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Testing Potential Effect of Environmental Endocrine Disruptors in Cow Milk on Reproductive Index in Female Rats

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Objective To study the effect of endocrine disruptor chemicals in cow milk on female reproductive system. **Methods** A two-generation reproduction was conducted according to U. S. FDA standard. Milk was fed in special bottle to Wistar rats of both sexes through two successive generations (F_0 and F_1) in the milk group while artificial milk was fed to rats in the control group. Twenty-four rats of each sex were mated in each group. Measurements were made according to this guideline. **Results** Reproductive parameters in the milk group such as fertility index, gestation index, weights of uterus and ovary, days of vaginal opening, estrous cycles, histological morphological changes were comparable to those in the control group. However, the means of body weight had some differences. The body weight gains increased significantly in the milk-treated group in F_1 and F_2 generation compared with those in the control group. The concentration of insulin-like growth factor-1 (IGF-1) in blood in the milk group was comparable to that in the control group, but the standard deviation changed greatly in the milk-treated rats. **Conclusion** Endocrine disruptor chemicals in milk have no severe effects on the female reproductive system.

Key words: Two-generation testing; Female reproductive system; Endocrine disruptor

INTRODUCTION

Cow milk harbors a significant amount of environmental endocrine disruptors, including pituitary hormones (PRL, GH, TSH, FSH, LH, ACTH), steroid hormones (estrogen, progesterone, testosterone, *ect.*), hypothalamic hormones (TRH, LHRH, GnRH, GRH), gastrointestinal peptides, halogenated aromatic hydrocarbons (HAHs). Some of them are known to have ability to cross the placenta and are present in large quantities in cow milk during lactation[1]. The relationships between the incidence rates of female reproductive cancers and cow milk consumptions are found by retrospective analysis in epidemiological studies. The limited epidemiological data in cow milk-consuming cohorts indicate that such chemicals may pose a threat to human health[2-4]. However, evidence to demonstrate association of endocrine-disrupting substances at natural concentrations in cow milk with female reproductive disorder have not been found in animal experiments. When selecting an *in vivo* model for testing the potential health effects of contaminants in cow milk, the test organism should be exposed to all the contaminants in the foods, including those that have been identified and those that remain unidentified, and the exposure period should be from conception. The parameters chosen for monitoring are both sensitive and subtle. The model chosen is a two-generation rat reproduction study wherein the offsprings would be examined for effects upon growth and reproduction.

MATERIALS AND METHODS

Determination of Insulin-like Growth Factor-1

It was found that insulin-like growth factor (IGF-1) is an important determinant of tumor growth, especially for breast cancer. Cow milk contains considerable IGF-1 especially when cow is treated by recombinant bovine growth hormone (rBGH). IGF-1 can be absorbed in gastrointestinal tracts. Hence, IGF-1 in plasma of female rats was assayed by the kit of ELISA, purchased from Sigma Company at the end of the experiment.

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Animals

Standard Wister rats (24 males and 24 females), closed colony, biological surveillances conforming to the standard of conventional animals, coming from the Animal Center of Yamanashi Medical College in Japan, were selected. The rats were numbered with a unique ear tag for identification at the time when they were placed in the study. The male and female rats were assigned by weight to either a control group or a cow milk treatment group by a stratified random experimental assignment procedure. Each group had 24 male and 24 female rats respectively. Before the formal experiment was carried out, the animals were allowed to be acclimated to the standard animal house condition for at least 1 week. During the course of this study the temperature of standard animal house was under 20℃-26℃, humidity was 40%-70%.

Rats received cow milk *ad lib*. in their special bottles, produced by a Japanese company, for approximately 20 wk (10 wk pretreatment, 3 wk mating, 1 wk post-mating, 3 wk gestation, 3 wk lactation) while the rats from the control group received "artificial milk". The cow milk was purchased from the local market and only one brand cow milk was used in the experiment. The animals in both groups drank clean water *ad lib*. from the local city water supply. The average food consumption and the average milk or "artificial milk" consumption were measured at least one time a week for each F_0 and F_1 generation rat.

