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Original Article

PRENATAL DEVELOPMENTAL TOXICITY EVALUATION OF FUROSTANOL SAPONIN GLYCOSIDE BASED STANDARDIZED FENUGREEK SEED EXTRACT DURING ORGANOGENESIS PERIOD OF PREGNANCY IN RATS

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ABSTRACT

Objective: To evaluate prenatal safety of furostanol saponin glycoside based standardized fenugreek seed extract (Fenu-FG) on pregnant female Wistar rats on embryo–fetal development organogenesis period in accordance with OECD guideline (No. 414).

Methods: Fenu-FG was administered to pregnant rats by gavage at 250, 500, and 1000 mg/kg/day over the exposure period of gestational days 5–19. The vehicle control (VC) group was also maintained. All dams were subjected to a cesarean section on gestational day 20 and the fetuses were examined for external, visceral, and skeletal alterations.

Results: There was no significant difference found during maternal examination (body weights, food consumption, numbers of pregnant and non-pregnant female rats) or reproductive parameters (gravid uterus weights, litter size and weights, number of fetuses, sex ratio (male/female numbers of implantations and resorption, number of implantation per female, pre-and post-implantation loss (%), dead and live fetuses (%), numbers and weights of corpora lutea) in Fenu-FG-treated as compared to VC group. Furthermore, the few incidental and non-significant malformations were observed in Fenu-FG-treated as well as VC group during external, visceral or skeletal examinations.

Conclusion: The prenatal oral exposure of Fenu-FG during organogenesis period to pregnant female rats was devoid of maternal or developmental (fetotoxicity or teratogenicity) with "No Observed Adverse Effect Level" (NOAEL) greater than 1000 mg/kg.

Keywords: Developmental toxicity, Fenugreek seeds extract, Furostanol saponin glycosides, OECD Test No. 414

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INTRODUCTION

There is a growing interest worldwide to identify novel functional foods and food supplement with biological activity with limited toxicity [1]. The natural botanical substances and derived products are being used as food supplements for their purported medical benefits [2] or as a safe and natural food grade additives for formulations [3]. These supplements may comprise the whole plant, extracts or purified components for optimum health benefits [4, 5], which needs extensive extraction, fractionation or purification [6]. However, the presence of unknown and diversified phyto-constituents in natural products has a risk of unknown toxicity that can harm to human health [7, 8]. Therefore, safety evaluations of natural products in their standardized form (extract or fractions) became important for their safe use.

Fenugreek (*Trigonella foenum-graecum* L.) seeds (Family: Fabaceae) is a popular natural resource used as an ingredient of marketed food supplements. Because of long term and safe medicinal use, fenugreek seeds derived ingredients have a generally regarded as safe (GRAS) status in the United States. Fenugreek has a long history of medical uses in Ayurvedic and Chinese medicine as a general health tonic to improve metabolism and health. The use of fenugreek seeds as a remedy for many disorders is cited in the traditional and modern literature [9, 10]. Fenugreek seeds are also integral parts of many ayurvedic formulations [11]. The consumption of fenugreek seeds is highly recommended for many conditions for women health improvement [12] especially during lactation is highly recommended in Indian tradition [13, 14].

Fenugreek seeds are a rich source of a many phyto-constituents with varied biological properties. Glycosides are most notable amongst them. Feugreek seed contains a variety of furostanol [15] and flavonol [16-18] glycosides. Furostanol glycosides from fenugreek seed are reported to have anti-inflammatory [19], anti-melanogenic [19] and androgenic [20] potential. Flavonoid glycosides from fenugreek seeds are reported as anti-inflammatory, antinociceptive [21], anti-oxidant [22, 23], and lipid lowering properties [23]. Saponins are amphipathic glycosides which form a soap-like foaming when shaken in aqueous solutions in which one or more hydrophilic glycoside moieties combined with a lipophilic triterpene derivative. Saponins from a variety of natural sources are reported to exhibit a range of biological properties [24].

