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Teratological Study of LBD-001, a Recombinant Human Interferon γ, in Rats

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Abstract – LBD-001, a recombinant human interferon γ produced by genetically engineered yeast as a host system, was intravenously administered to pregnant female rats (Sprague-Dawley) from day 7 to 17 of gestation at dose levels of 0.35×10^6, 0.69×10^6, and 1.38×10^6 I.U./kg/day. As the control groups, hydrocortisone sodium succinate (5 or 10 mg/kg/day) was also similarly administered. Teratological effects of the test agents on fetuses and development of offsprings (F1 rats) were investigated. (1) No significant changes by the treatment of LBD-001 were observed in body weight, food and water consumption, feeding and nursing behaviors, and autopsy of pregnant or lactating mother rats. However, in hydrocortisone sodium succinate (10 mg/kg/day)-treated group, significant decreases of body weight on day 16, 18, and 20 of gestation and food consumption on day 20 of gestation and outstanding atrophy of thymus and adrenals were observed in two rats autopsied on day 20 of gestation. (2) No significant changes in resorption rate, skeletal or visceral development of fetuses, and physical or sensory development of offsprings (F1) by the treatment of LBD-001 were detected. In hydrocortisone sodium succinate (10 mg/kg/day)-treated group, however, there were significant decreases of body weight of fetuses, delay of ossification, temporary delay of body weights of offsprings (F1) on day 1 and 3 of lactation, and increased tendency of stillborn rate and malformation rate of bone. The results show that LBD-001 at the dose of 1.38×10^6 I.U./kg/day or less is not teratogenic in organogenesis of fetuses and the development of offsprings (F1). Meanwhile, hydrocortisone sodium succinate (10 mg/kg/day) seems to delay ossification of fetuses and temporarily retard the development of offsprings (F1).

Keywords □ LBD-001, recombinant human interferon γ, hydrocortisone sodium succinate, teratological study, rats, intravenous injection.

LBD-001 is a recombinant human interferon γ produced by fermentation of genetically engineered yeast containing the DNA which encodes for the human protein. The main action mechanism of naturally occuring interferon γ, a biological response modifier which is secreted from antigen-stimulated T-lymphocyte is related with enhancement of phagocytic function. LBD-001 was developed by Biotech Research Institute, Lucky Chemical Ltd. (84 Jang-Dong, Yousung-Ku, Daejeon, Korea). As a part of toxicological tests of LBD-001, teratological study was carried out in Sprague-Dawley rats (National Institute of Safety Research of Korea, 1986). The fertility study of LBD-001 was reported previously (Lee, E. B. and Cho, S. I., 1996). This study is concerned with assessment of the test substance on the potential toxic effects on visceral or skeletal organogenesis of fetuses and normal development of offsprings (F1).

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MATERIALS AND METHODS

Treatment of the test substance

LBD-001 was supplied by Biotech Research Institute, Lucky Chemical Ltd. and serially diluted in 5% dextrose in phosphate buffered saline (PBS; pH 7.4). The dose of LBD-001 was 0.35×10^6, 0.69×10^6, or 1.38×10^6 I.U./kg/day. In vehicle-treated group, 5% dextrose in PBS (1 ml/kg/day) was treated. In nontreated group, none was treated. As a reference drug, hydrocortisone sodium succinate (Solu-Cortef, Upjohn Co.; shortly hydrocortisone) which was diluted by 5% dextrose in PBS shortly before the treatment was administered at a dose of 5 or 10 mg/kg/day. The test substance or hydrocortisone was administered intravenously to the tail vein of pregnant female rats from day 7 to 17 of gestation. The number of

animals per group ranged from 27 to 30.

Animal maintenance

Sprague-Dawley rats (over 40-day-old male rats and over 42-day-old female rats) bred in our Institute were kept at the temperature of 22-25 ℃ and under the constant bright (6 *a.m.* to 7 *p.m.*) and dark (7 *p.m.* to 6 *a.m.*) cycle. Standard laboratory rodent diet (Samyang Food Co.) and sterilized water were fed *ad libitum*.

Observation method

(1) Observation of mother rats

Body weight, food consumption, and water consumption of mother rats were measured on day 0, 3, 7, 14, 16, 18, and 20 of gestation or additionally on day 1, 3, 7, 10, 14, and 21 of lactation. General behavior and lactating or nursing behavior of mother rats was observed every day. Among 27 to 30 rats per group, 17 to 20 rats were used for observation of fetal development and 10 animals were used for observation of their offsprings after delivery. On day 21 of gestation, or after finishing the lactation period, the mothers were anesthetized by ether and their abdominal cavity and uterine horns were opened for observation of abnormalities. The number of implantation sites was counted from the mothers which were finished the lactation period and the delivery rate (=the number of delivered offsprings/the number of implantation sites × 100) was calculated.

