A Chemically Derived Milk Substitute that is Compatible with Mouse Milk for Artificial Rearing of Mouse Pups

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Abstract: The object of this study was to prepare a chemically derived milk substitute that is compatible with mouse-milk. Milk was independently collected from ICR, BALB/c, and FVB/N mice, and analyzed for the protein, fat, and mineral contents to formulate a milk substitute. Thereafter, ICR mouse pups were artificially reared on the milk substitute to evaluate the rate of increase of their body weights. A gastric cannula tube was placed through the esophageal way into 8-day-old ICR pups, and the mice were fed with the milk substitute by computer-regulated infusion pumping by the pup-in-a-cup method. The analytical mean values of total protein and total fat in milk from ICR, BALB/c, and FVB/N mice were $10.23 \pm 0.49\%$ and $21.34 \pm 1.31\%$, respectively. The milk substitute was constituted from purified bovine casein and whey proteins, five edible oils, including MCT oil, minerals, and vitamins. After 8 days of artificial rearing with the new milk substitute, 36 of the 42 pups had survived, and the growth rate of these mice was not significantly different from that of maternally reared littermate pups. In conclusion, we have succeeded in the preparation of a chemically derived milk substitute for mice pups which is available for clarifying the roles of dietary components such as milk-bone substance during the suckling period in mice pups including those of knockout and transgenic mice.

Key words: artificial rearing, chemical milk substitute, mouse

Introduction

A practical method of artificially rearing mouse pups is desirable for neonatal nutrition and genetic studies because mice are used more commonly than rats in experiments using transgenic and knockout animals. Furthermore, antibody reagents of mice are commercially available. Recently, Beierle *et al.* reported artificial rearing of mice pups using gastrotomy cath-

eters [2], and Hoshiba developed hand-feeding tools for mice, including a surrogate nipple with a nursing bottle [4]. They both used milk substitutes, such as that for rodents or a commercially available dog formula.

Although a chemically derived milk substitute to support rat pup growth has already been developed [5–7], no chemically derived milk substitute for rearing mice which achieves sufficient growth has been developed

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yet. The aim of this study was to prepare a chemically derived milk substitute for mice, and to rear mouse pups using the new milk substitute and compare them with maternally reared pups.

Materials and Methods

Meiji Dairies Corporation's Committee for Research on Experimental Animals approved the experimental protocol. Experiments were conducted in accordance with the *NRC Guide for the Care and Use of Laboratory Animals* (NRC, 1985).

Animals

Pregnant ICR and BALB/c mice were purchased from Japan SLC (Shizuoka, Japan), and pregnant FVB/N mice from CLEA JAPAN, Inc. (Tokyo Japan). All animals were kept individually under environmentally controlled temperature ($25 \pm 2^\circ\text{C}$), humidity ($50 \pm 5\%$ relative humidity), and light (12-h light-dark cycle) conditions. They had free access to water and chow (CA-1; Japan Clea, Tokyo, Japan) and were allowed to deliver spontaneously.

Analyses of the composition of mouse milk

Four dams of each species of mouse, ICR, BALB/c, and FVB/N, were milked during 4–12 days post partum. The milking was done every other day after intra-peritoneal stimulation by injection of 0.05 ml of 0.01% - oxytocin (Peptide Institute Inc., Osaka, Japan) with pentobarbital sodium anesthetic.

The milk of each species was pooled until analysis. The total protein was measured with an automatic analyzer, Kjeltac 2300 (FOSS Japan, Tokyo). Total milk fat was measured by using lipid extraction and gravimetric methods. One milliliter of milk was diluted to 10 ml with distilled and deionized water (DW). Then, 2 ml of ammonium and 10 ml of ethanol were then added and mixed. In addition, 25 ml of diethyl ether were added to the mixture, followed by vigorous mixing, and then, 25 ml of petroleum ether were added, followed by more vigorous mixing. After standing the mix for more than 45 min, the diethyl ether in the upper layer was placed in a pre-dried and weighed flask. After that, the second extraction was done by adding 15 ml of fresh diethyl ether and 15 ml of petroleum ether. The two portions of diethyl ether extracts were

