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Lack of Promotion of Estrogen-dependent Mammary Gland Tumors *in vivo* by an Isopropanolic *Cimicifuga racemosa* Extract

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ABSTRACT

***Cimicifuga racemosa* (CR) is widely used in the treatment of menopausal symptoms. Mechanistic studies suggest that unlike hormone-replacement therapy, CR does not stimulate estrogen-receptor positive breast cancer cells. To evaluate CR safety, we performed an *in vivo* investigation of a clinically tested isopropanolic CR extract. Mammary tumors were induced in Sprague Dawley rats (n = 75) by the application of 7,12-dimethylbenz[a]anthracene. Five to nine weeks later, the animals were ovariectomized, allowed to recover, and administered daily doses of CR extract (0.714, 7.14, or 71.4 mg/kg body weight per day) or control substances (estrogen/positive control: 450 μg/kg/day mestranol; or CR vehicle/negative control). The animals were sacrificed 6 weeks later, and tumor number, size, plasma hormone levels, and the weight of estrogen-sensitive organs were analyzed. In contrast to mestranol treatment, CR treatment did not stimulate cancerous growth. There were no significant differences in tumor number or size between the CR groups and the vehicle control. Likewise, prolactin, follicle-stimulating hormone, and luteinizing hormone levels and organ weights and endometrial proliferation were unaffected. The lack of mammary tumor-stimulating effects of this extract is of great significance in establishing the safety of CR extracts for treatment of menopausal symptoms in women with a history of breast cancer in which hormone-replacement therapy is contraindicated.**

INTRODUCTION

HRT[2] is a widely prescribed for the management of menopausal symptoms. However, the safety of estrogen replacement for women with endometrial, ovarian, and breast cancers has been questioned after reports that estrogen stimulates proliferation of estrogen-receptor positive tumors (1). On the basis of this concern, it has been recommended that women with estrogen-sensitive cancers seek alternative methods for alleviating menopausal symptoms.

Phytoestrogens, including soy and red clover, are popular alternative treatment options available for relief of menopausal symptoms. On the basis of their mechanism of action, however, soy and red clover may have side effects similar to estrogen (2), thus increasing the risk of tumor growth and estrogen-receptor positive cell proliferation (3). As such, it is important to evaluate the estrogenic effects of alternative therapies.

CR, commonly known as black cohosh, black snakeroot, and rattlesnake root, has been indicated as an effective medicinal herb offering relief of menopausal symptoms such as hot flashes, sweating, mood swings, irritability, and sleeplessness (4–10). Although CR has been proven to be effective for menopausal symptom relief, its mechanism of action is unclear.

In vitro studies of isopropanolic CR extracts on estrogen-receptor positive breast cancer cell lines suggest that CR, unlike estrogen and soy, does not stimulate cell proliferation (11–13). Likewise, clinical evaluations of menopausal women in which levels of gynecologically relevant hormones are measured support the lack of estrogenic effect of isopropanolic CR-extract formulations (14–16). In contradiction to these data, some studies suggest that other CR formulations (methanolic or ethanolic extracts) have estrogen-like action (17–21).

To further study the estrogenic activity of an isopropanolic CR extract (Remifemin; Schaper & Brümmer GmbH & Co KG, Salzgitter, Germany), which has been noted to be effective in relieving clinical symptoms of menopause (16), we performed an *in vivo* experiment in ovariectomized female rats simulating the estrogen-deprived environment of menopausal women. To investigate the effects of CR on estrogen-receptor positive mammary gland cells, mammary tumor growth was DMBA-induced in female rats. Using this *in vivo* estrogen-receptor positive breast cancer model, we sought to test for estrogen-like activity of a commercially available isopropanolic CR extract (Remifemin) on tumor development, hormone levels, and organ weight. The model used in this study, which has been previously validated and frequently used for toxicity studies of this type (22–28), was designed to evaluate the safety of CR extracts for treatment of menopausal complaints in women with a history of breast cancer. As such, the CR extract was administered after the development of a mammary tumor.

