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RESEARCH ARTICLE

TO STUDY THE EFFECTS ON PHYSICAL DEVELOPMENT/SEXUAL MATURITY OF WISTAR RAT PUPS EXPOSED TO *CROCUS SATIVUS* (SAFFRON) DURING GESTATION AND LACTATION

[1,*]Ramesh Edamula, [2]Deecaraman, M., [2]Vijayalakshmi, M., [1]Santhosh Kumar, D.P., [1]Krishnamurthy, H.N. and [1]Krishnappa, H.

[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, India

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ABSTRACT

The effects of post natal saffron exposure on physical development milestones such as pinna unfolding, incisor eruption, ear/eye opening including the land mark signs of attainment of sexual maturity with reference to preputial separation in males and vaginal opening were studied in Wistar rat offspring's whose dams received saffron administered as an oral gavage from implantation (day 5 post coitus) through lactation up to lactation day (LD) 20 at the doses of 50 (low dose), 250 (mid dose) and 1000 (high dose) mg/kg/day. A total of 23 litters (6 each in control, low and high doses and 5 in mid dose) born after 21 days of gestation were used. The results showed that the ages of pinna unfolding, incisor eruption, ear/eye opening including the land mark signs of attainment of sexual maturity were not affected by saffron exposure.

INTRODUCTION

Crocus sativus is a herbaceous perennial cormous plant belonging to family Iridaceae and is commonly referred to as saffron. The flower styles basically form commercial saffron used by most people either for medicinal or culinary purposes. Saffron contains more than 150 volatile and aroma yielding compounds, among these the important pharmacologically active principles are – Crocin and its derivatives which are responsible for colour, Picrocrocin responsible for the bitter taste and Safranal responsible for the odour/aroma (Rio's *et al*.,1996). Saffron and its constituents were widely evaluated for their pharmacological activities and are found to have anti-inflammatory, anti-diabetic, anti-spasmodic, anti-seizure, hypolipaemic effects including anti-cancer effects (Abdullaev *et al*., 2004). It is noted a review of information on toxicology and safety of saffron is not consistent. Crocetin, a carotenoid isolated from saffron has been found to be a teratogen (Martin *et al*., 2002). 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 toxic symptoms. As the toxicity information is not consistent, the present study was conducted to detect adverse effects of orally administered saffron on the physical development milestones such as pinna unfolding, incisor eruption and ear/eye opening of the Wistar rat offspring's whose dams received saffron administered as an oral gavage from implantation (day 5 post coitus) throughout the lactation period. Since manifestation of effect induced during this period may be delayed, observation was continued to monitor the land mark signs of attainment of sexual maturity with reference to preputial separation in males and vaginal opening in female offspring.

Corresponding author: Ramesh Edamula
Department of Safety Assessment, Advinus Therapeutics Limited, Peenya II Phase, Bengaluru - 560058, India

MATERIALS AND METHODS

Saffron

The material for the study, the saffron (dried stigma of flower) was obtained from Indian Saffron Industry, Bagander, Pampore, Kashmir – 192121, India. The material was authenticated at Central Food Technological Research Institute, Mysore-570020, India by spectrophotometric method as per International Organization for Standardization (ISO) method (5453, Part II, 1996). The results of the analysis indicated that the three main pharmacologically active components present in the material on dry basis were:

Picrocrocin – 72.7 %, Safranal – 51.6 % and colouring strength – 142.5 %. No added artificial colour was present in the material.

Animals and methodology

Wistar rats, obtained from Department of Safety Assessment, Advinus Therapeutics Limited, Peenya Industrial Area, Bengaluru – 560058, India were used in the experiment. 24 presumed '0' day pregnant rats confirmed mated by vaginal smear examination with weight ranging from 185 to 238 grams and about 84 days of age were randomly distributed to 4 groups of 6 animals each. These rats were housed individually in standard polysulfone rat cages in a barrier facility with standard laboratory condition of 12 – 15 filtered fresh air changes, temperature controlled (20 to 23 °C), relative humidity of 30 to 70 % and artificially lighted rooms with 12 hours fluorescent light and 12 hours dark cycle and with free access to food and water. The experimental project was approved by the Institutional Animal Ethics Committee (Proposal No. 023, dated 21 March, 2012).

