EFFECTS OF SEX SELECTION DRUGS (SSD) ON OUTCOME OF PREGNANCY AND REPRODUCTIVE BEHAVIOUR OF NEXT GENERATION- PROTOCOL FOR AN ANIMAL STUDY

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ABSTRACT
Intake of indigenous sex selection drugs (SSDs) to have a male child is a practice reported from certain parts of India. Population based observational studies have indicated a possible association of intake of SSDs with birth defects and stillbirths. This study will aim to establish the effects of SSDs on outcome of pregnancy and effects on the reproductive system of growing male and female foetuses. Evaluation of prenatal developmental toxicity of SSDs will be done by administering SSDs to primigravidae rats during a period that corresponds to the period when SSDs are consumed by pregnant women. Structural abnormalities and pathological changes will be observed by sacrificing the rats close to the date of delivery. Evaluation of peri and post natal developmental toxicity will be done by administering SSDs to rats during pre-specified gestational period and assessing the pups of F1 generation. The various parameters will include changes in the reproductive organs and sexual maturation. Approval has been sought from Ethics Committee of the agency conducting animal study and Ethics Committee of the Institute. OECD guidelines will be adhered at every stage of the project.

KEYWORDS: sex selection drugs, animal models, prenatal toxicity, perinatal toxicity.
BACKGROUND
Apart from improving the amenities for diagnosis and treatment of diseases, science is also committed to exploring facts that have become an integral part of societal norms. Gender preference gave rise to myriad of practices ranging from infanticide to feticide. A concurrent desire to restrict the family size and have a son at the same time have compelled families to follow sex selection measures. This innate desire has made different sex selection procedures - be it traditional or modern, popular in the community. One such example is the practice of intake of sex selection drugs (SSDs) reported to be taken by pregnant women during initial months of pregnancy to beget a male child.[1,2,3]

SSDs are indigenous preparations that contain certain herbal and non-herbal ingredients and are rarely sold over-the-counter. Stringent rituals are supposed to be followed for its success. A preliminary community based study indicated that 46% of the women gave a history of intake of SSDs.[4] Risk to the fetus might be involved as these are consumed at a time when sexual differentiation takes place at 6-10 weeks of pregnancy. Recent evidence suggests that women who take SSDs are 3 times more likely to give birth to babies with birth defects and 2.5 times more likely to have stillborn babies.[5,6] Preliminary results indicate that these preparations contain phytoestrogens in quantities that is much higher than the permissible limits.[6,7,8,9] Limited studies indicate that birth defects could be major malformations of internal organs like urogenital or renal malformations or trachea-esophageal fistula or could be visible defects like spina bifida, cleft lip/palate and imperforate anus.[10] Studies also indicate that a single exposure during pregnancy can be deleterious.[11]

In fetal life when reproductive organs are developing, androgenic and estrogenic hormones can exert ‘organizational’ effects that permanently shape the reproductive system and its functions. Limited evidence on exposure of fetus to androgens is obtained from studies conducted on patients suffering from adrenogenital syndrome.[12] In such patients, disbalance in hormone levels and over-production of androgens leads to partial masculinization of external genitalia and behavior.[13] Exposure to female fetus to phytoestrogens during this critical period may permanently alter sensitivity to hormonal signals such that the endocrine system may never function properly. eg. Inappropriate exposure of female rodents to estrogens alters the ‘setting up’ of their hypothalamic pituitary ovarian axis such that in adulthood, they are unable to exhibit normal ovarian cycle. Furthermore, sexual differentiation in the brain (which is dependent on the action of androgens and estrogens)
occurs in utero in humans during this crucial period. Any insult due to exogenous estrogens or androgens may have long term implications.[14,15]

Intake of phytoestrogens and testosterone during a critical period of development of fetus is fraught with dangers that may or may not be visible at birth.[6,16] Apart from still births and birth defects, the other complications may be premature thelarche, early onset of puberty, decline in fertility, disruption of balance between androgens and estrogens, degree of masculinization, altered socio sexual behaviour, to name a few. These are based on observations in animal models (rodents primarily and primates in some). Stages of sexual maturation in rodents do not correspond to the equivalent time periods in humans. Timing of exposure and levels of circulating estrogens and androgens are two important determinants that affect the growing fetus. Moreover, there are differences in the development process when sensitivities to circulating hormones vary.[17]

Despite all these, rodents form the most appropriate animal model for establishing the harmful effects that can result from administration of any drug. Some of the limitations observed by the previous studies include administration of the drug through subcutaneous route that bypasses the gut microflora and hepatic first pass metabolism and may have a major impact on biological potency of phytoestrogens. Some of the studies have administered the purified phytoestrogens or in combination with natural sources like soy products and also in much higher doses. Most studies do not report doses on body weight (mg of compound/kg of body weight/day).[12]

Although our preliminary analysis shows that SSDs contain phytoestrogens and testosterone, there could be a possibility of the presence of some other compound that is still unknown. Therefore, there is a need to conduct studies on animal model to establish the spectrum of effects that SSDs can cause keeping in mind the limitations reported so far.

