Silymarin BIO-C®, an extract from *Silybum marianum* fruits, induces hyperprolactinemia in intact female rats

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**Abstract**

Breastfeeding is widely acknowledged to have important health benefits for infants and mothers. Milk thistle (*Silybum marianum* fruits) has been recently proposed to be used by nursing mothers for stimulating milk production; however, the mode of action of this herbal drug is still unknown. In this paper, we have evaluated the effect of a micronized standardized extract of *S. marianum* (Silymarin BIO-C® = Piülette®) on the serum levels of prolactin in female rats. A 14-day treatment with Silymarin BIO-C® (25–200 mg/kg, given orally) increased, in a dose dependent manner, the serum prolactin levels. Moreover, after a 66-day discontinuation of Silymarin BIO-C® treatment, prolactin levels were still significantly elevated although we observed a trend to decrease that was counteracted by a further 7-day treatment with Silymarin BIO-C®. Bromocriptine, a dopamine D<sub>2</sub> receptor agonist, (1–10 mg/kg, os) significantly and in a dose dependent manner, reduced the serum prolactin levels; bromocriptine, at the dose of 1 mg/kg, significantly reduced the high serum prolactin levels induced by Silymarin BIO-C®. In conclusion, we have shown that an extract from *S. marianum* fruits significantly increases circulating prolactin levels in female rats; this effect seems to involve, at least in part, dopamine D<sub>2</sub> receptors.

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**Keywords:** Milk thistle; *Silybum marianum*; Silymarin; Prolactin; Lactation; Galactogen

**Introduction**

Breastfeeding is widely acknowledged to have important health benefits for infants and mothers. Early benefits include reduced mortality in preterm infants, reduced infant morbidity from gastro-intestinal, respiratory, urinary tract and middle-ear infections and less atopic illness (Britton et al. 2007; Hoddinott et al. 2008; Katona and Katona-Apte 2008). As well as health benefits to infants, breastfeeding has an impact on maternal health too; indeed, studies have demonstrated a low incidence of breast cancer, ovarian cancer and hip fractures in those women who have breastfed (Labbok 2001; Danforth et al. 2007; Shantakumar et al. 2007; Ip et al. 2007). The World Health Organization (WHO) recommends exclusive breast feeding (breast milk only, with no water, other fluids, or solids) for six months, with supplemental breast feeding continuing for two years and beyond (WHO 2003). During pregnancy, high circulating concentrations of estrogen promote prolactin production which initiates the growth of the mammary glands and triggers production of milk (lactation). After childbirth, estrogen and progesterone levels decrease and the production of prolactin declines; nipple sucking then promotes prolactin release which reaches a peak
about 1–3 weeks after childbirth. Some factors such as preterm delivery, alcohol abuse and use of drugs, can lead to an insufficient milk production (Mennella 2001; Ilett and Kristensen 2005); moreover, it has been estimated that about 5% of women experience primary lactation insufficiency (Neifert 2001). Considering the importance of breastfeeding for mothers and infants, galactogens of natural or synthetic origin are recommended to stimulate milk production. Among herbal drugs, *Silybum marianum* is traditionally used to stimulate milk production (Barnes et al. 2002)

*Silybum marianum* (L.) Gaertn (Family Compositae), commonly known as milk thistle or lady’s thistle, is a herbaceous plant, native to the Mediterranean countries. Extract of *S. marianum* fruits and its active substance silymarin (standardized mixture of flavanoids) have been shown to exert hepatoprotective, anti-inflammatory, antioxidant and choleretic properties (Gupta et al. 2000; Giese 2001; Nencini et al. 2007). Traditionally, *S. marianum* fruits have been used by nursing mothers for stimulating milk production (Newall et al. 1996) and recently it has been demonstrated that milk thistle increases lactation in cows (Tedesco et al. 1996) and women (Carotenuto and Di Pierro 2005). However, the biochemical mechanisms that led to its effects on lactation have not yet been established. Since prolactin is the principal lactogenic hormone, we have evaluated the concentration of circulating prolactin in female rats treated or not with silymarin. In our experiments we have used a micronized extract of milk thistle (Silymarin BIO-C® = Piulatte®).