(2) Observation of fetuses

Seventeen to twenty pregnant rats were anesthetized by ether, and their abdominal cavities and uterine horns were opened for observation of the number of corpora lutea, the number of implantation sites, the number of live or dead fetuses, their external malformations, the number of fetuses resorbed at early or late period of gestation, sex, and body weights of live fetuses. Fetuses delivered from about half number of mothers were used for skeletal observation after staining the fetuses according to the method of Dawson (1926) using alizarin red S (Sigma Chem. Co.). The methods of Saito *et al.* (1984) and Aliverti *et al.* (1979) were used as criteria of skeletal variation. Another fetuses delivered from another half number of mothers were used for visceral observation under the stereoscope (Wilson, 1965; Shirasu and Matsuoka, 1981; Manson *et al.*, 1994).

(3) Observation of delivered offsprings (F1)

Mean of the gestation period was calculated from 10 pregnant females. After delivery, survival state (whether live or stillborn), sex, and external malformations of offsprings were checked. Body weight of offsprings was measured on day 1, 3, 5, 7, 10, 14, 17, and 21 of lactation, respectively. Survival rate (the number of offsprings on a observed day of lactation/the number of live offsprings on day 1 of lactation × 100) was also calculated. The birth days of offsprings were counted as day 1 of lactation. During lactation period, detachment of ears, eruption of teeth, and opening of eyelids were observed to check the physical development of offsprings. In addition, in order to observe the development of sensory function of offsprings, visual placing reflex, preyer reflex, righting reflex, response to pain which was added to the tail by pinching with an aortic clamp, and free fall reflex were cheked in offsprings on day 20 of lactation (Irwin, 1968; Buelke-Sam and Kimmel, 1979). On day 28 of lactation, some of the offsprings received the above-mentioned reflex tests were sacrificed by ether and the weights of their organs such as spleen, thymus, testes, and ovaries were measured.

Statistical analyses

To analyze the statistical significance, Student's *t*-test or χ^2-test was used. Experimental data in a group were compared with those of vehicle-treated group. When a difference shows $p<0.05$, the data were considered significantly different.

RESULTS AND DISCUSSION

Influences on mother rats

(1) General signs

Any abnormal symptoms in general behavior by the treatment of LBD-001 were not detected.

(2) Changes on body weight and consumption of food and water

Changes of body weight were shown in Fig. 1. Consumption of food and water was shown in Fig. 2 and Fig. 3. Body weight gains were similar among non-treated group, vehicle-treated group, and LBD-001-treated groups. However, in hydrocortisone (10 mg/kg)-treated group, significant decreases of body weights were observed on day 16, 18, and 20 of gestation against the vehicle-treated group ($p<0.05$). In consumption of food and water, there was no significant differences among the groups except the hydrocortisone (10 mg/kg)-treated group in which significant decrease ($p<0.05$) of food consumption and slightly decreased tendency of water consumption were observed on day 20 of gestation.

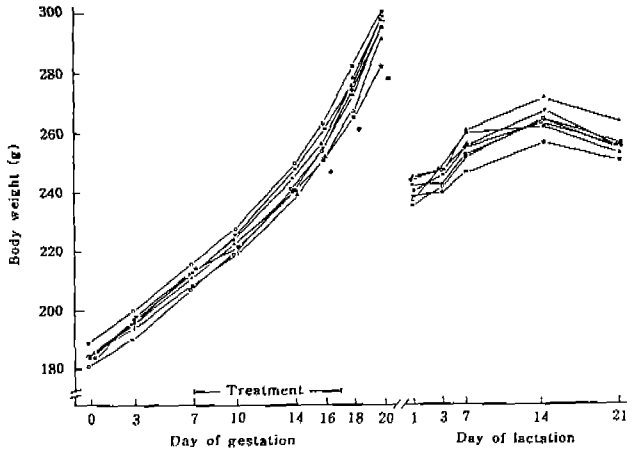

Fig. 1. Body weight changes of female rats treated with LBD-001 from day 7 to 17 of gestation. ○: Nontreated group, ●: Vehicle-treated group, ▲: LBD-001 (0.35×10^6 I.U./kg/day), □: LBD-001 (0.69×10^6 I.U./kg/day), △: LBD-001 (1.38×10^6 I.U./kg/day), ■: Hydrocortisone (5 mg/kg/day), ★: Hydrocortisone (10 mg/kg/day). *$p<0.05$; Significantly different from the vehicle-treated group.

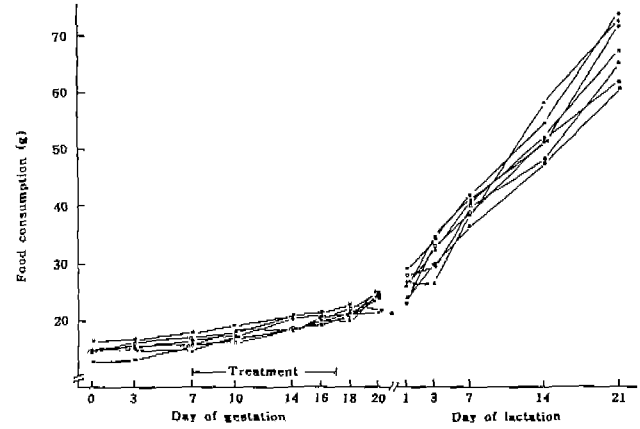

Fig. 2. Changes of food consumption in rats treated with LBD-001 from day 7 to 17 of gestation. ○: Nontreated group, ●: Vehicle-treated group, ▲: LBD-001 (0.35×10^6 I.U./kg/day), □: LBD-001 (0.69×10^6 I.U./kg/day), △: LBD-001 (1.38×10^6 I.U./kg/day), ■: Hydrocortisone (5 mg/kg/day), ★: Hydrocortisone (10 mg/kg/day). *$p<0.05$; Significantly different from the vehicle-treated group.

