FOLIA HISTOCHEMICA ET CYTOBIOLOGICA
Vol. 47, No. 2, 2009
pp. 281-287

Age-related changes in the expression of 11β-hydroxysteroid dehydrogenase type 2 in rat Leydig cells

Yvetta Koeva[1], Mariana Bakalska[2], Nina Atanassova[2], Katerina Georgieva[3] and Michail Davidoff[4]

[1]Department of Anatomy and Histology, Medical University, Plovdiv, Bulgaria
[2]Institute of Experimental Morphology and Anthropology with Museum, Bulgarian Academy of Sciences, Sofia, Bulgaria
[3]Department of Physiology, Medical University, Plovdiv, Bulgaria
[4]Institute of Anatomy 1, UKE, University of Hamburg, Germany

Abstract: Previous studies in rats have shown that the ability of Leydig cells (LCs) to produce testosterone significantly declines with age. To address the possible mechanisms by which aging LCs lose their steroidogenic function, we determined the effect of aging on the expression of 11β-hydroxysteroid dehydrogenase (11β-HSD) type 2. The enzyme plays a protective role in blunting the suppressive effects of glucocorticoids on LCs steroidogenesis. Our immunohistochemical analysis revealed progressive decline in 11β-HDS type 2 expression in LCs of the 18 months of age rats and the most significant reduction in 11β-HSD2 immunoreactivity was evident in the testicular interstitium of 24- month-old rats. The decrease in the 11β-HDS type 2 immunostaining in LCs during aging coincided with decline in insulin-like 3/relaxin-like factor (INSL3/RLF) expression, an independent marker for LCs differentiation status. Concomitant with the age-related decrease of 11β-HDS type 2 immunoreactivity in the LCs population, the immunoexpression of 3β-hydroxysteroid dehydrogenase (3β-HSD), marker for LCs steroidogenic activity, was greatly reduced at 24 months compared to 3-month-old control. Similar pattern of expression exhibited also androgen receptor (AR) which is localized in the nuclei of Sertoli cells (SCs), LCs, and peritubular cells. During ages we observed progressive decrease in the immunoreactivity for AR in the testicular types and there was a loss of stage specificity in SCs at age of 24 months. It now seems evident that a variety of factors are likely to be involved in age-related decreases in LCs steroidogenesis, including 11β-HSD type 2. The observed reduction in 11β-HSD type 2 expression in aging LCs reflects the decline in their protection ability, opposing the suppressive effect of glucocorticoids on testosterone production.

Key words: 11β-HSD2, Leydig cells, testis, aging

Introduction

It has been established that circulating levels of testosterone (T) decrease with age in both male rodents and men [1]. Demonstration that declines in androgen levels result from specific age-related changes in the male reproductive system and not secondarily from increased disease frequency associated with the aging process, was achieved by analyzing cohorts of healthy men and rodents [2,3]. Using old rats treated with ethane dimethanesulphonate (EDS), which destroyed Leydig cells (LCs), Chen *et al*. [4] reported that the newly formed LCs restored high plasma T in the old rats, indicating that the hypothalamic-pituitary axis in the old testis environment were still intact. Indeed, it is unlikely that deficits of the hypothalamic-pituitary axis are primarily responsible for age-related changes in steroidogenesis.

According to Zirkin & H. Chen [5] the reduced ability of aging LCs to produce T might be caused by

Correspondence: N. Atanassova, Institute of Experimental Morphology and Anthropology with Museum, Bulgarian Academy of Sciences, Acad. G. Bonchev Str., Bl. 25, BG-1113 Sofia, Bulgaria; tel.: (+3592) 9792336, fax.: (+3592) 719007, e-mail: ninaatanassova@yahoo.com

Folia Histochem Cytobiol. 2009:47(2): 281 (281-287)
doi: 10.2478/v10042-009-0021-3

events occurring outside these cells that impinge upon them or by events that occur over time within LCs themselves involving accumulation of free oxygen radicals as by product of steroidogenesis. Aging LCs are characterized by reductions in luteinizing hormone (LH) receptor number, cyclic adenosine monophosphate (cAMP) production [6,7], steroidogenic acute regulatory (StaR) protein, peripheral benzodiazepine receptor (PBR), cholesterol transport, and conversion of cholesterol to T by enzymes residing in the mitochondria and smooth endoplasmic reticulum [8-10].

The main function of glucocorticoids in adult LCs is inhibition of T biosynthesis [11]. Glucocorticoids directly regulate T production in LCs through a glucocorticoid receptor (GR)-mediated repression of the genes that encode T biosynthetic enzymes [12,13]. 11β-hydroxysteroid dehydrogenase (11β-HSD) catalyses the interconversion of glucocorticoid [corticosterone in rat] to an inert metabolite (11-dehydrocorticosterone) and therefore the enzyme is an important determinant of the intracellular level of bioactive steroid. Predominance of oxidative activity results in glucocorticoid inactivation, whereas the reductive activity of the enzyme has an opposite effect [13]. Recent studies showed that reductase activity predominates in both human and rat type 1 11β-HSD [13]. In contrast, the other 11β-HSD isoform, type 2, has been found to be exclusively oxidative [14-16].

