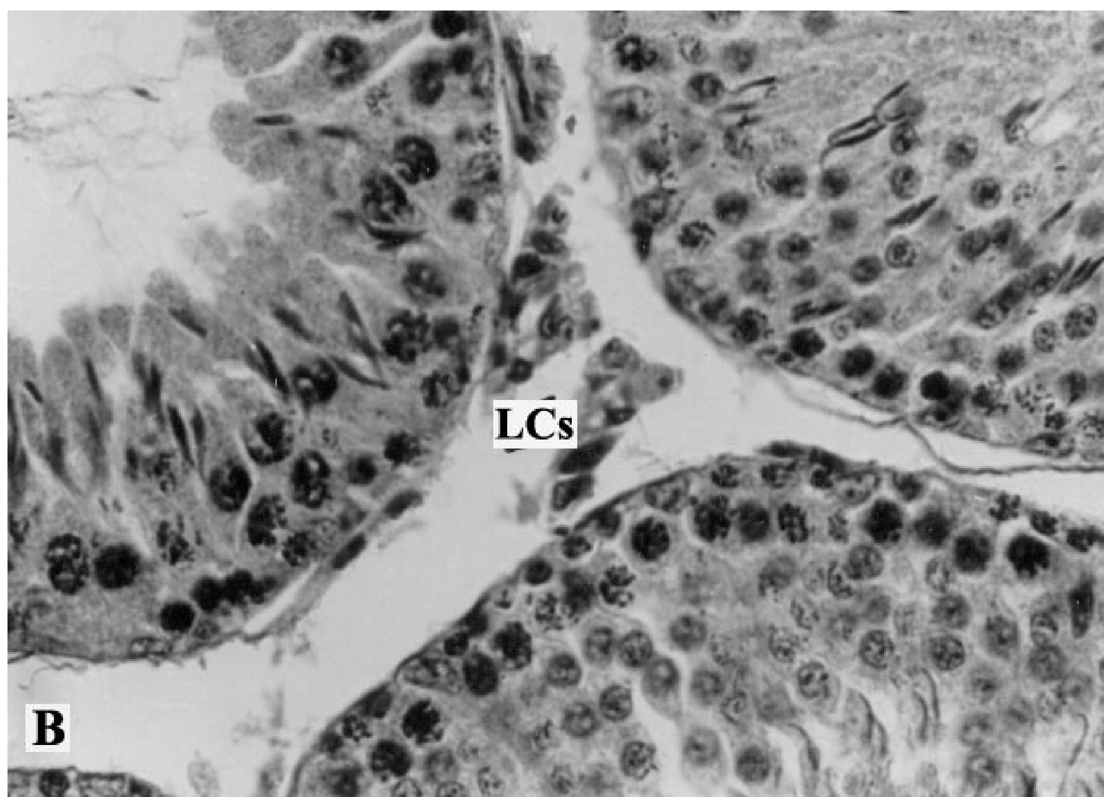

Fig.3 B – *first day after EDS. LCs in different stages of degeneration. x500*

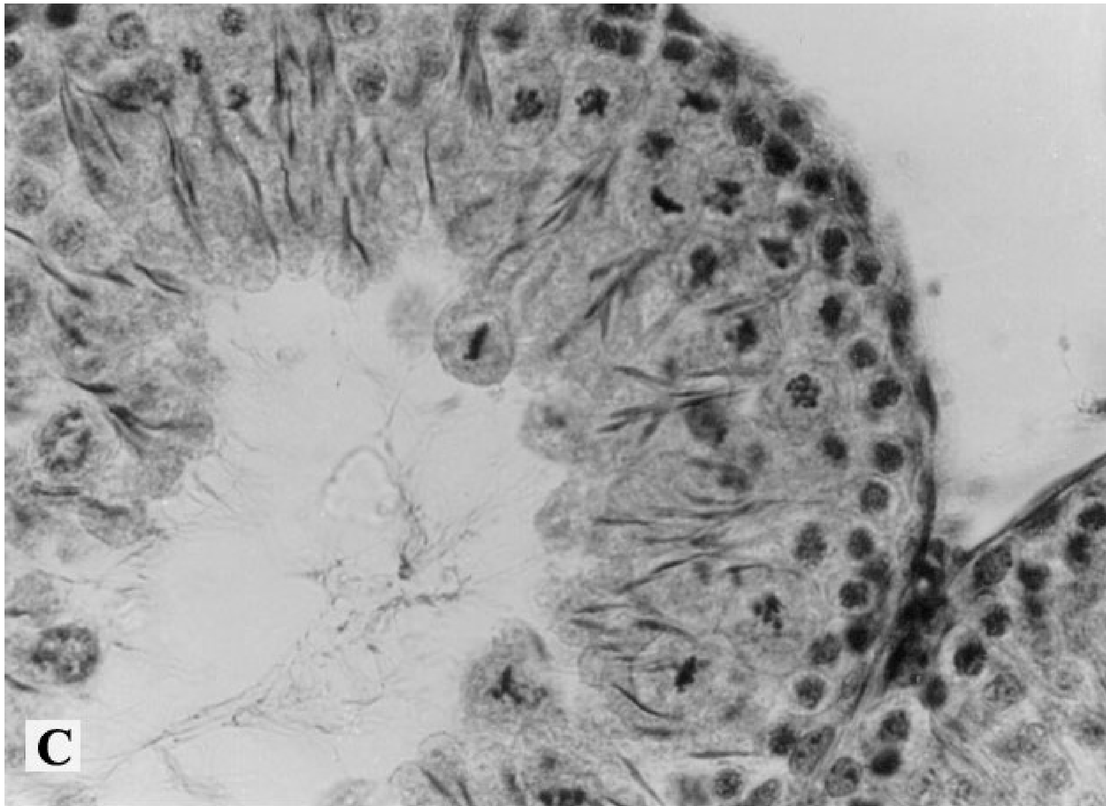

Fig.3 C – *On 7 day post-EDS. LCs were almost absent