(3) Postmortem of mother rats

No external abnormality was found in the organs autopsied on day 19 of lactation or after finishing the lactation period. However, two mother rats showed outstanding atrophy of thymus and adrenals in hydrocortisone (10 mg/kg)-treated group.

Influences on fetuses

(1) Intrauterine observation

The results were shown in Table I. In nontreated

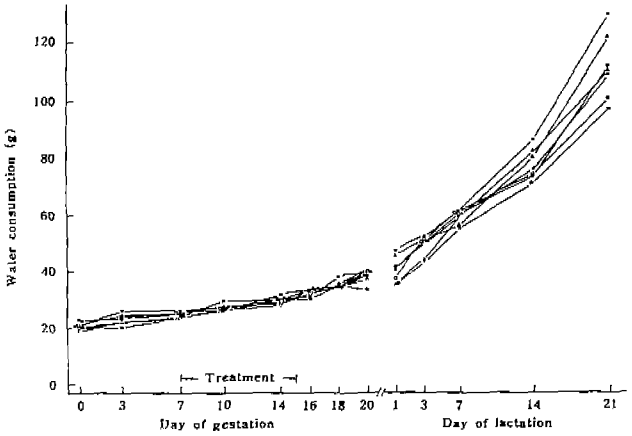

Fig. 3. Changes of water consumption in rats treated with LBD-001 from day 7 to 17 of gestation. ○: Nontreated group, ●: Vehicle-treated group, ▲: LBD-001 (0.35×10^6 I.U./kg/day), □: LBD-001 (0.69×10^6 I.U./kg/day), △: LBD-001 (1.38×10^6 I.U./kg/day), ■: Hydrocortisone (5 mg/kg/day), ★: Hydrocortisone (10 mg/kg/day).

group, the number of corpora lutea showed significant decrease compared with vehicle-treated group ($p<0.05$). However, the difference seems to be occasional because the numbers of implantation sites and implantation rate was not much different from vehicle-treated group. In hydrocortisone (10 mg/kg)-treated group, there were significant decreases in body weights of live fetuses irrespective of sex ($p<0.05$). The decreases of body weights of fetuses might be related with decreases of food and water consumption of mother rats (Fig. 2 and Fig. 3). In the rate of abnormal fetuses [=The number of abnormal (resorbed or dead) fetuses/(The number of live fetuses+The number of abnormal fetuses)×100], LBD-001-treated groups showed a little higher rates. That is, the rate was 5.6% in LBD-001 (0.35×10^6 I.U./kg)-treated group, 4.6% in LBD-001 (0.69×10^6 I.U./kg)-treated group, and 3.5% in LBD-001 (1.38×10^6 I.U./kg)-treated group, compared with 2.5% in vehicle-treated group. However, the tendency failed to show dose-dependent increases. Hydrocortisone-treated groups also showed higher rate of abnormal fetuses, even though no significant difference from vehicle-treated group was observed. Sex ratio (=No. of males/No. of females) in LBD-001 (0.35×10^6 I.U./kg)-treated group was 1.43 when observed on day 20 of gestation (Table I). However, in the records of naturally delivered mother rats, the sex ratio was 1.10 in LBD-001 (0.35×10^6 I.U./kg)-treated group (Table IV). It is hard to say that mainly female fetuses died between day 20 of gestation and delivery day because stillborn

Table I. Prenatal effect on fetuses of rats treated with LBD-001 from day 7 to 17 of gestation