As yet, the mechanisms by which the LCs become hypofunctional with aging remain uncertain. To address the possible intracellular mechanisms by which age-related reduction in T production occurs, we examined the expression of 11β-HSD type 2 in the LC. Our objective was to determine on which stage of LCs the 11β-HSD type 2 expression is compromised with aging, and whether there is a relation between the altered ability of the aging LCs to respond to glucocorticoids and the damage of the cell's steroidogenic machinery and respectively reduced steroidogenesis.

Materials and methods

Animals. Male Lewis rats at different ages (3, 12, 15, 18, 21 and 24 months) were used in the present study. The animals of each age group (n=6) were decapitated under narcosis and testes were sampled from each animal. Testicular fragments approximately 4-5 mm thick were fixed by immersion in Bouin's fluid for 24 hours at room temperature (RT), embedded in paraffin and prepared for routine histological analysis (hematoxylin-eosin staining) and immunohistochemistry.

Immunohistochemistry

a) for 11β-HSD type 2. Paraffin sections (6 μm thick) were incubated in 3% (v/v) H_2O_2 in methanol to inhibit endogenous peroxidase activity and treated with 2.0% normal rabbit serum to block non-specific binding. After that the sections were incubated with polyclonal sheep anti 11β-HSD type 2 antibody (Chemicon, USA, 1:1000) for 24 hours at 4°C in a humid chamber. For the detection of the antigen an amplification combination of the peroxidase anti-peroxidase (PAP) and the avidin-biotin-peroxidase complex (ABC) methods was applied [17]. Then biotinylated anti-goat IgG (Dakopatts, Denmark, 1:250) and goat PAP (Dakopatts, Denmark, 1:100) were used. In the last step ABC (Vector, USA, 1:250) was applied. The peroxidase activity was then developed by 3,3'-diaminobenzidine tetra-hydrochloride (DAB) chromogene substrate kit, (Vector, USA).

b) for INSL3/RLF. The pattern of INSL3/RLF expression in LC during aging was established using specific polyclonal rabbit anti-INSL3/RLF (Phoenix Peptide, USA; diluted 1:200) antibody. For visualization of the immune reactions a high sensitive Vectastain ® *Elite* ABC immunohistohemical kit (Vector, USA) with DAB as chromogen were applied.

c) for AR and 3β-HSD. Immunohistochemistry was performed on dewaxed sections with antigen retrieval (5 min in 0.01 M citrate buffer, pH 6.0) for visualization of AR or without antigen retrieval for 3β-HSD. For all washes between antibody or reagent incubations, 0.05 M TBS, pH 7.4, 2×5 min at RT was used. Tissue sections were blocked in TBS containing normal swine serum (NSS; 1:4) and 5% BSA before an overnight incubation at 4°C with primary antibodies diluted in NSS. For detection of AR and LCs a rabbit polyclonal antibody (Santa Cruz, USA, 1:200) and antibody against 3β-HSD, diluted 1:1000, kindly provided as a gift form Prof. I. Mason (Edinburgh University), were applied, respectively. A swine anti-rabbit biotinylated secondary antibody (E0353, DAKO), diluted 1:500 in NSS was applied for 30 min at RT. Bound antibodies were visualized by incubating the sections with ABC complex/HRP reagent (K0355, DAKO) for 30 min followed by color development with DAB chromogene substrate (K3468, Liquid DAB+ kit, DAKO), monitored microscopically. Sections were counterstained with hematoxylin, dehydrated and coverslipped using Pertex mounting medium (CellPath plc, Hemel Hempstead, UK). The specificity of immunostaining was checked for each antibody using previously established procedures. For AR, this involved preabsorption of the primary antibody with peptide immunogen (Santa Cruz N-20 peptide sc-816P) and for 3β-HSD it involved substitution of the primary antibody by NSS.

Measurement of seminiferous tubules (ST) diameter. Measurement of ST area and diameter was performed using image analysis software Axio Vision LE Rel. 4.1. At least 100 ST with circular cross section were measured per animal.

Quantification of immunohistochemistry and statistics. The intensity of the immune reactions for 11β-HSD2, INSL3/RLF, 3β-HSD and AR in LCs were assessed through the special software DP-Soft (Olympus, Japan, version 3.2 for Windows) on Microphot-SA (Nikon, Japan) microscope, equipped with a Camedia-5050Z (Olympus, Japan) digital camera. The values of intensity were in the interval 0÷256, where 0 represents -white and 256 - black. The course of the reaction intensity in positive LCs was evaluated in different microscopic fields of each testicular section (at least 100 cells). Mean intensity of antigen expression for each animal of group was calculated. The results were analyzed using one-way ANOVA and Tukey *post hoc* test for intergroup differences. Statistical significance was set at $P<0.05$.

