EFFECT OF ETRETINATE (AROMATIC RETINOID) TREATMENT DURING GESTATION AND LACTATION PERIODS ON VIABILITY AND SOMATIC GROWTH IN F1 RATS

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ABSTRACT — When female SD rats were continuously treated with Etretinate throughout pre-mating, gestation, and lactation periods, the resulting F1 pups exhibited low viability and inhibition of somatic growth after birth (Hummler *et al.*, 1981). Nevertheless, these pups showed no notable change in body weight and external appearance at birth. We used the cross-fostering (between control and treated groups) method and investigate the neonatal viability and the growth hormonal changes in order to assess which treatment period of gestation or lactation was mainly involved in these effects and what changes were actually induced in the F1 pups.

The results showed that low viability and inhibition of somatic growth after birth were mainly related to treatment during the gestation period, and these effects were augmented by treatment during the lactation period. Serum GH and IGF-I levels were increased on day 21 in F1 pups groups in which inhibition of somatic growth was observed. These results indicated that treatment with Etretinate during the gestation period might induce a decrease in the number of receptors of GH and IGF-I or other changes, such as a poor-response in target tissues due to a down-regulation, with an increase of serum GH and IGF-I levels.

KEY WORDS : Etretinate, Vitamin A, GH, IGF-I, Neonatal viability, Reproduction study.

INTRODUCTION

Ro 10-9359 (Etretinate) is a retinoid compound and is used clinically as an antipsoriatic. It is well known in the experimental animals that retinoids lead to teratogenicity involving skeletal anomalies such as craniofacial and limb anomalies (Aikawa *et al.*, 1982 ; Kalter, 1989 ; Thaller *et al.*,

1991 ; Hummler *et al.*, 1981 ; Agnish *et al.*, 1993), and behavioral alterations in terms of the tests in active avoidance and T-maze (Adams, 1993). Teratogenic effects in human have also reported (Rosa, 1993 ; Lammer, 1985 ; Stern, 1984) and elucidated the problem regarding the long-term accumulation in the living maternal body (Klein *et al.*, 1993).

Etretinate showed frank malformations (cleft palate, anophthalmia, exencephaly) at 8 mg/kg and the decrease of postnatal survival of pups in

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rats, malformations consisting of digital defects, cleft palate, and variations of the thirteenth rib at 2 mg/kg in rabbits, and malformations typical of hypervitaminosis-A syndrome (cleft palate, exencephaly, eye defects) in mice (Agnish, 1993).

Continuous dosing of Etretinate to dams through pre-mating, gestation, and lactation periods (FDA type Seg I study) resulted in low viability of F1 pups and inhibition of their somatic growth after birth. Nevertheless, no notable changes in body weight and external anomalies were observed in F1 pups at birth (Hummler *et al.*, 1981). We used a cross-fostering method to investigate which dosing during gestation and lactation periods was mainly involved in these effects and what changes were induced in the F1 pups.

MATERIALS AND METHODS

1. Animals.

Twenty female SD-slc rats confirmed to be mated were obtained from a "specific pathogen free" colony at SLC Corp., Shizuoka, Japan, on gestation day 1 (The day on which vaginal sperm was observed was designated as day 0 of gestation). After about 1 week acclimatization, healthy eight or seven dams per group (body weight 200 g to 290 g) were supplied for the experiment. They were housed in individual metal cages in a room with a temperature of $22 \pm 2^\circ\text{C}$ and a relative humidity of $55 \pm 5\%$, and 12-hr light/dark cycle (light, 7:00–19:00). Standard diet CRF-1 (Charles River Japan, Ltd.) and tap water were given *ad libitum*. For littering dams, a metal tray with autoclaved chips was provided for bedding.

