INTRODUCTION

Parathyroid glands (PTG) have a major role in the regulation and maintenance of calcium (Ca^{2+}) and phosphorus (Pi) homeostasis. Small decreases in serum Ca^{2+} and increases in serum Pi concentrations stimulate PTG to synthesize and secrete parathyroid hormone (PTH). In bone and kidneys PTH binds to its receptors in order to maintain normal levels of Ca^{2+} and Pi (1). PTH is one of numerous factors responsible for regulation of the abundance of sodium phosphate cotransporter type 2a (NaPi 2a) besides 1.25(OH)_{2} vitamin D3, glucocorticoids and fibroblast growth factor 23 (FGF23) actions, or dietary phosphate intake (2). NaPi 2a is located in the epithelial cells of kidney proximal tubules, on the apical brush border membrane (BBM), and it is responsible for reabsorbing most of the Pi from primary urine.

The andropause, the culminating phase of ageing in men, is associated with a decline in serum testosterone level and an increased incidence of cardiovascular issues, benign and malignant prostate diseases, and osteoporosis (3). During ageing PTG volume and serum PTH level increase in both rats and humans, which both contribute to bone loss and osteoporosis (4, 5). PTH affirmed bone loss, together with its opposite effect on NaPi 2a expression, induces phosphaturia and disturbance of mineral homeostasis (6, 2). Vanderschueren et al. (7) suggested that orchidectomized (Orx) male rats, as an animal model of the osteoporosis, very well reflects the potentially harmful aspects of hormone replacement therapy, increasing emphasis is being placed on alternative, plant-originated therapeutics for osteoporosis.

This study aimed to examine the effects of genistein on the structural and functional changes in parathyroid glands (PTG) and sodium phosphate cotransporter 2a (NaPi 2a) in orchidectomized rats. Sixteen-month-old Wistar rats were divided into sham-operated (SO) and orchidectomized (Orx) groups. Genistein (30 mg/kg/day) was administered subcutaneously for 3 weeks, while the controls received vehicle alone. PTG was analyzed histomorphometrically, while the expressions of NaPi 2a mRNA/protein levels from kidneys were determined by real time PCR and Western blots. Serum and urine parameters were determined biochemically. The PTG volume in Orx rats was increased by 30% (p<0.05), compared to the SO group. Orx+G treatment increased the PTG volume by 35% and 75% (p<0.05) respectively, compared to Orx and SO animals. Orchidectomy led to increment of serum PTH by 27% (p<0.05) compared to the SO group, Orx+G decreased it by 18% (p<0.05) comparing to Orx animals. NaPi 2a expression in Orx animals was reduced in regards to its abundance in SO animals, although it was increased in Orx+G group compared to the Orx. Phosphorus urine content of Orx animals was raised by 12% (p<0.05) compared to that for the SO group, while Orx+G induced a 17% reduction (p<0.05) in regards to Orx animals. Our study shows that Orx increases PTH volume and serum PTH level, while protein expression of NaPi 2a is reduced. Application of genistein attenuates the orchidectomy-induced changes in serum PTH level, stimulates the expression of NaPi 2a and reduces urinary Pi excretion, implying potential beneficial effects on andropausal symptoms.

Key words: andropause, genistein, parathyroid gland, NaPi 2a, orchidectomy, serum calcium, osteoblasts
In light of the above mentioned we assume that genistein could modulate the activity of PTG and NaPi 2a and thus make an impact on homeostasis of Ca\(^{2+}\) and Pi. The aim of this study was to detect and analyze the structure and function of PTG and NaPi 2a cotransporter, key regulators in Ca\(^{2+}\) and Pi homeostasis in middle-aged Orx rats, as an animal model of the andropause, before and after treatment with genistein.