mixed in the same flask and dried at $96\text{--}98^\circ\text{C}$ for 2 h in a vacuum oven. The total fat was weighed after cooling in plastic desiccators. The minerals were measured by atomic absorption spectrophotometry after appropriate dilution directly after heating at 550°C for over 5 h. The osmotic pressure of the milk was determined by measuring the freezing point depression (Osmostat-6020, Daiichi Kagaku Co., Ltd., Kyoto, Japan). The micelle size of the milk was determined with a laser diffraction particle size analyzer (SALD-2000, Shimadzu, Kyoto, Japan).

Composition and preparation of the mouse formula (MCW64)

The protein and fat in the milk substitute were prepared to mimic the contents of those found in the three species of mice milk analyzed in this study. The formula consisted of proteins such as purified bovine casein and whey proteins. Five edible oils, including MCT oil, were added to the composition as in milk substitute for rats [5]; however, the oil contents were approximately 1.3 times higher than those used in the rat formula prepared by Kanno *et al.* [5]. Vitamins were added at approximately the same concentration as those used in the rat formula prepared by Auested *et al.* [1].

Artificial rearing of ICR mice using the milk substitute for mice

Ten mice were allowed to deliver spontaneously. At 8 days after birth, the pups were weighed and divided into 2 groups at random in order to eliminate the influence of inter-litter variation. In the first group, a litter of 10 pups was reared by one of the dams throughout the study (maternally reared: MR). In the second group, an intragastric cannula was implanted in the gastric wall through the esophageal way in each pup using Hall's method [3], and they were then artificially reared (AR) on the milk substitute. The volume of the formula which was fed to the AR pups was determined daily for comparison with the growth of the MR pups and increased from 0.6 ml on day 8 to 1.2 ml on day 15. The syringe on the pump was placed in a refrigerator (5°C) to prevent bacterial growth in the milk formula inside the tubes. If the cannula fell off during the artificial rearing, the mice were sacrificed.

Cannula for pups

The artificial rearing method used in this study involved implanting a cannula in the stomach. The milk-pumping side of the cannula tube passed from the inside to the outside of the walls of the stomach and abdomen using a piano wire as a guide, and the inner gastric side of the cannula was fixed by a rim with a thin plastic disk of 3 mm in diameter to prevent it from coming out of the stomach wall. The cannula was made of a clear vinyl tube that was 0.2 mm in inner diameter and 0.5 mm in outer diameter (SV8, Natume Seisakusho Co., Ltd., Tokyo, Japan) and was attached to a 5-ml syringe pump connected to a polyethylene tube (PE50).

Results

Protein, fat, and mineral contents of the milk in ICR, BALB/c, and FVB/N mice

The concentrations of protein, fat and some minerals in the milks from ICR, BALB/c and FVB/N mice are shown in Table 1. The fat concentrations were 22.8%, 21.1%, and 20.2% in ICR, BALB/c and FVB/N mice, respectively, and the average value of the fat concentration was $21.3 \pm 1.3\%$. The protein concentrations were 11.3%, 10.3%, and 10.9%, in ICR, BALB/c and FVB/N mice, respectively, and the average value of the

protein concentration was $10.8 \pm 0.5\%$. We did not analyze the concentrations of vitamins, sugars, and fatty acids.

Composition and preparation of the chemically derived milk substitute for mice pups

The composition of the milk substitute for mice pups is listed in Table 2. The concentrations of vitamins in the mouse milk substitute were those used by Auestad *et al.* [1] with the exception of enriched ascorbic sodium, and the fats used were the same as those used in a rat milk substitute [3], though the concentrations were different.

Table 3 is a flow table for the preparation (4L) of the milk substitute, MCW64. The preparation procedure is similar to that for the rat milk substitute by Kanno *et al.* [5].