Material and methods

Animals

Wistar female four-days cycling rats (11–15 weeks old) weighting 200–225 g were used (Harlan, Italy). They were caged individually, isolated from males, and maintained in a room with a light/dark period of 12L:12D, at a temperature of 23±2°C and a relative humidity of 50±2%. Animals had free access to standard food, purchase from Mucedola Mangimi (Settimo Milanese, Italy) and water *ad libitum*. Rats were allowed to acclimate and all experiments complied with the Italian D.L. no. 116 of 27 January 1992 and associated guidelines in the European communities Council Directive of 24 November 1986 (86/609/ECC).

Drug administration and serum preparation

After oestrous-guided selection, female rats were treated with Silymarin BIO-C® at the doses of 25–200 mg/kg (orally) or metoclopramide at the dose of 2.2 mg/kg (intraperitoneally) (drug used as a positive control). Vehicles (saline or water), Silymarin BIO-C® and metoclopramide were administered daily for 14 days. Some animals after a 66-day break from Silymarin BIO-C (200 mg/kg, *os*) or metoclopramide (2.2 mg/kg, *ip*) treatment were re-treated with this drugs for others 7 days. The body weight of rats was measured on the first day of treatment and after 14 days of treatment. In some experiments, animals were treated orally with bromocriptine at the doses of 1–10 mg/kg. In another set of experiments the animals were randomly divided into three groups as follow: group 1 was treated with vehicles; group 2 was extract (consisted of 46.5% silybin, 16.3% isosilybin and 37.2% silydianin plus silychristin, provided by Indena S.p.A., Milan, Italy) subject to micronization to increase the biodisponibility of the drug. Bromocriptine mesylate salt and metoclopramide hydrochloride were purchased from Sigma (Milan, Italy). Silymarin BIO-C® and metoclopramide were dissolved in water and saline (0.9% NaCl), respectively; bromocriptine was suspended in 0.5% sodium carboxymethylcellulose. The vehicles (200 μl/rat water or carboxymethylcellulose and 100 μl/rat saline) had no significant effects on the responses under study.

Oestrous cycle induction and evaluation

The synchronization of oestrous cycle in sexually mature rats was induced using the Whitten effect (Whitten 1956; Whitten et al. 1968). It consists in the stimulation of oestrous cycle positioning female rats individually caged nearby a male rat; in particular this method utilizes the capacity of inducing oestrous through the closeness of male rat pherormone-laden urine.

Stage of oestrous cycle was determined by daily examination of fresh vaginal smears as previously described (van Zutphen et al. 1993). The vaginal smears were scored according to the following classification: vaginal epithelial and keratinized cells were considered to indicate oestrous phase (E); traces of secretory material with cellular debris, few intact cells and few leucocyte were considered to indicate dioestrous phase (D); presence of intermediary cells, with rare parabasal cells and leucocytes were considered pro-oestrous phase (P); presence of leucocytes, often in large numbers, and intermediary cells were considered to indicate metoestro phase (M).
treated with Silymarin BIO-C® (200 mg/kg, os for 14 days); group 3 was treated with Silymarin BIO-C® (200 mg/kg, os for 14 days) plus bromocriptine (1 mg/kg, os, on day 14, three hours before the collection of the bloods).

After nitrous oxide/isoflurane anaesthesia, blood samples were collected from rats at the beginning and the end of treatment (corresponding to days 1 and 14), after 14 and 66 days break period from drugs administrations and after others 7 days period of treatment (corresponding to day 87). For the experiment with bromocriptine alone, the blood samples were collected on day 1 (before the treatment with bromocriptine) and on day 2 (three hours after the administration of bromocriptine). Briefly, through intracardiac injection, blood (about 500 μl) was transferred in sterile 2 ml plain centrifuge tubes and allowed to clot at 4°C for 1 hour. Subsequently blood samples were centrifuged at 1200 g for 5 min at 4°C. Supernatants still containing platelets and white/red blood cells fractions, were transferred in new sterile 2 ml plain centrifuge tubes and subjected to a second centrifugation at 1200 g for 5 min a 4°C. The supernatants (serum) were collected and stored at −80°C until use.

