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Developmental, Behavioral And Reproductive Consequences In Female Progeny Exposed To *Artemisia Annua* L Leaf Extract *In Utero*

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ABSTRACT

The present study examines the postnatal effects of *in utero* exposure to artemisinin in the rat. Pregnant Wistar rats were administered with methanolic leaf extract of *Artemisia annua* (AAL) with 100, 200, or 400 mg/kg body weight from gestation day (GD) 7 to 9. At parturition, newborns were observed for clinical signs and survival. Male and female pups from control and AAL exposed animals were weaned and maintained up to postnatal day (PD) 100. Litter size and birth weights of control and experimental pups were comparable. Survival index and developmental landmarks were decreased in AAL-exposed rats when compared to controls. Elapsed time (days) for vaginal opening was significantly delayed in experimental pups when compared to control pups. Behavioral observations such as cliff avoidance, negative geotaxis, surface rightening activity, and ascending wire mesh were impaired in experimental pups. Decrease in total duration of estrous cycle, increase in conception time and post implantation loss were observed in 200 mg/kg body weight administered group. These results indicate that *in utero* exposure to AAL compromised postnatal developments and fertility efficiency in female progeny.

KEYWORDS: *Artemisia annua*; Progeny; Behavior; Estrus cycle

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1. INTRODUCTION

The jeopardy of malaria during pregnancy is devastating not only to the mother, but also to the developing foetus. More than 50 million pregnant women suffer with malaria every year. World Health Organization (WHO) estimates that 80% of the world populations currently use herbal drugs for healthcare[1,2,3]. Since fetuses are more sensitive to drugs, selection of drugs during pregnancy should be done with caution. Generally pregnant women prefer herbal medicines rather than pharmaceutical drugs for an accurate foetus development[4]. *Artemisia annua* L. (asteraceae) is a medicinal herb that has been used for more than 2000 years for the treatment of malaria and other disorders. Artemisinin, the principal product of *Artemisia annua*, approved by the US Food and Drug Administration and currently preferred antimalarial drug widely used in combination therapies[5]. In addition to its antimalarial activity, *A. annua* also possesses anti-inflammatory, antibacterial and cytotoxic phytochemicals[6,7]. Though previous studies warranted exposure to even low concentrations of artemisinin at different stages of pregnancy causes foetal resorptions in rats and rabbits[8,9,10,11], systematic studies on post natal development of progeny exposed to artemisinin during embryonic development is lacking. In the present study, we investigated the pregnancy outcomes of dams exposed to AAL and developmental, behavioral and reproductive consequences in female progeny.

2. MATERIALS AND METHODS

2.1. Plant extract preparation and Phytochemical analysis

Artemisia annua seedlings were obtained from the Central Institute of Medicinal and Aromatic Plant (CIMAP), Lucknow and grown up to flowering stage in Sri Padmavathi Mahila Visvavidyalayam (Women's University), Tirupati (A.P). The leaves were collected, washed thoroughly in water, and air-dried for two weeks at 35°C. Dried leaves were ground into powder using an electric blender. Powder was stored in air tight container at 4°C. Extraction was carried out with 100 g of *A. annua* leaf powder in 500 mL of 70% methanol by soxhlation for 18 h by using Soxhlet apparatus. The extracts were concentrated using rotary flash evaporator under reduced pressure and controlled temperature (60°C), and stored at 4°C in air-tight containers for further studies.

2.2. Animals and housing

Female Wistar rats (body weight 190–210 g) with a normal estrus cyclic pattern were selected and purchased from an authorized vendor (M/S Raghavendra Enterprises, Bengaluru, India). The rats were housed in polypropylene cages (18" x 10" x 8") lined with sterilized paddy husk as bedding material. The animals were provided with filtered tap water and standard rodent feed (purchased from Sai Durga Agencies, Bengaluru, India) *ad libitum* throughout the study. The rats

were maintained in well-controlled laboratory conditions (temperature 25 ± 2 °C; 12-h light and 12-h dark cycle, humidity 50 ± 10 %). The experiments were carried out in accordance with the guidelines of the Committee for the Purpose of Control and Supervision on Experiments on Animals, Government of India (CPCSEA, 2003) and approved by the Institutional Animal Ethical Committee at Sri Venkateswara University, Tirupati, India (vide no. IAEC/No- 438/01/a/CPSEA).

