Effect of tibolone on cytochrome c oxidase I, beta-2-microblobulin and vascular endothelial growth factor gene expression in the lower urinary tract of castrated rats

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Summary

Introduction: The objective of this study was to evaluate the effect of tibolone on cytochrome oxidase I (COX I), beta-2-microglobulin (B2M) and vascular endothelial growth factor (VEGF) gene expression in the lower urinary tract of castrated rats. These genes are related to cell energy, cellular immunity and vascularization processes.

Methods: Fifty adult castrated rats remained at rest for 28 days. Thereafter they were randomly divided into two groups of 25 animals each. The lower urinary tract (bladder and urethra) was extracted in animals of one group and the other group received tibolone at a dose of 0.25 μg/animal/day for another 28 days followed by removal of the lower urinary tract. Total RNA was extracted from animals of both groups, forming two pools. After RT-PCR (reverse transcriptase polymerase chain reaction), expression of COX I, B2M and VEGF genes was evaluated by agarose gel electrophoresis, visualized by UV illumination.

Results: Expression of the three genes (COX I, B2M and VEGF) was greater in the group treated with tibolone.

Conclusion: The use of tibolone increases the expression of COX, B2M and VEGF genes in the lower urinary tract as compared with that in castrated rats.

Key words: Urinary tract; Tibolone; Gene expression; Cytochrome c oxidase I; Beta-2-microglobulin; Vascular endothelial growth factor.

Introduction

The influence of hypoestrogenism on the lower urinary tract is well known with increase in incidence of infections, urgency-frequency syndrome, and urinary stress incontinence after menopause [1]. This is due to alterations such as atrophy of the mucosa and loss in elasticity of the urinary tract, reduction in blood supply, inflammatory response and cellular and humoral immunity [2].

In contrast, estrogen therapy reverses a great part of these symptoms. Although the role of estrogen replacement in the management of postmenopausal urinary incontinence remains controversial, its use in the treatment of women with urogenital atrophy is well-established [3, 4]. A thickening of the urethra and bladder epithelium and increase in periurethral vessels, important components of urinary continence, is observed. Humoral and cellular immunity increase and contribute to the decrease in incidence of urinary tract infections and consistently improving irritant symptoms such as urgency, frequency and urinary urgency-incontinence [3].

On the other hand, estrogen side-effects, such as genital bleeding and risk for breast cancer stimulate the discontinuance of the treatment by a great number of women [5, 6]. The use of new compounds has been encouraged in an attempt to minimize the undesirable effects of estrogen replacement therapy. One of those compounds is tibolone, a synthetic progestogen derived from 19-nortestosterone (norethinodrel), with pharmacokinetic characteristics that include weak estrogenic, progestational and androgenic activities [7].

After oral administration of tibolone, it is rapidly absorbed and converted into two estrogenic metabolites (3α and 3β -OH- tibolone), which bind the estrogen receptor, and a third metabolite, the Δ^4-isomer, which demonstrates binding to the progesterone and androgen receptors. It seems that tibolone also has a regulating effect on enzyme activities especially those involved in estradiol metabolism, events that occur in a tissue-selective manner, and determines whether a tissue is affected or not [7, 8].

There are relatively modest randomized clinical trials comparing the efficacy of tibolone to other hormone preparations for treatment of climacteric symptoms. Most studies report that tibolone is effective in preventing bone loss, but it is not known which hormone activity is responsible for this effect. It is supposed that tibolone leads to improvement of climacteric symptoms such as hot flashes, mood and insomnia and vaginal symptoms explained by its estrogen activity. Tibolone has been reported to provide beneficial effects on sexual function and decrease of HDL cholesterol and triglycerides due to its androgenic activity. Moreover, the endometrium is not stimulated by tibolone action attributed to the action of a highly stable progestinic metabolite (Δ^4-isomer) and does not seem to stimulate the mammary gland [8].

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In recent years, after the emergence of genomics, a term designating a diversified series of scientific and technological methods used for the identification of genes, the philosophy of drug discovery has changed [12]. Today the identification of some medicines can begin with the recognition of the genes which would be involved in pathologic processes and thus could be qualified as targets for future pharmacologic interventions [13]. In addition to identifying target drugs, genomics may also monitor in vivo effects of pharmacologically active compounds on genetic expression in different tissues or organs. This method, known as pharmacogenomics, is useful in elucidating the mechanism of action of the drugs as well as their efficacy and toxicity. In practice, experimental animals are treated with the investigated compound, RNA samples are extracted from the different tissues and gene expressions are compared before and after the treatment [9-11].

