Perinatal exposure of biochanin-A induced abnormalities in offspring of rats

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Abstract

Estrogenic activity is found with many phytoestrogens, including Biochanin-A (BC-A). The present study is to examine the effect of prenatal and perinatal exposure of BC-A on developmental landmarks and behavior in Wistar rats. The mated female rats were injected with 25, 50 and 100 mg BC-A/Kg body weight on 12th, 14th, 16th and 18th day of gestation. The exposure was continued up to completion of weaning period. After weaning, male and female pups were separated and maintained in separate cages with normal pellet diet and tap water ad libitum. The developmental landmarks and behavior of young ones were recorded starting from birth to completion of weaning. Observed significant decrease (p<0.05) in the number of live pups and sex (male/female) ratio of rat pups exposed transplacentally to BC-A when compared to control group. Body weights of young ones were measured on post natal day (PND) 1, 7, 14 and 21 and observed significant decrease (p<0.005) in 50 and 100 mg BC-A perinatally exposed young ones than the control and 25 mg BC-A exposed groups. No significant changes (p>0.05) were recorded in fur development and ear opening in exposed young ones. Whereas significant increase in pinna detachment (p<0.05), upper incisor eruption (p<0.05) and preputial separation (p<0.005) were observed in pups perinatally exposed to 100 mg BC-A only. In contrast, significant decrease in anogenital distance (AGD) (p<0.05) in male and female pups was observed at 100 mg BC-A trans-placental exposure. In case of crown rump length (CRL), we observed significance (p<0.05) at 50 and 100 mg BC-A trans-placental exposure. Decrease in survival index was also observed on PND 1 and 14 in young rats exposed perinatally to BC-A. Besides above results, rat pups perinatally exposed to 50 and 100 mg BC-A showed significant increase in lower incisor eruption (p<0.05), eye opening (p<0.005), vaginal opening (p<0.005) and testis descent (p<0.05). These data demonstrate that significant alterations in developmental landmarks of young rats exposed transplacentally and perinatally to BC-A.

Keywords: Biochanin-A, Trans-placental, Perinatal, Pups evaluation, Developmental landmarks

1. Introduction

Endocrine disruption in humans and animals is increasing by the release of chemical compounds into nature due to various human activities and these chemicals are collectively called as endocrine disrupting chemicals (EDCs).

The adverse effects of EDCs are in focus by researches especially on development and reproduction in humans. EDCs acts on the developing gonad and subsequently influence germ cell development (1, 2).

During developmental period especially central nervous system is susceptible to EDCs and shows toxic effects (3, 4). Maternal exposure of many EDCs during gestation and/or lactation caused developmental neurotoxicity and/or behavioral abnormalities in the offspring that may persist throughout the lifetime of the animal (5, 6).

Phytoestrogens are natural, predominantly occurring compounds identified in plants which show estrogenic effect in humans and animals after consumption. The environmental protection agency’s defined the phytoestrogens as EDCs which cause adverse effects on human health. It includes
alterations in lactation, the timing of puberty, the ability to produce viable/fertile offspring, sex specific behavior, per mature reproductive senescence and compromised fertility (7). The attention of scientific community has been directed towards phytoestrogens due to their potent estrogenic activity (8-10).

Many environmental chemicals interfere with the synthesis and/or action of androgens that could result in abnormalities of both female and male reproduction. The gonadotrophic axis awakening at puberty stage is the main end-point of a complex cascade of sex developmental mechanisms which leads to the attainment of reproductive capacity. Dietary phytoestrogens are well known to have potential hormone effects by showing their interaction with estrogen receptors (11).

Abnormalities in reproductive health, due to high intake of phytoestrogens have been reported in women (12, 13). Interestingly, the phytoestrogen genistein is known to have estrogenic properties and showed effects on various hormones in women (14-18). Adverse effects caused by phytoestrogens are not only reported in humans on various physiological aspects, but also available on male and female reproduction in rat and mice (19-22).

Red clover, a botanical dietary supplement has received much attention recently for their potential use in maintenance and improvement of cardiovascular health. The products of red clover are rich with the phytoestrogen BC-A (23). Isoflavonoid, BC-A is a derivative of methyl genistein and acts as EDC. Though many reports are available on the adverse effects caused by the isoflavones, not much literature is available on the developmental landmarks such as incisor eruption, total unfolding of the external ears, fur development, ear opening, body weight, anogenital distance, crown rump length and behavioral abnormalities.

