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## A B S T R A C T

*It has been shown that adult human Leydig cells express a number of neuroendocrine markers, and, therefore, could be considered as a part of the neuroendocrine system in the adult. A limited number of studies have dealt with the dynamics of development of human foetal Leydig cells, whereas studies on their neuroendocrine nature are still extremely rare. Therefore, the aim of our study was to investigate the development of human foetal Leydig cells in different weeks of gestation (wg) and to check if these cells express certain markers characteristic of the diffuse neuroendocrine system (DNS). Qualitative, quantitative histological studies and immunohistochemical analyses of human foetal testicular tissue have been performed. According to our data, Leydig cells formed a dynamic population of cells within the interstitum of testes in the period between 15 and 36 wg. The total number of Leydig cells of human foetal testes changed through different stages of gestation by means of 'pulsatile' dynamics (most likely, by following the variable level of gonadotropins). At early stages of development (15–17 wg) immunohistochemical reactions for the expression of neuron specific enolase (NSE) were positive within sex cords and between them, in the interstitum. Pro-spermatogonia in the sex cords were positive, as well as elongated spindle-shaped cells of the interstitum (very likely, precursors of Leydig cells). During the later stages of development (28–36 wg), excluding the pro-spermatogonia, the interstitial Leydig cells were also positive. The results of the immunohistochemical analyses (the expression of NSE) confirmed the hypothesis that human foetal Leydig cells were of neuroendocrine nature.*

*Key words: Leydig cells, human, foetus, development, neuroendocrine system*

## Introduction

Leydig cells are situated within the interstitial compartment of testes, between the seminiferous tubules. In the adult, these cells produce testosterone, a male sex hormone that influences a wide range of organs, including the brain, bones, bone marrow, skin, liver, kidney as well as seminiferous tubules of the testis. Observed at ultrastructural level, these cells have round or oval nucleus with a lot of euchromatin, abundant cisternae of smooth endoplasmic reticulum and mitochondria with cristae, glycogen granules and a moderate number of lipid droplets[1,2]. A special feature of adult Leydig cells are Reinke's crystals found in humans, primates and New Zealand rabbit[3]. By producing foetal testosterone, Leydig cells play a key role during the human development in the differentiation of sex cords and Wolffian ducts (from which ductus deferens and epididymis arise). Another form of testosterone, dihydrotestosterone, stimulates masculinisation of external genitalia (penis, scrotum) and the prostate[4,5].

It seems that testicular macrophages largely influence the function of Leydig cells, especially testosterone production. Morphological studies on the rat testis have revealed a specific ultrastructural coupling of macrophages and Leydig cells. Namely, Leydig cells project slender cytoplasmic processes towards deep coated channels of macrophages. The channels are limited by an electron dense part of the macrophage membrane. It can be supposed that these channels could be sites of an intensive exchange of molecules/signals between the two cell

populations[6–8]. Leydig cells are the major source of testosterone[9], but are capable of producing many non-steroidal (»neuroendocrine«) factors like β endorphin[10] and prodymorphin[11] which are known to act as paracrine factors affecting macrophage function. In the human testis with an impairment of spermatogenesis, the structure of Leydig cells, macrophages and the production of testosterone are altered when compared to controls[12–15].

Surprisingly, a number of studies have shown that adult human Leydig cells, apart form their steroidogenic characteristics, express a number of molecules typical for neurons and glial cells. Thus, the presence of neuronal markers, synaptic and storage vesicle proteins (synaptophisin and chromogranin), neurofilament cytoskeletal proteins, enzymes involved in the synthesis of catecholamines, neurohormones and their receptors, neuropeptides and their receptors (substance P, neurokinin, β-endrophin, methoinine-enkephalin, neuropeptide tyrosine etc.) as well as components of NO/cGMP system have been demonstrated in these cells. Moreover, a significant number of glial cells markers such as galactocerebrozide, 2'-3'cyclic nucleotide 3-phosphodiesterase, glial fibrillary acidic protein and A2B5 protein are expressed in the adult human Leydig cells (in addition, some of these antigens were discovered in the mouse, rat and hamster Leydig cells)[16]. Although the significance of the above-mentioned neuronal and glial markers is not fully understood, it has been shown that some of these molecules could influence the level of testosterone production (for example, substance P and NO)[17,18].

