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The effect of a phytoestrogen, genistein, on the hormonal and metabolic status of pregnant rats*

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ABSTRACT

Genistein, a plant-derived compound, is a phytoestrogen and has a quite well-documented effect on carbohydrate and lipid metabolism in animals. Data is lacking, however, about the influence of this compound when the level of endogenous oestrogens is high, for example in pregnancy. The aim of the performed experiment was to investigate the effect of dietary genistein (100 mg/kg of feed) on some blood hormone concentrations and metabolic parameters in pregnant rats. Two groups of pregnant Wistar rats were used: the animals in one group were fed a diet without genistein and in the other, a genistein-supplemented diet. Animals from both groups were sacrificed on day 13 or 18 of gestation or on the first day after delivery. Additionally, one group of control non-pregnant rats was fed a diet without genistein and used to compare with pregnant rats. The blood sera, livers and skeletal muscles of animals were collected and stored (-80 °C) until analysis.

Genistein caused a substantial decrease of the serum leptin concentration on day 18 of gestation and of leptin and ACTH concentrations on the first day after delivery. The serum insulin, glucagon and corticosterone concentrations remained unaffected by genistein. The tested compound did not influence serum glucose, triglycerides, free fatty acids, cholesterol or liver and muscle glycogen, triglycerides and cholesterol. These results suggest that genistein, despite its well-described oestrogenic potency, can not manifest its action during pregnancy because of high levels of endogenous oestrogens.

KEY WORDS: genistein, hormones, metabolism, pregnancy

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INTRODUCTION

Genistein, a well known isoflavonoid, belongs to phytoestrogens, compounds that demonstrate oestrogenic activity. Its oestrogenic potency is significant, especially for oestrogen receptor β. Soya, the richest source of this isoflavonoid, may contain up to 250 μg of genistein per gram of soya protein (Dixon and Ferreira, 2002). High concentrations of genistein appear in the urine after consumption of a soya-rich diet (King et al., 1996). The consumption of isoflavone-rich products can account for the numerous biological effects attributed to oestrogens. Shutt and Cox (1972) discovered that reproductive disorders and infertility in sheep consuming clover are evoked by two isoflavones, genistein and daidzein. Since that time, many laboratories have investigated the influence of dietary isoflavones on reproductive processes in animals in relation to their oestrogenic potency. Currently, it is acknowledged that genistein influences many physiological and biochemical processes, moreover, its action also manifests in an oestrogen-independent manner (Szkudelska et al., 2003). Many of the effects exerted by genistein are considered to be health protective and are well documented in the literature. Therefore, products containing large amounts of genistein are desirable dietary components.

Recently, several papers were published concerning the effects of genistein in very young organisms. It was demonstrated that the fetus and neonate are sensitive to oestrogen exposure (Faber and Hughes, 1993; Nagao et al., 2001). Genistein, as a phytoestrogen, may also evoke oestrogen-related changes in developing organisms. Prenatal and neonatal exposure to this compound can affect sexual differentiation. The prenatal treatment of female rats with genistein decreases birth weights, reduced the anogenital distance and delays the onset of vaginal openings. Moreover, genistein-treated females have delayed puberty (Levy et al., 1995). Subcutaneous injections of a small (10 μg) dose of genistein to rat neonates caused increased pituitary response to gonadotropin releasing hormone, while treatment with high doses (500-1000 μg) of this compound resulted in an increased volume of the sexually dimorphic nucleus in the preoptic area of hypothalamus (Faber and Hughes, 1993). Oestrus cycle irregularities and fertility disorders in female rats are also consequences of neonatal exposure to genistein (Nagao et al., 2001). Hughes et al. (2004) demonstrated that dietary genistein can modify the expression of the oestrogen-regulated progesterone receptor in the uterus of sexually mature rats during pregnancy and lactation and, consequently, may have long-term reproductive health consequences. There are also some positive aspects of genistein in young animals. It was found that perinatal exposure to this isoflavonoid may protect against mammary cancer in rats. The anticancerogenic effects of genistein in adults are well known and are considered oestrogen-independent (Wang and Kurzer, 1998).