The milk group: the rats received the same standard food as the control group. In addition, these rats received about 20 mL cow milk per day. The control group: in addition to the standard food, the control group received an "artificial milk mixture" in order to secure that the cow milk exposed had a comparable energy and nutrition intake. The artificial cow milk was given, which consisted of the same nutrients as the milk did except endocrine disruptors in distilled water.

After 10 weeks of treatment, male and female rats in the same treatment group were mated (one: one mating). The mating took place over a 3 week period. Pregnant F_0 generation female rats (pregnancy was determined by the presence of sperm plugs in the cage and the presence of sperm in the vagina) continued their exposure to cow milk from day 0 of gestation until the end of lactation. On post-partum day 4, litter was culled to 8 pups (4 males and 4 females) per litter using a random number table. On day 21 male and female rats were randomly selected to represent the F_1 generation. The weanlings remained in the same treatment group as their parents and female rats also were exposed to cow milk for about 20 wk.

Body weight Prior to mating, body weight was measured once a week and on the day of necropsy. The measurements were taken on the same calendar day for each female rat.

For the dam, body weights were measured on gestational and lactation days 0, 7, 14, and 20 days. For pup body weights were measured on days 4, 7, 14, 21 after birth.

Organ weight The weights of uterus and ovary were recorded and expressed as g/kg (reproductive organ weight/body weight) after the organ weights were adjusted for terminal body weight differences and as absolute weight in g.

Tissue preparation and histopathology of ovary Ovary was fixed in 10% neutral buffered formalin, embedded in paraffin, thinly sectioned and stained with hematoxylin and eosin. Five random sections of the ovary were examined under microscope.

Fertility index, gestation index, vaginal opening and estrous cycle For each group, the fertility index was calculated as the ratio of the number of impregnated females to the number for mating. A female was declared as having been impregnated if she littered or did not litter and at least one uterine implant scar was observed at necropsy, which represented the percent of matings that resulted in pregnancies. The formula was as follows: (number of pregnancies/number of matings)×100. This index could reflect the total number of dams that achieved pregnancy, including those that was delivered at term, aborted, or had fully resorted litters.

The gestation index was calculated as the ratio of the number of dams who resulted in at least one live offspring to the number who were impregnated. In this index, the litter with one live offspring was counted. The index was calculated as follows: (number of litters with live pups/ number of pregnancies) ×100.

Vaginal smears were collected every morning to evaluate estrous cycle length and pattern for 3 weeks prior to mating for each generation. Vaginal opening was checked every day when pups were born.

Statistical Methods

All the data were input into computer. All statistical analyses were performed by SPSS 8.0 software. Quantitative continuous data were analyzed using *t*-test if the data were subordinated to normal distribution, but if not, rank sum test (Mann-Whitney Test) was used. Chi-squire test was used to analyze the frequency of data for differences between the milk group and the control group.

RESULTS

IGF-1

For F_0 generation, IGF-1 concentrations in female rat plasma of the milk and control groups were 17.98±19.1561 and 9.61±4.0470 ng/mL, respectively. The IGF-1 variation in the milk group was more significant (the standard deviation was more than half of the mean). On the other hand, the variation of IGF-1 in the control group changed less (the standard deviation was less than half of the mean). The maximum and minimum variations in the milk group were 66.04 ng/mL and 1.29 ng/mL respectively and 14.20 ng/mL and 2.19 ng/mL in the control group. The median (9.97 ng/mL) in the milk group was not significantly different from that in the control group (10.24 ng/mL) (Mann-Whitney Test, U=0.459, *P*=0.646).

Body Weight

When food consumption in g/day did not exhibit any great differences between the two group, body weights of the female rats during the prebreed study day were not altered by the milk in F_0 generation. However, in F_1 generation, the body weight was significantly increased after 5 weeks compared to that in the control group (Fig. 1).

Similar to the body weight before mating, significant differences in the body weight during gestation and lactation also were observed in F_1 generation between the milk and control groups (Tables 1 and 2).