MATERIALS AND METHODS

Investigational Products and Control Substances. This study was conducted in correspondence with Good Laboratory Practices. The local German animal care and oversight committee approved the animal experiments in this study. The CR-extract formulation and the control vehicle (CR control), which was identical in all respects to the CR extract except that it was devoid of the active substance, were manufactured according to a validated manufacturing process and supplied by Schaper & Brümmer GmbH & Co KG. Mestranol (17 α-ethinylestradiol-3-methyl ether; CAS no. 72-33-3), an active estrogenic steroid administered p.o., was used as positive control (product no. E 5001; SIGMA-Chemie, Deisenhofen, Germany).

CR extract and CR control were suspended in fresh water at a concentration of 100 mg/ml immediately before the intragastric administration by gavage (10 ml/kg body weight). The CR extract was prepared and administered daily at 1-fold, 10-fold, and 100-fold the human therapeutic dose (0.714 mg/kg, 7.14 mg/kg, and 71.4 mg/kg, respectively). Mestranol was dissolved in sesame oil at a concentration of 225 μg/ml and prepared in an intragastric application volume of 2 ml/kg body weight.

Animals, DMBA Application, Tumor Measurement, Ovariectomy and Treatment. Fifty days after birth, single intragastric doses (20 mg) of DMBA (CAS no. 57-97-6; Batch 20Z 85H0296; product no. D 3254; SIGMA-Chemie) dissolved in sesame oil were administered to female Sprague Dawley rats (Mol:SPRD; Mollegaard Breeding, Ejby, Denmark; n = 75). All of the animals were housed in identical environments (two females per 800-cm^2 polycarbonate cage; softwood bedding; room temperature, 22 ± 2°C; 55 ± 15% relative humidity; light on, 12 h; light off, 12 h), and pelleted food (altromin 1324 N; Altromin, Lage, Germany) and water were offered *ad libitum*. After DMBA treatment, the test animals were palpated weekly for mammary tumor development. Mammary tumors were measured in mm (length × width) using a caliper. The two-dimensional tumor area was calculated as an ellipse. on identification of mammary tumors >5 mm in diameter (5–9 weeks after tumor induction), the rats were bilaterally ovariectomized (under Ketamin/Xylazin anesthesia).

The animals were not randomized before ovariectomy because the development of a tumor could not be predicted; however, measures were

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[2] The abbreviations used are: HRT, hormone-replacement therapy; CR, *Cimicifuga racemosa*; DMBA, 7,12-dimethylbenz[a]anthracene.

taken to randomize the animals in treatment groups. Mammary tumors were again measured 9 days after ovariectomy and the animals were randomly distributed according to tumor occurrence, size, and location to the following treatment groups: (*a*) CR-extract vehicle control (group V; $n = 14$); (*b*) 0.714 mg/kg CR extract (group I; $n = 14$); (*c*) 7.14 mg/kg CR extract (group II; $n = 14$); (*d*) 71.4 mg/kg CR extract (group III; $n = 15$); and 450 μg/kg mestranol (group M; $n = 14$). After 10 days of recovery from ovariectomy, the rats were given intragastricly the test and reference compounds daily for 6 weeks.

The rats were monitored daily for general clinical symptoms and examined weekly for mammary tumor and body weight changes. After completion of 6 weeks of experimental treatment, all surviving rats were euthanized and necropsied. Before necropsy, blood samples were drawn from the retrobulbar vein plexus (under halothane anesthesia from 7:00–8:30 a.m.) for clinical evaluation of plasma follicle stimulating hormone, luteinizing hormone, and prolactin levels.

Necropsy. All animals were anesthetized with an overdose of CO_2 and were exsanguinated. All major organs with a special emphasis on macroscopically altered organs were preserved for analysis. The organs were fixated in a 10% neutrally buffered formalin solution.

The following organs were weighed before fixation (pairs of organs were weighed separately): brain, pituitary, liver, spleen, kidneys, suprarenal glands, and uterus. Relative organ weight data in reference to the terminal body weight were computed.

