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Morphometric Studies of Leydig Cells in Chinchillas (*Chinchilla lanigera*) during High and Low Fertility Seasons

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The aim of this study was to examine morphometric data of Leydig cells of 10 male chinchillas. Testes, cut into 5-μm thick sections, were stained using the p.a.S. and Masson's methods. Some 3800 Leydig cells have been evaluated. Their dimensions, as well as the diameters of their nuclei and the distances of the nuclei from the boundaries of the cells, have been measured. The areas of the surface and volumes of the nuclei of Leydig cells have been calculated, as well as the areas of the surface of the Leydig cells themselves. The following data have been obtained. The Nuclei of Leydig Cells. The largest diameters: longer cells – 12 μm; shorter cells – 10 μm. Mean diameters: longer cells – 5.67 ± 3.44 μm, shorter cells – 4.45 ± 3.44 μm. The largest surface area – 120 μm^2, the mean surface area – 28.27 ± 11.21 μm^2. The largest volume – 1200 μm^3, the mean volume of nucleus – 171.8 ± 65.82 μm^3. Mean distances of Leydig cell nuclei from the opposite boundaries of the cells amounted to 1.29 ± 1.41 μm, 4.24 ± 2.39 μm, 4.09 ± 2.23 μm, 6.12 ± 2.33 μm. Leydig cells. The largest diameters: longer cells – 24 μm, shorter cells – 22 μm. Mean dimensions: longer cells – 13.86 ± 2.76 μm, shorter cells – 10.89 ± 2.44 μm. The largest area of surface – 528 μm^2, the mean area of surface – 155.44 ± 59.78 μm^2. Morphometric analysis confirmed cytologic observations that the shape of the nuclei of Leydig cells is somewhat ellipsoidal. The nuclei are located off-centre and are not situated in the greatest agglomeration of cytoplasm. The shape of Leydig cells is irregular. The obtained results may provide insight on the infertility of chinchilla males, as well as in research on the annual cyclic fertility of these animals. They may be put to use in practice for the purpose of improving breeding of this species.

Key words: *Chinchilla lanigera*, Leydig cells, reproduction.

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Leydig cells represent the key factor regulating the process of spermatogenesis in seminiferous tubules. This function can involve secretion of testosterone (CARREAU *et al.* 1988; ROBERTS & GRISWOLD 1990), derivatives of POMC (pro-opiomelanocortin), and oxitocin (PICKERING *et al*. 1990), and can be reversely regulated by a number of other factors (BILIŃSKA 1992). Seasonal changes in Leydig cells can be observed in some species, including the camel (ZAYED *et al.* 1995), roebuck (SHORT *et al*. 1966), and golden (Syrian) hamster (HAKIM *et al*. 1989), and involve reduced area and volume of cellular organelle, including nuclei, reduced area and volume of Leydig cells, as well as lower total volume and number of cells in the gonad.

Chinchillas, despite being polyoestrous mammals, display a clearly delimited seasonality in their reproduction. The seasonality can be observed both in their natural habitat and in farm environments. Wild chinchillas deliver their young during the season which is the most favouable in terms of raising the offspring. Under man-made housing conditions, the seasonality is not as distinct as in nature. Nevertheless, enhanced sexual activity in a temperate climate occurs from November until May (GROMADZKA-OSTROWSKA 1998; JAROSZ & RŻEWSKA 1996; NEIRA *et al*. 1989).

Recognition of basic morphometric parameters of Leydig cells can be crucial for research on mechanisms that regulate the seasonal fertility of

males. There are few reports on basic histological studies of this species, whereas the morphological structures of some organs have not been thoroughly described so far. The aim of this study was to describe basic morphometric measurements of Leydig cells in chinchillas during fertile periods and periods of seasonal decrease in fertility. Studies on seasonal fertility fluctuation provide understanding of the natural model for experimental and comparative studies related to the effects of various factors (including pharmaceuticals) on fertility. It is possible that hormonal methods of sexual cycle stimulation may be developed that could enable improvement of the reproduction potential of farm animals with seasonal fertility changes.