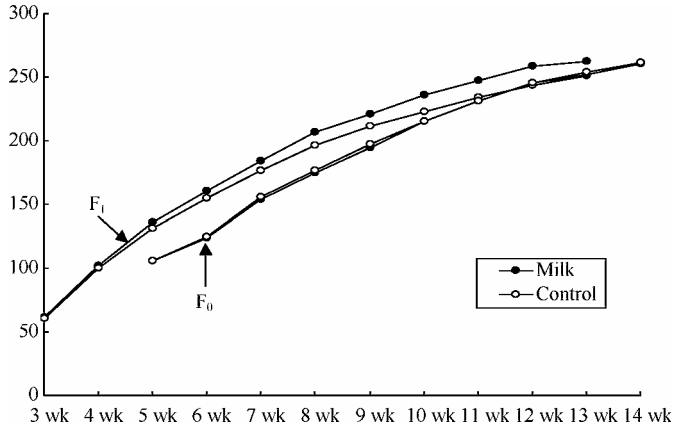

FIG. 1. Changes of body weight prior to mating in F_0 and F_1 generation.

TABLE 1

Body Weight During Gestation

	Gestational Day	Milk		Control		*t*	*P*
		n	$\bar{x} \pm s$	*n*	$\bar{x} \pm s$		
F_0	0 d	24	269.79±20.893	23	271.48±20.935	0.276	0.784
	7 d	22	291.91±21.271	21	291.62±19.931	0.046	0.963
	14 d	22	318.14±23.528	22	320.27±20.684	0.320	0.751
	20 d	23	376.52±33.977	23	375.35±30.647	0.123	0.903
F_1	0 d	21	264.19±17.3742	22	252.09±17.0235	2.306	0.026
	7 d	21	289.48±19.0148	22	275.05±14.7535	2.788	0.008
	14 d	21	313.48±18.2253	22	298.64±13.5209	3.042	0.004
	20 d	20	365.35±37.3014	22	348.41±28.7860	1.656	0.105

TABLE 2

Body Weight During Lactation

	Postnatal Day	Milk		Control		*t*	*P*
		n	$\bar{x} \pm s$	*n*	$\bar{x} \pm s$		
F_0	7 d	21	308.20±24.956	22	303.95±21.542	0.597	0.554
	14 d	21	316.14±24.036	22	310.41±19.786	0.856	0.397
	21 d	21	283.62±24.204	22	283.45±17.388	0.026	0.980
F_1	0 d	20	345.80±27.6645	19	326.00±23.4876	2.403	0.021
	7 d	20	356.55±21.9341	19	333.11±20.1354	3.472	0.001
	14 d	20	360.15±21.0044	19	340.44±19.2162	3.047	0.004
	20 d	20	333.85±23.6849	19	318.79±20.0488	2.138	0.039

Table 3 shows the pup body weight per litter during lactational period. In the F_1 generation, the body weights in the milk group sometimes were higher than those in the control group, and sometime were lower, although no significant differences were observed between the two groups. However, in F_2 generation, this change was different from that in F_1. From days 0 to 7 after birth, pup body weight in the milk group was consistently less than that in control group, but from days 14 to 21, the body weight gains initially increased in the milk group compared to that in control group, and significant difference was found even on day 21.

TABLE 3

Changes of Female Pup Body Weight (g)

	Day After Birth	Milk		Control		*t*	*P*
		Litters	$\bar{x} \pm s$	Litters	$\bar{x} \pm s$		
F_1	4 d	21	10.82±0.9057	22	10.64±1.3637	0.511	0.612
	7 d	21	17.75±1.0763	22	17.17±2.3369	1.037	0.306
	14 d	20	34.70±3.0622	22	35.16±3.2530	0.475	0.637
	21 d	21	53.89±4.2751	22	53.92±3.9678	0.023	0.982
F_2	0 d	16	5.83±0.3477	18	6.09±0.4708	1.857	0.073
	4 d	16	10.92±0.8775	18	11.08±1.2756	0.425	0.674
	7 d	16	17.73±0.9143	18	17.98±1.5515	0.558	0.581
	14 d	16	36.72±1.1573	18	35.57±2.1272	1.986	0.057
	21 d	16	55.94±4.2521	18	51.36±3.1238	3.607	0.001

Fertility Index, Gestation Index, Vaginal Opening and Estrous Cycle

For F_0 generation, 1 female rat had “no mating” in 24 female rats and the fertility index was 95.8% in the milk and control groups respectively. However, for F_1 generation, the proportion of mating (4/24) in the milk group was lower than that in the control group (2/24), but the fertility index (83.33%) in the milk group was not significantly different from that (91.67%) in the control group (P>0.05).

