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# Evidence of prenatal toxicity of herbal based indigenous formulations for sex selection in rat models

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ABSTRACT

Indigenous preparations(IPs) for a male child is reported from some parts of India. The present study aims to explore the effects of IPs for sex selection or sex selection drugs (SSDs) on pregnancy outcomes in rat models. SSDs contain *Bryonia laciniosa, Quercus infectoria and Putranjiva roxburghii* along with other ingredients.

*Methods:* An experimental design with successfully mated female rats were randomized into control and treatment groups. Phase 1 had 2 interventional arms while phase 2 had 3 interventional arms (12 rats/arm) besides control arm. In phase-1, pregnant females were dosed two SSDs(1000 mg/kg) on gestation days 1–5 whereas, in phase-2, on gestation days 6–19 to correlate the effect of the SSDs (500/1000/1500 mg/kg) consumption during different stages of pregnancy. Pregnant females were observed for clinical signs following treatment. The rats were sacrificed one day before expected day of delivery for evaluation. Pregnancy rate, gestation index, number of corpora lutea, and litter size were assessed. Foetuses were examined for sex, skeletal and soft tissue alterations.

*Discussion and conclusion:* In phase 1, no appreciable findings were there with SSD exposure. In phase 2, intrauterine growth and survival of foetuses were affected when SSDs were administered during organogenesis period. Decreased number of live foetuses and increased incidence of early and late resorption, reduced fetal growth with significant alteration in skeleton and viscera were found in treatment groups in a dose-dependent manner. This correlates well with findings from observational studies in pregnant women. However, such treatment at any dose did not effect sex differentiation.



## 1. Introduction

Traditional or indigenous preparations (IPs) are getting considerable attention in global health debates.[1] It continues to be used widely in several countries and its consumption is rapidly increasing.[2,3] Many reports have documented the use of such preparations. For instance, in China, traditional herbal medicines played a critical role during the epidemic of severe acute respiratory syndrome (SARS).[4] In fact, many drugs in modern medicine had their roots in the traditional system of medicine. Drugs like Penicillin (from *Penicillium* fungi) and Digoxin (from *Digitalis purpurea*) are some such examples. One of the promising therapies for Methicillin Resistant *Staphylococcus aureus* (MRSA) infection is a

*Abbreviations:* ANOVA, Analysis Of Variance (ANOVA); ARRIVE, Animal Research: Reporting of In Vivo Experiments; CPCSEA, Committee for the Purpose of Control and Supervision of Experiments on Animals; GD, Gestation Day; GLP, Good Laboratory Practice; HED, Human Equivalent Dose; HPLC, High Performance Liquid Chromatography; IPs, Indigenous Preparations; MRSA, Methicillin Resistant Staphylococcus Aureus; OECD, Organisation For Economic Co-operation and Development; SARS, Severe Acute Respiratory Syndrome; SSD, Sex Selection Drugs; VC, Vehicle Control; WEC, Whole Embryo Culture.

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ninth century treatment for eye infections which is a recipe consisting of garlic and onions, wine, and bile from a cow's stomach brewed in a brass cauldron.[5]

It is however important to note that culture specific practices are not always safe. Digitalis is a classic example where herbalists use this plant in allopathic treatment strategies; while in some countries such as Britain, law restricts their vast availability.[6] A report from Australia describes the adverse effects of 'herbal' preparations or IPs.[7] Problems arise when people do not report about the consumption of such preparations assuming that these are devoid of any side effects by virtue of them being natural products.[3] The need for research in this field is huge and scientific research into the quality, safety, molecular effects and clinical efficacy is crucial.[3,8]

Pregnancy is considered a time of minimal intervention, and a period when herbal medicines are usually contraindicated.[9] It is reported that many women consume indigenous medicines (that contains *Bryonia laciniosa*, *Quercus infectoria* and *Putranjiva roxburghii* along with other ingredients) during early pregnancy to beget a male child in India. These preparations, often called sex selection drugs (SSD) contain herbal ingredients but these are off the label prescriptions and hence cannot be certified as 'herbal' medicines. The prevalence of consumption varies from 7% to as high as 46%.[10–13] Studies have indicated that there is 3.5–4 times higher risk of birth defects including major malformations of internal organs like urogenital and renal malformation, trachea-esophageal fistula, and visible defects like spina bifida, cleft lip/palate and imperforate anus.[12,13] Risk of stillbirths also increases 2.5 folds. Studies suggest that a single exposure during pregnancy can be deleterious.[12,14,15] SSDs are reported to contain phytoestrogens, steroids and heavy metals.[12,16,17] It is also known that although placenta serves as a barrier, most drugs and environmental chemicals enter the foetal circulation by passive diffusion or active transport.[18] However, despite growing evidence on deleterious effects of these herbal formulations, few studies have attempted to systematically understand the risk of such preparations using animal models. Genetic sex of a child is determined at the time of conception by the fertilization of the ovum with X or Y sperms and intake of so called preparations for sex selection can never alter that.

