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Histological parameters of the adrenal cortex after testosterone application in a rat model of the andropause

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Summary. Histological analysis of the adrenal cortex, after testosterone application in a rat model of the andropause, was the main subject of the present study. Middle-aged Wistar rats were divided into sham-operated (SO; n=8), orchidectomized (Orx; n=8) and testosterone treated orchidectomized (Orx+T; n=8) groups. Testosterone propionate (5 mg/kg b.m. /day) was administered for three weeks, while SO and Orx groups received the vehicle alone. Histological objectives were achieved using stereology, histochemistry and steroid receptor immunostaining. The concentrations of testosterone, aldosterone, corticosterone and DHEA were determined by immunoassays. Expectedly, increased ($p<0.05$) serum concentration of testosterone was observed in Orx+T group. The volume of ZG cells and nuclei increased in Orx+T animals by 50% and 25% ($p<0.05$) respectively, but the serum concentrations of aldosterone decreased ($p<0.05$) by 60%, all compared to the same parameters in Orx group. The immunostaining for androgen receptors (ARs) suggested their cytoplasmic localization in ZG cells of Orx+T rats. Volume of the ZF cell nuclei in Orx+T group decreased ($p<0.05$) by 17%, which was followed by the significant ($p<0.05$) fall in corticosterone production and secretion, all in comparison with Orx animals. Also, nuclear immunolocalization of ARs of high optical density was observed through the ZF of Orx+T group. In Orx+T rats volume of ZR cells and nuclei, and circulating DHEA concentration increased ($p<0.05$) by 68%, 22% and about 6.6 times respectively, compared to Orx animals. Besides the extra-receptor actions in adrenal cortex, testosterone supposedly affects some steroidogenesis-related gene expression, as indicated by centripetal rise in the number of nuclear ARs.

Key words: Histology, Adrenal cortex, Testosterone, Rats, Andropause

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Introduction

As a complement to the previous research, where we examined the capacity for adrenocorticotropic hormone (ACTH) and corticosterone secretion *i.e.* the operability of hypothalamic-pituitary-adrenal (HPA) axis after testosterone application to ageing male rats (Ajdžanović et al., 2015), in the present study we aimed to investigate testosterone-caused histological changes of the adrenal cortex in the same rat model of andropause. Actually, the idea was to emphasize the quantitative as well as the qualitative histological parameters of middle-aged rat adrenal cortex, structured and complexly regulated HPA axis effector, in the light of testosterone supplementation as a potential treatment of andropausal symptoms. Ageing in males culminates in the andropause, the specific multi-symptomatic syndrome implying an irregular activity of the HPA axis (Hatzinger et al., 2000; Vance, 2003; Morales, 2004). Besides impaired central regulation of adrenocortical function due to the age-related loss of hippocampal, hypothalamic and limbic

neurons and synapses (Mani et al., 1986; Ferrari et al., 2001; Nichols et al., 2001), some considerable changes, including loss of steroid-containing lipids, mitochondrial fragmentation, hemorrhage, etc., also occur in the adrenal cortex tissue (Andres and Tobin, 1977; Hatzinger et al., 2000), all of which cause the corticosteroid secretion disturbance in that period of life (Vermeulen, 1983; Morales et al., 1994; Harper et al., 1999; Ajdžanović et al., 2012). Keeping in mind that low free testosterone remarkably characterizes the hormonal status of the aging male (Gray et al., 1991; Ajdžanović et al., 2015), a symptomatic approach based on testosterone supplementation during andropause appears to be an opportunity (Beg et al., 2008), although careful supervision for safety is advised (Myers and Meacham, 2003). When it comes to the mainstream HPA axis function (corticosterone secretion from adrenal cortex zona fasciculata - ZF) in the light of endogenous testosterone levels or upon its application, generally inhibitory influence was proposed in male rats (Handa et al., 1994; Evuarherhe et al., 2009; Ajdžanović et al., 2015). Bearing the other adrenal cortex zones in focus, it should be emphasized that Kau et al. (1999) found decreased ACTH-stimulated accumulation of cAMP in rat zona glomerulosa (ZG) cells and lowered aldosterone secretion, after testosterone treatment. Long ago, Malendowicz (1974) revealed the considerable increase in the percentage of parenchymal zona reticularis (ZR) cells in the adrenal cortex of orchidectomized and testosterone treated rats. However, a thorough quantitative and qualitative histological analysis of the adrenal cortex, after testosterone supplementation during andropause, was unduly neglected among investigators.

In order to achieve the set histological objectives pertinent to the adrenal cortex and its zones we used a well-founded stereological approach (Ajdžanović et al., 2009b; Milošević et al., 2011), combined with the histochemistry, steroid receptor immunostaining/immunohistochemistry quantification and hormonal/mineral analyses. The exploited experimental set-up, considering the usage of orchidectomized middle-aged rats in order to mimic the andropause, represents a pattern of good practice in examining the potential effects of synthetic steroids/steroid-like compounds on endocrine homeostasis (Ajdžanović et al., 2009a,b, 2011, 2014, 2015; Filipović et al., 2013).

Materials and methods

Animals and experimental design

The experiments involved 24 middle-aged (16-month-old) Wistar rats. They were bred at the Institute for Biological Research "Siniša Stanković", Belgrade, Serbia; housed one *per* cage, and maintained under constant laboratory conditions (22±2°C, 12-12 h light-dark cycle) with free access to food (standard diet; the chemical composition was previously reported - Ajdžanović et al., 2015) and water. At the age of 15 months, the experimental animals were randomly bilaterally orchidectomized (Orx; n=16 animals) or sham-operated (SO; n=8 animals) under Ketamine anesthesia (15 mg/kg b.m.; Richter Pharma, Wels, Austria). Recovery period was two weeks. Orchidectomized rats were then divided into two groups of eight animals (n=8) each. The first group was subcutaneously treated with testosterone propionate (Fluka Chemie AG, Buchs, Switzerland; Orx+T) in a dose of 5 mg/kg b.m. (Filipović et al., 2013; Ajdžanović et al., 2015) every day except on Sundays, for 3 weeks. The final injected volume was 0.5 ml *per* animal. The second orchidectomized group (Orx) and the SO group were given the same volume (0.5 ml) of vehicle (sterile olive oil) alone, and served as controls. There was no spontaneous death of the animals during the experiment. All the animals (n=24) were sacrificed by decapitation under ether anesthesia (*ether ad narcosis* Ph. Iug. III., Lek, Ljubljana, Slovenia) 24 h after the last injection. All experimental procedures were in compliance with Directive 2010/63/EU on the protection of animals used for experimental and other scientific purposes and were approved by the Ethical Committee for the Use of Laboratory Animals of the Institute for Biological Research "Siniša Stanković", University of Belgrade.