Gestational index: In F_0 generation, the index in the milk group was 95.5% (21/22) and was 100% in the control group, but no significant difference was found. In F_1 generation, the index in both groups was 100% (24/24).

The time for vaginal opening was not significantly changed in the milk group compared with the control group in the F_1 and F_2 generations. The number of estrous cycles during a 21-day evaluation period was comparable in the F_1 and F_2 generations of the milk and control groups. No significant differences in the percentages of the normal cycling were found in both generations,

approximately 92% of the estrous cycles were normal.

Litter Survival

The viability indices are measures of the offspring's ability to survive during specific brief intervals of their lives, from birth (day zero) to day our. As shown in Table 5, viability indices had no statistical differences between the milk-treated group and the control group (Table 4).

Weights and Histopathologies of the Ovary and Uterus

Table 5 shows the ovary and uterus weights at termination. The weights of the uterus and ovary were not significantly different between the milk and the control groups either in F_0 or F_1 generation. In both groups normal follicles and corpus lutea were observed. Histopathological changes were not found in the ovary of milk-treated rats in F_0 or F_1 generation.

TABLE 4

Viability Indices

	Day After Birth	Milk				Control				x^2	P
		n	Alive	Died	Viability Indices (%)	*n*	Alive	Died	Viability Indices (%)		
F_1	0 d	251	247	4	98.41	258	257	1	99.62	0.865	0.352
	4 d	247	246	1	99.60	257	254	3	98.83	0.214	0.644
	Total	251	246	5	98.01	258	254	4	98.45	0.002	0.483
F_2	0 d	207	203	4	98.10	206	203	3	98.50		
	4 d	203	200	3	98.50	203	203	0	100	1.343	0.246
	Total	207	200	7	96.62	206	203	3	98.54	0.908	0.341

TABLE 5

Weights of Reproductive Organs

		Milk		Control		t	P
		n	$\bar{x}\pm s$	*n*	$\bar{x}\pm s$		
F_0	Uterus						
	R	24	2.9759±0.6948	24	2.8980±0.8130	0.357	0.723
	A		0.8417±0.1886		0.8138±0.2003	0.496	0.622
	Ovary						
	R	24	0.39±0.0521	24	0.37±0.0588	1.362	0.180
	A		0.1111±0.0126		0.10±0.0161	1.536	0.132
	Implantation Rate (%)	22	95.21±10.3371	22	92.53±11.0688	0.830	0.411
F_1	Uterus						
	R	24	2.64±0.5792	24	2.74±0.9155	0.451	0.654
	A		0.8735±0.1784		0.8547±0.2662	0.287	0.776
	Ovary						
	R	24	0.37±0.0680	24	0.38±0.0721	0.439	0.663
	A		0.12±0.0200		0.12±0.0181	0.706	0.484
	Implantation Rate (%)	22	90.82±11.2350	21	91.50±9.4032	0.216	0.830

Note. R: (Organ weight relative to terminal body weight) ×1000; A: Absolute weight in g.

DISCUSSION

Cow milk contains considerable amounts of endocrine disruptors, particularly estrogens. Many epidemiological studies have indicated a positive correlation between the consumption of meat/milk/dairy products and breast cancer risk[5]. According to La Vecclia and Pampallona[2], milk and cheese are the only dietary variables which remain significantly positive after the correlation is adjusted for women's age at the birth of their first child and economic variables.