In view of these, the current study was conducted with an objective to determine the embryo-foetal and development toxicity of SSDs following maternal exposure during critical period of organogenesis using a small animal model.

## 2. Material and methods

### 2.1. Ethical considerations

The study was guided by the OECD guidelines for testing of chemicals (Guideline no: 414, adopted on 22nd January 2001, Prenatal Developmental Toxicity Study)[19] and was based on a mutually approved protocol. Personal protection equipment was employed as required while handling the test system.

The test facility is registered and renewed (No. 1266/PO/RcBi/S/09/CPCSEA dated July 14, 2015) for breeding and to conduct research on animals for commercial purpose by the committee for the purpose of control and supervision of experiments on animals (CPCSEA), Ministry of Environment, Forests and Climate Change, Government of India. The study was approved by the Institutional Animal Ethics Committee, Venus Medicine Research Center. Due permission was taken from the Ethics Committee of Indian Institute of Public Health Delhi.

### 2.2. Test system information and animal husbandry

For this experiment, strain of Sprague Dawley of rats (*Rattus norvegicus)* was used. The number of dams per group was 12 instead of 20 as laid down under OECD guidelines. Rats of both sexes (200–230 g) were obtained from National Institute of Biologicals, India at 9–11 weeks of age, acclimatized for five days and then subjected to the experiment at 10–12 weeks of age. They were housed in standard polypropylene rat cages with stainless steel top grill (one dam per cage) at a temperature of 23 ± 4 °C, with 30–70% relative humidity. Bedding material and cages were changed once in six days and 12 hourly light dark cycle was followed. Animals were identified by their existing ear numbering. Cages were labelled by tags indicating the study number, sex, group of the animals, experimental start and completion dates. Standard pelleted feed (from Ashirwad Industries, Mohali, India) was provided *ad libitum* to all the animals. They also had free access to purified water via drinking bottles throughout the study. The feed, cages and drinking bottles were not analysed for the presence of phytoestrogens and bisphenol A. However, similar materials were used for all the rats across all groups.

### 2.3. Experimental drug

The drug comprised of IPs for sex selection (SSD). These non branded locally made preparations are made available 1–2 days after placing an order and are never sold openly. These preparations essentially contain herbal ingredients such as Shivlingi (*Bryonia laciniosa*), Majuphal (*Quercus infectoria*), Putrajeevak (*Putranjiva roxburghii*), Nagkesar *(Mesua ferrea)* and feather of peacock (*Pavo cristatus*). A total of 48 such samples of SSDs were procured from varied sources like '*pansari*' shop owners (grocers), local alternative medicine practitioners, rickshaw pullers, drivers and commoners from different states. SSDs are advised to be consumed between 6 and 10 weeks of pregnancy.

The samples were categorized into 18 groups based on redundancy of the preparations and common ingredients reported. This was followed by chemical analysis in an accredited laboratory to detect the presence of phytoestrogens and steroids.[20]

Since we explored the toxic effects of these preparations we wanted to select two samples that would be most predictive of toxicity. Due to resource constraints, we did not want to subject all the 18 samples for animal study. Hence, three samples of SSDs, found to be most rich in phenols and steroids were selected for toxicity study.[20] All the three samples were also tested for heavy metals which suggested that Lead and Mercury were in very high quantities.[17] The toxicity was examined using C. elegans, an alternate animal model. Survival analysis and reproductive toxicity was assessed from the brood size and progeny count assay.[21] The two SSDs that were found to be most toxic were selected as test items for the subsequent study in rat models. SSD 1 was reported to contain *Shivlingi* and *Putrajeevak* while SSD 2 contained Shivlingi, Majuphal, Putrajeevak, Nagkesar and feathers of peacock. Both the samples were obtained from grocers. Local preparations like these are bound to have certain degree of variability in concentrations but more often than not, these remain the major ingredients.

According to studies reported, women consume such preparations along with cow's milk in dosages and schedule that are quite varied. While some advise these IPs to be consumed for one day, others prescribe it for 30 days, to be taken thrice a day. We considered the amount of SSDs most commonly reported by pregnant women (weighing 50 kg) which was 7–8 g daily for 15 days. The dose of the test item (1000 mg/kg) was calculated by using conversion factor of 6.2.

Conversion factor is the ratio of the correction factors (estimated by dividing the average body weight in kgs of species by the body surface area in $m^2$) of the species ($K_m$). Human equivalent dose (HED in mg/kg) = Animal dose (mg/kg) X correction factor ($K_m$

animal/$K_m$ human). $K_m$s of every species are constant. For humans it is 37 (assuming average weight of adults to be 60 kg/a surface area of 1.62 $m^2$) and for rats it is 6.

Therefore, HED (8000 mg/50 kg) = animal dose X (6/37). Or, animal dose = 8000/50 × 6.2 mg/kg (6.2 = 37/6) = 1000 mg/kg.

