Numeric and volumetric changes in Leydig cells during aging of rats[1]

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Abstract

Purpose: To analyze the effects of aging in rats on the nuclear volume, cytoplasmic volume, and total volume of Leydig cells, as well as their number.
Methods: Seventy-two Wistar rats were divided into six subgroups of 12 rats, which underwent right orchiectomy at 3, 6, 9, 12, 18, and 24 months of age. The weight and volume of the resected testicles were assessed. A stereological study of Leydig cells was conducted, which included measurements of cell number and nuclear, cytoplasmic, and total cell volumes.
Results: The weight and volume of the resected testicles showed reductions with age. Only the subgroup composed of 24-month old rats showed a decrease in the nuclear volume of Leydig cells. Significant reductions in the cytoplasmic volume and total volume of Leydig cells were observed in 18- and 24-month old rats. The number of Leydig cells did not vary significantly with age.
Conclusions: Aging in rats resulted in reduction of the nuclear, cytoplasmic, and total cell volumes of Leydig cells. There was no change in the total number of these cells during aging.
Key words: Aging. Leydig Cells. Testis. Hypogonadism. Testosterone. Rats.

■ Introduction

A decline in gonadal function is one change that occurs in aging men[1]. Even in the absence of disease, testosterone (TT) produced by testicles decreases progressively with age[2]. This phenomenon, known as senile hypogonadism, has also been observed in rats[3].

Leydig cells (LCs), first discovered in 1850 by Franz Leydig, are closely associated with production of the hormone TT[4]. Given their role in the production of androgens, these cells are important for differentiation and development of the male genital tract[5]. However, LCs suffer the deleterious effects of cell aging, and can gradually lose their capacity for TT synthesis[6].

The mechanisms that cause this drop in steroidogenesis are still poorly understood[7]. Stereology, the histological examination of cell structures[8], might facilitate a better understanding of this process. This study aims to evaluate volumetric and numeric changes in Leydig cells during aging by examining rats of different ages.

■ Methods

Animal husbandry and surgery

This study followed the ethical principles established by the Brazilian College of Animal Experimentation (COBEA) and was approved by the Universidade Federal do Paraná (UFPR) Committee for Ethics in Research (number 23075.032620/2010-10).

Seventy-two male albino Wistar rats (*Rattus norvegicus* var. *albinus*) were used for the study. Relative humidity was maintained between 50% and 60%, and the ambient temperature was maintained at 22°C, with a 12-hour light/dark cycle and minimal external noise. Access to water and standard food was provided on an *ad libitum* basis.

The rats were divided into six groups—designated G1 to G6—with 12 animals in each group, which underwent orchiectomy at 3, 6, 9, 12, 18 and 24 months of age, respectively.

For surgical procedures, the rats were anesthetized intraperitoneally with ketamine hydrochloride (57.67 mg/ml) and xylazine hydrochloride (2% w/v) at a dose of 1 ml/kg body weight. A one-punch cardiac puncture was carried out in the morning for blood sampling and induction of cardiac arrest by exsanguination. This was followed by resection of testicles, kidneys, bladder, liver, brain, heart, aorta, and penis. The right testicle was weighed on a high-precision electronic balance, and its dimensions (length, width, height) were measured with a digital caliper. The remaining organs were used in other studies.

Morphometric and histological analysis

Testicular volume was calculated using the following formula: length × width × height × 0.523[9].

Incisions 2 mm in length and depth were made in the top, middle, and lower poles of the testicles, to improve penetration of the fixative (80% alcohol, formaldehyde, and acetic acid). The testicles were kept in the fixative solution for 16 hours, then dehydrated by passage through solutions of xylene and alcohol. Since the testicle is a non-isotropic organ, the orientator method[10] was employed to obtain uniform sections. The pieces of fixed testicle were embedded in paraffin and sliced 5-µm thick using a microtome (American Optical Spencer AO 820). Three sections per blade were prepared and stained with hematoxylin and eosin (HE).

