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SCIENCE DIRECT
Journal of Steroid Biochemistry & Molecular Biology 97 (2005) 271–277
The Journal of Steroid Biochemistry & Molecular Biology
www.elsevier.com/locate/jsbmb

No estrogen-like effects of an isopropanolic extract of *Rhizoma Cimicifugae racemosae* on uterus and vena cava of rats after 17 day treatment

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Received 20 December 2004; accepted 20 May 2005

Abstract

The effects of black cohosh extracts (*Rhizoma Cimicifugae racemosae*) on primary estrogen target organs, like mammary gland and endometrium are better described then those on other estrogen-sensitive systems e.g. the vasculature. We therefore treated ovariectomized DA/Han rats for 17 days with an isopropanolic *Cimicifuga racemosa* rhizoma extract (iCR) alone and in combination with the pure antiestrogen fulvestrant. As control groups vehicle, estradiol, fulvestrant, and estradiol fulvestrant cotreatment were used. Effects of all substances were investigated by vena cava and uterine gene expression analysis using real-time-PCR.

Uterus wet weight was increased after estradiol treatment compared to the negative controls but none of the other treatments including the treatment with iCR had a uterotrophic effect. While estradiol-induced changes in uterine gene expression were mainly analogous to those detectable in shorter term experiments, iCR showed no or slightly antiestrogenic effects on gene expression in the uterus. This is mirrored in the vena cava where iCR had a very minor impact on the expression of the genes analyzed. While *C. racemosa* is effectively used for treatment of peri- and post-menopausal symptoms for a long time its mechanism of action remains unresolved. Contrary to earlier suggestions *C. racemosa* does not seem to act as an estrogen agonist, but possibly as a weak antiestrogen.
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Keywords: *Cimicifuga racemosa*; Phytoestrogen; Uterus; Vascular system; Gene expression

1. Introduction

Ethanolic and isopropanolic extracts of black cohosh (*Cimicifuga racemosa*) rhizomes have been widely and for a long time used for treatment of general menopause symptoms like hot flushes. Several clinical studies support the clinical efficacy of this plant extract [1–3]. Despite this, the mechanism of action remains unknown. Although *C. racemosa* has long been assumed to contain phytoestrogenic isoflavones, this could not be confirmed in either plant specimen from 13 different geographical locations nor in commercially available extracts [4]. Short term animal experiments have been conducted without being able to confirm estrogenic effects [5]. More recent in vitro experiments suggest antiestrogenic rather than estrogenic properties [6]. The primary active constituents of *C. racemosa* are believed to be part of the triterpene glycoside and the cinnamic acid ester fractions. Whereas both of these fractions are able to induce apoptosis in estrogen receptor positive human breast cancer cells [7], direct binding of these constituents to the estrogen receptor α or β is still a matter of controversial dicussions [8].

Menopause in women is associated with an increase in risk of cardiovascular disease [9]. Classical hormone replacement therapy (HRT) using a combination of estrogenic and gestagenic compounds has become more questionable after various prospective and retrospective clinical studies demonstrated an increased risk of cardiovascular disease and of mammary cancer during and after menopause [10,11]. This led to the recommendation for former breast cancer patients or women at risk of developing breast cancer not to use classical HRT at all. Despite its use for curing menopause

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0960-0760/$ – see front matter © 2005 Published by Elsevier Ltd.
doi:10.1016/j.jsbmb.2005.05.008

symptoms for many years, little is known about possible vasoprotective aspects of *C. racemosa*.

Our study thus aimed at investigating whether an isopropanolic extract of the rhizome of *C. racemosa* (*iCR*) in a prolonged treatment regimen shows any effect on the vascular system. Therefore a treatment time of 17 days was chosen as a modification of the usual 3 day standard uterotrophic assay. Six groups of ovariectomized dark agouti (DA/Han) rats were treated with 60 μl/kg body weight iCR extracts (i.e. 60 mg herbal drug/kg b.w.) or 60 μl/kg iCR extracts in combination with 5 mg/kg of the pure antiestrogen fulvestrant (formerly known as ICI 178,780) or the vehicle only as a negative control or 200 μg/kg estradiol (E2) as a positive control or 200 μg/kg E2 combined with 5 mg/kg of fulvestrant or with 5 mg/kg fulvestrant alone. The endpoints used as indicators for estrogenic effects in this experiment were the uterus wet weight as well as the expression of estrogen regulated genes in the uterus and in the vena cava.

