Short Communication

Steroidogenic and Structural Differentiation of New Leydig Cell Population Following Exposure of Adult Rats to Ethane Dimethanesulphonate

(EDS / Leydig cell / ultrastructure / histochemistry)

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Abstract. EDS alkylating agent has been shown to selectively and temporarily kill LCs in adult rats. The first newly formed single LCs appeared at 14th day post ESD and showed detectable activity for 3β-HSD and NADH2-diaphorase, which became progressively stronger with time after treatment. The ultrastructural study revealed that the progenitor LCs differentiated into immature LCs within a week, and two weeks later they were transformed into mature LCs. Therefore, the restoration of new LC population after EDS treatment repeated the dynamics of normal LC development within a similar time range. The dynamics of enzyme activity correlated with structural differentiation of the new LC population.

The biosynthesis of androgens from cholesterol depends upon action of several enzymes located almost entirely in Leydig cells (LCs). The enzyme 3β-hydroxysteroid dehydrogenase (3β-HSD) catalyzes an essential step in the biosynthesis of all steroid hormones that requires the reduced form of nicotinamide adenine dinucleotide (NADH2) as a cofactor. This enzyme is the most active of the enzymes involved in testosterone biosynthesis (O'Shaughnessy and Murphy, 1991).

Ethane dimethanesulphonate (EDS) specifically and temporarily destroys Leydig cells, which results in a drop in testosterone levels and disturbance of pituitary-testicular hormonal axis reflected by gross elevation of serum gonadotropin levels (Bartlett et al., 1986; Kerr et al., 1987). This substance can, therefore, be used to study the role of Leydig cells in normal testicular function. Within two weeks of Leydig cell destruction by EDS, regeneration of new Leydig cell population has begun from precursor cells within the interstitium (Teerds, 1996; Morris et al., 1997; Kancheva et al., 2000). The morphology of the regenerating Leydig cells has been well described (Kerr et al., 1987; Hatier and Grignon, 1997), but their functional features during the same period are largely unclear. The Leydig cell precursors that differentiate into mature Leydig cells should also at the same time undergo functional differentiation. In this respect two questions arise: 1) when the steroidogenic enzyme activity could be first detected and 2) whether this activity shows a normal pattern of development during Leydig cell regeneration after EDS.

In this respect the design of the present study was to establish changes in steroidogenic enzyme activity in Leydig cells in relation to their structural differentiation during the period of Leydig cell renewal after EDS.

Material and Methods

Adult Wistar male rats received a single intraperitoneal injection of EDS at a dose of 75 mg/kg body weight dissolved in dimethylsulphoxide and water (1 : 3, v/v). Rats were killed on days 14, 21 and 30 after initial treatment (N = 4 per group). One testis was fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, and 1% osmium tetraoxide and embedded in Durcupan. Ultrathin sections were observed in an Opton electron microscope 109. For enzyme histochemistry, the other testis was frozen and enzyme reactions were performed with 7-μm frozen sections according to Levy et al. (1959) for visualization of 3β-HSD with substrate dehydroepiandrosterone and to

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Abbreviations: EDS – ethane dimethanesulphonate, HSD – hydroxysteroid dehydrogenase, NADH2 – reduced form of nicotinamide adenine dinucleotide, LC – Leydig cell, cyt P-450scc – cytochrome P450 cholesterol side chain cleavage.

Nachlass et al. (1958) for NADH2-diaphorase activity using β-nicotinamide adenine dinucleotide, reduced form, as a substrate. The sections were observed and documented using a Zeiss light microscope.

