

Estrogenic effects of silymarin in ovariectomized rats

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ABSTRACT: The objective of this study was to test whether silymarin induces changes indicative of estrogenic effects in gonadal organs of ovariectomized (OVX) rats. Silymarin was administered in two experimental groups of OVX rats ($n = 7 + 7$) for 30 days at the doses of 25 or 50 mg per animal per day. OVX rats ($n = 7$) receiving 5 µg of 17β-estradiol (E_2) for the last three days before killing and untreated OVX rats ($n = 7$) were used as the positive and the negative controls, respectively. Uterine and blood samples were collected immediately after killing. Compared with the negative controls, total and normalized uterine weights were significantly higher in the two experimental groups ($P < 0.01$ and $P < 0.05$, respectively). Uterotrophic effects of silymarin were also evident from increased heights of the luminal epithelium ($P < 0.01$) and the endometrium ($P < 0.05$). The response was dose-independent within the tested range. The strongest uterine response was observed in the OVX rats treated with E_2 . A highly significant decrease in mean density of estrogen receptor (ERα) immunostaining in the luminal and the glandular endometrial epithelia ($P < 0.01$) and a stronger ERα immunostaining in stromal cells were observed in the two experimental and the positive control groups. The activities of alkaline and acid phosphatases were significantly increased in the luminal ($P < 0.05$) and the glandular ($P < 0.01$) epithelia only in the rats treated with E_2 . Both silymarin and E_2 induced an increase in thyroid hormone concentrations in blood serum. The rises of free T_3 and T_4 were significant ($P < 0.05$) in the group receiving 50 mg of silymarin per day. Hepatic oxidative metabolism of steroids was assumed to be another target of the action of silymarin. The mitochondrial cytochrome P450-dependent testosterone hydroxylase activity and the cytochrome P4501A-dependent 7-ethoxyresorufin O-deethylase activity were significantly increased ($P < 0.05$) in the group receiving 50 mg of silymarin per day and in the E_2 -treated control group, respectively. However, the modulations of the CYP enzymes played only a minor role in the overall estrogenic effect of silymarin. Histological and functional alterations in the OVX rats treated orally with silymarin for 30 days were consistent with those seen in E_2 -treated rats and were indicative of estrogenic effects of silymarin.

Keywords: silymarin; rat; uterus; estrogenic effect; estrogen receptor; cytochrome P450; thyroid hormones

INTRODUCTION

Silymarin is a mixture of flavonoids extracted from seeds of milk thistle (*Silybum marianum* [L.] Gaertn.) which contains silybin, silydianin, and silychristin as the major fractions (Morazzoni and Bombardelli, 1995). Extracts of *S. marianum* have been used in the treatment of liver diseases for more than 2 000 years. It has been established that the hepatoprotective effects of silymarin results from a strong antioxidant activity (inhibition of generation and scavenging of free radicals, inhibition of lipid peroxidation in cell membranes), in the stimulation of RNA-polymerase and biosynthesis of cell proteins, and in a strong inhibition of enzymes catalyzing the production of leukotrienes and prostaglandins, such as 5-lipoxygenase and cyclooxygenase (Valenzuela and Garrido, 1994; Robak and Gryglewski, 1996; de Groot and Rauen, 1998).

Flavonoids are a large group of polycyclic phenols of plant origin exerting multiple effects on mammalian organisms. So far more than 4 000 flavonoids have been identified (de Groot and Rauen, 1998). Intensive attention has been paid to flavonoids displaying estrogenic effects (nonsteroidal estrogens, phytoestrogens). Similarity of their chemical structure with that of estradiol (Miksicek, 1995) allows them to bind to and activate estrogen receptor (ER) of mammalian target cells. Because of their low binding affinity, they are classified as “weak estrogens” with a biological activity on the order of 10^{-2} to 10^{-5} that of 17β-estradiol. Nevertheless, long-term intake of feeds with a higher content of phytoestrogens can induce transient or permanent infertility. A typical example thereof is the ovine estrogenization syndrome, also known as “clover disease”, which massively occurred in western Australia (Bennetts *et al.*, 1946) and was caused by a

high content of phytoestrogens in subterranean clover.

