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Genistein Precipitated Hypothyroidism, Altered Leptin and C-Reactive Protein Synthesis in Pregnant Rats

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Summary: Genistein is an isoflavone constituent of soya. This study examined the mechanism by which genistein produced adverse effects in pregnant laboratory rats. Pregnant rats were divided into control (Con) and genistein (Gen) force fed (2 mg/kg) groups. At terminal gestation day (GD) ranging from 0-20, the rats were sacrificed, and blood samples and amniotic fluids were collected. Thyroid hormone, C-reactive protein (CRP) and leptin assay was carried using the blood samples. Leptin was also assayed in the placenta and amniotic fluid supernatant. Oral exposure of pregnant rats to genistein significantly altered maternal T3, (GD18; Con 1.65 ± 0.01, Gen 1.03 ± 0.04 nmol/L), T4 (GD6; Con 29.60 ± 0.00, Gen 36.04 ± 1.29 nmol/L), Leptin (Placenta GD20; Con 0.08 ± 0.01, Gen 0.31 ± 0.02 ng/ml, amniotic fluid ;GD 20; Con 0.02 ± 0.00, Gen 0.35 ± 0.05 ng/ml) in genistein group. These changes were accompanied with loss of embryonic implants and a decrease in fetal and placental weights. The CRP level was significantly decreased and increased at the onset and toward late pregnancy respectively. Oral exposure of pregnant rats to genistein precipitated hypothyroidism, altered some metabolic hormones with a reduction in fetal and placental growth and increased resorption of embryonic implants.

Keywords*:* Genistein, embryonic implants, pregnancy, thyroid hormone, leptin, C - reactive protein.

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INTRODUCTION

Genistein is a non-steroidal phytoestrogen (4, 5, 7-trihydroxyisoflavone) derived from soya and soya products. Although it is also present in other legumes, the highest concentration is found in soya and soya products (Price and Fenwick 1998). Infant soya formulae contain approximately 65% of genistein (Chen and Donovan 2004). Genistein exerts estrogenic effects, having a higher affinity for the beta than for alpha estrogenic receptors (Santell et al. 1997). It is a known tyrosine kinase inhibitor (Akiyama et al. 1987) and has the potential to inhibit several intracellular enzymes (Degen 1990).

Early exposure of female rats to genistein before sexual maturity resulted in marked structural and functional changes in their reproductive organs before the normal sexual maturity age (Awoniyi et al. 1998; Awobajo et al. 2013). Genistein has also been shown to exert anti-obesity effects with prompt weight reduction and reduced fat deposit during exposure in ovariectomized mice and non-pregnant female rats (Naaz et al. 2003, Kim et al. 2006, Nwicka-Stanczk et al. 2012). This effect has been adduced to its ability to down regulate lipoprotein lipase gene expression in adipose tissue with a possible decrease in blood lipid profile (Yousef et al. 2004). Genistein has also been reported to influence two body hormones, i.e. insulin and leptin, closely linked to body metabolism and food consumption. Exposure to genistein precipitated a reduction in insulin and leptin level in sexually immature female rats (Nogowski et al. 2007) and at higher doses in mature non pregnant rats (Nwicka-Stanczk et al 2012).

Establishment of pregnancy is dependent upon successful completion of fertilization leading to blastocyst implantation in the endometrium. Several bioactive agents produced within the maternal environment and those found to have originated from the environment can influence implantation, uterine receptivity and embryonic development. Several substances, including leukocyte inhibitory factor, heparin-binding epidermal growth factor (EGF), colony stimulating factor-1, interleukin-1 (Pampfer et al. 1991; Stewart et al. 1994; Simon et al. 1994), calcitonin (Ding et al 1995), and thyroid hormones (Zhang et al. 1997; Ashkar et al. 2009) have been observed to actively participate in the regulation of the implantation process. Reduced fetal and placental weight was reported as part of the consequences of genistein exposure in pregnant rats at a dose of 2 mg/kg body weight (Santell et al. 1997). It is well established that thyroid hormones play a significant role during in-utero development and even during neonatal and infant stages of development (Legrand 1986). They also have a long term impact on the behavior, locomotors ability, speech, hearing, and cognition of the offspring (Legrand 1986; Biondi and

Cooper 2008). The thyroid hormones; thyroxine (T4), and triiodothronine (T3), are tyrosine based hormones that are involved in the regulation of cellular metabolism. Their deficiency during pregnancy has been linked to reduction in placental and fetal growth (Evers 2012). To the best of our knowledge, most experimental and clinical trials on genistein and thyroid hormone to date have not included a pregnant model. There have been reports on healthy adult male and female subjects (Hampl et al. 2008), prostate cancer patients (Lazarevic et al. 2011), oophorectomized women (Mittal et al. 2011), and osteopenia postmenopausal women (Bitto et al. 2010). There have also been experimental reports in mature healthy male and female rats (Nwicka-Stanczk et al. 2012).