2. Test/Control Articles and Treatment.

The chemical name of Etretinate (Ro 10-9359) is Ethyl (all-E)-9-(4-methoxy-2,3,6-trimethylphenyl)-3,7-dimethyl-2,4,6,8-nonatetraenoate (Fig. 1). It was obtained from F.Hoffmann-La Roche Inc. (Basel, Switzerland),

Fig. 1. Chemical structure of Etretinate (Ro 10-9359).

and the purity was over 99.9%. The test or control articles (vehicle: rape seed oil) were administered to the dams by forced oral intubation from gestation day 7 to lactation day 20 at doses of 0 (vehicle) and 6 mg/kg/day with an administration volume of 0.2 ml/100 g body weight. Administration volumes were calculated based on the body weight on day 7 of gestation during gestation period, and on the latest body weight during lactation period.

3. Experimental Design.

Dams were treated with the test article or vehicle from gestation day 7 to lactation day 20. After parturition (lactation day 0), one half of the pups were cross-fostered from control dams to treated dams and vice versa up to lactation day 21. There were four experimental groups, as follows: group 1 (0-0 group), the pups delivered from control dams and nursed by control dams, and not cross-fostered between control dams; group 2 (E-0 group), the pups delivered from Etretinate-treated dams and nursed by control dams; group 3 (0-E group), the pups delivered from control dams and nursed by Etretinate-treated dams; and group 4 (E-E group), the pups delivered from Etretinate-treated dams and nursed by Etretinate-treated dams, and not cross-fostered between Etretinate-treated dams (Fig. 2).

Pups were identified up to lactation day 4 only by a group distinction marking (black marking ink) on the heads of pups delivered from the control dams. After standardization (maximally eight pups: two pups/sex/group, if possible) on day 4, pups were marked on the back with numbers in black marking ink or picric acid. The group distinction marking of pups delivered from the control dams was continued up to the end of lactation. The markings were rewritten once a day in black marking ink.

Observation of dams: All dams were observed at least once daily for signs of ill-health or overt signs of toxicity. Body weight was determined on gestation day 1, 7, 14 and 21, and on lactation day 0, 4, 7, 14 and 21. Food consumption was determined on gestation day 7, 14 and 21, and on lactation day 0, 4, 7, 14 and 21. After weaning, dams were sacrificed and examined macroscopically for pathological changes. Mammary glands were removed and examined

Experimental design

Fig. 2. Diagram of experiment designed to evaluate effects of Etretinate on F1 pups in rats.

histopathologically.

Observation of F1 pups : All selected F1 pups were observed at least once daily for signs of ill-health or overt signs of toxicity. Body weight was determined on day 0, 4, 7, 14 and 21 during the lactation period, and weekly after weaning up to day 49. Food consumption was determined weekly after weaning up to day 49.

Examination on lactation day 21 : One half of the F1 pups in each group were dissected and examined on lactation day 21. After 3 hr starvation, pups were anesthetized with ether, and their blood samples were collected through cardiac puncture. Serum growth hormone and IGF-I levels were determined. The pituitary and liver were removed and fixed in 15% buffered formalin (pH 6.8), embedded in paraffin, sectioned, stained with hematoxylin-eosin, and examined under a light microscope.

Serum growth hormone levels were determined by RIA (Rat growth hormone [125] assay system : Amersham Co. Ltd.). Serum IGF-I

levels were determined as described by Maiter (Maiter *et al.*, 1988). In brief, we extracted the serum samples using ODC silicic acid columns (C18 Sep-Pak cartridge, Waters Associates, Milford, MA), eluting binding proteins with 7% acetic acid and IGF-I with 100% methanol. Samples without binding protein were measured by RIA (IGF-1 reagent pack for RIA : Amersham Co. Ltd.). Human IGF-1 (Boehringer Mannheim Biochemica, Mannheim, Germany) was used as the standard.

Examination on lactation day 49 : The remaining half of the F1 pups in each group were dissected and examined for hormonal levels and histopathological changes on lactation day 49, the same as on lactation day 21.

4. Statistical Evaluation.

The data on body weight, growth hormone level, IGF-I level, and organ weight were analyzed statistically using Student's t-test. The data on viability were analyzed using categorical tests (Chi-Square and Fischer exact test).

