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EVALUATION OF EMBRYO TOXICITY OF *CROCUS SATIVUS* (SAFFRON) IN SPRAGUE DAWLEY RATS

RAMESH EDAMULA[1,2*],M.DEECARAMAN[2], M.VIJAYALAKSHMI[2] AND KRISHNAPPA.H[1]

[1]Department of Safety Assessment, Advinus Therapeutics Limited, Peenya II Phase, Bengaluru - 560058, India
[2]Department of Biotechnology, Dr.M.G.R Educational and Research Institute, Maduravoyal, Chennai - 600 095, India

ABSTRACT

Wistar and Sprague Dawley rats are 2 different rat strains used widely for toxicity study for evaluation of safety of new chemical entity/food additive/generic drugs in various areas like toxicology, pharmacology and mutagenicity assay. However it is evident that genetic variation and differences in the biotransformation enzymes controlled by genes have been well documented among strains resulting in differences in sensitivity to chemicals. *Crocus sativus* (saffron) widely used as a food additive and as active components in many traditional medicine and modern pharmacology has been found to be quite safe without any evidence of maternal and embryo toxicity in Wistar strain rats up to the highest dose of 1000 mg/kg bodyweight. Hence the present study was aimed to evaluate if any differences are observed when using Sprague Dawley strain of rats. In the present investigation results indicated that saffron was safe in pregnant Sprague Dawley strain of rats to the dose level of 1000 mg/kg body weight without any maternal and embryo toxicity. Thus the results indicated that there were no strain differences between pregnant Wistar and Sprague Dawley strain of rats in the response to the exposure of saffron administered orally.

KEYWORDS: **Saffron, Strain, Wistar rats, Sprague Dawley rats, embryo toxicity**

RAMESH EDAMULA
Department of Safety Assessment, Advinus Therapeutics Limited,
Peenya II Phase, Bengaluru - 560058, India

1. INTRODUCTION

Wistar and Sprague Dawley rats are 2 different rat strains used widely for toxicity study for evaluation of safety of new chemical entity/food additive/generic drugs in various areas like toxicology, pharmacology and mutagenicity assay. However it is evident that genetic variation and differences in the biotransformation enzymes controlled by genes have been well documented among strains resulting in differences in sensitivity to chemicals. To cite some examples are: 2,3,7,8-tetrachlorodibenzo-ρ-dioxin (TCDD) induced a decrease in body weight and plasma β-endorphin as well as an increase in brain serotonin metabolism and plasma free tryptophan in Long-Evans rats without any effect in Hanover Wistar rats[6,12], Bracken fern induced urinary bladder tumors in F344 rats while Sprague Dawley rats were resistant[5], female Sprague Dawley rats were resistant to hepatotoxicity of the insecticide N-hydroxy-2-acetylaminoflurene when compared to Wistar or F344 rats [7]. Thus the present investigation was carried out in Sprague Dawley strain of rats to investigate the embryo toxicity potential of Saffron, a spice derived from stigma of *Crocus sativus Linn* flowers which is widely consumed by pregnant women mainly in the belief to increase fairness in newborn. The previous investigation carried out in Wistar strain rats indicated no evidence of maternal and fetotoxicity up to the highest dose of 1000 mg/kg/day dose [11].

2. MATERIALS AND METHODS

2.1. Test substance and dose formulation

Saffron (Indian Saffron Industry, Bagander, Pampore, Kashmir – 192121, India), dried stigma of the flower with the three main active components present in the material on dry basis at the level of: Picrocrocin – 72.7 %, Safranal – 51.6 % and colouring strength – 142.5 % was used in the study. No added artificial colour was present in the material. Dose formulations were prepared in Milli-Q water on daily basis. The doses were administered as oral gavage at a volume of 10 mL/kg body weight/day. The controls received Milli-Q water alone. The formulations were stirred continuously throughout the dose administration procedures and the prepared dose formulations were administered within 15 – 30 minutes of its preparation.