Since pregnant women consume these orally, these were administered to rats by oral route.

The test items were coded as SSD 1 and SSD 2 and were provided to the experimental laboratory (Venus Medicine Research Center, Baddi) in powder form. On the day of dosing, the doses were administered between 10:00–13:00 h. The test items were administered in fresh cow's milk at a final volume of 10 ml/kg. The doses were calculated on the basis of the latest body weight recorded for the study. A stainless steel gavage cannula fitted to a calibrated syringe was used for dosing the animals.

For control animals, cow's milk was used as a vehicle while feeding.

### 2.4. Experimental design

The study was performed in two phases to evaluate the effect of SSD consumption duration during gestation period.

Adult, nulliparous female rats were mated with males of same species and strain in 1:1. Each morning, dams were examined for the presence of a vaginal plug and vaginal smear was taken from each dam by using pipette smear technique. The day of vaginal plug formation or sperms observed in vaginal smear (also known as successful mating) were considered as GD0 (Gestation day 0). The mated females were housed individually in clear polycarbonate cages with stainless steel wire lids. Mating process was carried out to result in 12 pregnant animals per group. Successfully mated females were randomized into control and treatment groups. All the rats were sacrificed on GD 20. According to OECD guidelines, the test chemical should be administered daily from implantation to the day prior to scheduled caesarean section in order to assess toxicity. Test chemicals were administered to pregnant animals and sacrificed as per the schedule, which was as close as possible to the normal day of delivery without risking loss of data resulting from early delivery(i.e GD 20). The females were killed, before caesarean section, the uterine contents were examined, and the fetuses were evaluated for soft tissue and skeletal changes.

### 2.5. First phase

The pregnant rats were randomized to two intervention and one control groups. SSD was given during pre-implantation to implantation stage (GD 1–5). Each group of rats was treated either with vehicle (G1: VC), SSD-1 (G2: 1000 mg/kg) or SSD-2 (G3: 1000 mg/kg).

### 2.6. Second phase

SSDs were given from GD 6–19. Each group of rats was treated with either vehicle or SSD-1 or SSD-2. Allocation of animals was randomized to the following groups: G1 (Control: VC), G2 (SSD-1: 500 mg/kg), G3 (SSD-1: 1000 mg/kg), G4 (SSD-1: 1500 mg/kg), G5 (SSD-2: 500 mg/kg), G6(SSD-2: 1000 mg/kg), G7 (SSD-2: 1500 mg/kg).

### 2.7. Experimental outcomes

The following parameters were observed:

- Clinical Signs- Animals were observed once after successful mating or on GD0 and after administration of the first dose. Cage side observations for clinical signs of toxicity in the animals were recorded once daily until the day before necropsy.
- Body weight- Body weight of all experimental animals were recorded once before mating (initial body weight), and then after confirmation of mating. Body weights were recorded on days 0, 3, 6, 9, 12, 15, 18 and on terminal day before sacrifice. These recorded body weights were used for calculating the dose/volume to be administered.
- Food consumption- Food consumption was evaluated once after successful mating or on day 0 of gestation (the day of vaginal plug formation or sperms observed in vaginal smear) and on days 3, 6, 9, 12, 15, 18 and one day prior to sacrifice. Calculated amount of feed were placed in the trough of each cage and the quantity of feed remaining after approximately 24 h were weighed and recorded.
- Morbidity/Mortality- All the animals were observed twice daily (once in the morning between 8.30 and 9.30 h and once in the evening between 16.00 and 17.00 h) for incidence of morbidity and/or mortality.
- Maternal examination related parameters- At the end of the observation period, all the dams were sequentially euthanized (by CO2 asphyxiation followed by cervical dislocation) and subjected to gross necropsy. After termination, dams were examined macroscopically for any structural abnormalities or pathological changes. Blinding was done while evaluation of the dams during caesarean section and subsequent foetal analyses to minimize bias. Pregnancy rate was calculated as the proportion of mated pairs that had produced at least one pregnancy within a fixed period where pregnancy was determined by the earliest available evidence that fertilization has occurred. Gestation index was calculated to indicate number of females with live born as a proportion of number of females with evidence of pregnancy.
- Examination of uterine contents- Immediately after termination, the uteri were removed and the pregnancy status of the animals ascertained. Gravid uteri including the cervix were weighed, and number of corpora lutea were determined. The uterine contents were examined for numbers of embryonic or foetal deaths and live foetuses. The degree of resorption was described in order to estimate the relative time of death of the conceptus. Any increase in the number of resorption and/or implantation loss is an indicator of litter size for the individual dam.
- Examination of foetuses- The sex and weight of each foetus were determined. Approximately one-half of each litter were prepared and examined for skeletal alterations from selected dams. The remainder were prepared and examined for soft tissue alterations, using accepted or appropriate serial sectioning methods or careful gross dissection techniques. Each foetus was examined for external alterations.

