Journal of Anatomy

*J. Anat.* (2015) **226**, pp489–496 doi: 10.1111/joa.12298

# Tamoxifen stimulates calcitonin-producing thyroid C-cells and prevents trabecular bone loss in a rat model of androgen deficiency

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## Abstract

Thyroid C-cells produce calcitonin (CT), a hypocalcemic hormone, that acts as an inhibitor of bone resorption. In this study, we investigated the effects of tamoxifen (TAM) as a selective estrogen receptor modulator on thyroid C-cells, trabecular bone and biochemical markers of bone metabolism in an animal model of androgen deficiency, represented by middle-aged orchidectomized (Orx) rats. Fifteen-month-old male Wistar rats were divided into: Orx and sham-operated (SO) groups. Rats from one Orx group were injected subcutaneously with TAM citrate (Orx + TAM; 0.3 mg $kg^{-1}$ b.w.), while the rats from SO and a second Orx group received vehicle alone, once a day for 3 weeks. The peroxidase–antiperoxidase method was applied for localization of CT in C-cells. Thyroid C-cells were morphometrically and ultrastructurally analyzed. An ImageJ image-processing program was used to measure bone histomorphometric parameters. Blood serum samples were analyzed for CT, osteocalcin (OC), calcium ($Ca^{2+}$) and phosphorus (P). Urinary $Ca^{2+}$ concentrations were measured. TAM treatment significantly increased thyroid C-cell volume ($V_c$) and serum CT when compared with vehicle-treated Orx rats. Analysis of trabecular microarchitecture of the tibia showed that administration of TAM significantly increased cancellous bone area, trabecular thickness and trabecular number, whereas trabecular separation was significantly decreased compared with vehicle-treated Orx rats. Serum OC and urinary $Ca^{2+}$ concentrations were significantly lower in comparison with the control Orx group. These results indicate that in our rat model of androgen deficiency, TAM stimulated calcitonin-producing thyroid C-cells and increased trabecular bone mass.
**Key words:** androgen deficiency; bone; calcitonin; rats; tamoxifen; thyroid C-cells.

## Introduction

Osteoporosis is a disease characterized by low bone mass and microarchitectural deterioration of bone tissue. Age-related bone loss due to gonadal steroid deficiency is greater in women than in men (Khosla & Riggs, 2005). Anti-resorptive drugs used in the treatment of osteoporosis include bisphosphonates, hormone replacement therapy, selective estrogen-receptor modulators (SERMs) and the peptide hormone calcitonin (CT).

Natural CT produced by thyroid C-cells (Zaidi et al. 2002) acts as an acute hypocalcemic hormone and also as a potent inhibitor of osteoclastic bone resorption (Chambers & Magnus, 1982). The function of thyroid C-cells and corresponding level of plasma CT are influenced by aging, including age-related sex hormonal changes. Unlike humans, in which gonadal steroids, plasma CT levels and number of thyroid C-cells decline with age, physiological hyperplasia of C-cells and hypercalcitoninemia were observed in old rats (Delverdier et al. 1990; Sekulić et al. 1998; Lu et al. 2000).

In the state of gonadal steroid deficiency, an appropriate estrogen or testosterone administration has bone-protective effects and stimulates the activity of CT-producing thyroid C-cells in rats (Catherwood et al. 1983; Yamazaki & Kinoshita, 1986; Filipović et al. 2003, 2013). Clinical

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Accepted for publication *13 February 2015*
Article published online *7 April 2015*

investigations indicate that, together with the lack of testosterone, estrogen deficiency plays an important role in age-related bone loss in elderly men (Slemenda et al. 1997). However, exposure to estrogen increases the risk of hormone-associated cancers, such as uterine or breast cancers in women and men (Anelli et al. 1994; Colditz et al. 1995; Grady et al. 1995). Moreover, the use of exogenous testosterone in men is associated with the development of prostate cancer (Gaylis et al. 2005) and possibly with breast cancer (Thomas et al. 2008).

Because the effects of estrogens in target tissues are mediated by specific estrogen receptors (ERs; Kuiper et al. 1996), the use of SERMs that lack the adverse effects of estrogen may be beneficial for treatment of age-related bone loss, with little risk of breast growth in elderly women and men. SERMs may have estrogenic and anti-estrogenic activities in different tissues (Gottardis & Jordan, 1987; Turner et al. 1988).