2.2. Animals, housing, diet and methodology

The study was conducted in compliance with Organization for Economic Co-operation and Development guidelines for the care and use of Laboratory animals. The study was carried out in an Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) International certified facility (Registration number:2/PO/RcBi/SL/1999/CPCSEA). The experimental procedures were approved by the Institutional Animal Ethics Committee (Proposal No. 048, dated 05 December, 2014) at Advinus Therapeutics Limited. Sprague Dawley rats were procured from Advinus Therapeutic Limited, Bengaluru, India. Twelve '0' day pregnant rats confirmed mated by vaginal smear examination with weight ranging from 200 to 243 grams and 10 to 11 weeks old were distributed to 2 groups of 6 rats each based on body weight stratification such that the body weight mean were within ± 20% in each group. All animals were housed individually in sterilized suspended polysulfone cages in an environmentally controlled room set to maintain temperature of 22 ± 3^0C and 50 ± 20% relative humidity. The fluorescent lighting provided illumination for 12 hour light/12 hour dark period. Animals were fed *ad libitum* Teklad Certified (2014C) Global 14% protein Rodent Maintenance Diet – pellet (Harlan Laboratories, Venray, Netherlands). Activated, charcoal filtered, ultraviolet light irradiated drinking water was provided *ad libitum*.

2.3. Study Design

The control group consists of 6 presumed pregnant rats were dosed with Milli-Q water, while the other remaining 6 rats received saffron suspended in Milli-Q water at the dose of 1000 mg/kg/day. The vehicle/saffron was administered from Day 5 of gestation (Day of implantation) until Day 19 of gestation. Animals were observed for survival and clinical signs daily. Body weights were taken twice weekly along with food consumption measurement. On Day 20 of gestation, all the rats were euthanized under isoflurane anesthesia. At sacrifice blood was collected from abdominal aorta for clinical chemistry analysis using Roche/Hitachi 902 (Hitachi High-Technologies Corporation, Tokyo, Japan) Automatic Analyzer. At necropsy, maternal viscera were examined macroscopically for gross lesions, liver and kidneys were collected in 10% buffered neutral formalin for microscopic evaluation. The ovaries were removed and the corpora lutea count was taken under an illuminated magnifying lens at a magnification of 5X.The gravid uterus was cut open along the ante-mesometrial side exposing the amniotic sacs. The sacs were ruptured and the number and position of implantation, early or late resorptions and dead or live fetuses were recorded. The umbilical cord of each fetus was cut and fetuses removed in a sequential order as present in the uterus, blotted dry and placed in a tray. The fetuses were then sexed, individually weighed and the crown-rump length measured using a digital vernier caliper. External examination of fetuses for morphological abnormalities was carried out under an illuminated magnifying lens at a magnification of 5X/10X. All the live fetuses were euthanized under isoflurane anesthesia and 50 % of fetuses were transferred into 70 % ethyl alcohol for visceral/soft tissue evaluation under an illuminated magnifying lens at a magnification of 5X/10X [8] and the remaining 50 % fetuses were skinned, eviscerated and processed and stained using alizarin red stain in order to evaluate for skeletal abnormalities [9]. The skeletal specimens were evaluated under a Stereoscopic Zoom microscope with typical magnification levels of 8X to 80X.

3. Statistical analysis

Students't' test was applied for single group comparisons for parameters related to maternal body weight gains, food consumption, gravid uterine weight, number of corpora lutea, number of implantations, litter size, litter weight, length and fetus number. The non- parametric test, Kruskal Wallis was used to analyze the differences in the incidences of pre and post implantation loss, number of early and late resorptions. The percentages of visceral and skeletal malformations and sex ratio were analyzed using 2X2 contingency table. A probability of 0.05 ($P \leq 0.05$) was accepted as statistically significant for all the tests applied.

4. EXPERIMENTAL RESULTS

4.1. Mortality and clinical signs

In general, no mortality or clinical signs of toxicity were found in the rodent dams throughout the treatment period except for the slight yellowish coloured feces at 1000 mg/kg/day dose which are considered to be related to the colour of saffron which is administered and are non-adverse in nature. No gross abnormalities were detected in the dams at caesarean section.

4.2. Maternal body weight during pregnancy and Fertility

Maternal body weights and fertility were unaffected by the administration of saffron (Table 1).