The estrogenic activity of flavonoids can be demonstrated by methods based on a comparison of effects of the tested substance with those of natural estrogens and expressed in terms of proliferation of gonadal tissues and increase of uterine weight (Santell *et al.*, 1997; Tinwell *et al.*, 2000), cell proliferation, stimulation of DNA synthesis, and protein pS2 expression in the estrogen-dependent line MCF-7 of human breast carcinoma cells (Zawa and Duwe, 1997; Breinholt and Larsen, 1998, Wang and Kurzer, 1998). Estrogenicity can further be tested by the method of competitive binding to ER using 17β -[^3H]estradiol (Miksicek, 1995; Kuiper *et al.*, 1998), by measurement of stimulation of β -galactosidase activity of yeasts with incorporated ER (Arnold *et al.*, 1996), or by methods detecting disruption of steroid synthesis or increased catabolism of steroids (Santti *et al.*, 1998; Machala and Vondráček, 1998).

Available data on possible estrogenic effects of silymarin or its biologically active components is rather scarce. Therefore, the aim of our study was to test whether oral administration of silymarin to ovariectomized rats can induce changes indicative of estrogenic effects.

MATERIAL AND METHODS

Animals and treatment scheme

Eleven-week-old female Wistar rats with a liveweight of 258 ± 6 g were ovariectomized under standard xylazine and ketamine anaesthesia 14 days before the treatment. The animals were kept at 20 to 24°C individually in separate rearing boxes in a facility with a 12-h day/night cycle and had free access to water and a commercial pelleted diet.

The rats were divided into four groups of seven and treated as follows:

- oral administration of 25 mg of silymarin (Sigma-Aldrich, Prague) per animal per day; this dosage corresponded to 96.9 mg and 74.6 mg per kg live weight per day at the beginning and at the end of the experiment, respectively;
- oral administration of 50 mg of silymarin per animal per day; this dosage corresponded to 193.8 mg and 149.2 mg per kg live weight per day at the beginning and at the end of the experiment, respectively;
- oral administration of 5 μg of 17β -estradiol (Sigma-Aldrich, Prague) on days –3, –2, and –1 before killing; this dosage corresponded to 15 μg per 1 kg live weight per day (positive controls);
- administration of oil vehicle alone (negative controls).

The tested substances were administered by gavage dissolved in 250 μl of corn oil. The rats were killed by

decapitation in ether anesthesia on day 30. The final live weight was 335 ± 7 g.

Sampling and test methods

Immediately after killing, the rats were necropsied and uteri were collected, stripped of adipose and connective tissues, and weighed. Samples of both uterine horns, intended for histological investigations, were fixed in 10% neutral buffered formaline and sections, prepared by the conventional paraffin technique, were stained with green Masson's trichrome. Samples for histochemical investigations were fixed in cold Baker's solution and cryosectioned. Alkaline (ALP) and acid (ACP) phosphatases were determined by the azocoupling method and nonspecific esterase (NSPE) by the indigogenic method (Vacek, 1990). Immunohistochemical detection of estrogen receptor, carried out in paraffin sections, was preceded by heat-induced antigen retrieval in a pressure cooker. The prediluted monoclonal anti-ER antibody 1D5 (reacting with ER α) and the visualization system EnVision TM+ (DAKO A/S, Denmark) with 3,3'-diaminobenzidine (Fluka Chemie, Germany) as the chromogen were used according to the instructions of the manufacturers. The sections were counterstained with hematoxylin.

The intensity of histochemical reactions was evaluated semiquantitatively by a single person using the following classification score: 0 – negative; 10 – weak; 20 – moderate; 30 – strong. The enzymatic activities in the luminal and glandular epithelia were expressed as means of reaction intensities in the supra-, peri-, and subnuclear cell parts. Structural changes (heights of luminal epithelium and endometrium) and the intensity of ER α immunostaining were determined by computer image analysis using the LUCIA G system (Laboratory Imaging, Prague).

Blood samples for endocrinological analyses were collected at decapitation. The concentrations of thyroid hormones (total and free T_3 and T_4) were determined by RIA using the kits supplied by Immunotech (Prague).