**OBJECTIVE**

The objectives of the study would be to establish the effects of administration of SSDs on:

- Outcome of pregnancy
- Effects on the growing male and female foetuses
- Development and sexual behaviour of offspring exposed to SSDs
METHODOLOGY

Mammalian development stages are sensitive to circulating hormones. Timing of exposure is an important determinant. Sexual differentiation occurs between 5-19 weeks of pregnancy in humans but in rodents, it occurs during a relatively short period from gestational days 12-20, with some aspects developing during neonatal period. (Figure 1) On the other hand, sexual differentiation of brain is dependent on the action of androgens and estrogens that occurs in-utero in humans, but the corresponding period is the neonatal period in rodents. Moreover, there are differences in time span of sexual maturation between species. Therefore a complete study on the possible effects in human beings would entail administration of the drug not only during prenatal period but also during perinatal period.

The protocol therefore has been split into sections for detailed observations: Prenatal and perinatal development toxicity.

All the experiments will be conducted in CPCSEA approved animal house with adequate facilities in accordance with guidelines approved by OECD. The experiments will be performed in accordance with OECD guidelines.[18]

Ethical considerations

An approval has been sought from the Animal Ethics Committee constituted by the Institution conducting animal studies and guidelines will be followed at every step. A regular and periodic update will be given to the Ethics Committee. For any amendment that may happen in due course, the Committee will be informed and an endorsement taken.

Apart from this, the Institutional Ethics Committee of the Investigator has also been informed about the study and they will be updated from time to time till the completion of the study.

Assessment of Pre-natal developmental toxicity

Evaluation of pre-natal developmental toxicity of SSD will involve administration of test compound/s to primigravida rats. The duration of administration will be closely related to the expected human exposure.

Procedure

Adult, nulliparous female rats will be mated with males of same species and strain. Mating process will be carried out adequately to result in at least 40 females with implantation sites/group at the time of scheduled necropsy. The day of vaginal plug formation (termed as
‘successful mating’) will be considered as day 0 of gestation. All the successfully mated females will be randomized into control and treatment groups as shown in Figure 2.

Pregnant females will be dosed once daily by oral intubation starting on appropriate days of gestation. Frequency may range from a single dose only to daily administration for a few days based on the prescription pattern in humans for specific drugs. The SSD will be administered in raw form and not as extract.

Human equivalent doses will be converted into animal dose based on the standard body surface area conversions mentioned in US FDA regulatory guidelines. Depending on the solubility, the calculated amount of test item may be dissolved in vehicle or administered as a suspension. Clinical observations will be performed and recorded once a day till the period of sacrifice. Body weights and food consumption will be recorded on days 0, 3, 6, 9, 12, 15, 18 and on terminal day before sacrifice. Pregnant females will be sacrificed approximately one day before expected day of delivery. Females showing signs of abortion or premature delivery prior to scheduled necropsy will be sacrificed and subjected to a thorough macroscopic examination. At the time of termination or death during the study, the dams will be examined macroscopically for any structural abnormalities or pathological changes. Observation parameters are summarized in Table 1.

**Table 1: Outcomes for prenatal toxicity**

<table>
<thead>
<tr>
<th>General</th>
<th>Body weight, food intake, premature delivery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gross pathology with reference to reproductive organs</td>
<td>Uterus- gravid/ non gravid, gravid uterine weight with cervix</td>
</tr>
<tr>
<td></td>
<td>Number of corpus lutea, uterine contents- number of implantation sites, number of embryonic/ fetal deaths, degree of resorption, number of viable fetuses</td>
</tr>
<tr>
<td>Fetus</td>
<td>Sex, body weight of each fetus, crown to rump length, external, skeletal alterations (confirmed by stained preparations) and soft tissue alterations, categorization of alterations.</td>
</tr>
<tr>
<td>Other parameters that may be derived (but are not limited to)</td>
<td>Body weight change corrected for gravid uterine weight, gestation index, percentage of dams with stillborn pups, live born pups, percentage of viable foetuses/ implantation sites, sex ratio, any other toxic effects</td>
</tr>
</tbody>
</table>

**Assessment of Peri- and Post-natal developmental toxicity**

Evaluation of peri- and post-natal developmental toxicity will involve administration of test compound/s to *primigravida* rats during specific gestational period that correspond to human exposure. Animals will be allowed to undergo normal delivery of the F1 generation. F1 generation animals will be selected for continued evaluation. F1 generation animals will not
receive the test item directly but potentially would have been exposed to the test item in utero.