The vena cava was selected for practical reasons as a large enough and accessible part of the vascular system. Further, the ratio between endothelic and other tissue parts in comparison to the aorta is higher. The uterus was selected because it is commonly recommended as the standard reference organ for estrogenic responses due to its high sensitivity to estrogenic substances. Hence, even small estrogenic effects of the treatment with the tested substances should be detectable in this organ. Furthermore, the uterus is the major target organ for detrimental estrogenic effects in post menopausal women, by inducing both uterine cyclicity and bleeding as well as promoting precancerous lesions and endometrial tumors.

2. Materials and methods

2.1. Materials

17β-Estradiol was purchased from Sigma (Deissenhofen, Germany). Fulvestrant was purchased from TOCRIS Cookson Ltd. (Bristol, UK). Isopropanolic extracts of the rhizome of *C. racemosa* were prepared according to standard procedures by Schaper und Brümmer GmbH & Co. KG, Salzgitter-Ringelheim, Germany. Extracts were prepared so that 60 μl liquid extract correspond to ∼60 mg herbal drug, calculated as and based on the detection of triterpene glycosides as one major, putatively pharmacologically active, component class.

TRIzol® was purchased from Life Technologies, Karlsruhe, Germany as were the PCR kits and SybrGreenI. MMLV reverse transcriptase was purchased from Promega GmbH, Mannheim, Germany. For real-time-PCR, the iCycler from Bio-Rad Laboratories, Hercules, CA, USA, was used.

2.2. Animal experiments

Female DA/Han rats ($n = 35$, 5–6 per group) were used to evaluate putative activities of iCR on uterus and vasculature.

At an age of approximately 90 days and a body weight of 150–210 g, the animals were bilaterally ovariectomized (under ketamin/xylazin anesthesia). All the animals were housed in identical environments and food and water was offered ad libitum. Two weeks after ovariectomy when residual endogenous estrogens had been washed out, treatments started and animals were treated daily with either 60 μl iCR/kg b.w., or 5 mg fulvestrant/kg b.w. or a combination of 60 μl iCR/kg b.w. and 5 mg fulvestrant/kg b.w., or 200 μg /kg b.w., or a combination of 200 μg E_2/kg b.w. and 5 mg fulvestrant/kg b.w. A sixth group remained untreated. iCR was administered via gavage, fulvestrant was injected intramuscularly, whereas E_2 was injected subcutaneously.

The animals were inspected weekly for general appearance. Upon completion of 17 days of experimental treatment, all animals were anaesthesized with an overdose of CO_2 and were exsanguinated. General pathological examinations were performed. Uteri and venae cavae were excised within 10 min after exsanguination and preserved in liquid N_2 for analysis. Before asservation, uteri were weighed and relative organ weights in reference to the body weight were calculated. Venae cavae were ligated and excised from 25 mm cranial of the bifurcation encompassing 5 mm of both branches caudal of the bifurcation.

All conditions of animal husbandry were in accordance with local regulations, and experimental procedures were approved and conducted under the auspices of a local German animal care and oversight committee.

2.3. RNA extraction

Total cytoplasmic RNA was extracted from homogenized rat uteri and venae cavae by the standard TRIzol® method. Concentration and purity of the extracted RNA was determined by measuring extinction at 260 and 280 nm. The quality of the extracted RNA was checked using gel electrophoresis. Based on the results of the gel electrophoresis some of the RNA-samples extracted from the venae cavae had to be discarded. Hence only five samples of the negative control group and only three samples of all other groups have been used. Samples belonging to one treatment group have been pooled.

2.4. cDNA synthesis

For further processing, 3 μg of RNA per sample were used at a time. DNase digestion was necessary to remove residual genomic DNA. Incubation time was 1 h at 37 °C. DNase was then inactivated by shortly raising the temperature to 80 °C after addition of EDTA to a final concentration of 1.75 mM. Verification of the successful RNA preparation was done in a PCR reaction using primers for the cytochrome c oxidase subunit I. This protein is encoded in mitochondria and therefore a higher copy number per cell is present. This ensures high sensitivity of the reaction. In the subsequent cDNA synthesis utilizing MMLV reverse transcriptase, OligodT-primers were used for initiating the reaction.

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