表1　陰茎外観における不満

	尿道下裂群（n＝22）	対照群（n＝38）	
不満あり	40.9%（9/22）	34.2%（13/38）	p＝0.809
理由			
サイズが小さいこと	9例（100%）	9例（69.2%）	
包茎	1例（11.1%）	6例（46.2%）	
屈曲	1例（11.1%）	3例（23.1%）	
尿道口の位置	1例（11.1%）		
全体の形	1例（11.1%）	1例（7.7%）	
亀頭の形	1例（11.1%）		
瘢痕		2例（15.4%）	
色調		1例（7.7%）	
陰茎体部の発毛		1例（7.7%）	

複数回答あり

外観に対する不満は下裂群で40.9%，対照群で34.2%と差はなかった。不満の理由は両群とも陰茎のサイズに伴うものが最多であったが，下裂群では不満のある症例全例で陰茎のサイズの不満を訴えていたのに対して，対照群ではサイズが69.2%，包茎が46.2%と不満の内容は様々であった。

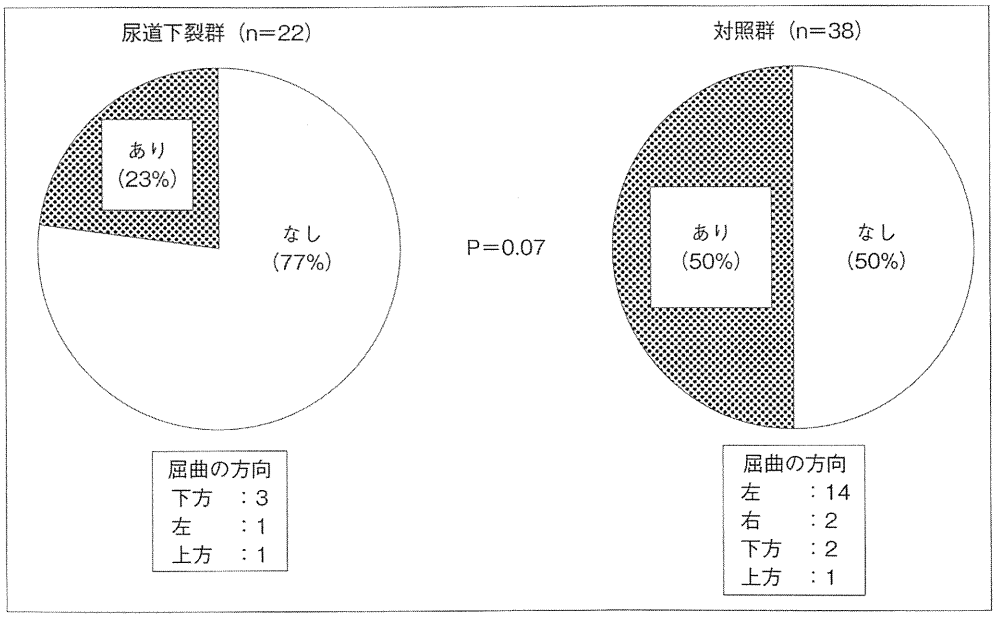

図3　性機能の長期予後：陰茎屈曲の有無とその方向
勃起時の陰茎の屈曲の頻度は，尿道下裂群・対照群で差はなかったものの，屈曲の方向は下裂群では下方への屈曲が最も多かったのに対し，対照群では左方向であった。

性的活動性について質問を行った。外観に対する不満は下裂群で40.9%・対照群で34.2%と差はなかったものの，不満の内容については下裂群では不満を有する症例全例が陰茎のサイズに不満を訴えていたのに対して，対照群ではサイズが69.2%，包茎が46.2%と不満の内容は様々であった(**表1**)。機能に関しては，勃起の強さや性欲には差を認めなかった。勃起時の陰茎の屈曲は，両群でその頻度には差はなかったものの，屈曲の方向は下裂群では下方への屈曲が最も多かったのに対し，対照群では左方向であった（**図3**)。また，勃起時の問題は下裂群で有意に多くみられ（73% vs 45%），その主たる理由は下裂群ではサイズ，対照群では屈曲や勃起が持続しないことであった（**図4**）射精の問題を訴えていたのは下裂群の13.6%で射精時のmilkingやdribblingであった。性交時の問題を訴えていたのはすでに性交渉を経験した症例のうち両群とも15%程度であったが（下裂群18.2%，対照群14.3%），具体的な問題点は下裂群ではサイズであり，対照群では勃起の持続しないことや性交痛，早漏など様々であった(**表2**）性行動に関しては，自慰の回数や開始時期，