Material and Methods

Animals

The study involved 10 chinchilla males (*Chinchilla lanigera*) during their full fertility (November-May, group 1) and 10 males during their seasonal fertility depression (June-October, group 2). A period of lower fertility in chinchillas is observed from June until October, the period of full fertlity – from November until May. Reduced fertility does not mean its entire inhibition.

Morphology and histology

A series of testis samples were dissected from the gland along its longitudinal axis through the largest diameter, and treated according to the rules given by GUNDERSEN *et al.* (1988a). The sections of the testes, after fixation in Bouins solution, 5 μm thick, were stained with haematoxylin and eosin as well as with the PASH (periodic acid Schiff and haematoxylin) and Masson's methods, and scanned for fields for analysis in meander-shape movements in the direction from the largest diameter of the section to its side.

Only those cells were classified for the analysis whose borders where sharp and easily distinguished.

Morphometric measurements

A total of 3800 Leydig cells were analysed in each group. The sample size was established for 0.99 probability, following an initial calculation of standard deviation for a sample of 30 analyses and for measurement error equal or less than 1 μm (MILLER & ORZESZYNA 1982). The measurements were done under light microscopy using Hauge's planimetric grid with 1 μm side dimension of the measuring squares as well as the grid according to CRUZ-ORIVE & HUNZIKER (1986). Measurements with Hauge's grid, performed according to Figure 1, involved the diameter (long and short) of Leydig cell nuclei as well as the long and short dimensions of the cells. The distances (A, B, C, and D) from the cell borders to the largest diameters of the nuclei (max_1 and max_2) did not include these diameters. The nuclei were considered circular if both diameters (long and short) were equal or the difference between them did not exceed 1 μm. The nuclei were classified as oval if the difference between their long and short diameters were more than 1 μm.

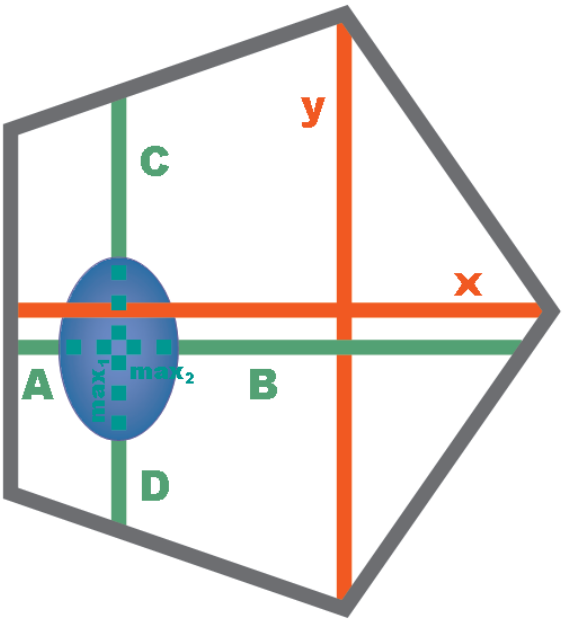

Fig. 1. Measurements of the distance between nucleus and cell borders, the longest distance between cell borders, as well as the longest major and minor axes of cell nuclei; max_1, max_2 – the longest major and minor diameters of the cell nucleus; A, B, C, D – distances between nucleus and cell borders measured as extensions of major and minor diameters of the nucleus; x – the longest "horizontal" distance between cell borders; y – the longest "vertical" distance between cell borders.

Surface area and volume of Leydig cell nuclei, surface area and volume of Leydig cells, as well as total values for the gland, i.e. surface area and volume of Leydig cell nuclei, surface area and volume of Leydig cells, were determined according to GUNDERSEN (1988b). Surface area of Leydig cells and their nuclei (μm^2) were derived from the formula $A = \pi \times l^2 / 2$, whereas their volume according to the equation $V = 4/3\ \pi \times l^3$. While archiving the data to a spreadsheet, shrinkage allowance, 0.9, was taken into account (ZIELIŃSKI & STRZELECKI 2002).

