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Pregestation and gestation exposure to an isoflavone: Impact on maternal reproductive health and postnatal development of neonatal mice

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ABSTRACT

Objective: To investigate the effects of pregestational and gestational exposure to an isoflavone of sexually mature female mice on their reproductive health and on the postnatal development of their offspring. **Methods:** Sexually-mature ICR female mice were randomly segregated into Control group 1 that was given the normal diet consisting of food pellets, Control group 2 that was given food pellets with additional corn oil of 2 mL/kg bodyweight. The three (3) isoflavone treatment groups were given 50 mg/kg body weight, 100 mg/kg body weight and 150 mg/kg body weight for low dose (LD), medium dose (MD) and high dose (HD) respectively. Treatments were administered 2 weeks prior to mating and gestation and thereafter until parturition. The delivered pups were weaned up until 21 days. On the 21st day, postnatal development were determined, excluding the birth weight of pups which was measured one day post-parturition. The dams were sacrificed as well and the markers of maternal health were determined. **Results:** There were no significant differences found between the control groups and the treatment groups in terms of the markers of maternal reproductive health. For postnatal development, only HD group displayed a significantly higher mean AGD from the other groups. **Conclusions:** The data implies that exposure to the isoflavone genestein, with the given dosages, does not impact the maternal reproductive health while the high dose brings about masculinization of the pups which implies that isoflavone exerts its action as an endocrine disruptor affecting postnatal development. This could be attributed to the decrease in estrogen due to the inhibition of aromatase, an enzyme involved in estrogen synthesis.

1. Introduction

Isoflavones are organic compounds that can be found naturally occurring in soybeans, chickpeas and other legumes [1]. Soybeans have the highest concentration of isoflavones, which are almost exclusively found in the Fabaceae family. The consumption of isoflavones, through soy products, has been suggested to aid in the prevention of several chronic diseases [2]. Some of these include the protection of the cardiovascular system from Low Density Lipoprotein oxidation, the prevention of osteoporosis as well as the prevention of cancer [1]. These perceived benefits have led to an increase in the popularity and consumption of soy products in recent years [3].

One example among the many products and supplements that have been produced to meet the demand for soy is the Soy-Based Infant Formula (SBIF) that serves to address the nutrition needs of infants who are unable to consume regular formula due to clinical reasons [4]. Comprehensive studies of infants fed SBIF have resolved questions with respect to nutritional adequacy, sexual development, neurobehavioral development, immune development or thyroid disease [5]. However, some critics claim that isoflavones can increase the incidence of epithelial hyperplasia causing goiter and hyperthyroidism [6]. SBIF and other soy products mostly contain high levels of the isoflavones genistein and daidzein, which are commonly referred to as phytoestrogens. These are non-steroidal chemicals with structures similar to estrogen [7]. These compounds, being endocrine-active, have the capability to affect the sexual development of the organisms that consume them [8].

Recent in-depth reviews of the safety of soy show no conclusive evidence of adverse effects of dietary isoflavones on health development and reproduction in animal or human adult or infant populations [5]. However, since 2010, there has been a call for further study into the effects of isoflavones in SBIF on infants due to the lack of comprehensive research on the area [4].

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In line with addressing the lack of information of isoflavone in terms of developmental effects, this research aims to provide more information on the effect of the intake of isoflavone during the prenatal and postnatal stages of development. These effects can be determined through testing using rodent models [9].

This study could be relevant and timely in providing information on the possible implications on reproductive health considering the increase in availability and consumption of soy-based infant formula in recent years.

This study aimed to determine if the pregestational and gestational exposure of mice to isoflavones could have any observable effects on the reproductive health of dams as well as on the postnatal development of their offspring.

2. Materials and methods

2.1. Test animals and setup

Six-week-old high quality ICR male and female mice with weight averaging 25 g were used in the experiment. The animals were obtained from the Bureau of Animal Industry in Diliman, Quezon City, Philippines. These were kept in separated, standard-sized cages in the animal house of De La Salle University-Manila. All cages were sanitized weekly and bedded with paddy husk that was autoclaved. The plates and bottles were washed and dried twice a week. The mice were allowed to acclimatize for one week to adjust to the light-dark cycle that they were exposed to, which was 12 h light: 12 h dark at 28-30 ℃[10]. They were given mineral water and food pellets ad libitum throughout the period of experimentation. Proper handling and maintenance of the mice was guided by the safety standards set by the Philippine Veterinary Medical Association.