RESULTS

1. Effect on dams.

No drug related changes were observed in clinical signs. No differences were observed in body weight development and food consumption between the control and drug-treated dams (Fig. 3). No drug related changes were observed in pregnant period and parturient conditions (Table 1). No drug related changes were observed in

Fig. 3. Body weight and food consumption of dams treated with Etretinate during gestation and lactation periods. Dams (N=8 in control group (vehicle: rape seed oil), N=7 in treated group (Etretinate 6 mg/kg)) were dosing from day 7 of gestation to day 20 of lactation.

gross necropsy findings, and in mammary gland histopathology.

2. Effect on F1 pups.

Viability: The total number of alive pups delivered from Etretinate-treated dams (Lactation day 0) was slightly lower than that from control dams. The total number of dead pups delivered from Etretinate-treated dams (Lactation day 0) was only five in both sexes, but all dead pups showed external anomaly (protruding tongue and cleft palate). A few alive pups delivered from Etretinate-treated dams also showed external anomaly (protruding tongue and cleft palate) (Table 1).

Pups in both E-0 and E-E groups showed low viability (Fig. 4), although viability in the E-E group was lower than that in the E-0 group. A minimal slight decrease in viability was observed in the 0-E group; however, it was within the normal range. Viability in female

Fig. 4. Viability of F1 pups which were cross-fostered between control and treated dams. 0-0 group, pups delivered from control dams (vehicle: rape seed oil) and nursed by control dam; E-0 group, pups delivered from Etretinate-treated dams (6 mg/kg) and nursed by control dam; 0-E group, pups delivered from control dams and nursed by Etretinate-treated dams; E-E group, pups delivered from Etretinate-treated dams and nursed by Etretinate-treated dams. Data for male and female pups were combined.

*Significantly different from the control (0-0 group), $P < 0.05$.

**Significantly different from the control (0-0 group), $P < 0.01$.

Table 1. Effect of Etretinate on pregnant period, parturition and pups.

Group	Number of dosing dams	Number of parturient dams	Pregnant period		Parturient conditions		Number of alive pups at lactation day 0		Number of dead pups at lactation day 0	
			Day 21	Day 22	Normal	Abnormal	Male	Female	Male	Female
Control	8	8	4/8	4/8	7/8	0/8	45	51	0	0
Etretinate 6 mg/kg	7	7	2/7	5/7	7/7	0/7	30 (4) ^{b)}	40(2) ^{a)} (5) ^{b)}	2(2) ^{a)}	3(3) ^{a)}

Dams were dosed vehicle (rape seed oil) or Etretinate 6 mg/kg from day 7 of gestation to day 20 of lactation.

a) : Figure in parenthesis shows number of pups with external anomaly (protruding tongue).

b) : Figure in parenthesis shows number of pups with external anomaly (cleft palate).

Fig. 5. Body weight of F1 pups (male and female) after birth. Pups in 0-0 group (Male ; N=32-20, #7, Female ; N=35-27, #10) were delivered from control dams (vehicle : rape seed oil) and nursed by control dams. Pups in E-0 group (Male ; N=11, #5, Female : N=11-4, #1-0) were delivered from Etretinate-treated dams (6 mg/kg) and nursed by control dams. Pups in 0-E group (Male ; N=13-10, #6, Female ; N=16-10, #5) were delivered from control dams and nursed by Etretinate-treated dams. Pups in E-E group (Male ; N=19-11, #4-2, Female ; N=29-9, #3-2) were delivered from Etretinate-treated dams and nursed by Etretinate-treated dams.

#Number of pups after weaning.

*Significantly different from the control (0-0 group), $P < 0.05$.

**Significantly different from the control (0-0 group), $P < 0.01$.

Fig. 6. Food consumption of F1 pups (male and female) after birth. Pups in 0-0 group (Male ; N=7, Female ; N=10) were delivered from control dams (vehicle : rape seed oil) and nursed by control dams. Pups in E-0 group (Male ; N=5, Female ; N=0) were delivered from Etretinate-treated dams (6 mg/kg) and nursed by control dams. Pups in 0-E group (Male ; N=6, Female ; N=5) were delivered from control dams and nursed by Etretinate-treated dams. Pups in E-E group (Male ; N=2, Female ; N=2) were delivered from Etretinate-treated dams and nursed by Etretinate-treated dams. Data could not be evaluated statistically because one data point consisted of food consumed by two pups housed in the same cage.

