Altered male reproduction in rats exposed perinatally to biochanin-A

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Abstract

Phytoestrogens are the plant extract compounds, primarily isoflavones that mimic or modulate endogenous estrogens, usually by binding to estrogen receptors. Biochanin- A (BC-A) is an isoflavone found in red clover and is a selective agonist of estrogen receptor-β (ER-β) and have the capacity to alter functioning of male reproduction. The present study focused on the changes of reproduction in male rats exposed to BC-A during the perinatal period. Normal adult male and female rats were co-habitated and the pregnant female rats were injected with 25mg, 50mg and 100 mg BC-A/Kg body weight on window period 12th, 14th, 16th and 18th day. Rats were allowed to deliver the pups and the BC-A exposure was continued up to weaning period. After weaning male and female pups were separated and maintained in separate cages in a dose wise manner. To check the reproductive performance of adult males (90 days old) exposed perinatally to BC-A are co-habited with sexually mature normal females. Female rats were sacrificed by cervical dis-location and autopsy was performed on 8th and 19th day of gestation. Uterus and ovaries were examined for the numbers of corpora lutea, implantations, dead, live foetuses, resorptions and fetal weight. Significant increase was noticed in the conception time, pre and post implantation loss (p< 0.0001) and decrease in the number of implantations (p<0.0001). Further observed significant decrease in fertility rate as measured by counting numbers of live fetuses (p<0.005) and resorptions (p<0.05) in the females mated with males exposed to BC-A. Females mated with BC-A exposed males were shown significant reduction (p<0.0001) in resorptions, and significant decrease (p<0.05) in fetal weight and number of live fetuses per rat. The present study elucidated the significance of perinatal BC-A exposure on male reproductive health.

Keywords: Biochanin-A, Isoflavonoid, Fertility rate, Implantation loss, Testosterone, Male wistar rats

1. Introduction

Consumption of phytoestrogens (also called as endocrine-disrupting chemicals; EDCs) is associated with beneficial health effects and potential adverse effects on development, fertility, reproductive and endocrine system. Hence, in recent year’s interest on undesirable effects of phytoestrogens on male fertility has increased as it has been shown that estrogens play a vital role in the male reproduction (1, 2). Initial reports on soy consumption are found to be beneficial on human health (3) and later found the possible endocrine disrupting properties of isoflavonoids, genistein and daidzein (4). Cao et al. (5) found that the exposure of genistein and daidzein is risky to infants. It is also found that genistein and daidzein also causes infertility in cheetahs (6). Neonatal exposure of genistein and other phytoestrogens are capable of crossing the placenta and might show prospective consequences on adult fertility (7). Exposure of genistein at neonatal stage affects sexual behaviour of adult male rats and an effect likely mediated by ER-β (8, 9).
Male rats exposed chronically to genistein showed abnormalities in spermatogenesis (10, 11). Genistein induced infertility was tested in adult male rats and observed alterations in sperm motility and reduction in litter size as evidenced by loss of post-implantation embryo (10). In low doses, daidzein, BC-A and genistein stimulate growth of some types of cancer cells, but at higher doses cell growth is inhibited (12, 13).

BC-A is the predominant dietary phytoestrogen source found in red clover. Perinatal exposure to phytoestrogen diet can decrease steroidogenesis and androgen secretion by decreased testicular Leydig cells in the adult rats (14). Long term administration of daidzein to male rats causes decrease in plasma levels of testosterone as well as erectile dysfunction and these effects are possibly mediated through estrogen receptor (15). Reduced testosterone levels have been reported in adult male rats exposed to high-dose of phytoestrogen coumestrol (16).

Dietary administration of genistein in rats, suppresses the steroidogenic response of Leydig cells. Reduction in the initiation of steroidogenesis in Leydig cells exposed to EDCs is due to reduced serum testosterone and its substrate androstenedione (17, 18).