The animals in Group I received only the vehicle (Milli-Q water) at 10 mL/kg body weight through oral gavage. The animals in Group II, Group III and Group IV received saffron suspended in Milli-Q water at the doses of 50 mg/kg/day, 250 mg/kg/day and 1000 mg/kg/day, respectively at 10 mL/kg body weight through oral gavage. The vehicle/test item was administered to the respective dose group rats from implantation (day 5 post coitus) through lactation up to lactation day (LD) 20. The rats were weighed on specified intervals of gestation and lactation and food intake was also measured at similar intervals as body weight. Daily records of activity with reference to appearance and behavior were maintained. All the presumed pregnant dams in each group littered expect for one dam at 250 mg/kg/day dose group which was found non-pregnant. At parturition, litter size (number of pups born), sex of pups, external deformities, weight of individual pup were recorded for each dam. On LD 4, standardization of the litter size to 8 pups was made and the pups were weighed at frequent intervals till weaning sacrifice. Physical development and maturation of pups within each group was evaluated by observing the day of onset and completion of the following land marks:

1. Pinna unfolding: Beginning on postnatal day (PND) 1, each pup in every litter was observed individually until all pups in the litter met the criterion for pinna unfolding i.e. the point of pinna detached from the head.
2. Incisor eruption: Beginning on PND 7, each pup in every litter was observed for eruption of upper incisor through the gums until the criterion was met in all the pups.
3. Ear opening: Beginning on PND 10, all pups in every litter was observed until all pups in the litter met the criterion for ear opening.
4. Eye opening: Beginning on PND 13, all pups in every litter was observed until all pups in the litter met the criterion for eye opening, i.e. the separation of upper eye lid from the lower eye lid in both eyes.

After completion of the 21 day post-partum period, the dams were euthanized under isoflurane anesthesia and from each dam, 2 male and 2 female pups were retained to evaluate the effects on attainment of sexual maturity by assessing the following parameters:

1. Balano-preputial separation: It is the complete retraction of prepuce from the penis when gentle pressure is applied to the animal's prepuce. This criterion was assessed by examining each male rat starting from 38 days of age.
2. Vaginal opening (Patency): It is the visible break in the membranous sheath covering the vaginal orifice which results in separation of vaginal edges. This criterion was assessed by examining each female rat starting from 28 days of age.

The animals were weighed on the day the criterion was met following which they were euthanized and examined macroscopically for gross lesions. The pups not selected for continued evaluation were sacrificed and examined macroscopically for gross lesions.

Statistical analysis

Comparisons were made between the saffron exposure groups and the control using Dunnett's method following one way analysis of variance (ANOVA) for physical developmental and sexual maturity land mark parameters. A probability of 0.05 was accepted as statistically significant for all the applied tests.

Experimental Results

Mortality, clinical signs, body weight and food intake

No mortality or clinical signs of toxicity were found in the rodent dams throughout gestation (Ramesh Edamula *et al*., 2014) and lactation period (Ramesh Edamula *et al*., 2015) except for the slight yellowish coloured feces at the highest dose of 1000 mg/kg/day dose which is 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 day 21 post-partum sacrifice. The maternal body weight and food intake were unaffected during gestation and lactation period (data not shown).

Body weight of pups and survival index

Body weight of pups and survival indices of pups showed no significant differences between treated groups and controls (data not shown).

Postnatal developmental observation in pups

To determine the possible effects of maternal saffron exposure on physical development of pups, assessment on important physical development milestones such as pinna unfolding, incisor eruption, ear opening and eye opening were made. Saffron exposure did not influence the onset and completion of pinna unfolding, ear opening and eye opening across the treated groups in both genders, while incisor eruption was delayed in all the treated groups (Table 1).

