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Antigestational effects of Icon®, a pyrethroid insecticide on late pregnancy of rats

S. S. K. Ratnayake, W. D. Ratnasooriya and Y. N. A. Jayatunga

Department of Zoology, University of Colombo, Sri Lanka

Abstract

Icon® is a type II synthetic pyrethroid insecticide based on active ingredient Lambda cyhalothrin (10% w/w) and used as an adulticidal indoor spray against malaria vector mosquitoes in Sri Lanka. The purpose of this study was to review the effects of Icon® on pregnancy outcome of rats when exposed during late pregnancy (days 15-21). Icon® was orally administered for 7 consecutive days in three different doses 63, 83, or 125 mg/kg/day respectively. Oral exposure to Icon® during late pregnancy was detrimental to pregnancy outcome (in terms of number of viable pups, gestation index, live birth index, pups survival ratio, viability index) and developmental parameters of pups (such as body weight, cranial length, cranial diameter, tail length, cranial – sacral length and viability at postnatal day 1) but induced no detectable congenital malformations. Further, there was a significant prolongation of pregnancy. The mechanism of Icon® induced deaths and growth retardation of foetuses appear to be due to Intra Uterine Growth Retardation (IUGR) mediated by a combination of specific and non specific modes of actions such as reduced food consumption, maternal and embryo/foetotoxicity, stress and uterotropic activity.

Key words: Icon®, Insecticides, Intra Uterine Growth Retardation (IUGR), Lambda cyhalothrin, Low birth weights, Pregnancy outcome, Pyrethroids, .

1. Introduction

Several currently used insecticides, especially those having endocrine disruptive properties, are known to adversely affect the pregnancy outcome of rats [1, 2, 3, 4]. Some of these agents are among the most commonly used insecticides in developing countries including Sri Lanka. Icon®, produced by the Public Health Division of the Imperial Chemical Industries in England [5] is a water miscible type II synthetic pyrethroid insecticide having Lambda Cyhalothrin (10% w/w; information on inert fillers, adjuvant, excipient, wetting agents and purity are not available) as the active ingredient [5]. Icon® has been recently introduced to Sri

Lanka as an adulticidal indoor spray against malaria vector mosquitoes (Manuweera G., Registrar of Pesticides, Sri Lanka, personal communication). Its effective spray dose is 312 mg/ m^2 (Gunasekara K., Parasitologiest, Anti malaria campaign, Sri Lanka, personal communications). Pesticide residues in indoor environments are not subjected to degradation by sun, rain and soil microbes and are more persistent than in the environment at large [6]. Therefore, there is a potential risk that inhabitants in Icon® sprayed houses are continually subjected to dietary, respiratory and cutaneuos exposures. Recently it was shown that Icon® exposure to male rats impairs sexual competence [7] and in female rats when exposed during early gestation [8] and mid gestation [9] was detrimental to their pregnancy outcome. Several pyrethroid insecticides when exposed during late pregnancy are known to interrupt pregnancy [3, 2, 10, 11]. However, the potential anti reproductive effects of Icon® exposure during late pregnancy are still unknown and are worth examining. The aim of this study was therefore to assess the potential impacts of Icon® exposure on late pregnancy of rats.

2. Materials and methods

2.1. Animals

Healthy adult crossbred female and male rats (weight: 200 – 250 g) of proven fertility from the colony maintained at the Department of Zoology, University of Colombo were used. They were maintained singly in plastic cages under standardized animal house conditions (temperature: 28 – 30 °C; photoperiod: approximately 12 h light / 12 h dark; and relative humidity: 50 – 55 %) with free access to pelleted food (Master Feeds Lanka Ltd., Colombo, Sri Lanka) and tap water. Except at the time of experiment the animals were handled only during cage cleaning.

2.2. Icon® preparation

Icon® was obtained from Anti-Malaria Campaign, Narahenpita, Sri Lanka. Three desired doses [63 (low), 83 (mid) and 125 (high) mg/kg/day; (containing active ingredient Lambda cyhalothrine 6.3, 8.3,12.5 mg /kg/day respectively)] of Icon® in 1ml aliquots were prepared by mixing Icon® in Distilled water (DW). These doses were comparable to what had been used previously by us to investigate the potential reproductive effects on male rats [7] and identical to what had been used to test the anti reproductive effects on female rats when exposed during early pregnancy [8] and mid pregnancy [9]. The reported oral No–Observed-Effect Level of Lambda cyhalothrine was 50 mg/kg [12].

