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Stereological Analysis of Reinke's Crystals in Human Leydig Cells *

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Summary. Reinke's crystals in human Leydig cells were analyzed stereologically to assess their functional role. Testicular tissues were taken from seven older men (57–82 years old) with prostatic carcinoma and also from seven younger men (26–38 y.o.) complaining of male infertility. Sections 0.5 μm thick, stained with toluidine blue or Heidenhein's iron-hematoxylin were examined by a point-counting method and with a Particle Measurement Computor System (*ΠMC*). When the patients were grouped by age, the mean crystal volume, the number of crystals per cell, the volume of crystals per cell and the volume ratio of crystals to cell were significantly larger in the older age group than in the younger age group. In particular, the latter three variables correlated well with the age of subjects, with correlation coefficients of $r = 0.66$–0.85. On the other hand, none of these variables had any correlation with the concentration of plasma testosterone. These results indicate that Reinke's crystals can be considered as degenerative products in cell life but not as facultative constituents for testosterone production.

Key words: Stereology – Reinke's crystal – Leydig cell – Testis – Testosterone.

Introduction

Human Leydig cells characteristically have Reinke's crystals in their cytoplasm, sometimes in the nucleus. Cells identical to Leydig cells may be found in the

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human ovary as a hilus cell tumor or hyperplasia (Roth and Sternberg, 1973; Salm, 1974). A rare case of ganglioneuroma of the adrenal medulla containing cells identical to hilus cells was reported by Scully and Cohen (1961). Magalhães (1972) suggested the possibility that Leydig cells may be a constant component of the adrenal cortex in human males. Identification of these cells as Leydig cells or hilus cells at light microscopic level has been based on the presence of Reinke's crystals in their cytoplasm. The Reinke's crystals are, thus, one of the most important characteristics for human Leydig cells. Their fine structure has been studied by Fawcett and Burgos (1960) and Yamada (1965), and has been shown to be a hexagonal prism consisting of 50-A-thick filaments by Nagano and Ohtsuki (1971). The functional role of the crystals, however, remains obscure because of the difficulty in obtaining testicular tissues, paucity of the crystals in the tissue and the difficulty of purification. Reinke (1896) originally reported that they occurred in the testes of human males between 15 and 65 years of age, whose testes showed spermatogenesis. Hence it has been suggested that they may be involved in testosterone production, because testosterone is actively produced in the testes of those individuals.

Testicular biopsies being examined in our laboratory, however, gave us the impressions that Reinke's crystals were degenerative products. They were not important constituents of Leydig cells in terms of testosterone production and both their number and the volume of crystals increased with age. The purpose of this study is to assess the functional role of the crystals in correlation with aging and testosterone production.

Materials and Methods

Collection of Samples

Testicular tissues were collected from seven patients with prostatic carcinoma and from seven individuals complaining of male infertility. The subjects with prostatic carcinoma, which had been diagnosed by trans-rectal biopsy in our laboratory, were 57 to 82 years old, averaging 69.4 years, and those with male infertility were 26 to 38, averaging 29.9 years. All patients agreed either to total orchiectomy as radical therapy for cancer or to open biopsy for histological examination of spermatogenesis.

Tissues cut into small pieces were fixed with 1% osmium tetroxide and 1.5% glutaraldehyde mixture buffered with phosphate as described previously (Mori and Matsumoto, 1974). Tissue blocks were stained with 2% uranyl acetate and embedded in Epon. Sections 0.5 μm thick were cut with a Porter-Blum MT-1 ultramicrotome. The exact thickness of the sections was determined by the method of Small (1968).

Plasma testosterone levels were estimated in the peripheral blood taken at 11 to 14 h, one to three hours before collection of tissues, using the method of Mayes and Nugent (1968).