Though there is copious literature available on direct effect of phytoestrogens on altered male reproduction in humans and laboratory animal models, still there is lack of knowledge on phytoestrogen caused reproductive abnormalities in trans-generations. Especially no reports are available on BC-A induced trans-generational reproductive abnormalities in humans and animals. Therefore the present investigation was initiated to elucidate the effect of perinatal exposure of BC-A on reproductive performance of male rats.

2. Materials and methods

2.1. Test chemical

The test chemical BC-A was purchased from Sigma Aldrich, St Louis, MO, USA and all other chemicals were purchased from Merck, Mumbai, India and HiMedia Private Limited Laboratories, Mumbai, India.

2.2. Animals and Maintenance

Wistar rats of 90 days old were purchased from Sri Venkateswara Traders, Bangalore, India. Rats were housed in well ventilated, clean and air-conditioned room (12 h: 12 h light: dark cycle; at 25 ± 2°C with a relative humidity of 50 ± 5%). The animals were fed with Rodent feed (manufactured by Godrej Agrovet Limited, Mumbai, India) and with tap water ad libitum.

The experiments were carried out in accordance with the guidelines made by the Committee for the Purpose of Control and Supervision on Experiments on Animals (CPCSEA), Government of India (18) and care and use of laboratory animals (NRC, 1996) which were approved by the Institutional Animal Ethical Committee at Sri Venkateswara University, Tirupati, India (Resolution No: 10/(ii)/a/CPCSEA/ IAEC/SVU/PSR/NG/Dt. 18-10-2010).

2.3. Experimental design

On the evening of proestrus, virgin female rats (with a body weight of 180 ± 10 g) were mated over night with fertile male rats. The day when sperm was detected in the vaginal smear was considered as gestation day (GD) one. Successfully mated females were randomly distributed into four groups of 6 rats each and housed individually. Group I served as control group and group II, III and IV were served as experimental groups. Groups II, III and IV were injected intra-perinatally with BC-A which is dissolved in 100% DMSO at a dose of 25 mg, 50 mg and 100 mg/Kg body weight respectively on GD 12, 14, 16 and 18 in 100 µl volume. Control rats were injected with 100 µl of Di-methyl sulfoxide (DMSO). All the rats were allowed to deliver pups; pups were individually identified and observed everyday for clinical signs of toxicity until completion of lactation period. After weaning, male and female young ones from each group were separated, maintained in separate cages. To assess the fertility of perinatal BC-A exposed male rats, on post natal day 90 they were mated with normal female rats. After successful mating, the females on GD 8 and 19 performed autopsy. During autopsy uterus was examined for number of pre-implantation sites on GD 8 and post-implantations, fetal weight, number of resorptions, live and dead foetuses on GD 19. Number of corpora lutea was also examined from ovaries on GD 8 and 19.

2.4. Progeny fertility parameters

2.4.1. Conception time

Reproductive efficiency of perinatally BC-A exposed male rats was tested by measuring conception time. The length of time required by male rat to mate with female after the launch of co-
habitation is determined. An increase in the duration between initiation of co-habitation and evidence of mating reflects impaired sexual performance of male rats.

2.4.2. Mating index
In order to check the mating performance of BC-A exposed males, mating index was calculated by using the following formula:

\[
\text{Mating Index (%)} = \frac{\text{Number of sperm positive females}}{\text{Number of pairing}} \times 100
\]

2.4.3. Fertility index
The fertility capacities of perinatally BC-A exposed males was calculated as fertility index by using the formula.

\[
\text{Fertility Index (%)} = \frac{\text{Number of pregnant females}}{\text{Number of sperm positive females}} \times 100
\]

2.4.4. Counts of corpora lutea
The corpus luteum is a temporary endocrine structure in mammals involved in production of progesterone, estradiol and inhibin. Corpora lutea is a light pink colour round shaped structure developed from an ovarian follicle during the luteal phase of the estrous cycle. Corpora lutea can be counted with the naked eye to determine the number of eggs ovulated.