Table 1. Physical development milestone observation in pups

Parameter	Treatment			
	Control	50 mg/kg/day	250 mg/kg/day	1000 mg/kg/day
Pinna unfolding	3.41±0.89 (59)	3.68±0.49* (82)	3.52±0.50 (58)	3.66±0.84 (71)
Incisor eruption	10.62±0.94 (45)	11.15±1.07* (48)	11.26±1.19* (39)	11.46±1.07* (48)
Ear opening	14.16±0.85 (45)	14.46±0.68 (48)	13.82±0.76 (39)	14.46±0.71 (48)
Eye opening	15.60±1.05 (45)	15.90±0.66 (48)	15.59±0.50 (39)	15.71±0.90 (48)

Values: Mean±SD ( ): Value in parenthesis indicate number of pups*: Significantly different from control group, P≤0.05

Table 2. Sexual maturity landmarks

End Point	Treatment			
	Control	50 mg/kg/day	250 mg/kg/day	1000 mg/kg/day
No. of male/female pups	12/12	12/12	10/10	12/12
Balano-preputial separation (BPS)				
1.Mean age (days)	46.08±1.08	46.33±1.15	45.7±0.67	46.83±1.27
2.Mean weight (g) at BPS	148.02±24.85	161.7±13.37	149.39±8.05	155.67±15.25
Vaginal patency (VP)				
1.Mean age (days)	35.83±1.75	36.58±1.62	35.00±2.26	37.00±2.00
2.Mean weight (g) at VP	92.84±9.28	96.49±9.96	89.11±7.32	98.38±11.7

Values: Mean ± SDEither F-test (ANOVA) or the Dunnett's t-test is not statistically significant

Sexual maturity landmarks

To evaluate the effects on attainment of sexual maturity, assessment was made by examining each male rat starting from 38 days of age for balano-preputial separation. Balano-preputial separation is the complete retraction of prepuce from the penis when gentle pressure is applied to the animal's prepuce. Similarly, each female rat was assessed for vaginal patency/opening starting from 28 days of age.Vaginal patency is the visible break in the membranous sheath covering the vaginal orifice which results in separation of vaginal edges. The animals were weighed on the day the criterion was met. Attainment of these land marks were uninfluenced by exposure to saffron at any of the treated levels and the data was statistically comparable to the control group (Table 2).

DISCUSSION

Commercially saffron constitutes the dried stigma of the flower. The pharmacologically important active constituents of saffron comprise the volatile agents (safranal), bitter principles (picrocrocin) and the colour component (crocetin and its glycoside, crocin). Saffron is widely consumed by pregnant women mainly in the belief to increase fairness in newborn. It has been reported that saffron can stimulate uterine contractions in pregnant women leading to abortions. Also it is reported that high concentrations of crocetin, a carotenoid component giving a characteristic golden yellow orange colour to saffron was found to be teratogenic in frogs, *Xenopus laevis*. As the toxicity information on toxicology and safety of saffron is not consistent, the objective of the present investigation was to detect adverse effects of orally administered saffron on the physical development milestones such as pinna unfolding, incisor eruption and ear/eye opening of the offspring whose dams received saffron administered as an oral gavage from implantation (day 5 post coitus) throughout the lactation period. Since manifestation of effect induced during this period may be delayed, observation was continued to monitor the land mark signs of attainment of sexual maturity with reference to preputial separation in males and vaginal opening in female offspring. Rat was selected as the model due its common and wide use in toxicity testing. The oral route was selected to administer the test material as it simulated the exposure pattern of the human population. The highest dose selected for the study was 1000 mg/kg/day which was the maximum feasible dose and also referred to as the limit dose by regulatory toxicity guidelines related to reproduction toxicity testing [Guidelines ICH S5 (R2)]. $1/4^{th}$ the highest dose (250 mg/kg/day) was selected as the mid dose and $1/5^{th}$ the mid dose (50 mg/kg/day) was selected as the low dose which closely relates to human dose. The control rats received the Milli-Q water, which was used to suspend the saffron at low, mid and high doses. Treatment with saffron up to the highest dose of 1000 mg/kg/day did not elicit any adverse effects on several land marks of physical development such as opening of eyes/ears and pinna unfolding which were comparable across the 3 dose groups. The age for eruption of incisor was delayed by a day in all the treated groups. However this delay was comparable to the results reported by Gandhi *et al*., 2012.The land mark signs of attainment of sexual maturity with reference to preputial separation in males occurred between days 44 to 49 (Stoker *et al*., 2000, Korenbrot *et al*., 1977) and vaginal opening in females occurred between days 32 to 39 (Law *et al*., 2003). These sexual maturity land marks were comparable across the 3 dose groups and the published literature.

Conclusion

The present findings indicated that exposure of saffron through gestation and lactation up to 1000 mg/kg/day did not pose any potential health risk related to the several land marks of physical development of rat off springs up to attainment of sexual maturity.

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