EFFECTS OF A DAILY FEEDING-RESTRICTION ON THE REPRODUCTIVE AND DEVELOPMENT TOXICITY PARAMETERS IN FEMALE RATS

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ABSTRACT — On the assumption of that the oral administration of an acid-unstable test compound into the empty stomach could enhance the systemic exposure to the test compound, the non-pregnant and pregnant rats had free access to the diet only for five hours per day. Female rats under the restricted feeding for a period of 21 days took diet at two thirds of the daily food-intake by the control animals, and lost their weight more than 10%. The vaginal smear test in these animals revealed a prolonged estrous cycle and diestrous period over four days. On the other hand, the restricted feeding from Day 0 to Day 17 of gestation suppressed the weight gain of pregnant animals but did not cause any significant influence upon the litter data, incidence of external anomalies or fetal skeletal development. The restricted feeding from Day 0 of gestation to Day 7 of lactation seriously disturbed the nursing behavior and the growth of offspring. These results suggested that the dosing method under the above-mentioned restricted feeding might be applicable to the teratology study but could not be applied to the fertility study nor to the peri- and post-natal study.

KEY WORDS : Feeding-restriction, Reproductive toxicity study, Estrous cycle, Nursing behavior, Female rats.

INTRODUCTION

It is generally recommended in the guidelines for the toxicity studies that the route of administration of test drugs should be the same as the intended clinical route. However, it is sometimes very difficult to conduct toxicity studies in accordance with the guidelines. This is a case where the intended clinical route of administration is oral, but a test compound is unstable in an acid solution and easily decomposed by the gas-

tric juice when it is orally administered. The systemic exposure to a test compound, a hydrogen-potassium ATPase inhibitor, could be satisfactorily achieved by preparing an enteric coated capsule for toxicity studies in dogs but not for toxicity studies in rats (unpublished). The bioavailability of the test compound orally given in the general feeding condition in rats was nearly zero percent. After a lot of try-and-error, we established a dosing method for rat studies by application of a feeding-restriction. Our study results indicated that a feeding-restriction in which rats were deprived of food for 19 hours and the test compound was administered into the

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empty stomach could enhance the relative bioavailability of the test compound to a level of 20% of the systemic exposure achieved by intraduodenal administration. Some repeated dose toxicity studies of this test compound in rats were successfully carried out by the oral administration under the feeding-restriction, where the animals had a free access to the diet only for five hours per day.

The present study aimed to examine whether or not the above-mentioned dosing under the daily feeding-restriction is applicable for the reproductive and developmental toxicity studies. For the purpose, effects of the daily feeding for only five hours on the female fertility, embryo-fetus development, delivery and nursing performance were examined.

MATERIALS AND METHODS

Animals and general conditions of experiments: Sprague-Dawley rats purchased from Japan SLC, Inc., Inasa Branch, were used for the present studies. They were housed throughout the study period in the animal room under the following conditions: temperature, 22–24°C; humidity, 50–60%; lighting 12-h light (7:00–19:00)/dark cycle. A commercial pellet diet for rodent (MF, diet for breeding; Oriental Yeast Co., Ltd.) was given to the animals. To prepare the pregnant animals, female rats were caged with male rats overnight for mating. The day when copulatory plugs were found in the next morning was defined as Day 0 of gestation, and the day of delivery as Day 0 of lactation.

Feeding-restriction: During the feeding-restriction period, the animals had free access to the diet only for five hours (9:00–14:00) per day, while they had free access to drinking water throughout the study period. Because reproductive and developmental toxicity studies consist of three-segment studies in which test drugs are administered a) prior to and in the early stages of pregnancy, b) during the period of organogenesis, and c) during the perinatal and lactation periods, the feeding-restriction periods for the studies are described in detail in respective parts of STUDY DESIGNS AND RESULTS.

Statistical analysis: Chi-square test was applied to mortality rate, pregnancy ratio, implanta-

tion ratio, live birth ratio, incidence of types of resorbed embryos and dead fetuses, survival ratio and incidence of 14th rib. Wilcoxon's rank sum test was applied to the incidences of resorbed embryos and dead fetuses, fetuses with non-ossification, and with skeletal variations. The other quantitative data were tested by Bartlett's analysis, and if the variances were homogeneous, Student's t-test was applied, and if the variances were heterogeneous, Aspin-Welch's t-test was applied. The levels of significance were designated as 5% and 1% for all cases.