Results

By routine histological and immunohisotochemical analysis we found that the interstitium of aging testes appeared increased in size as a consequence of age-related tubular regression without any evidence for loss or hyperplasia of LCs. Instead, it seems that LCs undergo atrophic changes in size. During aging the LCs were rarely arranged in clusters or sheets of cells

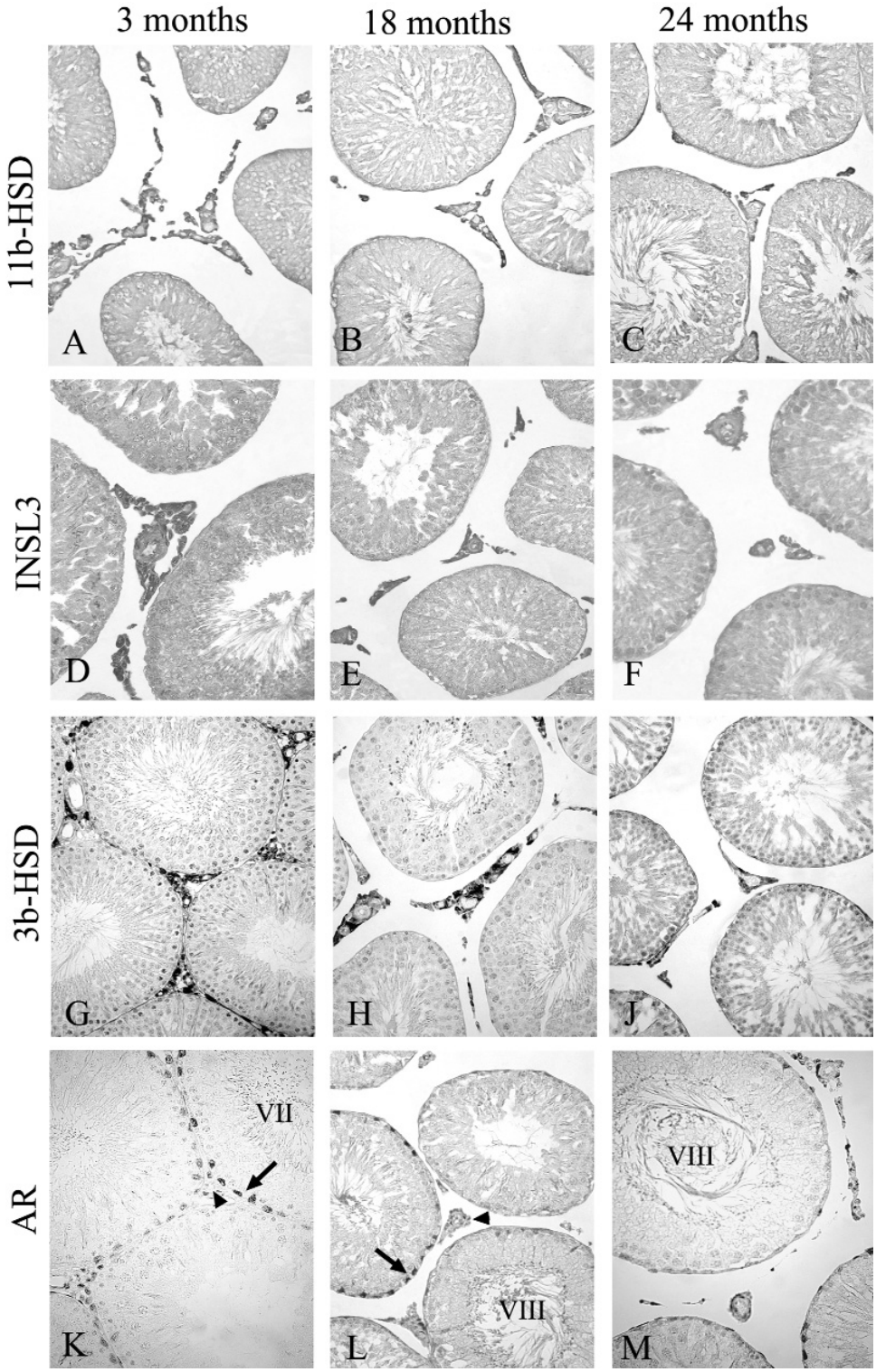

Fig 1. Immunohistochemical expression of 11β-HSD type 2 **(A, B, C)**; INSL3/RLF **(D, E, F)**; 3β-HSD **(G, H, J)**; androgen receptor (K, L, M) in Leydig cells of aging rats (3, 18 and 24-months of age). The Leydig cell immunostaining decreases during aging. There is a progressive reduction in the immunoreactivity for androgen receptor in Leydig cells (*arrowhead*) and Sertoli cells (*arrow*) **(L)**, and a lack of stage specificity in Sertoli cells at age of 24 months **(M)** (original magnification ×400).

that surround the tubules and follow the course of blood vessels. Isolated forms in peritubular and perivascular positions were more often seen in aged rats (21 and 24- month- old), (Fig. 1C, I).