The present study is focused to elucidate the role of isoflavone BC-A on number of live pups, body weight along with developmental landmarks and behavior in F1 rats exposed transplacentally and perinatally.

2. Materials and methods
2.1. Procurement and maintenance of experimental animals
The experimental animal model selected for present study is Wistar strain rats. Rats (90 days old) were purchased from Sri Venkateswara Traders, Bangalore, India. Animals were housed in clean, well ventilated and air-conditioned room (12 h:12 h light: dark cycle; 25 ± 2°C with a relative humidity of 50 ± 5%). Rodent feed and tap water were provided ad libitum. The experiments were carried out in accordance with the guidelines of the Committee for the Purpose of Control and Supervision on Experiments on Animals (CPCSEA), Government of India (24). This study was also carried out according to the guidelines for the care and use of laboratory animals (NRC, 1996) and approved by the Institutional Animal Ethical Committee at Sri Venkateswara University, Tirupati, India (Resolution No: 10/(i)/a/CPCSEA/IAEC/ SVU/PSR/NG/Dt. 18-10-2010).

2.2. Test Chemical (Biochanin-A)
The test chemical used in the present study is Biochanin-A (BC-A) purchased from Sigma Chemical Company, St Louis, MO, USA. Chemically BC-A is 5, 7-Di hydroxyl–4-methoxy isoflavone (Fig. 1). The molecular mass of BC-A is 284. 26g/mol. BC-A is soluble in DMSO (80 to 100%) and ethanol (95 %). DMSO (100%) was selected as a vehicle for BC-A due to its low toxicity than ethanol.

Figure 1. Structure of Biochanin-A

2.3. Vaginal smear cytology
Cytological observation of vaginal smear has been done for identification of different stages of estrus cycle in the female rats before co-habitation with males. The vaginal smear was examined according to the method described by Zarrow et al. (25) and reviewed by Cooper et al. (26).

2.4. Treatment and dosage
Healthy female rats of body weights 190 ± 10g were cohabitated with healthy males of body weight 210 ± 10g in 1:1 ratio. Copulation was examined every morning and was confirmed by the presence of a vaginal plug or sperm in a vaginal smear and
was considered as gestational day 1 (GD1). Pregnant rats were separated from males and maintained in separate cages after dividing them into four groups of each four. The first group served as control and received 100 µl of 100% DMSO (purchased from Merck, Mumbai, India) on par with experimental groups. Second, third and fourth groups were served as experimental groups, received 25, 50 and 100 mg BCA (dissolved in 100% DMSO) per Kg body weight on 12th, 14th, 16th and 18th day of gestation respectively in a final volume of 100 µl each.

All the rats were fed with normal pellet diet and were allowed to deliver pups. Number of live pups was recorded for each group. Starting from day 1 (PND 1), abnormalities in the developmental landmarks and clinical signs of toxicity were observed in young ones (Figure 2A) of both experimental and control groups during and after weaning.

2.5. Pups evaluation

The weight of young ones was measured on 1st, 7th, 14th and 21st day of weaning by weighing each pup. CRL and AGD were measured on the day of parturition with the help of vernier calipers and measuring scale (Fig. 2C-E). For measuring CRL, each pup was placed on its side on a flat surface and the distance from the top of head to base of tail was determined. Pups were not stretched or compressed during measurement of CRL. AGD is a sexually dimorphic measure of distance from the anus to the penopubic junction (genital tubercle) and is lengthier in males than females. Survival index was measured on post natal day (PND) 4 and 21. Survival index (%) on 4th day was measured by using the formula: [(Number of live off springs at lactation day 4/Number of live off springs delivered) X 100] and 21st day survival index (%) is with: [(Number of live off springs at lactation day 21/Number of live off springs delivered) X 100].

2.6. Developmental landmarks

The developmental landmarks such as pinna unfolding, lower and upper incisor eruption, fur development, ear opening, eye opening, vaginal opening, testes descent and preputial separation (PPS) were undertaken for the present study (Figure 2E-P). Pinna unfolding of pups was examined individually when the tip of the ear is separated from the head and days required for pinna detachment was recorded. Lower and upper incisor eruptions were examined every day until the first appearance of upper and lower incisors and time required for the incipience of incisors was recorded. Fur development was observed every day until the fur (bristles) appears on the dorsal surface of pups and days required for fur development was recorded. Opening of ear was examined and recorded every day until the opening of ear and time required for the opening of ear.