The sections were studied using a photomicroscope (Zeiss Axiophot D-7082) at x400 magnification. Digital images of ten

random histological fields from each section were captured. Images were analyzed in MetaSystem VSViewer software.

Stereological study of Leydig cells

Leydig cell nuclear volume (LCNV)

To obtain the mean nuclear diameter of LCs, ten nuclear cores were overlaid with a circular contour. The following formula was used for this calculation: LCNV = 4⁄3 R^3, where R = nuclear radius.

Leydig cell cytoplasmic volume (LCCV)

LCCV was calculated by applying the following formula: LCCV = %cytoplasm × LCNV %nucleus.

Leydig cell total volume (LCTV)

LCTV was evaluated as the sum of LCNV and LCCV, which was expressed in cubic micrometers (μm^3).

Leydig cell absolute number (LCAN)

LCAN, which corresponds to the numerical density (N_v) of LCs, or the number of LCs per unit volume of testis, was estimated using the disector method[11]. The disectors were compiled by joining photomicrographs of ten equal microscopic fields of each histological section of the testicle. The disector method involves counting the nuclei that are observable in a reference section and *not* observable in a look-up section separated from the reference section by a known distance. We carried out this procedure in both directions (i.e., moving from the reference section to the look-up section and *vice versa*) to increase the number of disectors. Disectors were overlaid systematically and randomly to cover the area encompassing all testicle sections.

N_v was obtained by dividing the number of disectors ($\sum Q^-$) by the sum of their volumes ($\sum V_{dis}$), which was obtained from the product of the test area and the disector height:

$$V_{dis} = a \times h$$

Where V_{dis} is the total volume of the disector, *a* is the test area, and *h* is the disector height.

Thus, N_v can be obtained from the following formula:

$$N_v = \sum Q^- / \sum V_{dis}$$

Where $\sum Q^-$ is the number of disectors and $\sum V_{dis}$ is the sum of the disectors volumes.

Statistical analysis

All parameters were statistically analyzed by one-way analysis of variance (ANOVA). In cases where a significant difference between groups was indicated, the results were compared using the Student-Newman-Keuls (SNK) post-hoc test. All analyses and graphs were generated using GraphPad Prism 6 statistical software. The significance threshold was 95% ($p < 0.05$).

■ Results

There was no loss of subjects; all rats remained alive until the predetermined time at which all the members of the group were sacrificed. Figure 1 shows the morphological characteristics of the animals and Figure 2 shows the morphometric characteristics of the resected testicles.

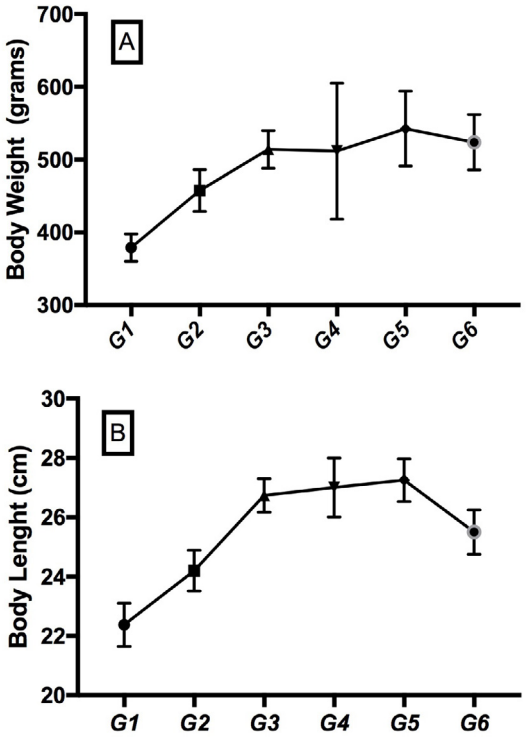

Figure 1 - **A**, mean body weight of rats in different age groups; **B**, mean body length of rats in different age groups.