Histopathology. Pairs of cervical, cranial, caudal thoracic, abdominal, cranial inguinal, and caudal inguinal glandular complexes in the mammary glands, uteri, and ovaries were prepared for histological examination. Tissues were trimmed according to standardized procedures (29), embedded in paraffin, sectioned at 3–4 μm, and routinely stained with H&E. The mammary glands were evaluated as described previously (30, 31). The preserved uteri were examined for hyperplasia, and the ovarian regions were evaluated for residual ovarian tissue.

Data Analysis. Animal body weights, mortality, and clinical chemistry were recorded and evaluated with the Datatox (version rC.10; Instem, Stone, United Kingdom) and the SAS statistical program. Body and organ weight data, gynecological hormone (follicle stimulating hormone, luteinizing hormone, and prolactin) levels, and mammary tumor sizes were evaluated using ANOVA as a global test. Pair-wise comparison of the means of the treatment groups with the means of the CR control group was performed with Dunnett's modification of the *t* test. The histopathology slides were examined by light microscopy and the observations were recorded with the on-line computer program PLACES 2000.1 ("Pathology Lexicon, Acquisition, Correlation, and Evaluation System"). Tumors of the mammary glands were classified according to WHO-IARC criteria (32).

RESULTS

Animal Health and Mortality. There were no statistically significant differences between the experimental groups (V, I, II, III, M) with respect to tumor latency, number of tumor carriers, and the distribution of tumor sizes (ANOVA + Dunnett's Test). Life expectancy, evaluated using the Kaplan-Meier survival analysis technique, did not statistically differ among the CR extract (I, II, III) and control groups (V, M), and the general health of the animals was satisfactory throughout the study. Of the 71 animals, 8 died or had to be euthanized prematurely because of their moribund state. In two of these cases (both in the mestranol group), premature euthanasia was motivated by ethical considerations because of large mammary tumors.

Body Weight. Mestranol treatment caused a significant reduction in weight gain within 1 week of the start of treatment ($P \leq 0.05$) as compared with the control and CR-extract treated groups (Fig. 1). No significant weight differences were noted between the CR-extract (I, II, III) and CR-control (V) groups.

Mammary Tumor Growth. Mammary tumor growth was significantly reduced after ovariectomy and the subsequent loss of natural estrogen secretion (Fig. 2). Mestranol, which induced tumor regrowth after 7 days of treatment, resulted in tumors significantly larger than those of the three CR-extract or CR-control groups ($P \leq 0.05$; ANOVA + Dunnett's test).

The three CR-extract and CR-control groups did not show significant differences in mammary tumor size. In CR-treated groups, decreased growth of the mammary tumors was observed compared with those of the CR controls, suggesting a possible inhibitory effect. However, statistical analysis (Ryan-Einot-Gabriel-Welsch multiple test) failed to demonstrate statistical significance.

Clinical Chemistry (Prolactin, Luteinizing Hormone, and Follicle Stimulating Hormone). The three doses of CR extract did not alter prolactin, luteinizing hormone or follicle stimulating hormone levels as compared with the CR-control group (Fig. 3). By contrast, and as expected for p.o. estrogen treatment, mestranol induced a significant increase in prolactin levels ($P \leq 0.001$) and a significant decrease in luteinizing hormone and follicle stimulating hormone levels ($P \leq 0.001$) compared with the CR-control animals (Fig. 3).

Postmortem Findings. Postmortem evaluations did not reveal significant differences between the CR-extract and CR-control groups. An apparent difference was noted between the animals treated with mestranol and the animals of the other four groups (CR vehicle and