2.3. Icon® administration

The rats were made pregnant by pairing pro-oestrus females overnight with a male rat individually and examining the vaginal smears on the following morning (8.00 – 9.00h) for the

presence of spermatozoa. On day 15, forty – eight pregnancy confirmed rats were assigned into 4 equal groups and orally administered with different doses of Icon® (low, mid, high: n = 12) as treatment and distilled water as control (n =12) for 7 consecutive days. The doses used were approximately 2 ½ - 4 times lower than the recommended spray dose in Sri Lanka, which is 312 mg/m^2 (Gunasekara K., Parasitologist, Anti - malaria campaign, Sri Lanka, personnel communication).

2.4. Adverse effects

Following every dosing, cage side observations were made on each rat continuously for 3 – 5 h for mortality, overt signs of toxicity (ataxia, tremors, convulsions, salivation, lacrymation, coughing, and changes in fur colour), stress (exophthalmia and pilo-erection), lethargy (reduction of spontaneous walking movements, climbing in cage, cleaning of fur) recumbence, aversive behaviours (self biting and scratching, licking of tail and/or paw, intense self grooming behaviour and vocalization) diarrhoea, colour and odour of urine and vaginal bleeding. In addition, the rectal temperature was recorded on day 15 of pregnancy before dosing and on day 21 of pregnancy (5 h after dosing) using a clinical thermometer (Oson Duopris Company Ltd., Berlin, Germany).

2.5. Effect on food and water consumption

Food and water intake of Icon® or vehicle treated rats were determined on day 15, day 17, day 19 and day 21 of pregnancy using conventional laboratory techniques [13].

2.6. Effect on body weight

The body weights of Icon® or vehicle treated rats were determined on day 15 of pregnancy before dosing and 5 h after dosing on day 21 of pregnancy using an electronic balance (MP6000, chyo YMC & Corporation Ltd., Japan).

2.7. Effect on righting reflex

The time for righting reflex of rats treated with Icon® or vehicle was determined 2 h prior to the onset of treatment on day 15 and after about 6h of last dosing on day 21 as described by Mortin *et al.*[14].

2.8. Effect on pregnancy outcome and post - natal development of pups

Rats were allowed to litter and the gestation length was recorded. After delivery, pups were closely observed and total number of pups and their viability were recorded. On postnatal day 1, again, the viability of pups and their body weights (using an electronic balance), cranial

length, cranial diameter, cranial-sacral length and tail length (using a venire calipers) were determined. The presence of gross external congenital abnormalities was also noted (such as anomalies of tail, amelia, clubfoot, oligodactyly or syndactyly). Then the pups were observed once daily for the appearance of fur and opening of eyes. The following reproductive indices were then computed using the reproductive findings. Gestation index= (# live litter / # pregnant) x 100; Live birth index (%) = (# viable pups / # littered pups) x 100; Pups' survival ratio (%) = (# surviving pups / # pups) x 100 and Viability index (%) = (# day 1 surviving animals / # live pups per animal) x 100.

2.9. Statistical analysis

Data was expressed as mean ± standard error of mean (SEM). Mann - Whitney *U*-test and Student's G-test were used as appropriate. $P < 0.05$ was considered as statistically significant.

3. Results

3.1 Adverse effects

On day 21 of pregnancy, 2 out of 12 deaths were evident with the high dose before parturition. As shown in the Table 1, Icon® induced ataxia (in all groups), exophthalmia (high dose), piloerection (high dose), salivation (low dose: around facial region; mid dose: upto neck region; and high dose: throughout the entire body), diarrohea (mid and high doses) and dark yellow coloured urine with an odour similar to that of Icon® (mid and high dose) between days 15 – 21 of pregnancy. These toxic effects appeared 30 – 60 min after the administration of Icon® and were completely abolished by 12 – 15h except with the highest dose where, the effects lasted upto 3 -5 days of cessation of treatment. None of the treated rats showed tremors, convulsions lacrymation, coughing, yellowing of fur, lethargy, recumbence or aversive behaviours. Further, Icon® administration significantly increased ($p<0.05$; Mann-Whitney *U*–test) the rectal temperature of high dose treated rats.