Days at both eyes were fully opened in the pups was observed and recorded. Vaginal opening is seen as a central perforation, which will be enlarged laterally until completion of vaginal opening and time point of vaginal opening was recorded. The time taken for descent of testis from the lower kidney pole to the scrotum was recorded. Separation of the foreskin of the penis from the glans is known as preputial separation and the time required for this was recorded.

2.7. Statistical analysis

The data were analyzed statistically with two tailed paired student’s t-Test at 95% confidence intervals by using the statistical programme GraphPad Prism Version 5.0.3.477. The experimental values at p<0.05 was considered as significant from control.

3. Results

No mortality or any clinical signs of toxicity were observed in mothers exposed by BC-A during pregnancy. Delivered pups are normal and no general toxicity like head flicking, head searching, biting, circling and walking backwards were recorded. The behaviour observed in the young ones exposed perinatally to BC-A is not significant from the control group. Except number of live pups, male and female ratio no significant change was observed in any parameter studied in this study at 25 mg BC-A exposed pups with control group.

Significant decrease (p<0.05) in the number of live pups per rat and male/female ratio were observed in the pups exposed transplacentally to BC-A when compared to control pups (Table 1).

The body weights of perinatally BC-A exposed (50 and 100 mg/ Kg body weight) rat pups on PND 1, 7, 14 and 21 are significantly (p<0.005) differ from the body weights of control and 25 mg BC-A exposed group (Table 2). The evaluation of rat pups was determined by considering the end points such as AGD, CRL and survival index (Table 3). The mean AGD measured on PND 1 in both male (0.33
± 0.016) and female (0.27 ± 0.016) rat pups exposed transplacentally to 100 mg BC-A was decreased significantly (p<0.05) when compared to control and other experimental groups. Significant decrease was also observed in CRL on PND 1 in rat pups transplacentally exposed to 50 (p<0.05) and 100 mg (p<0.005) BC-A than control and 25 mg BC-A exposed rat pups.

The mean CRL of 50 and 100 mg BC-A exposed young rats is 4.51 ± 0.071 and 4.35 ± 0.05 respectively. Decrease in the survival index on PND 4 and 21 was observed in the pups perinatally exposed to BC-A. Decrease in survival rate of rat pups exposed to BC-A was increased by 21st PND than PND 4.

No significant changes were observed in developmental landmarks such as fur development and ear opening in the BC-A exposed rat pups when compared to controls (Table 4).

Increase in the mean values of pinna detachment (3.75 ± 0.125), upper incisor eruption (10.75 ± 0.164) and preputial separation (44.38 ± 0.323) were found significant (p<0.05) at 100 mg BC-A perinatally exposed rat pups when compared to control and other experimental groups. Significant increase in lower incisor eruption (p<0.05), eye opening (p<0.05), vaginal opening (p<0.005) and testes descent (p<0.05) were observed in rat pups at all experimental groups with an exception at 25 mg BC-A perinatally exposed group (Table 4).

![Figure 2. Developmental landmarks of rat pups studied in the present study.](image)

Table 1. Effect of embryonic biochanin-A exposure on number of live pups/rat and sex ratio of rat pups

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Controls</th>
<th>25 mg/Kg body weight</th>
<th>50 mg/Kg body weight</th>
<th>100 mg/Kg bodyweight</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of live pups/rat</td>
<td>11 ± 0.408</td>
<td>9.5 ± 0.288</td>
<td>8.5 ± 0.288</td>
<td>6.25 ± 0.478</td>
</tr>
<tr>
<td></td>
<td>(-13.64), p&lt;0.05</td>
<td>(-22.73), p&lt;0.05</td>
<td>(-43.18), p&lt;0.05</td>
<td></td>
</tr>
<tr>
<td>Male:Female pups</td>
<td>23:21</td>
<td>18:20</td>
<td>18:17</td>
<td>15:10</td>
</tr>
</tbody>
</table>

Values are mean ± SEM of pups delivered by 4 pregnant rats.
Values in parenthesis are percent change from controls. ‘p’ at >0.05 was considered as significant.
### Table 2. Perinatal exposure of biochanin-A on body weights of young ones