Light microscopy, histochemistry and immunohistochemistry

Left adrenal glands were excised, weighed (the relative weight of the adrenal to the body mass was also determined), fixed in 4% paraformaldehyde for 24 h, dehydrated in a series of increasing concentrations of ethanol, enlightened in xylol and embedded in paraplast. Serial 5-μm thick tissue sections were deparaffinized in xylol and rehydrated in a series of decreasing concentrations of ethanol. For the light microscopy analysis, adrenals were histochemically stained following the H&E or Heidenhain's AZAN trichrome stain procedure, while for evaluation of androgen receptor (AR) and estrogen receptor alpha (ERα) expression, sections of the adrenal gland were stained immunohistochemically. Prior to the immunostaining procedure, heat-induced antigen retrieval was performed in a microwave on high power (750 W) for 21 min (3×7 min) in 0.01 mol/L citrate buffer, pH 6.0. After washing with PBS, sections were incubated with 0.3% hydrogen peroxide in methanol for 15 min at room temperature, for blocking of endogenous peroxidase activity. Adrenal gland sections were then treated with normal swine serum (Dako, Denmark) diluted in PBS (1:10). After blocking, sections were incubated overnight at room temperature with rabbit anti-AR primary antibody (1:100; Santa Cruz Biotechnology), and rabbit anti-ERα primary antibody (1:100; Santa Cruz Biotechnology). After rinsing in PBS, sections were incubated with swine anti-rabbit secondary antibody (DAKO, Glostrup, Denmark), diluted in PBS (1:100) for 1 h at room temperature. Binding sites were visualized using 0.05%

diaminobenzidine (DAB; Serva, Heidelberg, Germany), followed by counterstaining with hematoxylin and mounted with DPX (Sigma-Aldrich, Co., USA). Negative controls were obtained by replacement of primary antibody for a PBS.

Digital images of both the adrenal gland AZAN stained and immunohistochemically stained sections were taken using a LEITZ DM RB light microscope (Leica Mikroskopie & Systems GmbH, Wetzlar, Germany), a LEICA DFC320 CCD camera (Leica Microsystems Ltd., Heerbrugg, Switzerland) and the Leica DFC Twain Software (Leica, Germany).

Stereological analyses

Stage 1. Volumes and zonation of the adrenal gland

The absolute volume of the adrenal glands was calculated on the basis of their weight, assuming 1.039 gcm^{-3} as the average specific gravity of the adrenal (Swinyard, 1938). In order to estimate the volumes of the adrenal cortex and its' zones, every tenth H&E stained section (5 μm thick) of the gland was analysed at 100x magnification with the multipurpose test system M_{42}, by scanning all the adrenal cortex section surface. Keeping in mind that the adrenal cortex surface changed with progression through the tissue (increased and then decreased), the number of test fields consequently altered (Weibel, 1979; Ajdžanović et al., 2009b; Milošević et al., 2011).

Stage 2. Volume of adrenocortical cells and their nuclei

M_{42} system was also used to estimate the nuclear and cytoplasmic volumes of parenchymal adrenocortical cells on 5 μm thick H&E stained sections, under the light microscope at 1000x magnification (Weibel, 1979; Ajdžanović et al., 2009b; Milošević et al., 2011). For each adrenal gland, from the animals belonging to the experimental groups, a single paraffin section containing the *zona medullaris* was chosen and 30 test fields of the zona glomerulosa (ZG) and 50 test fields of both the zona fasciculata (ZF) and zona reticularis (ZR) were analysed. On the basis of earlier karyometric studies (Malendowicz, 1974), the shape coefficient β was assumed to be 1.382 for the ZF and 1.500 for the ZG. It relates Nv (number of cells counted *per* unit volume) to Na (number of cells counted *per* mm^2) and Vv (volume density) and depends on the axial ratio of the estimated nuclei.

Quantitative analyses of digital immunohistochemistry images

Analyses were performed using the Windows based ImageJ program (Image J, Version 1.50f). As a first step, we adopted the spectral deconvolution method of DAB/Hematoxylin color spectra, by using optimized optical density (OD) vectors of the color deconvolution plugin for the proper separation of the DAB color spectra. To determine the OD for the RGB channel of Hematoxylin and DAB, we followed the protocol as previously described by Ruifrok and Johnston, (2001) and Varghese et al., (2014). Since the OD is proportional to the concentration of the stain, the amount of stain present will be a factor determining the optical density at a wavelength specific to the stain, as *per* the Lambert-Beer law (Jähne, 1997). In brief, the OD for each channel is defined as:

$$OD = - \log10 (I_C/I_{0.C}),$$

wherein I is the transmitted light, I_C represents the intensity of the detected light after passing through the specimen and $I_{0.C}$ is the intensity of light entering the specimen.

Stained percentage color area for either cytoplasmic or nuclear fraction of steroid receptors was evaluated using ImageJ plugin named as IHC profiler according to the previously described procedures by Varghese et al., (2014). For both cytoplasmic and nuclear staining pattern, 30 unbiasedly captured images (the microscopic tool has already been described; 2088x1550 pixels, 63x objective magnification) *per* adrenocortical zone *per* animal were analyzed.

Biochemical analyses

Blood was collected from the trunk, and separated sera samples of all the animals were stored at the same time at -70°C until assayed. Serum testosterone concentrations were determined without dilution, using a competitive immunoenzymatic colorimetric method (EIAgen Testosterone Kit, Adaltis Italia S.p.A., Bologna, Italy), in duplicate samples within a single assay, with an intra-assay coefficient of variation (CV) of 6.2%. Serum aldosterone concentrations were determined by enzyme immunoassay for direct quantitative determination (Aldosterone ELISA, IBL Hamburg, Germany), in duplicate samples within a single assay, with an intra-assay CV of 4.1% (128.67 pg/ml). Serum corticosterone concentrations were measured without dilution by immunoassay (R&D Systems Inc., Minneapolis, USA), in duplicate within single assays, with an intra-assay CV of 8.0% (171 pg/ml). Serum DHEA concentrations were determined by enzyme immunoassay for quantitative determination (DHEA ELISA, IBL Hamburg, Germany), in duplicate samples within a single assay, with an intra-assay CV of 6.92% (0.58 ng/ml). Serum Na^+ and K^+ concentrations were determined using the ion-selective electrodes on cobas® 6000 analyzer, equipped with c501 module (Roche Diagnostics, Indianapolis, IN, USA).

Adrenal tissue corticosterone assay

The right adrenal glands were excised, weighed and immediately shredded on ice. Shredded tissue was then

homogenized in TRIS-saccharose buffer (pH 7.9; 1 mg of tissue: 1 µl of buffer) using a dispersion system (Ultra - Turax T25, Janke and Kunkel, IKA-Labortechnik, Staufen, Germany) at 8000 rpm. The homogenate was centrifuged at 35000 rpm (105000 g) for 1 h (BECKMAN ultracentrifuge, L7-55) and corticosterone concentration in the supernatant was determined by immunoassay (R&D Systems Inc., Minneapolis, USA) (Ajdžanović et al., 2009b, 2015).

Statistical analysis

STATISTICA® version 7.0 (StatSoft, Inc) was used for the statistical analysis. Stereological and biochemical data obtained for the experimental groups were subjected to one-way analyses of variance (ANOVA). Duncan's multiple range tests were used for *post hoc* comparisons between the groups. A confidence level of $p<0.05$ was considered statistically significant. The data are presented as means ± SD.

Results

Body mass and adrenal gland weights

Data for the body mass, absolute and relative weights of adrenal glands, as well as absolute adrenal gland volume are summarized in Table 1. It can be seen that orchidectomy (Orx), as well as subsequent treatment with testosterone (Orx+T) did not cause statistically significant changes in the measured parameters.