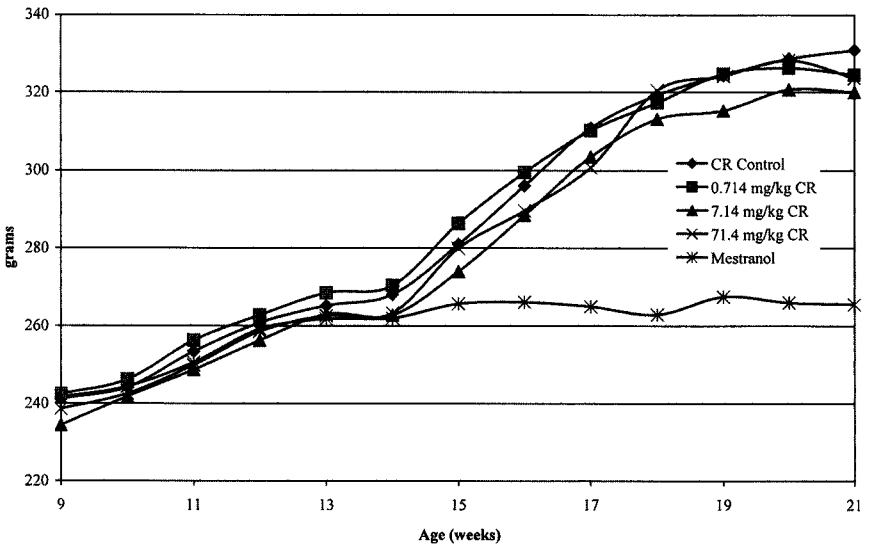

Fig. 1. Development of the body weight. Mestranol treatment inhibits weight gain, whereas CR extract does not significantly affect normal weight gain. Between weeks 17 and 21, there was a significant difference between the body weight of mestranol-treated animals and control animals ($P \leq 0.05$).

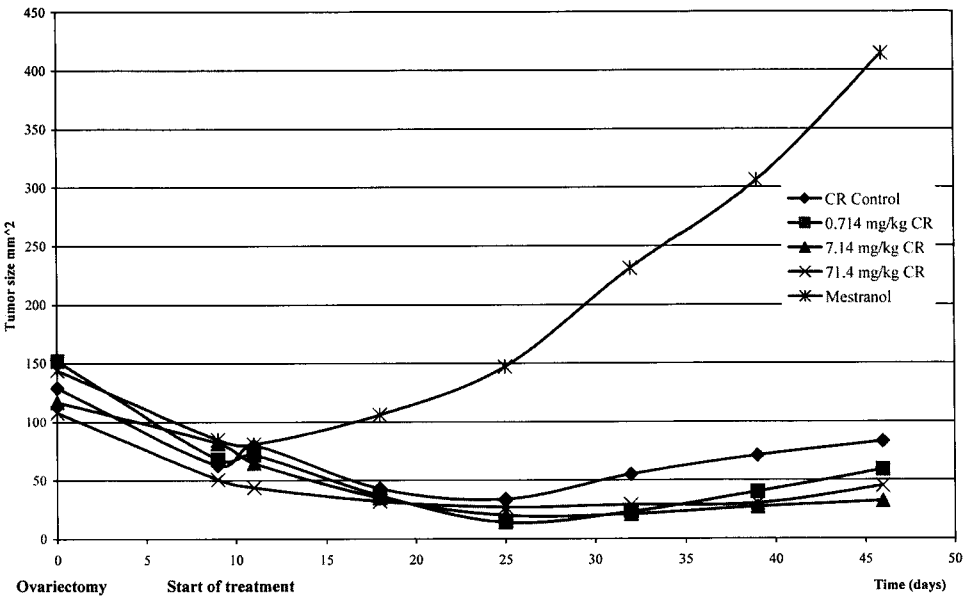

Fig. 2. Development of the mammary tumors. Mestranol treatment induced significantly greater tumor regrowth, noted by tumor size, than CR and control treatments. *Time 0* represents the time at which ovariectomies were complete. *Time 10* signifies the start of the experimental treatment.