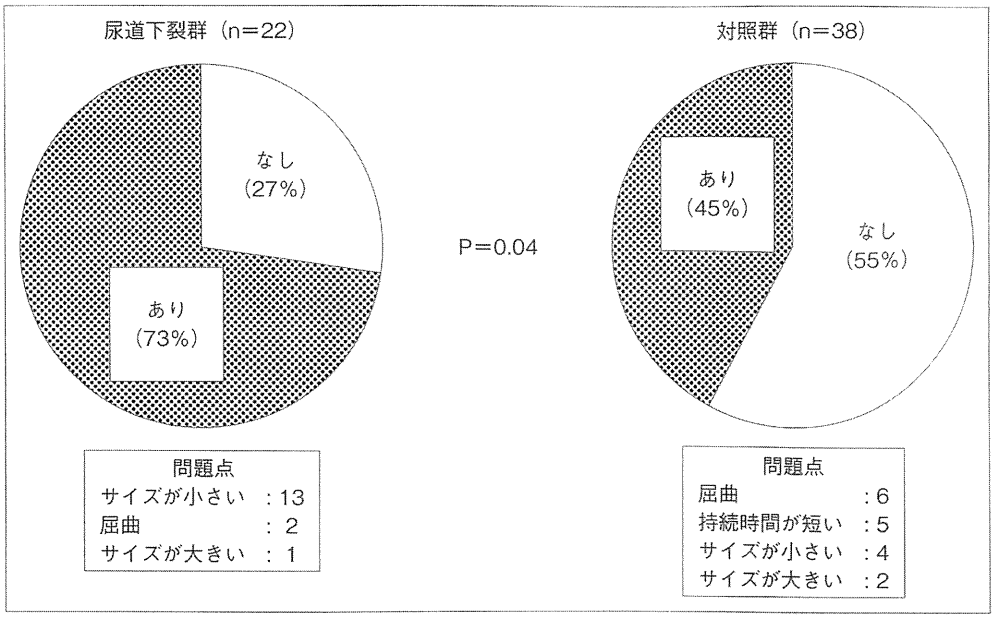

図4　性機能の長期予後：勃起時の問題点
勃起時の問題は尿道下裂群で有意に多くみられ，その主たる理由は下裂群ではサイズ，対照群では屈曲や勃起が持続しないことであった。

表2　射精・性交における問題点

問題点		尿道下裂群	対照群
射精		13.6%（3/22）	0%（0/38）
	Milking	2例	
	Dribbling	1例	
性交		18.2%（2/11）	14.3%（3/21）
	サイズが小さいこと	2例	
	勃起が持続しないこと	1例	1例
	性交痛		1例
	挿入困難		1例
	早漏		1例

複数回答あり

射精の問題を訴えていたのは尿道下裂群の13.6%のみで射精時のmilkingやdribblingであった。性交時の問題を訴えていたのは既に性交渉を経験した症例のうち両群とも15%程度であったが（下裂群18.2%，対照群14.3%），具体的な問題点は下裂群ではサイズであり，対照群では勃起の持続しないことや性交痛，早漏など様々であった。

性交渉の経験率や開始時期，これまでのパートナーの数などに両群間で差はなかった（**表3**）。また，下裂群を近位型，遠位型に分けてみても性行動に差はみられなかった。

われわれの検討をまとめてみると，尿道下裂に対して加療を受けた症例では陰茎のサイズに不満はあるものの，おおむね対照群と同様の性機能・性的活動を有していた。過去の報告を見てみると比較的低年齢で手術を行った場合，思春期には外観に対する不満，特に陰茎のサイズに対する不満はあるものの，性機能および性的活動性に関しては対照群と遜色ない結果となるとされている。しかしながら，手術時年齢が上昇すると性行為への躊躇が強くなるという報告があり[7]，平均初回手術時年齢が11.7歳と高年齢の検討では性行動の一部に差があるという報告がなされている[8]。アメリカ小児科学会では心理学的な面を考慮し尿道下裂の手術時期を生後6ヵ月から1歳程度と推奨しており[9]，本邦でも現在では1歳頃に手術を行うことが多い。このような早期に手術が完了すれば，手術自体の性的活動性への影響はきわめて少ないこと想像に難くない。しかし，それが明らかになるにはもう少し時間が必要であろう。さらに，今回の検討では射精時のmilking・dribblingの訴えがみられた。これは，排尿症状における尿の切れの悪さと同様に現在用いられている手術手技の

表3　性的活動性

		尿道下裂群	対照群
自慰			
	経験済み	100%（21/21）	97.4%（37/38）
	開始時年齢（平均）	13.4±1.4	13.0±1.9
	平均回数／週（中央値）	2.5±1.5	2.5±2.2
性交			
	経験済み	52.4%（11/21）	55.3%（21/38）
	開始時年齢（平均）	16.6±1.8	17.3±1.3
	これまでのパートナー数（中央値）	2.0±2.2	2.5±3.3
	現在パートナーあり	36.4%（4/11）	35.0%（7/20）