Therefore, we have attempted to determine the thyroid hormone profile, and leptin level in amniotic fluid, placental tissue, and plasma C-reactive protein (CRP) levels during pregnancy in rats orally exposed to genistein. Embryo implantation sites were also counted while fetal, and placenta growth were quantified with the respective weights.

MATERIALS AND METHODS

Chemical: Genistein (purity 98.2% was purchase from Chengdu Biopurify Phytochemicals ltd. China

Animals grouping and drug administration: Fifty-four adult regularly cycling female Sprague-Dawley rats, weighing between 150-160 g were used for this study. At proestrous stage, they were cohabited overnight with male rats (ratio; 1 male: 2 females). The presence of sperm cells in the vaginal smear confirmed successful mating and the day this was observed was recorded as day zero of pregnancy (Marcondes et al. 2002). The pregnant rats were divided into the following two groups; control (Cont) and genistein treated (Gen). Each group was further sub-divided into the following five sub-groups; pregnancy day 0, 6, 12, 18 and 20 of six rats per sub-group (the two groups shared day zero as there was no treatment done on this day). Rats in the Gen group were orally treated with genistein suspended in dissolved in distilled water at a dose of 2 mg/kg body weight per day. Our earlier studies using 1 mg and 2 mg/kg body weight showed significant pregnancy outcome impairment at 2 mg/kg body weight (Awobajo et al. 2013) throughout the gestational period. There are several reports of usage of wide dosages of genistein ranging from 0.2 mg to 100 mg/kg body weight in rodents (Elsa and Wendy 2010). The control group received equal volume of distilled water; the vehicle for genistein administration. Clean water and phytoestrogen-free rat chow was provided ad libitum throughout the experimental period. All protocols used including animal welfare, dissection, and humane euthanasia were approved by the Research and Ethics Committee of the Institution and they conformed to the Guidelines for Care and Use of Laboratory Animals in Biomedical Research (National Institute for Health 1985).

Blood and internal organs collection: Six pregnant rats from each sub-group were stunned and sacrificed by cervical dislocation at GDI, 6, 12, 18, and 20 respectively. Blood samples were collected into heparinized sample bottles via cardiac puncture, and centrifuged at 3000 rpm for 15 min to separate plasma. After bilateral ovariectomy, amniotic fluid was carefully syphoned out of the amniotic sac using a 13-gauge needle into sterile sample bottles, spun and filtered. Uterine horns containing conceptuses were removed and immediately placed on ice while number of embryonic implants and resorption sites were recorded for each rat. Placental were separated and placental tissues from each litter were pooled (four placental tissue from each of the six rats to make 21 placental tissues per sub-group). The ovaries were dissected out and the number of corpus luteum counted and recorded. Placental homogenates (10.0% w/v) were prepared in cold phosphate buffer solution (PBS), using a mechanically driven homogenizer, immersed in an ice pack, and then centrifuged at 3000 rpm for 20 min to obtain the homogenate. The homogenate was subsequently filtered with 40 gauge filter and used for the leptin assay. All the samples for assays were stored at -80^{o}C temperature until used.

Hormonal analysis: Thyroid hormones (T3, T4) were assayed in the plasma using an Enzyme-linked Immunosorbent assay kit (ELISA) while TSH was assayed using Enzyme Immunoassay (EIA) kit (ELISA) according to the manufacturer specifications. Leptin was assayed in the plasma, amniotic fluid and placental tissue homogenate using rat leptin ELISA kit (Crystal Chem Inc, USA) while rat CRP ELISA kit (Immunology Consultants Laboratory Inc. USA) was used for the CRP assay.

Statistical Analysis: Results are presented as mean ± standard error of mean (SEM), differences between each group of rats were statistically evaluated using two-way analysis of variance and post hoc Duncan's multiple range tests. Differences were considered significant at $P \leq 0.05$. A line chart was also used for graphical representation.

RESULTS

Number of embryonic implants, fetal and placenta weights obtained from genistein exposed rats at different gestational days compared to control rats (Table 1):

There was a significant decrease in the number of implanted fetuses or developing embryo at GD 12, 18

and 20 in genistein exposed rats as compared with the number recorded in control rats. There was however, no significant difference in the within group results of number of implanted fetuses or developing embryo between GD 6, 12, 18 and 20 either in the genistein exposed or control group. There was also a significant reduction in the weights of all fetuses and placentas harvested at GD 6, 12, 18 and 20 in the genistein exposed rats compared with those of the control rats.