Histochemical findings

Three cortical zones were clearly visible in all examined AZAN stained adrenal gland preparations (Fig. 1A-I). The ZG in SO middle-aged rats was arranged in closely packed cell clusters of medium sized spheroidal and small, columnar or pyramidal cells with oval nuclei (Fig. 1A). The overall shape of the ZG cells did not change in Orx group (Fig. 1D), while a noticeable feature of the ZG in Orx+T animals was sporadic clustering of small cells and vacuolization as a result of lipid-droplet pull down during fixation (Fig. 1G). The ZF cells in SO rats were large, with clearly visible, single, nucleoli-containing, round or oval nuclei, and appeared arranged in long, straight cords with intercalated blood vessels (Fig. 1B). The shapes and positions of the ZF cells did not change after orchidectomy (Orx) (Fig. 1E). Testosterone treatment of orchidectomized rats (Orx+T) caused massive vacuolization of darker ZF cells and induced vasodilatation (Fig. 1H). Cells of the ZR in SO middle-aged animals were dark and arranged net-like around the blood vessels (Fig. 1C). In Orx group these cells were smaller, while the connective elements within the cell net were slightly more pronounced (Fig. 1F). The ZR of Orx+T rats consisted of dark mononuclear cells that were frequently vacuolized, some of the ZR cell nuclei were more intensely stained and the connective tissue hypertrophied (Fig. 1I).

Stereological results

The stereological analysis revealed significant orchidectomy-induced (Orx) increases ($p<0.05$) in absolute volume of the adrenal cortex, amounting to 27% in comparison with the same parameter in SO group (Fig. 2A). The absolute and relative volumes of the ZG remained unaltered in Orx group, while in Orx+T middle-aged rats relative volume of the ZG significantly decreased by 14% and 11% ($p<0.05$) compared to SO and Orx groups, respectively (Fig. 2A,B). When it comes to ZF, the only significant change ($p<0.05$) pertinent to the volumes was the 24% increase in the absolute volume after orchidectomy (Orx), in comparison with the SO rats (Fig. 2A). Orchidectomy (Orx) also significantly increased ($p<0.05$) the absolute volume of ZR by 40% when compared to the same parameter in SO group (Fig. 2A). In Orx+T animals, the absolute volume of ZR was significantly increased by 55% ($p<0.05$) compared to the corresponding parameter in SO group (Fig. 2A). Similarly, the relative volume of ZR in Orx+T middle-aged rats was significantly increased ($p<0.05$) by 30% and 19% in comparison with the same parameter in SO and Orx groups, respectively (Fig. 2 B).

Further stereological analysis, performed at a finer level (Table 2), determined a significant decrease of the ZG cells and nuclei volume in Orx rats, of 43% and 38% ($p<0.05$) respectively, when compared to the corresponding parameters in SO group. Testosterone

Table 1. Body mass, absolute and relative weights of adrenal glands, as well as absolute adrenal gland volume in sham-operated (SO), orchidectomized (Orx) and testosterone treated orchidectomized (Orx+T) middle-aged rats.

Experimental group	Body mass after the treatment (g)	Absolute adrenal gland weight (mg)	Relative adrenal gland weight (mg%)	Absolute adrenal gland volume (mm^3)
SO	722±65	31.60±4.23	4.25±0.56	28.49±3.47
Orx	670±14	37.80±2.95	4.70±0.71	36.38±2.84
Orx+T	688±65	35.20±4.56	4.47±0.65	33.88±4.28

All values are the means ± SD, n=8 animals *per* group.

treatment of orchidectomized rats (Orx+T) caused a significant decrease ($p<0.05$) of ZG cells and nuclei volume by 15% and 23% respectively, in comparison with SO group. On the contrary, the volume of ZG cells and nuclei significantly increased in Orx+T animals by 50% and 25% ($p<0.05$) respectively, compared to the same parameters in Orx group (Table 2). After orchidectomy (Orx), the volume of ZF cells did not significantly change, but the volume of their nuclei significantly increased ($p<0.05$) by 35%, when compared to SO group. Orchidectomy followed with testosterone treatment (Orx+T) significantly increased

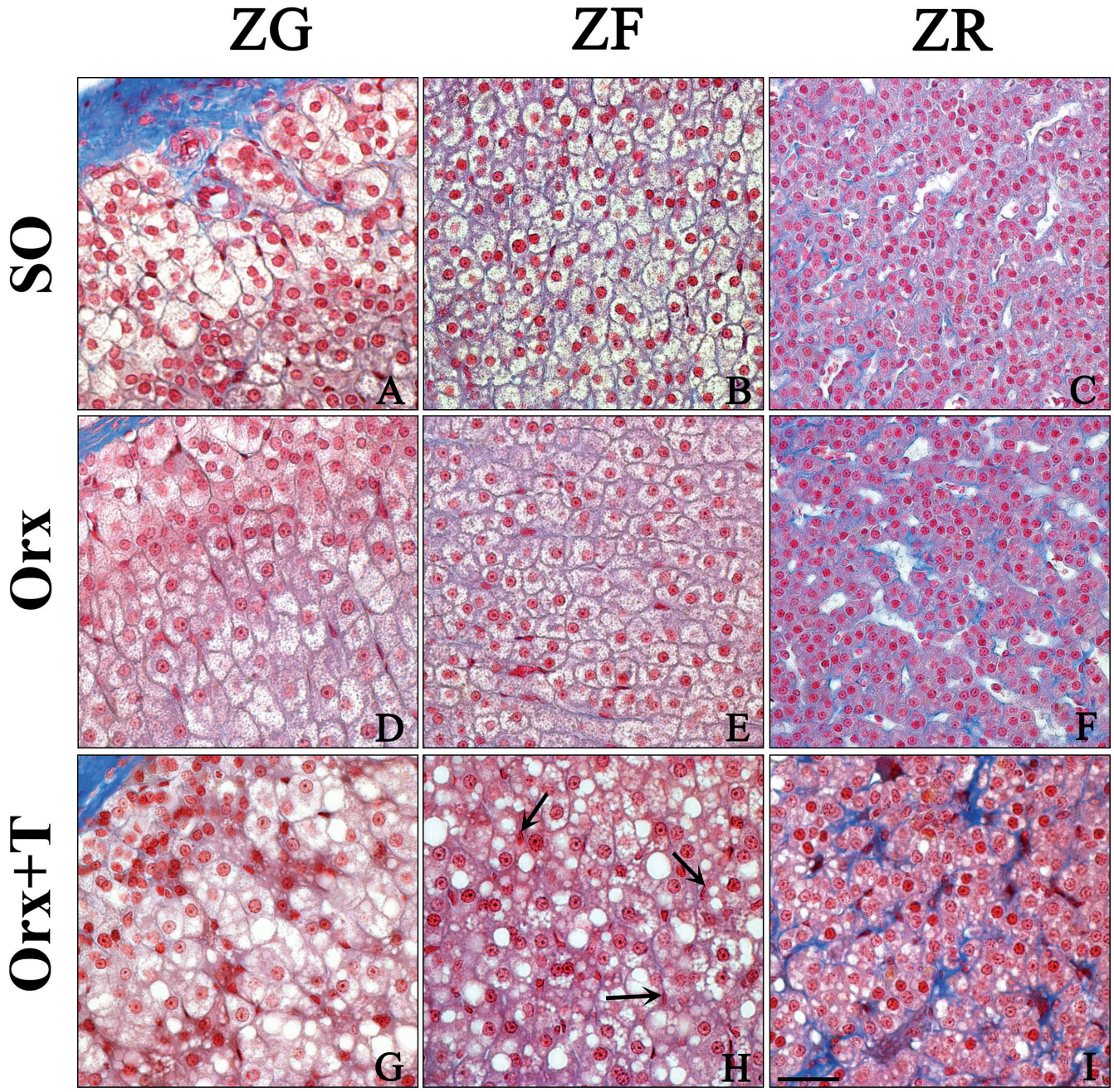

Fig. 1. Adrenal cortex in: sham-operated (SO) (**A-C**), orchidectomized (Orx) (**D-F**) and testosterone treated orchidectomized (Orx+T) (**G-I**) middle-aged rats. ZG: zona glomerulosa, ZF: zona fasciculata, ZR: zona reticularis. Black arrows: dilated blood vessels in ZF of Orx+T middle-aged rats. Heidenhain's AZAN trichrome stain. Scale bar: 25 μm.