Strong intensity of 11β-HDS type 2 immunoreactivity was detected in the cytoplasm of LCs of the rats of 3-15 months of age (Fig. 1A). By 18 month of age a decrease in the 11β-HDS type 2 immunostaining in

Folia Histochem Cytobiol. 2009:47(2): 283 (281-287)
doi: 10.2478/v10042-009-0021-3

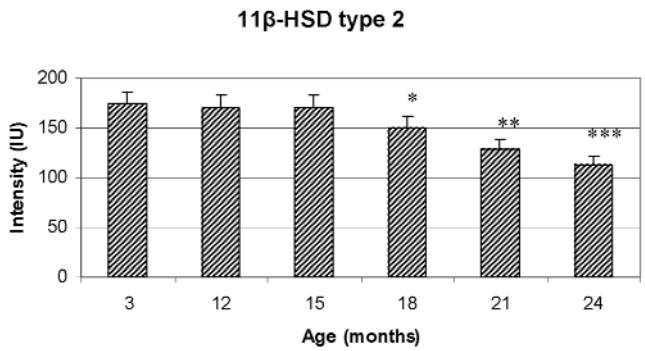

Fig. 2. Intensity of the immune reaction for 11β-HSD type 2 in rat Leydig cells during aging. *P<0.001 (in comparison with 3, 12, 15, 21 and 24 months of age); **P<0.001 (in comparison with 3, 12, 15, 18 and 24 months of age); ***P<0.001 (in comparison with 3, 12, 15, 18 and 21 months of age).

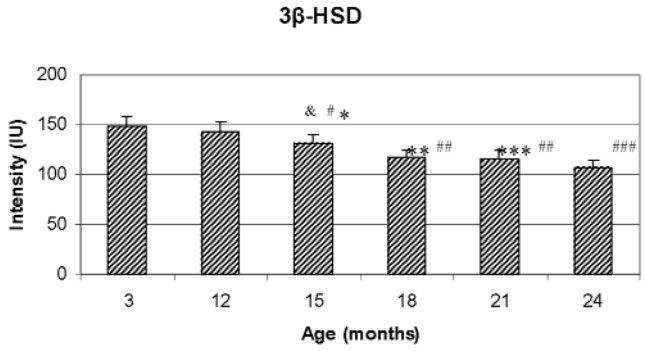

Fig. 4. Intensity of the immune reaction for 3β-HSD in rat Leydig cells during aging. &P<0.01 (in comparison with 3 months of age); #P<0.05 (in comparison with 18 and 21 months of age); *P<0.001 (in comparison with 24 months of age); **P<0.001 (in comparison with 3 and 12 months of age); ##P<0.05 (in comparison with 15 months of age); ***P<0.001 (in comparison with 3 and 12 months of age); ###P<0.001 (in comparison with 3, 12 and 15 months of age).

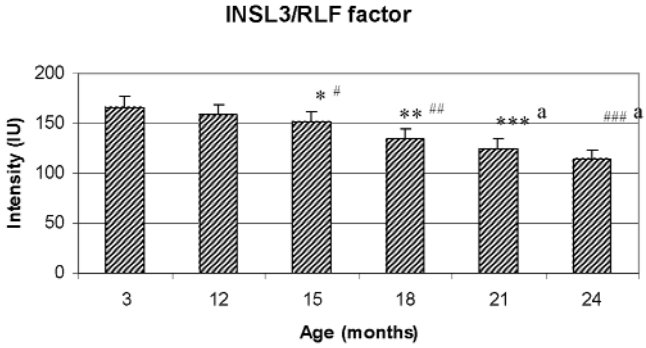

Fig. 3. Intensity of the immune expression for INSL3/RLF in rat Leydig cells during aging. *P<0.01 (in comparison with 3 and 18 months of age); #P<0.001 (in comparison with 21 and 24 months of age); **P<0.001 (in comparison with 3, 12 and 24 months of age); ##P<0.01 (in comparison with 15 months of age); ***P<0.001 (in comparison with 3, 12 and 15 months of age); ###P<0.001 (in comparison with 3, 12, 15 and 18 months of age); aP=0.05 (21 months group vs. 24 months group).

LCs coincided with decline in insulin-like 3/relaxin-like factor (INSL3/RLF) expression, an independent marker for LCs differentiation status (steroidogenic ability and number), (Fig. 1B, E). The most prominent reduction in 11β-HSD2 expression in LCs was observed in the testicular interstitium of 24- month-old rats (Fig. 1C). INSL3/RLF was also dramatically reduced in aged rats compared with younger animals (3-12-month-old), (Fig. 1D-F).

No 11β-HSD2 immunoreactivity was evident in peritubular, Sertoli cells (SCs) and germ cells of the testes from control and aging rats.

Immunoexpression of 3β-hydroxysteroid dehydrogenase (3β-HSD), marker for LCs steroidogenic activity, declined after 18 months of age and cytoplasm staining was greatly reduced at 24 months compared to 3-month-old control (Fig. 1G-I).

Androgen receptor (AR) is localized in the nuclei of SCs, LCs, peritubular cells but not in germ cells. There was a stage specific pattern of immunoexpression of AR in SCs with maximal staining in stages VII-VIII of the seminiferous epithelium cycle and lowest intensity in late (IX-XIV) stages, as reported previously [18,19]. During ages we observed progressive decrease in immunoreactivity for AR in the testicular types and there was lack of stage specificity in SCs at age of 24 months (Fig. 1K-M). Quantitative measurements of immunohistochemical reactions using DP-Soft *image system* revealed similar changes in the immunoreactivity for antigens under study (11β-HSD2, INSL3/RLF, 3β-HSD and AR) in LCs (Fig.2-5). Decreasing curves of the reactions intensity in LCs are evident during aging (Fig. 6).