Parameters		Nontreated group	Vehicle group	LBD-001 (I.U./kg/day)			Hydrocortisone (mg/kg/day)	
				0.35×10^6	0.69×10^6	1.38×10^6	5	10
No. of dams		19	20	20	20	19	17	20
Corpora lutea	total no.	181	214	215	205	185	178	225
	mean±S.D	9.5±1.23*	10.7±1.55	10.8±1.51	10.3±2.00	9.7±1.55	10.5±1.88	11.3±1.76
Implantation	total no.	175	198	196	194	173	170	202
sites	mean±S.D	9.2±1.58	9.9±2.34	9.8±1.72	9.7±2.45	9.1±1.41	10.0±1.85	101.±1.89
Implantation rate (%)[a]		96.7	92.5	91.2	94.6	93.5	95.5	89.8
No. of abnormal	total	4	5	11	9	6	4	8
(resorbed or dead)	early	4	5	9	8	6	3	7
fetuses	late	0	0	2	1	0	1	1
Rate of abnormal fetuses (%)[b]		2.3	2.5	5.6	4.6	3.5	2.4	4.0
Live fetuses	total no.	171	193	185	185	167	166	194
	mean±S.D	9.0±1.52	9.7±2.31	9.3±1.87	9.3±2.49	8.8±1.96	9.8±1.86	10.1±1.89
Sex ratio		0.99	0.91	1.43	0.93	0.90	1.10	1.04
(no. of males/no. of females)		(85/86)	(92/101)	(109/76)	(89/96)	(79/88)	(87/79)	(98/94)
Body weight of	male	2.60±0.20	2.63±0.23	2.61±0.24	2.66±0.30	2.57±0.25	2.59±01.19	2.57±0.23*
live fetuses (g, mean±S.D.)	female	2.49±0.24	2.51±0.19	2.49±0.17	2.54±0.23	2.48±0.21	2.48±0.30	2.46±0.24*

[a]No. of corpora lutea/No. of implantation sitex×100. [b]No. of abnormal fetuses/(No. of live fetuses+No. of abnormal fetuses)×100. *$p<0.05$; Significantly different from the vehicle-treated group.

Table II. External, skeletal, and visceral findings in fetuses of rats treated with LBD0-001 from day 7 to 17 of gestation

Parameters	Nontreated group	Vehicle group	LBD-001 (I.U./kg/day)			Hydrocortisone (mg/kg/day)	
			0.35×10^6	0.69×10^6	1.38×10^6	5	10
External malfomations/examined (%)	0/171 (0)	1/193 (0.5)	0/185 (1.1)	2/185(1.1)	0/167 (0)	0/166 (0)	0/194 (0)
General edema	0	1	0	0	0	0	0
Umbilical hernia	0	0	0	1	0	0	0
Subcutaneous hemorrhage around the dorasl part of cervical vertebrae	0	0	0	1	0	0	0
Skeletal abnomalities/examined (%)	1/68 (1.5)	0/87 (0)	0/89 (0)	1/83 (1.2)	1/71 (1.4)	1/79 (1.3)	3/92 (3.3)
Wavy ribs	1	0	0	1	1	1	2
Branched ribs	0	0	0	0	0	0	1
Skeletal variations/examined (%)	2/68 (2.9)	1/87 (1.1)	3/89 (3.4)	2/83 (2.4)	3/71 (4.2)	5/79 (6.3)	9/92 (9.8)
Asymmetrical sternebrae	2	1	1	2	1	3	5
Shortening of 13th ribs	0	0	1	0	1	0	1
Lumbar ribs	0	0	0	0	1	2	3
Hyperplasia of ossification center of 8th thoracic vertebra	0	0	0	0	1	0	0
Hypoplasia ossification center of 9th thoracic vertebra	0	0	0	0	1	0	0
Hypoplasia of ossification center of 11th thoracic vertebra	0	0	1	0	0	0	0
Visceral abnormalities/examined (%)	0/103 (0)	0/105 (0)	0/96 (0)	0/100 (0)	1/96 (1.0)	2.87 (2.3)	2/102 (2.0)
Hydronephrosis	0	0	0	0	1	1	0
Ventricular or Periocular hemorrhage	0	0	0	0	0	1	2

Values represent the number of fetuses or percent in case of the figures in parentheses.

rate of abnormal fetuses was 5.6%. The difference in the sex ratio rather seems to be occasional. Except these, the number of corpora lutea, the number of implantation sites, the number of implantation rate, the number of fetuses resorbed or dead, the number of live fetuses, sex ratio, and body weights of live fetuses did not show sig-

nificant differences compared with vehicle-treated group.

(2) External, skeletal, and visceral observation

The results were shown in Table II. The skeletal findings of ossification of fetuses were shown in Table III and Photo 1~4. As an external malformation, one case of general edema was observed in the vehicle-treated group. An umbilical hernia (Photo 1) and a subcutaneous hemorrhage around the dorsal part of cervical vertebrae were observed in LBD-001 (0.69×10^6 I.U./kg)-treated group. Since the abnomalities were found only in the low dose of LBD-001-treated group, the effects were not dose-dependent. Skeletal abnormalities with wavy ribs were found in one case of LBD-001 (0.69×10^6 I.U./kg)-treated group, one case of LBD-001 (1.38×10^6 I.U./kg)-treated group, one case of of hydrocortisone (5 mg/kg)-treated group, and two cases of hydrocortisone (10 mg/kg)-treated group (Photo 2). However, one case of wavy ribs was also found in nontreated group. These mean that the occurrences of wavy ribs might come from natural variation. One case of branched ribs was found in hydrocortisone (10 mg/kg)-treated group. Occurence rates of skeletal abnormality were not greatly different among the groups.