2.2. Chemicals and reagents

Genistein Soy Complex in powdered form was obtained from Source Naturals USA . This was made from isoflavone-rich soybean powder yielding approximately 42.5 mg of Daidzein, 25 mg of Glycitein and 10 mg of Genistein, with a total isoflavone amount of 77.5 mg per 2.5 g. A 2% NaOH solution was used as an indicator for the determination of the implantation sites.

2.3. Supplementation

After the acclimatization period, the mice were randomly assigned to five (5) test groups, from which their respective diets were determined and given for two (2) weeks. There were two control groups for this study. The control group 1 (C1) was provided with food pellets only and drinking water while control group 2 (C2) was provided with food pellets mixed with 0.05 mL corn oil and drinking water while the Isoflavone was administered to the low dosage (LD), medium dosage (MD) and high dosage (HD) groups through dietary supplementation. The doses were adapted[4], as the recommended doses for oral administration in rodent models studying reproductive health. Doses of isoflavone were as follows: 50 mg/kg body weight, 100 mg/kg body weight and 150 mg/kg body weight for LD, MD and HD respectively. The isoflavone powder was coated onto food pellets using water. Each mouse received 1.0 g of food pellets coated with the corresponding dose before being given food pellets *ad libitum* to ensure the consumption of the isoflavones.

2.4. Mating and checking of positive coitus

At the culmination of the two-week supplementation period, male and female mice (1:2) were joined in one cage. Female mice were monitored daily from 0700 to 0800 hours for the presence of copulatory plug.

The presence of the plug in the vagina after mating was used to determine pregnancy of the mice. The checking process was adapted from Deb, *et al.* (n.d.). The process was as follows: (1) Grasp the tail of the mouse. (2) Hold the mouse in one hand with its face up. (3) Locate the vagina and use a small pair of curved forceps to spread the lips of the vulva to identify the plug. (4) When gently touched with a pair of forceps, the plug feels solid and blocks the vagina.

Female mice that were positive for plugs were considered to have embryos aged at 0.5 days post coitus (dpc). The females that were negative for plugs were set aside to undergo mating again in order to increase the sample size. All the male mice were kept supplied with food pellets and water until the end of the study.

2.5. Determination of successful pregnancy rate

The successful pregnancy rate was determined by taking into account the number of dams that were impregnated based on the presence of the vaginal plugs and the number of dams that gave birth for each treatment group. The rate was calculated using the following formula:

$$\text{Successful pregnancy rate} = \frac{\text{Number of pregnant mice with delivery}}{\text{Total number of pregnant mice}} \times 100$$

2.6. Identification of implantation sites and determination of successful implantation rate

When the pups have weaned after 21 days, the dams were sacrificed through cervical dislocation and their uterus were retrieved. The number of implantation sites was determined by immersing the uterus in 2% sodium hydroxide (NaOH) solution for over one hour until the sites appear. The implantation traces will appear to be stained yellowish-brown [11]. The number of implantation sites that appeared on the dissected uterus after staining was recorded to determine successful implantation rate.

The number of implantation sites was determined for all dams and compared to the number of pups born to determine the successful implantation rate using the following formula:

$$\text{Successful implantation rate} = \frac{\text{Number of pups at birth}}{\text{Number of implantation sites}} \times 100$$

2.7. Accounting of litter size

After each dam gave birth, the pups were then given one (1) day to stabilize. The number of pups per litter was counted after the stabilization period to determine the litter size. The mean of all the litter sizes were then averaged.

2.8. Determination of birth weight, AGD and postnatal survival rate

Birth weight, postnatal survival rate and anogenital distance were evaluated to identify the effects of isoflavones on postnatal reproductive development.

The individual birth weights of the pups were also recorded one (1) day after parturition. Birth weights were determined to the nearest gram using a Sartorius analytical top-loading balance (Model #8 1205).

Postnatal survival rate was determined by counting the number of pups per litter that survived after the 21-day weaning. The survival rate for each litter was calculated using the following formula:

$$\text{Postnatal survival rate} = \frac{\text{Number of pups after weaning}}{\text{Number of pups upon birth}} \times 100$$

The Anogenital Distance (AGD) was also measured after the weaning period with the use of a Vernier caliper. The measurements were recorded to the nearest millimeter.