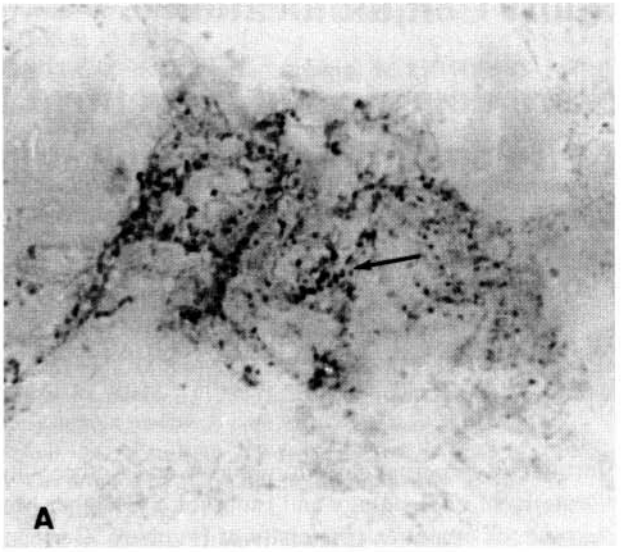
Results

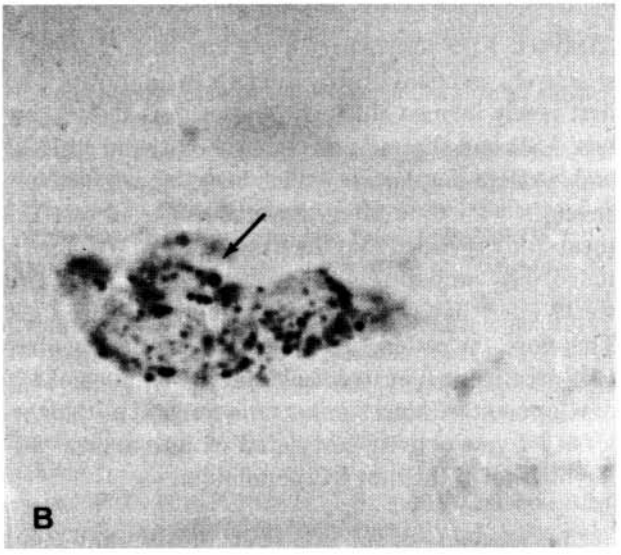
Our observations of control adult rat testis showed a strong activity in LCs for both enzymes, 3β-HSD and NADH2-diaphorase. The reaction product in LC cytoplasm was visualized as fine granules that were more abundant for NADH2-diaphorase in comparison to 3β-HSD (Fig. 1A and 1D). The 3β-HSD staining was specific for LCs whereas NADH2-diaphorase activity could be seen in the seminiferous tubules as well. The highly steroidogenic LCs were mature, adult-type, organized in clusters between the seminiferous tubules, or were in peritubular position.

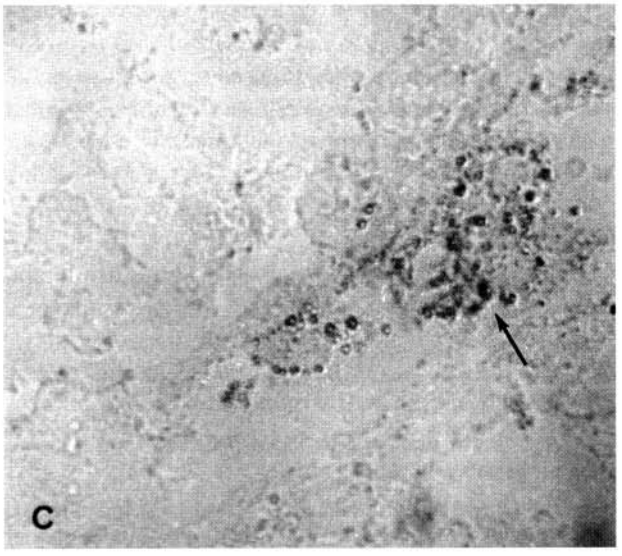
Two weeks after EDS treatment, single cells weakly stained for 3β-HSD or NADH2-diaphorase (not shown) could be found in the interstitial space of the testis. Electron-microscopical observation revealed the presence of a progenitor type of LCs with elongated spindle shape (Fig. 2A). They had little cytoplasm due to the virtual absence of the smooth endoplasmic reticulum (SER) and poor presence of the other cellular organelles in this stage. LC progenitors are intermediates in the LC lineage and they exist for only a brief interval (until at 21 days after EDS treatment), and after that they are transformed into immature LCs.