the volume of ZF cells and nuclei by 16% and 12% ($p<0.05$) respectively, in comparison with the corresponding parameters in SO rats. On the other hand, volume of the ZF cell nuclei in Orx+T group significantly decreased ($p<0.05$) by 17%, compared to Orx group (Table 2). When it comes to the ZR cells, it is observable that orchidectomy (Orx) caused a significant decrease of its cell and nuclei volume by 29% and 24% ($p<0.05$) respectively, in comparison with the same parameters in SO group. In Orx+T middle-aged rats volume of ZR cells significantly increased ($p<0.05$) by 19% and 68%, when compared to SO and Orx rats respectively. The volume of ZR cell nuclei significantly increased by 22% ($p<0.05$) after orchidectomy and testosterone treatment (Orx+T), in comparison with orchidectomy (Orx) alone (Table 2).

Immunohistochemical findings

Immunostaining of androgen receptors (ARs) in the adrenal cortex sections of SO (Fig. 3A) animals showed dominantly cytoplasmic localization and moderate intensity in ZG and ZF, while marked nuclear as well as cytoplasmic immunoreactivity was observed in ZR (Fig. 3A insets). The lack of steroid hormones induced with orchidectomy (Orx) did not change the optical density (OD) of imunolabeled ARs in ZG, while the same parameter in the ZF was significantly increased ($p<0.05$) by 12% when compared to SO group (Fig. 3C; Table 3). Testosterone treatment of orchidectomized middle-aged rats (Orx+T; Fig. 3E insets, Table 3) led to a significant increase ($p<0.05$) of OD of immunostained ARs in ZG and ZF, by 47% and 41% when compared to SO, and by 66% and 26% when compared to Orx group, respectively.

Estrogen receptor alpha (ERα) expression was not detected by the immunohistochemistry in the adrenal cortex of SO rats (Fig. 3B). Moreover, orchidectomy (Orx) did not induce changes in OD values in all adrenal cortex cells (Fig. 3D, Table 3). However, in Orx+T rats OD of ERα staining was significantly increased ($p<0.05$) in all zones of the adrenal cortex (ZG, ZF, ZR) by 158%, 73% and 104% respectively, in comparison with SO; and by 51%, 40% and 82% respectively, in comparison with Orx (Fig. 3F, Table 3).

The precise analysis of localization and percentage of contribution of AR and ERα immunostaning are presented in detail in Fig. 4. In the adrenal cortex sections of SO animals, ARs showed dominantly cytoplasmic localization. Orchidectomy (Orx) did not

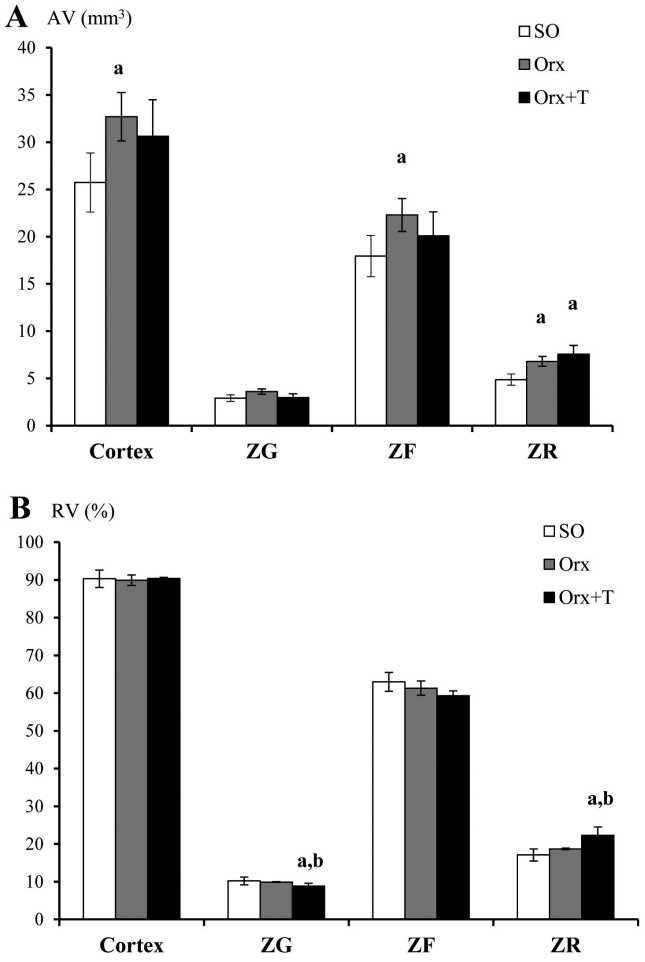

Fig. 2. The absolute volume (AV; mm^3) (**A**) and relative volume (RV; %) (**B**) of the adrenal gland cortex, zona glomerulosa (ZG), zona fasciculata (ZF) and zona reticularis (ZR) in sham-operated (SO), orchidectomized (Orx) and testosterone treated orchidectomized (Orx+T) middle-aged rats. All values are the means ± SD, n=5 animals *per* group; [a]$p<0.05$ vs. SO, [b]$p<0.05$ vs. Orx rats.

Table 2. Volumes of cells and nuclei (μm^3) of the adrenal gland zona glomerulosa (ZG), zona fasciculata (ZF) and zona reticularis (ZR) in sham-operated (SO), orchidectomized (Orx) and testosterone treated orchidectomized (Orx+T) middle-aged rats.

Groups	Volume of cells (μm^3)			Volume of nuclei (μm^3)		
	ZG	ZF	ZR	ZG	ZF	ZR
SO	1448±53	1887±25	943±76	217±6	234±6	224±14
Orx	821±27[a]↓	2113±99	668±9[a]↓	134±7[a]↓	315±6[a]↑	171±8[a]↓
Orx+T	1230±9[a,b]↓↑	2193±202[a]↑	1124±103[a,b]↑	168±14[a,b]↓↑	262±17[a,b]↓↑	208±19[b]↑

All values are the means ± SD, n=5 animals *per* group; [a]$p<0.05$ vs. SO, [b]$p<0.05$ vs. Orx rats.