Foetuses were examined for skeletal alterations (using Alizarin Red S staining method). The fetuses for skeletal staining were kept in pre-labelled plastic containers containing 90% ethanol before staining. After skinning, we processed the foetus for staining by immersing in 0.01% alcian blue 8 GX for three days, then performed rehydration through a gradient series of ethanol (70% ethanol, 2–3 h twice; 40% ethanol, 2–3 h;15% ethanol, 2–3 h), distilled water until the samples sank to the bottom of a conical tube. Samples were further treated with 0.001% KOH for 1–2 days or until it became clear and again treated with 0.001% alizarin red for next 2–3 days until bones became purple. Samples were rinsed 3 times in 1% KOH, for 12–15 h at each time point. Samples were further treated with a gradient series of glycerol/1% KOH, 24 h 100% glycerol 24 h X 2.

Visceral examination was done by micro dissection technique. Fetuses were preserved in 10% formalin until they became fixed. An illuminated magnifier was used for visceral examination. We placed the foetus in a supine position on a paraffin wax block tray covered with paraffin wax, secured the fetal limbs by paper pins to the paraffin wax block, made a ventral midline incision from the umbilicus, cutting caudally to the genital tubercle and cranially to the diaphragm. Once incision was completed, we located the ventral attachment of the diaphragm and lifted the liver carefully to examine the diaphragm for abnormal opening. After observation, the diaphragm was clipped. We gave a longitudinal cut to open the rib cage slightly lateral to and on the right of the sternebrae and then extended the cut anteriorly to neck region. The rib cage was opened gently and secured to the paraffin block/petridish/tray with pins.

Foetuses were examined for soft tissue alterations (e.g. variations and malformations or anomalies) using Wilson's Technique. The fetal viscera were examined sequentially, beginning with thoracic organs and moving caudally. We performed hearts cuts after completion of other observations. We examined the bilobed thymus for size, shape, coloration and presence of haemorrhages and then removed it for observation of trachea and esophagus. We observed the lungs for size, color and number of lobes, observed the trachea, esophagus for normal alignment and presence of fistula, opened the pericardial sac and cut off pericardium to expose the heart for observation. We checked the size, shape, color of the heart and normal development of major blood vessels. After observation of thoracic viscera, we observed the abdominal viscera and liver for size, shape, color, texture, and number of lobes. External anatomy of heart was examined by making two incisions using micro dissecting scissors.

We examined the stomach, spleen, pancreas, small and large intestines for size, position or any other developmental anomalies. The intestines were moved aside after observation for seeing the underlying structures. Ureters were checked for normal size and location and for continuity from the renal hilus to the urinary bladder. One kidney was cut transversely and another was longitudinally to examine renal papilla and renal pelvis. To confirm the sex of the foetus, the gonads were inspected carefully. We examined the reproductive organs for size, shape and location.

### 2.8. Statistical analysis

GraphPad Prism® statistical software version 5.01 was used to analyze the data and all the results were represented as mean ± S.E.M. Intergroup variance for most of the parameters was calculated by one-way analysis of variance (ANOVA) followed by Tukey's test. A value of $p < 0.05$ was regarded statistically significant. Linear regression was performed to examine the weight change of dams during pregnancy for each group comparing with the control after adjusting for the litter size. The malformations were analysed by performing Chi square test for trend for two groups: SSD1- G1, G2, G3 G4 and SSD2- G1, G5, G6, G7.

The ARRIVE guidelines were followed to report the findings.[22]

## 3. Results

In phase 1 pre-natal study, all the dams dosed with SSD-1 and SSD-2 had normal food intake and weight gain. The average gain in weight from GD0 to GD 20 was 100.35 ± 2.75 gms for controls while it was 103.46 ± 4.16 gms in SSD 1 and 105.36 ± 3.82 gms in SSD2. (Webtable 1). No test item related mortality or clinical sign of toxicity was encountered during the study. All dams in treatment groups and control group survived to scheduled terminal sacrifice. They showed normal behaviour until euthanized. There were no differences in mean maternal body weights, body weight gains, pregnancy rate, gestational index, pre-and post-implantation loss, and resorptions. No significant differences were observed in foetal sex ratio, foetal body weights. However, one dam from SSD-1 had all male foetuses. No abnormality was detected in skeletal and visceral examination.

In phase 2, controls and SSD-1 and SSD-2 treated dams exhibited a comparable food intake (Table 1). However, a dose response relationship was observed with reduced weight gain with higher doses of SSDs. (Table 2). Two dams, one in G6 (SSD-2, 1000 mg/kg) and the other in G7 (SSD-2, 1500 mg/kg) categories showed decreased or consistent body weight GD12 onwards. On an average, the weight on GD 20 was 316.83 ± 3.61 gms in controls while it was 292.56 ± 2.58 with SSD1 (1500 mg/kg) and 291.81 ± 2.84 gms with SSD2 (1500 mg/kg). The adjusted analysis indicated that except in G5 there was a significant reduction in weight gain in all the intervention groups as compared to the control group after controlling for litter size (Table 3).