Statistical analysis

Statistical analysis of the results was carried out with the coefficient of correlation, equations of correlation and regression, Spearman's rank correlation coefficient, chi-square test, Kolmogorov-Smirnov test, test for means of large samples, for the probability 0.95 and $\alpha = 0.01$.

Note

This study does not cover planimetric measurements of entire glands, nor did we analyse blood vessels, lymphatic vessels, or cells (fibroblasts, mast cells) found in lymphatic spaces near Leydig cells. The main reason behind this was that the texture of the gland easily went apart while cutting the tunica albuginea before preservation. Neither standard nor perfusion pre-preservation without cutting the tunica albuginea gave satisfactory results, since they did not prevent parting of the testicle texture after its preservation and, moreover, left unpreserved places in its central part. Perfusion treatment allowed preservation of the testicle parts near tunica albuginea; those parts in the centre of the gland, however, were insufficiently preserved or were not preserved at all and were useless for examination.

Due to the aim of the study, two types of grids were used for measurements. Houge's grid was very good for measuring the distance between the nucleus border and the cell border. We have not used it, however, to measure surface areas or volumes of Leydig cell nuclei due to their regular shapes; its application in examining cells would lead to excessive simplification (GUNDERSEN *et al*. 1988b; HARDY *et al*. 1992). The planimetric grid method by CRUZ-ORIVE & HUNZIKER (1986) best met the requirements for area and volume estimation.

Results

Leydig cells are polygonal in shape during the period of fertility. Small vacuoles are present in the cytoplasm. Normally fertile chinchilla males have 35% circular and 65% oval cross-sections of cellular nuclei. Average Leydig cell nucleus dimensions are 6.46 ± 1.25 μm by 5.61 ± 1.13 μm. Average dimensions of a Leydig cell are 13.86 ± 2.76 μm by 10.89 ± 2.44 μm. Leydig cells and their nuclei become smaller in size during the season of low fertility. The cells are smaller and their cytoplasm mostly contains large empty vacuoles. Single cells are more common than clusters. Both long and short dimensions decrease (by 2 μm and 3 μm, respectively) as well as all distances between the nucleus and the borders of a Leydig cell. Degenerative changes occur as a result of which total surface area and volume of the cells decrease by 27% and large empty vacuoles appear in the cytoplasm. The nuclei of the cells are smaller (by 23%) and are circular in shape rather than oval.

Morphology of Leydig cell nuclei

Normally fertile chinchilla males had 35% circular and 65% oval cross-sections of cellular nuclei. Distribution of longer cell diameters ranged between 3 and 10 μm, while distribution of shorter cell diameters ranged between 2 and 8 μm. Mean dimensions of the nuclei were 6.46 ± 1.25 μm by 5.61 ± 1.13 μm. Measurements of distances between nucleus border and cell border have demonstrated that normally fertile animals had the lowest (A) average distance between nucleus border from cell border at the level of 1.29 ± 1.141 μm, and the highest (D) was 6.12 ± 2.33 μm.

During the season of reduced fertility, i.e. between June and October, an increase in the percentage of circular nuclei cross-sections was observed up to 43%, whereas the percentage of oval ones decreased down to 56.7%. The altered shape of the dimension distribution curve during the low-fertility season speaks for a higher number of circular nuclei cross-sections of lower diameter, compared to animals in normal fertility (Fig. 2). Distances between nuclei borders and their cell borders also decreased (Table 1). The distances A and B are slightly reduced, while the distance C increased. The distance D decreased nearly twofold. All the nucleus dimensions decreased. The mean surface area and mean volume decreased by 23% (Table 2).