There is substantial evidence that hormones, particularly estrogens, are involved in the development of breast cancer in postmenopausal women[6-7]. According to Hartmann *et al.*[8], animal-derived foods, like meat, eggs and milk, contain considerable amounts of estrogens. The major sources of estrogens in the human diet are milk and dairy products accounting for 60%-70% of the estrogens. Present-day cow milk differs from milk consumed 100 years ago, in that milk is now produced from cows in late pregnancy, when estrogen levels are markedly elevated[9]. The main reason is that a lot of additives, animal drugs, even hormones are artificially, even not restrictively applied to diary cow to raise yield in modern dairy farming.

However, in our study, no severe effects on female reproductive system in the rats were found in the milk-treated group. But the body weight had some different changes. In F_0 generation, the change of body weight in the milk group was comparable to that in the control group, whereas the body weight increased significantly in the milk-treated group compared to that in the control group in F_1 generation. An increase of change in body weight also was observed in the pups of the milk group, indicating that whole-life exposure to endocrine disruptors in the cow milk could promote body growth. So far as the cow milk consumption and breast and ovarian cancer rate are concerned, it is most probable that the relations are indirect because breast and ovarian cancers occur in over weight women, especially in prepubescent girls[10-13].

In addition to estrogens, milk contains insulin-like growth factor-1 (IGF-1), which stimulates the proliferation of human breast cancer cell line MCF-7 at nanomolar concentrations[14]. IGF-1 maintains the malignancy of human breast cancer cells, including their invasiveness and ability to spread to distant organs. In addition, IGF-1 promotes transformation of normal breast cellular activity to breast cancers. The prenatal and infant breast is particularly susceptible to hormonal influence. Such imprinting by IGF-1 may increase future breast cancer risk, and sensitivity of the breast to subsequent unrelated risks such as carcinogens or estrogen-like chemicals in food, particularly in premenopausal women. IGF-1, a peptide, is not deactivated by pasteurization[15] and can survive digestion in the gastrointestinal tract. Instead, IGF-l is readily absorbed across the intestinal wall[16-17]. Research has shown that it can be absorbed into the bloodstream where it can affect other hormones. However, in our study, IGF-1 in female rat blood from the milk group was not significantly different from that in the control group. However, the standard deviation of IGF-1 in the milk-treated rats changed more significantly than that in the control, suggesting that individual sensitivity to IGF-1 is different. So, considering the relationships between the incidence of malignant tumors and milk consumption, breast and ovarian cancers may occur in women who are more sensitive to IGF-1 if they drink large amount of cow milk.

The relationship between cow milk consumption and female reproductive disorders has been contentious. Two-generation testing is one of the multi-end points of animal experiments. According to the results of the two-generation testing, no obvious adverse effects on the reproductive system were observed in female rats. Component of endocrine disruptors in cow milk is very complicated and the concentration is very low. Based on the report by Safe[18], a famous American toxicologist, human beings are exposed to both natural and industrial chemicals, which exhibit estrogenic and antiestrogenic activities. Many weak estrogenic compounds, including bioflavonoids, are also antiestrogenic at certain concentrations. Large amounts of natural or artificial antiestrogenic products can protect against the effects of environmental estrogen hormone and make the net effect equal to zero. Cow milk is a very intricate colloid containing a great deal (about 3000) of other bioactivity products, such as hypothalamic, pituitary, steroid hormones and gastrointestinal peptides *etc.*, other than fats, carbohydrate, protein, vitamin and minerals, *etc*. It is possible that these bioactive substances protect against the effects of estrogen-like products or that the estrogen-like products exert antagonism each other.

We often obtain the results from an animal experiment after the animals are treated with a high dosage of test substances. Therefore, the extensibility of the results is worth discussing. As a result, some persons may criticize this experiment when it is utilized to assess the potential risk of human exposure levels. However, in the present study, F_0 generation

male and female rats were given 20-30 mL cow milk daily at the age of about 35 days for approximately 14 weeks. F_1 generation male and female rats were exposed to cow milk in utero during lactation and for approximately 14 weeks after weaning. These F_1 generation animals, after weaning, received the same treatment as their parents. With respect to the results of the two-generation testing, endocrine disruptor compounds in the cow milk have no severe effects on the reproductive system in female rats.

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