2.9. Statistical analysis

The data on the birth weight, litter size and anogenital distances expressed as means were subjected to One-way Analysis of Variance (ANOVA) and the means were compared by Tukey's test using Statistical Package for the Social Sciences (SPSS) Version 20 to determine significant differences among treatment groups. The level of significance in all cases was $P < 0.05$.

3. Results

3.1. Maternal reproductive health

3.1.1. Successful pregnancy rate

There were no treatment groups in which all of the females positive with vaginal plugs gave birth. Both the C1 and LD displayed the highest successful pregnancy rate among the treatment groups which was 60% (Figure 1). The pregnancy rates of the rest of the treatment groups did not vary greatly, meaning that only an average of 50%-60% of the dams which showed positive vaginal plugs remained pregnant and gave birth.

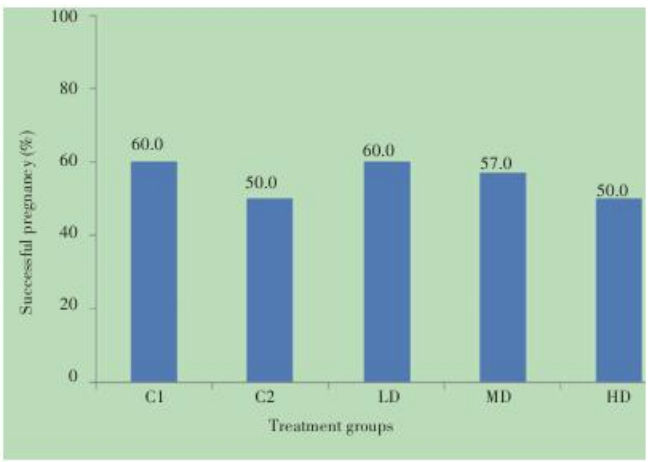

Figure 1. The successful pregnancy rate of different treatment groups (expressed as %).
Control 1 (C1); Control 2 (C2); Low dose (LD); Medium Dose (MD) and High dose (HD).

3.1.2. Successful implantation rate

The successful implantation (SI) rate of the two (2) control groups and the three (3) isoflavone treatment groups are shown in Figure 2. The SI rate for C1 was comparably similar with those of all three (3) isoflavone groups-LD, MD and HD. It was also observed that the SI rate for C2 was remarkably lower than those for the other treatment groups. However, the difference between C2 and the other treatment groups was not enough to be considered significant (Figure 2 & 3). Figure 3 shows uterine implantation sites.

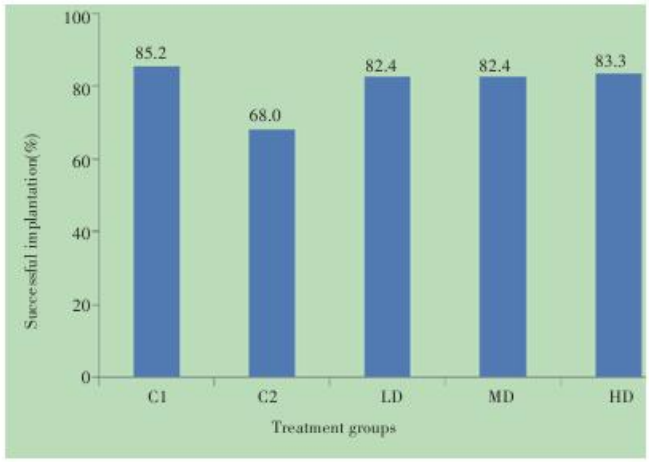

Figure 2. The successful implantation rate of different treatment groups (expressed as %).
Control 1 (C1); Control 2 (aC2); Low dose (LD); Medium Dose (MD) and High dose (HD).

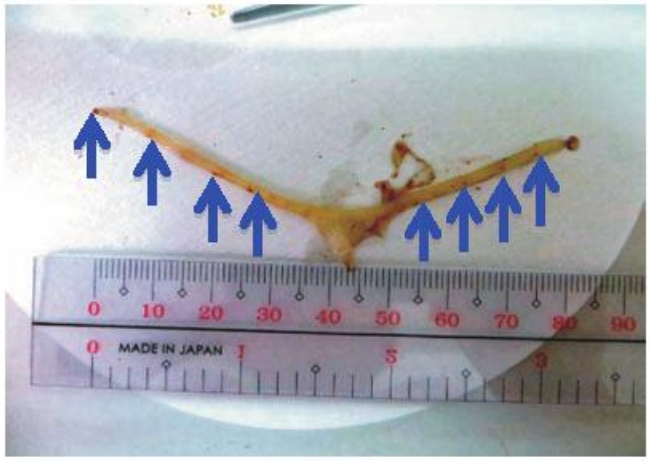

Figure 3. Uterine sample showing implantation sites (in arrows).