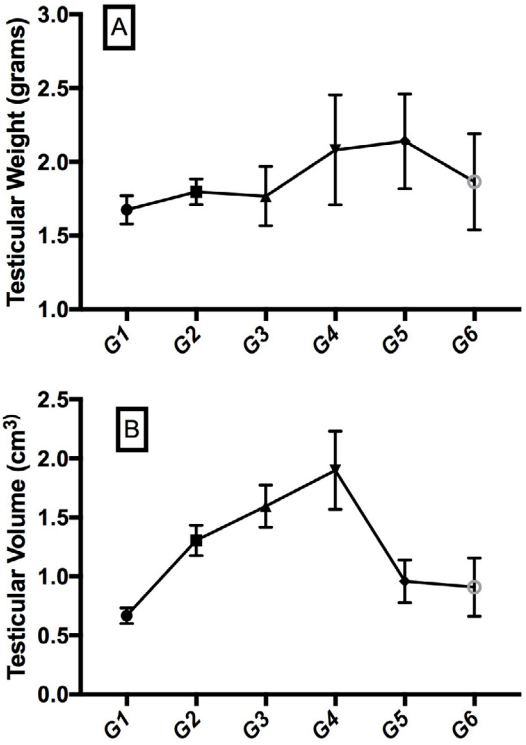

Figure 2 - **A**, mean testicular weight of rats in different age groups; **B**, mean testicular volume of rats in different age groups.

LCNV was generally constant across age groups, except for G6, which exhibited a statistically significant reduction in LCNV (Figure 3 and Table 1). However, LCCV values were observed to decrease progressively as the age of the rats increased, as demonstrated in Figure 4; statistically significant differences in LCCV were found when G1 was compared with the other groups (Table 2). Consequently, LCTV also declined gradually with age (Figure 5 and Table 3). In contrast, LCAN varied minimally between the groups; no statistically significant differences were observed (Figure 6).

In Figure 7, all groups were shown. As the LC's becomes old its nuclear, cytoplasmic and total volume decrease. The last group have demonstrated significant hypotrofic findings.

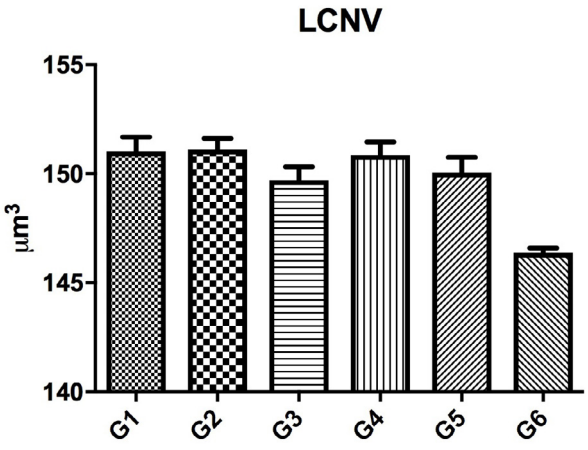

Figure 3 – Leydig cell nuclear volume (LCNV) of rats in different age groups.

Table 1 – Comparison of mean Leydig cell nuclear volume (LCNV) between groups with different ages.

Group	Versus Group	Absolute difference between groups	p	Mean LCNV (μm^3)	±SD
G1	G2	0.08	ns	151.05	±4.36
	G3	1.32	ns		
	G4	0.17	ns		
	G5	0.97	ns		
	G6	4.64	*		
G2	G3	1.4	ns	151.09	±4.33
	G4	0.25	ns		
	G5	1.05	ns		
	G6	4.7	*		
G3	G4	1.14	ns	149.69	±3.97
	G5	0.34	ns		
	G6	3.32	*		
G4	G5	0.79	ns	150.85	±4.30
	G6	4.46	*		
G5	G6	3.67	ns	150.04	±4.21
G6	-	-	-	146.37	±2.06

ns, not significant; * p<0.05; ±SD, standart deviation; post-hoc Student-Newman-Keuls test.

