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Long-term inhibition of PDE5 ameliorates aging-induced changes in rat testis

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ABSTRACT

NO-cGMP signaling pathway has been implicated in reduction of testicular steroidogenesis during aging. Here we analyzed the effect of PDE5 inhibition on old testicular phenotype formation. The old phenotype exhibited low testosterone and increased nitrite levels in circulation, increased cGMP accumulation in testicular interstitial fluid (TIF), progressive atrophy of testicular seminiferous tubules and enlargement of interstitial area followed by rise in blood vessel density and slight increase in the number of Leydig cells and macrophages. Leydig cells have reduced steroidogenic capacity, increased MAP kinases expression (MEK, ERK1/2, JNK) and antiapoptotic PRKG1 and AKT, suggesting increased proliferation/survival and accumulation of senescent Leydig cells in testis. In 12 month-old rats, a long-term treatment with sildenafil (PDE5 inhibitor) normalized testosterone/nitrite levels in circulation and cGMP accumulation in TIF; improved Leydig cell steroidogenic capacity; decreased MEK, ERK1/2 and PRKG1 expression; prevented an increase in the Leydig cells number and atrophy of seminiferous tubules leading to histological appearance of young rat testes. In 18 month-old rats, long-term PDE5 inhibition partially recovered testosterone and nitrite levels in serum; normalized PRKG1 expression without effect on MEK and ERK1/2; and slowed down Leydig cell and macrophage accumulation and regressive tubular changes. Culturing of primary Leydig cells from aged rats in presence of PDE5-inhibitor stimulated steroidogenic and MAPK gene expression. Taking together, results indicate that cGMP targeting alter both steroidogenesis and signaling pathways associated with cell proliferation/survival. The long-term PDE5 inhibition improves testicular steroidogenesis and slows-down regressive changes in testes during aging.

1. Introduction

Aging includes a series of physiological changes of function and structure at molecular, cellular, tissue and organism levels (Waaijer et al., 2016). It is thought to begin after reproductive maturation and is followed by a gradual loss of homeostatic mechanisms leading to lower fertility, reduced tissue functionality, increased sensitivity to disease and increased risk of dying (Rando and Chang, 2012). The fact that aging, as part of the developmental process of the individual, begins after reproductive maturation indicates the interference of reproductive hormones in the processes of the old phenotype formation.

Male reproductive aging is associated with a decrease in serum testosterone even in the absence of disease (Harman et al., 2001; Mohr et al., 2005). However, the consequences of low testosterone may be a cause of various disorders like osteoporosis, reduced muscle strength, reduced libido, declined fertility and mood changes (Basaria, 2013).

Abbreviations: *Actb*/ACTB, gene/protein for β-actin; AKT, protein for protein kinase B (AKT/PRKGB); BSA, bovine serum albumin; cAMP, cyclic adenosine monophosphate; cGMP, cyclic guanosine monophosphate; *Cyp11a1*, gene for cytochrome P450 side chain cleavage enzyme; *Cyp17a1*, gene for 17α-gydroxylase/C17-20 lyase; DAB, 3,3′-diaminobenzidine; DMEM/F12, Dulbecco's modified eagle medium; EDTA, ethylenediaminetetraacetic; EIA, enzyme immunoassay; *Erk*/ERK, gene/protein for extracellular signal-regulated kinases; FasL, fas ligand; hCG, human chorionic gonadotropin; *Hmgcr*, gene for 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase; HPF, high power field; HSD3B1/2, protein for hydroxysteroid dehydrogenase 3β 1 and 2; IFN-γ, interferon γ; *Igf*, gene for insulin-like growth factor; *Igfr*, gene for insulin-like growth factor receptor; IL-6, interleukin 6; JNK, protein for c-Jun N-terminal kinase; LH, luteinizing hormone; *Lipe*, gene for hormone-sensitive lipase; M199, medium 199; Mapk/MAPK, gene/protein for mitogen-activated protein kinases; *Mek2*/MEK2, gene/protein for mitogen-activated protein kinase kinase 2; mTOR, protein for mammalian target of rapamycin; NO, nitric oxide; NP-40, niaproof4; P70S6K, protein for ribosomal protein S6 kinase; PAS, periodic acid-Schiff; PBS, phosphate-buffered saline; *Pde*/PDE, gene/protein for phosphodiesterase; PRKG1, protein for cGMP-dependent protein kinas 1; PVDF, polyvinylide difluoride; *Ras*, gen for small GTPase; RIA, radioimmunoassay; RQ-PCR, relative quantification polymerase chain reaction; SDS, sodium dodecyl sulfate; SF-1, protein for steroidogenic factor 1; SMA, antibody for actin, smooth muscle; *Star*/StAR, gene/protein for steroidogenic acute regulatory protein; T + DHT, testosterone + dihydrotestosterone; TBS, *tris*-buffered saline; TIF, testicular interstitial fluid; TNF-α, protein for tumor necrosis factor α; *Tspo*, gene for translocator protein