Fig. 7. Serum growth hormone and IGF-I levels of F1 pups on day 21 after birth. Serum samples were collected and hormone levels were measured by RIA in duplicate. Values are the means $\pm$ S.E. Pups in 0-0 group (Male ; N=10, Female ; N=10) were delivered from control dams (vehicle : rape seed oil) and nursed by control dams. Pups in E-0 group (Male ; N=6, Female ; N=3) were delivered from Etretinate-treated dams (6 mg/kg) and nursed by control dams. Pups in 0-E group (Male ; N=5, Female ; N=5) were delivered from control dams and nursed by Etretinate-treated dams. Pups in E-E group (Male ; N=8, Female ; N=5) were delivered from Etretinate-treated dams and nursed by Etretinate-treated dams.

*Significantly different from the control (0-0 group), $P < 0.05$.

**Significantly different from the control (0-0 group), $P < 0.01$.

Fig. 8. Serum growth hormone and IGF-I levels of F1 pups on day 49 after birth. Serum samples were collected and hormone levels were measured by RIA in duplicate. Values are the means $\pm$ S.E. Pups in 0-0 group (Male ; N=7, Female ; N=9) were delivered from control dams (vehicle : rape seed oil) and nursed by control dams. Pups in E-0 group (Male ; N=5, Female ; N=0) were delivered from Etretinate-treated dams (6 mg/kg) and nursed by control dams. Pups in 0-E group (Male ; N=6, Female ; N=5) were delivered from control dams and nursed by Etretinate-treated dams. Pups in E-E group (Male ; N=2, Female ; N=2) were delivered from Etretinate-treated dams and nursed by Etretinate-treated dams.

*Significantly different from the control (0-0 group), $P < 0.05$.

**Significantly different from the control (0-0 group), $P < 0.01$.

Table 2. Effect of Etretinate on relative organ weight of F1 rat pups.

<i>Examination on day 21</i>				
Group	0-0	E-0	0-E	E-E
Male (n)	(10)	(6)	(4)	(8)
Pituitary (mg/100 gBW)	5.14±0.88	5.20±0.82	5.53±1.62	7.41±1.79**
Liver (g/100 gBW)	3.706±0.187	3.542±0.082	3.803±0.196	3.681±0.385
Female (n)	(9)	(3)	(5)	(5)
Pituitary (mg/100 gBW)	6.58±0.98	9.13±1.68**	5.84±0.64	8.10±1.67
Liver (g/100 gBW)	3.740±0.117	3.610±0.121	3.812±0.163	3.762±0.217
<i>Examination on day 49</i>				
Group	0-0	E-0	0-E	E-E
Male (n)	(7)	(5)	(6)	(2)
Pituitary (mg/100 gBW)	4.23±0.52	4.40±0.30	3.93±0.49	4.55±0.35
Liver (g/100 gBW)	4.936±0.205	4.500±0.127**	4.932±0.167	4.060±0.368
Female (n)	(10)	(0)	(5)	(2)
Pituitary (mg/100 gBW)	5.59±0.68	—	5.64±0.57	5.65±1.06
Liver (g/100 gBW)	4.743±0.321	—	4.380±0.087*	4.210±0.113

Pups in 0-0 group were delivered from control dams (vehicle : rape seed oil) and nursed by control dams.

Pups in E-0 group were delivered from Etretinate-treated dams (6 mg/kg) and nursed by control dams.

Pups in 0-E group were delivered from control dams and nursed by Etretinate-treated dams.

Pups in E-E group were delivered from Etretinate-treated dams and nursed by Etretinate-treated dams.

* Significantly different from the control (0-0 group), $P<0.05$.

** Significantly different from the control (0-0 group), $P<0.01$.

BW : Body Weight

pups was lower than that in males in both E-0 and E-E groups. Nearly dead pups in the E-0 group and many dead pups in the E-E group were observed until day 4 after birth. There were also some dead pups observed in the E-E group immediately after weaning.