Stereological Principles

Point-Counting Method. Volume density V_{vi}, the total volume of a component i within the unit volume of the tissue, is determined in sections by comparing the number of hits P_i from an array of superimposed test points on the component i with the total number of test points P_T lying on the tissue (Weibel and Bolender, 1973),

$$V_{vi} = k_i \cdot \frac{P_i}{P_T}$$

where k_i is a correction factor for section thickness (Weibel and Paumgartner, 1978). The volume density is also equal to the product of the average volume $\bar{v}_i$ of the component i per cell and the numerical density N_v of cells.

$$V_{vi} = \bar{v}_i \cdot N_v$$

Average cell volume $\bar{v}_c$ of an individual cell can, therefore, be estimated from the ratio of hit points P_c on either the cytoplasm or the nucleus to those P_n on the nucleus, if the average volume $\bar{v}_n$ of an individual nucleus is known,

$$\bar{v}_c = \bar{v}_n \cdot \frac{P_c}{P_n} \cdot \frac{k_c}{k_n}$$

where k_c and k_n are the correction factors of the cell and the nucleus for section thickness, respectively. The average volume $\bar{v}_n$ of an individual nucleus is estimated from the mean diameter $\bar{d}$ of the nucleus.

$$\bar{v}_n = \tfrac{1}{6} \pi \bar{d}^3$$

Reconstruction Method From Serial Sections. Reinke's crystals are considered to be hexagonal prisms. If a structure such as a column or a pillar is cut in serial sections, the volume V can be estimated from the area A_i on each section,

$$V = \sum_{i=1}^{n-1} \frac{h}{3} (A_i + \sqrt{A_i \cdot A_{i+1}} + A_{i+1})$$

where h is the height from one section to the next.

Stereological Applications

A set of preparations for point-counting consisted of 5 to 10 sections per testis, a single section being obtained from a single block. Sections stained with toluidine blue were viewed through a 40× objective lens and an eyepiece equipped with a lattice grid containing 400 test points (Fig. 1). The number of fields examined was from 100 to 390, so that the relative error of the volume of the Leydig cells fell below 2%. Hit points P_i on the profiles of the nucleus, the crystal and the cytoplasm were separately recorded and compared with total test points P_T within the tissue. Long and short axes of 200 nuclear profiles per tissue were measured. From a histogram of the diameter of nuclear profiles, the mean diameter $\bar{d}$ of the nucleus of each tissue was determined by the method of Giger and Riedwyl (see reference of Weibel and Bolender, 1973).

Another set of preparations consisted of serial sections stained with Heidenhein's iron-hematoxylin after removal of embedding resin (Imai et al., 1968). This staining gave the crystals a very dark appearance and allowed the crystals to be easily recognized (Fig. 2). Selection of Leydig cell nests representative of tissue from the standpoint of frequency of crystals was done by a preliminary survey view throughout the serial sections. The Leydig cell nests consisting of a total of more than 300 cells per testis were photographed consecutively on serial sections throughout the nests. Negative films were then projected onto a glass screen. Profiles of the crystals, nuclei and boundary lines of the cell nests were drawn on paper. Each crystal was then identified, numbered consecutively and painted black (Fig. 3). Area A_i of each profile of the crystal was measured by a Particle Measurement Computor System (*IIMC*, Millipore-Olympus, Tokyo). The area of the cell nests in which the crystals examined above occurred was measured by cutting and weighing. The number of total Leydig cells in the nests was counted by identifying each nuclear profile. Subsequent calculation was made with a Hitachi Hitac 10 computor.