3.2. Effects on food and water intake

Icon® administration caused significant reduction ($p<0.05$; Mann-Whitney *U*-test) in food consumption through the study period: on day 15 (low: by 35%, mid: by 65%, high: by 80%), day 17 (mid by 51% and high by 48%), day 19 (high by 44%) and day 21 (high by 64%) (Table 2).

3.3. Effects on body weight

Icon® caused significant reduction ($p<0.05$; Mann-Whitney *U*-test) in body weight gain in mid (by 46%) and the high (by 59%) doses of Icon® treated groups (Table 3).

3.4. Righting reflex

Only the highest dose of Icon® significantly ($p<0.05$; Mann - Whitney U- test) increased (by 67 %) the righting reflex time (control vs. high dose: 0.3 0.2 vs. 0.5 ± 1.0* s) when compared with the control.

3.5. Reproductive out come and post- natal development of pups

The results obtained were summarized in Tables 4 and 5. Administration of low dose of Icon® on days 15 - 21 of pregnancy did not significantly ($p>0.05$) alter any of the investigated parameters. The highest dose induced a significant prolongation ($p<0.05$; Mann-Whitney *U*-test) in gestation length. Further, with the high dose, except 2 rats all the others delivered their pups on day 23 of pregnancy. One rat delivered pups on day 26 and the other on day 28 of pregnancy. In contrast, mid dose treated rats did not show any significant prolongations ($p>0.05$) in gestation length. Only the highest dose of Icon® significantly ($p<0.05$; Mann-Whitney *U*-test) impaired the viability of pups (by 45 %), gestation index (by 48%), live birth index (by 37%) and viability index (by 55%). Pups' survivals on postnatal day 1 and pups' survival ratio were significantly reduced ($p<0.05$ & $p<0.01$; Mann-Whitney *U*-test) in both mid and high dose treated groups: (pups' survivals on post natal day 1: mid by 28% and high by 62% and pups' survival ratio: mid by 17 % and high by 57 % respectively).

Pups born to females treated with both mid and high doses of Icon® showed growth retardation: these were significant reduction ($p<0.05$; Mann-Whitney *U*-test) in body weights (mid: by 13% and high: by 24%), cranial length (mid: by 6% and high: by 7%), cranial diameters (mid: by 5% and high: by 7%), tail length (mid: by 7% and high: by 10%), and cranial – sacral length (mid: by 5% and high: by 9%) of pups. In contrast, fur appearance time and eye opening time of pups were not significantly altered ($p>0.05$; Mann-Whitney *U*–test) due to Icon® administration (Table 4).

4. Discussion

The results demonstrated clearly for the first time, that in rats, repeated oral exposure of Icon® during late pregnancy (days 15 –21) was detrimental to pregnancy outcome (in terms of number of viable pups, gestation index, live birth index, pups survival ratio, viability index) and developmental parameters of pups (as judged by body weight, cranial length, cranial diameter, tail length, cranial – sacral length and viability at postnatal day 1). However, some post-natal developmental parameters such as appearance of fur and opening of eyelids were not altered by Icon®. At birth, gross external congenital abnormalities (such as anomalies of tail, amelia, clubfoot, oligodactyly or syndactyly) were absent in this study. In addition, there was a significant increment in gestation length. In contrast, Icon® exposure during early [8] and mid pregnancy [9] did not cause any impairment in pre - or post-natal development of pups or alter the

gestation length. Both teratogenic and developmental inhibitions of late pregnancy exposures of insecticides such as pyrethroids [3, 2, 10, 11, 15, 16], carbamates [17], organophosphates [18, 19] and organochlorines [20, 21] were reported.

Icon® exposure during early gestation interrupted pregnancy by increasing both pre- and post-implantation losses [8] and in mid pregnancy by increasing post-implantation loss [9]. In the rat, uterine implantation occurs on day 4 of pregnancy [22]. Obviously, therefore, pre-implantation loss cannot account for any of the antireproductive effects seen in this study. Further, all treated animal's littered pups and the numbers of pups born were comparable to that of control. Therefore, potentiation of post – implantation losses is also unlikely to be operative in this study. Icon® administration caused stillbirths. Further, the survived pups in high and mid doses had Low Birth Weights (LBW) and retarded growth.