Table 2 – Comparison of mean Leydig cell cytoplasmic volume (LCCV) between groups with different ages.

Group	Versus Group	Absolute difference between groups	p	Mean LCCV (μm^3)	±SD
G1	G2	15.08	*	666.36	±21.63
	G3	15.24	*		
	G4	19.23	*		
	G5	29.57	*		
	G6	35.37	*		
G2	G3	0.16	ns	651.28	±4.55
	G4	4.15	ns		
	G5	14.49	*		
	G6	29.30	*		
G3	G4	3.99	ns	651.12	±4.46
	G5	14.33	*		
	G6	20.14	*		
G4	G5	10.34	*	647.13	±4.76
	G6	16.14	*		
G5	G6	5.805	ns	636.79	±7.31
G6	-	-	-	630.98	±6.42

ns, not significant; * p<0.05; ±SD, standart deviation; post-hoc Student-Newman-Keuls test.

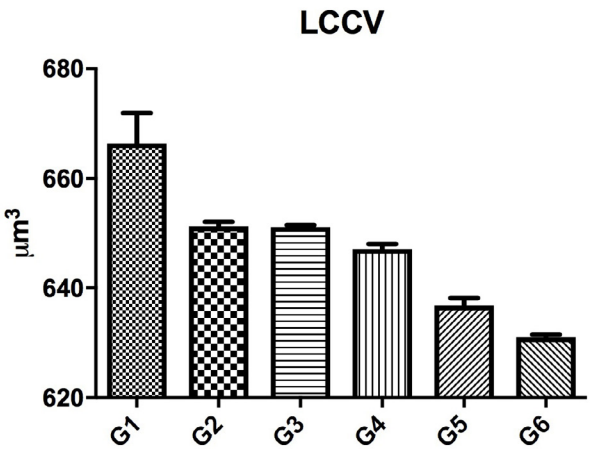

Figure 4 – Leydig cell cytoplasmic volume (LCCV) of rats in different age groups.

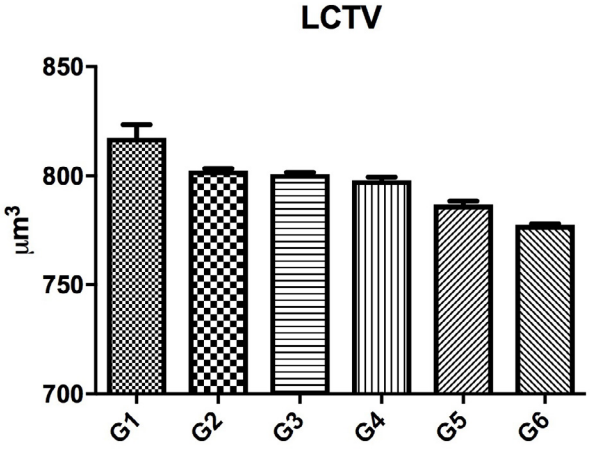

Figure 5 – Leydig cell total volume (LCTV) of rats in different age groups.

Table 3 – Comparison of mean Leydig cell total volume (LCTV) between groups with different ages.

Group	Versus Group	Absolute difference between groups	p	Mean LCTV (μm^3)	±SD
G1	G2	14.99	*	817.39	22.9
	G3	16.56	*		
	G4	19.41	*		
	G5	30.54	*		
	G6	40.01	*		
G2	G3	1.56	ns	802.40	6.37
	G4	4.41	ns		
	G5	11.13	*		
	G6	25.02	*		
G3	G4	2.84	ns	800.84	6.12
	G5	13.98	*		
	G6	23.46	*		
G4	G5	11.13	*		7.03
	G6	20.61	*	797.99	
G5	G6	9.47	*	786.85	8.40
G6	-	-	-	777.38	6.59

ns, not significant; * p<0.05; ±SD, standart deviation; post-hoc Student-Newman-Keuls test.