Procedure
Young, adult, nulliparous female rats will be mated with males of same species and strain. Mating process will be carried out adequately to result in at least 12 pregnant females/group. The day of vaginal plug formation will be considered as day 0 of gestation. All the successfully mated females will be randomized into control and treatment groups as shown in Figure 2.

The pregnant female animals will be dosed with the SSD formulation (raw form) by oral intubation. Duration of administration will be based on the directions for human use (usually 3-21 days of gestation); frequency may range from a single dose to daily administration for a few days based on the prescription pattern in humans for specific drugs. Human equivalent doses will be converted into animal dose based on the standard body surface area conversions mentioned in US FDA regulatory guidelines. Depending on the solubility, the calculated amount of test item may be dissolved in vehicle or administered as a suspension. Clinical observations (including mortality, if any) of the dams will be performed and recorded once a day upto the day of delivery. Body weights and food consumption by the dams will be recorded on days 0, 3, 6, 9, 12, 15, 18 and on the day of delivery. Duration of gestation will be recorded for each dam.

All the pregnant female animals will be allowed to undergo normal delivery of the F1 generation. Total number of animals delivered per dam will be recorded for each group along with the number of live stillbirths per dam. Animals from the F1 generation will be observed upto day-70. F1 generation animals obtained from females of each group will be randomized into 3 different categories for observation of gross abnormalities, behavioral and functional parameters and reproductive performance.

All animals will be sexed on PND-1. On PND-1, 4, 7 and once a week thereafter, F1 generation animals from each group will be weighed and any abnormal behavioral parameters will be recorded. A set of F1 generation animals from each group will be sacrificed on PND-4 (Arm-A) and carefully examined externally for gross abnormalities. Percentage mortality/survival of pups per group during PND 0-4 and PND 5-35 will be determined.
Sexual maturation of the animals will be checked periodically (balanopreputial separation for males and vaginal opening for females).

At least 12 animals/group in the F1 generation (Arm-B) will be subjected to behavioural tests on PND-35 and PND-70. Additionally, for F1 generation animals in the Arm-B, blood samples will be collected and subjected to hematological and biochemical analysis on PND-70.

Table 2: Outcome measures for peri- and post-natal toxicity

<table>
<thead>
<tr>
<th>General</th>
<th>Body weight and food intake, behavioural parameters, gross pathology (with special reference to reproductive organs),</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dams</td>
<td>Abortions/ premature deliveries, clinical signs and duration of gestation</td>
</tr>
<tr>
<td>Litter (F1 generation)</td>
<td>Stillbirths/ livebirths, number of runts/litters per group, necropsy findings, incidence of gross abnormalities at birth or later, survival/ death of F1 animals during PND 0-4 and PND 5-35, sexual maturity, weights of testis and prostate, weight and morphology of ovary and uterus, age of vaginal opening, age of balanopreputial separation</td>
</tr>
<tr>
<td>Other parameters that may be derived but not limited to:</td>
<td>Body weight change, gestation index, viability index, lactation index, percentage of dams with stillbirths per group, sex ratio, percentage of surviving pups per group on days 1,4,35,70; percentage mortality per group on days 1,4,35,70; Average pup weight/ group on days 1,4,35,70</td>
</tr>
</tbody>
</table>

Effects of SSD on reproductive behaviour of F1 generation animals

A set of animals from the F1 generation (Arm C), 6 male and 6 female rats will be chosen to assess their reproductive behaviour. Since these animals would already have been exposed to the effect of SSD in utero, males from this set will be mated with unexposed females and females will be mated with unexposed males to determine the effect of SSD on male and female reproductive behaviour in the F1 generation. The day of vaginal plug formation will be considered as day 0 of gestation. Pregnant females will be sacrificed approximately one day before expected day of delivery. Females showing signs of abortion or premature delivery prior to scheduled necropsy will be sacrificed and subjected to a thorough macroscopic examination. At the time of termination or death during the study, the dams will be examined macroscopically for any structural abnormalities or pathological changes. Outcomes would be observed as given in Table 3.
Table 3: Outcomes measures to assess effects on reproductive behavior