The objective of this study was to analyze the expression of COX I, β2 M and VEGF genes of the lower urinary tract under the action of tibolone in view of the few studies in the literature related to its action on the bladder and urethra.

Materials and Methods

The study was performed in the Molecular Gynecology Laboratory of the Department of Gynecology and at the Discipline of Histology and Structural Biology of the Department of Morphology, Federal University of São Paulo – Paulista School of Medicine (UNIFESP-EPM) and was approved by the Ethics Committee of the institution.

Wistar EPM-1 rats *(Rattus norvegicus albinus – Rodentia, Mammalia)* from the Center of Development of Experimental Models for Medicine and Biology of the same institution, were kept in cages at a room temperature of 22°C and artificial light (fluorescent *Philips* 40 W daylight bulbs). The animals were submitted to light photoperiods for 12 hours from 7:00 a.m. to 7:00 p.m. followed by dark periods for 12 hours. The rats received standard rations (*Labina-Purina,* SP, BR) and water ad libitum. Fifty three-month-old virgin rats were divided into two groups: Group I consisted of 25 adult castrated rats that had their urethra and bladder extracted 28 days after castration and were then sacrificed, and Group II 25 castrated rats that received tibolone at a dose of 0.25 µg/animal/day for 28 days [13]. On the first day after the end of medication, the bladder and urethra were removed and the animals sacrificed thereafter. The material was rapidly frozen in liquid nitrogen for RNA extraction.

Total RNA was extracted using the Trizol® technique *(GIBCO/BRL Life Technologies Inc.)* according to the manufacturer's instructions. Two pools of total RNA were then obtained (Groups I and II) by the mixture of identical RNA amounts extracted from each group, to form 40 µg total RNA pools. RNA from the two groups was purified again in silica columns of the Rneasy (QIAGEN) kit extolled by the manufacturer. Concentration and purity of nucleic acids was determined by spectrophotometry, with absorbance read at 260 and 280 nm.

The semi-quantitative RT-PCR (reverse transcriptase polymerase chain reaction) was used with the housekeeping gene beta-actin for normalization of the assay. With a 11 µl RNA sample from the pool of each study group (Groups I and II) a reverse transcriptase reaction catalyzed by the reverse transcriptase enzyme of the Superscript kit *(Life Technologies)* was performed, in addition to random primers according to the manufacturer's instructions. The reaction was amplified by PCR, along 20 cycles (94°C for 3 min; 55°C for 30 sec; 72°C for 30 sec) in a thermocycler (*MJ Research PTC-200 Thermocycles Watertown,* MA, USA) using the Mastermix (Eppendorf) kit. The obtained PCR products were submitted to agarose gel and ethidium bromide electrophoresis and the light intensity of the bands was evaluated with *Kodak Digital Science EDAS 120"* software.

After normalization, the definitive PCR amplification reaction was performed with three samples of RNA pools using specific primers for the chosen genes (Prime3 program-http://www-genome.wi.mit.edu/cgi-bin/primer/primer3.cgi/primer3-_www.cgi) at the same conditions for the housekeeping gene.

β2 M - S: 5' GAATTCACACCCACCGAGAC-3'; AS: 3' CCGGATCTGGAGTAAACTGG-5'

COX I - S: 5' TCGCATCAAAACGAGAAGTG-3'; AS: 3' GGGTTCGAATCCTTCCTTTC-5'

VEGF - S: 5' TGCACCCACGACAGAAGGC-3'; AS: 3' TCACCCCCTTGGCTTGTCACAT-5'

ACTB - S: 5' CGTGACATTAAGGAGAAGCTG-3'; AS:3' CTCAGGAGGAGCATGATCTTGA-5'

Results

COX I, β2 M and VEGF gene expression was greater in the lower urinary tract of the rats that received tibolone (Group II) as compared with the castrated group without medication (Group I) as shown in Figure l. In this figure, the gene used for normalization of the reaction, cytoplasmic beta-actin (ACTB), is also shown. For the evaluation of the results we considered the light intensity of the bands in the gel electrophoresis of RT-PCR reaction product of the three genes, comparing the castrated group (G I) with the medicated group (G II).