from the interstitial space. x500*

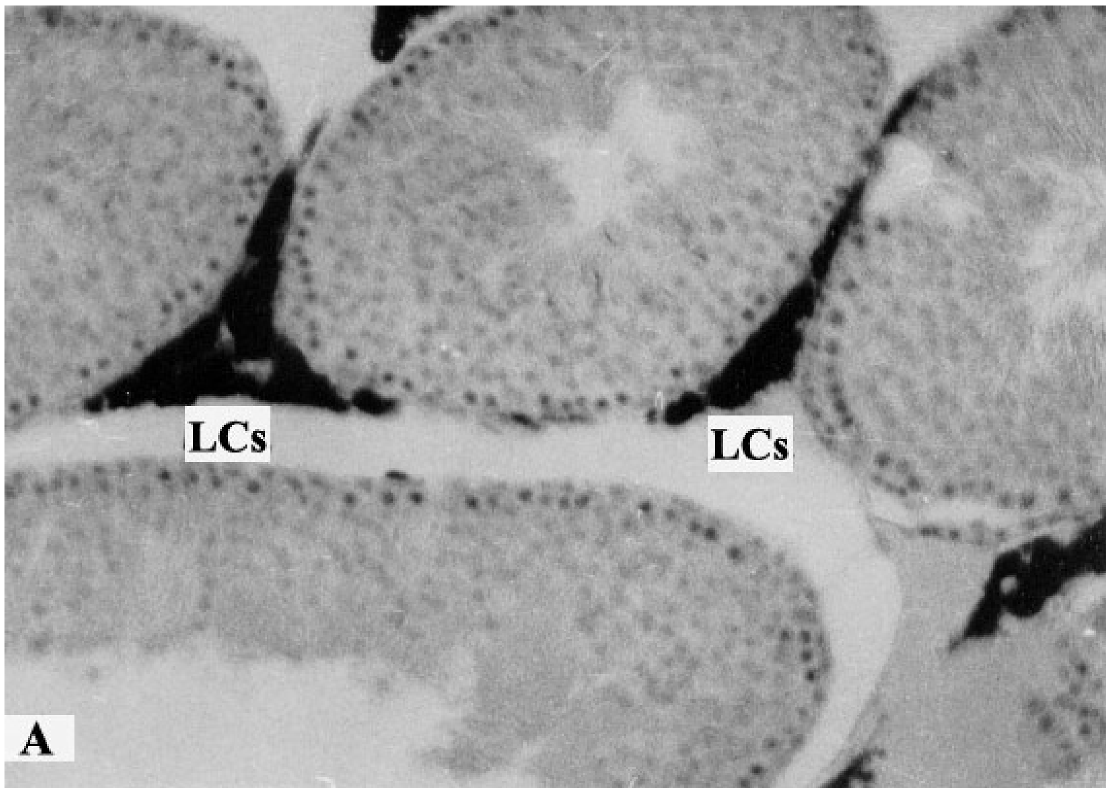

Fig. 4. *Immunohistochemical visualization of 3β-HSD activity:*
Fig. 4 A – *in control testis the ntense immunostaining was evident. x 200*