3.1.3. Litter size

The litter size upon delivery per dam ranged from 3-16 pups (Table 1). The highest number of total pups per group belonged to MD and HD with 42 and 30, respectively. Despite this and the fact that the highest individual litter size belonged to a dam in HD, the mean litter size of C1 was comparable with those of LD, MD and HD and the mean litter sizes of the three isoflavone treated groups did not vary significantly from the controls.

3.2. Offspring postnatal development

3.2.1. Birth weight

The means of the litter birth weights per treatment group are shown in Table 2. Although statistical analysis revealed that all three mean birth weights of the three groups treated

with isoflavone did not vary significantly from the controls, the litter weight for C1 was slightly higher than the rest. This data coincides with previous studies [11,12] by Bucci, *et al.* that showed a decreased gain in body weight by pups whose mothers were treated with genistein.

Table 1

Mean number of pups per treatment group (pulled from at least three dams per treatment group).

Groups	Dams(n)	Pups(n)	Mean litter size
C1	3	23	7.700 ± 2.309
C2	3	17	5.700 ± 3.055
LD	3	20	6.700 ± 2.517 ^{ns}
MD	4	42	10.500 ± 4.655 ^{ns}
HD	3	30	10.000 ± 2.646 ^{ns}

ns = not significantly different from other groups. $P < 0.05$, n = total number, Mean litter size were expressed as Mean ± S.D.

Table 2

The mean birth weight measurements (grams) of pups (pulled from at least three dams per treatment group).

Group	Pups delivered(n)	Weight (g)
C1	23	2.069 0 ± 0.309 8
C2	17	1.663 0 ± 0.519 6
LD	20	1.780 0 ± 0.366 6^{ns}
MD	42	1.674 0 ± 0.317 7^{ns}
HD	30	1.917 0 ± 0.832 7^{ns}

ns = not significantly different from other groups. $P<0.05$, n=total number, Mean litter size were expressed as Mean ± S.D.

3.2.2. Survival rate

None of the litters in the control groups had any instances of infanticide, with 100% postnatal survival rate. Figure 4 illustrates that the three (3) isoflavone treatment groups suffered loss of pups with less than 100% survival. Surprisingly, the groups treated with isoflavone had lower postnatal survival rate than those of the control groups. A total of 13 pups from the treatment groups died at varying days during the weaning period of 21 days.

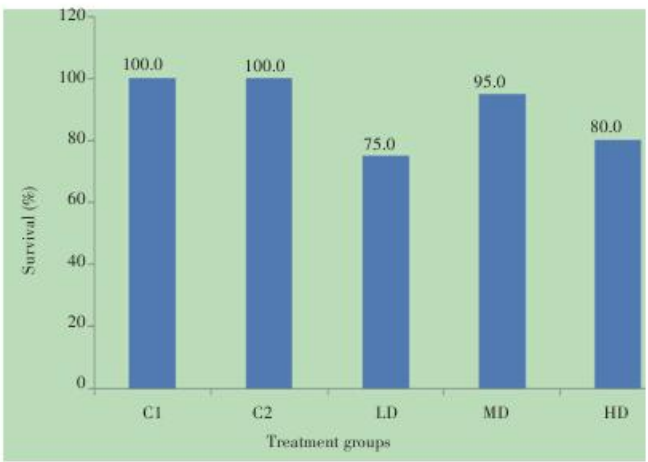

Figure 4. The postnatal survival rate of pups from the different treatment groups (expressed as %).
Control 1 (C1); Control 2 (C2); Low dose (LD); Medium dose (MD) and High dose (HD).

3.2.3. Anogenital distance measurements (AGD)

The only significant variation that came from the statistical analysis was from the measurement of the AGD. The data from the experimental groups showed the mean AGD of HD was significantly higher than those of C1 and LD (Table 3). While it is true that only HD showed a significant difference, there was a consistent trend of increase of mean AGD measurement with the higher doses of isoflavone.