Tamoxifen (TAM) is a SERM used for treatment of ER-positive breast cancer in both women and men (Ribeiro, 1983; Osborne, 1998). Laboratory studies show that TAM acts as an estrogen in bone, but also as an anti-estrogen in mammary tissue (Gottardis & Jordan, 1987; Turner et al. 1988). Clinical and experimental data related to the effects of TAM mainly concern the female sex. There is little information on the action of TAM in men and male animals, despite the fact that TAM is used to treat breast cancer in men (Anelli et al. 1994). Thus, in this study we have investigated the effects of TAM as a potent inhibitor of bone resorption on thyroid C-cells and trabecular bone microarchitecture, by using orchidectomized (Orx) male rats as a suitable animal model for male osteoporosis.

## Materials and methods

### Animals and experimental groups

Twenty-four 15-month-old male Wistar rats, bred at the Institute for Biological Research 'Siniša Stanković', University of Belgrade, Serbia, were maintained under constant laboratory conditions (room temperature 22 °C, 12 h/12 h light/dark cycle), with free access to food and water. Sixteen rats were randomly bilaterally Orx under ketamine anesthesia (intramuscularly injected at a dose of 15 mg $kg^{-1}$ body weight). Eight rats were sham-operated (SO). Two weeks after the surgery the rats were divided into three groups (eight rats per group). One Orx group was injected subcutaneously (s.c.) with TAM citrate (Sigma-Aldrich, St Louis, MO, USA) at a dose of 0.3 mg $kg^{-1}$ b.w. daily for 3 weeks. This dose of TAM was used to mimic the standard dose of TAM used for patients with breast cancer (Makris et al. 1998). The SO and other Orx rats were s.c. administrated the same volume of vehicle alone (controls). Urine was collected for 24 h before death. Sera were separated from trunk blood after decapitation and stored at −70 °C until analyzed biochemically. All animal procedures were in compliance with the EEC 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, Serbia.

### Immunohistohemical stains

Thyroid lobes were isolated and fixed in Bouin's solution, embedded in paraplast, and cut into 5-μm-thick longitudinal sections. Deparaffinized sections were stained immunohistochemically with rabbit antisera directed against human CT (Dakopatts, Copenhagen, Denmark; 1 : 500), using the peroxidase–antiperoxidase method.

### Stereological analysis of CT-labeled thyroid C-cells

Immunocytochemically stained C-cells in the central part of each rat thyroid were stereologically analyzed by a point-counting method described by Filipović et al. (2007), using a M42 multipurpose test grid inserted into the ocular of a Zeiss light microscope (Jena, Germany). The cell volume of thyroid C-cells ($V_c$) and their volume density ($V_V$) were estimated on 50 test fields per section at 1000 × magnification.

### Electron microscopy

Samples of thyroid gland lobes were fixed in 4% glutaraldehyde solution in 0.1 M phosphate buffer (pH 7.4) for 24 h. Samples were postfixed in 1% osmium tetroxide dissolved in the same buffer, dehydrated in a graded series of ethyl alcohol and embedded in Araldite resin. Ultrathin sections of thyroid gland were stained with uranyl acetate and lead citrate, and viewed with a transmission electron microscope (MORGAGNI 268; FEI Company, USA).

### Trabecular bone histomorphometry

The right tibiae were cleaned of soft tissue, fixed in Bouin's solution, decalcified in 20% ethylenediaminetetraacetic acid disodium salt (EDTA, pH 7.4), routinely processed and embedded in paraplast. Longitudinal sections of the proximal tibiae were cut through the middle part of the bone using a rotation microtome (RM 2125; Leica, Germany) with microtome blades for hard sections (Sakura Finetek, Japan). These 5-μm-thick sections were stained by the Azan method, as previously described (Filipović et al. 2007).

An ImageJ public domain image-processing program was used for histomorphometric measurements on a trabecular area of the tibia, starting 1 mm below the epiphyseal growth plate. Histomorphometry terminology and the calculations used are those described in the *Report of the American Society for Bone and Mineral Research Committee Histomorphometry Nomenclature* (Parfitt et al. 1987). The data automatically determined by the image analysis system were used to calculate the cancellous bone area (B.Ar) and cancellous bone perimeter (Evans et al. 1994). Trabecular thickness (Tb.Th), trabecular number (Tb.N) and trabecular separation (Tb.Sp) were derived from these parameters as described earlier (Filipović et al. 2007).