The furostanol saponin glycosides (FG), a major constituents from fenugreek seed, demonstrated excellent potential as a food supplement due to its biological activities [20, 25-28]. The efficacy of FG-based standardized fenugreek seeds extract (Fenu-FG) as an androgenic and anabolic supplementation in male rats [20] and human subjects [25] has been reported earlier. Furthermore, double-blind, randomized, placebo-controlled studies confirmed libido enhancement properties of Fenu-FG in young and aging males [26, 27] and menstruating female subjects [28] with good safety profile. Considering applications of Fenu-FG as a food supplement in female subjects, evaluation of reproductive safety profile of Fenu-FG supplementation in females, especially during prenatal exposure with well-accepted international guidelines is needed.

During past preclinical studies, a crude form of fenugreek seed powder or extract was found to be safe for long-term administration [29, 30] even during pregnancy period [31, 32]. However, there have been few reports of reproductive toxicity of crude extract of fenugreek seeds during prenatal exposure in rabbit [33], mice [34, 35] and rats [33, 36]. However, these reports utilized whole and crude extract with unknown composition and not standardized to any marker compounds. The safety evaluations of fenugreek seed extract standardized to specific marker compound is lacking. Recently, Prenatal developmental toxicity evaluation of low molecular weight

galactomannans based standardized fenugreek seed extract during pregnancy period of rats has been reported [37]. However, such safety study on Fenu-FG, which as different marker compound (FG), has not yet been reported. Therefore, the present study was undertaken with the objective of safety evaluation of Fenu-FG on pregnant female rats and their fetuses during prenatal development, following oral exposure, especially during the gestation period. The well accepted international guidelines for such study is set forth by Organization for Economic Co-operation and Development (OECD) guideline, OECD. Test No. 414 [38] was followed.

MATERIALS AND METHODS

Animals

The study was conducted in accordance with the principles of Good Laboratory Practice as set forth by OECD and 'Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) Guidelines for Laboratory Animal Facility'. The study protocol was approved by Institutional Animal Ethics Committee (IAEC) of INTOX Pvt Ltd, Pune, India (Approval number: 14160/14161). The nulliparous and non-pregnant Wistar female rats (aged 12 to 16 w) bred in-house were used for the present study. After 5 d of acclimatization, female rats were housed overnight with adult fertile male rats. The day on which sperms was detected in the vaginal smear was considered to be Gestational Day 0 (GD0) for that specific female rat. Mated females were singly housed in solid polypropylene cages with stainless steel grill tops and bedding of clean and sterilized paddy husk.

The pelleted feed (M/s Provimi Animal Nutrition India Pvt. Ltd., Bangalore, India) and filtered water were provided *ad libitum*. Animal rooms were maintained in air-conditioned rooms with 10 to 15 air changes per h and temperature between 22±3 °C, a relative humidity of 50±20%, and illumination cycle set to 12 h light and 12 h dark. After mating is confirmed (GD0), pregnant rats were randomly assigned to the vehicle control (VC) and treatment groups.

Chemicals

The test compound, Fenu-FG (standardized to 75.23% of furostanol saponin glycosides as a marker compound) was prepared and characterized by earlier reported method [20] and provided by Indus Biotech Private Limited (Pune, India). Fenu-FG is also an active component of marketed supplementation, Testofen® (Gencor Pacific Limited, Hong Kong). The solution of Fenu-FG was prepared fresh every day before dosing using analytical grade water (a vehicle). Dosage volume of 5 ml/kg body weight was maintained.

Method

The OECD Guidelines (Test No. 414:Prenatal Development Toxicity Study) was followed [38]. The main study was done after the conduct of the dose-range finding study.