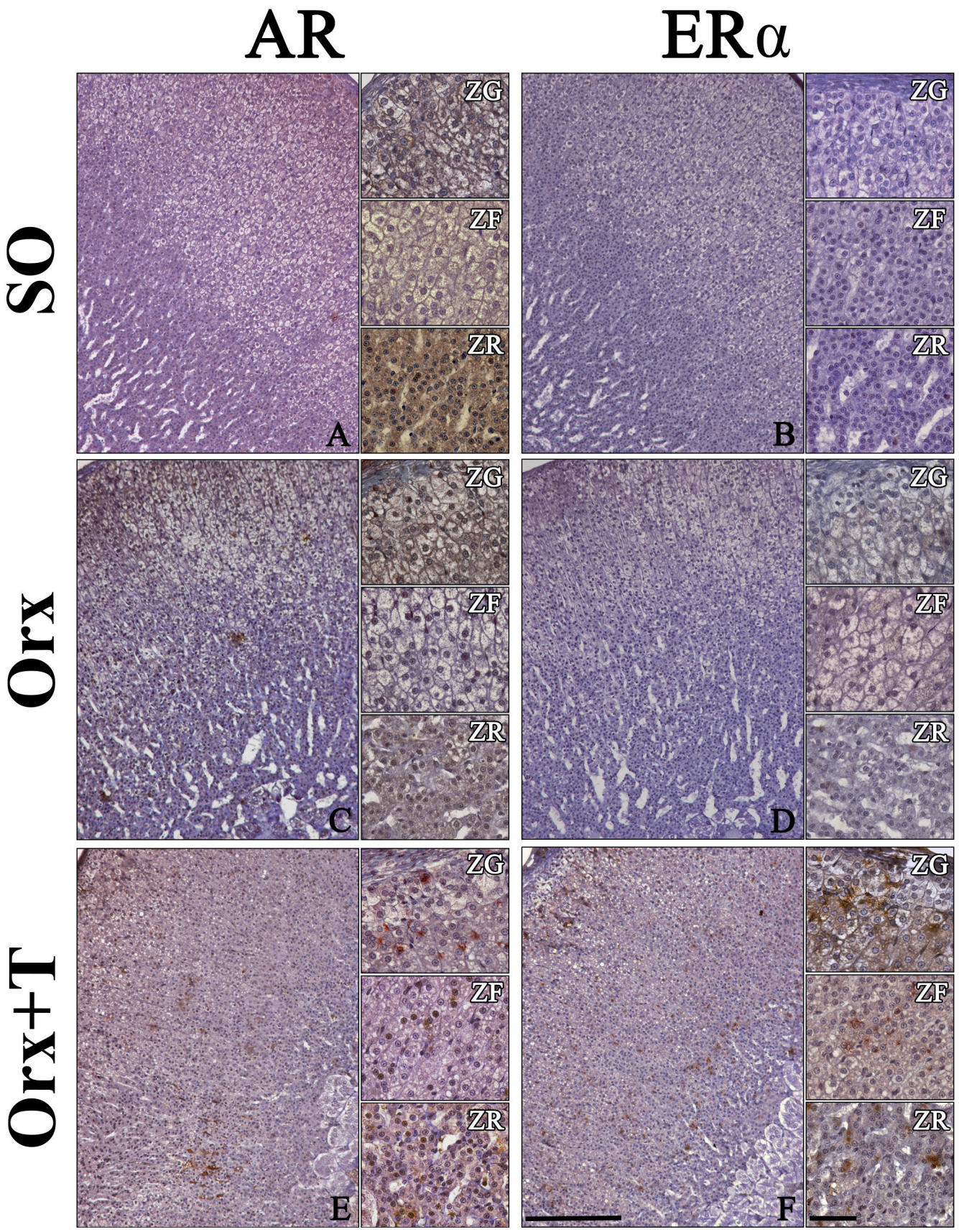

Fig. 3. Immunohistochemical staining illustrating the expression of androgen receptor (AR) and estrogen receptor α (ERα) in the adrenal cortex of: sham-operated (SO) (**A,B**), orchidectomized (Orx) (**C,D**) and testosterone treated orchidectomized (Orx+T) (**E,F**) middle-aged rats. Panoramic overview representing the ZG: zona glomerulosa, ZF: zona fasciculata, ZR: zona reticularis. Scale bars: 40 μm; insets, 16 μm.

induce changes in contribution percentage of AR in ZG, while a significant increase ($p<0.05$) in percentage of immunoreactivity was noticed in the nuclear domain of ZF and a decrease ($p<0.05$) in the same domen of ZR cells (Fig. 4A). Testosterone treatment of orchidectomized middle-aged rats (Orx+T) induced a significant ($p<0.05$) decrease in nuclear AR immunoreactivity of ZG cells, which was accompanied by a significant increase ($p<0.05$) in abundance of nuclear AR immunostaning in ZF and ZR cells (Fig. 4A).

Cytoplasmic as well as nuclear immunohistochemical staining of estrogen receptor alpha (ERα) was negative in the adrenal cortex of SO rats (Fig. 4B). Moreover, most of the adrenal cortex cells were not positively stained after orchidectomy (Orx) (Fig. 4B), but a significant increase ($p<0.05$) of cytoplasmic ZF

Table 3. Optical density (OD) of immunostaining of androgen receptor (AR) and estrogen receptor α (ERα) of the adrenal gland zona glomerulosa (ZG), zona fasciculata (ZF) and zona reticularis (ZR) in sham-operated (SO), orchidectomized (Orx) and testosterone treated orchidectomized (Orx+T) middle-aged rats.

Optical density		SO	Orx	Orx+T
AR	ZG	2.48±0.18	2.19±0.13	3.64±0.08[a,b]↑
	ZF	2.34±0.11	2.62±0.11[a]↑	3.31±0.31[a,b]↑
	ZR	2.35±0.22	2.81±0.04	2.53±0.18
ERα	ZG	1.12±0.11	1.92±0.06	2.89±0.09[a,b]↑
	ZF	1.41±0.13	1.75±0.15	2.45±0.07[a,b]↑
	ZR	1.46±0.12	1.64±0.12	2.98±0.36[a,b]↑

All values are the means ± SD, n=5 animals *per* group; [a]$p<0.05$ vs. SO, [b]$p<0.05$ vs. Orx rats.