**MATERIAL AND METHODS**

**Animals and diets**

The experiment was performed on middle-aged (16-month-old at the time of sacrificing) Wistar male rats, bred in the Institute for Biological Research, Belgrade, Serbia, under constant laboratory conditions (22±2°C, 12/12h light/dark cycle). Animals were fed a soy-free diet prepared in cooperation with the Institute for Biological Research, Belgrade, Serbia, under old at the time of sacrificing) Wistar male rats, bred in the Institute for Biological Research, Belgrade, Serbia, under same regime and they served as controls. Before sacrifice, urine samples were collected for Ca\(^{2+}\) and Pi analyses. The rats were decapitated 24 h after the last injection. Blood samples were then collected from the trunk and the separated serum samples were stored at –80°C until analyzed. The experimental protocols were approved by the Local Animal Care Committee of the Institute of Physiology, University of Zurich, Zurich, Switzerland. After rinsing in PBS, the sections were covered for 2 h at room temperature with secondary antibody Alexa Fluor 555 donkey anti-rabbit IgG (1:200; Molecular Probes, Inc., USA). Finally, they were rinsed five times in PBS, and incubated with DAPI (1:1000, Molecular Probes, Inc., USA) for 5 minutes and rinsed six times in PBS. Sections were cover slipped using Mowiol 4-88 (Sigma-Aldrich Co., USA). Transverse immunofluorescently stained kidney sections were analyzed per animal, using Carl Zeiss AxioVision software module for generating optical sections through fluorescence samples.

**Histological and electron microscopy analysis**

Thyroid-parathyroid tissue was excised from six animals per group and fixed in Bouin’s solution for 48 h. After dehydration through a series of alcohols of increasing concentration, the tissue was embedded in paraplast. The parathyroid glands were cut serially, using a rotational microtome (Leica, Germany), at 3 µm thickness. The sections were stained with hematoxylin-eosin and mounted with DPX (Sigma-Aldrich, Co., USA).

Kidneys were fixed in formalin solution at room temperature for 48 h, embedded in paraplast, and sectioned at 3 µm. For immunofluorescence staining, sections were deparaffinised and dehydrated, while antigen retrieval was performed in 0.1 M citrate buffer solution pH 6.0. Sections were washed in PBS and pretreated with blocking normal donkey serum (Dako, Denmark) as indicated: 2 minutes at 50°C for dUTP quantification by spectrophotometry, and cDNA was synthesized using reagents from cDNA Reverse Transcription kit (Applied Biosystems, USA). PCR amplification of cDNA was performed in a real-time PCR machine ABI Prism 7000 (Applied Biosystems) with SYBRgreen PCR master mix (Applied Biosystems) as indicated: 2 minutes at 50°C for dUTP activation, 10 minutes at 95°C for initial denaturation of cDNA, followed by 40 cycles, each consisting of 15 s of denaturation at 95°C and 60 s at 60°C for primer annealing and chain extension. Primer pairs were the following: NaPi 2a, forward, 5'-GCCACTTCTTCTCAACATC-3'; reverse, 5'-
CACACGAGGAGGTAGAGG-3'; cyclo A forward 5'-CAAAGTTCCAAAGACAGCAGAAAA-3'; reverse, 5'-CCACCCTGGCACATGAAT-3'. The expression level of each gene was calculated using formula 2^(-Cti-Cta), where Cti is the cycle threshold value of the gene of interest and Cta was the cycle threshold value of cyclophilin A. All of the data were calculated from triplicate reactions. RNA data are presented as average relative levels versus cyclophilin A ± S.D.

Western blot analysis

Immunoblot analyses were performed on isolated BBM vesicles from rat kidney cortex (6 animals per group were used) using Mg2+ precipitation technique as previously described (26). BBM proteins were solubilized in Laemmli sample buffer, and SDS-PAGE was performed on 12% polyacrylamide gels. Proteins were transferred electrophoretically to polyvinylidene difluoride membranes at 5 mA/cm² with a semidry blotting system (Fastblot B43; Bio-Rad, Goettingen, Germany). The membranes were blocked with 5% BSA in PBS with 0.1% Tween 20 for overnight, followed by incubation with rabbit anti-rat NaPi 2a primary antibody (1:2000), and rabbit anti-rat β-actin (Abcam, Cambridge, USA) overnight at 4°C. After washing, blots were incubated with secondary antibody (1:1000; ECL donkey anti-rabbit horse-radish peroxidase-linked; GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK) for 1 h at room temperature. Antibody binding was detected using a chemiluminescence detection system (ECL; GE Healthcare).

Stereological measurements

The volume of PTG was estimated using Cavalieri’s principle (27) with a newCAST stereological software package (VIS-Visiopharm Integrator System, version 2.12.1.0; Visiopharm; Denmark). Every 30th section from each of the tissue blocks was analyzed. The PTG volume (Vptg) was calculated by the formula:

\[ V_{ptg} = a(p) \cdot d \cdot \sum_{i=1}^{n} \sum_{j=1}^{n} \]

where a(p) is the area associated with each sampling point (10956.52 µm²); d is the mean distance between two consecutively studied sections (90 µm); n is the number of sections studied for each PTG; and ΣP is the sum of points hitting a given target. The percentages of chief cells and interstitium (blood vessels and connective tissue) were determined for every sampled section.