Although many researchers have focused their attention on the effects of genistein on foetuses and neonates, little is still known about its influence on pregnant animals. Only sporadic reports have appeared concerning this problem. It has recently been documented that genistein evokes endocrine and metabolic changes. These effects were, however, investigated predominantly in normal or ovariectomized rats (Nogowski et al., 1998, 2002). The aim of the presented study was to determine whether dietary genistein could affect serum insulin, leptin, glucagon, corticosterone and adrenocorticotropic hormone concentrations and some carbohydrate and lipid parameters in pregnant rats.

MATERIAL AND METHODS

Sixty-three female Wistar rats with an initial body weight of 190±10 g were used in the experiment. The animals were kept at constant temperature (22±1°C) with a 12-h dark-light cycle in an air-conditioned room. Feed and water were provided *ad libitum*. Females were mated overnight and when spermatozoa were detected in the vaginal smear at the morning, this day was treated as the first day of pregnancy. Each pregnant rat was housed individually in a cage. The pregnant animals were divided into group fed a diet without natural phytoestrogens and a group of rats fed a diet with genistein. Moreover, a non-pregnant control group of rats was kept. Each group consisted of nine rats. Genistein was added to the diet in the amount of 100 mg/kg of feed. The animals were fed the control or genistein-supplemented diets for the entire pregnancy. Rats were sacrificed on day 13 (n=9) and 18 (n=9) of gestation and on the first day after delivery (n=9) and their blood serum, liver and muscle (*m. biceps femoris*) samples were collected and stored (-80ºC) until analysis. Serum hormones were assayed radioimmunologically using kits specific for rat hormones: insulin, leptin, glucagon from Linco Research Inc. (USA); corticosterone, MP Biomedicals, LLC (USA); adrenocorticotropic hormone, DSL (USA). Glucose was determined photocolorimetrically by means of an enzymatic method with glucose oxidase and o-dianisidine. Free fatty acids were assayed by the method of Duncombe (1964), serum triglycerides were measured according to Foster and Dunn (1973), and serum cholesterol, by the enzymatic method of Richmond (1973). HDL-cholesterol was assayed in serum after separation of high density lipoproteins using polyethylene glycol (PEG 6000). Liver and muscle glycogen content was determined as described previously (Szkudelska et al., 2003). Liver and muscle triglycerides and cholesterol were determined similarly as in serum after extraction of total lipids using the method of Folch et al. (1975). Solvent was evaporated from obtained extracts before cholesterol was determined. The experiment was performed according to the rules accepted by the Local Ethics Committee for Investigations on Animals.

All results were evaluated statistically by analysis of variance and Duncan's multiple range test at $P \leq 0.05$.

RESULTS

In pregnant rats fed a diet without genistein, some changes in serum concentrations of hormones were found: decreased insulin concentration on the first day after delivery, increased glucagon concentration on day 18 of gestation and on the first day after delivery, and increased leptin concentration on day 18 of gestation (Table 1). Genistein had no effect on serum insulin on days 13 and 18 of gestation and on the day after delivery. The serum concentration of glucagon was not affected by genistein in any treated group. Serum leptin was unchanged on day 13 of gestation by genistein but on day 18 and on the first day after delivery, this hormone's concentration was significantly lower in isoflavonoid-treated animals. The adrenocorticotropic hormone (ACTH) concentration was markedly decreased only on the first day after delivery in animals consuming the genistein-supplemented diet compared with the appropriate group of rats fed a diet without genistein. In the groups of pregnant animals, ACTH was not influenced by this compound. No effect of genistein was demonstrated on the serum corticosterone concentration during pregnancy. None of the metabolic parameters were substantially affected by genistein in the performed experiment in any of the analysed tissues, however, some changes occurred among no-genistein groups of pregnant animals: lower serum glucose concentrations and increased serum triglycerides on day 18 of gestation (Table 2), increased concentration of free fatty acids, HDL-cholesterol and the content of liver triglycerides on the first day after delivery (Tables 2 and 3) and between genistein treated pregnant animals (serum glucose, total and HDL cholesterol, liver and muscle triglycerides).