Using the criteria of Saito *et al.* (1984), skeletal variations were also observed such as asymmetrical sternebrae, shortening of the 13th ribs, lumbar ribs, hyperplasia of ossification center of 9th thoracic vertebra, hyperplasia of ossification center of 11th thoracic vertebra, and bipartite vertebral body were observed. The skeletal variation rates (the number of fetuses with skeletal variations/the number of fetuses examined $\times$ 100) ranged from 1.1 to 4.2% in nontreated group, vehicle-treated group, and LBD-001-treated groups. The rates were not significantly different among the groups. However, in hydrocortisone-treated groups, The skeletal variation rates showed dose-dependently increased tendency. That is, the rate was 6.3% at lower dose and 9.8% at higher dose.

Table III. Skeletal development (ossification) in fetuses of rats treated with LBD-001 from day 7 to 17 of gestation

Parameters	Nontreated group	Vehicle group	LBD-001 (I.U./kg/day)			Hydrocortisone (mg/kg/day)	
			0.35×10^6	0.69×10^6	1.38×10^6	5	10
No. of examined fetuses	68	87	89	83	71	79	92
No. of fetuses with poorly ossified supraoccipital bone (%)[a]	0(0)	1(1.1)	0(0)	2(2.4)	1(1.4)	2(2.5)	5(5.4)
No. of sternebrae[b]	4.0±0.59	3.8±1.07	3.7±1.04	3.8±0.62	3.6±1.05	3.6±0.62	3.5±0.86*
No. of sacral and coccygeal vertebrae[b]	5.5±0.94	5.2±1.22	5.2±1.36	5.5±1.03	4.9±1.58	5.0±1.17	4.8±1.33*
No. of metacarpus[b]	5.9±0.10	5.8±0.70	5.8±1.12	6.0±0.88	5.8±0.66	5.8±0.11	5.5±0.89*
No. of metatarsus[b]	7.3±0.92	7.1±1.01	6.9±1.00	7.5±0.34	7.2±1.36	6.9±0.94	6.7±1.13*

[a]The figures in the parentheses indicate the percent. [b]Mean±S.D. *$p<0.05$; Significantly different from the vehicle-treated group.

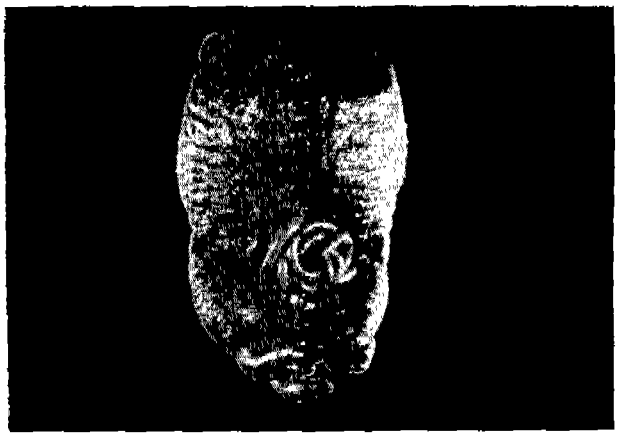

Photo 1. Umbilical hernia found in LBD-001 (0.69×10^6 I.U./kg/day)-treated group.

Photo 2. Wavy ribs found in hydrocortisone (10 mg/kg/day)-treated group.

As visceral abnormalities, hydronephrosis was observed in one case of LBD-001 (1.38×10^6 I.U./kg)-treated group and in one case of hydrocortisone (5 mg/kg)-treated group (Photo 3.) Ventricular or periocular hemorrhage (Photo 4.) was also observed in one case of hydrocortisone (5 mg/kg)-treated group and in two cases of hydrocortisone (10 mg/kg)-treated group. The abnormality rate (=the number of fetuses with visceral abnormalities/ the number of examined fetuses $\times$ 100) rate was slightly higher in hydrocortisone-treated groups even though the occurrence rates (about 2%) were low and negligible.

Ossification was not much different among the groups except the results that hydrocortisone (10 mg/kg)-treated group showed significant decreases in the ossification of the sternebrae, sacral and coccygeal vertebrae, metacarpus, and metatarsus ($p<0.05$; Table III). In hydrocortisone (10 mg/kg)-treated group, the number of poorly ossified supraoccipital bone was also increased.

Altogether, external malformation, skeletal abnormalities, and skeletal or visceral abnormalities in LBD-001-treated groups might be negligible. Only hydrocortisone (10 mg/kg) increased the significant delay of ossification and showed the tendency of skeletal abnormalities.

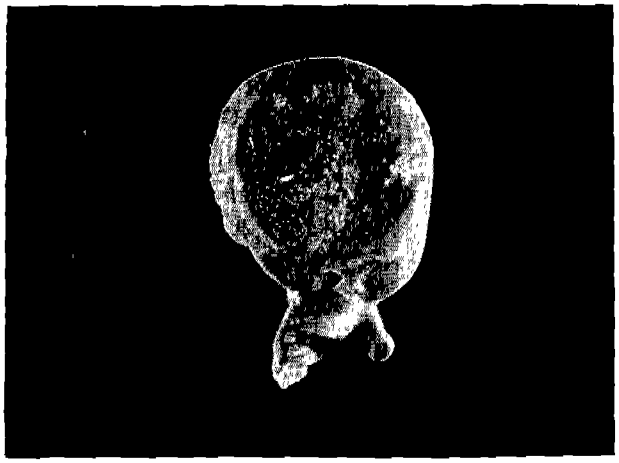

Photo 3. Hydronephrosis found in hydrocortisone (10 mg/kg/day)-treated group.