During follow up, one dam from SSD1 (500 mg/kg) and one from SSD2 (1500 mg/kg) showed haematuria during gestational day 11–14. However, no another clinical sign of toxicity was encountered during the study. All the dams in treatment and control groups showed normal behaviour and survived to scheduled terminal sacrifice. In phase 2, pregnancy rate and gestational index were found to be 100% at all dose levels with SSD1 whereas, in SSD-2, it decreased to 83.33% in G6 (SSD-2, 1000 mg/kg) and G7 (SSD-2, 1500 mg/kg) groups. One dam from G6 (SSD-2, 1000 mg/kg) and another one from G7 (SSD-2, 1500 mg/kg) group showed mating confirmation during vaginal smear examination and showed positive sign in initial days of pregnancy but during necropsy on expected day of delivery (GD 20) no foetus was found. Body weights of dams increased in the initial stages that became constant at a later period. To reconfirm it, non-gravid uterus was stained in salewski strain and presence of conceptus was found. This indicated the possibility of miscarriage.

Increased pre-and post-implantation losses as well as resorptions and reduced live foetuses were observed with both SSD-1

**Table 1**
Average food consumption of dams when exposed to SSDs during Gestational Days (GD) 6–19 in different experimental groups (n = 12 in each group) (in mean ± SE of means in gms).

| Days → Groups ↓ | Day-0 | Day-3 | Day-6 | Day-9 | Day-12 | Day-15 | Day-18 | Day-20 |
|---|---|---|---|---|---|---|---|---|
| G1 (Control) | 15.29 ± 0.71 | 17.33 ± 0.71 | 19.13 ± 0.70 | 20.31 ± 0.79 | 21.99 ± 0.89 | 22.31 ± 0.84 | 24.92 ± 0.75 | 25.07 ± 0.98 |
| G2 (D-1/500) | 15.12 ± 0.32 | 17.32 ± 0.74 | 19.12 ± 0.41 | 20.30 ± 0.50 | 21.95 ± 0.64 | 22.30 ± 0.75 | 24.89 ± 0.73 | 25.06 ± 0.84 |
| G3 (D-1/1000) | 15.11 ± 0.23 | 17.28 ± 0.66 | 19.11 ± 1.08 | 20.28 ± 0.43 | 21.96 ± 0.44 | 22.28 ± 0.77 | 24.87 ± 1.08 | 25.05 ± 0.66 |
| G4 (D-1/1500) | 15.13 ± 0.50 | 17.26 ± 0.82 | 19.10 ± 0.85 | 20.26 ± 0.68 | 21.96 ± 1.41 | 22.26 ± 1.03 | 24.86 ± 0.49 | 25.04 ± 0.62 |
| G5 (D-2/500) | 15.12 ± 0.71 | 17.31 ± 0.40 | 19.12 ± 0.54 | 20.30 ± 1.30 | 21.98 ± 1.19 | 22.30 ± 1.12 | 24.89 ± 0.84 | 25.05 ± 0.86 |
| G6 (D-2/1000) | 15.18 ± 0.36 | 17.27 ± 0.68 | 19.10 ± 1.04 | 20.29 ± 1.04 | 21.96 ± 1.24 | 22.29 ± 1.36 | 24.88 ± 1.09 | 25.06 ± 1.09 |
| G7 (D-2/1500) | 15.16 ± 0.31 | 17.28 ± 0.85 | 19.13 ± 0.83 | 20.28 ± 0.97 | 21.95 ± 1.26 | 22.27 ± 1.61 | 24.87 ± 0.98 | 25.04 ± 0.40 |

**Table 2**
Average gain in body weight of dams when exposed to SSDs during Gestational Days (GD) 6–19 in different experimental groups (n = 12 in each group) (in mean ± SE of means in gms).