性的活動性に関しては，自慰の回数や開始時期，性交渉の経験率や開始時期，これまでのパートナーの数などに両群間で差はなかった。

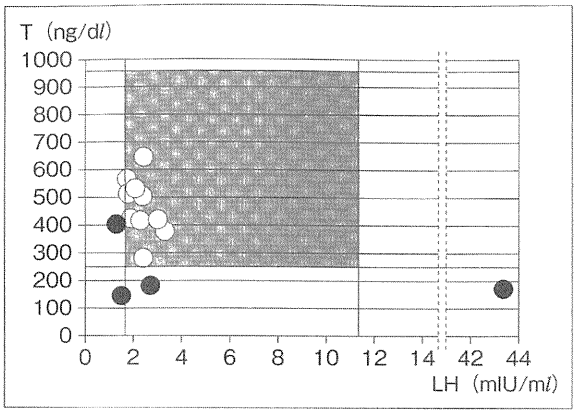

図5　遠位型尿道下裂症例のテストステロン（T）とLHの関係
14例中4例（●）で異常（Hypogonadotropic hypogonadism　1例・hypergonadotropic hypogonadism　1例・低LH血症1例・低テストステロン血症1例）を認めた。（▒：正常域）

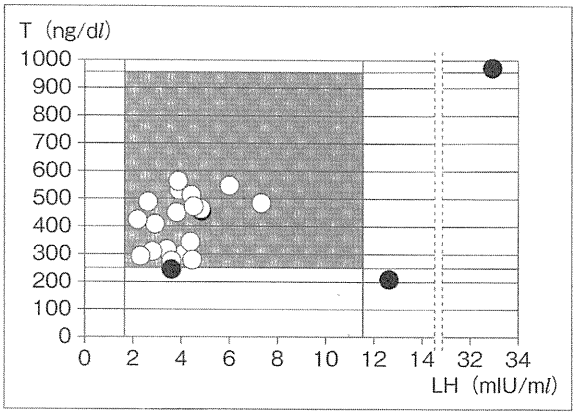

図6　停留精巣のない近位型尿道下裂症例のテストステロン（T）とLHの関係
21例中3例（●）で異常（Hypergonadotropic hypogonadism　1例・低LH血症1例・高LH/高テストステロン（partial androgen insensitivity）1例）がみられた。

持つ問題点と考えられる[4, 5]。

Ⅲ　内分泌学的予後

当科で加療した尿道下裂症例のうち15歳以上で内分泌検査を行った症例の検討を行った[10]。患者背景は遠位型14例，近位型29例で近位型の8例で停留精巣（両側6例，片側2例）を伴っていた。テストステロンとLHの関係を見てみると，遠位型では4例（29%），停留精巣のない近位型では3例（14%），停留精巣合併した近位型では50%で異常を認めた（**図5～7**）。また，造精機能障害のリスクファクターである精巣の委縮（10ml未満）を伴う高FSH血症は全体で7例認められ，その内訳は遠位型では14例中1例(7%)，停留精巣を伴わない近位型では21例中2例(10%)，停留精巣を合併した近位型では8例中4例（50%）であった。尿道下裂の発生には様々な

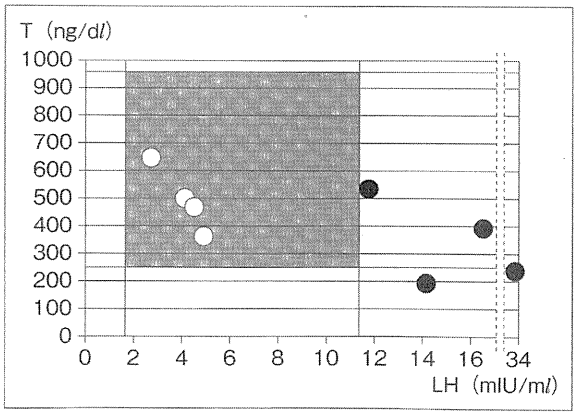

図7　停留精巣を伴う近位型尿道下裂症例のテストステロン（T）とLHの関係
8例中4例（●）で異常（Hypergonadotropic hypogonadism　2例・高LH血症2例）を認めた。

要因が関与しているが，その一つに内因性の内分泌異常が知られている。従来より高度の尿道下裂症例や停留精巣・矮小陰茎などを伴う症例の一部には内分泌学的異常を有する症例がいることが知

られていたが，これまで幼少期の内分泌動態についての報告はいくつかの報告あるものの[11]，思春期以降の報告はごく僅かであった[12, 13]。本検討の結果が示すように，尿道下裂症例の一部には思春期においても内分泌学的異常を呈する症例が存在する。その発生頻度についてはフォローアップの問題もあり今回の検討のみでは明らかではないものの，少なくとも近位型，遠位型ともに異常を認める症例が存在することから，尿道下裂の程度のみで長期的予後を予想することは困難であると考えられた。その半面，停留精巣を伴う症例については内分泌異常や造精機能・父性獲得に障害が疑われる症例が高頻度に存在すると考えられた。