Dose-range finding study

Male and Female rats (1:2 ratio) cohabited overnight. They were examined daily for the presence of spermatozoa in vaginal smear next day morning. The day on which the sperms were found in the vagina was considered gestational day 0 (GD0). On GD0, the female rats with the presence of spermatozoa in the vagina (dams) were randomized into 4 groups of seven rats each. From day 5 of gestation (GD05) GD5 to day 19 of gestation (GD19), they were administered either with vehicle (Water) or Fenu-FG (250 mg/kg, 500 mg/kg or 1000 mg/kg) respectively. These rats were checked for systemic toxicity, body weight, and food consumption. All dams were sacrificed on day 20 of gestation (GD20) and subjected to necropsy examination for ovaries and uterine contents. Fetal abnormalities by external examination were made. The dose level of 1000 mg/kg body weight (the limit dose) did not result in any remarkable maternal or fetal toxicity in the exposed rats. Therefore, the doses of 250 mg/kg, 500 mg/kg and 1000 mg/kg body weight were selected for the main study.

Main study

On GD0, 120 pregnant female rats were selected and randomized into 4 groups of 30 rats each. The rats were gavaged daily from GD5 to GD19 with either vehicle (G1:VC) or Fenu-FG (G2:250 mg/kg, G3:500 mg/kg or G4:1000 mg/kg). The female rats were observed at least once daily. Starting from GD5, food consumption was measured at 3 d intervals. Body weights of each female dam (maternal) were recorded on GD0, GD5, GD8, GD11, GD14, GD17 and just prior to their terminal sacrifice on GD20. All rats were euthanized on GD20 by CO_2 asphyxiation. The uterus from each female dams was examined for the number and placement of uterine implantation sites, the number of fetuses (live or dead), the number of resorptions (early or late) and any abnormalities of the uterus or embryonic sac, the number of corpora lutea of ovaries. Pre-implantation and post-implantation loss was calculated as follows: pre-implantations loss = [(no. of corpora lutea – no. of implantations)/no. of corpora lutea] × 100 and post-implantation loss = [(no. of dead implants)/no. of total implantations] ×100 [39]. Fetal examination for sex (gender) and the body weight of each fetus were determined. Uteri which had no visible implantation sites were stained with ammonium sulfide (10%) to detect very early resorptions [40].

All live fetuses were individually weighed; sex was determined and counted and examined for external anomalies in a uniform order (from head to tail) for external malformations. Live fetuses were euthanized by using diethyl ether vapors. Visceral examination of one-half of the fetuses from each litter female rat (random selection) by Wilson's Technique was performed for assessment of soft tissue development [41]. The remaining half number of the fetuses were sacrificed, eviscerated and processed for skeletal examination (Alizarin Red S staining method) [42].

Statistical analysis

The data was presented as mean±standard deviation (SD). Gestational body weights, body weight change, and food consumption/day, gravid uterus weight, pre-and post-implantation loss (%), dead and live fetuses (%) and litter weight (total, male and female) were analyzed by one-way analysis of variance, followed by Dunnett's test. Numbers of corpora lutea, implantation per female, early resorptions and late resorptions, litter size, number of fetuses (total, live and dead), sex ratio (male/female), incidences of malformations (fetal visceral and skeletal) were analyzed by using the Kruskal–Wallis test, followed by the Mann–Whitney test. Numbers of pregnant and non-pregnant female rats, the number of live and dead rats were analyzed by using Fisher's test. Treated groups were compared with respective VC group. The values were considered significance at $P<0.05$.

RESULTS

Maternal examination related parameters

No treatment-related clinical signs, effects on body weight or gross pathological changes were observed in any of treatment groups during the study period except the incidental death of 2 dams in each of Fenu-FG (250 and 1000 mg/kg) treated group. There was not causative pathological observations in dead dams were observed, so these observations were not treatment related. All other females in treatment groups and VC group survived to scheduled terminal sacrifice. The daily examination did not reveal any treatment-related clinical signs, in any dams, from any dose group, during the period of the study. There were no statistically significant changes in mean maternal body weights and body weight gains throughout the study (table 1). Maternal food consumption was unaffected by treatment (table 1).

Reproductive parameters related to embryo–fetal examination

There were no effects of Fenu-FG on pre-and post-implantation loss, resorptions, live fetuses, fetal sex ratio (percent male fetuses per litter), fetal body weights. There was an increase in percent post-implantation loss in the G4 (1000 mg/kg), but it was not found to be statistically significant (table 2).