2.4.5. Pre-implantation loss
Pregnant female rats were scarified on the day 8th of gestation. The number of corpora lutea and implantations were determined to calculate the pre-implantation loss by using the following formula:

\[
\text{Pre-implantation loss} = \frac{\text{Number of implantations} - \text{No. of implantations}}{\text{Number of Corpus Lutea}} \times 100
\]

2.4.6. Post-implantation loss
Pregnant female rats were scarified on the day 19th of gestation. The number of corpora lutea and the number of implantations were determined to calculate the post-implantation loss by using the following formula.

\[
\text{Post-implantation Loss} = \frac{\text{Number of implantations} - \text{Number of fetuses}}{\text{Number of implantations}} \times 100
\]

2.4.7. Count of resorptions
Autolysis of an implanted embryo is termed as resorption. In general fetal characterization can be done by using late resorptions than early resorptions. Both early and late resorptions were counted in the present study. An early resorption was visualized and recorded in the uterus of 8th day old pregnant rats, where as late resorption in the uterus of 19th day pregnant rats.

2.4.8. Resorption index (%)
Resorption index was measured by calculating the total number of resorption sites by total number of implantation sites and multiplying with 100 (20).

2.5. Statistical analysis
The data were analyzed statistically by using the statistical programme GraphPad. Prism. Version 5.0.3.477 and data were processed with Two-way ANOVA followed by student’s t-Test. The ‘p’ value <0.05 was considered as significant.

3. Results
Throughout the experiment no mortality was observed both in control and treated groups. No significant net body weight gain and food consumption were observed in rats mated with exposed males when compared to control males. No significant changes in lacrimation, urination, respiration, vocalization, postural or gait abnormalities were found in control and treated groups. All the treated and experimental rats were apparently normal and no unusual behavior resembling head flicking, head searching, biting, licking, self-mutilation, circling, and walking backwards were recorded.

The reproductive performance of BC-A exposed male rats were determined by considering the end points such as mating index, fertility index, number of implantations per rat, pre- and post-implantation losses (Table 1). The mating ratio of control females with control and experimental males in the present experiment is 1:1. But, there is a delay in the mating time required for conception of pregnancy in the experimental males when compared to control males. Significant increase (p<0.0001) in the conception time was observed in all the females mated with BC-A exposed male rats (experimental groups) when compared to the control group. No significance was observed in the mating index and fertility index of control and treated groups. Significance was also not recorded in the mean number of corpora lutea in females co-
habited with treated and control males. The mean number of implantations in females mated with 100 mg BC-A/Kg body weight exposed male rats was decreased significantly (p<0.0001), whereas no significance was observed in 25mg and 50mg BC-A/Kg body weight exposed males when compared to controls.

Significance (p<0.0001) in decrease of implantation sites were observed in control females mated with BC-A exposed males (Table 1). Significance in decrease of implantation sites were also found among the experimental groups (Controls > 25 mg BC-A/Kg body weight received > 50 mg BC-A/Kg body weight received > 100 mg BC-A/Kg body weight received; 13 ± 0.21 > 10.17 ± 0.31 > 7.83 ± 0.31 respectively).

The pre-implantation and post-implantation losses were increased significantly in females mated with BC-A treated males (Table 1). It is about 42.0% in 100 mg BC-A/Kg body weight exposed males mated with females in terms of pre-implantation loss. The figure 1 clearly showing the pre-implantation loss in females mated with BC-A exposed males. But in case of post implantation loss significant increase was observed only at females mated with 100 mg BC-A/Kg body weight (14.81%) exposed males (Table 1 and figure 2). Besides this, significant (p<0.005) reduction in the number of live fetuses per rat and significant increase (p<0.05) in the number of resorptions were observed in females mated with experimental males. The increase in the percentage of resorption index is significant in females mated with 50 mg and 100 mg BC-A/Kg body weight exposed males (16.42% and 29.76% respectively) when compared to control and 25 mg BC-A/Kg body weight exposed male rats (0 and 5.74% respectively).