From day 21 to day 30 the intensity of histochemical staining for 3β-HSD increased in the cytoplasm of LCs (Fig. 1B and 1C) and reached the control pattern. The enzyme reaction for NADH2-diaphorase became more pronounced in the period investigated, but on day 30 it still remained weaker compared to the control (Fig. 1E and 1F). The changes in both enzyme activities coincided with numerous lipid droplets, mitochondria and appearance of SER – the specific ultrastructural characteristics for immature LCs (Fig. 2B).

On day 30 some of immature cells during their further differentiation obtained functional and morphological characteristics of mature adult-type LCs. The transition between immature and mature adult LCs was marked by a decline in cytoplasmic lipid droplets, abundance of SER and higher numbers of mitochondria with tubular cristae (Fig. 2C). An intimate association of mitochondria with SER was also observed.



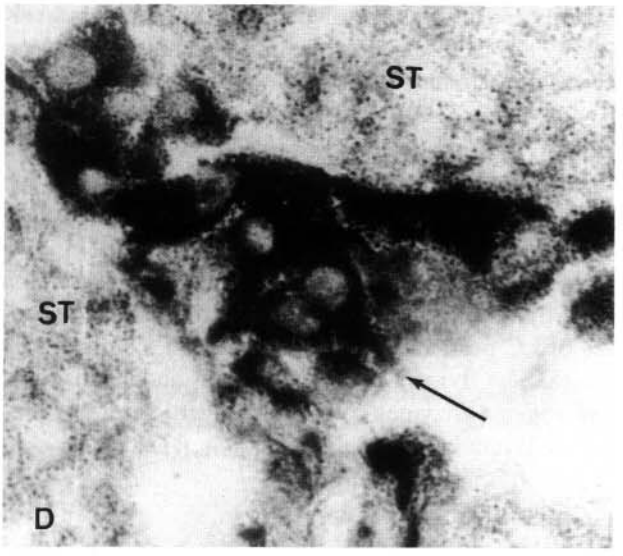


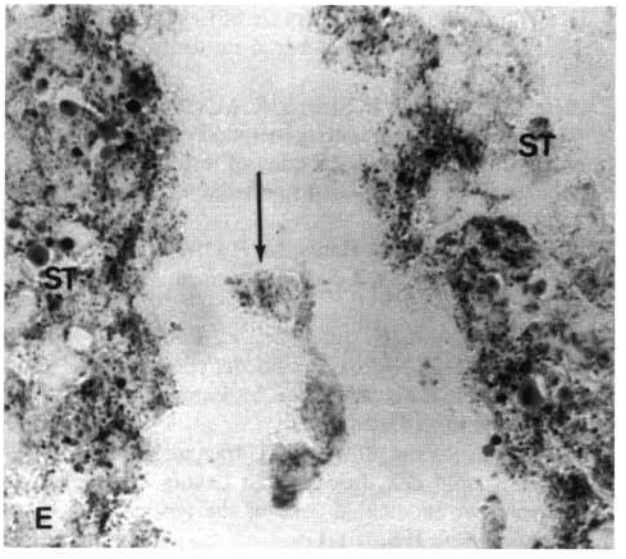


Discussion

The EDS-treated rats have become a widely used model for studies on LC development, as well as for investigation of testicular function in the absence of LCs (Tena-Sempere, 1997).