### Biochemical analyses of serum and urine samples

Blood serum was collected for determination of CT, osteocalcin (OC), calcium ($Ca^{2+}$) and phosphorus (P) concentrations. Twenty-four hour urine samples were collected for measurement of $Ca^{2+}$ concentration. Serum CT levels were assayed immunochemiluminometrically (Nichols, USA) using a mouse monoclonal anti-human CT antibody marker with acridium ester. The luminescence was quantified with a semi-automated MLA 1 hemiluminescence analyzer

(Ciba-Corning). A Roche Elecsys 2010 immunoassay analyzer (Roche Diagnostics GmbH, Mannheim, Germany) was used to quantify serum OC. Serum $Ca^{2+}$ and P, and urinary $Ca^{2+}$ were determined on a Hitachi 912 analyzer (Roche Diagnostics GmbH).

### Statistical analysis

All data were analyzed using STATISTICA 6.0 software (Statsoft, Tulsa, Oklahoma, USA). The Kolmogorov–Smirnov test was used to determine potential deviation from normal distribution, followed by one-way analysis of variance (ANOVA). Duncan's multiple range test was employed for *post hoc* comparisons. Differences between mean values were considered to be significant at $P < 0.05$. The data are presented as means ± standard error of the mean (SEM).

## Results

### Immunohistomorphometric and ultrastructural assessment of thyroid C-cells

Thyroid C-cells in the SO rats were numerous, large and mainly occurred in clusters. Their cytoplasm was lightly immunostained for CT (Fig. 1a,b). In Orx rats, C-cells were present in smaller groups or individually. They were smaller, with stronger immunoreaction to CT compared with C-cells in SO rats (Fig. 1c,d). After TAM treatment of Orx rats, the C-cells were larger, with granular lighter immunostaining within the cytoplasm than in the vehicle-treated Orx animals (Fig. 1f,g).

The ultrastructure of thyroid C-cells in the SO animals was predominantly characterized by the presence of granules of lower electron density. Mitochondria were numerous, oval and dispersed in the cytoplasm. A well-developed Golgi complex and long profiles of rough endoplasmic reticulum were observed (Fig. 2, SO). The relative abundance of high electron density granules in C-cells of Orx rats was increased. The cytoplasm contained fewer organelles than in SO animals (Fig. 2, Orx). After TAM treatment of Orx rats, the ultrastructure of the C-cells was similar to that of SO rats and revealed cytoplasm rich in organelles (Fig. 2, Orx + TAM).

We have previously reported that Orx induces a significant decrease in $V_c$ and $V_v$ of thyroid C-cells when compared with SO rats (Filipović et al. 2007). Treatment of Orx animals with TAM increased C-cell $V_c$ by 17% ($P < 0.05$), with $V_v$ not significantly altered in relation to the values obtained for control Orx rats. No significant differences in $V_c$ and $V_v$ of thyroid C-cells were detected between TAM-treated and SO rats (Fig. 3a,b).