DISCUSSION

Changes in hormones regulating metabolic processes are well recognized in pregnant animals. In our experiment, serum insulin during pregnancy was elevated and then lowered on the first day after delivery (Table 1). Genistein had no effect on this hormone's concentrations during pregnancy and after delivery. This observation seems to be interesting in relation to our previous results demonstrating that genistein substantially decreases serum insulin levels in adult male rats (Szkudelska et al., 2003) and in female, sexually immature rats (Nowicka et al., personal communication). It is known that the increased levels of the sex steroids

Table 1. The effect of genistein on some hormones in blood of pregnant rats

Parameter	Non-pregnant no-genistein	13 gestation day		18 gestation day		1 day after delivery	
		no-genistein	genistein	no-genistein	genistein	no-genistein	genistein
Insulin, ng/ml	1.99 ± 0.23	3.11 ± 0.29x	3.47 ± 0.46p	3.18 ± 0.55x	2.83 ± 0.42p	1.85 ± 0.23y	1.51 ± 0.25q
Glucagon, pg/ml	117.48 ± 10.15	104.52 ± 5.04x 1	12.49 ± 5.64p	134.36 ± 11.31y	140.02 ± 10.00	188.94 ± 17.64z	172.02 ± 18.63q
Leptin, ng/ml	2.55 ± 0.83	3.51 ± 0.27x	3.28 ± 0.19	5.54 ± 0.53ay	3.80 ± 0.18b	4.54 ± 0.32ax	3.63 ± 0.25b
ACTH, pg/ml	27.53 ± 1.01	65.97 ± 6.71	63.49 ± 5.02	58.11 ± 6.09	50.65 ± 3.79	65.52 ± 4.30a	52.17 ± 2.96b
Corticosterone, ng/ml	108.34 ± 20.31	387.85 ± 30.72	406.65 ± 42.10	423 ± 23.82	385.43 ± 51.13	374.29 ± 44.53	343.71 ± 41.09

rats were fed *ad libitum* a diet without genistein ("no-genistein") or supplemented with genistein ("genistein") at the dose 100 mg/kg from the beginning of pregnancy. Results are means ±SEM from nine rats; a, b – differences statistically significant (P≤0.05) between "no-genistein" and genistein consuming rats on the same day of gestation and on 1 day after delivery; x, y, z - differences statistically significant (P≤0.05) between "no-genistein" rats on day 13 and 18 of gestation and on 1 day after delivery; p, q - differences statistically significant (P≤0.05) between genistein treated rats on day 13 and 18 of gestation and on1 day after delivery; ACTH - adrenocorticotropic hormone; in each group fed a diet with or without genistein n=9

Table 2. The effect of genistein on some metabolic parameters in blood serum of pregnant rats

Parameter	Non-pregnant no-genistein	13 gestation day		18 gestation day		1 day after delivery	
		no-genistein	genistein	no-genistein	genistein	no-genistein	genistein
Glucose, mmol/l	5.38 ± 0.36	5.58 ± 0.18x	5.76 ± 0.16p	4.64 ± 0.25y	4.42 ± 0.19q	5.32 ± 0.31	5.20 ± 0.20
Triglycerides, mmol/l	3.35 ± 0.20	3.59 ± 0.23x	4.16 ± 0.36	5.31 ± 0.35y	4.97 ± 0.39	4.38 ± 0.30	4.43 ± 0.33
FFA, mmol/l	0.23 ± 0.03	0.30 ± 0.02x	0.34 ± 0.02	0.37 ± 0.04	0.32 ± 0.03	0.49 ± 0.04y	0.40 ± 0.04
Total cholesterol, mmol/l	1.33 ± 0.12	1.30 ± 0.06	1.32 ± 0.07p	1.62 ± 0.11	1.56 ± 0.07	1.62 ± 0.07	1.72 ± 0.14q
Free cholesterol, mmol/l	0.16 ± 0.02	0.27 ± 0.03	0.31 ± 0.05	0,35 ± 0.06	0.30 ± 0.04	0.37 ± 0.04	0.37 ± 0.03
HDL cholesterol, mmol/l	0.92 ± 0.10	0.78 ± 0.05x	0.86 ± 0.05p	0.96 ± 0.04	0.94 ± 0.05p	1.22 ± 0.05y	1.26 ± 0.07q