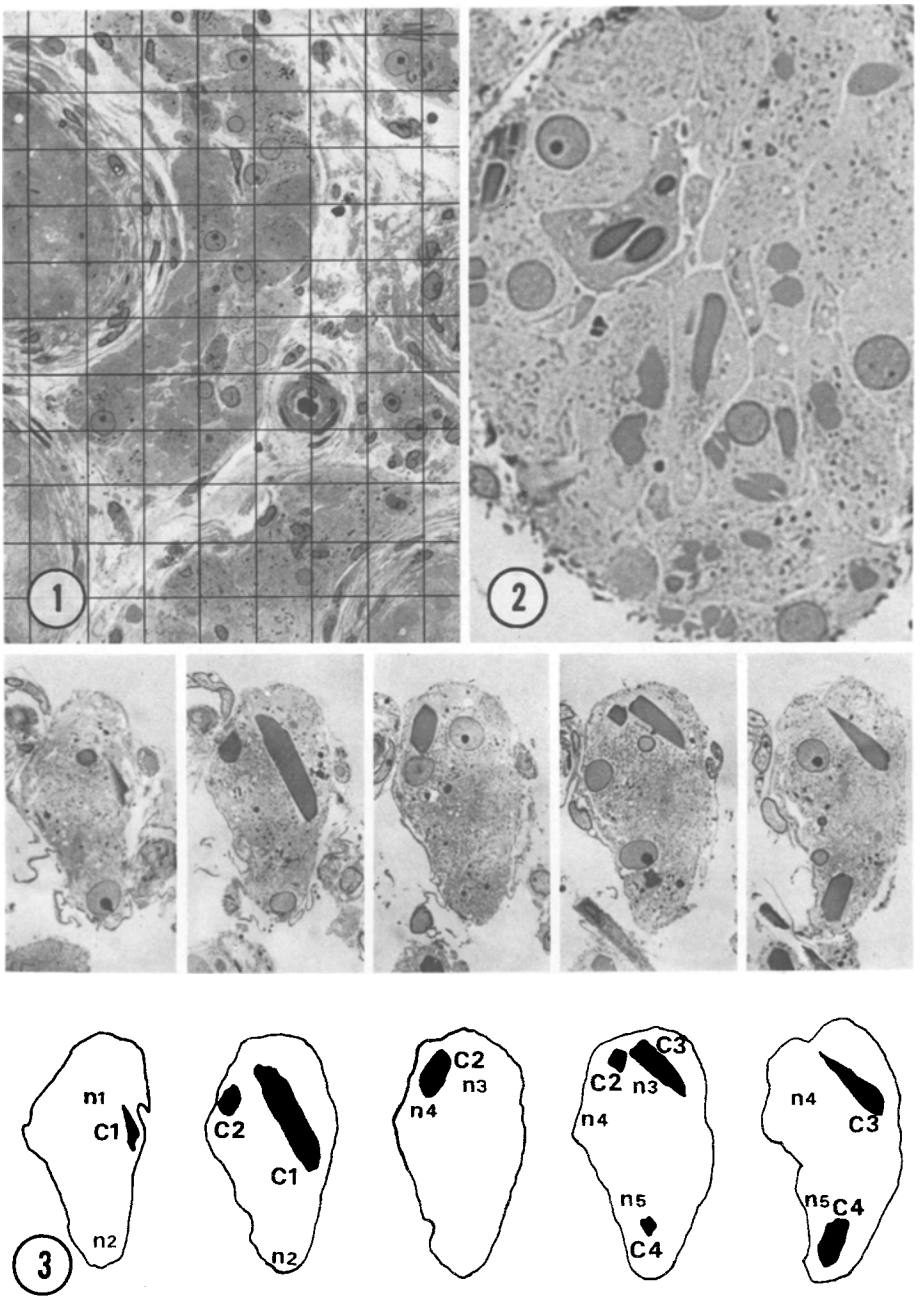

Fig. 1. Light micrograph of the testis from case No. 9. This lattice grid was used for volume density measurements of Leydig cells and Reinke's crystals. Toluidine blue stain, × 330

Fig. 2. A Leydig cell nest from case No. 2, stained with Heidenhein's iron-hematoxylin, containing numerous Reinke's crystals

Fig. 3. A portion of serial sections shown at intervals of about 4 μm. Reinke's crystals were numbered consecutively and painted black. × 500

Table 1. Comparison of parameters of Leydig cells and Reinke's crystals by the age