The first step was the preparation of casein mixture. Three amino acids, serine (1.15 g), cystine (0.90 g), and tryptophan (1.08 g), were added to an alkaline solution (1.35 L) of NaOH (2.4 g) and KOH (6.8 g), under continuous stirring with a motor-driven stirrer (three-one motor, Heidon, Tokyo, Japan) at 60–70°C before the addition of casein. Then, bovine casein (266.05 g) was slowly dissolved in the alkaline solution. The casein solution was sterilized by boiling in a water bath for 30 min. In the second step, calcium and

Table 1. Concentrations of milk fat, protein, and minerals in mouse milks

concentration		mouse milk			
		ICR	BALB/c	FVB/N	average $\pm$ SD
fat	g/100 ml	22.8	21.1	20.2	21.3 ± 1.3
protein	g/100 ml	11.3	10.3	10.9	10.8 ± 0.5
Fe	mg/100 ml	4.91	n.t.	1.0	3.0 ± 2.8
Na	mg/100 ml	84.23	n.t.	186.7	135.5 ± 72.5
Ca	mg/100 ml	360.00	n.t.	n.t.	
P	mg/100 ml	216.00	n.t.	n.t.	
K	mg/100 ml	109.00	n.t.	n.t.	
Cl	mg/100 ml	147.00	n.t.	n.t.	
Mg	mg/100 ml	24.00	n.t.	n.t.	
Zn	mg/100 ml	1.12	n.t.	n.t.	
Cu	mg/100 ml	0.26	n.t.	n.t.	
Mn	mg/100 ml	0.01	n.t.	n.t.	
Al	mg/100 ml	0.03	n.t.	n.t.	

Milk was obtained from ICR, FVB/N and BALB/c mice to determine the concentrations of fat, total fat, total protein, iron (Fe), sodium (Na), calcium (Ca), phosphorus (P), potassium (K), chloride (Cl), magnesium (Mg), zinc (Zn), copper (Cu), manganese (Mn) and aluminum (Al). Abbreviations used: n.t., not tested; SD, standard deviation.

Table 2. Nutrient composition and physical properties of the milk substitutes for mouse and rat pups

nutrients (weight / 100 ml)	milk substitutes	
	for mice	for rats [‡]
Protein (g)	9.40	9.00
Casein	5.75	5.40
Whey	3.64	3.60
Carbohydrate (g)		
lactose	2.40	2.50
Fat (g)	16.00	12.00
Palm oil	4.79	3.60
Coconut oil	3.99	3.00
Soya oil	3.19	2.40
MCT oil	2.39	1.80
Corn oil	1.60	1.20
Cholesterol	0.04	0.04
Minerals (mg)		
Calcium	320.00	315.00
Phosphorus	193.00	207.00
Sodium	96.00	128.00
Potassium	125.00	122.00
Chloride	110.00	160.00
Magnesium	20.00	21.00
Zinc	1.37	2.40
Copper	0.41	0.0006
Fluorine	0.029	0.07
Iodine	0.139	0.14
Iron	4.62	
Manganese	0.02	
Aluminium	0.027	
Vitamins (mg)		
Cyanocobalamin	0.00119	0.00119
Biotin	0.018	0.018
Folic acid	0.079	0.079
Thiamin hydrochloride	0.881	0.881
Pyridoxin hydrochloride	1.25	1.25
Riboflavin sodium phosphate	1.415	1.415
Calcium pantothenic acid	2.643	2.643
p-Amino-benzoic acid	4.404	4.404
Nicotinic acid	4.988	4.988
Sodium ascorbate	47.4	47.4
4-Inositol	49.725	49.725
Choline citrate	0.14	0.14
Vit.E: α -tocopherol	2.35	2.346
Vit.K	1.98	1.982
Vit.A (I.U.)	42.8	42.8
Vit.D (I.U.)	10	10
Others (mg)		
Carnitine	4	3.5
Picolinate	2	2
Ethanolamine	3.4	3.5
Taurine	15	15
Cysteine	22.5	22.5
Serine	28.75	27.5
Tryptophan	27	27.5
Energy contents (Kj/100 ml of milk)	790.2 c	637
Osmolarity (mOsm/kg H ₂ O)	327*	343 $\pm$ 12*
pH	6.51*	6.5 $\pm$ 0.1*
Size of micelles (microns)	1.5*	1.1 $\pm$ 0.1*