Table 1

Distances between nuclei border and opposite Leydig cell borders in animals with normal fertility (Group 1) and with seasonally decreased fertility (Group 2)

	Group 1			Group 2	
	means	SD	DSS*	means	SD
Distance A	1.29	1.14	+	1.14	1.09
Distance B	4.24	2.39	+	3.55	1.81
Distance C	4.09	2.23	+	4.80	1.82
Distance D	6.12	2.33	+	2.97	1.77

*DSS – statistically significant difference for two large sample means at $\alpha = 0.01$
All means differ from each other significantly

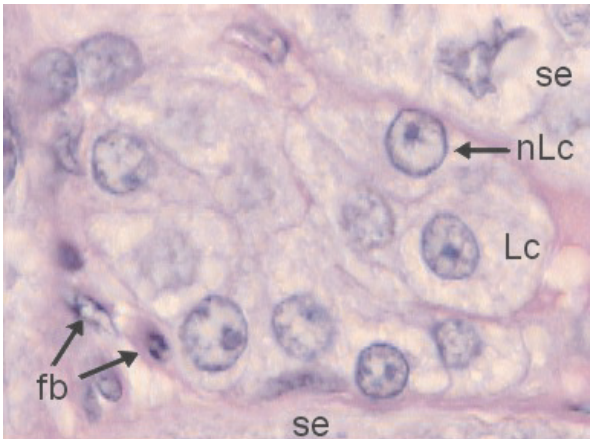

Fig. 2. Leydig cells in chinchillas during high fertility seasons. p.a.S. Mag. 100×. Lc – Leydig cell, nLc – nucleus of Leydig cell, se – seminiferous epitheplim, fb – fibroblast.

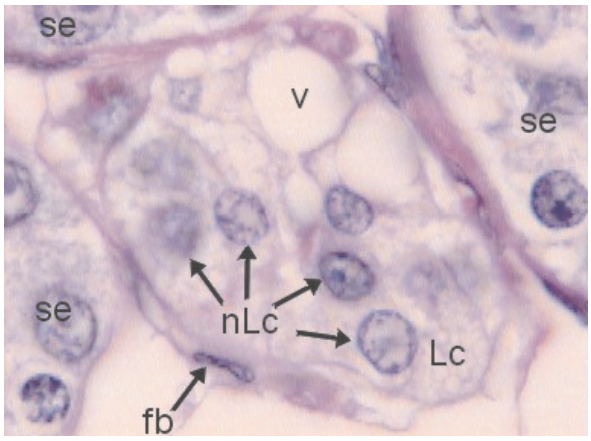

Fig. 3. Leydig cells in chinchillas during low fertility seasons. p.a.S. Mag. 100×. Lc – Leydig cell, nLc – nucleus of Leydig cell, sc – seminiferous epitheplim, fb – fibroblast, v – empty vacuoles.

Table 2

Morphometric parameters of Leydig cell nuclei and the gonad in animals with normal fertility (Group 1) and with seasonally decreased fertility (Group 2)

	Group 1		Group 2
	a	DSS*	b
Leydig cell nucleus			
minimum major diameter	3.00 μm		3.00 μm
maximum major diameter	10.00 μm		12.00 μm
minimum minor diameter	2.00 μm		3.00 μm
maximum minor diameter	8.00 μm		10.00 μm
minimum area	3.53 μm^2		6.28 μm^2
maximum area	113.43 μm^2		71.53 μm^2
minimum nucleus volume	18.84 μm^3		33.49 μm^3
maximum nucleus volume	604.97 μm^3		381.51 μm^3
major diameter	6.46±1.25 μm	ab	5.56 ±0.98 μm
minor diameter	5.61±1.13 μm	ab	4.83 ±0.80 μm
nucleus area	34.87±13.30 μm^2	ab	25.57 ±8.30 μm^2
nucleus volume	185.95±70.95 μm^2	ab	136.39 ±44.28 μm^3
Leydig cell			
minimum major dimension	6.00 μm		7.00 μm
maximum major dimension	26.00 μm		24.00 μm
minimum minor dimension	5.00 μm		5.0 μm
maximum minor dimension	17.00 μm		14.0 μm
Minimum area	28.36 μm^2		28.36 μm^2
Maximum area	453.73 μm^2		427.43 μm^2
minimum cell volume	151.24 μm^3		151.24 μm^3
maximum cell volume	2419.90 μm^3		2279.64 μm^3
major dimension	13.86 ±2.76 μm	ab	13.29 ±2.89 μm
minor dimension	10.89 ±2.44 μm	ab	9.65 ±2.33 μm
cell area	151.66 ±57.42 μm^2	ab	134.13 ±55.88 μm^2
cell volume	808.87 ±306.26 μm^3	ab	715.41 ±298.03 μm^3
Gonad			
nuclear area	132789.20 μm^2		97175.15 μm^2
volume of nuclear	706609.42 μm^2		518267.88 μm^3
Leydig cells area	576317.37 μm^2		509681.86 μm^2
volume of Leydig cells	3073695.10 μm^3		2717834.40 μm^3

