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Comparative effects of estrogen, raloxifene and tamoxifen on endothelial dysfunction, inflammatory markers and oxidative stress in ovariectomized rats

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ABSTRACT

Aim: Endothelial dysfunction is considered a premature indication of atherosclerosis and vessel damage and is present in the postmenopausal period. This study compares the influence of estrogen, raloxifene and tamoxifen on factors that affect endothelial function in ovariectomized (OVX) rats.
Main methods: The rats were divided into: SHAM; OVX; OVX + estrogen (0.5 μg/kg/day); OVX + raloxifene (2 mg/kg/day) and OVX + tamoxifen (1 mg/kg/day) groups. The acetylcholine vasorelaxation response was evaluated in the mesenteric vascular bed. The vascular oxidative stress and serum inflammatory cytokine levels were monitored, and analyses of eNOS and iNOS were performed.
Key findings: The acetylcholine-induced responses obtained in the OVX were lower than those obtained in the SHAM, and all treatments restored this response. L-NAME reduced and equalized the acetylcholine-induced response in all groups. The attenuation of the acetylcholine-induced responses by aminoguanidine was greater in the OVX. Endothelial dysfunction in OVX was associated with oxidative stress and an increase in iNOS and decrease in eNOS expression. Except for the production of reactive oxidative species (ROS) in the OVX + tamoxifen, treatments improved the nitric oxide component of the relaxation response and normalized both the oxidative stress and the expression of those signaling pathway enzymes. Serum levels of TNF-α and IL-6 were increased in OVX, and treatments normalized these levels.
Significance: Raloxifene and tamoxifen have similar anti-inflammatory effects that may be important in improving vascular dysfunction. Tamoxifen did not affect the ROS but improved endothelial dysfunction. The protective effect on endothelial function by these treatments provides evidence of their potential cardiovascular benefits in the postmenopausal period.

Introduction

In the postmenopausal period, the decrease in ovarian hormones can influence inflammation in many tissues, including the vasculature [3]. The inflammatory activation of the endothelium, which plays a critical role in vascular homeostasis, induces a broad range of local and systemic responses, including the expression of adhesion molecules, the production of chemotactic factors (such as cytokines) and the secretion of chemical mediators (such as free radicals) [12,25].

Evidence demonstrates that after cytokine stimulation, endothelial cells undergo morphological alterations [2]. An important factor for these endothelial changes is the decreased availability of nitric oxide (NO). This reduction occurs before the structural manifestation of vascular disease, such as atherosclerosis, and may thus represent an independent predictor of potential cardiovascular events [61]. Another factor that influences the atherosclerotic process and vessel damage is an increase in oxidative stress [19,26].

Therapies that involve selective estrogen receptor modulators (SERMs) have shown protective effects in the treatment of menopausal symptoms in clinical situations [69,70]. SERMs are a class of synthetic compounds that act as estrogen receptor antagonists or agonists, depending on the target tissues [50,58]. Tamoxifen is the most widely used anti-estrogen for managing breast cancer [23,50]. Raloxifene is used for the treatment and prevention of post-menopausal osteoporosis [58]. In addition to these specific indications, these drugs could be used to reduce cardiovascular problems that arise in post-menopausal women. Some clinical and experimental studies have shown that

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raloxifene and tamoxifen provide protective effects on cardiovascular function by increasing the synthesis and bioactivity of endothelial factors [9,16,62], thereby reducing oxidative stress or inflammatory markers [14,17,36,52]. SERMs have acquired a safety profile and have been shown to exhibit a very low incidence of adverse side effects. However, along with estrogen therapy, these drugs increase the risk of venous thromboembolism in postmenopausal women. It is noteworthy that treatment with estrogen, raloxifene and tamoxifen shifts the coagulation pattern toward prothrombosis. Patients should therefore be exhaustively informed about the risks associated with these therapies [21,22,24,28,40,59,66].

Although long-term *in vitro* [29] and *in vivo* [52] studies have confirmed that SERMs improve cardiovascular dysfunction, several variables such as gender, endothelial status, vessel type and animal species appear to affect treatment outcomes. Moreover, the direct effects of raloxifene and tamoxifen on vascular dysfunction and inflammatory or oxidative patterns in an estrogen-deficient animal have not been compared.

Therefore, in this study, we first examined whether estrogen, raloxifene and tamoxifen equally restored the vascular dysfunction observed in the isolated mesenteric vascular bed from ovariectomized rats. We then evaluated whether these drugs were able to modify the participation of endothelial factors in the vascular responses, induce changes in the oxidative status of the vessel and alter the levels of inflammatory circulating cytokines in an animal model of estrogen deficiency.

Material and methods

Ethical approval

The investigation was conducted in accordance with the biomedical research guidelines for the care and use of laboratory animals, and the experimental protocol was approved by the Ethics Committee in Animal Experimentation of the Physiology Sciences Department of the Federal University of Espirito Santo (no. 012/2008). The experiments were performed using eight week-old female Wistar rats weighing 180 to 200 g. Throughout the experiment, the animals were housed in groups in a temperature-controlled room (22 °C) with a 12-h (light)–12-h (dark) cycle. Standard rat chow and tap water were available *ad libitum*.