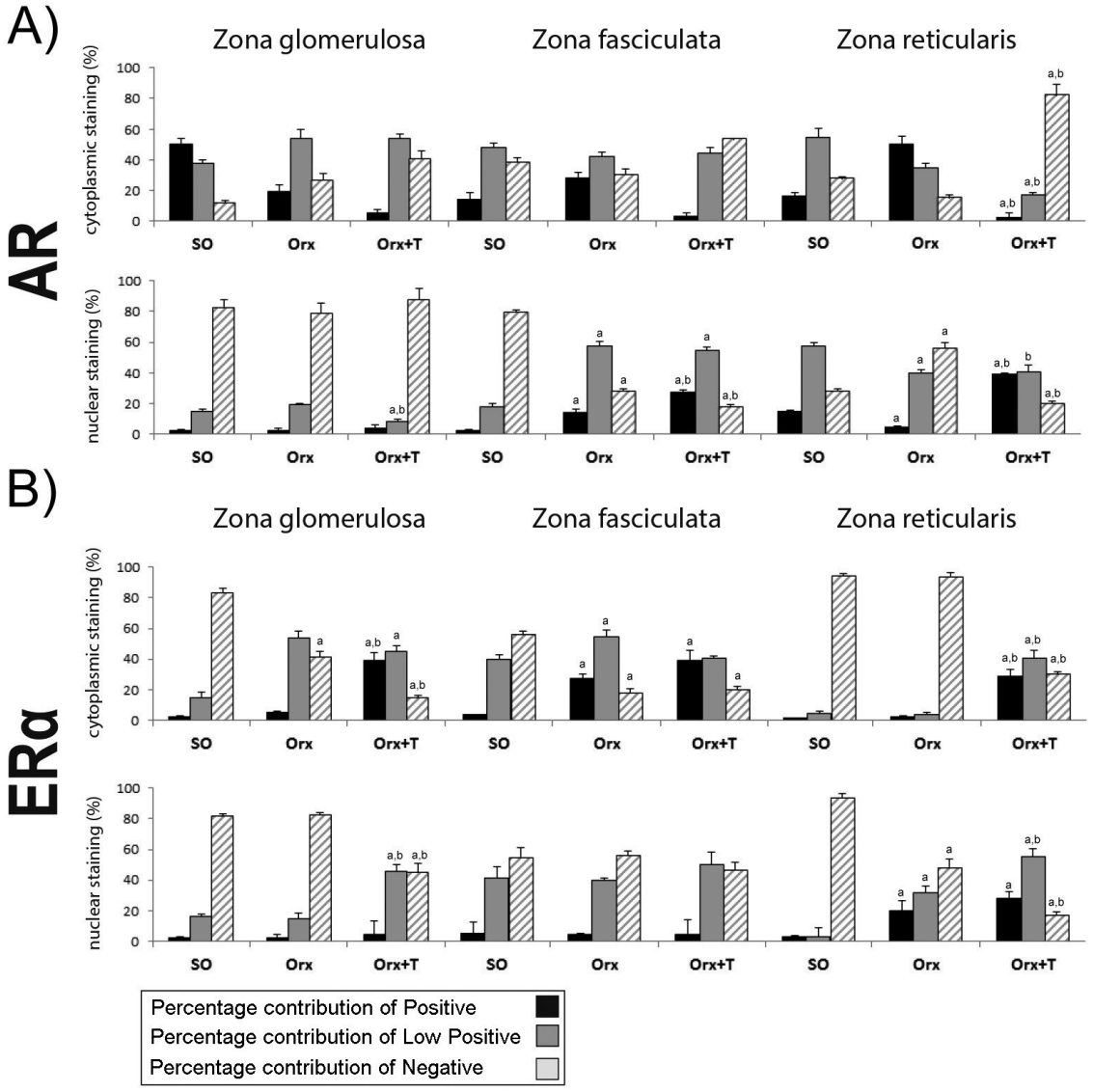

Fig. 4. Percentage contribution of positive, low positive and negative immunohistochemical staining and expression of cytoplasmic and nuclear distribution of androgen receptor (AR) (**A**) and estrogen receptor α (ERα) (**B**), in the adrenal cortex of: sham-operated (SO), orchidectomized (Orx) and testosterone treated orchidectomized (Orx+T) middle-aged rats. For both cytoplasmic and nuclear staining pattern, 30 unbiasedly captured images *per* adrenocortical zone *per* animal were analyzed. All values are the means ± SD, n=5 animals *per* group; [a]$p<0.05$ vs. SO, [b]$p<0.05$ vs. Orx rats.

Table 4. Serum concentrations of testosterone, aldosterone, corticosterone, DHEA, Na^+ and K^+, as well as adrenal tissue level of corticosterone in sham-operated (SO), orchidectomized (Orx) and testosterone treated orchidectomized (Orx+T) middle-aged rats.

Experimental group	SO	Orx	Orx+T
Testosterone (ng/ml)	0.12±0.01	0.10±0.01[a]↓	2.44±0.18[a,b]↑
Aldosterone (pg/ml)	386±36	336±28[a]↓	135±9[a,b]↓
Corticosterone (ng/ml)	175±12	168±15	157±6[a]↓
Adrenal tissue level of corticosterone (ng/mg)	0.80±0.04	0.77±0.07	0.53±0.05[a,b]↓
DHEA (ng/mg)	0.064±0.005	0.038±0.003[a]↓	0.290±0.025[a,b]↑
Na^+ (mmol/L)	151.6±2.4	151.4±3.1	147.6±3.9
K^+ (mmol/L)	6.4±0.2	6.3±0.6	6.6±0.3

All values are the means ± SD, n=8 animals *per* group, [a]$p<0.05$ vs. SO, [b]$p<0.05$ vs. Orx rats.

and nuclear ZR immunostaning was observed (Fig. 4B).

In contrast, in Orx+T rats contribution percentage of cytoplasmic ERα staining was significantly increased ($p<0.05$) in all zones of the adrenal cortex (Fig. 4B). Increased ($p<0.05$) nuclear ERα immunopositivity in ZG and ZR of the same group was observed.

Biochemical data

The biochemical data are presented in Table 4. In the current study, orchidectomy caused a significant decrease in the serum concentrations of testosterone (by 17%; $p<0.05$), while in the Orx+T animals this parameter was considerably increased ($p<0.05$) by about 20 and 24 times when compared to SO and Orx groups, respectively. Also, orchidectomy (Orx) led to a significant decrease in the serum concentrations of aldosterone by 13% ($p<0.05$), while in Orx+T group this parameter was further decreased ($p<0.05$) by 65% and 60% when compared to SO and Orx groups, respectively. Serum Na^+ and K^+ did not significantly change after orchidectomy (Orx) or orchidectomy followed with testosterone treatment (Orx+T), although there was a slight trend of Na^+ decrease and K^+ increase in Orx+T group. Orchidectomy (Orx) did not cause significant effects pertinent to the serum and adrenal tissue corticosterone values, while in Orx+T rats serum corticosterone concentrations were significantly decreased by 10% ($p<0.05$) in comparison with SO group. Furthermore, testosterone treatment of orchidectomized middle-aged rats (Orx+T) led to a significant decrease ($p<0.05$) in adrenal tissue corticosterone levels by 34% and 31% compared to SO and Orx groups, respectively. Serum DHEA concentration in Orx rats was significantly decreased ($p<0.05$) by 41% in comparison with the same parameter in SO group. On the other hand, testosterone treatment of orchidectomized rats (Orx+T) caused a massive increase in DHEA values, by about 3.5 and 6.6 times ($p<0.05$), when compared to SO and Orx groups respectively.

Discussion

Various high circulating testosterone-caused histological changes of the adrenal cortex, in a rat model of the andropause, were the focus of the present study. Special attention was devoted to the quantification of changes when it comes to AR and ERα immunohistochemistry.

Initially, we should mention some expected fall in the body mass after orchidectomy, that is in accordance with previous reports, and supposedly is the consequence of skeletal muscle atrophy induced by testosterone deprivation (Malendowicz, 1976; Antonio et al., 1999; Ajdžanović et al., 2009a,b, 2011, 2014, 2015). On the other hand, testosterone application to andropausal rats had a certain recovering body mass effect, which may be in line with its adipogenic gene transcription stimulating and bone loss preventing role (Varlamov et al., 2012; Filipović et al., 2013).

In addition to the confirmation of our previous findings when it comes to orchidectomy-induced decrease of the ZG cell and nuclear volume as well as serum aldosterone concentrations in middle-aged rats (Ajdžanović et al., 2009b), herein we also observed dominantly cytoplasmic localization of immunostained steroid receptors (AR and ERα). It seems that low circulating sex steroids in our model of the andropause (Ajdžanović et al., 2015) preserve a small involvement of AR and ERα in the ZG gene expression regulation, so low testosterone-induced activation of steroid 5α-reductase, followed by aldosterone synthesis reduction, represents the crucial event in ZG after orchidectomy (Kitay, 1968; Ajdžanović et al., 2009b). Further application of testosterone in the model and its high circulating levels led to an increase of the ZG cell and nuclear volume, while the circulating aldosterone concentration was decreased even more. Consequently, a slight trend of serum Na^+ decrease and K^+ increase was noted. AR and ERα immunoreactivity appeared also cytoplasmatically localized, but the optical density upon staining was a bit more pronounced compared to orchidectomized animals. Some previous studies suggest that testosterone application to rats has a stronger inhibitory effect on aldosterone secretion than the orchidectomy alone (Kau et al., 1999). Actually, testosterone was shown to inhibit the basal, angiotensin II- and ACTH- stimulated secretion of aldosterone by means of cytochrome P450scc and aldosterone synthase enzymes activity reduction, as well as to decrease ACTH-stimulated cAMP accumulation in rat ZG cells (Kau et al., 1999). In this respect, the ACTH levels fall in testosterone treated andropausal rats (Ajdžanović et al., 2015) with reference to the important agonistic role of ACTH in aldosterone secretion (Hattangady et al., 2012), should not be disregarded herein. The observed ZG cell volume increase and vacuolization, after orchidectomy and testosterone treatment in our study,

represent the histological reflection of steroidogenesis precursors deposition and their droplets pull down during fixation (Ajdžanović et al., 2009b, 2015). Considering the cytoplasmatical localization of immunostained AR and ERα (inactive in transcription) in ZG, it seems that direct testosterone effects remain largely extra receptor in this adrenal cortex zone of our andropausal model.