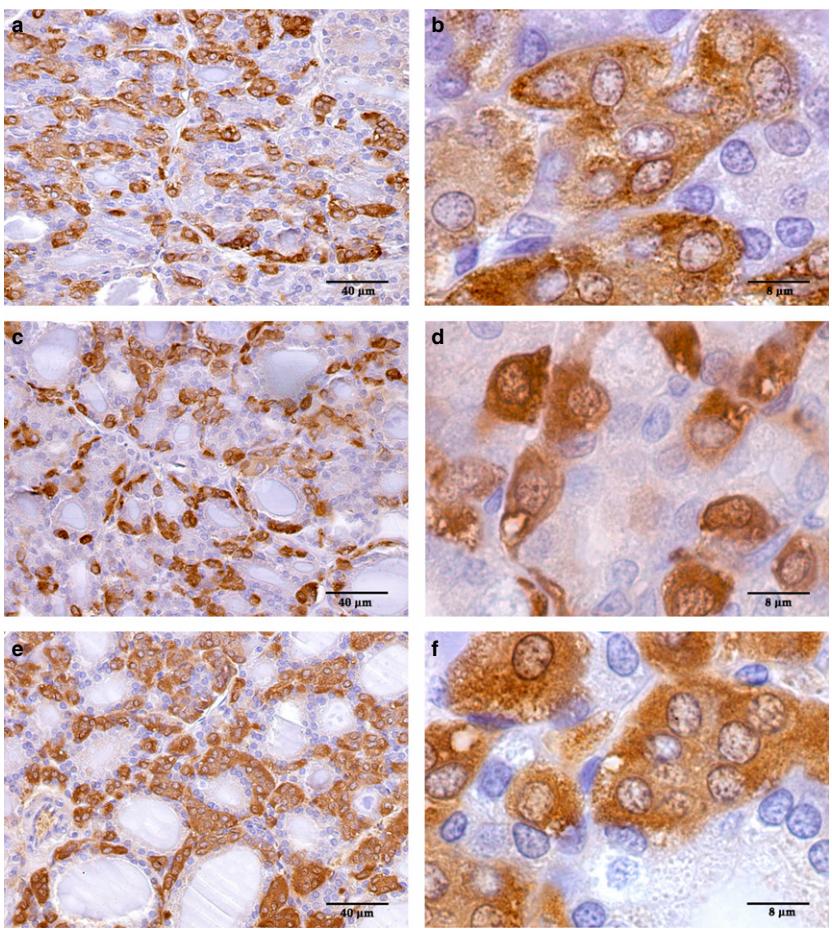


**Fig. 1** Calcitonin-producing thyroid C-cells. In SO animals, C-cells are numerous and large, with light cytoplasm (a and b). Small groups or individual, smaller C-cells with darker cytoplasm in Orx rats (c and d). Large clusters of C-cells with lighter cytoplasm in Orx rats treated with TAM (Orx + TAM) (e and f). Immunoperoxidase staining specific for calcitonin.

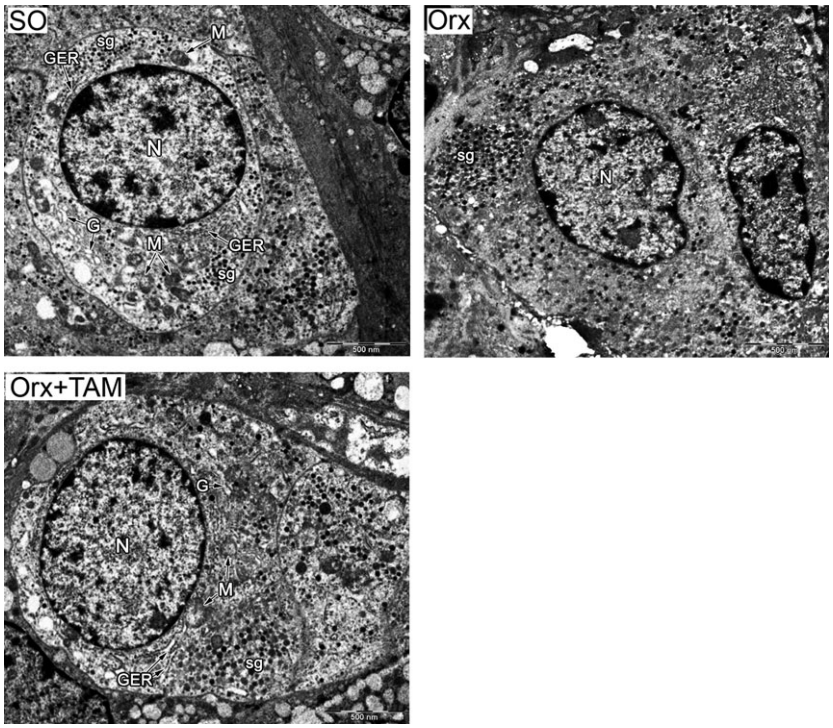


**Fig. 2** Ultrastructure of thyroid C-cells in sham-operated (SO), orchidectomized (Orx) and Orx rats treated with tamoxifen (Orx + TAM). G, Golgi complex; GER, granular endoplasmic reticulum; M, mitochondria; N, nucleus; sg, secretory granules.