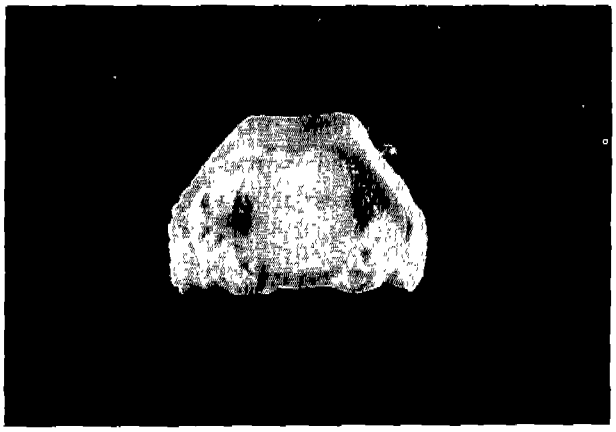

Photo 4. Ventricular hemorrhage found in hydrocortisone (10 mg/kg/day)-treated group.

Table IV. Delivery records in rats treated with LBD-001 from day 7 to 17 of gestation

Parameters	Nontreated group	Vehicle group	LBD-001 (I.U./kg/day)			Hydrocortisone (mg/kg/day)	
			0.35×10^6	0.69×10^6	1.38×10^6	5	10
No. of dams	10	10	10	10	10	10	10
Duration of gestation (days, mean±S.D.)	21.7±0.46	21.7±0.46	21.7±0.45	21.6±0.45	21.8±0.60	21.7±0.46	21.5±0.50
Implantation total no.	105	113	103	94	100	113	101
traces mean±S.D.	10.5±1.36	11.3±1.35	10.3±1.35	9.4±1.74	11.0±1.61	11.3±1.42	10.1±2.66
Total no. of newborns	102	109	101	91	105	111	91
No. of newborns per litter (mean±S.D.)	10.2±1.17	10.9±0.83	10.1±1.37	9.1±1.51**	10.5±1.63	11.1±1.22	9.1±2.17*
Delivery rate (%)[a]	97.1	96.5	98.1	96.3	95.2	96.5	90.1
Total no. of stillborns	1	0	0	1	0	4	6
Stillborn rate (%)[b]	1	0	0	1.1	0	3.6	6.6
Total no. of live offsprings	101	109	101	90	105	107	85
Live offsprings per litter (mean±S.D.)	10.1±1.14	10.9±0.83	10.1±1.37	9.0±1.41**	10.5±1.63	10.7±1.73	8.5±2.06**
Sex ratio (no. of males/no. of females)	1.06 (52/49)	0.98 (54/55)	1.10 (53/48)	1.14 (48/42)	1.98 (52/53)	1.10 (56/51)	0.89 (40/45)
No. of newborns with external malformations	0	0	0	0	0	0	0

[a]No. of newborns/No. of implantation traces $\times$ 100. [b]No. of stillborns/No. of newborns $\times$ 100. *$p<0.05$, **$p<0.01$: Significantly different from the vehicle-treated group.

Table V. Viability of offsprings (F1) born from rats treated with LBD-001 from day 7 to 17 of gestation

Parameters		Nontreated group		Vehicle group		LBD-001 (I.U./kg/day)						Hydrocortisone (mg/kg/day)			
						0.35×10^6		0.69×10^6		1.38×10^6		5		10	
No. of dams		10		10		10		10		10		10		10	
Sex of offsprings		male	female	male	female	male	female	male	female	male	female	male	female	male	female
day	1[a]	52	49	54	55	53	48	48	42	52	53	56	51	40	45
		(100)	(100)	(100)	(100)	(100)	(100)	(100)	(100)	(100)	(100)	(100)	(100)	(100)	(100)
No. of	3	51	49	54	55	52	48	47	41	52	53	55	51	40	45
survived		(98.1)	(100)	(100)	(100)	(100)	(100)	(97.9)	(97.6)	(100)	(100)	(98.2)	(100)	(100)	(100)
offsprings	7	51	49	53	55	52	48	47	40	51	53	55	51	38	45
(% of		(98.1)	(100)	(98.1)	(100)	(100)	(100)	(97.9)	(95.2)	(98.1)	(100)	(98.2)	(100)	(95)	(100)
survival)	14	48	49	53	55	52	48	45	38	50	53	55	51	36	41
		(92.3)	(100)	(98.1)	(100)	(100)	(100)	(93.8)	(90.5)	(96.2)	(100)	(98.2)	(100)	(90)	(91.1)
	21	46	46	52	54	51	48	43	37	48	53	55	51	36	41
		(88.5)	(93.9)	(96.3)	(98.2)	(96.2)	(100)	(89.6)	(88.1)	(92.3)	(100)	(98.2)	(100)	(90)	(91.1)

[a]Day of lactation; Birthday is regarded as day 1 of lactation.