c conversion factors in mice: 1 g protein=17 kj, 1 g carbohydrate=16 kj, 1 g fat=37 kj. * values (values $\pm$ SD) obtained by analysis. [‡]nutrient composition of milk substitute for rat pups quoted from Kanno *et al.* [3].

Table 3. Preparation of formula for mouse pups

Steps		
process No.	reagents	weights or volume
1a	NaOH	2.4 g
	KOH	6.8 g
		D.W. 1350 ml
1b	Serine	1.15 g
	Cystine	0.90 g
	Tryptophan	1.08 g
1c	Casein	266.1 g
2	CaCl ₂ ·2H ₂ O	4.4 g
	GlyCaPO ₄	32.0 g
	MgCl ₂ ·6H ₂ O	6.4 g
		D.W. 200 ml
3	CaCO ₃	10 g
	Ca ₃ Citrate·4H ₂ O	4.8 g
		D.W. 100 ml
4	Na ₂ HPO ₄	3.2 g
	KH ₂ PO ₄	0.32 g
		D.W. 50 ml
5	Lactose·H ₂ O	74.05 g
		D.W. 220 ml
6	WPI	160g
		D.W. 600 ml
7	FeSO ₄ ·7H ₂ O	0.92 g
	Citrate·H ₂ O	0.02 g
		D.W. 5 ml
8	ZnSO ₄ ·7H ₂ O	0.24 g
	CuSO ₄ ·5H ₂ O	0.06 g
	MnSO ₄ ·5H ₂ O	0.003 g
		D.W. 5 ml
9	NaF	0.006 g
	KI	0.01g
		D.W. 5 ml
10	Carnitine	0.16 g
	Picolinate	0.08 g
	Ethanolamine	0.136 g
	Taurine	0.6 g
		D.W. 10 ml
11	Na-Choline-Dihydrogencitrate	5.6 g
		D.W. 70.0 ml
	10N-NaOH	1.82 ml
	Vitamin Mix.	20 g
12	Palm oil	191.5 g
	Coconut oil	159.6 g
	Corn oil	63.84 g
	MCToil	95.76 g
	Soya oil	127.68 g
	Cholesterol	0.0016 g
	Vit.K	0.0793 g
	Vit.E	0.0938 g
	Vit A and D mixture	0.0057 g
13		D.W. [138.5]ml

magnesium were added to the casein solution to obtain micelles of casein salts as follows. The salts of $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (4.4 g), glycerophosphoric acid calcium salt (32 g), and $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ (6.4 g) were mixed in 200 ml DW, which was autoclaved (121°C for 10 min) and then homogenized with a Polytron-like mixer (Nihonseiki Kaisha Ltd., Tokyo, Japan). After that, the mixture was slowly added to the protein solution under continuous mixing with an Ultra-Turrax (IKA Labortechnik, Germany). Then, CaCO_3 (10.0 g) and calcium citrate (4.8 g) in 100 ml of DW were also slowly added to the protein solution after autoclaving and homogenization. A mixture of Na_2HPO_4 (3.2 g) and KH_2PO_4 (0.32 g) in 50 ml of DW, sterilized by an autoclave in advance, was added to the casein micelle suspension. Then, a lactose solution (74.5 g of lactose in 220 ml) was slowly added to avoid foaming. Solutions of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (0.92 g) and citrate (0.02 g), ZnSO_4 (0.24 g), CuSO_4 (0.06 g), and MnSO_4 (0.003 g), and NaF (0.0062 g) and KI (0.01 g) were consecutively added to the casein micelle suspension under continuous stirring after sterilization by Millipore filtration (0.45 micrometer). Whey protein (160 g), which was sterilized by gamma-irradiation (30 kGy) in advance, was dissolved in DW (600 ml) and then added to the casein and salt mixture after cooling to below 40°C under continuous stirring. A mixed solution of carnitine (0.16 g), picolinate (0.08 g), ethanolamine (0.136 g), taurine (0.6 g), and a mixture of water-soluble vitamins (20 g) was added after Millipore filter sterilization (0.45 micrometer). Then, fatty vitamins and a mixture of five edible oils were added to the above mixture under continuous stirring after sterilization by boiling in a water bath for 30 min. Finally, the total mixture was weighted up to 4,000 g by adding DW (No.13).