Table 1
Maternal body weight and fertility

End Point	Treatment	
	Vehicle Control	**Saffron**
Dose (mg/kg/day)	**0**	**1000**
No. of females	6	6
Fertility Index[a]	100	100
Gestation Index[b]	100	100
Maternal Body Weight Gain during Pregnancy (g)[c]		
Pre-treatment period (Days 0 – 5)	22.43±4.65	17.84±6.32
Treatment period (Days 5 – 20)	108.22±17.62	112.74±14.86
Throughout Gestation period (Days 0 – 20)	130.65±21.49	130.57±19.67

[a:] No. of pregnancies/No. of animals with successful copulation X 100
[b:] No. of females with live new borns/No. of pregnancies X 100
[c:] Mean ± SD

4.3. Maternal food intake during pregnancy

The food intake was also statistically similar when compared with the control group (Table 2).

Table 2
Maternal food intake (g/rat/day)

End Point	Treatment	
	Vehicle Control	**Saffron**
Dose (mg/kg/day)	**0**	**1000**
No. of females	6	6
Pre-treatment period (Days 0 – 5)	18.10±2.69	18.47±1.17
Treatment period (Days 5 – 20)	21.46±2.65	21.38±1.95
Throughout Gestation period (Days 0 – 20)	20.62±2.54	20.65±1.64

4.4. Maternal Parameters

The total number of corpora lutea, the total number of implantations and the percentage of pre and post implantation loss were statistically comparable with the control group (Table 3).

Table 3
Maternal parameters

End Point	Treatment	
	Vehicle Control	Saffron
Dose (mg/kg/day)	**0**	**1000**
No. of Dams	6	6
Gravid Uterine weight (g)	79.99±9.26	81.18±15.57
No. of Corpora Lutea[a]	16.50±3.15	17.83±1.94
No. of Implantations[a]	14.50±2.26	16.17±2.71
No. of resorptions[a]		
1. Early resorptions	0.67±0.82	0.67±0.82
2. Late resorptions	0.17±0.41	1.17±2.86
% Implantation Loss		
1. Early	4.60	4.12
2. Late	1.15	7.22
Pre-implantation Loss (%)	12.12	9.35
Post-implantation Loss (%)	5.75	11.34

[a:] Mean ± SD

4.5. Litter parameters

The length of male fetuses at 1000 mg/kg/day was significantly lower (Table 4).

Table 4
Litter parameters

End Point	Treatment	
	Vehicle Control	Saffron
Dose (mg/kg/day)	**0**	**1000**
No. of Litters	6	6
Total No. of Fetuses	82	86
Dead Fetuses	0	0
Mean Litter Size	13.7	14.3
Fetal Body weights (g)[a]		
1. Males	4.12±0.29	3.84±0.31
2. Females	4.02±0.26	3.63±0.42
Fetal length (mm)[a]		
1. Males	38.87±2.21	35.97±2.15*
2. Females	38.07±1.11	35.78±2.37
Sex Ratio (Male : Female)	1:0.74	1:1

[a:] Mean ± SD
*: Significantly different from control group, P≤0.05

4.6. Fetal morphological observations

a) External examination

External examination of fetuses did not reveal any morphological abnormalities at1000 mg/kg/day dose (Table 5).

Table 5
Fetal external observations

End Point	Treatment	
	Vehicle Control	Saffron
Dose (mg/kg/day)	**0**	**1000**
No. of Litters	6	6
Total No. of Fetuses	82	86
External Examination[a]	82 (6)	86 (6)
Abnormalities[b]	0 (0)	0 (0)

[a:] Number of fetuses (litters)
[b:] Total Number of fetuses (litters) exhibiting variations/malformations

b) Visceral/soft tissue examination

The visceral/soft tissue evaluation also revealed no abnormalities in any of the organs (Table 6) except for extra lobation of liver and minimal dilatation of renal pelvis of kidneys which were comparable between the treated and control group.