It is reported that rats have low sensitivity to corticosteroids than mice in teratogenesis and mainly show both increase of fetal death and retardation of fetal growth (Ingalls and Curley, 1957; Aoyama *et al.*, 1974). It was also reported that hydrocortisone-17α-butyrate showed delay of ossification, but which was compensated to the normal state in the following postnatal period (Aoyama *et al.*, 1974). Meanwhile, more potent derivatives of corticosteroids such as betamethasone and dexamethasone show teratogenesis such as cleft palate, wavy ribs and so on (Hasegawa *et al.*, 1974; Miyamoto *et al.*, 1975). Our results showed similar results to the previous reports using hydrocortisone.

(3) Effects on offsprings

Duration of gestation, delivery rate, sex ratio of offsprings, and number of offsprings with external malformations were shown in Table IV. Mean number of newborns per litter was significantly low in LBD-001 (0.69×10^6 I.U./kg)-treated group ($p<0.05$). The significant low number might result from occasionally low number of offsprings itself, because stillborn rate was not much different from that in the vehicle-treated group and the effect of LBD-001 was not dose-dependent. Meanwhile, significant decrease of mean number of newborns per litter ($p<0.05$) and significant low number of live pups per litter ($p<0.01$) which were observed in hydrocortisone (10 mg/kg)-treated group might be related with dose-dependent increase of the stillborn rate which shows 3.6% at lower dose and 6.6% at higher dose of hydrocortisone.

In the duration of gestation, the number of implantation sites, delivery rate, sex ratio of offsprings, and the number of newborn rats with malformations, there were no significant differences.

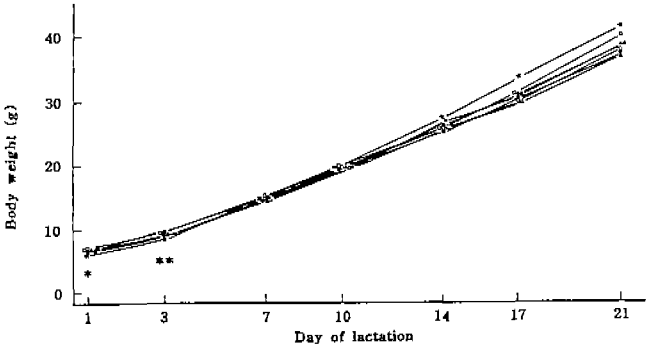

Fig. 4. Body weight changes of male offsprings (F1) born from rats treated with LBD-001 from day 7 to 17 of gestation. ○: Nontreated group, ●: Vehicle-treated group, ▲: LBD-001 (0.35×106 I.U./kg/day), □: LBD-001 (0.69×10^6 I.U./kg/day), △: LBD-001 (1.38×10^6 I.U./kg/day), ■: Hydrocortisone (5 mg/kg/day), ★: Hydrocortisone (10 mg/kg/day). *$p<0.05$, **$p<0.01$; Significantly different from the vehicle-treated group.

(4) Effects on viability of offsprings (F1)

The results were shown in Table V. On day 3 of lactation, survival rate was not different among the groups. On day 21 of lactation, survival rate was a little lower in LBD-001-treated groups and hydrocortisone (10 mg/kg)-treated group. In both cases, all the number of dead offsprings was originated from one mother rat. The death of offsprings might be explained by poor lactation or nursing behavior of the mother rats. However, the absence of dose-response relationship suggests that the viability of offsprings is not significantly affected by the treatment of LBD-001.

(5) Effects on body weight changes of offsprings

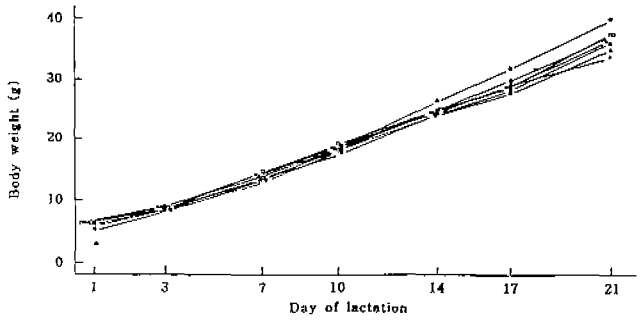

Fig. 5. Body weight changes of female offsprings (F1) born from rats treated with LBD-001 from day 7 to 17 of gestation. ○: Nontreated group, ●: Vehicle-treated group, ▲: LBD-001 (0.35×10^6 I.U./kg/day), □: LBD-001 (0.69×10^6 I.U./kg/day), △: LBD-001 (1.38×10^6 I.U./kg/day), ■: Hydrocortisone (5 mg/kg/day), ★: Hydrocortisone (10 mg/kg/day). *$p<0.05$; Significantly different from the vehicle-treated group.