Biochemical analyses

Serum PTH concentration was measured in duplicate samples without dilution, using a Rat Intact PTH ELISA Kit (Immunotopics, Inc., San Clemente, CA, USA), within a single assay. The intra-assay coefficient of variation (CV) was 2.4%. The lowest concentration of rat intact PTH measurable by this kit was 1.6 pg/mL (assay sensitivity). Serum concentrations of P and Ca²⁺, and urinary concentration of P, were determined on a Hitachi 912 analyzer (Roche Diagnostics GmbH, Mannheim, Germany).

Statistical analysis

STATISTICA® version 6.0 (StatSoft, Inc) was used for the statistical analysis. All results were expressed as mean ± S.D. Differences between the groups were assessed by one-way analyses of variance (ANOVA) followed by Duncan’s multiple range tests for post hoc comparisons between groups. Values of p<0.05 were considered statistically significant.

RESULTS

Parathyroid glands volume

In sham-operated (SO) middle aged male rats PTG volume was 0.14±0.01 mm³. The value was 30% (p<0.05) greater in Orx rats than in the SO group (Fig. 1A). After treatment with genistein (Orx+G) the PTG volume was increased by 35% and 75% (p<0.05) respectively, when compared with values for the Orx and SO animals (Fig. 1A). The volume density of the PTG chief cells was raised by 2% (p<0.05) in the Orx group of animals when compared with the SO group, while treatment with genistein (Orx+G) induced a 4% decrease of chief cell volume (p<0.05), when compared to Orx animals (Fig. 1B). After genistein treatment the presence of interstitium in PTG (Orx+G) was increased by 20% (p<0.05), in comparison to the Orx group of animals (Fig. 1B).

![Fig. 1.](image-url) Fig. 1. [A] - The volume of PTG in sham-operated (SO), orchidectomized (Orx) and orchidectomized rats treated with genistein (Orx+G) group. All values are presented as mean ± S.D.; *p<0.05 versus SO, **p<0.05 versus Orx. [B] - Relative representation of chief cells and interstitium in sham-operated (SO), orchidectomized (Orx) and orchidectomized rats treated with genistein (Orx+G). All values are presented as mean ± S.D.; *p<0.05 versus SO, **p<0.05 versus Orx.
Histological findings in the parathyroid glands

The parathyroid glands (PTG) are located laterally to the thyroid gland lobes. They typically possess an oval shape and are surrounded with a connective tissue capsule. These glands are composed of one cell type, the chief cells, densely packed in cords or clusters around and along capillaries, with spherical to oval or elongated nuclei. PTG in the sham-operated (SO) rats had an apparent connective tissue capsule, while the chief cells (Fig. 2A, white arrows) were separated with a delicate stroma of connective tissue and blood vessels (Fig. 2A, black arrows). In comparison with this, PTG in the Orx group were larger, with numerous chief cells (Fig. 2B, white arrows) and noticeable interstitium (Fig. 2B, black arrows). After genistein treatment (Orx+G) PTGs were even larger, with massive interstitium (black arrows), in relation to the glands in Orx animals (Fig. 2C).

Ultrastructural observations in the parathyroid glands

Ultrathin sections of PTG in the SO group showed compactly arranged chief cells, with numerous interdigitations of the cell membrane. Rough endoplasmatic reticulum (RER; white arrows) and the Golgi complex (black arrows) were moderately developed. Mitochondria (white arrow head) were

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Fig. 2. Sections of PTG from [A] sham-operated (SO), [B] orchidectomized (Orx) and [C] orchidectomized rats treated with genistein (Orx+G). Chief cells (white arrows) and interstitium (black arrows) are clearly visible. Hematoxylin-eosin staining, scale bar – 100 µm.

Fig. 3. Ultrathin sections of PTG chief cells from [A] sham-operated (SO), [B] orchidectomized (Orx) and [C] orchidectomized rats treated with genistein (Orx+G). RER (white arrows), Golgi complex (black arrows) and mitochondria (white arrow head) are marked as the representative structures. Scale bar – 500 nm.