Case number	Age	Plasma testosterone	Volume density of Leydig cells	Mean cell volume	Mean crystal volume	Number of crystals per cell	Volume of crystals per cell	Volume ratio of crystals to cell
High age group								
1	82	2.0	0.8	1607	73.2	1.14	83.4	5.2
2	77	3.0	5.1	5020	58.4	2.33	136.3	2.7
3	74	6.8	6.0	4268	125.1	1.34	167.0	3.9
4	74	6.8	0.7	3091	68.6	1.77	121.5	3.9
5	63	4.3	2.7	3744	136.4	0.81	110.7	3.0
6	59	3.0	3.2	6402	152.3	0.63	96.4	1.5
7	57	3.7	5.1	3974	77.9	2.07	161.1	4.1
Mean ±S.E.	69.4 ±3.7	4.2 ±0.7	3.4 ±0.8	4015 ± 567	98.8 ± 14.3	1.44 ±0.24	125.2 ± 11.9	3.5 ±0.5
Lower age group								
8	38	5.0	2.1	2916	55.8	0.73	40.6	1.4
9	32	4.5	17.5	4095	65.6	0.99	64.8	1.6
10	31	3.7	5.8	3474	93.1	0.88	81.4	2.3
11	29	3.7	2.7	2958	49.8	0.03	1.3	0.0
12	27	8.0	4.5	3879	70.1	0.45	31.5	0.8
13	26	3.6	6.3	3323	33.3	1.03	34.3	1.0
14	26	6.5	4.1	3761	51.2	0.37	18.9	0.5
Mean ±S.E.	29.9 ±1.6	5.0 ±0.6	6.1 ±2.0	3487 ± 171	59.8 ±7.1	0.64 ±0.14	39.0 ± 10.2	1.1 ±0.3
t Test			$p<0.25$	<0.50	<0.05	<0.025	<0.005	<0.005

Results

Concentrations of plasma testosterone of 14 individuals were from 2.0 to 8.0 ng/ml with an average of 4.6 ± 0.5 (S.E.) ng/ml (normal values obtained in our laboratory: 3.5–11.0 ng/ml). The concentration of plasma testosterone tended to be higher in younger subjects than in older ones, although it varied from case to case.

Seminiferous tubules of the patients with prostatic carcinoma showed fibrosis and hyalinization of the peritubular boundary layer and a decrease in number of tubular cells. Male infertility was caused by either germ cell maturation arrest or hypoplasia of the tubules. Although proliferation of Leydig cells was seen in some cases of tubular hypoplasia, there was no instance of severe degeneration or atrophy of the Leydig cells.

The tissue relationship of the Leydig cells to the seminiferous tubules in the testes used in this study was generally maintained. Relative volume of the Leydig cells to the whole testis may be underestimated, because the interstitial space was somewhat widened during tissue preparation in some cases. It varied

greatly from case to case, being 0.7% to 17.5% with an average of 4.8 ± 1.1 (S.E.)%, but the structure of the Leydig cells was well preserved. The Leydig cells were polygonal in shape with a round, vesicular nucleus (Figs. 1, 2) and abundant cytoplasm which stained densely with toluidine blue. The nucleus showed chromatin distribution predominantly toward the periphery of the nucleus and had a membrane of considerable thickness. Reinke's crystals varied in shape but usually were rod-shaped and occurred mainly in the cytoplasm. With these characteristics Leydig cells were distinguished from other cells in the interstitial tissue, particularly from macrophages.

The mean volume of individual Leydig cells from 14 patients ranged from 1607 to 6402 μm^3 (3751 ± 294 μm^3: Mean $\pm$ S.E.). Mean crystal volume was 33.3 to 152.3 μm^3 (79.3 ± 9.4). Number of crystals per cell was 0.03 to 2.33 (1.04 ± 0.17) and volume of crystals per cell was 1.3 to 167.0 μm^3 (82.1 ± 14.1). Reinke's crystals occupied 0.0 to 5.2% (2.3 ± 0.4) of the volume of the Leydig cell.