Hepatic tissue was homogenized and the microsomal fraction was separated by differential centrifugation (Machala *et al.*, 1998). Microsomes were stored at –80°C up to the analysis. The monooxygenase activity towards testosterone was determined after incubation with 250 μM testosterone (final concentration) at 37°C for 10 min by analysis of HPLC products (Reinerink *et al.*, 1991; Machala *et al.*, 1998). The activity of 7-ethoxoresorufin-O-deethylase (EROD) was determined fluorimetrically using 2 μM 7-ethoxoresorufine as the substrate (Prough *et al.*, 1978). Protein concentrations were determined by the bicinchoninic acid assay (Smith *et al.*, 1985). Testosterone and its monooxygenized products, resorufin, and bicinchoninic acid were sup-

plied by Sigma-Aldrich (Prague) and 7-ethoxyresorufin by Molecular Probes (Eugene, USA).

The results are presented as means \pm SEM. Significance of among-group differences in mean values was examined by the Student's *t*-test.

RESULTS

Oral administration of 25 or 50 mg of silymarin per animal per day to OVX rats for 30 days induced a significant increase in total (136.00 ± 5.93 mg and 134.57 ± 6.61 mg, respectively, vs. 107.71 ± 6.96 mg; $P < 0.01$) and normalized (40.60 ± 1.87 mg and 40.05 ± 1.33 mg, respectively, vs. 33.65 ± 2.25 mg; $P < 0.05$) uterine weight (Figure 1). The uterotrophic effects of silymarin were also evident from increases in the height of the luminal epithelium (12.47 ± 0.53 μ m and 12.79 ± 0.64 μ m, respectively vs. 8.85 ± 0.83 μ m; $P < 0.01$) and endometrium (291.83 ± 8.68 μ m and 271.06 ± 18.43 μ m, respectively, vs. 237.60 ± 19.22 μ m; $P < 0.05$). The differences between the two groups treated with different doses of silymarin were nonsignificant. The most pronounced uterine responses in terms of total and normalized weights (173.14 ± 13.05 mg and 49.86 ± 3.62 mg, respectively) and heights of luminal epithelium (14.18 ± 0.30 μ m) and endometrium (339.75 ± 19.70 μ m) were observed in the positive control group treated with 5 μ g of E_2 per day for the last three days before killing. All the differences between the positive and the negative control groups were highly significant and those between the positive control group and any of the experimental groups were significant ($P < 0.05$). Additional manifestations of estrogenic effects on endometrium, including hyperemia and edema of stroma and secretion of cells of the luminal and glandular epithelia, were also most marked in the positive control group.

The immunohistochemical response for ER α in the endometrium of OVX rats of all the groups was most pronounced in nuclei of the luminal and glandular epithelial cells (Figure 2A). Nuclear positive immunostaining was observed in all epithelial cells with only small individual variations in the intensity. On the other hand

the immunostaining intensity in stromal cells were not thus uniform. Positive ER α immunoreactivity was detected in only 40 to 80% of the cells and the intensity of immunostaining varied within a wider range. While both the experimental and the positive control groups showed a highly significant decrease ($P < 0.01$) in the mean density of immunostaining for ER α in the luminal and glandular epithelia (Figure 2B, Table 1), the mean density of immunostaining and the number of positive stromal cells, expressed as the ER+ nuclei area fraction of the total stromal area, were significantly higher in the two experimental groups ($P < 0.05$ and $P < 0.01$, respectively). No marked difference in the intensity of expression of ER α was observed between the groups treated with different doses of silymarin.

No salient changes in the activities of ALP, ACP, and NSPE were found in the groups treated with silymarin. Compared with the negative control group, the E_2 -treated group showed an increase of the index of ALP activity in the luminal epithelium from 14.5 ± 0.6 to 20.5 ± 1.7 ($P < 0.05$) and in the glandular epithelium from 15.9 ± 1.1 to 21.4 ± 0.7 ($P < 0.01$). The index of activity of ACP increased in the luminal epithelium from 16.0 ± 0.7 to 21.0 ± 1.9 ($P < 0.05$) and in the glandular epithelium from 11.2 ± 1.0 to 16.6 ± 2.2 ($P < 0.01$).

The administration of silymarin or E_2 to OVX rats resulted in an increase of thyroid hormone concentrations in blood serum (Table 2). A significant difference ($P < 0.05$) in comparison with the negative control group was found for the concentrations of free T_3 and free T_4 in the group receiving 50 mg of silymarin per day.