rats were fed *ad libitum* a diet without genistein ("no-genistein") or supplemented with genistein ("genistein") at the dose 100 mg/kg from the beginning of pregnancy. Results are means ±SEM from nine rats; x, y - differences statistically significant (P≤0.05) between "no-genistein" rats on day 13 and 18 of gestation and on 1 day after delivery; p, q - differences statistically significant (P≤0.05) between genistein treated rats on day 13 and 18 of gestation and on 1 day after delivery; FFA – free fatty acids; in each group fed a diet with or without genistein n=9

Table 3. The effect of genistein on some metabolic parameters in liver and skeletal muscle of pregnant rats

Parameter	Non-pregnant no-genistein	13 gestation day		18 gestation day		1 day after delivery	
		no-genistein	genistein	no-genistein	genistein	no-genistein	genistein
Liver							
glycogen, mg/g	28.15 ± 3.15	28.86 ± 1.61	25.44 ± 3.25	26.14 ± 1.65	24.47 ± 1.79	21.67 ± 1.98	19.02 ± 2.15
triglycerides, mg/g	13.61 ± 1.59	12.68 ± 0.62x 1	14.69 ± 1.09p	13.29 ± 0.66	10.64 ± 0.98q	17.25 ± 0.85y	18.51 ± 1.14 p
cholesterol, mg/g	2.59 ± 0.09	2.64 ± 0.05	2.59 ± 0.04	2.66 ± 0.04	2.70 ± 0.10	2.79 ± 0.12	2.50 ± 0.05
Muscle							
glycogen, mg/g	4.47 ± 0.33	4.23 ± 0.18	4.34 ± 0.31	3.98 ± 0.16	4.94 ± 0.27	4.08 ± 0.33	4.63 ± 0.24
triglycerides, mg/g	13.58 ± 1.59	14.91 ± 2.18	14.08 ± 2.9 p	20.00 ± 3.42	30.63 ± 3.70q	15.91 ± 1.58	22.56 ± 3.74
cholesterol, mg/g	0.40 ± 0.04	0.37 ± 0.02	0.43 ± 0.02	0.39 ± 0.02	0.39 ± 0.02	0.34 ± 0.02	0.34 ± 0.02

rats were fed *ad libitum* a diet without genistein ("no-genistein") or supplemented with genistein ("genistein") at the dose 100 mg/kg from the beginning of pregnancy. Results are means ±SEM from nine rats; x, y - differences statistically significant (P≤0.05) between "no-genistein" rats on day 13 and 18 of gestation and on 1 day after delivery; p, q - differences statistically significant (P≤0.05) between genistein treated rats on day 13 and 18 of gestation and on 1 day after delivery; in each group fed a diet with or without genistein n=9

estradiol and progesterone are responsible for augmentation of plasma insulin concentrations, hypertrophy of pancreatic islets and enhanced insulin secretion during pregnancy (Kalkhoff and Kim, 1978). Genistein, like endogenous oestrogens, has an affinity to oestrogen receptors, so many of its effects can be mediated through this receptor. However, this affinity is lower for genistein than for estradiol (Kuiper et al., 1998). Therefore, some aspects of genistein action during gestation may not be manifested due to enhanced concentrations of endogenous oestrogens.