Body weight development : The post-natal development of body weight was inhibited in both sexes of the E-0 and E-E groups, in spite of their normal body weight at birth. Inhibition in the E-E group was more severe than that in the E-0 group. F1 pups in the 0-E group had body weights similar to those in the 0-0 group (Fig. 5).

Food consumption : Food consumption was inhibited in both sexes of the E-0 and E-E groups similarly as was body weight development ; the inhibition in the E-E group was more severe than that in the E-0 group. Food consumption was the same for F1 pups in both the 0-E and 0-0 groups (Fig. 6).

3. Examination on day 21.

Trends toward increasing serum growth hor-

mone and IGF-I levels were observed in both sexes of the E-0 and E-E groups. No notable changes were observed in the 0-E group (Fig. 7). These data for male and female pups were exhibited together, because the results were nearly the same. No drug related changes were observed in serum T3 and T4 levels in any group (data not shown).

The females of the E-0 group and both sexes of the E-E group showed a trend toward increased pituitary organ weight (Table 2). No such change was noted in the 0-E group.

Reduction of cytoplasmic eosinophilia of acidophils in pituitary was observed in the E-0 and E-E groups, whereas no such drug related change was observed in the 0-E group. No drug related changes were observed in the liver for any group.

4. Examination on day 49.

No significant change was observed in the serum growth hormone levels among all groups, because there was a large variation among individuals (Fig. 8). No change was observed in the

IGF-I levels in any group. No definitive change was observed in serum T3 and T4 levels (data not shown). The pituitary and liver showed no definitive changes in organ weight and in histopathological examination, although a few statistically significant changes were observed (Table 2).

DISCUSSION

The E-0 and E-E groups, which were treated with Etretinate during the gestation period, showed decreased viability after birth the same as that observed in the Seg I study (FDA type) of Etretinate. Almost all deaths in these pups occurred from day 0 to 4 after birth, although their body weight at birth was in the normal range. We can consider three possible reasons for these deaths. The first involves external anomalies such as protruding tongue and cleft palate. These anomalies were observed in only a few pups, but they may be concerned with decrease of viability. The second involves Central Nervous System (CNS) anomalies. A few of the pups delivered from Etretinate treated dams showed an external anomaly (protruding tongue/likely caused by an incomplete jaw and cleft palate). Craniofacial anomalies have been frequently observed in retinoid treated animals (Aikawa *et al.*, 1982; Kalter, 1989). Even though no definitive external anomaly was observed at birth, asymmetry of the skull (curving of the nasal, frontal, and occipital bone) was observed on day 49 after birth. Such skeletal anomalies in the skull may be present from birth; the slight changes in the skull may lead to anomalies in the brain and pituitary tissues and in the vascular system. It is also possible that retinoids exert a direct effect on the brain or pituitary tissues. A third possible reason for deaths involves Cardiovascular System (CVS) anomalies in the F1 pups. It has been reported that Isotretinoin induced defects of visceral arch development in 9.5-day rat embryos (Ritchie and Webster, 1991).

The decrease of viability in pups that were treated with retinoid during gestation and lactation periods was mainly caused by the treatment during gestation, but the effect was amplified by the treatment during the lactation period. It is supposed that exposure to retinoid during some

Fig. 9. Expected relationship between GH, IGF-I and their receptors in pups treated with Etretinate during gestation period.

period after birth may be concerned with pups death, because development and growth of some tissues and organs continue after birth (Rodier, 1980; Fujii, 1991).

Both E-0 and E-E groups showed an inhibition in body weight development in spite of their normal body weight at birth. Fostering by the treated dams was sufficient, because their food consumption was in the normal range, the histopathology of their mammary glands showed no change, and the pups, which were delivered from control dams, that they fostered showed normal body weight development. It is reported that malnourished neonates show a decrease in serum IGF-I levels during the lactation period (Donovan *et al.*, 1991). In our study, pups in the E-0 and E-E groups showed increased serum IGF-I level on day 21. Thus, the inhibition of body weight development in pups treated during the gestation period is likely caused by some anomalies in the pups rather than by that in the dams.