The administration of silymarin resulted in a slight increase in the hepatic oxidative catabolism of steroids. The increase was significant ($P < 0.05$) in the group receiving 50 mg of silymarin per day. More than 90 % of testosterone used as the substrate were degraded (data not shown herein) to 6 β -hydroxytestosterone and 4-androsten-3,17-dione. The EROD activity, which is specifically associated with the P4501A cytochromes, was not modulated by silymarin. A significant increase ($P < 0.05$) was found only in the E_2 -treated group. The data on testosterone hydroxylase and EROD activities in the hepatic microsomal fraction are presented in Figure 3.

Table 1. Intensity of immunostaining for ER α in the endometrium of OVX rats treated with silymarin for 30 days ($x \pm$ SEM)

Group ($n = 7$)	Mean density of ER+ nuclei			ER+ nuclei area fraction of the total stromal area (%)
	luminal epithelium	glandular epithelium	stroma	
Controls	7.30 ± 0.46^a	6.48 ± 0.82^a	5.91 ± 0.21^a	10.47 ± 0.71^a
Estradiol (5 μ g/d for 3 days)	5.05 ± 0.86^b	3.94 ± 0.85^b	6.57 ± 0.23	14.55 ± 1.26^c
Silymarin (25 mg/d)	5.23 ± 0.81^b	3.57 ± 0.17^b	6.59 ± 0.20^c	16.08 ± 1.20^b
Silymarin (50 mg/d)	5.50 ± 0.78^b	4.38 ± 0.87^b	6.63 ± 0.13^c	15.03 ± 1.87^b