Fig. 4. Localization of NaPi 2a cotransporter in epithelial cells of proximal tubules. [A] In the sham-operated group of animals (SO) NaPi 2a was strongly labeled in the brush border membrane of proximal tubule epithelial cells, [B] NaPi 2a staining intensity in the apical domain of proximal tubule cells was decreased after orchidectomy (Orx). The signal was also detected in the subapical region of the cells, [C] After genistein treatment (Orx+G) the intensity of NaPi 2a labeling was increased. Paraffin sections, immunofluorescence, scale bar – 10 µm.
dispersed throughout the cytoplasm, while the nuclei were elongated and located near the apical domain of the cells (Fig. 3A). Interdigitations of the plasma membrane in Orx animals were more numerous than in the chief cells of SO animals. The RER and Golgi complex were more developed than in the SO group, with abundant mitochondria and larger centrally located nuclei (Fig. 3B). Ultrastructural micrographs of chief cells from Orx animals treated with genistein (Orx+G) showed less prominent plasma membrane interdigitations. The RER and Golgi complex were poorly represented with fewer and smaller mitochondria, in comparison to the Orx animals (Fig. 3C).

Immunofluorescent appearance of NaPi 2a

Kidneys sections of all experimental groups were immunofluorescently stained with the specific antibody for NaPi 2a. Kidney sections of SO rats NaPi 2a showed a strong signal in the microvilli of the BBM in proximal tubule cells (Fig. 4A). After Orx, the intensity of the NaPi 2a signal in BBM was reduced, in comparison with SO animals (Fig. 4B). Besides in the apical membrane, NaPi 2a was also localized in the subapical domain of epithelial cells of proximal tubules in Orx animals (Fig. 4B). Treatment with genistein (Orx+G) increased the abundance of NaPi 2a in the brush border of the proximal tubules, in comparison with the Orx group of animals (Fig. 4C).

NaPi 2a expression levels in epithelial cells of proximal tubules

Analysis of NaPi 2a mRNA level revealed that orchidectomy slightly decreased NaPi 2a mRNA compared to SO group of animals, while genistein administration led to an increase in NaPi 2a mRNA expression level in comparison with Orx animals (Fig. 5A).
Urinary Pi concentration was 17% lower (p<0.05) than for the SO control (Fig 6A). Orchidectomy increased excretion of Ca²⁺ and Pi in urine. However, this study first showed that genistein treatment of Orx rats significantly increased serum Ca²⁺ and Pi concentrations, and regulated the reabsorption of Pi from primary urine in kidney tubules. The exact mechanism by which genistein treatment influence on low PTH serum level, in the milieu of almost entirely without androgens, remains to be clarified. It should be considered that genistein might bind to ERs, with a higher binding affinity for ERβ (13), but the presence of ERs in PTG is still a controversial issue (34, 14). It is possible that the suppressing effect of genistein on PTG activity is achieved through an indirect mechanism. In one study, Ben-Dov et al. (16) showed that FGF23 has an inhibitory role in the synthesis and secretion of PTH through binding to the FGFR-Klotho receptor complex and activation of the MAPK signaling pathway. In addition, Carrillo-Lopez et al. (14) suggest that the factor involved in the possible indirect effect of estrogen on PTG could be FGF23. Namely, the authors demonstrated in vitro that estradiol increases FGF23 levels in osteoblast-like cells in a concentration and time-dependent manner. The presence of ERs in bone is well documented (35), as well as the importance of steroid hormones for bone metabolism, so some possibility arises that genistein, which is structurally similar to estradiol, might induce stimulation of FGF23 synthesis and secretion by binding to ERs in bone cells.

Consistent with our previous findings (33, 36), Orx induced a significant decrease in serum Ca²⁺ and P concentrations, together with increased excretion of Ca²⁺ and P, in urine. However, this study for the first time showed that genistein treatment of Orx rats significantly increased serum Ca²⁺ and P concentrations, and regulated the reabsorption of P, from primary urine in kidney tubules. Our findings indicated that the phosphaturia occurring after Orx was due to diminished gene and protein expression of NaPi 2a cotransporter in BBM of proximal tubules. Genistein treatment reduced P excretion in urine and recovered NaPi 2a expression in BBM of proximal tubules. Coherently, NaPi 2a mRNA levels were increased after genistein administration. Kempson et al. (37) showed that PTH decreased NaPi 2a protein content on the apical membrane of epithelial cells of proximal tubules, while Bacic et al. (38) observed that acute application of PTH induced withdrawal of NaPi 2a via receptor-mediated endocytosis. The augmented expression of NaPi 2a in genistein treated andropausal male rats in our study could have resulted from reduced PTH inhibition, since the level of this hormone declined significantly after genistein application. Moreover, the PTH and FGF23 induced downregulation of NaPi 2a is mediated through the ERK1/2 signaling pathway (21, 39). The tyrosine kinase inhibiting role of genistein suggests possible interference in the ERK1/2 signaling pathway and diminished PTH and model of the andropause to explore the effects of the soy isoflavone, genistein, on the structure and function of PTG and NaPi 2a cotransporter in BBM of proximal tubules.