Table 1: Effects of Fenu-FG on maternal parameters during organogenesis period in pregnant female rats

Parameters	G1	Fenu-FG treatment		
		G2	G3	G4
	VC	250 mg/kg	500 mg/kg	1000 mg/kg
Pregnancy data				
Rats per group	30	30	30	30
Confirmed pregnancy at necropsy	28	26	30	26
Pregnancy rate (%)	93.3	86.6	100	86.6
Maternal data				
Body weight (g) GD05	219.32±24.48	213.26±16.73	216.33±20.60	219.65±22.99
Body weight (g) GD20	290.32±33.22	273.65±27.12	277.83±34.22	282.30±37.29
Body weight change (g) during GD5 to GD20	71.0±16.6	60.4±19.9	61.5±19.8	62.7±20.4
Corrected Body weight (g)	241.80±24.50	227.00±15.20	230.80±21.40	237.40±23.70
Food consumption (g/day) during GD5 to GD20	14.5±1.9	13.5±2.4	13.2±2.6	13.7±2.1

Values are expressed as mean±SD. n = 30 female rats per group were randomized. VC-Vehicle control, GD-Gestational Day.

Table 2: Effects of Fenu-FG on reproductive parameters during organogenesis period in pregnant female rats

Parameter	Fenu-FG treatment			
	VC	250 mg/kg	500 mg/kg	1000 mg/kg
	G1	G2	G3	G4
All litters[a]	28	26	30	26
Gravid uterus weight (g)	48.5±14.3	46.7±16.4	47±17.7	44.9±19.8
Corpora lutea (no.)	10.4±2.5	10.2±2.2	9.8±1.9	9.7±3.1
Total implantation per female (no.)	9.4±2.3	9.2±2.4	9.2±1.8	8.3±3.4
Pre-implantation loss (%)[c]	19.1±14.50	17.3±10.8	13.1±15	27.9±19.60
Post-implantation loss (%)[d]	26.8±25.30	19±8.20	38.1±33	51.3±46.60
Early resorptions (no.)	1.7±1.5	1.9±1.1	2.6±3	2.4±1.6
Late resorptions (no.)	1.0±0.0	-	2.8±3.5	-
Litters available for evaluation	27	25	28	25
Litter size (no.)	8.89±1.93	8.76±2.35	8.36±2.41	8.08±3.19
Total live foetuses (no.)	8.85±1.99	8.76±2.35	8.36±2.41	8.08±3.19
Total dead foetuses (no.)	0.04±0.20	-	-	-
Live male foetuses (no.)	4.44±1.63	4.5±1.96	4.3±1.38	4.88±2.23
Live female foetuses (no.)	4.58±1.70	4.25±2.01	4±1.82	3.54±1.84
Male/female sex ratio (no.)	1.22±0.97	1.29±1.02	1.15±0.68	2.04±2.10
Average foetal weight (g)	3.58±0.33	3.52±0.41	3.82±0.58	3.75±0.25
Average male foetal weight (g)	3.71±0.30	3.73±0.30	3.97±0.52	3.81±0.33
Average female foetal weight (g)	3.5±0.29	3.36±0.58	3.73±0.59	3.68±0.31

[a]Females with confirmed pregnancy (including those died) (Nos.), [b]Values are expressed as means±standard deviation (SD), [c][(Number of Corpora Lutea-Number of implantations)/Number of Corpora Lutea]×100, [d][(Number of dead implants)/Total number of implantations]×100.