ab = $P < 0.01$; ac = $P < 0.05$

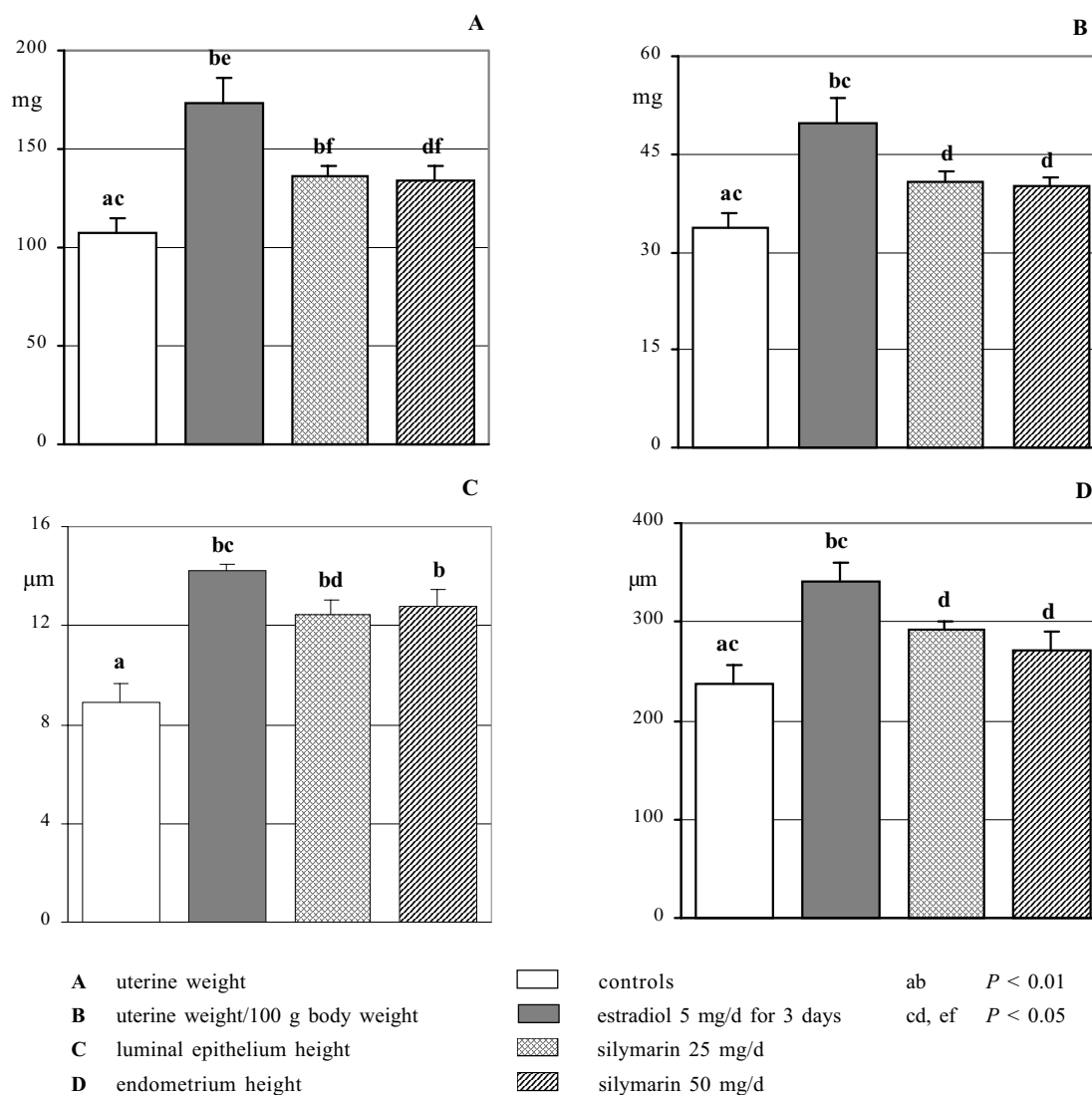


Figure 1. Uterine weight and structural alterations in the endometrium of OVX rats treated with silymarin for 30 days ($\bar{x} \pm \text{SEM}$)

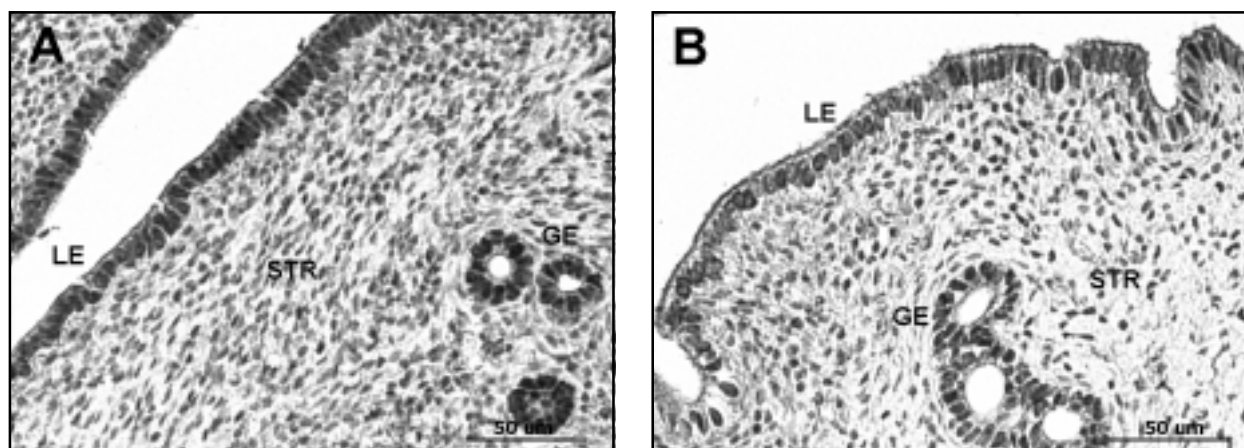


Figure 2. Immunohistochemical localization of ER α in OVX rat endometrium ($\times 400$)

A – Strong immunopositive reaction in nuclei of the luminal (LE) and glandular (GE) epithelia in negative control.; B – Weaker immunostaining in the luminal (LE) and glandular (GE) epithelia and stronger immunostaining for ER α in the stroma (STR) of rats treated with 50 mg of silymarin