<table>
<thead>
<tr>
<th>Post natal day</th>
<th>Control</th>
<th>25 mg/Kg body weight</th>
<th>50 mg/Kg body weight</th>
<th>100 mg/Kg body weight</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>5.93 ± 0.148</td>
<td>5.87 ± 0.156</td>
<td>5.34 ± 0.129</td>
</tr>
<tr>
<td></td>
<td>(-1.01), p=0.7586</td>
<td>(1.70), p=0.4758</td>
<td>(15.94), p&lt;0.005</td>
<td>6.07 ± 0.151</td>
</tr>
<tr>
<td>7</td>
<td>11.12 ± 0.205</td>
<td>10.93 ± 0.199</td>
<td>9.40 ± 0.257</td>
<td>9.0 ± 0.298</td>
</tr>
<tr>
<td></td>
<td>(-1.40), p=0.7586</td>
<td>(-5.27), p&lt;0.005</td>
<td>(-15.19), p&lt;0.005</td>
<td>(-19.06), p=0.005</td>
</tr>
<tr>
<td>14</td>
<td>17.81 ± 0.135</td>
<td>17.56 ± 0.175</td>
<td>16.87 ± 0.295</td>
<td>15.31 ± 0.339</td>
</tr>
<tr>
<td></td>
<td>(-1.32), p=0.2753</td>
<td>(-5.27), p&lt;0.05</td>
<td>(-14.03), p&lt;0.005</td>
<td>(-14.03), p&lt;0.005</td>
</tr>
<tr>
<td>21</td>
<td>22.50 ± 0.227</td>
<td>21.84 ± 0.238</td>
<td>21.65 ± 0.275</td>
<td>19.95 ± 0.322</td>
</tr>
<tr>
<td></td>
<td>(-2.93), p=0.1234</td>
<td>(-3.77), p&lt;0.05</td>
<td>(-11.33), p&lt;0.005</td>
<td>(-11.33), p&lt;0.005</td>
</tr>
</tbody>
</table>

Values are mean ± SEM of 8 young rats. Values in parenthesis are percent change from controls. ‘p’ at >0.05 was considered as significant.

### Table 3. Trans-placental exposure of biochanin-A on anogenital distance, CRL and survival index of rat pups

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Controls</th>
<th>25 mg/Kg body weight</th>
<th>50 mg/Kg body weight</th>
<th>100 mg/Kg body weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anogenital distance (cm) of male pups on PND 1</td>
<td>0.41 ± 0.022</td>
<td>0.39 ± 0.022</td>
<td>0.37 ± 0.016</td>
<td>0.33 ± 0.016</td>
</tr>
<tr>
<td>Anogenital distance (cm) of female pups on PND 1</td>
<td>0.33 ± 0.016</td>
<td>0.31 ± 0.022</td>
<td>0.29 ± 0.022</td>
<td>0.27 ± 0.016</td>
</tr>
<tr>
<td>Crown rump length (cm) of pups on PND 1</td>
<td>4.89 ± 0.0107</td>
<td>4.77 ± 0.097</td>
<td>4.51 ± 0.071</td>
<td>4.35 ± 0.05</td>
</tr>
<tr>
<td>Survival index of pups on PND 4 (%)</td>
<td>45/45 (100)</td>
<td>39/40 (97.5)</td>
<td>35/36 (87.5)</td>
<td>26/28 (70)</td>
</tr>
<tr>
<td>Survival index of pups on PND 21 (%)</td>
<td>44/45 (97.7)</td>
<td>38/40 (90.0)</td>
<td>34/36 (87.5)</td>
<td>25/28 (77.5)</td>
</tr>
</tbody>
</table>

Values are mean ± SEM of 8 young rats. Values in parenthesis are percent change from controls. ‘p’ at >0.05 was considered as significant.