LBW is the most significant indicator of risk to the survival, health and development of an offspring and also regarded as one of the most sensitive indicators of maternal health status [23]. LBW is caused either by short gestation period or Intra Uterine Growth Retardation (IUGR) or by combination of both [24]. In this study, pregnancy length was significantly prolonged, thus shortening of gestation length was unlikely to be responsible for the LBW. Therefore, IUGR may be considered as the main cause for the observed LBW. IUGR it self is reported to be associated with significant prenatal and childhood morbidity and mortality [25] and is currently considered as the second leading cause of foetal deaths in the world [26]. Further, 13.7 million infants are born with IUGR, comprising 11% of all births in developing countries annually [27].

Infants born with IUGR are five to ten times more likely to die in the first year of life than are average gestation age (AGA) infants [25]. The foetuses with IUGR often are unable to tolerate stress during pregnancy or during delivery [28]. Neonatal complications found to be associated with IUGR include hypoglycemia, hypothermia, hypocalcaemia, polycythemia, necrotizing enterocolitis, meconium aspiration, and persistent foetal circulation [29]. Further, children with IUGR are at high risk for intellectual deficit and permanent debilitating short stature [30].

In this study, as already mentioned, Icon® caused reduction in body weights, cranial length, cranial diameter, tail length and cranial – sacral length of pups. In humans, problems in postnatal development are reported in babies with small head and body length [28]. Thus, this is a matter that should be investigated in the future. Interestingly, some pyrethroids [15] and [2] and organochlorines [21] are known to cause mortality, as well as LBW and growth retardation in pups.

Numerous factors have been implicated as playing a role in IUGR and pregnancy wastage [31]. Nutritional factors play a key role in IUGR [32]. In this study, Icon® markedly reduced

the food consumption without altering the water consumption and caused significant maternal weight loss. Lambda cyhalothrin and other related pyrethroids are known to reduce food consumption and thereby caused maternal weight loss in rats [33, 34]. Stress is another factor which impairs food consumption [35]. Icon® was stressogenic: as evident by exophthalmia, pilo-erection and increment in adrenal weights [8]. If maternal nutrition conditions are very poor then the foetus adapts to nutritionally deprived environment in utero by reducing its growth [36]. Therefore, it is possible that retarded growth evident in this study could have arisen, at least partly, through the reduced food consumption of rats. Growth retardation of pups observed due to the suppressed food consumptions of mothers was also previously reported with pyrethroids [2] and [15].

Icon® induced uterine contractions *in vitro* [8]. Theoretically, this should also facilitate preterm delivery or induced abortions although it did not happen in this study. This may be because the Icon® induced contractions were not powerful enough and / or the characteristics of contractions were different to those required to initiate parturition. Icon® was antiprogestrogenic [8]. However, it is possible that the progesterone deprivation and induced uterine contractions could influence overall vascular regulation (causing reduction in blood flow to the uterus and to the foeto – placental unit) during pregnancy [37, 38]. Such impairment in uterine blood supply can result in IUGR in pups. Clinically, impaired placental blood flow is associated with poor foetal development, IUGR and LBW [39]. Further, Oxygen delivery to the gravid uterus is directly proportional to uterine blood flow [39]. Therefore, reduction in uteroplacental blood flow was also thought to disrupt oxygen and nutrient supply, thus resulting in causing abnormalities in growth of foetuses. Chemical agents can cause developmental toxicity by acting on the father, mother, on the foeto-placental unit or on the foetus directly [40]. In this study maternal toxicity was evident with Icon®: 2 rats died in the high dose and the remaining in the high dose and some animals in the mid dose exhibited marked overt clinical signs of toxicity such as ataxia, salivation, diarrhoea, hyperthermia, reduced body weights and depressed food consumption throughout the treatment period. Further, Icon® prolonged the righting reflex time suggesting disturbance in nervous co –ordination. Furthermore, Icon® had been shown to kill both human spermatozoa and *Artemia* nauplii *in vitro* [8] possibly indicating embryo/foetotoxicity [41]. Since Icon® was toxic and there was a correlation between maternal toxicity and developmental toxicity [32] the foetal deaths could have been due to a toxic action on the developing offspring. Some chemicals can cross the placental barrier and act directly on the embryo/ foetus, and cause developmental toxicity [32]. Interestingly, some insecticides are found in the follicular fluids and in ovarian mucus of women [42]. Further, some pyrethroids [2, 15] and organochlorines [21] act as maternal toxic compound and are reported to cause LBW and growth retardations in pups. However, the decreased viability of pups can account for the increase in gestation index, live birth index, pups' survival ratio, and viability index as evident in this study.