At the level of ZF, orchidectomy increased the cell and nuclei volumes, while the latter may indicate some gene expression-related events. To note, some hypertrophy in the rodent adrenal cortex upon androgen deprivation has already been demonstrated (Benmouloud et al., 2014). In our previous work, decreased capacity for corticosterone secretion after testosterone application to andropausal rats was thoroughly elaborated (Ajdžanović et al., 2015). Namely, the silenced pituitary ACTH inputs to ZF in parallel with cytochrome P450scc and 3β-hydroxysteroid dehydrogenase enzymes activity reduction caused by high circulating sex steroids (compromised cortico-steroidogenesis; Malendowicz, 1976; Chinoy and Rao, 1982), represent the crucial processes in this respect (Ajdžanović et al., 2015). Histological aspect of the changes, besides the ZF cells vacuolization and vasodilatation in this adrenal cortex region (Ajdžanović et al., 2015), is supplemented with the present finding of decreased ZF cell nuclei volume and clearly visible AR staining of high optical density at the nuclear domen of the ZF cells. In accordance with the above mentioned, the presence of AR in the rat adrenal gland is already known, while there is a great probability of testosterone actions *via* them on the expression of various genes (Hirst et al., 1992; Bentvelsen et al., 1996; Trejter et al., 2015). The significant ZF nuclei compression and the observed ARs optical density/immunolocalization possibly fit into the trend of corticosterone synthesis decrease after testosterone application to andropausal rats and may point to a certain gene expression down-regulation, which requires additional studies to clarify.

Herein we confirmed our previous report referring to the orchidectomy-induced increase of the whole ZR volume (comprising cell volumes + intercellular spaces), followed by a decrease of the ZR cell and nuclear volume, and the evident fall in serum DHEA concentration (Ajdžanović et al., 2009b). Orchidectomy was shown to downregulate the rate of apoptosis in the rodent ZR (Benmouloud et al., 2014). On the other hand, AZAN stained adrenal cortex sections from our orchidectomized rats depicted massive vasodilatation that presumably expanded the ZR volume, as earlier proposed (Ajdžanović et al., 2009b). Considering high levels of gonadothropins (LH and FSH) after orchidectomy and the ZR atrophy upon their exogenous administration to rats (Städtler and Langner, 1985; Ibrahim et al., 1986), vasodilatation through the attenuated ZR we observed is obviously the compensatory mechanism that tends to recover the circulating concentration of DHEA. Testosterone application to orchidectomized rats and its high levels definitely stimulated the ZR, *i.e.* increased the zonal relative volume, volume of the cells and nuclei as well as DHEA serum concentration, while connective tissue through the ZR hypertrophied and AR immunopositivity manifested nuclear localization. The significant increase in the percentage of parenchymal ZR cells in adrenal cortex of orchidectomized and testosterone treated rats was previously reported (Malendowicz, 1974). Given that pituitary ACTH has some role in the modulation of DHEA secretion (Odell and Parker, 1985), while orchidectomy and subsequent testosterone application markedly decrease ACTH release in middle-aged rats (Ajdžanović et al., 2015), the basis for ZR stimulation in the present study could partly be the mechanism of negative feedback. Pertinent to this, as well as to the logic of ARs activation, the observed cell nuclei volume rise and nuclear AR immunopositivity through the ZR may suggest some gene expression up-regulation, entwined with direct testosterone action.

Our current study highlighted numerous quantitative and qualitative histological changes of the adrenal cortex, in a rat model of the andropause after testosterone application. One detail in this respect appears to be very intriguing. Namely, the adrenal cortex AR immunolocalization depicted a centripetal rise in the proportion of nuclear ARs (active in transcription) in testosterone treated andropausal rats. It seems that the testosterone effects on male adrenal cortex are not only central (*via* pituitary) or through different steroidogenesis enzymes activity modulation, but may also include intraadrenal gene expression-related events, whose frequency increases in the tissue depth. On the other hand, the biomedical aspect of the conclusion implies many corticosteroid secretion-related benefits of testosterone application to ageing males. Aldosterone and corticosterone secretion decrease, in parallel with DHEA secretion increase, are promising regarding some cardiovascular and metabolic symptoms alleviation, or bone structure improvement in andropausal subjects receiving testosterone supplementation.

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References

Ajdžanović V., Šošić-Jurjević B., Filipović B., Trifunović S., Brkić D., Sekulić M. and Milošević V. (2009a). Genistein affects the morphology of pituitary ACTH cells and decreases circulating levels of ACTH and corticosterone in middle-aged male rats. Biol. Res. 42, 13-23.

Ajdžanović V., Šošić-Jurjević B., Filipović B., Trifunović S., Manojlović-Stojanoski M., Sekulić M. and Milošević V. (2009b). Genistein-induced histomorphometric and hormone secreting changes in the

adrenal cortex in middle-aged rats. Exp. Biol. Med. 234, 148-156.

Ajdžanović V.Z., Šošić-Jurjević B.T., Filipović B.R., Trifunović S.L. and Milošević V.Lj. (2011). Daidzein effects on ACTH cells: immunohistomorphometric and hormonal study in an animal model of the andropause. Histol. Histopathol. 26, 1257-1264.

Ajdžanović V.Z., Milošević V.Lj. and Spasojević I.B. (2012). Glucocorticoid excess and disturbed hemodynamics in advanced age: the extent to which soy isoflavones may be beneficial - mini review. Gen. Physiol. Biophys. 31, 367-374.

Ajdžanović V., Medigović I., Živanović J., Šošić-Jurjević B., Trifunović S., Tanić N. and Milošević V. (2014). Immuno-histomorphometric and -fluorescent characteristics of GH cells after treatment with genistein or daidzein in an animal model of andropause. Acta Vet. (Beograd) 64, 93-104.

Ajdžanović V., Jarić I., Živanović J., Filipović B., Ristić N., Miler M. and Milošević V. (2015). Testosterone application decreases the capacity for ACTH and corticosterone secretion in a rat model of the andropause. Acta Histochem. 117, 528-535.

Andres R. and Tobin J.D. (1977). Endocrine systems. In: Handbook of the biology of aging. Finch C.E. and Hayflick L. (eds). Van Nostrand Reinhold. New York. pp 367-387.

Antonio J., Wilson J.D. and George F.W. (1999). Effects of castration and androgen treatment on androgen-receptor levels in rat skeletal muscles. J. Appl. Physiol. 87, 2016-2019.

Beg S., Al-Khoury L. and Cunningham G.R. (2008). Testosterone replacement in men. Curr. Opin. Endocrinol. Diabetes Obes. 15, 364-370.