Body weight changes of male offsprings and female offsprings were shown in Fig. 4 and Fig. 5, respectively. In LBD-001-treated groups, body weight gains of male or female offsprings grown from day 1 to 21 of lactation were not significantly different from vehicle-treated group. In hydrocortisone (10 mg/kg)-treated group, significant decreases of body weights as compared with the vehicle-treated group were observed in both male offsprings on day 1 of lactation ($p<0.05$) and day 3 of lactation ($p<0.01$) and female offsprings on day 1 of lactation ($p<0.05$). The decreases were found only at the early stage of the development and disappeared after that. The decreases might be related with signicantly delayed ossification of fetuses at the late stage of gestation.

(6) Effects on physical and sensory development of

Table VI. Physical and sensory development of offsprings (F1) born from rats treated with LBD-001 from day 7 to 17 of gestation

Parameters	Nontreated group	Vehicle group	LBD-001 (I.U./kg/day)			Hydrocortisone (mg/kg/day)	
			0.35×10^6	0.69×10^6	1.38×10^6	5	10
Physical development[a]							
Detachment of ears	2.6±0.67 (100)	2.6±0.49 (110)	2.7±0.57 (100)	2.8±0.70 (87)	2.5±0.73 (104)	2.6±0.59 (106)	2.8±0.67 (84)
Eruption of teeth	10.6±0.94 (100)	10.9±0.90 (108)	11.0±0.82 (100)	11.0±1.11 (85)	10.6±0.76 (103)	10.6±0.81 (106)	10.4±1.70 (72)
Opening of eyelids	15.3±0.65 (96)	15.4±0.52 (106)	15.1±0.86 (100)	15.1±0.87 (82)	14.8±0.79 (103)	14.9±0.74 (106)	15.0±0.93 (72)
Sensory function on day 20 of lactation							
No. of offsprings	92	106	99	73	101	106	72
Visual placing reflex[b]	0	0	0	0	0	0	0
Preyer reflex[b]	0	0	0	0	0	0	0
Righting relfex[b]	0	0	0	0	0	0	0
Response to pain[b]	0	0	0	0	0	0	0
Free fall reflex[b]	0	0	0	0	0	0	0

[a]Values represent the mean±S.D. of days of lactation or total number of examined offsprings in case of the figures in parentheses.
[b]No. of offsprings with abnormal sensory function.

Table VII. Organ weight of 28-day-old offsprings (F1) born from rats treated with LBD-001 from day 7 to day 17 of gestation

Parameters		Nontreated group	Vehicle group	LBD-001 (I.U./kg/day)			Hydrocortisone (mg/kg/day)	
				0.35×10^6	0.69×10^6	1.38×10^6	5	10
No. of offsprings		10	10	10	10	10	10	10
Body weight (g)	male	96.5±10.6	69.8±5.5	69.7±7.8	73.8±10.7	70.8±5.5	73.1±7.9	70.5±7.8
	female	68.4±10.7	67.4±2.7	69.8±8.4	66.7±9.7	68.7±3.1	68.4±6.8	70.6±4.9
Adrenals (mg)	male	19.8±3.8	18.6±2.8	17.5±2.7	21.0±2.9	19.8±1.9	20.4±4.3	19.4±4.4
	female	18.2±2.9	18.3±2.5	18.6±2.2	19.8±2.5	19.2±1.7	17.9±4.6	19.4±3.2
Spleen (mg)	male	320.3±82.3	371.2±68.2	328.5±88.4	376.9±96.7	393.5±71.8	339.0±24.6	360.9±84.7
	female	361.8±52.3	341.9±51.5	286.3±59.9	322.2±68.9	374.2±73.7	318.1±8.2	337.1±55.1
Thymus (mg)	male	278.7±77.3	225.3±80.4	248.4±50.2	293.8±70.3	262.9±49.0	281.1±52.8	235.1±37.8
	female	296.1±80.3	264.3±29.5	269.9±48.5	255.7±64.9	266.0±53.3	284.9±82.6	256.3±46.1
Testes (mg)	male	520.2±139.7	535.4±58.0	532.7±49.0	560.5±102.3	574.9±85.6	592.3±162.0	478.9±101.5
Ovaries (mg)	female	16.3±2.8	16.4±2.5	17.3±2.0	15.6±2.3	17.8±1.9	16.8±1.0	14.4±3.0

Values represent the mean±S.D.

offsprings (F1)

The results were shown in Table VI. Physical development such as detachment of ears, eruption of teeth, and opening of eyelids was not much different among the groups. The numbers of abnormality in sensory functions of 20-day-old offsprings such as visual placing reflex, preyer reflex, righting reflex, response to pain, and free fall reflex were also not significantly different among the groups. The results show that LBD-001 or hydrocortisone does not affect the normal development of physical and sensory function of F1 offsprings.

(7) Effects on organ weights of offsprings

The results were shown in Table VII. The organ weights of some offsprings were measured on day 28 of lactation. No significant differences in the weights of adrenals, spleen, thymus, testes, and ovaries were observed among the groups. There were no abnormalities of the organs in macroscopic observation of autopsied offsprings.

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