### Table 4. Effect of trans-placental and weaning exposure of biochanin-A on developmental landmarks of rat pups

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Controls</th>
<th>25 mg/Kg body weight</th>
<th>50 mg/Kg body weight</th>
<th>100 mg/Kg body weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pinna detachment</td>
<td>3.12 ± 0.125</td>
<td>3.25 ± 0.163</td>
<td>3.50 ± 0.189</td>
<td>3.75 ± 0.125</td>
</tr>
<tr>
<td>Lower incisor eruption</td>
<td>3.12 ± 0.125</td>
<td>3.25 ± 0.163</td>
<td>3.75 ± 0.163</td>
<td>3.87 ± 0.125</td>
</tr>
<tr>
<td>Fur development</td>
<td>7.12 ± 0.125</td>
<td>7.25 ± 0.163</td>
<td>7.37 ± 0.183</td>
<td>7.62 ± 0.183</td>
</tr>
<tr>
<td>Upper incisor eruption</td>
<td>10.12 ± 0.125</td>
<td>10.25 ± 0.164</td>
<td>10.37 ± 0.183</td>
<td>10.75 ± 0.164</td>
</tr>
<tr>
<td>Ear opening</td>
<td>13.25 ± 0.163</td>
<td>13.38 ± 0.183</td>
<td>13.50 ± 0.189</td>
<td>13.88 ± 0.226</td>
</tr>
<tr>
<td>Eye opening</td>
<td>14.13 ± 0.125</td>
<td>14.25 ± 0.164</td>
<td>14.63 ± 0.183</td>
<td>14.75 ± 0.164</td>
</tr>
<tr>
<td>Vaginal opening</td>
<td>34.13 ± 0.295</td>
<td>34.88 ± 0.295</td>
<td>36.25 ± 0.250</td>
<td>37.88 ± 0.350</td>
</tr>
<tr>
<td>Testes descent</td>
<td>23.25 ± 0.366</td>
<td>23.63 ± 0.263</td>
<td>24.38 ± 0.263</td>
<td>25.13 ± 0.295</td>
</tr>
<tr>
<td>Preputial separation</td>
<td>42.50 ± 0.378</td>
<td>42.88 ± 0.295</td>
<td>43.13 ± 0.295</td>
<td>44.38 ± 0.323</td>
</tr>
</tbody>
</table>

Values are mean ± SEM of 8 young rats. Values in parenthesis are percent change from controls. ‘p’ at >0.05 was considered as significant.
4. Discussion

Biochanin-A is a plant isoflavone with polyphenolic compounds derived from a common class of phytoestrogens. The greatest estrogenic activity was found in isoflavones. Functionally, estrogens at physiological levels execute reproductive development and induce secondary sexual characters. But, at excess levels, estrogens exert detrimental consequences on development and remarkably on the development of reproductive organs thereby sexual performance of the animal (27, 28). Phytoestrogens binds to estrogen receptors and alters the physiologic and morphologic development of prenatal offspring (29, 30). Consumption of isoflavone rich plant-based food is increasing in developed and developing countries. In the present study, we tested the toxicity and developmental abnormalities of BC-A at 25, 50 and 100 mg/Kg body weight exposure transplacentally and perinatally. Decrease in number of live pups and male/female ratio were found in trans-placental BC-A exposed groups. Decreased litter size by different EDCs was reported in rat and mice (31-33). Pushpalatha (31) and Harini (32) reported decrease in the size of litter in pregnant rats and mice exposed in utero to proluton (hydroxylprogesterone) and progesterone respectively. Similarly, Romer et al. (33) found significant alterations in the number of live foetuses delivered by 10 and 100 mg of phytoestrogens (daidzein/genistein)/Kg body weight in rat. They observed the smaller number of live litter in their study.

In the present study, decreased male and female ratio from control is in a dose-wise manner which is in correlation with decreased number of live pups which may be due to the increased trans-placental fetal toxicity of BC-A towards increased number of resorptions and smaller number of implantations in a dose-dependent manner.

Perinatal exposure of BC-A resulted in significant (p<0.005) reduction in the mean body weight of 50, and 100 mg BC-A/Kg body weight exposed pups at PND 1, 7, 14 and 21. These results are in agreement with the results of Musameh et al. (28) and they found significant reduction in the mean body weights of rat pups exposed perinatally to genistein (10 and 100 mg) on PND 1, 7, 14 and 21. Significant decrease in the body weight of female rat pups exposed to daidzein/genistein on PND 14 and 21 was also reported (33). Several other studies were also found significant reduction in body weight of rat pups at the high concentration of genistein administration (35-37).