*DSS – statistically significant difference for two large sample means at $\alpha = 0.01$
All means differ from each other significantly

Table 3

Equal (a = b) and unequal (a b) diameters of Leydig cell nuclei in fertile animals (Group 1) and with seasonally decreased fertility (Group 2)

Group 1			Group 2		
diameters	number	%	diameters	number	%
equal	1340	35.26	equal	1646	43.30
unequal	2460	64.74	unequal	2154	56.70
total	3800	100.00	total	3800	100.00

Leydig cell morphology

In the lymphatic spaces, among seminiferous tubules, Leydig cells most often agglomerated in groups of several tightly packed cells adjoined to one another. The cells always gather closely to a nearby blood vessel.

During the fertile season, Leydig cells were most often polygonal or irregular in shape with foamy cytoplasm. Very seldom did we find oval shaped cells; thus, they are not of regular shape similar to an ellipsoid as has been reported for camels (ZAYED *et al*. 1995) and hamsters (HAKIM *et al*. 1989). Single, tiny vacuoles can be found in the foamy cytoplasm. The cytoplasm foaminess is due to the presence of numerous fine lipid bodies (ZAYED *et al.* 1995; ICHIHARA *et al.* 1993). A nucleus is located eccentrically near one of the sides of the cell. Circular nuclei contained two nucleoli more often than oval ones (Fig. 1). The distribution of long dimensions ranged from 6.0 to 26.0 μm, whereas the distribution of short dimensions ranged between 5.0-17.0 μm. The mean long and short dimensions of cells are, respectively, 13.86 ± 2.76 μm and 10.89 ± 2.44 μm. The remaining data are presented in Table 2.

The size and appearance of Leydig cells changed during the season of low fertility. The cells were smaller and their cytoplasm most often contained large empty vacuoles (Fig. 2). Single cells were more common than clusters. Both long and short dimensions decreased (by 2 μm and 3 μm, respectively) as well as all distances between the nucleus and the borders of a Leydig cell. The total surface area and total volume of a cell also decreased, by 12% on average. The surface areas and volumes of the largest cross-sections of Leydig cells during the low fertility season were 6% lower than during the full fertility season. The total surface area and total volume decreased by 11.6% during the low fertility season.

Discussion

Simply comparing the means of measurements may not cover the full specrum of changes under analysis. Comparison of the distributions of results and their extreme values allows better description of studied and compared results (ZIELIŃSKI & STRZELECKI 1994). Long and short dimensions of Leydig cells did not differ much in the studied groups (Table 1). Morphological or functional studies of Leydig cells in animals with seasonality of fertility are scarce (ZAYED *et al.* 1995), thus it is difficult to compare our results with those obtained by other authors.