The human foetal Leydig cells do not appear earlier than the 8 wg and are fully differentiated by the 10 wg. During the 15–16 wg, these cells increase in number, reaching approximately 24 million cells per testis[5,19]. The Leydig cell number remains stable until the 26 wg, but afterwards the number progressively decreases to approximately 9 million cells per testis just before birth[19]. This reduction coincides with low hCG plasma concentrations[20]. Leydig cell regression is thought to take place by degeneration and destruction of these cells[19].

Since there is a lack of data on the neuroendocrine characteristics of foetal human Leydig cells, in the current study we wanted to investigate the expression of neuron specific enolase (NSE), glial fibrillary acidic protein (GFAP), synaptophysin, protein S-100 and chromogranin in foetal human testicles. In addition, the developmental dynamics of foetal Leydig cells has been assessed by a qualitative and quantitative histological analysis.

## Materials and Methods

A total of 39 human foetal testicles from 15 to 36 wg were used in the study. The tissue samples were obtained during the routine paedopathological section of miscarried children during 1994 (approved by the Ethical Committee of Clinic for Gynaecology and Obstetrics, Clinical Hospital Centre »Zagreb«). Testicles have been weighed by means of a precise scale to obtain their mass/volume

for stereological analysis. The samples were fixed in Bouin's fluid, dehydrated and embedded in paraffin. Paraffin blocks were cut extensively in order to provide sections for hematoxylin and eosin (H+E), and immunohistochemical staining (IHC). Monoclonal antibodies to NSE (1:100), GFAP (1:200), synaptophysin (1:25), chromogranin (1:100) and S-100 (1:600) in combination with an appropriate EnVision kit (Dako, Glostrup, Denmark) were applied.

Stereological analysis of foetal human Leydig cells has been performed on H+E stained slides to assess the developmental dynamics of these cells (a total number of Leydig cells per testis /N/ has been determined). For this part of the analysis, a binocular light microscope Nikon Ecllipse 200 (Nikon, Japan) was used. A non-biased Weibel's 42-point multipurpose test system[21] has been applied at the magnification of 400x, where the length of the test lines (Lt) was 0.504 mm, whereas the test surface area (At) was be 0.02094 mm$^2$ for each analysed microscopic field[21,22]. An optical dissector principle[23,24] was applied to estimate the number of cells in the interstitial testicular compartment. The numerical density (Nv) of Leydig cells has been determined[21,22]. The absolute number of Leydig cells was estimated according to the following formula:

$$N = Na/Ni \times Vt \qquad (1)$$

where:

N – absolute number of Leydig cells
Na – numerical density of Leydig cells
Ni – numerical density of the cells in the interstitial compartment determined by optical dissector[24]
Vt – testis volume.

A pilot stereological measurement has been made in order to determine the number of microscopic fields (n) needed for a reliable data assessment. This measurement has been carried out on 20 microscopic fields. After the preliminary measurement, de Hoff's formula[25] has been applied:

$$n = (20 \times s/x)^2 \qquad (2)$$

where x is a mean value of Na and s is a standard deviation. In our case, the number of microscopic fields to be assessed for each slide was 20.

Statistical analysis has been done using a »SAS« biostatistical package (University of Zagreb, School of Public Health »Andrija Štampar«)[26]. Individual data were processed by a personal computer. In addition to general statistics, parts of the programme such as Pearson's coefficient of correlation and regression analysis were applied.