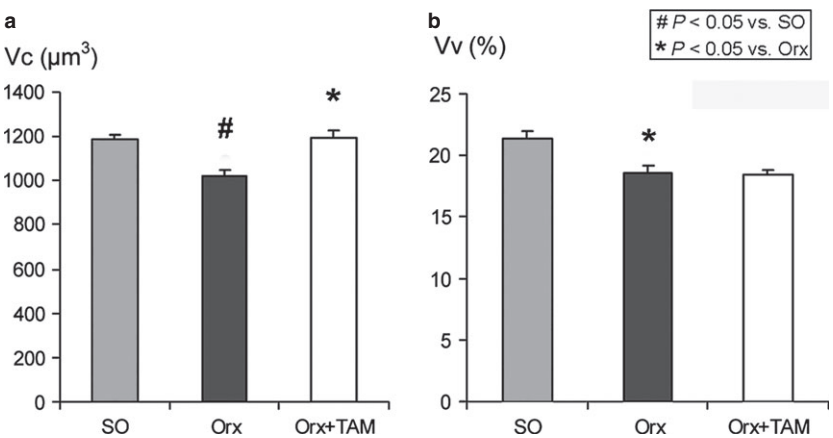


**Fig. 3** (a) Cellular volume ($V_c$) and (b) volume density ($V_v$) of CT-immunoreactive thyroid C-cells. Orx, orchidectomized rats; Orx + TAM, Orx rats treated with tamoxifen; SO, sham-operated control. All values are mean ± SEM. $^{\#}P < 0.05$ vs. SO and $^{*}P < 0.05$ vs. Orx.

### Histomorphometric assessment of trabecular bone

It has previously been reported that Orx in middle-aged rats markedly reduces trabecular bone tissues, and decreases the value of trabecular bone structural parameters, such as B.Ar, Tb.Th, Tb.N, and increases Tb.Sp of the tibia (Filipović et al. 2007). Administration of TAM to Orx rats reversed the effects of Orx and prevented structural deterioration of trabecular bone (Fig. 4, upper). Thus, in Orx rats injection of TAM increased B.Ar, Tb.Th and Tb.N by 113%, 29% and 26% ($P < 0.05$), respectively, when compared with the control Orx group. Tb.Sp was 24% lower ($P < 0.05$) in the Orx + TAM group than in the control Orx group. No differences in bone structural parameters of proximal tibia between Orx rats treated with TAM and SO animals (Fig. 4, below) were observed.

### Serum and urine parameters

In our previously published work (Filipović et al. 2007), we reported that Orx significantly decreases serum CT, $Ca^{2+}$ and P, and increases OC and urinary $Ca^{2+}$ concentration. TAM given to the Orx rats increased serum CT concentration, which was 44% ($P < 0.05$) higher than in the control Orx group, and 34% lower ($P < 0.05$) compared with the SO group (Fig. 5a). Administration of TAM decreased serum OC concentration by 51% ($P < 0.05$) in comparison with vehicle-treated Orx rats (Fig. 5b). Furthermore, after TAM