The fetal weight of females mated with exposed males was also measured in this study. Significant (p>0.0001) decrease in the fetal weight of normal females mated with males exposed perinatally to BC-A was observed (17%, 20.3% and 50.57% in 25 mg, 50 mg and 100 mg/ Kg body weight respectively) when compared to the normal females mated with control males.

### Table 1. Fertility output of perinatally Biochanin-A exposed male rats.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control</th>
<th>25 mg/kg body weight</th>
<th>50mg/kg body weight</th>
<th>100mg/kg body weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>Conception time (days)</td>
<td>1.5±0.22</td>
<td>2.67±0.21 (78)</td>
<td>3.33±0.21 (122)</td>
<td>4.83±0.40 (222)</td>
</tr>
<tr>
<td>Mating index (%)</td>
<td>100(6/6)</td>
<td>100(6/6)</td>
<td>100(6/6)</td>
<td>100(6/6)</td>
</tr>
<tr>
<td>Fertility index (%)</td>
<td>100(6/6)</td>
<td>100(6/6)</td>
<td>100(6/6)</td>
<td>100(6/6)</td>
</tr>
<tr>
<td>No. of corpora lutea/rat</td>
<td>13.67±0.42</td>
<td>13.17±0.40 (-3.66)</td>
<td>12.83±0.40 (-6.14)</td>
<td>13.5±0.34 (-1.24)</td>
</tr>
<tr>
<td>No. of implantations/rat</td>
<td>13.0±0.45</td>
<td>11.67±0.21 (-10.23)</td>
<td>10.17±0.31 (-21.77)</td>
<td>7.83±0.31 (-41.00)</td>
</tr>
<tr>
<td>Pre-implantation loss (%)</td>
<td>4.90</td>
<td>11.39</td>
<td>20.73</td>
<td>42.0</td>
</tr>
<tr>
<td>No. of live fetuses/rat</td>
<td>13.0±1.00</td>
<td>11.33±0.33 (-12.84)</td>
<td>9.67±0.67 (-25.61)</td>
<td>6.67±0.33 (-48.69)</td>
</tr>
<tr>
<td>No. of resorptions/rat</td>
<td>0</td>
<td>0.67±0.33 (-12.84)</td>
<td>1.67±0.33 (-21.77)</td>
<td>2.33±0.33 (-41.00)</td>
</tr>
<tr>
<td>Resorption index (%)</td>
<td>0</td>
<td>5.74</td>
<td>16.42</td>
<td>29.76</td>
</tr>
<tr>
<td>Post-implantation loss (%)</td>
<td>0</td>
<td>2.91</td>
<td>4.92</td>
<td>14.81</td>
</tr>
<tr>
<td>Fetal weight (gm)</td>
<td>3.99±0.09</td>
<td>3.41±0.07 (-17)</td>
<td>3.18±0.67 (-20.3)</td>
<td>1.97±0.06 (-50.57)</td>
</tr>
</tbody>
</table>

Values are mean ± S.E.M of six individuals. Values in the parentheses are percent changes from that of control. Mean values with the same superscript do not differ significantly from each other. The mean values with superscript ‘b’, ‘c’ and ‘d’ are significantly differ at p<0.05, p<0.005 and p<0.0001 respectively from ‘a’. The significance was considered at p<0.05.
4. Discussion