The milk substitute was then homogenized three times under high pressure (180 Kg/cm^2) using a homogenizer (Sanwa Machine Co., Ltd., Numazu, Japan) that had been sterilized twice by circulation of hot alkaline water for 20 min at 75°C heated by bubbling with steam and then cooled with sterilized DW to around room temperature. Portions of the milk substitute (20 or 40 ml) were then poured into 50-ml sterilized polypropylene bottles and stored at -40°C . If necessary, bottles containing milk substitute were gamma-irradiated (30 kGy) and stored at -40°C until use.

The osmotic pressure and pH of the formula were

327 mOsm and 6.51, respectively. No enterobacteria were detected in the milk.

Growth rates of ICR mice fed the milk substitute

The volume of milk substitute fed to the pups was changed daily according to their weight gain relative to the MR pups, and was increased from 0.6 ml on day 8 to 1.2 ml on day 15 (Fig. 1b). As shown by the growth curve in Fig. 1, the body weight of the artificially reared pups decreased on the day following the gastrostomy but increased thereafter. On day 16, the body weight of the AR pups matched that of their MR counterparts. Thirty-six AR pups survived to day 16 and 8 died during the artificial rearing from 8 to 16 days of age.

Discussion

This is the first report of a preparation of a chemically derived milk substitute for mouse pups which was devised to obtain good growth (Fig. 1).

As shown in Table 1, the average concentration of the protein in the milk of ICR, BALB/c and FVB/N mice (11.3%) was the same as that in the SD rat (10.5%) [5]; however, the average concentration of total fat was about two times higher (21.3%) than that in the SD rat milk (11.4%) [5]. With regard to the concentration of minerals, in ICR mice milk, sodium was 2.3 times lower, whereas potassium was 1.3 times higher than in rat's milk. On the other hand, the amounts of calcium and magnesium were almost identical to those found in rat's milk.

Fig 1. Weight gain of the artificially reared mice (AR: n=36) and maternally reared (MR: n=10) mice. AR mice were cannulated at 8 days after birth, and were artificially reared using the chemically derived milk substitute.

The concentrations of vitamins used in the mouse milk substitute were those reported by Auested *et al.* [1] as they were not analyzed in this study.

AR pups grew at almost the same growth rate as MR pups, and were successfully weaned and survived for a further 7 months until the animals were sacrificed (data not shown). However, we think that it will be necessary to determine the concentration of sugar in mouse milks to make a more fitting milk substitute.

We could not artificially rear pups younger than 7 days old, because the rim of the cannula was too big to insert through the esophagus in younger pups, the tube connecting the rim being the minimum diameter that could be commercially obtained. Therefore, if pups need to be examined immediately after birth, another method, such as that developed by Hoshiba [4], would be appropriate. In addition, some modification to the composition of the formula may be required for good digestion in newborn pups. It may be possible to substitute part of the casein content with whey protein hydrolyzate.

In summary, a chemically derived milk substitute compatible with mouse milk for artificially rearing mice pups was developed. This substitute will be useful not only for the artificial rearing of mice but also for identifying dietary components contributing to metabolic influence during the suckling period.

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