Fetal malformations and variations

In general, the embryo–fetal examination did not show any major abnormalities which could have caused any functional damage to these fetuses if allowed to grow in the normal course (table 3). Each fetus was externally examined for any abnormal findings with respect to length, cranium, eyes, palate, limbs, tail, genitals, sex, etc. Anasarca was observed in one fetus from VC group, and the small sized (runt) fetus was observed in G3 (500 mg/kg). In visceral examinations, hydrocephalus of lateral ventricles of the brain was observed in two fetuses from VC, three fetuses from G2 (250 mg/kg) and two fetuses from G3 (500 mg/kg). Retinal folding was observed in one fetus from G2 (250 mg/kg). Whole body distortion in one fetus was observed in VC group. Few incidences of fetal soft tissue abnormalities encountered in this study are all classified as either normal variants or minor anomalies and considered to be incidental and not treatment related. In skeletal examinations, as per skull anomaly is concerned, variation from the ossification patterns was seen like unossified, scrambled, poorly and incompletely ossified skulls. Such variations were observed in 14 fetuses from the VC, 6 from G2 (250 mg/kg), 20 fetuses from G3 (500 mg/kg) and 4 fetuses from G4 (1000 mg/kg) which were classified as normal variants. Six fetuses from VC, 18 from G2 (250 mg/kg) and one each from G3 (500 mg/kg) and G4 (1000 mg/kg) were observed with poor and incomplete ossifications of sternebrae. Unossified 5th and 6th sternebra were also encountered in all dose groups like 14 fetuses in VC, 21 in G2 (250 mg/kg), 8 in G3 (500 mg/kg) and one in G4 (1000 mg/kg). These were all classified as normal variants. Two fetuses in G3 (500 mg/kg) with poor ossification of cervical vertebrae and one fetus from G4 (1000 mg/kg) with unossified caudal vertebra was observed as normal variants.

Minor anomalies like branched, split, rudimentary, dumbbell shaped and asymmetrically shaped sternebrae were seen in all dose groups. These anomalies observed in 5 fetuses from VC, 8 fetuses from G2 (250 mg/kg), 6 from G3 (500 mg/kg) and 7 fetuses from G4 (1000 mg/kg). Minor anomalies in ribs like rudimentary, wavy, extra, accessory and asymmetrical ribs were observed in 3 fetuses from VC, 2 fetuses from G2 (250 mg/kg), 34 fetuses from G3 (500 mg/kg) and 7 fetuses from G4 (1000 mg/kg). Dumbbell shaped and fused vertebrae (thoracic, lumbar, sacral and caudal) were observed in 3 fetuses from VC, 9 fetuses from G3 (500 mg/kg) and one fetus from G4 (1000 mg/kg) (table 3). In general, the skeletal examination did not show any major abnormalities which could have caused any functional damage to these fetuses if allowed to grow in the normal course. The abnormalities noticed could be considered as the variations which might have occurred during development but got repaired and normalized during the normal course of development.

Table 3: Effects of Fenu-FG on fetal malformations and variations during organogenesis period in pregnant female rats

Parameter	Fenu-FG treatment			
	VC	250 mg/kg	500 mg/kg	1000 mg/kg
	G1	G2	G3	G4
Total No. Fetuses (litters) examined				
External	240	219	234	202
Visceral	116	102	110	96
Skeletal	124	117	124	106
External Malformation				
Anasarca	1	0	0	0
External Variations				
Small sized fetus (runt)	0	0	1	0
Visceral variations				
Brain: Hydrocephalus of lateral ventricles (slight)	2	3	2	0
Eye: Retinal folding (right)	0	1	0	0
Skeletal variations				
Skull ossification	14	6	20	4
Sternebra Variations				
Incomplete/Poor ossification	6	18 #	1	1
Un-ossified (5^{th} and 6^{th})	14	21 #	8	1
Branched, spilt, rudimentary, dumbbell and asymmetrical	5	8	6	7
Rib Variations				
Rudimentary (14^{th}), wavy, asymmetrical, extra, accessory	3	2	34 #	7
Vertebrae variations				
Cervical: Poor ossification	0	0	2	0
Caudal: Not ossified	0	0	0	1
Thoracic vertebra**:** centra dumbbell shaped	0	0	7	0
Caudal: Fused	1	0	0	0
Sacral: Fused	1	0	0	0
Lumbar: Fused	1	0	0	0

Fetuses from pregnant female rats per group were examined. The incidence of the individual defect is presented as a number of fetuses (numbers of litters). VC-Vehicle control, # $P<0.05$ as compared with VC (Mann-Whitney test).