2.3. Treatment

After a 2-weeks acclimatization period, the rats were housed as breeding pairs (one male and one female in proestrous stage). Female rats were examined every morning while evidence for mating was confirmed by the presence of a vaginal plug and/or sperm in a vaginal smear. The cohabitation period was 4 days. On pregnancy day 0 (determined by the presence of sperm in vaginal smear), the dams were divided into four groups, each group consisting of four animals. The first group served as control. Pregnant rats in the second, third, and fourth groups were daily administered with 100, 200, and 400 mg plant extract /Kg bw (dissolved in DMSO) respectively through oral gavage from days 7 to 9 of gestation (GD 7 – 9). The dosage levels of AAL were based on previous studies on rats[12,13,14], which caused decreased fertility efficiency. All rats were allowed to deliver pups. Litter size and live pups were counted and their sex and survival rate on postnatal day 21 was determined. Two days after birth, the offspring were cross-fostered. Ten pups (5 male and 5 females) were housed with a lactating dam and were weaned on postnatal day 22. Thereafter male and female animals were housed separately in groups of three to five.

2.4. Pregnancy outcomes

Pregnant animals were evaluated for mortality, morbidity and general clinical signs, such as behavioral changes such as agitation, lethargy, hyperactivity and cannibalism, neurological changes such as convulsions, tremors, muscle rigidity and hyper-reflexia and autonomic signs (e.g. lacrimation, piloerection, pupil size and unusual respiratory pattern). Additionally, pregnancy length, litter size, and pup birth weight were recorded. The viability index [(number of live offspring on PND 4/number of live offspring delivered) X 100], and the weaning index [(number of live offspring on PND 21/number of live offspring delivered) X 100] were also evaluated.

2.5. Developmental landmarks in pups

Pups were individually identified by paw tattoo and observed every day for clinical signs of toxicity until completion of the lactation period. The anogenital distance (AGD) and crown-rump length was measured using Vernier calipers. The age at which the incidences of pinna unfold, lower

and upper incisor eruption, fur development, eye opening, and vaginal opening was tracked by an investigator that was blindfolded to treatment.

2.6. *Behavioral studies*

All the behavioral tests were performed between 08:00 a.m.and 12:00 noon. Pups were submitted to behavioral evaluation only once in their lives. Litter mates were not used within a group, but they were used across groups.

2.6.a. Cliff avoidance

On PD 5, a pup was placed on a table edge with the forepaws and nose over the edge. The time required to complete backing and turning away from the edge of the table was recorded. The number of pups with successful responses within 30 s was recorded.

2.6.b. Surface rightening

Each pup was placed in a supine position and allowed a maximum time of 15 s to upright itself (two trials were given per day) on PD 6. Time of achievement of rightening reflex was recorded.

2.6.c. Negative geotaxis

The time taken to complete a 180° turn when placed in a head down position on a 25° inclined plywood surface was recorded. The number of pups with successful responses within 30 s on PD 7 was recorded.

2.6.d. Ascending wire mesh

The wire mesh (50×30 cm) was dipped in a water bath at 26°C such that it is 31 cm above the water surface. The pups on PD 16 were placed with their quarter hind and tail dipping in the water. The number of pups to reach the top within 60 s was recorded.

2.7. Vaginal smear cytology and description of estrous stages of female progeny

From the postnatal day 70, vaginal smears were prepared every morning (6.00 – 8.00 am) and observed under microscope to characterize the estrous cycle for 20 consecutive days. Different stages of estrous cycle were determined using the method described by Zarrow et al.[15] and reviewed by Cooper et al.[16].

2.8. Evaluation of reproductive performance of female progeny

On PND 100, female rats from control and experimental groups in pro-estrous stage were cohabited with untreated 100 day old male rats (1:1 ratio) to evaluate their fertility efficiency. Successful mating was confirmed by the presence of vaginal plug or sperm in vaginal washing. Pregnant rats were sacrificed on 18th day of gestation; both ovaries were removed and examined for the number of corpora lutea. Uterine horns from both sides were removed and numbers of implantations and live/dead fetuses were counted. Mating index (number of sperm positive females/number of pairings×100), fertility index (number of pregnant females/number of pairings×100), post-implantation loss (difference between the number of implantations and the number of live fetuses expressed as per number of implantations) were calculated. In addition, the conception time, the interval between the first day of cohabitation and the day of vaginal plug and/or sperm in vaginal smear, was recorded for each female.