## Results

Qualitative histological analysis on H+E slides demonstrated that human foetal Leydig cells appeared in 2 forms: one cell form was oval, with a centrally or eccentrically positioned nucleus (and sometimes well-visible nucleolus), whereas another was elongated with a round or oval nucleus situated mainly in the middle of the cell
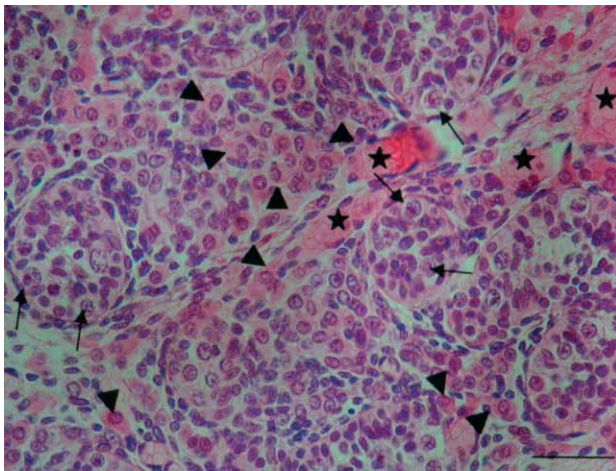
*Fig. 1. Human foetal testis in 19 wg. Within the interstitial tissue, Leydig cells (arrowheads) could be identified. These cells have an oval or elongated form. Sex cords are limited by a gentle layer of peritubular cells and are lined by pro-spermatogonia (arrows) and foetal Sertoli cells. Blood vessels (stars). H+E staining. 400x, bar = 100 μm.*

(Figures 1 and 2). The population of foetal Leydig cells changed with time/gestation weeks. Thus, in the period from 15–24 wg, as well as 27–36 wg, the cells could be easily recognized by their shape (Figure 1). In the period from 25–27 wg, foetal Leydig cells resembled fibroblasts i.e. their mesenchymal precursors (Figure 2). The described cells occupied the interstitial space between sex cords. Sex cords had 40–50 μm and were composed of pro-spermatogonia and foetal Sertoli cells. The future lamina propria consisted of several gentle layers of peritubular cells (Figures 1 and 2). In general, sex cords and associated blood vessels in the interstitium grew progressively from 15–36 wg having a rather tortuous structure (Figures 1 and 2).
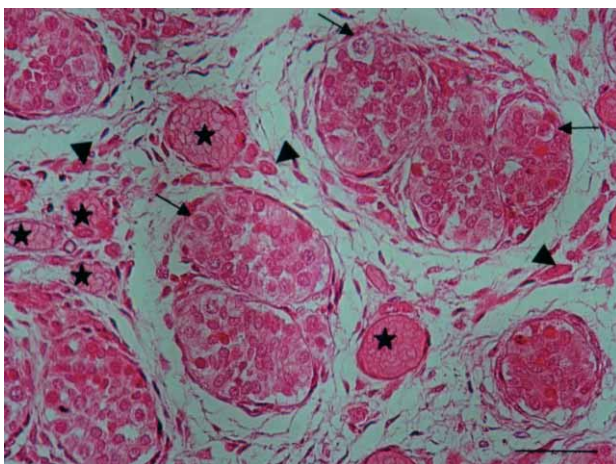


*Fig. 2. Foetal testis in the 25 wg. The majority of Leydig cells have elongated form (arrowheads) or fibroblast-like architecture. Pro-spermatogonia (arrows). Blood vessels (stars). H+E staining. 400x, bar = 100 μm.*

IHC analysis pointed out a positive expression of NSE in the foetal testis interstitium as well as within sex cords. In the early stages of foetal development (15–17 wg) elongated, spindle-shaped cells were positive (Figure 3). In the period of 18–36 wg, Leydig cells were positive for NSE (++) (Figure 4). Moreover, in both of the above--mentioned periods, a strong expression of NSE (+++) was discovered in pro-spermatogonia within sex cords, whereas foetal Sertoli and peritubular cells were negative (Figures 3 and 4). No positive results were obtained applying antibodies to S-100, GFAP, synaptophysin and chromogranin.

Stereological analyses demonstrated a pulsatile (oscillatory) development of foetal Leydig cells during the observed foetal period. However, N values steadily increa-
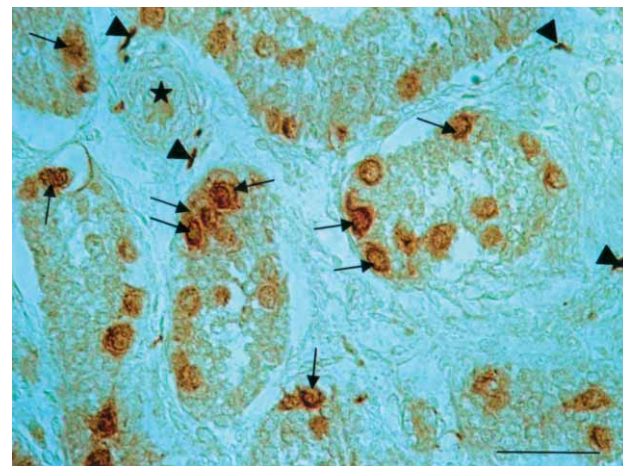