The present study used this model to provide data on functional properties of regenerating LCs related to their structural maturation, which involves development of specific cellular organelles for steroidogenesis (smooth endoplasmic reticulum, tubular mitochondria, lipid droplets). In this regard we compared the histochemical data with electron microscopic observation. Our histochemical study demonstrated an increasing





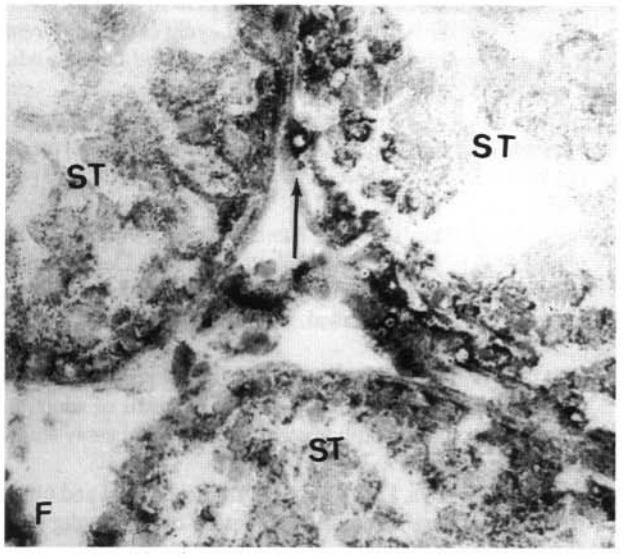


Fig. 1. Histochemical visualization of 3β-HSD and NADH2-diaphorase activity in the LCs on a frozen section of the rat testis. A. Control rat testis. A strong activity of 3β-HSD was found in the cytoplasm of mature adult-type LCs in the interstitium (arrow). Magnification 400x. B. By 21 days after EDS treatment, the presence of 3β-HSD activity was seen in the cytoplasm of newly formed cells corresponding to immature LCs (arrow). Magnification 600x. C. By 30 days after EDS treatment the 3β-HSD activity was confined to the cytoplasm of newly formed mature LCs (arrow). Magnification 400x. D. Control rat testis. Strong enzyme activity of NADH2-diaphorase was evident in the cytoplasm of mature LCs (arrow) and weaker staining was observed in seminiferous tubules (ST). Magnification 600x. E-F. A weak NADH2-diaphorase activity can be seen in single newly formed LCs after 21 and 30 days, respectively (arrow), as well as in the seminiferous tubules (ST). Magnification 200x, 400x.

activity for 3β-HSD, which is one of the most relevant steroidogenic markers for LC function. The developmental changes in histochemical staining for NADH2-diaphorase revealed the same pattern as for 3β-HSD, which suggested a close inter-relationship between this enzyme and NAD as an essential cofactor involved in the steroidogenic cycle.

Following EDS treatment, single morphologically recognizable LCs reappeared in the testis after about two weeks. The only steroidogenic enzyme to show an increase by day 14 post EDS was 3β-HSD (O'Shaughnessy and Murphy, 1991). Based on the biochemical data on enzyme activity the authors suggested that 3β-HSD was the first enzyme expressed during LC differentiation, although it is not clear whether this occured before or after the cells acquired specific morphological characteristics of LCs. However, new findings by Ariyaratne et al. (2000) provide evidence for first immunoreactivity for 3β-HSD, cyt P-450scc and P450c17 on the 11th postnatal day in elongated precursor mesenchyme-like cells. These results are in contrast to the enzyme histochemical data of Haider and Servos (1998), who established the first 3β-HSD active cells on day 13 postpartum. An explanation of this discrepancy might be that on the 11th day, progenitor LCs produce the enzyme protein (visualized by immunocytochemistry), but its enzyme activity appears two or three days later, as established by the enzyme histochemical technique. In this respect, our findings about the first 3β-HSD enzyme activity on the 14th day after EDS treatment and the strong staining intensity of this enzyme on day 21 post EDS and onwards coincide with the differentiation of progenitor LCs via immature type toward mature LCs acquiring highest capacity for production of testosterone (Ge et al., 1996). Therefore, the increase in steroidogenic enzyme activity occurs in tandem with the development of cellular organelles (mentioned above) responsible for steroidogenesis. Moreover, regeneration of LCs was reflected by elevated serum testosterone levels, which reached the normal range (Sharpe, 1994; Teerds, 1996). Our data