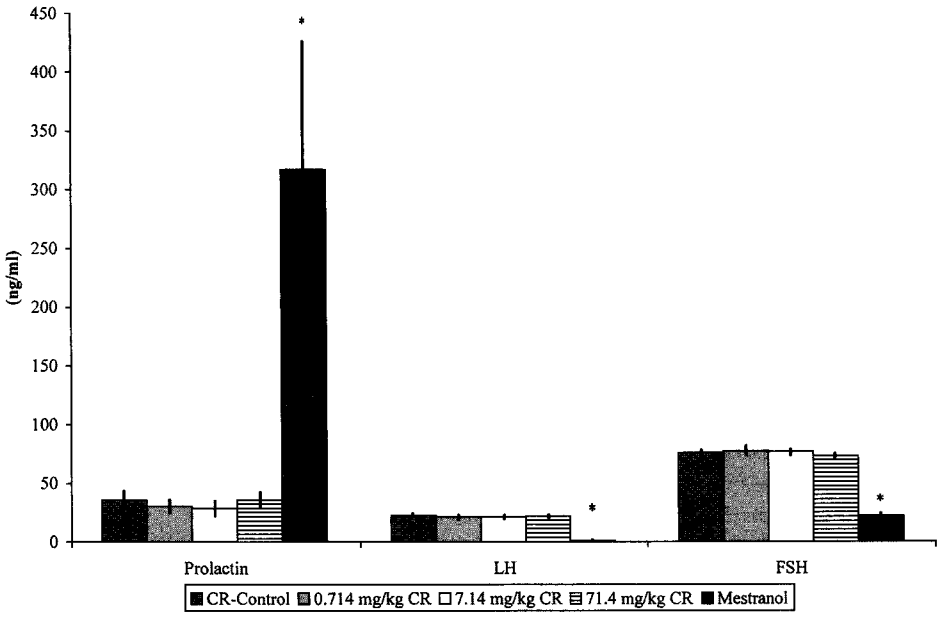

Fig. 3. Hormone levels. Unlike mestranol treatment, CR treatment did not significantly alter gynecological hormone levels [prolactin, luteinizing hormone (*LH*) or follicle stimulating hormone (*FSH*)]. *, $P \leq 0.001$ *versus* CR control (ANOVA + Dunnett's Test).

CR extract I, II, III) with respect to total tumor count as well as malignancy (Table 1). Of the mestranol animals, 86% showed malignant tumor growth, compared with the 50% in the control group and 50%, 64%, and 47% in the three CR-extract groups (I, II, III), respectively. The number of malignant tumors per animal was significantly higher in the mestranol group compared with the other four groups ($P < 0.01$; Mann-Whitney, exact test, two-sided). Unlike the CR-treated and control animals, 50% ($n = 7$) of the mestranol-treated animals presented with four or more mammary tumors (Table 1). There were no differences between the CR-treatment and the control animals with respect to tumor number or malignancy. There was, however, a slight tendency toward reduced tumor growth in CR-treated rats.

A histopathological examination of the ovarian region, which confirmed a complete bilateral ovariectomy in all animals, revealed foreign body granulomas and low-grade round cell infiltrates in the ovarian region. Such findings are likely to be reactions to the suture material used after ovariectomy. The bilateral ovariectomies also resulted in atrophic uteri in the CR-vehicle control and CR-extract groups, an observation that is typical in estrogen-deficient animals. Conversely, the mestranol-treated group presented signs of hyperestrogenism, with hypertrophic uteri showing

Table 1 *Number of animals presenting with multiple malignant mammary gland tumors*

	No. of tumors per animal									Total no. of
Treatment group	0	1	2	3	4	5	6	7	8	malignant tumors, M
CR control	7	4	2	0	1	0	0	0	0	12
CR treatment										
0.714 mg/kg	7	4	2	0	0	1	0	0	0	13
7.14 mg/kg	6	3	4	1	0	0	0	0	0	14
71.4 mg/kg	8	3	4	0	0	0	0	0	0	11
Mestranol	2	3	0	2	2	2	0	1	2	50

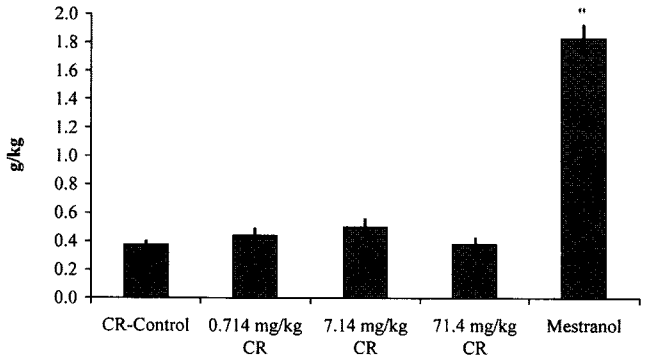

Fig. 4. Relative weight of uterus. Unlike mestranol treatment, CR treatment did not significantly affect uterus weight. KEY: **, $P \leq 0.001$ *versus* CR control (ANOVA + Dunnett's Test).

an increased incidence of endometrial squamous cell metaplasia and glandular-cystic hyperplasia.