2.9. Statistical treatment of the data

The data were statistically analyzed using One-way Analysis of Variance (ANOVA) followed by Tukey's test. The data were expressed as mean ± S.D. and '*p*' value < 0.05 was considered significant. All statistical tests were performed using Statistical Package for Social Sciences (SPSS Inc., Chertsey, UK).

3. RESULTS AND DISCUSSION

Administration of AAL did not cause any abortions in pregnant rats. No unusual behavior (viz. head flicking, biting, licking, self-mutilation, aggressiveness, redness around eyes) was observed in the control or experimental dams. None (0 %) of the animals in the control and AAL exposed groups died during treatment and none of the animals were excluded from the research.

Pregnancy length, Litter size (number of pups delivered) and the body weight of pups delivered to AAL exposed rats were comparable to those of control pups. Conversely, weaning- and viability indices were decreased in experimental pups when compared to control pups (Table 1). Our results are in agreement with those reported by Abolaji et al.[13,17]. All pups delivered to control and experimental dams were apparently normal. However, the mortality recorded before weaning suggests developmental toxicity of *A. annua* extract in the pups. This mortality could be due to *in utero* direct toxic effect of A. *annua* extract on the pups, or it may be due to maternal toxicity[18]. Adverse early-life experiences, including maternal exposure to stress during pregnancy, can ''programme'' persistent changes in several physiological systems and behaviors, probably via epigenetic mechanisms. In this study, developmental landmarks such as crown-rump length and ano-genital distance of pups were comparable in all experimental pups when compared to those of

control pups (Table 2). The elapsed time for eye opening, lower and upper incisor eruption, pinna unfolding, fur development and vaginal opening was also not delayed in experimental groups when compared to control group (Table 2).

It is well established that prenatal and/or neonatal exposure to toxicants results in long-term influences on brain, behavior, and reproductive functions[19,20]. In the present study, prenatal exposure to graded doses of AAL resulted in significant delay in the cliff avoidance, negative geotaxis, rightening reflex response, and ascending wire mesh activity, when compared to the control pups (Table 3). Previous studies with dogs, monkeys, rats and humans reported that artemisinin compounds revealed considerable neurotoxic potency (gait disturbance, loss of spinal and pain response reflexes, and prominent loss of brain stem and eye reflexes)[21,22,23].

The mean age of puberty in female rats and beginning of the estrous cycle is based on the occurrence of vaginal opening (VO)[24]. Disturbances in length of the estrous stages and decrease in estrous cycle duration (Fig. 1) were observed in experimental females when compared with controls. This might be due to disruption in endocrine circuits resulting in alterations in reproductive hormone levels. Previous studies suggested that artemisinin decreases the levels of estrogen[25,13]. In the present study, although mating and fertility index (100%) was not affected, post implantation loss was increased in experimental females (Fig. 2; Table 4). Previous findings demonstrated that oral administration of artemisinin can adversely affect post-implantation development and pregnancy in the rat[10,17]. Additional studies also highlighted that *Artemisia* species also possesses antifertility properties and could be used for contraceptive actions[26,14,17].

4. CONCLUSIONS

In conclusion, AAL treatment did not alter fertility output in the dams. Though AAL may be safe to mother, the survival and weaning indices of female progeny decreased indicating toxic effect of AAL. This early life toxicity also reflected in developmental, behavioral and reproductive consequences of female progeny. Besides antimalarial activity, AAL is also known for hypoglycemic activity. Furthermore due to the fact that artemisinin is considered as a relatively “new” antimalarial medication with unknown risks, more research is required, before prescription particularly to pregnant women.

5. CONFLICT OF INTEREST

The authors have no conflicting interests to declare.