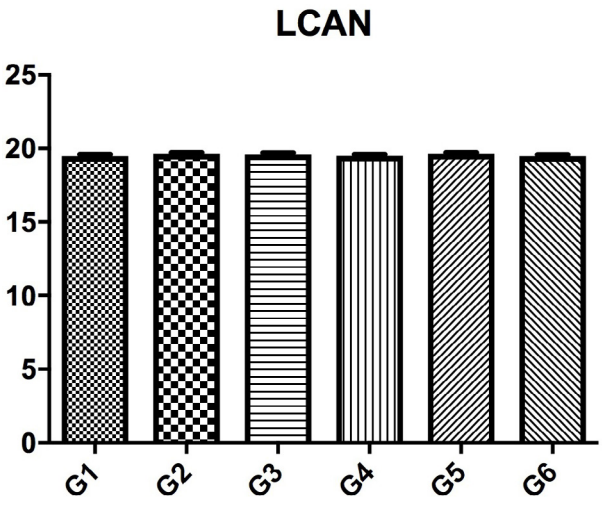

Figure 6 – Leydig cell absolute number (LCAN) of rats in different age groups.

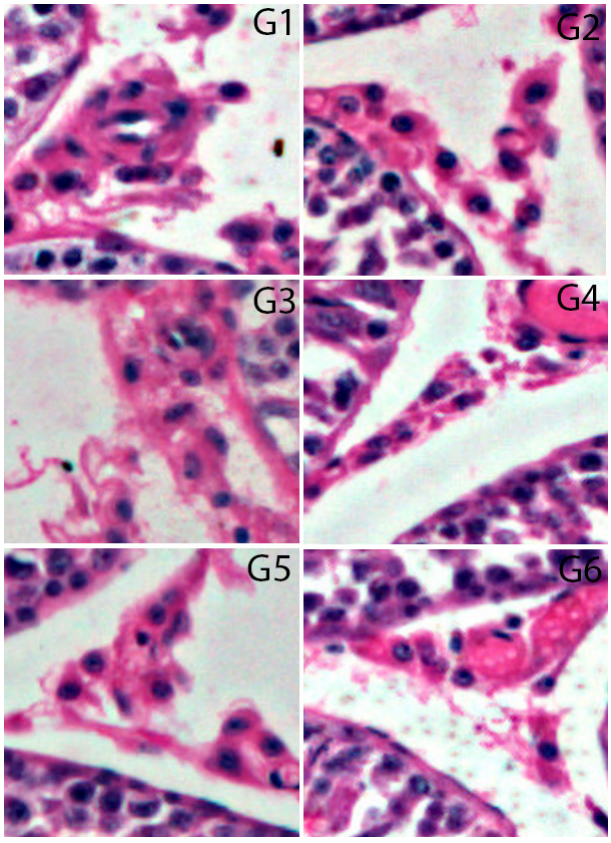

Figure 7 – Interstitial space and Leydig cells of rats in different age groups.

■ Discussion

During testicular development in rodents, LCs divide themselves into fetal and adult types. Fetal-type LCs ensure that the embryo develops as a male and undergo apoptosis after birth[12]. Adult-type LCs, which originate from mesenchymal precursor cells, begin to differentiate 14 days after birth and gradually mature over time[13].

In rats, adult-type LCs progress through four developmental stages: stem cells until the 14th day after birth, progenitor cells until the 28th day, immature cells until the 56th day, and adult cells, characterized by high hormone production, after the 56th day[14]. TT serum levels begin to rise on the seventh day after birth, and continue to increase until peaking on the 45th

day[15]. Rats achieve sexual and reproductive maturity between 40 and 60 days after birth and can live for up to three years. According to an editorial that established equivalencies between the ages of rats and humans, G1 and G6 rats may be considered equivalent in age to 27- and 61-year old humans, respectively[16].