The seminiferous epithelium of the rats of 3 and 12 months of age exhibited intact spermatogenesis whereas by 18 months of age both normal and altered tubules were observed. At 24 months tubules with regression of seminiferous epithelium and germ cell depletion were predominantly present. Sertoli cell-only tubules were also found. Significant and progressive age-related reduction (by 15-30%) of seminiferous tubules diameter was confirmed by quantitative analysis (Fig. 7). Often, germ cell-depleted tubules exhibited thickened basement membrane with irregular contour. As we previously have shown, intense reaction for α-smooth muscle actin was evident in thickened tubular basal lamina and small blood vessels wall in the interstitium of aged testis [20].

Discussion

It seems that changes in adult LCs during aging depend on the strain of rat used. Commonly in many

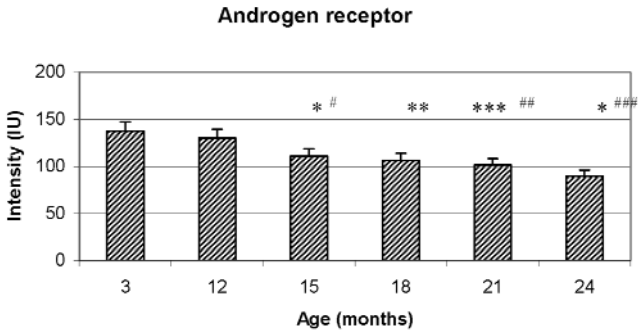

Fig. 5. Intensity of the immune expression for androgen receptor in rat Leydig cells during aging. *P<0.01 (in comparison with 21 months of age); #P<0.001 (in comparison with 3, 12 and 24 months of age); **P<0.001 (in comparison with 3, 12 and 24 months of age); ***P<0.001 (in comparison with 3 and 12 months of age); ##P<0.01 (in comparison with 15 and 24 months of age); ###P<0.001 (in comparison with 3, 12, 15 and 18 months of age).

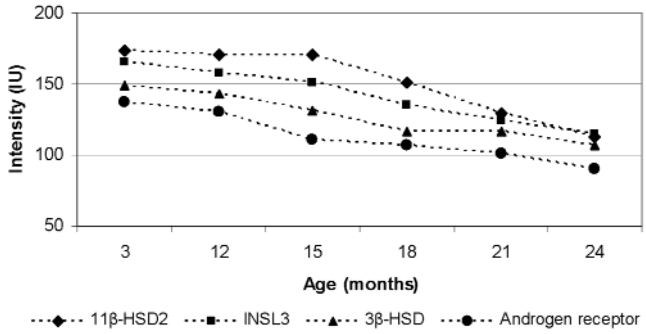

Fig. 6. Age related changes in the 11β-HSD type 2, INSL3/RL, 3β-HSD and androgen receptor expression in rat Leydig cells.

strains a reduction of T production as a consequence of decrease in LH was reported [21]. LH or hCG treatment does not reverse LH and T levels completely; it seems that functional changes in LCs themselves rather than their loss cause reduced steroidogenesis [22]. Our results revealed that aging affects T production not only through the direct suppression of 3β-HSD, a key marker for LCs steroidogenic activity but also through the inhibition of 11β-HSD type 2 and INSL3, factors involved in functional maturation of adult LCs. The current data are of clinical importance suggesting that increasing functional hypogonadism in older male rats is likely caused by a dedifferentiation of the LCs themselves. Our findings for reduced 11β-HSD type 2 expression in aging LCs provide new data about the functional properties of this enzyme in rat testis and bring an additional elucidation on the intracellular mechanisms underlying the decrease in T production that accompanies aging. The results obtained are consistent with the conclusion that compromise of production/action of 11β-HSD type 2 may play significant role in age-related decreases in LC steroidogenesis.

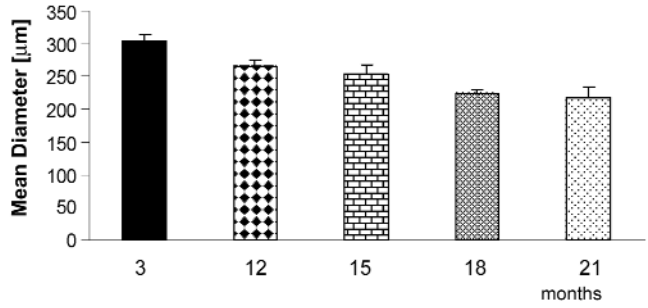

Fig. 7. Progressive age-related reduction in mean diameter of seminiferous tubules (μm). Data represent mean value ± SE. All data are significant compared to control.

The current paper demonstrated that aging of rat testis is accompanied by reduction of tubular diameter, disruption of spermatogenesis, thickening of tubular basement lamina and wall of blood vessels in the interstitium. These changes are indicative for disturbed communication between seminiferous epithelium and interstitium and imply the altered testicular trophic during aging that might be responsible for dysfunction of blood-testis barrier and germ cell depletion [23]. Our findings are in concert with data for increased collagen deposition in testicular interstitium [24] and decreased tubular volume, diameter and length as well as luminal volume [25] of aged testis.