<table>
<thead>
<tr>
<th>General</th>
<th>Fertility/infertility, Abortions/premature deliveries, clinical signs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uteri and their contents</td>
<td>Uterus - gravid/ non gravid, gravid uterine weight with cervix, number of corpus lutea, uterine contents - number of implantation sites, number of embryonic/fetal deaths, degree of resorption, number of viable fetuses</td>
</tr>
<tr>
<td>F2 fetus</td>
<td>Sex, body weight of each fetus, crown to rump length, external appearance, any other specific gross abnormality</td>
</tr>
</tbody>
</table>

Statistical Analysis

The outcome data at each stage of the experiment will be expressed in proportion for categorical variables (e.g., number of live births or stillbirths) and means, standard deviation for quantitative variables showing a normal distribution (e.g., weight of the uterus, age of thelarche). For skewed distribution, it will be expressed as median with interquartile ranges. The effect size will be determined using an appropriate method based on the type of variable. Each group will be considered as the unit for analysis. Quantitative data can be analysed using parametric tests, (t test will be used to compare 2 groups and ANOVA for comparing multiple groups) or non parametric tests (Mann-Whitney test).

Data management and reporting

Data will be recorded in predesigned forms developed for the study. These will be entered and stored electronically in SPSS version 21.0. The data will be stored in safe custody and will not be made accessible to anyone other than the investigating team.

Analysis will be done by the investigators and the results reported according to ARRIVE guidelines.19

Fig 1: Sexual differentiation in humans and rodents
Fig 2: Figure showing the protocol to assess the prenatal toxicity of sex selection drugs (SSDs) in rats
Fig 3: Figure showing the protocol to assess the peri and post natal toxicity of sex selection drugs (SSDs) in rats
**Fig 1: Sexual Differentiation in humans and rodents**

- Human: 3-19 weeks of pregnancy (complete), 12-20 gestational days
- Rodent: 3 or 2, Prenatal development

**Fig 2: Figure showing the protocol to assess the prenatal toxicity of Sex Selection Drugs (SSD) in rats**

1. Normal multiparous rats → X → Normal ♀ rats
2. At least 12 ♀ pregnant rats in each of the 3 groups
3. Day 0: Vaginal plug formation → "Successful mating" → G1 (Control) 12, G2(SSD) 12, G3(SSD) 12
4. Followed up BW and food consumption recorded on Day 0, 5, 12, 18, 25, 30, terminal day
5. Sacrificed ≈ 1 day before expected day of delivery
6. Macroscopic examination for any structural abnormalities or pathological changes in F1 generation

**Fig 3: Figure showing the protocol to assess the perinatal and postnatal toxicity of Sex Selection Drugs (SSD) in rats**

1. Normal multiparous ♀ rats → X → Normal ♂ rats
2. At least 12 ♀ pregnant rats for 3 groups each
3. Day 0: Vaginal plug formation → "Successful mating" → G1 (Control) 12, G2(SSD) 12, G3(SSD) 12
4. Followed on Normal delivery:
5. F1: No. of live/total births delivered per dam recorded
6. Followed up till Day 70
7. PND 1, 4, 7 and once a week thereafter: recording of any abnormal behavioral parameters
8. Arm A: sacrificed on PND 4, Arm B: subjected to Behavioral test on PND 45
9. Histological analysis
10. Arm C: subjected to reproductive behavioral tests

**Fig 3 Unclear**
CONCLUSION

The current study proposes to understand in totality the effects of SSDs, in particular phytoesterogens in reproductive ability of rodents. Since, these compounds are usually administered following conception in a realistic scenario, inclusion of first and second generation components of the study assume importance. A unique problem is being addressed, and the experimental design integrates SSDs following administration to pregnant mice and their subsequent effects on the newborn. We postulate that the end result would be adequate in understanding the mal-effects of SSDs on the reproductive ability as well as the translational effect on the newborn mice pups thereby enabling a near identical correlation to actual observed clinical cases. The designed experimental protocol, to the best of our knowledge has not been adopted or applied in any previous studies which attempted to address the deleterious effects of food additives/drugs/chemical or biological carcinogens on the mammalian( female) reproductive system.

Funding source

The study is being supported by Department of Science and Technology, New Delhi, India; Science and Technology Council, Haryana, India.

Conflict of Interest: None.

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