STUDY DESIGNS AND RESULTS

1. Adaptation to the feeding-restriction in non-pregnant rats.

Sixteen non-pregnant rats at 14 weeks of age were used for the experiment. They were divided into two groups, i.e. a control and a feeding-restricted group. Food consumption and body weights were measured once a day.

Fig. 1 shows changes in food consumption and body weight of the animals which had access to the diet for only five hours per day during a period of consecutive 10 days. The food consumption decreased to a level of one third of the controls on the first day of the feeding-restriction but gradually increased thereafter to a level of two thirds of the controls. The animals lost their body weight by about 15% during the first five days of the feeding-restriction, but kept their weight unchanged thereafter. The weight loss during the 10 days of the feeding-restriction was 33.9 g on the average, while the control animals had a weight gain of 0.6 g during the period. The animals under the feeding-restriction adapted to the study condition by greedy feeding during the restricted feeding hours. The autopsy at the end of study revealed a markedly enlarged stomach in the feeding-restricted animals.

2. Effects of the feeding-restriction on the estrous cycle.

Sixteen non-pregnant rats at 9–10 weeks of age were allocated to two groups, i.e. a group of control animals and a group of feeding-restricted animals. The feeding-restriction was conducted for consecutive 21 days. Food consumption and body weights were measured twice a week, and all animals were smeared daily to monitor the

Fig. 1. Effects of the restricted feeding on food consumption (A) and body weight (B) in non-pregnant female rats. The animals in the feeding-restricted group (open square) had free access to the diet only for five hours (9:00–14:00) per day for a period of consecutive 10 days. The control animals (closed circle) had free access to the diet throughout the experiment period. Each point represents the mean of 8 animals with SEM.

estrous cycle.

Mean food consumption during the study period of 21 days was 15 g/day for the control animals and 10 g/day for the feeding-restricted animals, and the control animals had a weight gain of 33.5 g but the feeding-restricted animals had a weight loss of 13.8 g during the 21 days. The feeding-restriction prolonged the estrous cycle in all eight animals. Five of them had a diestrous smear over 4 consecutive days (2 days in most control animals), and two of the five animals lost the estrous cycle completely from the fourth day of feeding-restriction.

3. Effects of the feeding-restriction on the embryo-fetus development.

Eighteen copulated female rats at 16 weeks of age were allocated to a control and a restricted group. The feeding-restriction was conducted for 18 days from Day 0 to 17 of gestation. Body weight and food consumption were measured daily from Day 0 through Day 20, excluding Day 18 of gestation. On Day 20 of gestation, all dams were killed by cervical dislocation to examine numbers of corpora lutea, live fetuses, resorbed embryos, and dead fetuses, and distribution of implantations in the uterus. Each fetus was weighed and grossly examined for the anomaly. All fetuses were initially fixed in 85% ethylalcohol, and examined for skeletal alterations after staining of both bone and cartilage with alizarin red S and alcian blue.

Fig. 2 shows changes in food consumption and body weight. The food consumption in the control dams on Day 0 of gestation was 15 g/day on the average, which gradually increased to 24 g/day on Day 20 of gestation. The control dams had body weight of 280 g at the start and 390 g at the end during the gestation period. On the other hand, the dams in the feeding-restricted group consumed the daily diet less than a half of the controls, and their body weights remained decreased throughout the gestation period.

Table 1 summarizes number of fetuses and fetal weight examined on Day 20 of gestation. There were no statistically significant differences in the numbers of corpora lutea, implantations, resorptions and live fetuses and in fetal weight between the control and the feeding restricted groups. Significant differences were found neither in the incidence of retarded ossification of sternebrae, odontoid process, 7th cervical centrum, metacarpals, metacarsals and skull bones, nor in the presence of cervical rib, 14th rib, asymmetry of sternebrae or dumbbell shape of ossification centers.

4. Effects of the feeding-restriction on the delivery and nursing.

Eighteen copulated female rats at 12 weeks of age were divided into three groups. A group of rats serving as the control had free access to the diets throughout the study period, while rats in two other groups were restricted their feeding to the five hours from Day 0 to 17 of gestation

Fig. 2. Effects of the restricted feeding on food consumption (A) and body weight (B) in pregnant female rats. The animals in the feeding-restricted group (open square) had free access to the diet only for five hours (9:00–14:00) per day during Days 0 to 17 of gestation. The control animals (closed circle) had free access to the diet throughout the experiment period. Each point represents the mean of 8 animals with SEM.