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Decline in serum testosterone is a due to decreased ability of Leydig cells to produce testosterone in the presence of unchanged or elevated serum luteinizing hormone (LH) levels (Chen et al., 2002; Tajer et al., 2010). Although mechanisms leading to age-associated hypofunction of Leydig cells have not been fully clarified, it is known to arise as a result of a reduced cAMP response to gonadotropin stimulation (Chen et al., 2002, 2004), increased oxygen (Chen et al., 2001; Midzak et al., 2009) and nitrogen free radical production (Sokanovic et al., 2013), disturbed mitochondrial functions, and reduced expression and activity of steroidogenic enzymes and elements (Chen et al., 2002; Culty et al., 2002; Luo et al., 2005; Sokanovic et al., 2013, 2014).

Leydig cells from old rats produce increased amount of NO and increased cGMP signaling was detected, suggesting the engagement of NO-cGMP signaling in decreased synthesis of testosterone and development of age-related hypogonadism (Sokanovic et al., 2013). Namely, this signaling pathway appears to act in opposition to modulate testicular steroidogenesis: NO at higher concentration directly inhibits steroidogenic enzymes activities (Del Punta et al., 1996; Drewett et al., 2002) while at lower concentration exerts stimulatory effect through cGMP-dependent activation of protein kinase G 1 (PRKG1; Andric et al., 2007; Davidoff et al., 1997; Gambaryan et al., 2003). Since cGMP-specific phosphodiesterase5 (PDE5, an enzyme that degrades the cGMP) is one of the important regulators of the cGMP level in the cell, PDE5 can be the target molecule for the pharmacological manipulation of the activity of the NO-cGMP signaling pathway. The results from our laboratory show that long- and short-term inhibition of PDE5 by using sildenafil in young male rats increases production of testosterone (Andric et al., 2010; Janjic et al., 2012). The positive effect of sildenafil on testosterone production was also confirmed by a clinical study, indicating testicles as the target tissue for sildenafil (Spitzer et al., 2013). In old rats, long-term inhibition of PDE5 causes partial restoration of aging-deranged testosterone, NO and cyclic nucleotide levels and the expression of steroidogenic and NO-cGMP signaling genes (Sokanovic et al., 2013).

Recently there are attempts to link unbalanced cellular growth and survival with senescence and aging (Ferbeyre, 2017). Aberrant kinases signaling involved in regulation of cell growth, proliferation, and survival, such as AKT, mTOR, p70S6K, MAP kinases underlie cell senescence and aging (Cardoso et al., 2017; Ferbeyre, 2017). Some of them have been found to be up-regulated in Leydig cells from aged rats (Sokanovic et al., 2014). Moreover, the main effector in NO-cGMP signaling pathway, the antiapoptotic and survival kinase, PRKG1, is also increased in Leydig cells from old rats (Sokanovic et al., 2013) suggested to be part of aberrant signaling networks connected with aging and cell senesce.

Based on these observations, the present study focuses on the effect of long-term PDE5 inhibition, by sildenafil treatment, on age-related changes in testicular tissue and correlates them with testosterone production and survival signaling in Leydig cells.