Many phytoestrogens show structural similarity with mammalian estrogens and exhibit competitive inhibition for binding with estrogen receptors thereby altering fertility. There is not enough available literature on phytoestrogens and their interaction with estrogen receptors. First time Bennetts et al. (21) demonstrated the role of genistein in infertility and estrogenicity in sheep. Since then, several studies were conducted pertaining to the possible health benefits and the developmental effects of phytoestrogens in various animal models (10-16). Despite of our understanding on the reproductive physiological events associated with phytoestrogens, relatively little is known about genistein and its effect on reproduction in adult rats. However the trans-generational effects of phytoestrogens on reproductive health of humans and animals are still in its infant stage. BC-A is a methyl derivative of genistein and systematic study has been undertaken to know the effect of BC-A exposure during perinatal period and alterations in reproduction at their adulthood using wistar rats as test model. The final reproductive end point tested is the male’s ability to sire offspring in a fixed time period. In the present study male rats exposed to BC-A are able to impregnate the unexposed females and the rate of fertility is also observed to be unaffected as compared to unexposed male rats. The fertility index in the 100 mg BC-A/Kg body weight exposed males was decreased (17%) when compared to the control and other BC-A exposed groups. Nagao et al. (22) observed decrease in fertility index in the neonatal genistein exposed rats mated with control females. Reports are also available on reduced fertility index in the direct administration of phytoestrogens in males and explained that the decrease in fertility index is due to low levels of testosterone which is altered by phytoestrogens. Perinatal exposure of phytoestrogens attribute the decrease in testosterone levels in circulation and increases oxidative stress in reproductive tissues leading to decreased sperm quality and density which ultimately decreases fertility index (23-29).

In the present study, number of implantations and live pups were observed in control females mated with control males are quite normal, but decrease in number of implantations, live pups and fetal weights were significant (p<0.0001) in females cohabited with BC-A exposed males. Meena and Reddy (30) reported the significant decrease in the number of implantations in female rats mated with perinatally 100 mg daidzein or genistein exposed males. They also observed significant decrease in number of live pups in females mated with 50 mg and 100 mg daidzein or genistein exposed males (30). Jefferson et al. (31) also reported that exposure of the pregnant females to genistein decreases the implantation sites and number of live pups. Similarly Nagao et al. (22) explained destruction of fertility in females exposed to 100 mg genistein/Kg body weight in rats.

BC-A exposed males mated with females showed increase in the mean value of pre- and post-implantation loss, number of resorptions and resorption index. Almedia and Lemonica (32) and Chang et al. (33) observed the loss of resorption index and post implantations in the female rats exposed to plant extracts during different periods of pregnancy. The treatment with 880 mg/kg per day of the extract of Coleus barbatus before embryo implantation caused delayed fetal development and an anti-implantation effect in rats (32). Similar findings were also reported by Dinesh and Varsha (20), Elbetieha et al. (34) and Yakabu et al. (35).
Abortifacient activity of phytosteroids found in *Pulmeria rubra* pod and *Senna alata* leaves in pregnant rats was reported (19, 34). Elbetieha et al. (32) reported the fetotoxic effects of *Globularia arabica* and *Globularia alypum* exposure in pregnant rats.

The decrease in the β-hydroxy steroid dehydrogenase and 17β-hydroxy steroid dehydrogenase activities were found in perinatally BC-A exposed males (Author’s unpublished data). Decrease in these enzymes may decreases the testosterone levels in exposed rats. Therefore we conclude that the decreased male fertility at levels of perinatal exposure by BC-A may be due to decreased testosterone levels in male rats.

Since testosterone is an essential hormone for spermatogenesis, the reduced testosterone levels decreases daily sperm count and quality of the sperm which may ultimately alters the various reproductive parameters in female (during pregnancy) after mating.

Moreover the effect of BC-A on spermatogenesis may also depends on the amount of regular plant based food intake, especially BC-A rich plant foods (Red Clover). These effects in human beings by BC-A is still in its infancy and present data may or may not be extrapolated to humans. However it is important to check the effect of BC-A on human reproductive health. Further in depth studies are required to know the reduced molecular mechanism of spermatogenesis by BC-A in men.

**Conflict of interest Statement**

The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

**References**