These variables were correlated with age and plasma testosterone level. At first, 14 males were divided into two groups by age (Table 1). The high age

Table 2. Comparison of parameters of Leydig cells and Reinke's crystals by the concentration of plasma testosterone

Case number	Plasma testosterone	Age	Volume density of Leydig cells	Mean cell volume	Mean crystal volume	Number of crystals per cell	Volume of crystals per cell	Volume ratio of crystals to cell
High testosterone group								
12	8.0	27	4.5	3879	70.1	0.45	31.5	0.8
3	6.8	74	6.0	4268	125.1	1.34	167.0	3.9
4	6.8	74	0.7	3091	68.6	1.77	121.5	3.9
14	6.5	26	4.1	3761	51.2	0.37	18.9	0.5
8	5.0	38	2.1	2916	55.8	0.73	40.6	1.4
9	4.4	32	17.5	4095	65.6	0.99	64.8	1.6
5	4.3	63	2.7	3744	136.4	0.81	110.7	3.0
Mean	6.0	47.7	5.4	3679	81.8	0.92	79.3	2.2
±S.E.	±0.5	± 8.3	±2.1	± 189	±13.0	±0.19	± 20.8	±0.5
Low testosterone group								
10	3.7	31	5.8	3474	93.1	0.88	81.4	2.3
11	3.7	29	2.7	2958	49.8	0.03	1.3	0.0
7	3.7	57	5.1	3974	77.9	2.07	161.1	4.1
13	3.6	26	6.3	3323	33.3	1.03	34.3	1.0
2	3.0	77	5.1	5020	58.4	2.33	136.3	2.7
6	3.0	59	3.2	6402	152.3	0.63	96.4	1.5
1	2.0	82	0.8	1607	73.2	1.14	83.4	5.2
Mean	3.2	51.6	4.1	3823	76.9	1.16	84.9	2.4
±S.E.	±0.2	± 8.8	±0.8	± 580	±14.6	±0.30	±20.8	±0.7
t Test			$p > 0.50$	> 0.50	> 0.50	> 0.50	> 0.50	> 0.50

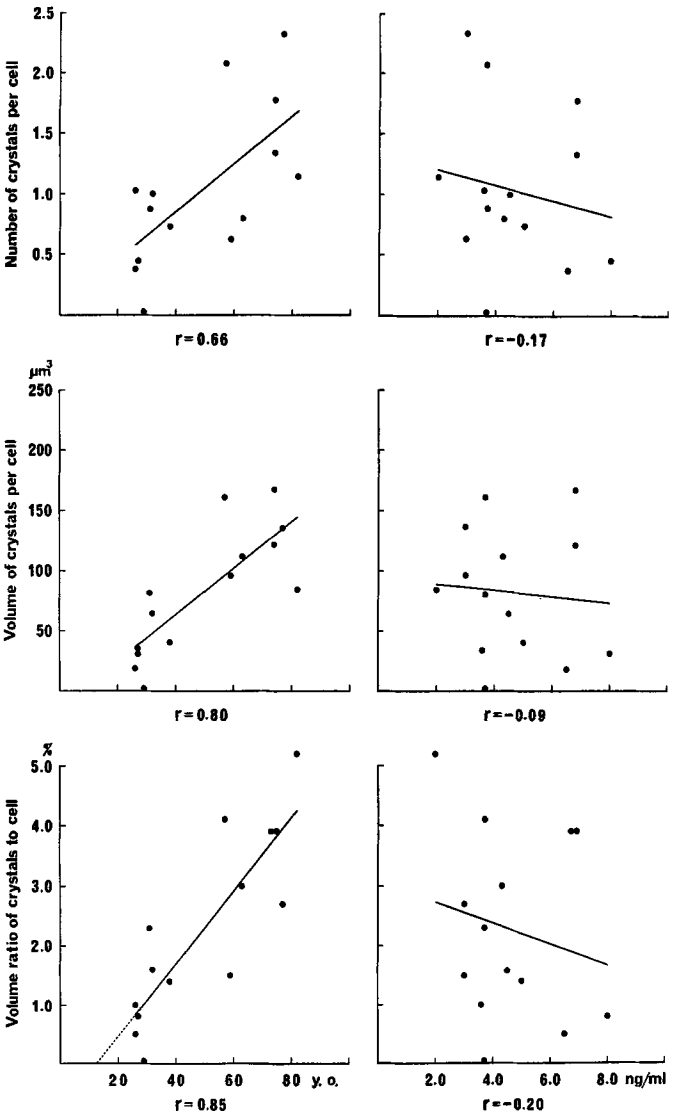

Fig. 4. Scatter diagrams and regression lines of number of crystals per cell, volume of crystals per cell and volume ratio of crystals to cell on the age (*left*) and on the plasma testosterone concentration (*right*). *r*: correlation coefficient