The blood samples collection was performed early in the morning (9.00 am) according to the circadian rhythm of prolactin (Kawakami and Arita 1981), while the drugs administration (Silymarin BIO-C® or metoclopramide) was performed later in the morning (2.00 pm).

**Serum rat prolactin determination**

Serum prolactin levels were measured using an EIA kit (Spi-Bio Bertin Group, France). This colorimetric assay is based on the competition between serum rat prolactin and acetylcholine linked to rat prolactin for limited specific rabbit anti-rat prolactin antiserum sites (Duhau et al. 1991). Results are expressed as ng of prolactin per ml of rat serum.

**Statistical analysis**

Results are expressed as mean±S.E.M. of n experiments. The analyses of results were carried out using GraphPad Prism Software (San Diego, USA). Comparisons between two sets of data were made by Student’s test for paired data. When multiple comparisons against a single control were made, one-way analyses of variance (ANOVA) was used, followed by Turkey–Kramer multiple comparisons test. A p value less than 0.05 was considered significant.

**Results**

**Effect of Silymarin BIO-C® on body weight**

At the start of the experiments the body weights (grams) of control female rats, Silymarin BIO-C® (SI, 25–200 mg/kg) or metoclopramide (ME, 2.2 mg/kg) treated animals were: control 223.7±3.7 g, SI 25 mg/kg 220.3±2.1 g, SI 50 mg/kg 225.7±3.1 g, SI 100 mg/kg 228.6±2.9 g, SI 200 mg/kg 230.5±4.2 g, ME 2.2 mg/kg 219.7±3.7 g. After 14 days of treatment, the increase in animals body weight was significantly more pronounced in the Silymarin BIO-C® (25–200 mg/kg) groups than in the control or metoclopramide (2.2 mg/kg) groups (Fig. 1).

**Effect of Silymarin BIO-C® on serum prolactin levels**

No significant differences in the basal levels of prolactin in the serum of female rats untreated or treated with Silymarin BIO-C® (SI, 25–200 mg/kg, os) or metoclopramide (ME, 2.2 mg/kg) were observed (Prolactin ng/ml: Control 22.2±0.60, SI 25 mg/kg 21.9±0.59, SI 50 mg/kg 22.4±0.45, SI 100 mg/kg 20.9±0.60, SI 200 mg/kg 23.1±0.52, ME 2.2 mg/kg 22.9±0–58). The treatment of animals for 14 days with Silymarin BIO-C® increased, in a dose dependent manner, the serum levels of prolactin (Fig. 2).
Significant increase in prolactin levels was observed at the Silymarin BIO-C® doses of 50, 100 and 200 mg/kg. Metoclopramide, used as a positive control, administered for 14 days at the dose of 2.2 mg/kg significantly \((p<0.01)\) increased the serum prolactin levels (Fig. 2). The stimulatory action of Silymarin BIO-C® on prolactin levels remained 66 days after the drug treatment discontinuation (Fig. 3). By contrast, 14 days after the interruption of metoclopramide administration, the prolactin levels showed values similar to the control (Fig. 3). A re-treatment of the animals for seven days with Silymarin BIO-C® (200 mg/kg, orally) after the break of 66 days restored prolactin high levels (Fig. 3).

**Effect of bromocriptine on serum prolactin levels**

Bromocriptine, a dopamine D2 receptor agonist, (BR, 1–10 mg/kg, orally) significantly and in a dose dependent manner, reduced the serum prolactin levels in the rats (prolactin ng/ml: control 21.0±0.57, BR 1 mg/kg 21.4±0.56, BR 3 mg/kg 19.6±0.42, BR 6 mg/kg 15.3±0.36, BR 10 mg/kg 9.2±0.32; \(n=6\), \(p<0.01\) at the 6 mg/kg dose and \(p<0.001\) at the 10 mg/kg dose).