No differences were observed by stereological analyses of LC number in the testes of young and old rats, suggesting that there must be deficit in the capacity of individual old LCs to produce T [25,26]. Our results demonstrated atrophy of LCs during aging rather than reduction in their number and are consistent with previously studies in rat regarding the effects of aging on LCs [24,26]. It seems that LCs number does not change during aging; instead, the steroidogenic capacity of LCs in old individuals declines. Obviously, age-related decrease in T levels result from defects in the steroidogenic pathway beyond the LH receptor-cAMP cascade. Our findings of reduced immunoexpression of 3β-HSD in aged rats are indicative for suppressed steroidogenic activity of aging LCs that correlate with data for reduced serum T levels [7].

Our results are strongly consistent with the hypothesis that age-related loss of steroidogenic function results at least in part from reduction in the levels and activities of each of the critical steroidogenic enzymes responsible for converting cholesterol to T, including 3β-HSD and not by differential regulation of these enzymes [8,27].

The response of LCs to glucocorticoid depends not only on the number of GR and the circulating concentration of glucocorticoid, but also on the ratio of 11β-HSD oxidative and reductive activities [28]. When oxidation predominates over reduction, 11β-HSD

Folia Histochem Cytobiol. 2009:47(2): 285 (281-287)
doi: 10.2478/v10042-009-0021-3

decreases the intracellular availability to active glucocorticoid, attenuating GR-mediated responses [29]. In this way, T production is maintained in the presence of normal serum concentrations of corticosterone and is inhibited only if 11β-HSD oxidative capacity in LCs is reduced. Moreover, developmental analysis of 11β-HSD in rat LCs revealed that 11β-HSD reductive activity predominated in LCs precursors, whereas in adult LCs, the enzyme was primarily oxidative [14]. The observed switch in the predominant direction of catalysis of 11β-HSD from reduction to oxidation in adult LCs may protect this cell type from glucocorticoid-mediated inhibition of steroidogenesis. Recently it was demonstrated that adult LCs expressed not only 11β-HSD type 1, an oxidoreductase but also type 2, an unidirectional oxidase [30-32]. 11β-HSD type 2 may also play a protective role in blunting the suppressive effects of glucocorticoids on LCs steroidogenesis due to its high affinity for glucocorticoid substrates and exclusively oxidative activity [32]. In the present study we found significant diminished expression of 11β-HSD type 2 in LCs with aging which implies suppression in 11β-HSD oxidative capacity resulting in elevated inhibitory potency of corticosterone on T production [32]. Moreover, our data are suggestive for decline in LCs protection ability as opposed to adverse effect of glucocorticoids on T production. Inhibition of 11β-HSD 2 oxidative activity by treatment with 11β-HSD 2 antisense oligomer results in excess of glucocorticoids due to lowering the rate of their inactivation [32]. On the other hand, the elevated levels of corticosterone decreased the oxidative activity of 11β-HSD leading to impaired LCs steroidogenesis [33]. Therefore, reduction of 11β-HSD type 2 oxidase occurred during LC aging appears to be a key event that leads to downstream deficits in the response of LCs to prevent glucocorticoid-mediated suppression of steroidogenesis.

In the present study we observed that the decrease in 11β-HSD type 2 immunostaining in aging LCs coincided with decline in INSL3/RLF expression, an independent measure of LC function both, qualitatively and quantitatively [34]. While INSL3 in fetal LCs regulates testicular descent [35], in adult LCs INSL3 still has not well defined function. However, its pattern of expression correlates temporally with the development of steroidogenic function and spermatogenesis [36]. The concentration of INSL3 in LCs is considered to reflect their functional status [37].

INSL3 secretion is dependent on the chronic differentiating effect of LH on LCs but independent of the acute steroidogenic LH-mediated action [38]. Our quantitative immunohistochemical analysis of INSL3 pattern revealed progressive decrease in the reaction intensity in LCs from rats of 18 months of age, and further significant reduction occurred thereafter. These findings are consistent with previous data demonstrating dramatically reduced INSL3 expression in the testes of 2 year-old rats at mRNA and protein levels [39]. Taken together, these data show that INSL3 appears to be independent of acute control via the hypothalamic-pituitary-gonadal axis and its decline with age reflects a decline in the properties of the LC population only, and emphasizes a gonadal component in the age-related decrease in T production [34].

Androgens are especially important for maintenance of spermatogenesis in adulthood [40] and their effects on germ cells are mediated via AR localized in SCs, LCs and peritubular myoid cells. There was a stage specific pattern of AR expression in the nuclei of adult SCs whereas in the LCs AR expression did not vary with the stage of the cycle of the seminiferous epithelium [18]. In our study we observed that during aging there is a decrease in immunoreactivity for AR in the testicular cell types and loss of stage specificity in SCs which probably reflected decreased responsiveness to androgens [41]. It is likely that SCs from aging testis are unable to provide adequate androgen support for germ cells and to respond to selective signal from them. These data are suggestive of altered Sertoli-germ cells communications and LCs autocrine regulation in aging testis.