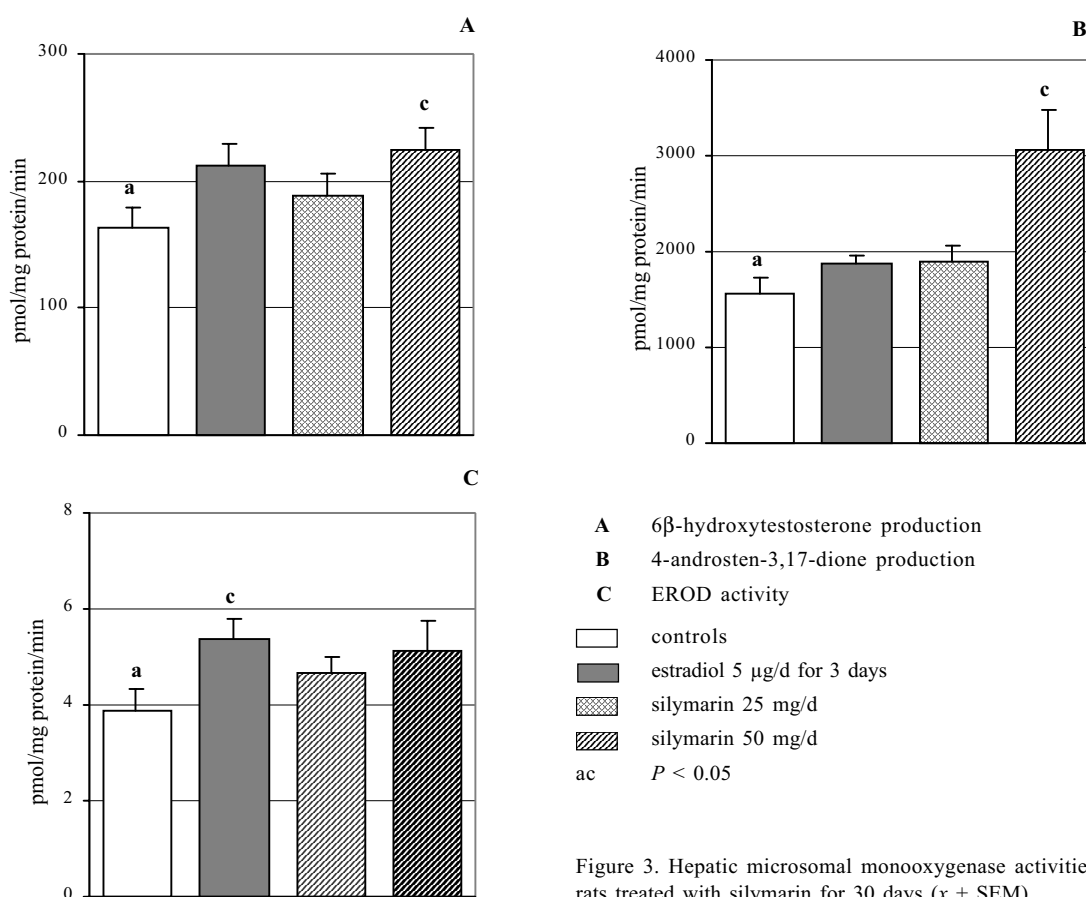


Figure 3. Hepatic microsomal monooxygenase activities in OVX rats treated with silymarin for 30 days ($\bar{x} \pm \text{SEM}$)

Table 2. Concentrations of thyroid hormones in blood serum of OVX rats treated with silymarin for 30 days ($\bar{x} \pm \text{SEM}$)

Group ($n = 7$)	Total T_3 (nmol/l)	Free T_3 (pmol/l)	Total T_4 (nmol/l)	Free T_4 (pmol/l)
Controls	0.91 ± 0.06	3.16 ± 0.27^a	41.87 ± 5.83	17.94 ± 1.99^a
Estradiol (5 µg/d for 3 days)	0.99 ± 0.05	3.76 ± 0.18	50.07 ± 4.31	21.78 ± 1.74
Silymarin (25 mg/d)	1.00 ± 0.08	4.01 ± 0.44	47.70 ± 4.50	22.62 ± 1.86
Silymarin (50 mg/d)	0.99 ± 0.06	4.02 ± 0.25^c	48.21 ± 0.74	23.43 ± 0.80^c

ac = $P < 0.05$

DISCUSSION

Our experiments in OVX rats demonstrated readily detectable uterine responses to silymarin administered orally for 30 days. Significant increases of uterine weight and endometrial height, as well as hypertrophy of the luminal epithelium, have been established as reliable indexes of estrogenic effects (Galand *et al.*, 1971; Korach and McLachlan, 1995; Tinwell *et al.*, 2000). The most pronounced uterotrophic effects were observed in the rats treated with E_2 .

Recent studies (Hiroi *et al.*, 1999; Wang *et al.*, 1999; Nephew *et al.*, 2000) indicate that the dominant receptor subtype in the rat endometrium is $ER\alpha$, which is localized

in nuclei of the luminal and glandular epithelium and in the stroma. The effect of E_2 on uterine ER in OVX rats is apparently biphasic. While the expression of the $ER\alpha$ protein is inhibited by E_2 during the first 24 hours after administration, immunostaining becomes more pronounced thereafter. Owing to the differences in experimental models, dosage of E_2 , and antibodies to $ER\alpha$ used in our experiments, a direct comparison of results is rather difficult. A significant decrease in the intensity of immunostaining for $ER\alpha$ in uterine epithelial cells was observed both in the groups treated with silymarin and in the positive control group treated with E_2 . On the other hand, both treatments increased the intensity of immunostaining for $ER\alpha$ and the number of ER-positive cells in the stroma.