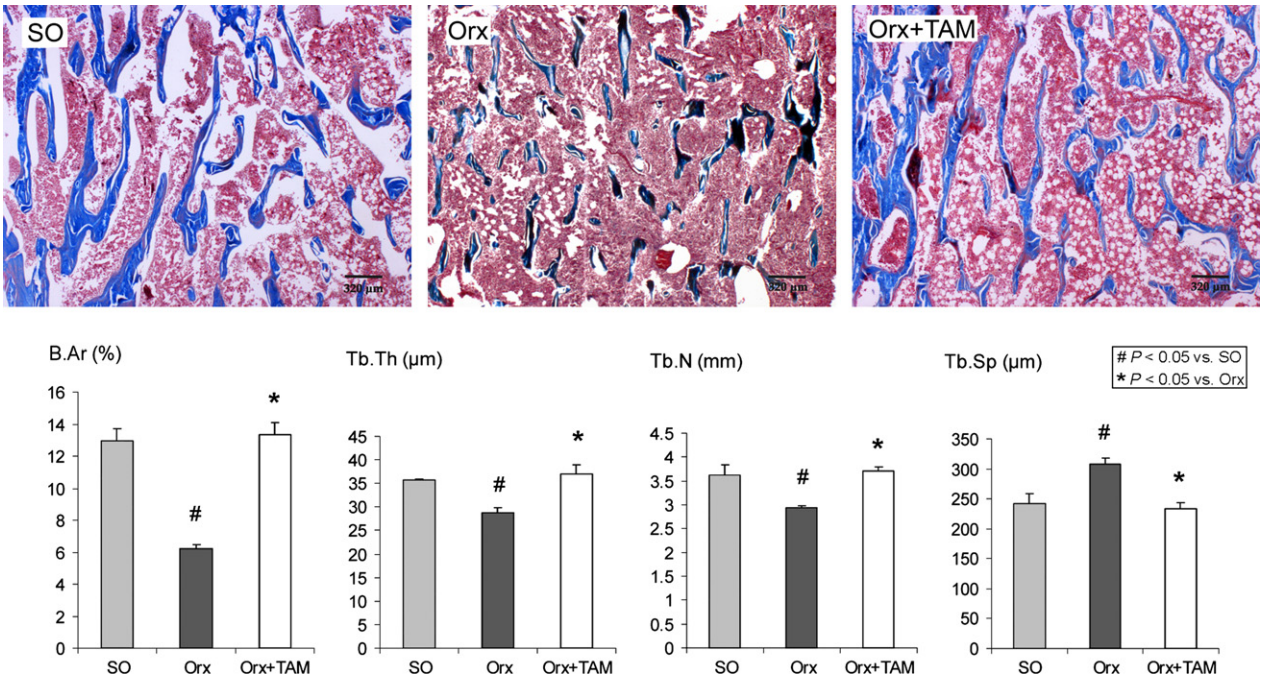


**Fig. 4** Upper: histological sections of trabecular bone from proximal tibia metaphysis in sham-operated (SO), orchidectomized (Orx) and Orx rats treated with tamoxifen (Orx + TAM). The metaphyseal region of the proximal tibia in SO rats contains well-developed blue-stained trabeculae. After Orx, the trabeculae are markedly attenuated and thinner. In the Orx + TAM animals, trabecular structure, destroyed after Orx, has recovered; 5-µm sections from the center of the specimen; Azan method stain. Below: histomorphometric parameters of bone structure. Cancellous bone area (B.Ar), trabecular thickness (Tb.Th), trabecular number (Tb.N) and trabecular separation (Tb.Sp). All values are mean ± SEM. $^{\#}P < 0.05$ vs. SO and $*P < 0.05$ vs. Orx.

administration, serum $Ca^{2+}$ and P were 9% and 15% lower ($P < 0.05$), respectively, compared with levels detected in SO rats (Fig. 5c). Urinary $Ca^{2+}$ concentration in TAM-treated rats was 71% ($P < 0.05$) lower than in the control Orx group, while the decrease of 34% in comparison with SO rats was not statistically significant (Fig. 5c).

## Discussion

Synthetic salmon CT is one of the therapeutic options for treatment of osteoporosis. This peptide regulates $Ca^{2+}$ and P metabolism, and is a powerful physiological inhibitor of osteoclast activity (Tuck & Datta, 2007). Natural CT is produced and secreted by thyroid C-cells, and these processes are influenced by gonadal steroids (Chambers & Magnus, 1982). In our previous papers we have reported that sex steroid deficiency induced by gonadectomy modulated the structure of CT-producing thyroid C-cells, and diminished CT secretion both in female and male rats (Filipović et al. 2003, 2007). In middle-aged male rats, Orx induced decreases of $V_c$ and $V_v$ of thyroid C-cells, and reduced serum CT levels (Filipović et al. 2007). Administration of estradiol or testosterone to Orx rats stimulated the secretory activity of thyroid C-cells (Filipović et al. 2013).

However, although gonadal steroids have a beneficial effect on bone tissue, estrogens may increase the risk of developing breast cancer in humans (Colditz et al. 1995), and promote mammary gland hyperplasia in female and male rats (Andrews et al. 2002). Therefore, we chose to use TAM for our study as a non-steroidal anti-estrogen, which is used as an essential drug for treatment of breast cancer. This SERM exerts anti-estrogenic effects on the breast but mimics them on bone (Gottardis & Jordan, 1987; Turner et al. 1988).