DISCUSSION

The safety of standardized extracts of natural products cannot be assumed or directly correlated for the safety of powder, fraction or crude unstandardized extract. For example, steviol glycosides (SGs), a well-known sweetener contains a fraction of *Stevia rebaudiana* leaves, did not show reproductive toxicity in rats [43] whereas crude stevia leaf extracts showed adverse effects (renal and cardiovascular) as well as reduced fertility of in male and female rats [44-47]. Therefore, the safety of each food supplement needs to be ensured during prenatal exposure during pregnancy period in order to ensure safety in female users [48].

The present study evaluated the toxicity potential of oral gavage exposure of a furostanol saponin glycoside based standardized fenugreek seed extract, Fenu-FG in 30 pregnant female rats during developmental period i.e. gestational days GD5 through GD19. On day 20 of gestation, the females were sacrificed to examine ovaries, uteri, and fetuses for assessment of toxicity in terms of maternal and fetal parameters. Fenu-FG did not show any adverse effect on fetal weight or number of fetuses and their skeletal and soft tissue alterations. Furthermore, Fenu-FG showed no harmful effects on maternal as well as fetal parameters during gestational days (GD1-GD21) in rats, Intrauterine growth and survival were unaffected by Fenu-FG. In addition to the absence of treatment-related malformations, there were no significant changes in the incidence of external, visceral or skeletal variations. Taken together, these results indicated the absence of teratogenicity or abortifacient potential during oral exposure Fenu-FG during the gestational period. Thus, the "No-Observed-Adverse-Effect-Level (NOAEL) of Fenu-FG for maternal toxicity was found to be greater than or equal to 1000 mg/kg/day.

Pregnancy rate is the proportion of mated pairs that have produced at least one pregnancy within a fixed period where pregnancy is determined by the earliest available evidence that fertilization has occurred. Our results showed no significant difference in pregnancy rate amongst the treatment groups. The G3 (500 mg/kg) showed 100 % pregnancy rate as compared to 93.3%pregnancy rate shown by VC group. Significant shortening of gestation period can lead to adverse outcomes of pregnancy such as decreased birth weight and offspring survival. Significantly longer gestation may be caused by the failure of the normal mechanism for parturition and may result in death or impairment of offspring. During the present study, Fenu-FG did not show any adverse effect percentage of pregnancy rate and retain the normal length of gestation. There was no change in body weights, body weight gains, and food consumption of pregnant rats in tested dose levels. Similarly, fertility related parameters such as a number of early and late resorptions, litter size, and a number of fetuses did not show significant changes during our study. Increase in a number of resorptions and/or implantation loss is an indicator of litter size for the individual dam. An increase in percent post-implantation loss was observed in Fenu-FG (1000 mg/kg/day) treated group. However, such changes were statistically not significant and therefore, considered incidental

The results of present study showed no evidence of teratogenic effects was observed after oral administration of Fenu-FG up to 1000 mg/kg/day. Human equivalent dose (HED) can be derived from NOAEL by using USFDA guidance for Industry [49]. Considering NOAEL of 1000 mg/kg in pregnant female rats, HED is approximately 9.7 g considering (considering the average human weight of 60 kg). The HED value of 9.7 g per day is much higher than effective efficacy dose of Fenu-FG (600 mg per day) in male volunteers [25, 27]. This safety information during a gestational period in pregnant female rats will form an important basis for the clinical development as a safe food supplement or botanical agent for female specific applications.

CONCLUSION

The prenatal exposure of Fenu-FG (dose up to 1000 mg/kg, oral) during organogenesis period to female rats did not show maternal

or embryo-fetal toxicity and no teratogenicity. The oral dose of 1000 mg/kg, was found as the NOAEL of Fenu-FG during the gestational period in pregnant female rats.

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CONFLICT OF INTERESTS

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