In contrast, repeated exposure of genistein and ingestion of dietary phytoestrogens to pregnant female rats has no effect on maternal body weight or their feed intake (38, 39). It is also reported that the decrease in the body weights of pups exposed perinatally to genistein was started increasing after PND 21 and weights are normalized to controls on PND 50. The decrease in the body weights of pups exposed perinatally to BC-A in this study is may be due to reduced food intake of pregnant dams and decreased appetite in young ones, leads to no deposition of fat during the lactation period which ultimate results in loss of body weight during perinatal period.

In humans AGD (distance from anus to the posterior base of the scrotum) plays an important role on sperm parameters. Men with AGDs of less than 51.7 mm showed 7.3 times reduction in the sperm concentration (40). However, the AGD in humans and rats is under the regulation of androgens. In the present study, rat pups treated with BC-A transplacentally at a dose of 100 mg/Kg body weight showed decrease in AGD of both male and female at PND 1. Similarly, reduction in AGD was observed in male offspring exposed prenatally to 10 and 100 mg genistein/Kg body weight on PND 1 to 21 and 1 to 15 respectively (28). Increased anogenital index in female rat pups exposed transplacentally to flaxseed meal (phytoestrogen rich diet) on PND 21 was reported (34). AGD is used as a marker for prenatal androgenization, which is related with the testosterone production in the pups (41, 42).

Changes in AGD among male infants and suppressions of androgen synthesis have been associated with anti-androgen exposure in humans (43). Suggested reason for the decrease in AGD of rat pups in this study may be due to interference of BC-A on external genitalia at an embryonic development by reducing the androgen production. In rat pups exposed transplacentally to 50 and 100 mg BC-A/Kg body weight showed significant decrease in crown rump length. Decreased crown rump length was observed in conceptus of albino rats exposed to ciprofloxacin 20 mg/Kg body weight on 18th day of gestation (44). Studied survival index of pups on PND 4 and 21 in this study and found decrease in the survival rate of pups exposed to BC-A perinatally. Though no difference is witnessed in fur development and ear opening, significant change is found in the pinna detachment, upper and lower incisor eruption, eye opening, vaginal opening, testes descent and preputial separation in the young rats exposed perinatally to BC-A.
The average age of vaginal opening was 33 days for female pups which falls in the natural pubertal age range, 32–38 days (45). Reasonable reports are available on delay in the vaginal opening in rats (28, 46-48). Delayed vaginal opening and testes descent was reported in perinatally genistein exposed female and male pups (28). In contrast, an earlier onset of vaginal opening was observed in mice exposed directly to genistein during the lactation period (49) and in rats neonates treated with 10 mg genistein/Kg body weight/day (50). Shortened eye opening, ears unfolding and vaginal opening in female offspring exposed perinatally to daidzein/genistein were also reported in rats by Romero et al. (33). Besides this, genistein exposure during pregnancy in offspring of rats showed the decreased birth weight, reduced anogenital distance (AGD), delayed puberty, altered mass of reproductive organs, and altered reproductive behavior (22, 51, 52). However, the reports are not sufficient about onset of puberty and sexual maturation in rats and mice on exposure during embryonic and lactation or continuous exposure to phytoestrogen diet. But the excess exposure to estrogens during critical window period of development may show detrimental abnormalities on development of the embryonic reproductive tissues (53-55). However the trans-placental and perinatal developmental abnormalities observed in the present study are confined to 50 and 100 mg BC-A exposure, but not at 25 mg concentration.

By the above results it has been shown that red clover isoflavones (BC-A) affect growth and development of the reproductive tissues in male and female offspring of rats which ultimately alters the physiological concentrations of growth and reproductive hormones. The effects caused by BC-A is depends on the period of life when the BC-A exposure applied. For all the observed abnormalities, the estrogenic mimicking action of BC-A is may be responsible. Though there is no evidence of BC-A at the recommended dosage can produce the similar effects in humans. So, the present data may or may not be extrapolated to humans. However, the pregnant mothers should be aware of these isoflavonoids present in red clover diets in order to overcome the infertility problems in offspring. Future studies with longer duration of BC-A exposure is necessary to better account for its effects and possible mechanisms as an endocrine disruptor to the reproductive system of both women and men. Thus, despite the indiscriminate recommendation on the use of red clover phytoestrogens and its derivatives, the results of this study show that Biochanin-A is not totally free from undesirable effects.

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

**References**