Pattern of thyroid hormones at different days of pregnancy in genistein treated compared with control rats (Table 2):

TSH concentration pattern in the control group within the gestational period measured showed an initial decrease from GD 0 to 6, followed by an increase towards GD 12 and a subsequent reduction in the plasma concentration towards GD 20. In genistein

Table 1: Number of embryonic/fetal implantation sites recorded at different stages of pregnancy along with the weights of the embryo/fetus and their placenta in pregnant rats orally treated with genistein at a concentration of 2mg/kg body weight compared with unexposed control pregnant rats. Results presented as mean ± SEM, $p \leq 0.05$, (n=6)

Gestation day	Embryo implantation		Fetal weight (g)		Placental weight (g)	
	Control	**Genistein**	**Control**	**Genistein**	**Control**	**Genistein**
0	NA	NA	NA	NA	NA	NA
6	9.20 ± 1.11	8.40 ± 1.03	0.78	NA	NA	NA
12	10.00 ± 0.71	7.80 ± 0.37[b]	1.35	0.24 ± 0.02[b]	1.67 ± 0.25	1.22 ± 0.01 [b]
18	9.80 ± 0.58	7.01 ± 0.45 [b]	1.42	0.84 ± 0.06 [b]	2.03 ± 0.11	1.50 ± 0.01 [b]
20	9.20 ± 0.66	7.02 ± 0.71 [b]	1.89	1.25 ± 0.04 [b]	2.20 ± 0.14	1.53 ± 0.15 [b]

b Significant decrease NA=Data not available

Table 2: Pattern of plasma thyroid hormones and the T3/T4 ratio at different stages of pregnancy in pregnant rats orally treated with genistein at a concentration of 2mg/kg body weight compared with unexposed control pregnant rats. Results presented as mean ± SEM, $p \leq 0.05$, (n=6)

Gestation day	Group (n=6)	TSH (pIU/L)(x 10^{-3})	T3 (nmol/L)	T4(nmol/ L)	%A in TSH within group(x10"^{3}) (after-before)	%A in T3 within group (x10^{-3})	%A in T4 within group (x10^{-3})	T3/T4 ratio
0	Control Genistein	2.15 ± 0.15	1.17 ± 0.03	57.27 ± 3.22				0.0204 ± 0.0006
6	Control	0.30 ± 0.00	1.10 ± 0.02	29.60 ± 0.00	-616.67 ± 50.00	-8.06 ± 3.34	-97.22 ± 7.31	0.0371 ± 0.0008
	Genistein	3.35 ± 0.15 [a]	2.10 ± 0.11[a]	36.04 ± 1.29	35.89 ± 1.61	69.45 ± 10.42	-63.44 ± 9.34	0.0584 ± 0.0009[a]
12	Control	4.75 ± 0.75	1.24 ± 0.04	30.89 ± 2.57	93.53 ± 1.02	12.90 ± 3.26	6.62 ± 5.60	0.0402 ± 0.0021
	Genistein	4.00 ± 0.00 [b]	1.27 ± 0.01[a]	36.04 ± 2.57[a]	16.25 ± 3.75	-73.65 ± 0.08	3.06 ± 7.30	0.0354 ± 0.0027 [b]
18	Control	0.55 ± 0.25	1.65 ± 0.01	30.25 ± 0.64	-1066.67 ± 666.67	24.37 ± 1.71	-6.01 ± 7.17	0.0546 ± 0.0009
	Genistein	2.15 ± 0.05[a]	1.04 ± 0.04 [b]	25.74 ± 0.00	-86.15 ± 4.33	-23.60 ± 2.51	-41.82 ± 6.05	0.0403 ± 0.0015[b]
20	Control	0.00 ± 0.00	1.96 ± 0.04	64.06 ± 3.86	0.00 ± 0.00	15.27 ± 0.83	50.53 ± 1.78	0.0311 ± 0.0013
	Genistein	3.95 ± 0.25[a]	1.77 ± 0.08 [b]	21.88 ± 0.00[b]	45.27 ± 4.73	68.44 ± 16.04	-18.01 ± 0.37	0.0807 ± 0.0035[a]

a Significant increase b Significant decrease

Table 3: Leptin concentration in the maternal plasma, placenta tissue homogenate and amniotic fluid at different stages of pregnancy in pregnant rats orally treated with genistein at a concentration of 2mg/kg body weight compared with unexposed control pregnant rats. Results presented as mean ± SEM, $p \leq 0.05$, (n = 6)

