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Altered spermatogenesis, steroidogenesis and suppressed fertility in adult male rats exposed to genistein, a non-steroidal phytoestrogen during embryonic development

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ABSTRACT

This article focuses on the effects of prenatal exposure to genistein on the mother, her pregnancy and reproductive functions of the male progeny, since these issues have ethological relevance in both animals and humans. Pregnant Wistar rats received i.p. injections of genistein at a dose level of 2, 20 or 100 mg/kg body weight daily from 12th to 19th day of gestation. Male pups from control and genistein exposed animals were weaned and allowed to develop until 100 days of age; however, when they were 90 days old, twelve males from each group were cohabited with untreated 90-day old females for 8 days. Results revealed a significant decrease in indices of reproductive organs in adult male rats exposed to genistein during embryonic development. Dose dependent reduction was observed in daily sperm production and epididymal sperm density and quality in genistein treated rats. Significant decrease was observed in the activity levels of 3β- and 17β-hydroxysteroid dehydrogenases in testis of experimental rats with a decline in plasma testosterone levels. Histological examination of testis of genistein treated rats indicated deterioration in testicular architecture. In the fertility study, the mean number of implantations and live fetuses per dam mated with 100 mg genistein exposed males was reduced.

1. Introduction

Flavonoids, are compounds belongs to a class of 'environmental estrogens', having significant hormonal potency both in *in vivo* and *in vitro* systems and are ubiquitously found in fruits, vegetables, grains, nuts, most abundant in soybeans and other legumes (Liggins et al., 2000; Akingbemi et al., 2007). Accessibility of flavonoids over-the-counter popularized consumption in recent years due to their potential curative, preventative, and nutritive value (Webb, 2010; Orf, 2013). Soybeans, and foods derived from soy, are a rich source of isoflavones amounting to 1.037 mg/g fresh weight (Thompson et al., 2006). Daidzein and genistein are the most abundant isoflavones in soybean. Previous studies reported that isoflavones play a pivotal role in prevention and treatment of hormone-dependent cancers, cardiovascular diseases, osteoporosis, menopausal symptoms, body weight homeostasis and age-related diseases (Cooke, 2006; Orgaard and Jensen, 2008; Liu et al., 2009). In addition to their positive effects, flavonoids has been shown to cause both direct and indirect adverse effects on the reproductive tract of animals. These effects include infertility, abortion, persistent vaginal cornification, hemorrhagic ovarian follicles, premature reproductive senescence and compromised fertility (Kim et al., 2011; Jefferson et al., 2012). Abnormalities in reproductive health due to high intake of soy products have also been reported in different species including rabbit, quail, ewe, cow, cheetah, grasshopper, parrot, trout and monkey (Bennetau et al., 2001; Sharpe et al., 2002).

Fetuses and infants are exposed to flavonoids through maternal dietary consumption, breast milk, soy-based infant formulas (Foster et al., 2002). Placenta is potential target of isoflavones during gestation and genistein readily crosses the placenta and accumulates in fetuses at levels higher than or comparable to circulating levels in the dam (Todaka et al., 2005; Soucy et al., 2006). Genistein and daidzein are detected in amniotic fluid of second trimester pregnant women at levels similar to those observed in adult serum and 10 to 20-fold higher than levels of the average amniotic fluid estradiol at that time of pregnancy (Foster

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et al., 2002). A higher incidence of retained testes, atrophic testes and sperm abnormalities has been reported in some male progeny of women who were exposed to synthetic estrogen, diethylstilbestrol during pregnancy (Gill et al., 1979). Similar disorders, as well as lower fertility, have been found in laboratory animals that were exposed to estrogenic chemical during neonatal/perinatal period. Studies reporting infertility in genetically altered mice lacking aromatase enzyme (Robertson et al., 1999) or estrogen receptor α (ERα) (Eddy et al., 1996) and describing poor sperm quality in male patients with a defective ERα (Smith et al., 1994) or aromatase gene (Carani et al., 1997) have indicated that deprivation of estrogen or its receptors impairs male reproductive functions. It is interesting that both deprivation of and excessive exposure to estrogen-like chemicals can lead to reproductive disorders. Considering the potential implications of the above review for human health, the aim of the present study is to determine effects of embryonic exposure to genistein on male reproductive parameters at adulthood in Wistar rats. In addition, this study broadened its scope by including developing milestones, which have not been well characterized following embryonic genistein exposure.