In conclusion, the present study demonstrated that the expression of 11β-HSD type 2 that has been suggested as a marker for the functional maturity of adult LCs [31] is reduced in aging testis. Moreover, the age-related decline in 11β-HSD type 2 in LCs coincided with reduction in the immunoreactivity for INSL3 that is expressed in the LCs in a differentiation-dependent manner and thus seems to be associated with their maturation [42]. The observed course of 11β-HSD type 2 in aging LC is very similar with the pattern of expression of both 3β-HSD and AR, indicating that there is an age-related reduction in the functional properties of the LCs, and thereby reduced T production.

Acknowledgements: This study was supported by the Medical University-Plovdiv, grant no. NO 16/2004.

References

[1] Hardy M and Schlegel P. Testosterone production in the aging male: Where does the slowdown occur? *Endocrinology*. 2004;145(10):4439-40.
[2] Harman SM, Metter EJ, Tobin JD, Pearson J, Blackman MR. Longitudinal effects of aging on serum total and free testosterone levels in healthy men. Baltimore Longitudinal Study of Aging. *J Clin Endocrinol Metab*. 2001;86:724-31.
[3] Wang C, Hikim AS, Ferrini M, *et al*. Male reproductive aging: using the brown Norway rat as a model for man. *Novartis Found Symposium*. 2002;242:82-95; discussion 85-87.
[4] Chen H, Huhtaniemi I, Zirkin BR. Depletion and repopulation of Leydig cells in the testes in aging Brown Norway rats. *Endocrinology*. 1996;137:3447-52.
[5] Zirkin BR and Chen H. Regulation of Leydig cell steroidogenic function during aging. *Biol Reprod*. 2000;63:977-81.