Also, C2 can be comparable with LD. However, the larger sample size of pups after weaning may be a factor of the lower mean AGD measurement of LD compared to C2.

Table 3

AGD measurements (millimeter) of pups (pulled from at least three dams per treatment group).

Groups	Pups after weaning(n)	Mean AGD measurement (mm)
C1	23	4.540 0 ± 0.385 9^{a}
C2	17	5.740 0 ± 1.821 4^{b}
LD	15	5.290 0 ± 1.630 5^{d}
MD	40	6.410 0 ± 0.553 2^{c}
HD	24	7.840 0 ± 0.433 1^{ad}

values with the same letter are significantly different from each other $P<0.05$, n=total number, Mean AGD were expressed as Mean ∫ S.D.

4. Discussion

The results in Figure 1 and Figure 2 which do not show dramatic differences among and between the control and treatment groups imply that the genistein treatments has not influenced the successful pregnancy rates of the dams. Similarly, the isoflavone genistein could neither enhance nor induce successful implantation rate. These results may indicate that the hormones necessary for implantation remain in a balance regardless of the presence of isoflavone. These conflicts with previous studies which showed that the exposure of dams to genistein led to decreased implantation and increased resorption [11]. However, the timing of exposure differs from what was conducted in this study. In Goulding, *et al.*[11], genistein exposure was given at neonatal days 1–5. These differing results suggest that the timing of exposure to genistein plays a role in how it affects implantation.

This data further shows that isoflavone also had a negligible effect on litter size. Given that a big litter size is an indicator of a high number of ovulated and fertilized oocytes, the absence of significant difference in litter size of the experimental groups from the control groups could imply that genistein does not trigger any effect on ovarian function of the dams. This does not coincide with previous findings that genistein, being a tyrosine kinase inhibitor, induces a decrease in oocyte maturation and development [6]. The contrasts between this study and that of previous study [6] in dosages as well as results may suggest that the dosages used in this study may not have been adequate to trigger the reaction.

The absence of significant difference between the birth weights of the experimental groups and the control groups implies that preimplantation and gestation exposure to the dosage levels of isoflavone that were used in this study did not have any positive influence on the mean birth weights of mice.

Regardless of the fluctuations of results from the three (3) isoflavone treatment groups, the data shows that the isoflavone treated groups had decreased survival rate. However, it is difficult to ascertain whether these results were caused solely by the exposure of dams to genistein. The litter size as a factor was initially hypothesized to affect the postnatal survival rate in that a smaller litter size would lead to a higher survival rate. Considering that LD, with the lowest survival rate, had a smaller litter size than C1, which had 100% survival rate, the hypothesis is unlikely. Similarly, the birth weight as a factor was also initially considered in that lower birth weights would result in lower survival rates. However, given that there were no significant differences in birth weight among the groups, it was also an unlikely explanation.

AGD has been commonly used as an external marker for hormonally sensitive development in mice [13]. According to the American Association for Laboratory Animal Science (2005), the anogenital distance is greater in the male than in the female for all ages. Therefore, it can be implied that with higher exposure to isoflavones due to increased doses, the more masculinization occurs in mice. Differentiation of the AGD is also influenced by the concentrations of testosterone exposure of the fetus [14].

The data shows that genistein has a significant effect on the postnatal development of mice in terms of AGD. This can be attributed to the ability of isoflavones to inhibit aromatase, an enzyme involved in estrogen synthesis [15]. With the inhibition of aromatase, there is reduced estrogen synthesis [16]. The decrease in estrogen production due to pregestational and gestational exposure to genistein could imply that the fetus is exposed to more testosterone, leading to masculinization during postnatal development.

These results show that genistein acts as endocrine disruptors. Endocrine disruptors affect endocrine pathways by causing estrogenic activities in place of endogenous steroidal chemicals [17].

In conclusion, the study found no significant influence of the isoflavone genestein on the markers of maternal health such as successful pregnancy rate, successful implantation rate and litter size. Of the markers of postnatal development, there were also no significant influence on birth weight and postnatal survival rate. The only marker which showed significant difference from the control groups was the AGD. The masculinization exhibited by the increased mean AGD from a high dosage implies that isoflavones like genistein may have exerted its function as an endocrine disruptor by inhibiting estrogen synthesis through the inhibition of aromatase.

Conflict of interest statement

We declare that we have no conflict of interest.

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