Mathur (1986) demonstrated that the increase in intensity of histochemical reactions for ALP in the endometrial epithelium in rats treated with selected plant extracts was an expression of estrogenic effects. In our experiments, significant increases in the activities of ALP and ACP were observed in the E_2 -treated group, but not in any of the experimental groups.

Estrogens are known to markedly modulate pituitary and thyroid functions. While ovariectomy in rats is followed by a suppression of thyroid functions (Gladkova and Karpenko, 1991), treatment with estrogens was found to decrease the intrapituitary amount of TSH and the serum T_4 concentration, and, at higher doses, to increase significantly the serum T_3 concentration. All the effects depended on dosage and length of the treatment period (Chen and Walfish, 1978; Boado *et al.*, 1983). It has been demonstrated (Lisboa *et al.*, 1997) that higher doses of estradiol strongly stimulate the activity of thyroid enzymes (5'-deiodinase) which are responsible for the generation of T_3 . In our experiments, the treatments with either silymarin, or E_2 , increased the serum concentrations of all thyroid hormones. However, this effect was significant only for free T_3 and T_4 and only in the group treated with the higher dose of silymarin.

Our study of possible effects of silymarin on the modulation of xenobiotic- and steroid-metabolizing enzymes demonstrated an increase in oxidative metabolism of steroid hormones in hepatic tissue in the group treated with the higher dose of silymarin only. An increase in the EROD activity, which is also indicative of increased hydroxylation of estrogens (Davis *et al.*, 1997), was observed in the group treated with E_2 . Although the steroid-transformation enzymes are known to be the critical regulators of *in vivo* steroid action (Song and Melner, 2000), the contribution of the modulation of steroid metabolism to the overall estrogenic effects of silymarin in OVX rats was minor only.

No quantitative data on *in vitro* estrogenic effects of silymarin have been published so far. Stimulatory effects of silybin, which is the principal component of silymarin, on ribosomal RNA and DNA synthesis resulting from direct interaction with RNA-polymerase were demonstrated repeatedly (Sonnenbichler and Zetl, 1986; Sonnenbichler *et al.*, 1999) and binding affinity of silybin to the ER was confirmed in an *in vitro* study (Sonnenbichler and Zetl, 1988).

These data encouraged us to assess estrogenic effects of *S. marianum* seed cake which is used as a supplement to rations for postparturient dairy cows to prevent the development of liver steatosis. In an earlier experiment, rations fed to OVX dairy cows were supplemented with 500 g of *S. marianum* seed cake, containing 4.1% of silymarin, for 28 or 56 days. The treatment resulted in endometrial alterations indicative of a weak estrogenic effect. Prolonged supplementation induced alterations which are typical of persisting follicular

cysts, including cystic uterine glands, hyperplasia of stroma, and proliferation of blood vessels with hyperplastic walls (Kummer *et al.*, 2000). Possible effects of additional biologically active components that may interfere with silymarin must be considered when feeding *S. marianum* seed cake (Morazzoni and Bombardelli, 1995).

Our study in OVX rats treated orally with silymarin for a period of 30 days demonstrated the development of histological and functional uterine alterations which were similar to those induced by oral treatment with E_2 . Findings typical of estrogenic effects included an increase in uterine weight and hypertrophy of the luminal epithelium. The estrogenic effects of silymarin were evident also from significant changes in the expression of ER and in concentrations of thyroid hormones in blood serum. The findings in the experimental groups were consistent with the dynamics of alterations observed in the control group treated with E_2 .

The experimental model used in our study allowed us only a tentative quantitative assessment of estrogenic effects of silymarin. The results indicate that the uterotrophic effects of daily doses of 25 or 50 mg of silymarin administered for 30 days were weaker than in the positive control group treated E_2 . It can therefore be estimated that the estrogenic activity of silymarin is approximately 10 000 to 25 000 times lower than that of E_2 and corresponds to those of the isoflavones genistein and daidzein (Farmakalidis *et al.*, 1985).

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