The peripheral glucagon concentration is usually reported to be unaffected during pregnancy, although its secretion may slightly increase in cultured pancreatic islets of pregnant rats (Moes et al., 1993). In the performed experiment, a rise in serum glucagon levels was observed during pregnancy, with the highest level recorded on the first day after delivery, but genistein did not alter this pattern (Table 1). There is no literature data concerning the possibility of glucagon being affected by isoflavones. Recently, the function of the adipocyte-derived hormone, leptin, as a factor regulating food intake and energy expenditure has been widely investigated. Moreover, leptin plays an important role in the regulation of several processes associated with reproduction: puberty onset, ovarian function and fetal/placental growth (Smith and Waddell, 2003). The leptin concentration increases throughout rodent gestation. This is probably due to its increased synthesis in the fat tissue or to an increased concentration of a leptin-binding protein in serum (Chien et al., 1997) because synthesis of leptin by rodent placenta is rather negligible. In our experiment, the leptin concentration increased markedly during pregnancy and decreased after delivery. Genistein reduced the serum leptin concentration on day 18 of gestation and on the first day after delivery (Table 1). On day 18 of gestation this effect could have been caused in part by a simultaneous decrease in the serum insulin level. However, since after delivery serum leptin was reduced by genistein despite insulin being unchanged, another reason must be pivotal for genistein-induced hypoleptinaemia. Our previous experiments and the literature data demonstrate that genistein directly affects some processes in adipocytes, resulting in reduced secretion of leptin. This compound was found to restrict insulin-stimulated glucose transport in isolated rat adipocytes (Smith et al., 1993), to inhibit insulin action in adipocytes and to reduce insulin-stimulated glucose metabolism (Abler et al., 1992). Experiments performed on isolated rat adipocytes also showed that genistein diminishes glucose conversion to lipids (Szkudelska et al., 2000). Moreover, our recent studies revealed that genistein abates leptin secretion from isolated adipocytes and it was also demonstrated that this phytoestrogen administered to adult rats diminishes the serum leptin level (Szkudelski et al., 2005). Therefore, it is possible that in pregnant rats, the serum leptin-lowering action of genistein is a consequence of its direct action on cells secreting this hormone.

Genistein appears to be an important factor decreasing steroidogenesis in the adrenal gland. This compound administered to rats (40 mg/kg) evoked a significant increase of adrenal weight and markedly decreased the serum corticosterone concentration. Concurrently, the serum adrenocorticotropic hormone (ACTH) level increased (Ohno et al., 2003). In early pregnancy, serum corticosterone can decline in comparison with non-pregnant rats and remain so until day 10, after which it begins to rise to an elevated level by day 16, and remains elevated to day 22 of gestation (Atkinson and Waddell, 1995). The ACTH concentration may drop in early pregnancy and does not change to parturition, but it is significantly augmented on day 4 of lactation. In our experiment, elevated corticosterone concentrations were observed during pregnancy (day 13 and 18) and after parturition, but genistein did not alter serum corticosterone in any group. The consumption of the genistein-supplemented diet evoked a slight decrease of the ACTH level during pregnancy, but this effect was statistically significant only on the first day after delivery (Table 1). The mechanisms of such genistein activity are difficult to explain. One can suggest that the influence of genistein on the ACTH level is more distinct when the placenta, a source of oestrogens, is removed. The important mechanism by which genistein may affect blood corticosterone levels is by lowering its synthesis—genistein is a competitive inhibitor of 21-hydroxylase *in vitro* (Ohno et al., 2003). The lack of influence of genistein on corticosterone levels in pregnant rats indicates that during pregnancy the isoflavone does not manifest its inhibitory action on the synthesis of this hormone, probably because of the higher concentration of progesterone, the endogenous substrate for 21-hydroxylase.

During pregnancy many metabolic parameters vary, but glucose changes seem to be the most broadly described and explained. Among many reports concerning elevated glucose concentrations during gestation, there are some which demonstrate a progressive fall of glucose in the second half of rat pregnancy (Liberati et al., 2004). Similarly to the latter results, in our studies the serum glucose concentration during pregnancy was unchanged in early pregnancy but diminished on day 18 and slightly decreased after delivery. Genistein did not affect glucose concentration during pregnancy or after delivery (Table 2). In our previous experiment, genistein administered intragastrically to male rats for three days evoked only a slight hyperglycemic effect. Moreover, in these animals the insulin concentration was lowered by the phytoestrogen (Szkudelska et al., 2003). In another experiment, glucose and insulin were unchanged in ovariectomized rats injected with genistein for three days (Nogowski et al., 2002). After two weeks of administration of genistein in a diet to ovariectomized female rats, the serum glucose concentration was markedly augmented in comparison with control animals (Nogowski et al., 1998). The above data suggest that the influence of genistein on serum glucose

is rather slight and differs depending on sex, endogenous oestrogens, time of phytoestrogen treatment, or even route of administration.