Icon® caused prolongation in pregnancy length. The causes of prolonged pregnancy are still

largely unknown [43]. Prolongation of gestation length may be due to anatomic functional disturbances, which prevent the activation of the foetal hypothalamic-hypophyseal-adrenal axis and the release of the birth initiating stimuli originating in the foetus [43]. Unfortunately, in this study investigations regarding anatomical functions of parturition were not carried out. Interestingly, similar prolongations in pregnancy length are reported in rats with paraquat [44], propanil [45], carbofuran [1] and heptachlor [46] exposures. The mechanisms triggering parturition are poorly understood but appear to involve a complex interplay between maternal and foetal factors [47] and require distinct as well as interdependent physiologic activities [48]. Oxytocin (OT) is a potent and specific stimulus to mayometrial contractility and is thought to be important in the mechanism of labor initiation [49]. OT and its receptor (OTR) are synthesized in the endometrium and myometrium of the pregnant rat during late gestation (this synthesis increase rapidly between day 14 and day 18 of pregnancy and remains at a high level during the last 3 – 4 days of gestation) [50]. In the rat, synthesis of OT and OTR is primarily regulated by oestrogen and progesterone [49]. In most animal species, including the rat, parturition is preceded by a significant increase in oestrogen and decrease in progesterone concentrations, resulting in a marked increase in oestrogen.

Progesterone ratio [51]. Critical oestrogen: progesterone ratio is necessary to initiate parturition [52]. These changes in oestrgen: progesterone ratio favor synthesis of OT and OTR within intrauterine tissues [49]. Therefore, antiprogestrogenic activity of Icon® [8] should advanced parturition rather than the delay, as seen in this study. This may be due to some direct action of Icon® on OT release or OTR activity. Alternatively, Icon® may impair relaxin release or its action and delay the onset of parturition: relaxin plays an important role in initiating parturition [53]. Usually, prolongation of gestation period complicates childbirth and may evoke debilitating life long injuries to foetus or even can cause death of a foetus [54].

Finally, if these data are applicable to women, exposure to Icon® during late pregnancy can have serious implications in countries like Sri Lanka, India and Nepal where more than 2/3 rd of all infants born are already small in size for their gestation age [55]. Hence if impacts of Icon® on birth weights are applicable to women, further increase in under weight births may be possible.

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Table 1: Number of rats (n=12/group) displaying overt clinical signs of toxicity after oral administration of Icon® or vehicle from days 15 –21 of pregnancy.

Parameter monitored	Number of rats displaying signs			
	Control Distilled Water	Treated Icon® mg/kg/body wt. /day		
		63	83	125
Mortality	0	0	0	2
Ataxia	0	1	4	10
Tremors	0	0	0	0
Convulsions	0	0	0	0
Pilo-erection	0	0	0	3
Exophthalmia	0	0	0	3
Salivation	0	1 (around mouth)	9 (around neck)	10 (Whole body)
Lacrymation	0	0	0	0
Coughing	0	0	0	0
Change in fur colour	0	0	0	0
Change in urine colour	0	0	3 (dark yellow)	6 (dark yellow)
Vaginal bleeding	0	0	0	0
Diarrhoea	0	0	4	7

Table 2: Food consumption of rats orally administrated with Icon® or vehicle from days 15 -21 of pregnancy. Data represented as means ± SEM, n =12.

Day	Amount consumed (g)			
	Control Distilled Water	Treated Icon® mg/kg/body wt. /day		
		63	83	125
Day 15	22.7 ± 0.2	15.1 ± 0.9*	7.9 ± 3.1**	4.5 ± 0.9**
Day17	15.8 ± 0.9	15.2 ± 2.3	7.8 ± 0.6**	8.2 ± 1.8**
Day 19	17.3 ± 1.9	13.1 ± 0.9	11.0 ± 0.6	9.6 ± 2.2*
Day21	17.9 ± 2.6	11.7 ± 2.3	9.2 ± 0.5	6.4 ± 1.6*

As compared with control, * $p < 0.05$, ** $p < 0.01$, Mann-Whitney U-test.

Table 3: Body weight gain of rats orally administrated with Icon® or vehicle from days 15 -21 of pregnancy. Data represented as means ± SEM, Ranges are given in parentheses, n=12

Duration	Body weight gain (g)			
	Control Distilled Water	Treated Icon® mg/kg/body wt. /day		
		63	83	125
Day 15 - 21	29.5 ± 4.1 (12.5 -61.5)	19.6 ± 1.5 (15.3 - 24.6)	16.0 ± 1.5* (11.6 - 21.5)	12.1 ± 5.4* (1.7 - 36.4)

As compared with control, * $p < 0.05$, Mann-Whitney U-test.