6. ACKNOWLEDGMENTS

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Table No. 1: Effect of AAL on progeny outcome of dams

Parameter	Control	100mg AAL	200mg AAL	400mg AAL
Pregnancy length# (days)	21.25±0.83	21.7±0.43 (2.12)	22±0.71 (3.53)	21.5±0.50 (1.18)
Litter size#	9.25±0.50	8.75±0.96 (-5.40)	8.75±1.26 (-5.40)	8.5±2.38 (-8.11)
Pup birth weight$ (g)	6.91±0.68	6.17±0.40 (-4.04)	5.84±0.37 (-9.17)	6.43±0.50 (-7.46)
Viability index$ (%)	100	92.31	77.14*	91.12
Weaning index$ (%)	100	92.31	74.29*	82.35

Values are expressed as mean ± S.D. n= #4; $130

Values in the parentheses are % change from control.

*p<0.05 when compared to control

Table No. 2: Effect of in utero exposure to AAL on developmental landmarks in pups

Parameters	Control	100mg AAL	200mg AAL	400mg AAL
Crown-rump Length (cm) (n=136)	5.57±0.20	5.39±0.39 (-3.23)	5.26±0.34 (-5.57)	5.43±0.14 (-2.51)
Anogenital Distance (cm) (n=136)	0.305±0.006	0.31±0.018 (0.33)	0.30±0.008 (-1.64)	0.29±0.025 (-4.25)
Pinnae Unfold (days) (n=134)	3.78±0.80	3.96±0.30 (4.76)	3.68±0.21 (-2.65)	4.03±0.26 (6.61)
Lower Incisor Eruption (days) (n=134)	4.44±0.57	4.45±0.70 (0.23)	4.62±0.18 (4.73)	4.78±0.29 (7.66)
Fur Development (days) (n=131)	11.31±0.74	10.94±0.09 (-3.27)	10.67±0.41 (-5.66)	11.30±0.41 (-0.09)
Upper Incisor Eruption (days) (n=131)	12.2±1.07	11.84±0.51 (-2.95)	11.80±0.47 (-3.28)	12.13±0.46 (-0.57)
Eye Opening (days) (n=130)	13.0±0.71	12.73±0.44 (-2.08)	13.07±0.29 (0.54)	12.71±0.30 (-2.23)
Vaginal Opening (days) (n=63)	37.30 ± 1.22	37.80 ± 2.62 (1.34)	40.40 ± 0.43 (8.31)	39.50 ± 1.91 (5.90)

Values are expressed as mean ± S.D.

Values in the parentheses are % change from control.

Table No. 3: Effect of *in utero* exposure to AAL on pre-weaning behavioral responses in pups

Parameter	Control	100mg AAL	200mg AAL	400mg AAL
Cliff Avoidance	$8.95^{a} \pm 1.44$	$14.44^{b} \pm 4.24$ (61.34)	$21.33^{c} \pm 4.21$ (138.32)	$16.74^{bc} \pm 4.19$ (87.04)
Surface Rightening	$1.81^{a} \pm 0.11$	$2.06^{b} \pm 0.76$ (13.81)	$4.47^{c} \pm 1.49$ (146.96)	$3.34^{bc} \pm 0.45$ (84.53)
Negative Geotaxis	$8.53^{a} \pm 1.66$	$12.89^{b} \pm 1.42$ (51.11)	$20.11^{c} \pm 4.35$ (135.76)	$22.25^{d} \pm 6.13$ (160.84)
Ascending wire mesh	$29.54^{a} \pm 2.49$	$31.59^{b} \pm 6.60$ (6.94)	$36.85^{c} \pm 8.77$ (24.75)	$34.28^{bc} \pm 3.20$ (16.05)

Values are expressed in sec

Values are expressed as mean ± S.D.

Values in the parentheses are % change from control.

Values with different superscripts in a row differ significantly from each other at $p<0.05$

Table No. 4: Fertility output of female progeny exposed to AAL during embryonic development

Parameters	Control	100mg AAL	200mg AAL	400mg AAL
Conception time (days)	1.5 ± 0.71	1.0 ± 0 (-33.33)	2.0 ± 0 (33.33)	1.5 ± 0.71 (0.00)
Mating index (%)	100%	100%	100%	100%
Fertility index (%)	100%	100%	100%	100%
No.of corpora leutia/rat	13.5 ± 0.71	13 ± 1.41 (-3.70)	13.5 ± 3.53 (0.00)	14 ± 1.41 (3.70)
No of live fetuses/rat	12.5 ± 0.71	12.5 ± 0.71 (0.00)	4.0 ± 5.66 (-68.00)	9.5 ± 2.12 (-24.00)
Post implantation loss (%)	7.41%	3.85%	70.37%	32.14%

Values are mean ± S.D. of 6 individuals.