| Days → Groups ↓ | Day-0 | Day-3 | Day-6 | Day-9 | Day-12 | Day-15 | Day-18 | Day-20 | Body weight change (g) during GD0 to GD 20 |
|---|---|---|---|---|---|---|---|---|---|
| G1 (Control) | 200.43 ± 3.47 | 210.16 ± 3.72 | 221.01 ± 3.26 | 235.48 ± 3.43 | 257.03 ± 2.77 | 274.92 ± 3.28 | 302.87 ± 4.46 | 316.83 ± 3.61 | 116.40 ± 1.43 |
| G2 (D-1/500) | 199.75 ± 3.47 | 214.16 ± 4.21 | 227.73 ± 3.77 | 243.18 ± 3.18 | 258.13 ± 3.19 | 271.83 ± 2.59 | 287.31 ± 2.19 | 306.93 ± 1.45[a] | 107.18 ± 2.61[a] |
| G3 (D-1/1000) | 206.27 ± 5.65 | 216.86 ± 6.13 | 237.54 ± 5.11 | 251.50 ± 5.27 | 265.87 ± 5.76 | 280.75 ± 5.79 | 296.60 ± 5.83 | 311.14 ± 5.78 | 104.87 ± 2.75[a] |
| G4 (D-1/1500) | 204.78 ± 3.37 | 215.91 ± 2.95 | 229.06 ± 3.31 | 242.55 ± 3.05 | 255.31 ± 2.90 | 267.32 ± 2.36 | 279.75 ± 2.49 | 292.56 ± 2.58[a] | 87.78 ± 2.03[a] |
| G5 (D-2/500) | 204.46 ± 7.36 | 218.19 ± 6.86 | 227.68 ± 6.48 | 239.89 ± 6.38 | 252.33 ± 6.35 | 263.44 ± 5.03 | 276.83 ± 5.98 | 289.88 ± 5.97 | 122.61 ± 6.66 |
| G6 (D-2/1000) | 209.72 ± 6.23 | 222.53 ± 6.40 | 236.15 ± 6.17 | 248.53 ± 6.21 | 258.47 ± 6.54 | 266.71 ± 8.54 | 277.40 ± 9.98 | 288.89 ± 10.9[a] | 79.17 ± 9.41[a] |
| G7 (D-2/1500) | 203.56 ± 2.46 | 216.11 ± 2.63 | 227.61 ± 3.11 | 240.09 ± 3.18 | 252.37 ± 2.61 | 265.45 ± 2.70 | 277.92 ± 2.68 | 291.81 ± 2.84[a] | 88.25 ± 0.80[a] |

[a] ·Difference in mean statistically significant as compared to the control ($p < 0.05$).

**Table 3**
Effects on reproductive parameters in pregnant female rats after exposure to SSDs during Gestational Days (GD) 6–19 in different experimental groups (n = 12 in each group).

| | G1 (Vehicle) | G2 (SSD-1; 500 mg/kg) | G3 (SSD-1; 1000 mg/kg) | G4 (SSD-1; 1500 mg/kg) | G5 (SSD-1; 500 mg/kg) | G6 (SSD-1; 1000 mg/kg) | G7 (SSD-1; 1500 mg/kg) |
|---|---|---|---|---|---|---|---|
| Number of dams | 12 | 12 | 12 | 12 | 12 | 12 | 12 |
| Dam body weight on GD 20 | 316.83 ± 3.61 | 306.93 ± 1.45[a] | 311.14 ± 5.78 | 292.56 ± 2.58[a] | 289.88 ± 5.97 | 288.89 ± 10.9[a] | 291.81 ± 2.84[a] |
| Uterus weight | 70.82 ± 1.73 | 74.56 ± 2.58 | 79.63 ± 2.14 | 78.18 ± 4.45 | 67.68 ± 1.49 | 77.78 ± 1.7 | 80.38 ± 1.85 |
| Corrected dam weight | 246.01 ± 4.82 | 232.37 ± 3.35[a] | 231.51 ± 6.86[a] | 214.38 ± 7.83[a] | 259.39 ± 8.47 | 211.12 ± 9.98[a] | 211.43 ± 4.96[a] |
| Number of corpora lutea | 14.67 ± 0.35 | 12.83 ± 0.46[a] | 14 ± 0.41 | 13.33 ± 0.89 | 13.5 ± 0.44 | 13.67 ± 0.35 | 13.5 ± 0.47 |
| Number of implantation sites | 12.17 ± 0.28 | 11.17 ± 0.28[a] | 10.83 ± 0.64[a] | 9.33 ± 0.6[a] | 11.33 ± 0.43 | 9.5 ± 0.57[a] | 9 ± 0.86[a] |
| Resorption: Early | 1 ± 0.26 | 0.67 ± 0.24 | 1.17 ± 0.12 | 0.83 ± 0.22 | 0.5 ± 0.16 | 0.83 ± 0.38 | 1.83 ± 0.34[a] |
| Resorption: Late | 0 ± 0.00 | 0.17 ± 0.12 | 1 ± 0.32[a] | 1.67 ± 0.35[a] | 0.33 ± 0.15 | 1.33 ± 0.35[a] | 1.83 ± 0.34[a] |
| Dead fetuses | 0 ± 0.00 | 0 ± 0.00 | 0 ± 0.00 | 0 ± 0.00 | 0 ± 0.00 | 0.17 ± 0.12 | 0.5 ± 0.35 |
| Litter size | 11.17 ± 0.34 | 10.33 ± 0.3[a] | 8.67 ± 0.39[a] | 6.83 ± 0.62[a] | 10.5 ± 0.3 | 7.33 ± 1.11[a] | 5.33 ± 0.85[a] |
| Gain in weight compared to control group (unadjusted)[#] | - | −9.22 | −11.52 | −28.62 | 6.21 | −37.23 | −28.15 |
| Gain in weight compared to control group (adjusted for litter size)[#] | - | −6.98 | −4.79 | −16.94 | 8.01 | −26.91 | −12.45 |
| Number of male fetuses | 5.67 ± 0.15 | 5 ± 0.41 | 4.17 ± 0.22[a] | 3.5 ± 0.44[a] | 5 ± 0.32[a] | 3.83 ± 0.59[a] | 2 ± 0.41[a] |
| Number of female fetuses | 5.5 ± 0.4 | 5.33 ± 0.24 | 4.5 ± 0.24[a] | 3.33 ± 0.3[a] | 5.5 ± 0.3 | 3.33 ± 0.57[a] | 2.83 ± 0.46[a] |
| Sex ratio (Male:Female) | 1.03 | 0.93 | 0.93 | 1.05 | 0.9 | 1.10 | 0.78 |
| Fetal weight (g): Male | 4.14 ± 0.039 | 3.76 ± 0.09 | 3.54 ± 0.05 | 3.45 ± 0.06 | 4.101 ± 0.01 | 3.98 ± 0.06 | 3.76 ± 0.13 |
| Fetal weight (g): Female | 3.87 ± 0.07 | 3.70 ± 0.09 | 3.53 ± 0.05 | 3.28 ± 0.05 | 3.66 ± 0.04 | 3.44 ± 0.07 | 3.19 ± 0.09 |