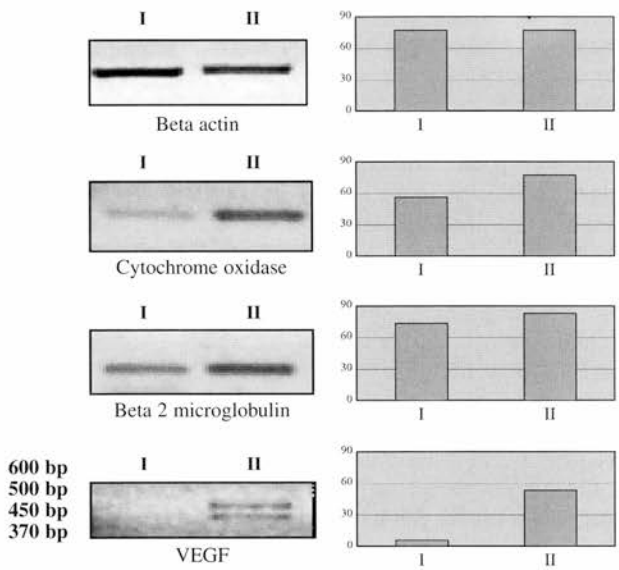

Figure 1. — Algorithm showing differential expression of cytochrome C oxidase I (COX I), beta-2 microglobulin (β2 M) and vascular endothelial growth factor (VEGF) genes in the lower urinary tract of castrated rats (Group I) and castrated rats treated with tibolone (Group II). Microphotograph of the gels.

Discussion and Conclusion

Significant improvement of the quality of life, social isolation and depression concomitant with urinary complaints of women is extremely important [11].

Thus, several clinical and experimental studies have been performed aiming at the evaluation of the action of drugs, mainly estrogen, on the postmenopausal period when there is an increase (or worse) in undesirable urinary symptoms due to ovarian hormone deprivation [4, 13, 14].

We decided to study the influence of tibolone therapy on gene expression of the same genes on the lower urinary tract of rats as one of the alternative drugs used to obtain expected benefit estrogen effects. We opted for the RT-PCR technique (repeated three times) to confirm the results once the methodology was available in our service, and also utilized the urethra and bladder as a whole and not separately as a first molecular analysis of the urinary tract.

Expression of the three genes was increased by tibolone therapy in this experimental model. We cannot affirm if the results were due to the estrogen, androgen or progestogen action of the drug with only these findings.

The VEGF gene is involved in the process of tissular angiogenesis [22] and thus, as with other growth factors, has its expression modulated by sexual steroids [15].

The VEGF protein is a polypeptide with a specific effect on cells of the vascular endothelium and vascular permeability and is the most potent mitogenic and proliferative factor of endothelial cells [25]. Six isoforms are generated as a result of alternative splicing from gene VEGF, consisting of 121, 145, 165, 183, 189 and 206 according to the quantity of amino acids [26]. Different types of cells or tissues express specific patterns of the different isoforms [15, 16].

Agrawal *et al.* [17] reported significantly higher serum VEGF levels in hysterectomized women treated with conjugated equine estrogen (CEE), transdermal estradiol (E2) and tibolone, and furthermore, that CEE was associated with higher VEGF levels as compared with transdermal E2 or tibolone.

Christodoulakos *et al.* [18] evaluated the effect of tibolone and raloxifene on healthy postmenopausal women and observed increased plasma circulating VEGF after continuous tibolone therapy for 12 months. They postulated tibolone increased VEGF via an increased presence of oxidized LDL, an effect attributed to the compound, or direct by an effect of the estrogen action of 3α and 3β - hydroxyl metabolites.

In our study, we found an increase of VEGF gene expression on the lower urinary tract after 28 days of tibolone replacement in ovariectomized rats. These results corroborate the hypothesis of an estrogenic activity of tibolone on the lower urinary tract, even though we cannot affirm it with only this experimental model. In this way, similar to the use of estrogen preparations, we can consider the fact of using this synthetic steroid to treat and prevent urogynecological disorders such as irritating bladder and urethral symptoms, urinary incontinence and urinary infection by increasing local circulation, oxygen uptake and immunological defense.

Regarding VEGF specifically, when analyzing the agarose gel photograph after RT-PCR, which allowed the detection of all the splice variants, we could observe the appearance of two transcripts, different from most studies when the gene is evaluated after the use of estrogens alone, where normally four isoforms appear [29]. We think that this fact is due to the weak estrogenic action of tibolone.