Animals and treatment

Five groups (n = 06/group) of female rats were studied: SHAM; ovariectomized (OVX); OVX treated with 17-β-estradiol (EST: 0.5 μg/kg/day; Sigma Chemical Co., St. Louis, MO, USA); OVX treated with raloxifene (RLX: 2.0 mg/kg/day; Eli Lilly, Indianapolis, IN) and OVX treated with tamoxifen (TAM; 1.0 mg/kg/day; Sandoz, Cambé, PR). 17β-Estradiol was dissolved in peanut oil and administered by subcutaneous injection, and the SERMs were pulverized, dissolved in water and administered by gavage. These treatments were initiated 21 days after bilateral ovariectomy and continued for 14 additional days. The effects of ovariectomy and estrogen treatment were confirmed by measuring the body and uterine weights at the time of the experiment.

Ovariectomy

Female Wistar rats underwent bilateral ovariectomy after they were anesthetized with an intraperitoneal injection (i.p.) of ketamine (70 mg/kg) and xylazine (10 mg/kg). A bilateral muscle wall incision was made, and both the ovaries and oviduct were exteriorized. A sterile silk ligature was placed around the oviduct and each ovary, and part of the oviduct was removed with a single cut through the oviduct near the ovary. The remaining tissue was returned to the peritoneal cavity, which was then sutured. The female sham group only underwent an incision.

Estrous cycle phase determination

Daily vaginal smears were taken from each female sham rat as previously described [11,18] to confirm that their estrous cycles were proceeding normally [estrus, metaestrus, diestrus and proestrus]. The vaginal epithelial cells were examined *via* a microscope for at least 7 consecutive days before the experiment. The swabs were performed between 8:00 and 10:00 A.M. to maintain consistency. The experiments were performed in the females with normal estrous cycle, during the proestrus phase.

Vascular reactivity in the mesenteric vascular bed

To test the vascular reactivity responses to acetylcholine (ACh), the rat mesenteric vascular bed (MVB) was isolated, as described by Mcgregor [42]. Initially, the superior mesenteric artery, with its branches, was isolated and perfused with a warmed (37 °C), gassed (5% CO_2 in 95% O_2) physiological salt solution with the following composition: 130 mM NaCl, 4.7 mM KCl, 1.6 mM $CaCl_2.2H_2O$, 1.17 mM $MgSO_4 \cdot 6H_2O$, 14.9 mM $NaHCO_3$, 1.18 mM KH_2PO_4, 0.026 mM EDTA, and 11.1 mM glucose, pH 7.4. The MVB was excised from the intestinal wall and placed in a chamber, and the preparations were allowed to stabilize for 30 min prior to beginning the experiments. The perfusion was maintained at a constant rate of 4 ml/min with a roller pump (Ismatec AS Laboratorium Stechnik, Switzerland). The perfusion pressure was recorded with a pressure transducer (Spectramed P23XL), and the data were digitized (Acqknowledge for Windows; Biopac Inc.). The basal perfusion pressure (approximately 40 mm Hg) was elevated by the addition of norepinephrine (0.1 to 0.3 mM) in the perfusion fluid to increase the tone by approximately 90–120 mm Hg. Once a stable tone was established, concentration–response curves to ACh (1.68×10^{-12} to 1.68×10^{-3} M) were determined in the MVB in the absence and presence of inhibitors. The ACh doses were administered randomly in the MVB. The ACh curves were obtained initially in each MVB without the presence of inhibitors. To evaluate the effect of NO availability on vascular reactivity, the preparations were treated with the nonspecific NOS inhibitor N^G-nitro-L-arginine methyl ester (L-NAME, 100 mM) and the inducible NO synthase (iNOS) inhibitor aminoguanidine (AG, 100 mM). The participation of endothelium-derived hyperpolarizing factor (EDHF) in modulating endothelial function was assessed by constructing concentration–response curves to ACh in the presence of L-NAME and the cyclooxygenase (COX) inhibitor indomethacin (INDO, 2.8 μM), thus excluding the involvement of NO and prostanoids, respectively. The responses obtained in the presence of indomethacin and L-NAME represented EDHF-mediated relaxation, as the remaining relaxation (indomethacin and L-NAME-resistant relaxation) was completely abolished by 30 mM K^+ physiological salt solution (data not shown). All of these drugs were added to the bath 30 min before obtaining the ACh concentration–response curves.

Western blot analysis

To achieve greater representation of the MVB, mesenteric arteries were carefully dissected to be free of surrounding adipose tissue. The samples were homogenized and centrifuged at 3000 *g* for 15 min (4 °C). Protein concentrations were determined using the method of Lowry [39,53]. The protein lysates [50 μg for eNOS and iNOS] were separated by 7.5% SDS-PAGE. The proteins were transferred to polyvinylidene difluoride (PVDF) membranes that were incubated with mouse monoclonal antibodies for endothelial nitric oxide synthase (eNOS, 1:2500, BD Transduction Laboratories, Lexington, KY, USA), inducible nitric oxide synthase (iNOS, 1:2000, BD Transduction Laboratories, Lexington, KY, USA) or β-actin (1:1500, Santa