In the present study we have demonstrated that TAM acts on the CT-producing thyroid C-cells in androgen-deficient male rats. Thus, TAM treatment significantly increased C-cell volume when compared with that in Orx rats. Ultrastructural analysis indicated mostly the presence of low electron density granules and cytoplasm richer in organelles than in Orx animals. These findings, together with elevated serum CT levels, suggest that TAM treatment stimulates CT synthesis and secretion from thyroid C-cells. To our knowledge, this is the first study to examine the effect of TAM administration on the structure and function of thyroid C-cells. However, TAM inhibited proliferation of a tumor cell line derived from human medullary thyroid carcinoma, a thyroid C-cell neoplasm (Yang et al. 1988).

The effect of TAM on thyroid C-cells may be mediated by ERs, which have been detected in both normal and hyperplastic C-cells (Naveh-Many et al. 1992; Blechet et al. 2007). The earlier report (Greenberg et al. 1986) suggested that

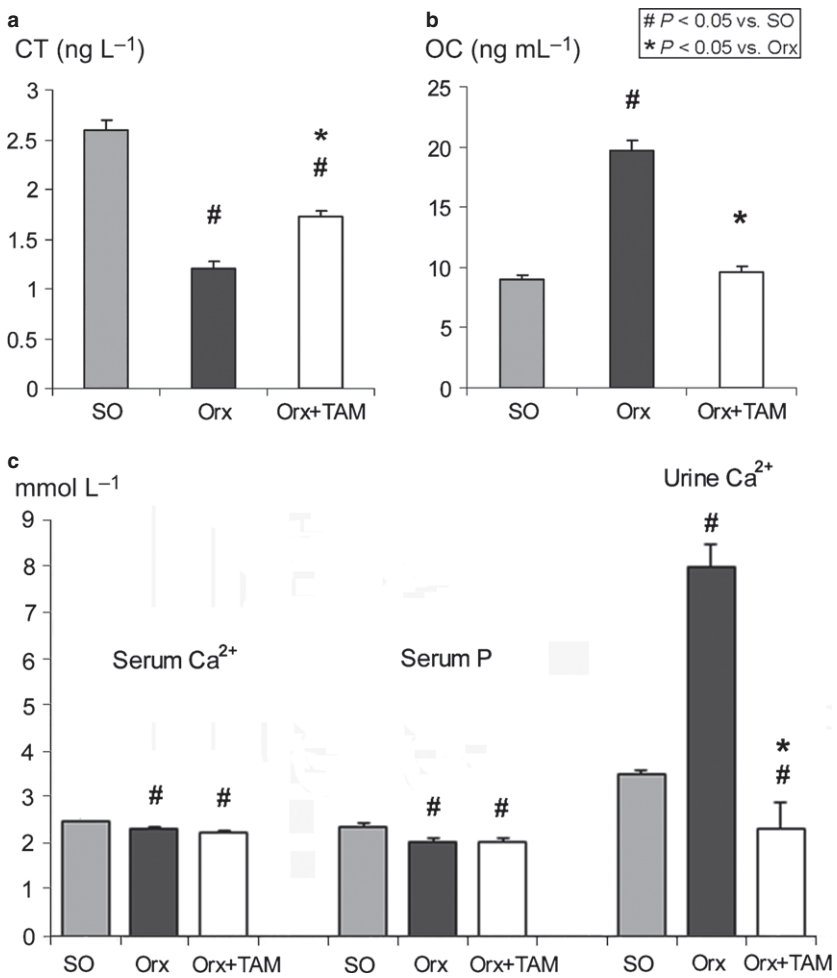


**Fig. 5** (a) Serum calcitonin (CT) concentration (ng $L^{-1}$). (b) Serum osteocalcin (OC) concentration (ng $mL^{-1}$). (c) Serum calcium ($Ca^{2+}$), serum phosphorus (P) and urine calcium (uCa) concentration (mmol $L^{-1}$). Orx, orchidectomized rats; Orx + TAM, Orx rats treated with tamoxifen; SO, sham-operated control. All values are mean ± SEM. $^{\#}P < 0.05$ vs. SO and $^{*}P < 0.05$ vs. Orx.