*Fig. 3. Immunohistochemical reactions for NSE. Human foetal testis in week 17 of development. Within the testis interstitium, elongated cells (arrowheads) are positive (putative Leydig cells precursors). Within the sex cords, pro-spermatogonia (arrows) strongly express NSE. Blood vessel (star). Diaminobenzidine (DAB) staining. 400x, bar = 100 μm*
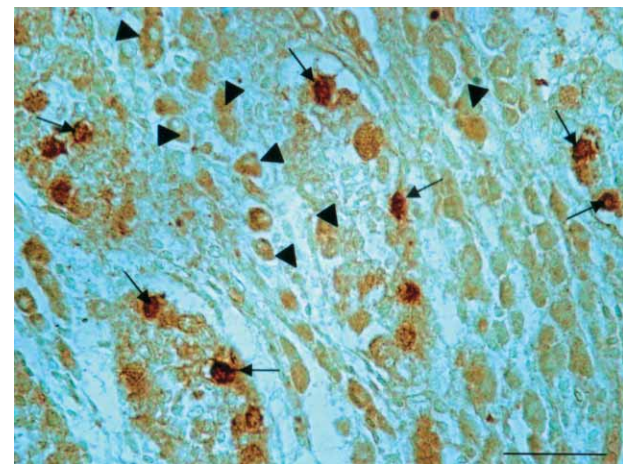


*Fig. 4. Foetal testis in 32$^{nd}$ wg. Leydig cells (arrowheads) are positive for NSE, as well as pro-spermatogonia (arrows). Diaminobenzidine (DAB) staining. 400x, bar = 100 μm*
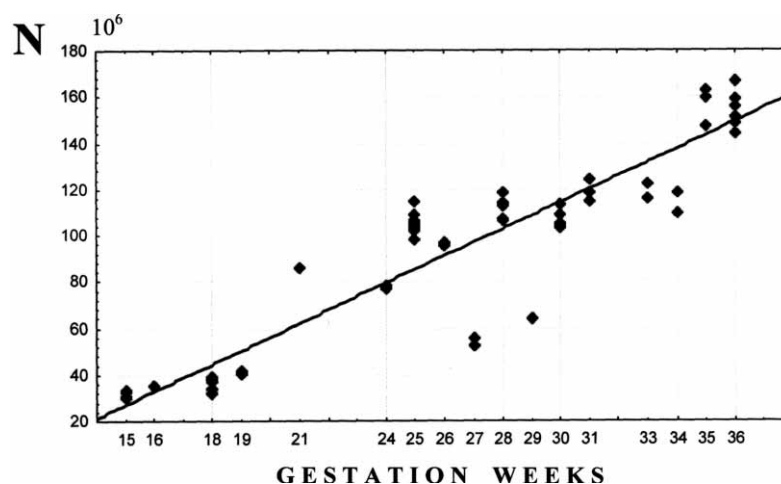
*Fig. 5. Total number of Leydig cells (N) in foetal testes is closely associated to the gestational weeks (Pearson's coefficient of correlation: r = 0.9321; P<0.001). According to the regression analysis, week of gestation is a significant predictor of N. Leydig cells demonstrate steady increase in number. However, the oscillations in N values (pulsatile dynamics) during the observed gestational period could be connected to oscillations in values of foetal gonadotropins and hCG produced by placenta.*

sed with gestational weeks. A strong positive correlation between N and weeks of gestation has been found (r= 0.9321; P<0.001) (Figure 5).