Bromocriptine, given orally at the dose of 1 mg/kg, significantly \((p<0.01)\) reduced the high serum prolactin levels induced by Silymarin BIO-C® (Fig. 4).

**Discussion**

In terms of health, the benefits of breast feeding for both the mother and the baby are well known. Therefore, a number of botanical remedies (e.g. anise, fennel, fenugreek seed, nettle and milk thistle) have been traditionally used to stimulate milk production (Capasso et al. 2003). Recently, the observation that an extract of milk thistle increases lactation in cows...
(Tedesco et al. 2004) and women (Carotenuto and Di Pierro 2005) has gained renewed interest in its galactogenic properties. However, the mode of action of S. marianum is still elusive.

In the present study we have demonstrated that a micronized extract of S. marianum (silymarin BIO-C® = Piùlatte®) increases prolactin levels in female rats too. More importantly, we have shown an unprecedented involvement of dopamine D2 receptor in Silymarin BIO-C® galactogenic action.

Previously studies have shown an increased body weight in hyperprolactinemic female (but not male) rats (Rehm et al. 2007). The effect is probably due to changes in metabolic activity. Consistently, we have here reported that Silymarin BIO-C® increased, in a dose-related manner, rats body weight. Interestingly, the increase in body weight has not been observed in animals treated with the D2 receptor antagonist metoclopramide.

Prolactin, a hormone produced by the pituitary gland, enhances two activities in the breast such as stimulation of breast cells growth during pregnancy and stimulation of milk production while nursing an infant (Grattan et al. 2001; Grattan 2002; Ben-Jonathan et al. 2006). Therefore, drugs stimulating prolactin production are generally used to enhance milk production (Gabay 2002). Our experiments have shown that Silymarin BIO-C®, similarly to metoclopramide (a drug with galactogenic properties), increases serum prolactin levels in female rats after a 14-day daily treatment; these results could explain the increased milk production previously observed in cow and humans (Tedesco et al. 2004; Carotenuto and Di Pierro 2005). Moreover, our results revealed that after a 66-day discontinuation of Silymarin BIO-C® treatment, prolactin levels were still significantly elevated although we observed a trend to decrease that was counteracted by a further 7-day treatment with Silymarin BIO-C® (Fig. 4). In contrast to Silymarin BIO-C® treatment, the effect of metoclopramide stopped 7 days after the discontinuation of the treatment.

To further characterize the action of Silymarin BIO-C®, we analyzed the effect of the botanical extract in the presence of bromocriptine, a selective dopamine D2 receptor agonist, which is known to reduce serum prolactin levels (Mancini et al. 2008; Chanson et al. 2007; Colao et al. 2002). As expected, we have shown that oral-administered bromocriptine reduced significantly and in a dose dependent manner serum prolactin levels in female rats. More importantly, a per-se non effective dose of bromocriptine – i.e. a dose (1 mg/kg) of bromocriptine which did not cause hypoprolactinemia – was able to reduce the hyperprolactinemia induced by Silymarin BIO-C® thus suggesting an involvement of the dopamine D2 receptor in the Silymarine BIO-C® action.

In conclusion, we have shown that Silymarin BIO-C® is able to produce a significant increase in circulating prolactin levels in female rats after oral administration. The effect could involve, at least in part, dopamine D2 receptor. Since the levels of prolactin remain elevated for up to 66 days after Silymarin BIO-C® discontinuation and in view of its safety records in humans (an average daily dose of about 420 mg/day for 41 months was found to be non-toxic, relative to placebo, in clinical trials) (Rainone 2005; Tamayo and Diamond 2007), Silymarin BIO-C® can be considered a good candidate for the treatment of lactation insufficiency.

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References