Table 6
Fetal visceral observations

End Point	Treatment	
	Vehicle Control	Saffron
Dose (mg/kg/day)	0	1000
No. of Litters	6	6
Soft tissue alterations[a]	41 (6)	43 (6)
Normalvariant		
Liver median lobe extra lobation[b]	1 (1)	1 (1)
Fetus (Litter) %	2.44 (2.38)	2.33 (1.85)
Kidneys renal pelvis dilatation (+)[b]	1 (1)	1 (1)
Fetus (Litter) %	2.44 (2.38)	2.33 (1.85)
MinorMalformations[b]	0 (0)	0 (0)
Major Malformations[b]	0 (0)	0 (0)

[a]: Number of fetuses (litters)
[b]: Total Number of fetuses (litters) exhibiting variations/malformations

c) Skeletal examination

The skeletal evaluation of the fetuses which were stained with alizarin red stain also did not show any major malformations (Table 7) except for some normal variations related to the ossification of some bone components like delayed/incomplete/poor ossification. In addition some minor anomalies like hypoplastic sternum, dumbbell thoracic vertebral centra, split thoracic vertebral centra, rudimentary/accessory ribs were seen which were in general comparable between the treated and control group.

Table 7
Fetal skeletal observations

End Point	Treatment	
	Vehicle Control	Saffron
Dose (mg/kg/day)	0	1000
No. of Litters	6	6
Skeletal Examination[a]	41 (6)	43 (6)
Minor anomalies		
Hypoplastic Sternum No. 2[b]	0 (0)	1 (1)
Fetus (Litter) %	0 (0)	2.33 (1.85)
Hypoplastic Sternum No. 5[b]	2 (2)	3 (3)
Fetus (Litter) %	4.88 (4.76)	6.98 (7.24)
Dumb bell: thoracic vertebral centra 9/13[b]	0 (0)	1 (1)
Fetus (Litter) %	0 (0)	2.33 (3.33)
Dumb bell: thoracic vertebral centra 10/13[b]	2 (1)	2 (2)
Fetus (Litter) %	4.88 (5.56)	4.65 (5.19)
Dumb bell: thoracic vertebral centra 11/13[b]	1 (1)	6 (4)
Fetus (Litter) %	2.44 (2.78)	13.95 (15.00)*
Dumb bell: thoracic vertebral centra 12/13[b]	2 (2)	6 (5)
Fetus (Litter) %	4.88 (5.16)	13.95 (14.28)*
Split: thoracic vertebral centra 9/13[b]	0 (0)	1 (1)
Fetus (Litter) %	0 (0)	2.33 (3.33)
Split: thoracic vertebral centra 10/13[b]	1 (1)	0 (0)
Fetus (Litter) %	2.44 (2.38)	0 (0)
Split: thoracic vertebral centra 11/13[b]	0 (0)	1 (1)
Fetus (Litter) %	0 (0)	2.33 (3.33)
Split: thoracic vertebral centra 12/13[b]	1 (1)	2 (2)
Fetus (Litter) %	2.44 (2.38)	4.65 (5.19)
Rudimentary Rib No. 14[b]	3 (3)	4 (3)
Fetus (Litter) %	7.32 (6.85)	9.30 (8.86)
Acessory Rib No. 14[b]	0 (0)	1 (1)
Fetus (Litter) %	0 (0)	2.33 (1.85)
Major Malformations[b]	0 (0)	0 (0)

[a]: Number of fetuses (litters)
[b]: Total Number of fetuses (litters) exhibiting variations/malformations
*: Significantly different from control group, P≤0.05

4.7. Biochemicalinvestigation

The blood collected at sacrifice was centrifuged to obtain plasma. Plasma was used for analysis of markers of normal liver function [Aspartate Amino Transferase (AST), Alanine Aminotransferase (ALT), Alkaline Phosphatase (ALP), Gamma Glutamyl Transpeptidase (GGT) and Total Bilirubin (T.Bil)], markers of normal kidney function [Blood Urea Nitrogen (BUN), Creatinine, Albumin], electrolyte levels (sodium, potassium, chloride, calcium) and general metabolism (glucose, total

cholesterol, total plasma protein) were measured. All these parameters were within the normal biological variation except for ALT and T.Bil levels which were significantly higher at 1000 mg/kg/day (Table 8).