Benmouloud A., Amirat Z., Khammar F., Patchev A.V., Exbrayat J.M. and Almeida O.F. (2014). Androgen receptor-mediated regulation of adrenocortical activity in the sand rat, Psammomys obesus. J. Comp. Physiol. B 184, 1055-1063.

Bentvelsen F.M., McPhaul M.J., Wilson C.M., Wilson J.D. and George F.W. (1996). Regulation of immunoreactive androgen receptor in the adrenal gland of the adult rat. Endocrinology 137, 2659-2663.

Chinoy N.J. and Rao M.V. (1982). Effect of estradiol benzoate on the hormonal profile and steroidogenic organs of immature and adult male rats. Endokrinologie 80, 287-293.

Evuarherhe O., Leggett J.D., Waite E.J., Kershaw Y.M., Atkinson H.C. and Lightman S.L. (2009). Organizational role for pubertal androgens on adult hypothalamic-pituitary-adrenal sensitivity to testosterone in the male rat. J. Physiol. 587, 2977-2985.

Ferrari E., Cravello L., Muzzoni B., Casarotti D., Paltro M., Solerte S.B., Fioravanti M., Cuzzoni G., Pontiggia B. and Magri F. (2001). Age-related changes of the hypothalamic-pituitary-adrenal axis: pathophysiological correlates. Eur. J. Endocrinol. 144, 319-329.

Filipović B., Šošić-Jurjević B., Ajdžanović V., Pantelić J., Nestorović N., Milošević V. and Sekulić M. (2013). The effects of sex steroids on thyroid C cells and trabecular bone structure in the rat model of male osteoporosis. J. Anat. 222, 313-320.

Gray A., Feldman H.A., McKinlay J.B. and Longcope C. (1991). Age, disease, and changing sex hormone levels in middle-aged men: results of the Massachusetts Male Aging Study. J. Clin. Endocrinol. Metab. 73, 1016-1025.

Handa R.J., Burgess L.H., Kerr J.E. and O'Keffe J.A. (1994). Gonadal steroid hormone receptors and sex differences in the hypothalamo-pituitary-adrenal axis. Horm. Behav. 28, 464-476.

Harper A.J., Buster J.E. and Casson P.R. (1999). Changes in adrenocortical function with aging and therapeutic implications. Semin. Reprod. Endocrinol. 17, 327-338.

Hattangady N.G., Olala L.O., Bollag W.B. and Rainey W.E. (2012). Acute and chronic regulation of aldosterone production. Mol. Cell. Endocrinol. 350, 151-162.

Hatzinger M., Wotjak C.T., Naruo T., Simchen R., Keck M.E., Langraf R., Holsboer F. and Neuman I.D. (2000). Endogenous vasopressin contributes to hypothalamic-pituitary-adrenocortical alterations in aged rats. J. Endocr. 164, 197-205.

Hirst J.J., West N.B., Brenner R.M., Novy M.J. (1992). Steroid hormone receptors in the adrenal glands of fetal and adult rhesus monkeys. J. Clin. Endocrinol. Metab. 75, 308-314.

Ibrahim S.N., Moussa S.M. and Childs G.V. (1986). Morphometric studies of rat anterior pituitary cells after gonadectomy: correlation of changes in gonadotropes with the serum levels of gonadotropins. Endocrinology 119, 629-637.

Jähne B. (1997). Practical handbook on image processing for scientific applications. CRC Press. Boca Raton, Florida, USA. pp 76-82.

Kau M.M., Lo M.J., Wang S.W., Tsai S.C., Chen J.J., Chiao Y.C., Yeh J.Y., Lin H., Shum A.Y., Fang V.S., Ho L.T. and Wang P.S. (1999). Inhibition of aldosterone production by testosterone in male rats. Metabolism 48, 1108-1114.

Kitay J.I. (1968). Effects of estrogen and androgen on the adrenal cortex of the rat. In: Functions of the adrenal cortex. Vol II. McKerns K.W. (eds). Appleton-Century-Crofts. New York. pp 775-811.

Malendowicz L.K. (1974). Sex differences in adrenocortical structure and function II. The effects of postpubertal gonadectomy and gonadal hormone replacement on the rat adrenal cortex evaluated by stereology at the light microscope level. Cell Tissue Res. 151, 537-547.

Malendowicz L.K. (1976). Sex differences in adrenocortical structure and function III. The effects of postpubertal gonadectomy and gonadal hormone replacement on adrenal cholesterol sidechain cleavage activity and on steroids biosynthesis by rat adrenal homogenates. Endokrinologie 67, 26-35.

Mani R., Lohr J. and Jeste D. (1986). Hippocampal pyramidal cells and aging in the human: a quantitative study of neuronal loss in sectors CA1to CA4. Exp. Neurol. 94, 29-40.

Milošević V., Ajdžanović V., Filipović B., Nestorović N., Manojlović-Stojanoski M., Pantelić J. and Sekulić M. (2011). The adrenal cortex in female rats after estradiol or calcium treatment. Cent. Eur. J. Biol. 6, 41-47.

Morales A. (2004). Andropause (or symptomatic late-onset hypogonadism): facts, fiction and controversies. Aging Male 7, 297-303.

Morales A.J., Nolan J.J., Nelson J.C. and Yen S.S. (1994). Effects of replacement dose of dehydroepiandrosterone in men and women of advancing age. J. Clin. Endocrinol. Metab. 78, 1360-1367.

Myers J.B. and Meacham R.B. (2003). Androgen replacement therapy in the aging male. Rev. Urol. 5, 216-226.

Nichols N.R., Zieba M. and Bye N. (2001). Do glucocorticoids contribute to brain aging? Brain Res. Rev. 37, 273-286.

Odell W.D. and Parker L.N. (1985). Control of adrenal androgen production. Endocr. Res. 10, 617-630.

Ruifrok A.C. and Johnston D.A. (2001). Quantification of histochemical staining by color deconvolution. Anal. Quant. Cytol. Histol. 23, 291-299.

Städtler F.A. and Langner V. (1985). The effect of cyproterone and gonadotrophins on the adrenal gland of juvenile and adult rats. A morphological and morphometrical study. Pathol. Res. Pract. 179,

493-498.
Swinyard C. (1938). Methods for volumetric determination of fresh endocrine glands. Anat. Res. 74, 71-78.
Trejter M., Jopek K., Celichowski P., Tyczewska M., Malendowicz L.K. and Rucinski M. (2015). Expression of estrogen, estrogen related and androgen receptors in adrenal cortex of intact adult male and female rats. Folia Histochem. Cytobiol. 53, 133-144.
Vance M.L. (2003). Andropause. GH&IGF Res. 13, S90-S92.
Varghese F., Bukhari A.B., Malhotra R. and De A. (2014). IHC Profiler: an open source plugin for the quantitative evaluation and automated scoring of immunohistochemistry images of human tissue samples. PLoS One 9, e96801.
Varlamov O., White A.E., Carroll J.M., Bethea C.L., Reddy A., Slayden O., O'Rourke R.W. and Roberts C.T. Jr (2012). Androgen effects on adipose tissue architecture and function in nonhuman primates. Endocrinology 153, 3100-3110.
Vermeulen A. (1983). Androgen secretion after age 50 in both sexes. Horm. Res. 18, 37-42.
Weibel E.R. (1979). 1. Practical methods for biological morphometry. In: Stereological methods. Academic Press. London, UK. pp 1-415.

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