It is known that somatic growth is pituitary-

independent during the gestation period and pituitary-dependent after birth (Cooke and Nicoll, 1983). Our study showed that the body weight of F1 pups was in the normal range at birth and that body weight development was inhibited in proportion to age. We can support the idea that dosing with Etretinate during the gestation period induces some anomalies in pituitary-dependent growth.

We summarized the results in this study and showed the expected relationship between GH, IGF-I, and their receptors in the pups treated with Etretinate during the gestation period (Fig. 9). Pups in the E-0 and E-E groups showed trends toward increases in serum growth hormone and IGF-I levels on day 21. An increase in the relative weight of the pituitary gland was also observed in females of the E-0 group and in both sexes of the E-E group. Histopathological examination showed reduction of cytoplasmic eosinophilia of acidophils in the pituitary in both E-0 and E-E groups.

Growth hormone secreted from the pituitary mainly acts in the liver to produce IGF-I. IGF-I acts on the peripheral tissues and stimulates somatic growth (Behringer *et al.*, 1990; Glasscock *et al.*, 1991; Humbel, 1990). Although decreases in serum growth hormone and IGF-I levels were presumed corresponding to the inhibition of body weight development, those levels were actually increased on day 21. It has also been reported that retinoic acid increased growth hormone gene expression in pituitary GH 1 cells (Bedo *et al.*, 1989).

It is supposed that the inhibition of somatic growth despite high levels of serum growth hormone and IGF-I is due to changes affecting the receptor response in target tissues (i.e., bone, pituitary and hypothalamus). If the receptors in bone are changed, the effects of growth hormone and IGF-I on bone may not be exhibited as somatic growth. If the receptors in the hypothalamus are changed, negative feedback may not occur and the excessive secretion of growth hormone may be continued.

Another possibility might involve qualitative or quantitative changes in the IGF-I binding protein. Changes in the IGF-I binding protein in blood may make it difficult for IGF-I to access its target tissues. For example, it is known that

retinoid inhibits the growth of cells by increasing the IGF-I binding protein level in a breast cancer cell line (Adamo *et al.*, 1992; Fontana *et al.*, 1991).

It has been shown that IGF-I alone does not play a physiologically important role in the feedback regulation of growth hormone secretion and that the synergistic interaction between IGF-I and IGF-II in the brain is important (Harel and Tannenbaum, 1992). Thus, although we did not determine the serum IGF-II level, it may be supposed to be decreased.

An increase in the relative pituitary organ weight and a reduction of cytoplasmic eosinophilia in the pituitary acidophils were observed histopathologically in both E-0 and E-E groups on day 21. Because growth hormone is secreted from acidophils in the pituitary, this reduction of cytoplasmic eosinophilia in the pituitary acidophils may be caused by the decrease of growth hormone in acidophils due to its excessive release into the circulation.

On day 49, trend toward decreasing serum growth hormone level was observed in male of the E-0 group. In our experiments, however, the individual difference in serum growth hormone levels among pups was very wide on day 49, and we could not determine that the changes were drug related. It is reported that growth hormone secretory patterns in both sexes were extremely variable and highly pulsatile (Clark *et al.*, 1987). In this study, we might suppose that serum growth hormone levels were recovered to the normal range, because IGF-I levels were recovered and no drug related changes were observed in the pituitary histopathology.

In conclusion, a decrease in viability and inhibition of body weight development was observed in F1 pups dosed with Etretinate during the gestation and lactation periods, in spite of the fact that their body weight was normal at birth. The cross-fostering study showed that these toxicological effects mainly resulted from some anomalies in the pups rather than those in the dams, and that these effects were amplified by dosing the dams during the lactation period. Growth retarded pups showed, conversely, an increase in the serum growth hormone and IGF-I levels on day 21. They also showed an increase in the relative pituitary weight and a reduction of cyto-

plasmic eosinophilia in the pituitary acidophils on day 21. Dosing of Etretinate during the gestation period might lead to some anomalies (changes in the target tissues, etc.) of those pups with increased serum growth hormone and IGF-I levels and changes in the pituitary gland.

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