Pregnancy Day	Leptin level (ng/ml)					
	Maternal plasma		Placenta homogenate		Amniotic fluid	
	Control (n=6)	Genistein (n=6)	Control (n=6)	Genistein (n=6)	Control (n=6)	Genistein (n=6)
0	0.32 ± 0.06		SNA		**SNA**	
6	0.24 ± 0.06	1.84 ± 0.00[a]	SNA	SNA	SNA	**SNA**
12	0.52 ± 0.02	1.78 ± 0.17 [a]	0.03 ± 0.01	0.16 ± 00.01[a]	0.01± 0.00	**0.16 ± 0.04 [a]**
18	0.29 ± 0.04	0.70 ± 0.10 [a]	0.06± 0.01	0.26 ± 0.03 [a]	0.01± 0.00	**0.24 ± 0.12 [a]**
20	0.31 ± 0.03	1.86 ± 0.13 [a]	0.08 ± 0.01	0.31 ± 0.02 [a]	0.02 ± 0.00	**0.35 ± 0.05 [a]**

[a] Significant increase at $P \leq 0.05$ [b] SNA= Sample not available

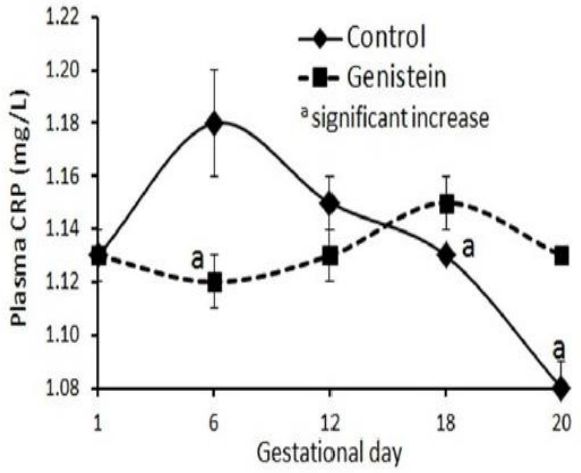

Fig. 1: Plasma C-reactive protein level at different days of pregnancy in rats exposed orally to genistein (2mg/kg body weight)

exposed, the pattern was an increase from GD 0 onward GD 6 and 12. This was followed by a reduction in the plasma TSH concentration towards GD 18, and with another increase towards GD 20 in genistein exposed rats. Plasma TSH level was significantly increased at GD 6, 18, and 20 respectively in genistein exposed rats compared to the control group. The plasma T3 and T4 level also recorded a significant increase at GD 6, 12, while the levels of the two thyroid hormones were significantly decreased at GD 18 and 20 in genistein exposed rats as compared with the plasma levels of the two hormones in control rats. T3/T4 ratio was only significantly increase at GD 6 and 20, while the ratio was significantly decreased at GD 12 and 18 in genistein exposed rats.

Leptin concentration in the maternal plasma, placenta homogenate and amniotic fluid along with plasma concentration of CRP at different stages of pregnancy in genistein treated along with control rats (Table 3):

Plasma leptin level was significantly increased at GD 6, 12, 18 and 20 in all genistein exposed rats compared with control rats. Leptin level in placenta homogenates and amniotic fluids were also significantly increased at GD 12, 18 and 20 in genistein exposed rats compared to control rats. The highest concentration of leptin hormone recorded was in the plasma throughout the gestational period monitored compared with the concentration recorded in the placenta homogenate and amniotic fluid at any of the gestational days monitored.

Plasma C-reactive protein level at different days of pregnancy in rats exposed orally to genistein (Fig 1)

Plasma C-reactive protein level was significantly reduced at gestational day 6, while its level was significantly increased at GD 18 and 20 in genistein exposed rats compared with control rats. The highest and the lowest plasma concentration of CRP were recorded in the control rats at GD 6 and GD 20 respectively. In genistein exposed group of pregnant rats, the highest and the lowest concentration of CRP was recorded at GD 18 and GD 6 respectively.