2. Materials and methods

2.1. Animals and housing

Timed-pregnant Wistar rats were housed individually in polypropylene cages (18″ × 10″ × 8″) containing sterilized paddy husk as bedding material, and provided filtered tap water and standard rodent feed (purchased from Sai Durga Agencies, Bengaluru, India) *ad libitum*. The feed composition (g/kg) used in the present study was made up of: corn starch, 500; casein, 200; sucrose, 100; fat, 100; cellulose, 50; mineral mix, 35; vitamin mix, 10; L-cysteine, 3; choline bitartarate, 2 and free from soy product. The level of genistein was zero as per the manufacturer. Animals were maintained under controlled conditions at 25 ± 2 °C ambient temperature with a 12-h light and 12-h dark cycle. The experiments were carried out in accordance with the guidelines of the Committee for the Purpose of Control and Supervision on Experiments in Animals, Government of India (CPCSEA, 2003). The experiments were also reviewed and approved by the Institutional Animal Ethical Committee at Sri Venkateswara University, Tirupati, India (Vide No. 04/2011-12/(i)/a/CPCSEA/IAES/SVU/PSR-RM/Dt.01-09-2011).

2.2. Chemicals

Genistein (>98% purity by HPLC) was purchased from Chengdu Biopurify Phytochemicals Ltd. China. Bovine serum albumin (BSA), nicotinamide adenine dinucleotide (NAD), nicotinamide adenine dinucleotide phosphate (NADP), dehydroepiandrosterone, androstenedione were obtained from Sigma Chemicals Co, St Louis, MO, USA. Thiobarbituric acid (purity ≥ 99%) and malondialdehyde (purity ≥ 98%) were obtained from Merck (Darmstadt, Germany). All other chemicals used in this study were of analytical grade and obtained from local commercial sources.

2.3. Experimental design

Pregnant rats were randomly distributed into four groups, each group consisting of eight animals. Animals in the first group served as control and injected intra-peritoneally with 50 μl 10% DMSO daily from 12th to 19th day of pregnancy. Pregnant rats in 2nd, 3rd and 4th groups were dosed once daily by intra-peritoneal injection on gestational days (GD) 12–19 with 50 μl 10% DMSO containing genistein at doses of 2, 20 or 100 mg/kg bw respectively. Although humans are exposed to soy-products or genistein orally, intraperitoneal injection of substances is a common route of administration in laboratory rodents to test their effects. Further, it enhances the bioavailability of substance. The doses of genistein were selected based on our earlier studies (Meena and Reddy, 2014).

Clinical signs such as genital swelling, redness and vaginal bleeding were also recorded in dams daily during the treatment period. Rats were allowed to deliver pups. Two days after birth, the litter size was standardized to nine pups per dam, with as many as eight males, if possible. Pups were evaluated in terms of litter size, birth weight, and survival rate. Out of pups maintained, the male pups for each group were as follows: control = 56; 2 mg group = 54; 20 mg group = 51; 100 mg group = 61. The pups were also observed for pinna unfolding, fur development, lower and upper incisor eruption, eye opening, and testis descend into scrotal sac besides behavioral abnormalities. Male pups were weaned on postnatal day 22 and were observed regularly until used for assessment for reproductive performance at 90 days of age and/or necropsied on 100 days of age. Reproductive performance of the male pups was evaluated by cohabiting them with normal fertile females. Testis descent was also determined by an investigator that was blinded to treatment. Schematic representation of experimental design is presented in Fig. 1.

2.4. Fertility and reproductive performance of F1 male rats

Ninety day old rats from control (n = 12) and treated groups (n = 12 from each group) were transferred to a mating cage and cohabited with untreated normally cycling 90-95 day-old female rat (1:1) for 8 days. These male rats were not used further for analysis. Females were checked for the presence of copulatory plugs, and vaginal washings were evaluated for the presence of sperm each morning during mating. The day on which evidence of copulation identified, was designated day zero of gestation (GD 0). Impregnated females were removed from the male's cage on GD 0 and housed, one or two per cage (with same GD 0 date), until scheduled sacrifice. On GD 6, six pregnant rats were killed by decapitation. After collection of the uterus and ovaries, the number of corpora lutea and the number of implantations were counted. On GD 18, another six rats were laparotomized and both uteri were removed and examined for number of live fetuses. Data were analyzed to determine the effects of embryonic genistein exposure on mating- and fertility-indices (mating index (%) = number of sperm positive females/number of pairings × 100; fertility index (%) = number of pregnant females/number of sperm positive females × 100); pre-implantation loss (%) [(number of corpora lutea − number of implantations)/number of corpora lutea × 100]; and post-implantation loss (%) [(number of implantations − number of live fetuses)/number of implantations × 100]. In addition, the conception time, the interval between the first day of cohabitation and the day of plug and/or sperm, was recorded for each female.

2.5. Necropsy and determination of tissue indices

Before termination of the experiment, control and experimental rats were fasted overnight, weighed and killed by cervical dislocation. The weights of the testes, different regions of epididymis, seminal vesicles, prostate glands, spleen, vas deferens and penis were recorded for each animal, and relative weights (weight of the organ per 100 g body weight) were calculated.

2.6. Testicular daily sperm production

Daily sperm production (DSP) was determined by the method of Blazak et al. (1993). Briefly, the left testis was decapsulated and the