There are some data suggesting that genistein is able to modify liver glycogen stores, another important parameter of carbohydrate metabolism. Keppens (1995) reported that the tested phytoestrogen slightly activated glycogen phosphorylase in cultured rat hepatocytes. It was also found that genistein decreased liver glycogen stores in ovariectomized female rats (Nogowski et al., 2002). However, such an effect was not seen in intact male rats (Szkudelska et al., 2003) or in pregnant rats, as observed in our experiment (Table 3).

Pregnancy induces changes in lipid metabolism. Plasma lipid components, i.e. triglycerides, cholesterol, free fatty acids increase during this period (Argiles and Herrera, 1981). The production of triglycerides by the liver is enhanced (Wasfi et al., 1980). The very low density lipoproteins (VLDL) in plasma are also elevated (Argiles and Herrera, 1981); these changes are partly the consequence of the altered activities of enzymes engaged in lipid metabolism. The activity of adipose tissue lipoprotein lipase (LPL) and its expression is decreased (Martin-Hidalgo et al., 1994). The activity of another enzyme responsible for lipid metabolism, hormone-sensitive lipase (HSL), and the expression of its gene are elevated, especially in late pregnancy (Martin-Hidalgo et al., 1994). Gestational changes also involve cholesterol metabolism. The results of our previous experiments indicate that genistein can modify lipid parameters in rats. In ovariectomized female Wistar rats, dietary genistein ingested by 14 days decreases serum triglyceride and free cholesterol concentrations and increased free fatty acid concentrations (Nogowski et al., 1998). Liver cholesterol was also increased, while muscle triglycerides were decreased in the same investigations. The three-day genistein treatment of male Wistar rats substantially reduced the content of muscle triglycerides (Szkudelska et al., 2003). Moreover, estradiol administration in the same experiment also resulted in decreased muscle triglycerides. *In vitro,* genistein and estradiol manifested an inhibitory effect on lipogenesis in isolated rat adipocytes and genistein stimulated lipolysis in these cells (Szkudelska et al., 2000). This compound is also engaged in cholesterol metabolism—it can reduce total cholesterol and LDL-cholesterol and augments HDL-cholesterol (Anthony et al., 1996). In spite of well-documented effects of genistein on lipid metabolism in non-pregnant animals, we did not observe any genistein-induced changes in the tested lipid parameters during pregnancy, although pregnancy itself evoked a slight increase in some of them (Tables 2 and 3). The most probable explanation for this finding is the high concentration of endogenous oestrogens resulting in values characteristic in pregnancy that mask the effect of genistein. The results of our previous experiments mentioned above support this hypothesis.

The influence of the phytoestrogen, genistein, on hormonal status and some metabolic parameters during pregnancy had never been previously studied. The results obtained in our experiment revealed for the first time that the action of genistein during pregnancy is rather weak and it is manifested only in slight hormonal changes, but it is noteworthy that the lowered serum leptin concentration after isoflavone treatment is particularly important, because the hormone may regulate mammary gland growth and development during pregnancy (Laud et al., 1999). These effects seem to be oestrogen-independent. The plasma glucose and lipid parameters as well as liver and muscle glycogen and lipid parameters were not altered by genistein in pregnant rats. Taking into consideration that under experimental conditions without endogenous oestrogens or in experiments with male rats, the changes evoked by genistein are evident, it can be stated that they are in great part oestrogen-dependent. The presented results support the conclusion that, although genistein affects the development of the fetus, its influence on maternal carbohydrate and lipid metabolism is quite weak.

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