DISCUSSION

The current study has shown that the administration of genistein to pregnant rats resulted in increased resorption of embryonic implants, and a decrease in placental and fetal weights. These adverse effects which have previously been reported by Ikegami et al. (2006) and Awobajo et al. (2013), indicated the possibilities of genistein adversely influencing some of the mechanisms that control placental and fetal metabolic processes. In addition, T4, a major metabolic hormone produced in the thyroid gland, is known to influence cell differentiation and growth during fetal life (Yen 2001). Therefore, the results of the thyroid hormonal analysis from day 1 to 20 of pregnancy revealed a significant decrease in the maternal secretion of T4, and T3 from mid-gestation onward (tab 2.). This also necessitated the changes recorded in the TSH secretion pattern via negative feedback mechanism. The T3/T4 ratio was significantly decreased most importantly between days 12-18 of pregnancy, indicating impairment of T4 to T3 conversion. Considering the observed genistein-induced hypothyroidism at the dose used, this has adversely affected normal growth of the fetus which may partly explain the reason for the observed reduction in fetal and litter weight. Genistein has been shown to inhibit thyroid-peroxidase, the enzyme responsible for catalyzing iodination of thyroglobulin and oxidative coupling of di-iodothyronine during the synthesis of thyroid hormones (Doerge and Sheehan 2002). Genistein has also been reported to interfere with iodide reutilization by inhibiting sulfotransferase enzymes (Ebmeier and Anderson 2004). This may partly explain the significant alteration recorded in thyroid hormone synthesis in the genistein exposed pregnant rats.

Genistein may also influence the metabolic hormone leptin, a peptide secreted by placental and white adipose tissue (Barr et al. 1997; Hoggard et al. 2000). This study revealed a significant increase in maternal plasma leptin concentration in the amniotic fluid and placental homogenate throughout pregnancy (tab 3). Linnemann et al. (2000) reported that about 98% of the leptin secreted by the placenta are released

into the maternal blood. This report corroborated our findings in which maternal plasma leptin level was increased along with the increase recorded in placenta leptin level (tab 3), throughout the period of gestation. Based on the wide spread expression of the leptin receptor gene in the fetus and placental tissues, it has been strongly suggested that leptin may play an important role in regulating fetal growth (Forhead and Fowden 2009). This was confirmed by the establishment of a correlation between umbilical cord leptin level and fetal birth weight (Tamura et al. 1998). However, contrary to previous reports on leptin and fetal growth (Valuniene et al. 2007), a significant decrease in placental and fetal weight was recorded in pregnant rats orally exposed to genistein from day 6 to 20 of pregnancy (tab 1.), despite the significant increase in plasma and placental leptin level. Other authors have reported a similar decrease in fetal weight when pregnant rats were exposed to genistein (Ikegami et al. 2006; Awobajo et al. 2013). Thyroid hormone is known to exert a negative feedback on leptin synthesis with increase in the synthesis of the latter during hypothyroidism (O'Connor et al. 2007). Therefore, the observed genistein-induced hypothyroidism may have silenced the negative feedback control of thyroid hormone on leptin with resultant increase secretion as observed in this study. Placental growth signified by increase weight as it strives to cope with the increased demand for gaseous, hormonal and nutrient supply to the developing fetus is a pre requisite for normal development of the fetus (Ishikawa et al. 2006). The persistent significant reduction in placental weight from gestational day 18-20 (a critical period in fetal weight gain) as recorded in the genistein exposed rats (tab 1.), will partly explain the reason for the reduction in fetal weights. However, further studies will be required to unravel other mechanism by which increased leptin secretion failed to promote fetal weight gain in genistein exposed rats.

Although, inflammatory activities are usually increased towards term with increased CRP (Mendelson 2009), genistein works in synergy with the process of parturition to significantly increase the plasma CRP level onward the day 20 of pregnancy. CRP is an example of the first acute phase response usually with increased production after any inflammatory reaction (Mackiewicz 1997). The significant decrease recorded in the level of CPR at the early stage of pregnancy (fig 2.), may be connected with the increased resorption rate of embryos reported by some authors (Awobajo et al. 2013). Implantation and placentation stages of pregnancy have the characteristics of an acute inflammatory response and therefore, it is referred to as a pro-inflammatory phase (Mor et al. 2011). It involves break down of the epithelial lining of the uterus by the blastocyst and the invasion of the uterine myometrium by giant trophoblast cells to initiate development of the placentation and angiogenesis. Therefore, the reduction in CPR within the first six days of pregnancy; (the period of implantation) may be an indication in the reduction in this necessary inflammatory process. Genistein has been reported to have anti-inflammatory property (Verdrengh et al. 2003). In summary, the evidence presented here, shows that genistein at a dose of 2 mg/kg body weight precipitated hypothyroidism in the pregnant rats. It also adversely affected the synthesis and leptin levels in maternal plasma, amniotic fluid and placental tissue. These along with a reduced inflammatory process at the period of implantation as indicated by reduced plasma CRP level precipitated increased resorption of embryonic implants, reduced growth of the placenta and development of the fetus.

Conflict of interest

The authors report no conflict of interest.

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