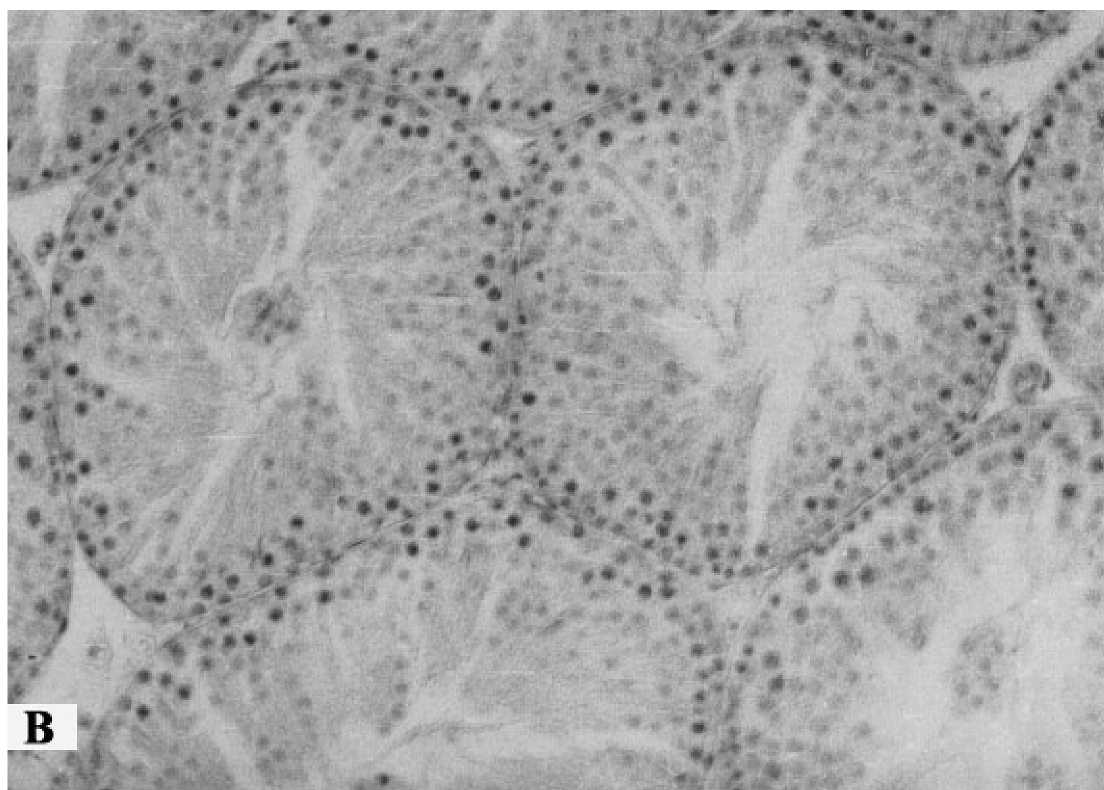

Fig. 4 B – *on 7 day lack 3β-HSD immunoreactivity indicated loss of LCs from the testis. x200.*

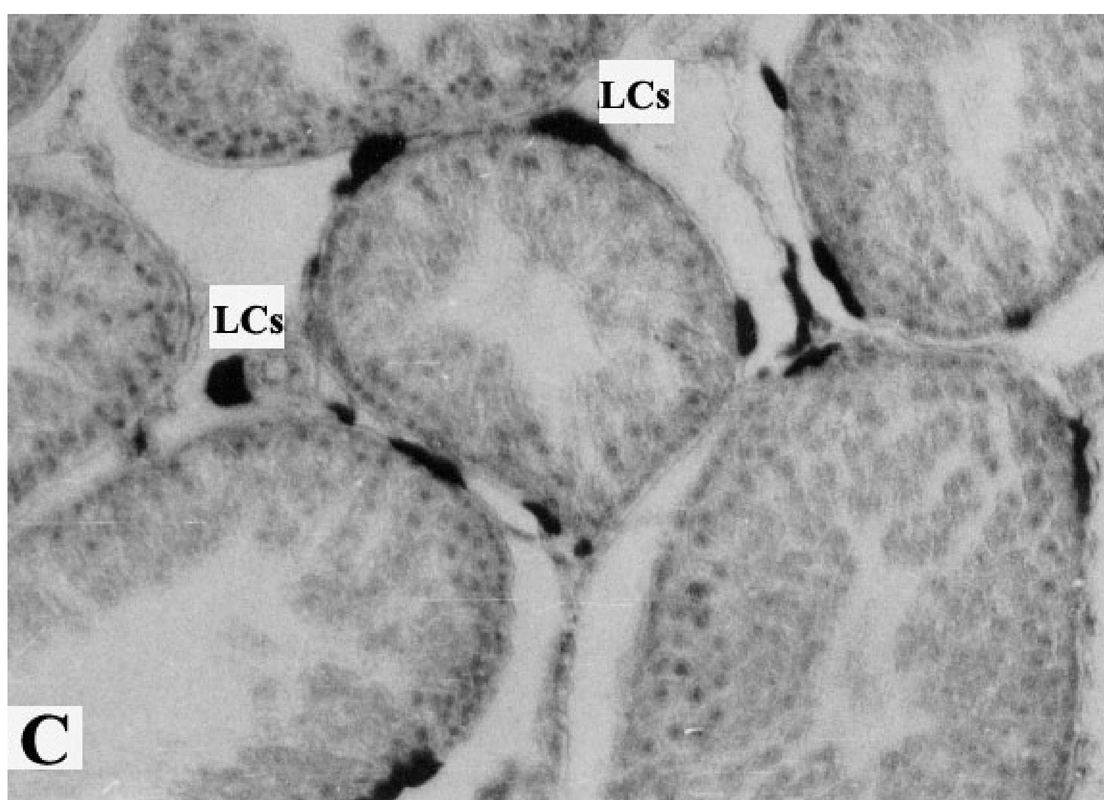

Fig. 4C – *on 14 day some 3β-HSD positive cells appeared in the interstitium. x200*