Table 8
Biochemical investigation

Parameter	Treatment	
	Vehicle Control	Saffron
Dose (mg/kg/day)	**0**	**1000**
No. of GD 20 dams →	6	6
Glucose (mmol/L)	6.27±0.62	6.69±0.53
Total Cholesterol (mmol/L)	2.78±0.34	2.47±0.18
Total Protein (g/L)	50.48±3.59	51.75±2.64
AST (U/L)	68.83±3.06	63.50±6.77
ALT (U/L)	65.67±14.14	81.17±5.53*
ALP (U/L)	62.33±24.70	74.83±13.89
GGT (U/L)	3.00±3.58	1.50±2.07
Total Bilirubin (µmol/L)	3.56±0.34	4.02±0.30*
BUN (mmol/L)	3.27±0.82	4.06±0.76
Creatinine (µmol/L)	25.83±2.14	26.33±3.39
Albumin (g/L)	32.38±2.95	32.85±2.05
Calcium (mEq/L)	2.88±0.19	3.01±0.20
Sodium (mEq/L)	131.88±2.52	132.32±2.28
Potassium (mEq/L)	3.55±0.14	3.44±0.22
Chloride (mEq/L)	93.72±3.72	94.42±1.67

Values: Mean±SD
*: Significantly different from control group, P≤0.05

5. DISCUSSION

Saffron is the dried and dark red stigma of *Crocus sativus Linn* flowers possessing active constituents comprising of the volatile agents (safranal), bitter principles (picrocrocin) and the colour component (crocetin and its glycosidic, crocin). These active components have demonstrated many therapeutic roles like: in treatment of cancer, anticonvulsant properties, anti-inflammatory and antinociceptive properties, radical scavenging effects, hypolipaemic properties, anti-diabetic effects, anti-genotoxic effects and many more. In contrary to beneficial effect, information related to saffron toxicity has also appeared [3]. Increased miscarriage rate in female farmers working in saffron fields have also been reported as at low doses saffron causes the stimulation of the pregnant uterus and in larger amounts can cause contraction and spasm leading to abortion and possible other toxic symptoms [1]. However, in pregnant Wistar strain rats saffron was found quite safe up to the dose of 500 [10] and 1000 mg/kg/day [11]. So the objective of the present investigation was to determine whether any strain differences are noticed when saffron is administered to Sprague Dawley rats. The highest dose of 1000 mg/kg used in the previous study was also used in the present investigation wherein saffron was administered orally to pregnant rats during gestation days 5 through 19 which were in-lines with the previous investigation. Treatment with saffron at1000 mg/kg/day did not elicit any adverse clinical signs, effects on gestation body weight or food intake. The maternal parameters comprising of gravid uterine weight, corpora lutea and implantation counts, early and late resorptions, pre and post implantation loss were all comparable to the control. The litter parameters comprising of the number of fetuses and weight of the fetuses were comparable to the control, while the length of the male fetuses were significantly lower in the saffron treated group. This decrease observed in the fetal body length was by 7 % without any effect on maternal body weight gain and gravid uterine weight is considered non-adverse. These findings were also in lines with the body length of control fetuses reported by various authors [2,4]. Fetal external and visceral examination revealed no signs of embryo toxicity at the dose of 1000 mg/kg/day. Skeletal examination also revealed no major malformations except for some minor anomalies like hypoplastic sternum, dumb bell thoracic vertebral centra, split thoracic vertebral centra, rudimentary/accessory ribs which are commonly seen in a fetus from day 20 gestation dam. Clinical Chemistry analysis carried out from the blood at caesarean section to detect any adverse biochemical effects indicative of abnormal liver and kidney functioning, electrolyte imbalances or general metabolism revealed that all the parameters were within the normal biological variation at the dose of1000 mg/kg/day dose when compared to the control except for increased ALT and T.Bil levels. This increase was not associated with any microscopic changes in liver and hence considered as non-adverse finding.

6. CONCLUSION

The study indicated that saffron did not induce any maternal toxicity and embryo toxicity in Sprague Dawley strain rats when saffron was administered orally daily by gavage during gestation days 5 to 19 at the tested dose of 1000 mg/kg/day. These observations were in lines to the observations reported in Wistar strain rats. Thus the results indicated that there were no strain differences between pregnant Wistar and Sprague Dawley rats in the response to the exposure of saffron administered orally.

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8. CONFLICT OF INTEREST STATEMENT

Authors declare that there are no conflicts of interest.

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