Large and empty vacuoles present in Leydig cell cytoplasm and a decrease in the cell surface area and volume strongly supports the opinion that endocrine functions of these cells decrease during summer, when new offspring are born. Close linkage between the amount of secreted hormones and surface area of endocrine cells has been demonstrated by FAWCETT *et al*. (1969), ICHIHARA *et al*. (1993), and FOUQUET *et al*. (1984). In Arctic fox (*Alopex lagopus*) males, seasonal changes in hormones were observed, including luteinizing hormone (LH), prolactin, testosterone, or follicle-stimulating hormone (FSH) (SMITH *et al*. 1985). Changes in Leydig cells related to fertility seasonality have been described in the European mole (*Talpa europaea*) (SUZUKI & RACY 1978) and the camel (SINGH & BHARADWAJ 1978). In the golden (Syrian) hamster, during seasonal total fertility inhibition, the surface area of the plasma membrane and the nuclear membrane were found to decrease by 58% and 33%, respectively. Reduced sizes can also be seen in the nucleolus (by 77.0%), mitochondria (by 50.0%), and endoplasmic reticulum and Golgi complex (by 69.4%). Also, the number of LH receptors decreases in the testicle and Leydig cells; however, their concentration per Leydig cell does not change (HAKIM *et al*. 1989). Testosterone, LH, oestrogens with a number of biologically active substances (BILIŃSKA 1992; GANCARCZYK *et al*. 2003) play important roles in the regulation of the spermatogenic epithelium (SHAN *et al.* 1995; HAKIM *et al*. 1989). Thus, reduced production of testosterone will lead to inhibition of the functions of testosterone-dependent cells of the spermatogenic epithelium, prostate gland, and epididymis.

Leydig cell nuclei distance cross-sections in the fertile season were oval in shape or, less often, circular. The nuclei are placed eccentrically in their cells and do not lie in the largest concentration of cytoplasm.

The population of Leydig cells becomes smaller because the part of the cytoplasm in which large empty vacuoles were previously found is missing.

Similar changes in nuclei dimensions during the annual fertility cycle have been described for the camel, which the difference, however, that only one such change a year occurs in the chinchilla (ZAYED *et al.* 1995).

We conclude that nuclei of Leydig cells during the season of low fertility are smaller in size, located closer to the border of a cell, and their cross-sections are more frequently circular than oval.

Based on analyses of other authors we can expect that in chinchillas (analogically to other animals), production and secretion of hormones and biologically active substances by Leydig cells is reduced during the period of low fertility.

The main reasons leading to size changes of Leydig cell nuclei include meiotic division, apoptosis, oncogenesis, and cell degeneration. In our case, we have excluded meiosis and apoptosis, since meiosis does not pertain to Leydig cells and the process of apoptosis leads to nucleus fragmentation and apoptotic body formation (GAO *et al*. 2002). Oncogenesis should rather also be excluded. It can therefore be presumed that Leydig cells undergo degeneration during the period of lower fertility which is visible in the form of reduced sizes of the cells and a change in their shape from oval to circular. A similar, seasonal degeneration of Leydig cells has been observed in the camel (ZAYED *et al*. 1995).

Irrespective of how fertility is reduced, we do agree that Leydig cells regenerate before the full fertility period starts (HARDY *et al*. 1992).

Studies on seasonal fertility fluctuation help unravel the natural model for experimental and comparative studies related to the effects of various factors (including pharmaceuticals) on fertility. It is possible that hormonal methods of sexual cycle stimulation may be developed that could enable improvement of reproduction potential of farm animals with seasonal fertility changes.

Conclusions

1. Leydig cells are polygonal in shape during the period of fertility. Small vacuoles are present in the cytoplasm. Cross-sections of the nuclei are usually oval rather than circular. Average dimensions of a Leydig cell are 13.86 ± 2.76 μm by 10.89 ± 2.44 μm. Average Leydig cell nucleus dimensions are 6.46 ± 1.25 μm by 5.61 ± 1.13 μm.

2. Leydig cells and their nuclei become smaller in size during the season of low fertility. Degeneration occurs as a result of which total surface area and volume of the cells decrease by 27% and large empty vacuoles appear in the cytoplasm. The nuclei of the cells are smaller (by 23%) and are circular in shape rather than oval.

3. Leydig cells take active part in seasonal reduction of fertility through reducing the production and secretion of hormones and biologically-active substances that regulate fertility.

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