COX I is a gene of mitochondrial DNA which encodes for cytochrome c oxidase subunit I belonged to the respiratory complex IV and involved in the process of oxidative phosphorylation of cells [20].

Tong-Long Lin *et al.* [21] studied the effect of age on mitochondrial enzyme activity in rat bladders while evaluating some enzyme activities in addition to phosphocreatine and ATP concentrations in the bladders of 24-month-old female rats. The authors observed significantly lower phosphocreatine and ATP levels and significantly lower activities of citrate synthetase, malate dehydrogenase, NADH-cytochrome c reductase, succinate-cytochrome c reductase and cytochrome c oxidase in the bladders of old rats. They concluded that age reduced the activity of mitochondrial enzymes in rat bladders, resulting in less ability of energy production which could explain some micturition disorders frequent in elderly patients.

Adequate energy supply is a prerequisite for good vesical functioning, muscle contraction and vesical emptying. Cell energy, in the form of ATP originating from mitochondrial oxidative phosphorylation, is the immediate energy used for contraction of detrusor muscles and other cell activities [21]. Levin *et al.* [22] demonstrated that interruption of the oxidative metabolism abolished the plateau component of detrusor muscle contraction, resulting in decreased ability of vesical emptying.

Some authors observed an influence of hormone therapies in mitochondrial genes and proteins. Bettini and Maggi [23], on administering subcutaneous estradiol to oophorectomized rats, observed a significant increase (30%) in levels of mRNA for COX III in the hippocampus and hypothalamus as well as an increase in cerebral neuronal activity under estrogenic action.

In addition, estrogen was associated with an increase in subunit II of the cytochrome oxidase complex in pituitary tumor cells, with expression of high levels of estrogen receptors [24].

Our experiment evidenced that COX I gene expression increased after tibolone replacement. The result agrees with the steroid hormone influence on the respiratory complex activity demonstrated by other authors, therefore we cannot be sure about which hormone action of tibolone was responsible for this effect.

Thus, we can postulate that an increase in expression of genes of cell respiration may be one of the mechanisms by which tibolone would act in urinary disorders by keeping an ideal metabolism of cells to obtain energy needed for physiological activities such as detrusor contraction.

The β2 M gene encodes for the protein of the same designation which is a component of HLA antigens (histocompatibility antigens) and essential for their expression. It is structurally similar to the amino acid sequences of immunoglobulin and is implied in immunologic functions [25].

β2 M function has not been completely clarified yet. However, it is believed that β2 M would be a link between antibodies or IgG and histocompatibility antigens. Therefore, it would have the function of cellular recognition of antigens and interaction of the cellular and humoral immune systems, by interaction between T and B lymphpocytes [25, 26].

Some clinical and experimental evidence support the hypothesis of immune function regulation by gonadal steroids: different behavior of immune systems between men and women, alterations in immune response after gonadectomy, estrogen replacement and in women during pregnancy. Even so, hormone steroid receptors are identified in immune system organs [25-27].

Most scientific researches demonstrate that estrogen acts as a stimulator of the humoral immune response mediated by B lymphocytes and immunoglobulin, and as an inhibitor of the cellular immune response mediated by cytotoxic T lymphocytes [27].

Decadurabolin, an anabolic steroid, has been suggested to be responsible for elevating serum levels of β2-M protein influencing its cell production in osteoporotic women [27].

The observation of increased β2 M gene expression in rats treated with tibolone therapy perhaps has some relationship with the prevention of urinary infection incidence seen in postmenopausal women. However, the real function of β2 M protein in immune responses and the participation of the immune system specifically in the genesis of urinary infections in the postmenopausal period have to be better elucidated. Any possible tibolone action, once again, cannot be associated with its estrogen, androgen or progestin action just with this assay.

According to our results, we can conclude that tibolone has a positive effect on the modulation of genes involved in important cell functions such as energy production (COX I), immunity (β2 M) and angiogenesis and increased vascularization (VEGF) in the lower urinary tract of female rats. However, further studies are needed in order to confirm the correlation between these genes, sexual hormones and the female urinary tract.

The study of drug action on gene expression and elucidation of the biomolecular interactions responsible for the pharmacologic effect is starting and progressing rapidly. We believe that in the near future, through pharmacogenomic research in the area of urogynecology, disclosing the function of genes involved in the lower urinary tract and the action of drugs thereon, a greater number of possibilities will be available for the treatment of urinary disorders during perimenopause, based on genetic variations for the individualization of drug administration.

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