Corrected weight = Dam weight- Uterus weight.
#$p < 0.001$.
[a] Difference in mean statistically significant as compared to the control ($p < 0.05$).

and SSD-2. No significant differences were observed in foetal sex ratio, but decreased foetal body weights were noted in both sexes. On an average one early resorption in a group was a normal observation. More than one late resorption was observed in all treatment groups while there was none in the control group. Total count of live foetuses count was significantly reduced with SSD-1 and SSD-2 treatment in a dose dependent manner. Total number of live foetuses were reduced up to 39% in SSD-1 at 1500 mg/kg treatment group and 57% in SSD-2 at 1500 mg/kg treatment group.

Both SSD-1 and SSD-2s at different dose levels showed decreased fetal weight in dose dependent manner, although the difference was not significant. Skeletal and soft tissue alterations were observed in both treatment groups. In general, the embryo–foetal examination showed abnormalities, which could have caused some functional damage to these foetuses if allowed to grow in the normal course. In visceral examinations, slight dilation of the renal pelvis, reduced papilla size, urinary bladder hypertrophy was observed in few foetuses from both SSD-1 and SSD-2 treatment groups. Few incidences of foetal soft tissue abnormalities encountered in this study were normal variants. Skeletal examination showed variations in the ossification patterns like incomplete or poorly ossified skulls, ossification of sternebra, cleavage ossification of thoracic centrum, asymmetric thoracic centrum, and supernumerary rib in many foetuses in SSD-1 and SSD-2 groups (Table 4). A trend was noted with increased incidence of certain malformations with increasing dosages of SSDs that was statistically significant for most malformations with SSD2.

## 4. Discussion

The results of this developmental toxicity study suggest that administration of SSDs to pregnant rats had significant impact on intrauterine foetal growth, early and late resorption as well as developmental anomalies. These formulations have teratogenic potential but does not affect sex ratio.

IPs contain phytoestrogens like diadzein and genistein.[12] In vitro rat whole embryo culture assay (WEC) studies of genistein in rats have indicated fetotoxic and teratogenic potential at concentration of ≥10 μg/mL.[23] However, in vivo, an oral (gavage) embryonic and fetal development pilot study could not demonstrate any teratogenic effect in the same study. These findings are different from our study probably because we used SSDs containing natural herbal and non-herbal ingredients unlike those studies where synthetic genistein was used. In both the studies, decreased body weight was observed indicating slight maternal toxicity. Food consumption was unaffected in our study which is contrary to what was reported.[23]

In another study, pregnant rats exposed to oral isoflavones (diadzein and genistein in varying combinations) showed alterations in the number of live foetuses, lysed foetuses, number of resorption sites, and implantation sites while no clinical signs suggestive of maternal toxicity (seizures, tremors and salivation) that was observed similar to our study. Maternal mass gain was significantly reduced in rats treated with 100 mg/kg of isoflavones.[24] Ratio of females to males in the litter of different groups was similar akin to our finding.[24] These observations clearly nullify

**Table 4**
Findings from skeletal and visceral examination of pups following exposure to SSDs in-utero during Gestational Days (GD) 6–19 in different experimental groups (n = 12 in each group).