TAM competitively binds to ERs but does not inhibit estrogen-induced CT release. It is possible that by binding to ERs as an estrogen agonist, TAM could affect thyroid C-cell activity. In addition, there are possible indirect effects of TAM on C-cells. Long-term TAM therapy in women with breast cancer may lead to increased serum dehydroepiandrosterone (DHEA) levels (Lum et al. 1997). This sex hormone precursor can be converted to testosterone by hydroxysteroid dehydrogenase action and finally to estradiol under the influence of P450 aromatase (Labrie et al. 2005). Estradiol and testosterone produced by conversion of DHEA may bind to their receptors on C-cells affecting their activity. Although ERs were detected in normal C-cells (Naveh-Many et al. 1992), we cannot be sure that androgen receptors (ARs) are functional in these cells. ARs were found in hyperplastic C-cells and medullary thyroid carcinoma (Zhai et al. 2003; Blechet et al. 2007). However, this does not exclude the possibility of aromatization of testosterone to estradiol and subsequent action through ERs in C-cells (Simpson & Dowsett, 2002).

In our study, TAM treatment increased the activity of thyroid C-cells, but these findings cannot illuminate the exact mechanism(s) of TAM action on these cells. Therefore, further research is needed to clarify the way in which this SERMs act on thyroid C-cells.

Patients with breast cancer have an increased risk of osteoporosis due to various factors, such as advanced age, chemotherapy or aromatase inhibitor therapy. Due to the high rate of ER positivity in male breast cancer, TAM therapy has been used in men (Anelli et al. 1994). Therefore, we have examined the effect of TAM treatment on trabecular bone in Orx rats as an excellent animal model of androgen deficiency (Vanderschueren et al. 1992). Our earlier published results demonstrate that androgen deficiency induced by Orx markedly decreases trabecular bone mass in male middle-aged rats (Filipović et al. 2007). In the current study, treatment of Orx rats with TAM significantly improved bone structural parameters, such as B.Ar, Tb.Th and Tb.N, whereas Tb.Sp was markedly lower than in the control Orx rats. We also obtained significant decreases in serum OC and urinary $Ca^{2+}$ excretion after TAM treatment. These findings suggest that TAM is an effective anti-resorptive agent that prevents trabecular bone loss and decreases bone turnover markers in our model. Other studies also demonstrated that TAM prevents bone loss in androgen-deficient male mice and rats (Broulik, 2000; Fitts et al. 2004), as well as in

ovariectomized rats (Turner et al. 1988) and postmenopausal women (Powles et al. 1996).

The mechanisms by which TAM exerts its effects on bone may involve ERs. Both ER-α and ER-β isoforms are present in osteoblasts and osteoclasts (Eriksen et al. 1988; Bord et al. 2001). As an estrogen agonist, TAM binds to an ER and mimics the effects of estrogen in bone cells. It has been suggested that *in vivo* in rats, TAM prevents the increase of osteoclast number and activity (Turner et al. 1988). As confirmation that the ER-mediated mechanism is involved in the direct inhibitory effect of TAM on osteoclast differentiation, an *in vitro* study showed that the anti-estrogen ICI 182 780 can prevent inhibition of differentiation of these cells (Michael et al. 2007). In addition to a direct effect on osteoclasts, TAM may also inhibit osteoclastogenesis by acting as an estrogen agonist on osteoprotegerin production in osteoblasts (Michael et al. 2007).

The significant increase in serum CT following TAM treatment, detected in the current study, suggests a possible indirect mechanism of TAM action on bone through increased activity of CT-producing thyroid C-cells. Namely, as a potent inhibitor of osteoclastic bone resorption, CT affects osteoclasts directly by binding to CT receptors located on these cells (Nicholson et al. 1986), reducing their number and mobility (Zaidi et al. 1990; Gao & Yamaguchi, 1999). Also, CT increases osteoblast proliferation and the activity of alkaline phosphatase. This action of CT is, at least in part, mediated via CT receptors located on osteoblasts (Villa et al. 2003).

In conclusion, the present study is the first to provide evidence that TAM stimulates thyroid C-cell activity and CT production in Orx male rats. Also, as an estrogen agonist, TAM prevented trabecular bone loss in our animal model of androgen deficiency. In addition to its known protective role in breast cancer, TAM treatment may have the additional beneficial effect on bone in male patients with breast cancer. Taken together, our results suggest that TAM may also affect bone tissue by regulating endogenous CT, produced by thyroid C-cells.

## Acknowledgements

This work was supported by the Ministry of Education, Science and Technological Development of the Republic of Serbia, Grant No. 173009. The authors wish to express their gratitude to Dr Anna Nikolić, PhD for language correction of the manuscript.

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