2. Material and methods

2.1. Animals

All experiments were performed with male Wistar rats raised and bred in the animal facility of the Faculty of Sciences, University of Novi Sad. Animals were cherishing under the controlled environmental conditions (12 h light, 12 h dark, 22 ± 2 °C) with unlimited access to water and food (*ad libitum*). All experiments were approved by local Ethical Committee on Animal Care and Use of the University of Novi Sad operating under the rules of the National Council for animal welfare and the National Low for Animal Welfare (March 2009) and in accordance with the National Research Council publication Guide for the Care and Use of Laboratory Animals (copyright 1996, National Academy of Science, Washington, DC) and the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications number 80 23, revised 1996, seventh edition).

6 and 12-month-old male rats were orally treated with phosphodiesterase 5 (PDE5) inhibitor sildenafil (1.25 mg/kg/day, Pfizer, New York) (experimental groups) or with water (control groups) for 6 months. At the end of the treatment animals were decapitated and blood and testes were collected. Serum was prepared and used for hormone and nitrite measurements while testes were used for testicular interstitial fluid (TIF) collection and Leydig cells purification. All procedures were performed exclusively with healthy old rats without eye visible morphological changes.

2.2. Histochemistry, immunohistochemistry and histomorphometrical analyses

The left testis of each animal was fixed in Bouin's solution for 24 h at 4 °C. After fixation, 5 mm thick midsagittal section of testis was dehydrated in isopropyl alcohol, embedded in paraffin (Histowax, the Netherland) and cut on a rotary microtome (Leica, Germany) at 5 μm. For each testis sample, the first slide section was stain with PAS histochemical method and next three serial sections were immunohistochemically stained. Immunohistochemical staining included primary antibodies: rabbit anti-SMA in a 1:100 dilution (Lab Vision; Thermo Scientific, Cat NoRB-9010-R7), rabbit anti-SF-1 in a 1:500 dilution (donation from Professor Ken-ichirou Morohashi, Meeks et al., 2003) and rabbit anti-HSD3B1/2 in a 1:500 dilution (donation from Professor Ian Masson, Bain et al., 1991) and visualization system: UltraVision LP Detection System HRP Polymer & AEC Chromogen (Lab Vision; Thermo Scientific). All the antibodies were applied for 30 min at room temperature but before application they required antigen retrieval using citrate buffer (pH 6.0) in a microwave oven at 850 W for 20 min. Visualization was performed using DAB Chromogen (Lab Vision; Thermo Scientific). Mayer's hematoxylin was used as a counterstain for immunohistochemistry followed by mounting and coverslipping (Bio-Optica, Italy) for slides. Prepared slides were analyzed using a Leica DMLB microscope (Leica, Germany) and photographed on a Leica MC 190 HD camera (Leica, Germany).

For estimating *areal density* of interstitium, normal and atrophic tubules per testicular tissue we photographed 30 randomly selected fields at a magnification of ×100 per slide. The areal density was obtained by counting points over mentioned structures and dividing by the total number of points (468) over analyzed photography. Also, on the same slides, we analyzed *blood vessels density*. Analysis was performed using ImageJ 1.45 image measurement software with CAST plug-in.

For estimating number of Leydig cells and macrophages the 10 randomly selected interstitial space at magnification of ×400 (high power field, HPF) per slide/rat was photographed. For each group mean number ± SEM of Leydig cells and macrophages per HPF were presented.

2.3. Testicular interstitial fluid (TIF) collection and Leydig cells purification

Right testes were quickly removed, decapsulated and placed on Falcon mesh No100 (Sigma, St. Louis, MO) in 50 mL plastic tube and centrifuged 7 min/100*g*/RT. A volume of the TIF was recorded and stored at −80 °C for testosterone and cyclic nucleotide measurements (Janjic et al., 2012; Sokanovic et al., 2013). After the TIF collection, testes from the same animal were digested by collagenase treatment (0.25 mg collagenase/ml-1.5%-BSA-20 mM HEPES-M199). Seminiferous tubules were separated by filtration; interstitial cells ($35–40 \times 10^6$) moved on the Percoll gradient and centrifuged 28 min/1100*g*. After centrifugation Leydig cells were harvest, washed with 0.1%-BSA-M199 and resuspended in DMEM medium. Presence of Leydig cells was 95 ± 2% respectively to HSD3B staining and viability was more than 90% (Andric et al., 2007, 2010). Purified Leydig cells were plated on the Petri dishes (3×10^6 cells/3 mL) or 24 well-plates