Values in the parentheses are % change from control.

Figure No. 1: Effect of embryonic exposure to AAL on duration of different stages of estrous cycle in control and experimental rats

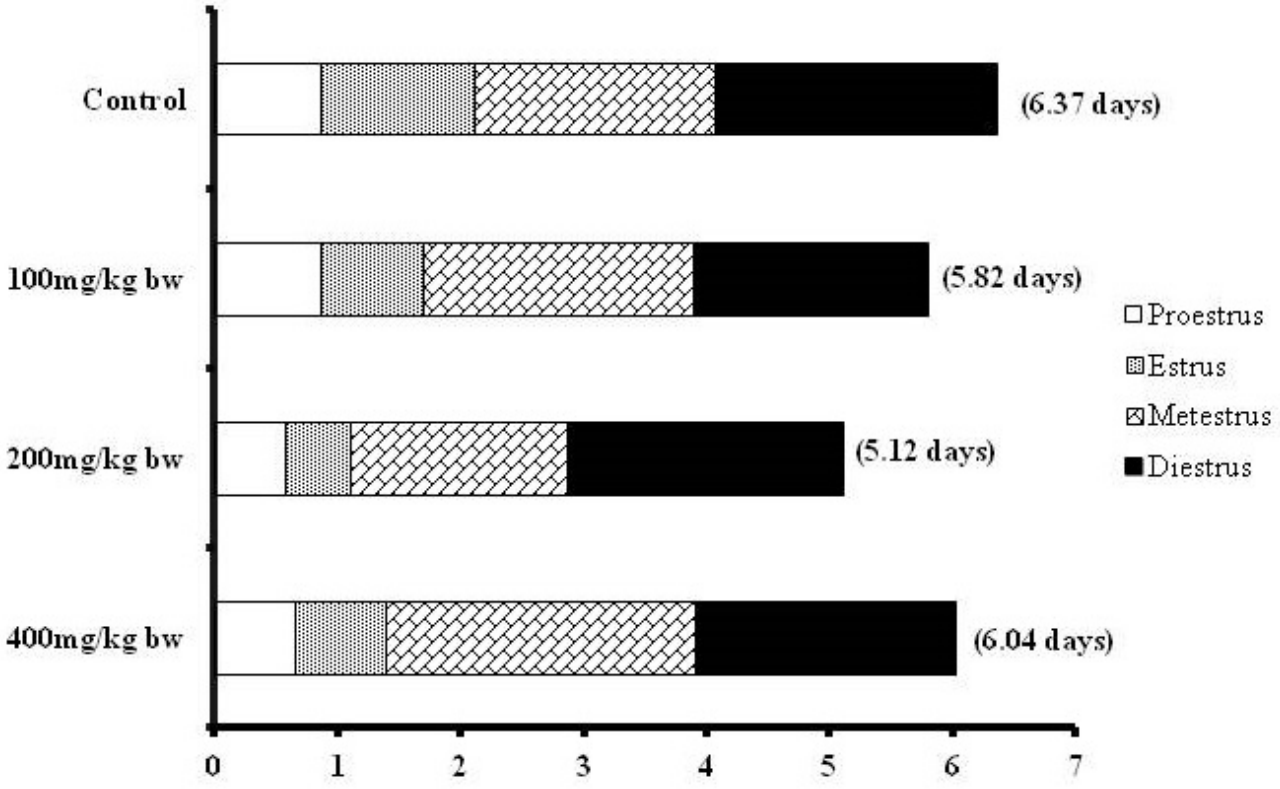

Figure No. 2: Uterus of control rats (A) and rats exposed to 100mg (B), 200mg (C) and 400mg (D)of AAL/kg bodyweight during embryonic development showing fetuses on 18th day of pregnancy