The CR-extract and CR-control animals presented with a decreased uterine size, whereas the mestranol-treated animals had significantly increased absolute and relative uterus weight in comparison with the CR-control group (Fig. 4). Significant increases in the relative weights of the brain, spleen, kidneys, and pituitary gland were also noted in the mestranol group compared with the CR-control group; however, such observations may be the result of a severely decreased total body weight at necropsy. There were no significant differences between the organ weights of the control animals as compared with the CR-extract treated animals.

DISCUSSION

The results of this study confirm *in vitro* (11, 12), *in vivo* (33), and clinical findings (4, 14–16, 34) suggesting that an isopropanolic CR extract does not exert estrogenic activity on hormone-responsive mammary or uterine tissues. In this study, 6 weeks of CR-extract treatment, in doses up to 100-fold the human therapeutic dose, did not exert growth-promoting effects on DMBA-induced mammary tumors in ovariectomized Sprague Dawley rats. In addition, the CR extract had neither a direct effect on uterine tissue proliferation nor an indirect effect on pituitary-secreted, estrogen-regulated hormones. As a result, hypophyseal feedback mechanisms involving prolactin as an alternative regulatory stimulus for DMBA-induced rat mammary tumors do not play a role in our experimental setting. These findings have significant importance for the evaluation of CR extract as a safe alternative to HRT and phytoestrogens, such as soy, which have been reported to exert estrogen-agonistic activities (2), for use in patients with a history of breast cancer.

As shown in other studies (35, 36), ovariectomy, which results in a loss of estrogen secretion, causes a clear tumor regression in all groups. As would be expected with the therapeutic replacement of estrogen (37, 38), the mestranol-treated group showed a significant growth of the mammary tumors. In addition to significantly promoting cell growth, the mestranol treatment also caused a higher prevalence of multiple mammary tumor growth and malignant adenocarcinomas as compared with the CR-treated and control animals. Furthermore, estrogen replacement resulted in an increase in uterus size, increased prolactin and decreased luteinizing hormone, and follicle stimulating hormone blood plasma levels in comparison with the other four treatment groups.

On the basis of the changes in the mestranol-treated animals compared with the CR-extract and control groups, we can verify the sensitivity of our experimental model in the measure of estrogenic parameters. Although our model clearly illustrates the effect of estrogen replacement on the growth of mammary tumors and the uterus, we did not find evidence of similar "estrogen-like' adverse events or dose-dependent effects after CR-extract treatment, thus indicating the lack of estrogenic action of CR on the respective target organs.

The conclusion that an isopropanolic CR extract does not exert estrogen-agonistic effects on female reproductive tissues is supported by a study on the uterine growth of immature mice and proliferation of the vaginal epithelium of ovariectomized rats (33). *In vitro* studies of estrogen-receptor and progesterone-receptor positive cell lines, MCF-7, MDA- MB-435S, T-47D, further support these findings (12, 34).

Further research is clearly warranted to fully understand the mechanism of action of CR. In our study, no significant differences could be found between the CR-treated animals and animals from the control group. In fact, the data suggest a trend toward reduced tumor growth in the CR-treated animals, further supporting that the CR extract studied does not promote further development of malignant mammary tumors as has been shown consistently for estrogen. Further research, however, is needed to determine the effect of CR extracts before the development of mammary tumors.

In summary, the treatment of ovariectomized Sprague Dawley rats with CR extract yielded no evidence of estrogenic effects on estrogen-sensitive hormone levels, the endometrium, or the growth of DMBA-induced mammary tumors. These data, in conjunction with the previously reported *in vitro*, *in vivo*, and clinical trial data, support the safe use of CR in estrogen-sensitive patients for which HRT is contraindicated.

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