| Dose (mg/kg/bw/day) | G1 (Vehicle) | G2 (SSD-1; 500 mg/kg) | G3 (SSD-1; 1000 mg/kg) | G4 (SSD-1; 1500 mg/kg) | G5 (SSD-2; 500 mg/kg) | G6 (SSD-2; 1000 mg/kg) | G7 (SSD-2; 1500 mg/kg) |
|---|---|---|---|---|---|---|---|
| Dams examined | 12 | 12 | 12 | 12 | 12 | 12 | 12 |
| Findings of Skeletal examination | | | | | | | |
| Fetuses examined | 67 | 62 | 52 | 41 | 63 | 44 | 32 |
| Incomplete/poorly ossified skulls (SSD1/SSD2) | 0 | 0 | 1 | 0 | 0 | 0 | 1 |
| Ossification of sternebra (SSD1[a]/SSD2[a]) | 2 | 0 | 4[a] | 7[a] | 3[a] | 5[a] | 8[a] |
| Cleavage ossification of thoracic centrum (SSD1/SSD2[a]) | 1 | 2 | 2 | 2 | 3[a] | 3[a] | 5[a] |
| Asymmetric thoracic centrum (SSD1/SSD2[a]) | 1 | 1 | 3 | 2 | 3[a] | 3[a] | 6[a] |
| Supernumerary rib (SSD1/SSD2[a]) | 3 | 8 | 8 | 2 | 5[a] | 5[a] | 9[a] |
| Findings of Visceral examination | | | | | | | |
| Fetuses examined (SSD1/SSD2) | 60 | 61 | 53 | 40 | 57 | 43 | 32 |
| Dilation of the renal pelvis (SSD1/SSD2[a]) | 0 | 1 | 1 | 2 | 3[a] | 3[a] | 4[a] |
| Reduced papilla size (SSD1[a]/SSD2[a]) | 0 | 1[a] | 3[a] | 3[a] | 4[a] | 2[a] | 4[a] |
| Urinary bladder hypertrophy (SSD1/SSD2[a]) | 1 | 3 | 3 | 2 | 3[a] | 4[a] | 4[a] |

[a] Statistically significant for trend, $p < 0.05$.

the claim that consumption of SDDs could alter the sex of the growing foetus. An analysis done by the same authors earlier revealed that SSDs contain testosterone and steroids.[12,16] The prenatal testosterone in pregnancy is known to affect sexual development adversely.[25–27] The principles of reproductive biology state that the genetic sex of an individual is determined at the time of conception and can never be altered under any circumstances. However, phenotypic sex of the growing foetus is likely to be influenced by the hormonal milieu of in utero environment. Our analysis was confined to phenotypic sex only and hence it is difficult to state if any differences existed with reference to genetic and phenotypic sex. This requires further inquiry.

Evidence on the effects of SSDs on humans should best be obtained from human beings. The findings of the current study corroborate well with the evidence generated from observational studies in human beings.[13–15] Presence of skeletal and visceral malformations could explain why stillbirths are likely to be more in women who consume SSDs. The dosing of the test drugs was intended not only to examine the period of organogenesis solely but to assess effects from preimplantation, through the entire period of gestation to the day before caesarean section. The results were based on observations of more than 40 female animals with implantation sites at necropsy that added to its strength. Unnecessary handling of pregnant animals as well as any stressors from outside was avoided. At least three dose levels and a concurrent vehicle control were used with dams randomly allocated to different treatment groups that support the validity of the results. However, absence of 20 rats per group does not support it to be a fully OECD compliant study. We did follow the principles of Good Laboratory Practices (GLP) but due to constraints of resources the study could not be conducted as a GLP compliant study, though this is optimal for academic interests.

There is a lack of systematic approach to assess the safety and effectiveness of herbal preparations. Ingredients for herbal medicines are often drawn from different sources and are used in combination, which may exhibit variability in terms of species, growing conditions, and biologically active constituents. To isolate each active ingredient from each herb and then to establish its toxicity is time consuming, resource intensive and not practical. Moreover, drug approval process does not accommodate undifferentiated mixtures.[3] Though important, it surpasses the herb-herb interaction and the potentiating effect of one compound over another. Therefore, in our study, we administered the herbal mixture to assess the effects. Real time evaluations have yielded important observations for several herbal drugs especially related to pregnancy. For instance, Wang et al. (2014) used mice model to evaluate the adverse pregnancy outcomes after maternal exposure to the herbal medicines, particularly during early pregnancy.[28] The major events included maternal and perinatal mortality. Maternal weight gain, embryo growth and post-natal weight gain were significantly decreased. Moreover, foetal resorption and skeletal malformations were increased, signifying potential toxicity of Chinese herbal medicines. This is comparable to our findings.

## 5. Conclusions

To conclude, the results of this developmental toxicity study suggest that administration of SSD-1 and SSD-2 to pregnant rats exerted significant impact on intrauterine foetal growth and development without any effect on sex ratio when administered during the period of organogenesis. The prenatal exposure of SSD-1 and SSD-2 at dosage level of 1000 mg/kg/day and 1500 mg/kg/day during gestational days 6–19 to female rats had demonstrable effect on maternal or embryo-foetal toxicity and teratogenicity.

## Contribution of each author

SBN and AG conceptualized the study, AS and DD developed the study protocol and conducted the experiment, analysed the data and interpreted the findings. SBN drafted the manuscript. AG did a literature review. RS and RG provided inputs to the manuscript. All the authors approved the final version of the manuscript.

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## Conflicts of interest

None.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jtcme.2019.09.005.

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