Table 4: Reproductive parameters of rats orally administrated with Icon® or vehicle from days 15 – 21of pregnancy. Data represented as means ± SEM, Ranges are given in parentheses.

Parameter monitored	Control Distilled Water	Treated Icon® mg/kg/body wt. /day		
	(n=12)	63 (n=12)	83 (n=12)	125 (n=10)
Gestation length (days)	22.6 ± 0.2 (22- 23)	22.6 ± 0.2 (22 - 23)	22.8 ± 0.4 (22 - 24)	24.6 ± 0.9* (23 - 28)
Number of pups born	8.4 ± 0.5 (6 - 13)	7.5 ± 0.6 (12 - 14)	7.7 ± 0.4 (5 - 10)	7.3 ± 0.7 (0-10)
Number of live pups	8.2 ± 0.5 (5 - 13)	7.5 ± 0.6 (4 - 12)	6.8 ± 0.5 (2 - 9)	4.5 ± 0.8** (0 - 9)
No. of pups survived on postnatal day 1	8.1 ± 0.5 (4 - 13)	7.2 ± 0.7 (4 - 12)	5.8 ± 0.7* (0 - 9)	3.1 ± 0.9** (0 - 9)
% Gestation index	879	800	764	453**
% Live birth index	97.9 ± 1.4 (83.3 - 100)	100 ± 00 (100 - 100)	88.4 ± 5.9 (20 - 100)	61.9 ± 10.2** (0 - 100)
% Pups survival ratio	94.4 ± 4.8 (33.3 - 100)	95.9 ± 3.3 (57.1 - 100)	78.3 ± 9.3* (0 - 100)	40.8 ± 11.9** (0 - 100)
% Viability Index	97.3 ± 2.7 (60 - 100)	95.9 ± 3.3 (57.1 - 100)	84.1 ± 9.7 (0 - 100)	43.3 ± 11.8** (0 - 100)

As compared with control, * $p < 0.05$, ** $p < 0.01$, Mann-Whitney U-test & G-test.

Table 5: Developmental parameters of pups, delivered by rats orally administered with Icon® or vehicle from days 15 -21 of pregnancy. Data represented as means ± SEM, Ranges are given in parentheses.

Parameter monitored	Control Distilled Water	Treated Icon® mg/kg/body wt. /day		
		63	83	125
Body weights (g)	5.5 ± 0.1 (4.9 - 6.4)	5.6 ± 0.4 (4.6 - 6.6)	4.8 ± 0.2** (3.8 - 5.6)	4.2 ± 0.2** (2.6 - 5.3)
Cranial length (mm)	14.2 ± 0.2 (13 - 15.5)	13.8 ± 0.1 (13.2 - 15)	13.3 ± 0.2* (12 - 14.3)	13.2 ± 0.2* (11 - 14)
Cranial diameter (mm)	10.3 ± 0.1 (9.8 - 12)	10.1 ± 0.1 (9.5- 11.0)	9.8 ± 0.1* (8.8 - 10.4)	9.5 ± 0.2* (8 - 10)
Tail length (mm)	16.6 ± 0.3 (15.1 - 18)	15.7 ± 0.4 (14 - 18.5)	15.4 ± 0.2* (13.6 - 17)	14.9 ± 0.4** (12 - 16.8)
Cranial - sacral length (mm)	41.9 ± 0.3 (40.2 - 44)	40.7 ± 0.4 (37.6 - 42.3)	39.9 ± 0.6* (35.8 - 42.7)	38.3 ± 0.8** (31 - 42.7)
Fur appearance time (Days)	2.4 ± 0.2 (2 - 3)	2.4 ± 0.2 (2 - 3)	2.0 ± 0 (2 - 2)	2.2 ± 0.2 (2 - 3)
Eye opening time (Days)	15.0 ± 0.3 (14 - 16)	14.6 ± 0.2 (14 - 15)	15.2 ± 0.2 (15 - 16)	15.0 ± 0.3 (14 - 16)

As compared with control, * $p < 0.05$, ** $p < 0.01$, Mann-Whitney U-test.