Zirkin *et al*.[3] have shown that serum levels of TT decrease with age in brown rats; this finding was confirmed by Wang *et al*.[17] by characterization of intratesticular TT levels in rats at 22 weeks of age.

In the present study, the animals gained weight and grew in length until an age of 18 months, and subsequently decreased. Testicular weight and volume followed an almost identical trend. Comparable decreases in testicular weight have also been observed in rats subjected to chronic stress[18].

LCNV reduction in elderly rats may be related to chromatin remodeling and telomere shortening, which preclude cells from replicating in old age. Reductions in LCCV and LCTV are possibly associated with hypotrophy of cytoplasmic organelles, which also impairs steroidogenesis. These volumetric reductions predominantly occurred as the animals reached an advanced age, when the negative effects of aging are more evident. In previous stereological studies, it was established that the mean volume of LCs in rats declines with age[19], and it was shown that the total volume of LCs per testicle and their mean cell volume remain stable until six months of age, with significant reductions observed only in 12- and 19-month old rats[20].

In this study, LCAN remained constant as the rats aged. Similarly, Wang *et al*.[18] demonstrated that the number of LCs in old rats (up to 21 months of age) is not lower than the number of LCs in young rats, and Chen *et al*.[19] detected no difference in the number of LCs upon stereological analysis. Thus, the decline in TT levels with age cannot be explained by loss of LCs[17,21]; this change is the result of LCs dysfunction rather than death[7].

In old age, adult-type LCs stop dividing. They exhibit defects in steroidogenic pathways and decreased hormone production[14,22]. However, decreases in TT serum levels are not accompanied by changes in luteinizing hormone (LH) serum levels, and must therefore be caused by impaired stimulation of LCs.

LH stimulation increases LC volume and the number of subcellular organelles (endoplasmic reticulum and mitochondria), in contrast deprivation leads to hypotrophic changes in cell structure[23].

Haolin *et al*.[24] conducted a study on in vitro cultures of young and old LCs, which were exposed directly to LH. Even when the LH concentration was maintained at the same level for both cultures, old LCs were unable to produce testosterone at levels comparable to those of young cells. The authors concluded that old LCs have defects in the LH-AMPc signaling cascade, which is involved in testosterone production.

Steroidogenesis by LCs is affected by age for multiple reasons: reduction of cAMP levels, decreased intracellular transport of cholesterol by the steroidogenic acute regulatory (StAR) and translocator (TSPO) proteins, and reduction of the activity of enzymes in mitochondria (CYP11A1, HSD3B) and the smooth endoplasmic reticulum (HSD3B, CYP17A1, HSD17B)[25].

Many potential causes of senescence in LCs have been proposed, including lesions by free radicals[26]. It could also be speculated that lesions in the cell membrane are the most likely cause of senescence in LCs[14,18]. Oxidative stress is known to occur during steroidogenesis by LCs, and this may explain the volumetric changes identified in this work. The free radical theory advances the idea that aging is the result of cumulative cellular damage over time[27]. This damage is caused by free radicals produced by catabolic pathways, and increases with age[28].

The presence of LCs with their

respective alterations were demonstrated in Figure 7. The most important observation were the hypotrophic findings like reductions in the nuclear volume, cytoplasmic volume and the whole cell. These results are direct involved in the old LCs dysfunction. As these cells lose its volume probably lose the capacity of TT production too.

Hormonal changes during aging are of great clinical and scientific interest. The future offers interesting possibilities of improvement in TT production, such as LCs replacement by stem cell transplantation[29]. New therapies currently under development, such as the use of drugs to stimulate hormone production in LCs, have shown encouraging results[30]. Additional structural and morphometric evaluations are needed to corroborate these findings, followed by assays of oxidative stress.

■ Conclusions

Aging in rats was accompanied by hypotrophic changes in Leydig cells. Stereological analyses revealed a reduction in the nuclear, cytoplasmic, and total volumes of these cells. There was no concomitant reduction in cell number.

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