group consisted of seven patients bearing prostatic carcinoma; their average age being 69.4 ± 3.7 (Mean $\pm$ S.E.) years. These patients complained dysuria and/or hematuria but were otherwise normal. The low age group consisted of seven individuals who complained of male infertility; the average age was 29.9 ± 1.6 years. The relative volume of Leydig cells in the testis was greater in the low age group (6.1%) than in the high age group (3.4%). There was no significant difference in mean cell volume ($P < 0.50$). Thus the number of

Leydig cells seems to decrease in older individuals. Differences between the two groups in variables for the crystals, however, were all significant. Mean crystal volume, number of crystals per cell, volume of crystals per cell as well as volume ratio of crystals to cell were greater in the high age group than in the low age group ($P < 0.05$–0.005). Grouped by high and low plasma testosterone levels, there were no significant differences in all six variables ($P > 0.50$, Table 2).

Scatter diagrams and regression lines of these variables are shown in Figure 4. Number of crystals per cell, volume of crystals per cell and volume ratio of crystals to cell correlated well with age, with a respective correlation coefficient of $r = 0.66, 0.80, 0.85$, but not with plasma testosterone level. From the regression lines, it can be roughly estimated that in a 30-year-old man, there is one crystal in every two Leydig cells and the crystals occupy 1% of cell volume; and in a 60-year-old man, one crystal appears in every Leydig cell, occupying 3% of the cell volume.

Discussion

The tissue preparations used here consisted of 0.5-μm-thick sections stained with toluidine blue or iron-hematoxylin and were suitable for morphometric analysis of Reinke's crystals, because of clear identification of their structure. Although the interstitial tissue in the testes examined was somewhat widened (due to fixation artefact), the structure of Reinke's crystals and Leydig cells was well preserved. The morphological variables of the crystals can be discussed in relation to the age of the subjects and testosterone production.

Our results indicate that the occurrence of Reinke's crystals correlates with the aging of individuals but not with the plasma testosterone level. When the individuals examined are grouped by age, the mean crystal volume, the number of crystals per cell, the volume of crystals per cell and the volume ratio of crystals to cell are all greater in the high age group than in the low age group with $P < 0.05$–0.005 (Table 1). On the contrary, the concentration of plasma testosterone has no relation to the occurrence of crystals (Table 2).

Hornstein et al. (1966) studied the frequency of Reinke's crystals in normal and injured testes. Examining 136 biopsied testes they counted the number of crystals in 100 unselected Leydig cells per biopsy and evaluated the correlation between the crystals and normal Leydig cells. They concluded that Reinke's crystals could be considered as facultative constituents of well stimulated and hormonally active cells and not as products of degenerate cells. The frequency of crystals in their study seems to be an underestimate because of the difficulty in recognizing small crystals in paraffin-embedded sections stained with hematoxylin and eosin. It also seems difficult to distinguish "pathological" Leydig cells from macrophages or fibroblasts in conventional sections. In Hornstein's study, the correlation coefficient between the number of crystals and that of normal Leydig cells was $r = 0.406$. This is much lower than our findings ($r = 0.66$–0.85). Testosterone is produced as the major androgen in the Leydig cells of most animals (Hall, 1970). Nevertheless the crystals occur only in human Leydig cells. It seems implausible that Reinke's crystals play an important role in testosterone production.

Interestingly, the regression line of volume ratio of crystals to cell by age indicates that the earliest Reinke's crystals may appear at about 13 years of age (Fig. 4). This result agrees with the general finding that Reinke's crystals are observed after puberty. In addition, with the value of $r=0.85$, it seems possible to use this variable as an index of the aging of the human males provided they are well nourished. From the finding that Leydig cells may decrease in number as the individual ages, and at the same time the relative volume of the crystals in the Leydig cells increases it can be postulated that the crystals disappear with the death of Leydig cells.

It is desirable to perform further studies on the clarification of the mechanism of development and also the biochemical nature of the crystals, which have been considered to be protein.

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