DISCUSSION

It has been established that PTG has a central role in regulating mineral and bone metabolism (28). Pertinent to this, the increased level of PTH during ageing (4), together with the age-related decline in serum testosterone, contribute to bone loss and osteoporosis. Hormone replacement therapy in elderly people may cause hyperphosphaturia (29) and increase the risk of cancer development (11), so finding alternative therapeutic solutions in osteoporosis treatment is definitely needed. Accumulating evidence suggests that soy isoflavones may represent a promising alternative remedy for aging symptoms in both genders (30, 31), but their role in PTG regulation has not been previously studied. Therefore, we have used an animal immunoblot of BBM preformed with NaPi 2a antibody showed decrease expression of NaPi 2a (p<0.05) cotransporter in Orx animals in comparison with SO control group (Fig 5B). Treatment with genistein significantly elevated expression of NaPi 2a (p<0.05) compared to Orx group (Fig 5B).

Biochemical findings

Serum PTH concentration in SO rats was 62.5±5.68 ng/L. Orchidectomy induced a 27% increase of serum PTH (p<0.05), when compared to SO animals (Fig 6). After treatment with genistein (Orx+G) serum PTH concentration was 18% lower (p<0.05) than in the Orx group (Fig 6). In SO animals serum Ca²⁺ was 2.33±0.05 mmol/L and serum P, 2.04±0.07 mmol/L. After Orx, concentrations were decreased by 5% (Ca²⁺) and 10% (P) (p<0.05) respectively, in comparison with the SO group (Fig 7). After genistein treatment (Orx+G) serum concentration of Ca²⁺ and P, were 7% and 12% (p<0.05) higher respectively, than for Orx animals (Fig 7). In SO animals urine P concentration was 35.15±1.85 mmol/L. Orchidectomy increased the P concentration in urine by 12% (p<0.05), in comparison with the SO control (Fig 8). After genistein treatment (Orx+G) urinary P concentration was 17% lower (p<0.05), than for the Orx group (Fig 8).
FGF23 induced inhibition of P uptake. Besides the inhibitory role of PTH in the regulation of NaPi 2a expression, glucocorticoids may also have some suppressing effect in the same field (40). Ajdzanovic et al. (24) demonstrated that genistein treatment inhibited corticosterone production and secretion in an animal model of the andropause, so a diminished glucocorticoid effect may be involved as well. Decreased corticosterone production, after genistein application in vitro, was also observed (41). However, we cannot exclude a possible direct mechanism of genistein action on the proximal tubule cells. Faroqui et al. (42) showed that estradiol has a significant impact on downregulation of NaPi 2a in the renal proximal tubule, but this effect probably was not mediated through ERs. Since genistein preferentially binds to ERβ, besides ERα (13), its effect may be mediated via such a mechanism. Rogers et al. (43) demonstrated that androgen deprivation induced significant elevation of ERβ content in the kidney cortex of male rats, while ERα remained the same. Additional molecular studies are needed to elucidate possible mechanisms of genistein action on the regulation of NaPi 2a expression in BBM.

In summary, this is the first report evaluating genistein effects on PTG and the functionally related NaPi 2a cotransporter in kidney tubules. Our results showed that Orx, emphasizing andropausal symptoms, led to elevation of PTG volume and serum PTH level, but also decreased expression of the NaPi 2a cotransporter in epithelial cells of proximal tubules. Genistein administration reduced the elevated serum PTH level, stimulated expression of NaPi 2a in the apical domain of proximal tubule cells and decreased urinary P excretion in Orx rats. These data credibly suggest that the soy isoflavone, genistein, inhibits the function of PTG and stimulates the NaPi 2a cotransporter activity, in an animal model of the andropause.

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REFERENCES

368


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