## Discussion

In general, Leydig cells are considered to be of mesodermal (mesenchyme) origin. The mesenchyme of the genital ridge is thought to give rise to the interstitium of the testis, including steroid producing Leydig cells. The two described forms of foetal Leydig cells observed on H+E slides indicate the possible dual nature of these cells: the oval much resembles the epithelial-derived population of cells, whereas the elongated or spindle-shaped form could be connected to the mesenchymal/fibroblast cell line. Epithelial-like Leydig cells could originate from other sources, not only from the mesoderm of the genital ridge. Thus, a possible source of these cells could be the neural crest/tube, since it is well known that it gives rise to dorsal root ganglions, medulla of suprarenal gland and enteric ganglia. A significant number of cells from neural tube undergo a rather complex migration to reach their »destination« in the suprarenal glands or the gut. It can be presumed that some of them, while populating the suprarenal medulla, migrate to the genital ridge, which is in the close vicinity. Our IHC results support this assumption, since the expression of NSE was shown in developing Leydig cells and their precursors. However, expression of GFAP, synaptophysin, S-100 and chromogranin was negative. This finding is in contrast to IHC results on the adult human Leydig cells (i.e. positive expression of GFAP, synaptophysin and chromogranin). One can assume that, due to the extremely low level of the above-mentioned antigens in foetal Leydig cells and application of commercially available antibodies (that are optimized for the detection of »high« concentrations of neuronal or glial antigens in central nervous system or tumours) IHC results were negative. Recent studies clearly indicated a presence of neuronal and glial markers in both the developing and adult human testis[27–29].

Stereological analyses pointed out a pulsatile developmental dynamics of foetal Leydig cells. This could be due to the activity of foetal hypophysis. It is known that gonadotropins are released from adenohypophysis in an oscillatory manner, which could strongly influence the development of the foetal testis. However, N showed a steady and stable increase in the observed gestational weeks. This could be the effect of human chorionic gonadotropin secreted by the placenta. This hormone has relatively stable levels during the foetal period (in addition to foetal LH and FSH), and could stimulate the growth of Leydig cells[30].

In conclusion, we propose that foetal Leydig cells have a dual nature: they are steroidogenic cells that produce significant levels of testosterone during the foetal development; however, since they express NSE (and other neuronal and glial markers found in other studies), they could be considered as a part of the neuroendocrine system or paraneurons.

## Acknowledgements

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## LJUDSKE FETALNE LEYDIGOVE STANICE – DA LI SU DIO FETALNOG NEUROENDOKRINOG SUSTAVA?

## SAŽETAK

Ljudske Leydigove stanice u odraslom sjemeniku posjeduju izražaj neuroendokrinih markera. Stoga se smatraju dijelom neuroendokrinog sustava odrasle individue. Manji broj istraživanja bavio se razvojem ljudskih fetalnih Leydigovih stanica, dok su studije o neuroendokrinim obilježjima tih stanica izrazito rijetke. Zato je cilj našeg istraživanja bio istražiti razvoj ljudskih fetalnih Leydigovih stanica tijekom različitih tjedana gestacije i provjeriti da li ove stanice izražavaju određene molekule/markere karakteristične za difuzni neuroendokrini sustav (DNS). Načinjena je kvalitativna, kvantitativna i imunohistokemijska analiza tkiva ljudskog fetalnog sjemenika. Prema našim podacima, fetalne Leydigove stanice u razdoblju od 15. do 36. tjedna gestacije čine dinamičnu skupinu stanica u intersticiju sjemenika. Ukupan broj Leydigovih stanica ljudskih sjemenika povećavao se »pulzatilno« (najvjerovatnije slijedeći promjenjivu razinu gonadotropina). U ranim stadijima razvoja (od 15. do 17. tjedna gestacije) imunohistokemijski izražaj neuron specifične enolaze (NSE) bio je pozitivan u stanicama spolnih tračaka i između njih, u intersticiju. Unutar spolnih tračaka pozitivne su bile prospermatogonije, dok su u intersticiju pozitivne bile izduljene stanice (najvjerovatnije preteče Leydigovih stanica). U kasnijim fazama razvoja (od 28. do 36. tjedna gestacije), osim prospermatogonija, pozitivne su bile i intersticijske Leydigove stanice. Rezultati imunohistokemijske analize (izražaj NSE) potvrdili su hipotezu o neuroendokrinoj prirodi ljudskih fetalnih Leydigovih stanica.