(RF-A group) or from Day 0 of gestation to Day 7 of lactation (FR-B group). Body weight and food consumption were determined on Days 17, 18, 19 and 20 of gestation and on Days 1, 4 and 7 of lactation. After delivery, the nursing behavior (eating of placenta, lactation, retrieving and cannibalism of neonates) was examined on Days 1, 4 and 7 of lactation. The neonates were sexed and observed grossly. Numbers of live and stillborn offspring and body weights were recorded on Days 0, 1, 4 and 7 of lactation. All surviving neonates and dams were killed by carbon dioxide inhalation on Day 7 of lactation.

Fig. 3 shows changes in food consumption and body weight. The animals in RF-A group which had the feeding-restriction until Day 17 of gestation consumed 164% food of the controls on Day 18 but the amount of food-intake was comparable to controls thereafter. They restored weight lost to a level of the controls after having the free access to the diet. The animals in RF-B which were still under the feeding-restriction after the delivery had a food consumption at about a half of the controls during the lactation period, and their weight loss during the 7 days were 3.8 g on the average.

As Table 2 summarizes, the feeding-restriction did not affect the length of gestation period nor the number of neonates delivered. All neonates were externally normal. However, two dams in RF-B group cannibalized some of their litters on Days 2, 3 and 6 of lactation. The FR-B dams lost 12.5% of their neonates during the first 7 days after the delivery, while no neonates in the control and RF-A groups died. The body weight of the neonates in RF-B group

Table 1. Reproductive performance in dams subjected to the restricted feeding (5 hours per day during days 0 to 17 of gestation).

	Control	Feeding restricted
No. of dams	9	9
No. of corpora lutea	14.9±0.7	14.9±0.5
No. of implantations	11.8±1.0	12.7±1.3
No. of resorptions	0.67±0.29	1.78±0.52
No. of live fetuses	11.1±1.1	10.9±1.2
Fetal weight	3.59±0.08	3.37±0.08
External anomaly	0	0

Data represent the mean±SEM of 9 dams.

Fig. 3. Effects of the restricted feeding on food consumption (A) and body weight (B) in rats during the peri- and postnatal period. The animals in RF-A group (open square) had free access to the diet only for five hours (9 : 00–14 : 00) per during Days 0 to 17 of gestation, and the animals in RF-B group (open triangle) were under the feeding restriction throughout the experiment period including the lactation. The control animals (closed circle) had free access to the diet throughout the experiment period. Each point represents the mean of 6 animals with SEM.

remained significantly reduced as compared to the controls.

DISCUSSION

The present study aimed to examine effects of the daily restriction of feeding on the female fertility, embryo-fetus development, delivery and nursing performance. From the assumption of that the oral administration of an acid-unstable

test compound into the empty stomach could enhance the systemic exposure to the test compound, the animals had free access to the diet only for five hours per day. The results of the present study with non-pregnant rats indicated that it took a period of more than six days for the animals to adapt to the feeding-restriction.

Female rats under the feeding-restriction for a period of 21 days had a diet at two thirds of the controls, and lost their weight more than 10%. A prolonged estrous cycle was recognized in all these animals, and a long diestrous over four days was found in five out of eight animals. There are several papers reporting the similar results. Knuth and Friesen (1983) reported that diestrous serum FSH levels increased, whereas serum LH levels decreased by starvation in rats, and Parshad (1990) suggested that feeding-restriction affected pituitary gonadotropins and ovarian steroids in rats. In brief, the feeding-restriction in the present study seriously disturbed the estrous cycle, and therefore it is concluded that the feeding-restriction is not applicable to the female fertility study.