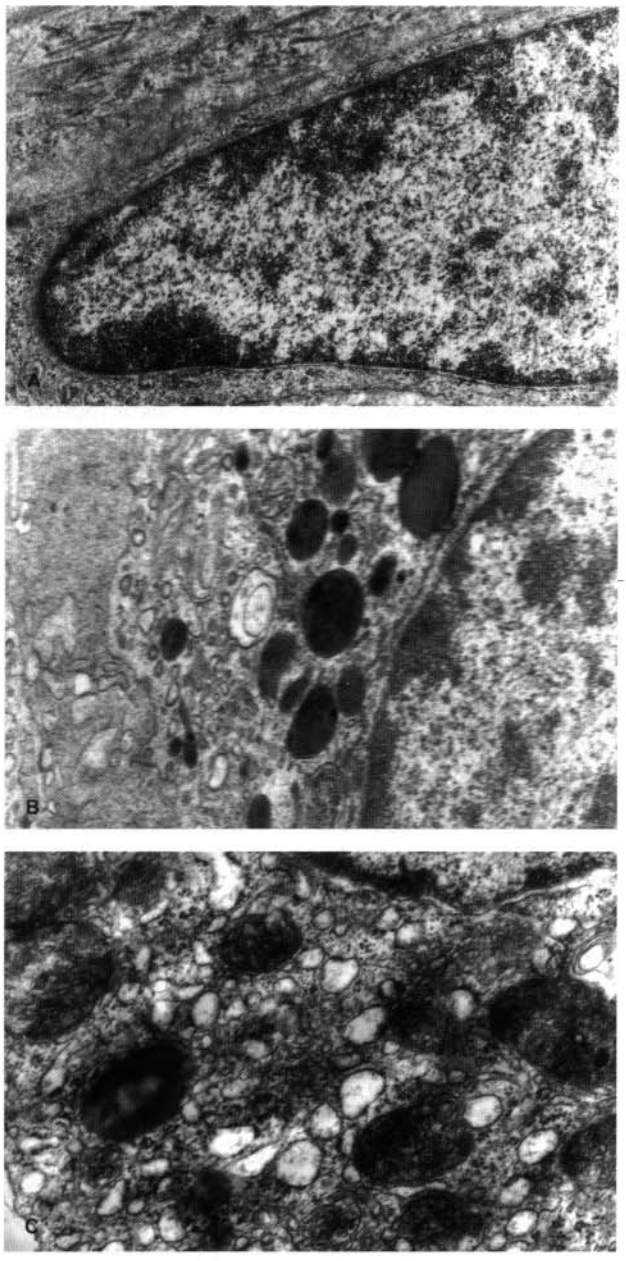


Fig. 2. Electron micrographs of newly formed LCs after EDS treatment. A. – 14 days after EDS. Fragment of progenitor LC with elongated spindle shape, irregular nuclei and little cytoplasm. Magnification 11 000x. B. – 21 days after EDS. Immature LC characterized by numerous lipid inclusions (L), appearance of smooth endoplasmic reticulum and mitochondria. Magnification 11 000x. C. – 30 days after EDS. LC transforming into the mature adult type. An abundance of smooth endoplasmic reticulum and tubular mitochondria was observed. Magnification 19 000x.

for the increasing enzyme activity of renewing LCs closely support this finding. A marked increase in the 3β-HSD activity also occurred between 21th and 30th postnatal days, when the population of mature LCs was developing (Dupont et al., 1993; Payne and O'Shaughnessy, 1996; Koeva and Popova, 1997). Therefore, the pattern of postnatal functional development of LCs seems to be similar to that seen during the recovery phase after EDS. In this respect, data by Teerds et al. (1999) indicating 3β-HSD as a marker for LC differentiation in adult rat testis post EDS bring additional support to the similarity between the steroidogenic enzyme pattern in normal and EDS-treated rats.

In conclusion, the restoration of new LC population after EDS repeats, to a great extent, the normal dynamics of LC postnatal development within a similar time range. The dynamics of appearance and intensity of investigated enzymes correlate with structural differentiation of the LC population.

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