[6] Chen H, Irizarry RA, Luo L, Zirkin BR. Leydig cell gene expression: effects of age and caloric restriction. *Exp Gerontol*. 2004;39(1):31-43.
[7] Chen H, Hardy MP, Zirkin BR. Age-related decreases in Leydig cell testosterone production are not restored by exposure to LH in vivo. *Endocrinology*. 2002;143:1637-1642.
[8] Luo L, Chen H, Zirkin BR. Leydig cell aging: steroidogenic acute regulatory protein (StAR) and cholesterol side-chain cleavage enzyme. *J Androl*. 2001;22:149-156.
[9] Culty M, Luo L, Yao Z, Chen H, Papadopoulos V, Zirkin B. Cholesterol transport, peripheral benzodiazepine receptor, and steroidogenesis in aging Leydig cells. *J Androl*. 2002;23:439-447.
[10] Luo L, Chen H, Zirkin BR. Temporal relationships among testosterone production, steroidogenic acute regulatory protein (StAR), and P450 side-chain cleavage enzyme (P450scc) during Leydig cell aging. *J Androl*. 2005;26(1):25-31.
[11] Monder C, Miroff Y, Marandici A, Hardy MP. 11ß-dehydrogenase alleviates glucocorticoid-mediated inhibition of steroidogenesis in rat Leydig cells. *Endocrinology*. 1994;134:1199-1204.
[12] Schultz R, Isola J, Parvinen M, *et al*. Localization of the glucocorticoid receptor in testis and accessory sexual organs of male rat. *Mol Cell Endocrinol*. 1993;95:115-20.
[13] Ge RS, Hardy DO, Catterall JE, Hardy MP. Developmental changes in glucocorticoid receptor and 11ß-hydroxysteroid dehydrogenase oxidative and reductive activities in rat Leydig cells. *Endocrinology*. 1997;138:5089-5095.
[14] Gao HB, Ge RS, Lakshmi V, Marandici A, Hardy MP. Hormonal regulation of oxidative and reductive activities of 11ß-hydroxysteroid dehydrogenase in rat Leydig cells. *Endocrinology*. 1997;138:156-161.
[15] Ge RS, Hardy MP. Initial predominance of the oxidative activity of type I 11ß-hydroxysteroid dehydrogenase in primary rat Leydig cells and transfected cell lines. *J Androl*. 2000;21:303-310.
[16] Ge RS, Dong Q, Sottas CM, Chen H, Zirkin BR, Hardy MP. Gene expression in rat Leydig cells during development from the progenitor to adult stage: a cluster analysis. *Biol Reprod*. 2005;72(6):1405-1415.
[17] Davidoff M, Schulze, W. Combination of the peroxidase antiperoxidase (PAP)- and avidin- biotin- peroxidase complex (ABC)- techniques: An amplification alternative in immunocytochemical staining. *Histochemistry*. 1990;93:531-536.
[18] Bremner WJ, Millar MR, Sharpe RM, Saunders PTK. Immunohistochemical localization of androgen receptors in the rat testis: evidence for stage-dependent expression and regulation by androgens. *Endocrinology*. 1994;135(3):1227-1234.
[19] Atanassova N, Koeva Y, Bakalska M, Pavlova E, Nikolov B, Davidoff M. Loss and recovery of androgen receptor protein expression in the adult rat testis following androgen withdrawal by ethane dimethanesulfonate. *Folia Histochem Cytobiol*. 2006;44(2):81-86.
[20] Bakalska M, Atanassova N, Pavlova E, Koeva Y, Nikolov B. Morphological alterations in rat testis during aging. *Acta Morphol et Anthropol*. 2008;13:82-6.
[21] Steiner RA, Bremner WJ, Clifton DK, Dorsa DM. Reduced pulsatile luteinizing hormone and testosterone secretion with aging in the male rat. *Biol Reprod*. 1984;31:251-8.
[22] Chen H, Luo L, Zirkin BR. Leydig cell structure and function during aging. In: Payne AH, Hardy MP, Russell LD, eds. *The Leydig cell*. Cache River Press, Vienna, IL; 1996:221-30.
[23] Levy S, Serre V, Hermo L, Robaire B. The effect of aging on the seminiferous epithelium and the blood-testis barrier of the Brown Norway rat. *J Androl*. 1999;20:356-65.
[24] Ichihara I, Kawamura H, Pelliniemi LJ. Ultrastructure and morphometry of testicular Leydig cells and the interstitial components correlated with testosterone in aging rats. *Cell Tissue Res*. 1993;271(2):241-55.
[25] Wang C, Leung A, Sinha-Hikim A. Reproductive aging in the male Brown Norway rat: a model for the human. *Endocrinology*. 1993;133:2773-81.
[26] Chen H, Hardy MP, Huhtaniemi I, Zirkin BR. Age-related decreased Leydig cell testosterone production in the Brown Norway rat. *J Androl*. 1994;15:551-57.
[27] Luo L, Chen H, Zirkin BR. Are Leydig cell steroidogenic enzymes differentially regulated with aging? *J Androl*. 1996;17:509-515.
[28] Monder C, White PC. 11 hydroxysteroid dehydrogenase. *Vitam Horm*. 1993;47:187-271.
[29] Walker BR, Connacher AA, Lindsay RM, Webb DJ, Edwards CR. Carbenoxolone increases hepatic insulin sensitivity in man: a novel role for 11-oxosteroid reductase in enhancing glucocorticoid receptor activation. *J Clin Endocrinol Metab*. 1995;80:3155-59.
[30] Hardy MP, Gao HB, Dong Q, *et al*. Stress hormone and male reproductive function. *Cell Tissue Res*. 2005;322(1):147-53.
[31] Koeva Y, Bakalska M, Atanassova N, Georgieva K, Davidoff M. 11β hydroxysteroid dehydrogenase type 2 expression in the newly formed Leydig cells after ethane dimethanesulphonate treatment of adult rats. *Folia Histochem Cytobiol*. 2007;45(4):381-6.
[32] Ge RS, Dong Q, Nin EM, *et al*. 11 beta-hydroxysteroid dehydrogenase 2 in rat Leydig cells: its role in blunting glucocorticoid action at physiological levels of substrate. *Endocrinology*. 2005;146(6):2657-2664.
[33] Sankar BR, Maran RR, Subha S, Govindarajulu P, Balasubramanian K. Chronic corticosterone treatment impairs Leydig cell 11beta-hydroxysteroid dehydrogenase activity and LH-stimulated testosterone production. *Horm Metab Res*. 2000;32(4):142-6.
[34] Anand-Ivell RJ, Relan V, Balvers M, *et al*. Expression of the insulin-like peptide 3 (INSL3) hormone-receptor (LGR8) system in the testis. *Biol Reprod*. 2006;74:945-953.
[35] Ivell R and Bathgate R. Reproductive biology of the relaxin-like factor (RLF/INSL3). *Biol Reprod*. 2002;67:699-705.
[36] Mendis- Handagama SM, Ariyaratne HB, Mrkonjich I, Ivell R. Expression of insulin-like peptide 3 in the postnatal Leydig cell lineage; timing and effects of triiodothyronine-treatment. *Reproduction*. 2007;133(2):479-85.
[37] Foresta C, Bettella A, Vinanzi C, *et al*. A novel circulating hormone of testis origin in human. *J Clin Endocrinol Metab*. 2004;89:5952-8.
[38] Sadeghian H, Anand-Ivell R, Balvers M, Relan V, Ivell R. Constitutive regulation of the Insl3 gene in rat Leydig cells. *Mol Cell Endocrinol*. 2005;124:10-20.
[39] Paust HJ, Wessels J, Ivell R, Mukhopadhyay AK. The expression of RLF/INSL3 gene is reduced in Leydig cells of the aging rat testis. *Exp Gerontol*. 2002;37:1461-67.
[40] Heinlein CA, Chang C. Androgen receptor (AR) coregulators: An overview. *Endocr Rev*. 2002;23:175-200.
[41] Syed V, Hecht N. Selective loss of Sertoli cell and germ cell function leads to a disruption in sertoli cell-germ cell communications during aging in the Brown Norway rat. *Biol Reprod*. 2001;64:107-112.
[42] Ivell R. Biology of the relaxin-like factor (RLF). *Rev Reprod*. 1997;2:133-138.

Submitted: 24 November, 2008
Accepted after reviews: 8 March, 2009