On the other hand, in the present study with pregnant rats which were under the feeding-restriction from Day 0 to Day 17 of gestation, there were no significant influence upon litter data, incidence of external anomalies or fetal skeletal development. There are several papers comparable to our findings (Beall and Klein, 1977; Pascalon and Bertrand, 1987). On comparison of responses in rats and mice to the same degrees of feeding-restriction, it has been shown that mice were distinctly more affected than rats (Chapin *et al.*, 1993a, 1993b). It has been reported that feeding-restriction in the pregnant mouse increased the incidence of malformations (Hemm *et al.*, 1977; Kalter, 1954; Runner, 1954; Runner and Miller, 1956). Beall and Klein (1977) reported that the feeding-restricted rats which received aspirin had more twice the incidence of malformations than those which received aspirin and were on an unrestricted diet. In the present study, body weights of fetuses in the feeding-restricted dams were marginally lower than in the *ad libitum*-fed dams but differences were not statistically significant. Because our feeding-restriction did not enhance congenital malformations nor seriously disturb the fetal

Table 2. Delivery and litter sizes of dams subjected to the restricted feeding (5 hours per day during days 0 to 17 of gestation (RF-A group) or during day 0 of gestation to day 7 of lactation (RF-B group)).

	Control	RF-A	RF-B
No. of dams	6	6	5
Gestation length (days)	21.8±0.2	22.0±0.0	22.0±0.0
No. of delivered neonates	12.8±1.0	13.0±0.8	12.8±0.7
Male/female ratio of neonates	0.79	1.11	1.07
No. of dams with cannibalism	0	0	2
Viability index (%)			
Day 1	100	100	96.9
Day 4	100	100	89.1
Day 7	100	100	87.5
Neonatal weight (g)			
Male			
Day 1	6.1±0.1	6.2±0.4	5.6±0.3
Day 4	9.2±0.2	9.0±0.5	7.2±0.3**
Day 7	13.2±0.5	12.8±0.7	8.2±0.4**
Female			
Day 1	6.0±0.1	5.9±0.3	5.3±0.2*
Day 4	9.0±0.3	8.7±0.4	6.9±0.3**
Day 7	12.9±0.4	12.5±0.6	7.8±0.4**
External anomaly	0	0	0

Data present the mean ± SEM.

* and ** indicate significant difference from the control at $P < 0.05$ and $P < 0.01$.

development, the dosing under the feeding-restriction may be applicable to the teratology study in rats, as an unavoidable and specific choice.

However, as indicated in the present paper, the feeding-restriction during the lactation period seriously disturbed the nursing performance and the growth of offspring, and the feeding-restriction is not applicable to the peri- and postnatal study.

As the conclusion of these studies, we decided to conduct the reproductive and development studies of this acid-unstable test compound by oral dosing under the feeding-restriction for the teratology study but by intravenous injection for the fertility study and the peri- and postnatal study.

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REFERENCES

- Beall, J. R. and Klein, M. F. (1977) : Enhancement of aspirin-induced teratogenicity by food restriction in rats. *Toxicol. Appl. Pharmacol.*, **39**, 489–495.
- Chapin, R. E., Gulati, D. K., Fail, P. A. *et al.* (1993a) : The effects of feed restriction on reproductive function in Swiss CD-1 mice. *Fundam. Appl. Pharmacol.*, **20**, 15–22.
- Chapin, R. E., Gulati, D. K., Barnes, L. H. *et al.* (1993b) : The effects of feed restriction on reproductive function in Sprague-Dawley rats. *Fundam. Appl. Pharmacol.*, **20**, 23–29.
- Hemm, R. D., Arslanoglou, L. and Pollock, J. J. (1977) : Cleft palate following prenatal food restriction in mice : Association with elevated maternal corticosteroids. *Teratology*, **15**, 243–248.
- Kalter, H. (1954) : Preliminary studies on the metabolic factors involved in the production of cleft palate in mice. *Genetics*, **39**, 1975.
- Knuth, U. A. and Friesen, H. G. (1983) : Starvation induced anoestrus : Effect of chronic food restriction on body weight, its influence on oestrous cycle and gonadotropin secretion in rats. *Acta*

- Endocrinol (Copenh), **104**, 402–409.
- Parshad, R. K. (1990) : Effect of restriction in daily feeding periods on reproduction in the female rat. *Acta Physiol. Hung.*, **76**, 205–209.
- Pascalon, A. and Bertrand, M. (1987) : Effects de la restriction alimentaire globale sur le developpment embryo-fetal chez la rate. *Ann. Rech. Vet.*, **18**, 379–388.
- Runner, M. N. (1954) : Inheritance of susceptibility to congenital deformity-embryonic instability. *J. Nat. Cancer Inst.*, **15**, 637–649.
- Runner, M. N. and Miller, J. R. (1956) : Congenital deformity in the mouse as a consequence of fasting. *Anat. Rec.*, **124**, 437–438.