おわりに

冒頭に述べたように尿道下裂治療の目的は外観の正常化とともに良好な排尿が行える尿道を形成すること，および将来の性交渉に問題のない陰茎を形成することにある。本稿で供覧した様に尿道下裂術後の長期予後はおおむね良好であり，手術の目的は多くの患者で達成されていると考えられる。その反面，幼少期に外科的治療を終了した患児の一部は，思春期以降になって顕在化する問題点を有することも明らかになってきている。その問題点は手術手技に起因する（尿の切れ・射精時の milking など）と考えられるものもあれば，尿道下裂と同じ原因によると考えられるもの（陰茎のサイズや内分泌学的問題）も存在する。尿道下裂は小児泌尿器科領域では比較的頻度の高い疾患であり，幼少期に外科的治療が完了することが多く長期間のフォローは困難な場合も少なくない。しかしながら，治療を行う際には上述のような長期的な問題点を医療者側が理解し，患児の家族が抱える長期的な不安に対して十分な説明を行うとともに，少なくとも思春期までのフォローアップが必要なことを説明すべきである。

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Pediatrics International (2010) **52**, 33–38 doi: 10.1111/j.1442-200X.2009.02890.x

Original Article

Exposure to exogenous estrogen through intake of commercial milk produced from pregnant cows

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Abstract ***Background***: Modern genetically improved dairy cows continue to lactate throughout almost the entire pregnancy. Therefore, recent commercial cow's milk contains large amounts of estrogens and progesterone. With regard to the exposure of prepubertal children to exogenous estrogens, the authors are particularly concerned about commercial milk produced from pregnant cows. The purpose of the present study was therefore to examine concentrations of serum and urine sex hormones after the intake of cow milk.

Methods: Subjects were seven men, six prepubertal children, and five women. The men and children drank 600 mL/m^2 of cow milk. Urine samples were collected 1 h before the milk intake and four times every hour after intake. In men the serum samples were obtained before and 15, 30, 45, 60, 90 and 120 min after milk intake. Women drank 500 mL of cow's milk every night for 21 days beginning on the first day of the second menstruation. In three successive menstrual cycles, the day of ovulation was examined using an ovulation checker.

Results: After the intake of cow milk, serum estrone (E1) and progesterone concentrations significantly increased, and serum luteinizing hormone, follicle-stimulating hormone and testosterone significantly decreased in men. Urine concentrations of E1, estradiol, estriol and pregnanediol significantly increased in all adults and children. In four out of five women, ovulation occurred during the milk intake, and the timing of ovulation was similar among the three menstrual cycles.

Conclusions: The present data on men and children indicate that estrogens in milk were absorbed, and gonadotropin secretion was suppressed, followed by a decrease in testosterone secretion. Sexual maturation of prepubertal children could be affected by the ordinary intake of cow milk.

Key words cow milk, estrogen, prepubertal child, sexual maturation, sexual precosity.

During the 1960s and 1970s, with the worldwide spread of the Green Revolution,[1] the possibility of year-round global milk production was realized. Modern genetically improved dairy cows, such as the Holstein, continue to lactate throughout almost the entire pregnancy, extending the milk-producing period to 305 days per year.[2] Therefore, recent commercial cow's milk contains large amounts of estrogens and progesterone.[3–5]

A dramatic increase in estrogen-dependent malignant diseases, such as ovarian, corpus uteri, breast, testicular and prostate cancers has been recognized.[5–8] Ganmaa *et al.* investigated the incidence and mortality of testicular and prostate cancers in relation to dietary practices. Among various food items, cow's milk and cheese had the highest correlation with incidence and mortality rate of these cancers.[5,7,9] They also investigated the correlation between food consumption and incidence rates of breast, ovarian and corpus uteri cancers. The intake of milk, meat and cheese was closely correlated with those cancers.[8]

Among the exposure of humans, especially prepubertal children, to exogenous estrogens, we are particularly concerned with commercial milk produced from pregnant cows. In Japan, milk is produced predominantly by lactating cattle, and approximately 80% of this milk originates from pregnant cows. In prepubertal children there is little secretion of estrogens, and serum 17β-estradiol (E2) concentration is undetectable (<2 pg/mL) in a conventional enzyme immunoassay.[10] Therefore, exposure to small doses of estrogens may have adverse effects on growth and maturation in prepubertal children. But because measuring the concentration of estrogens in milk is considerably difficult, concentrations reported by several analysts range widely from low to extremely high.[3–5,11–13]

In the present study we examined the concentration of estrogens and progesterone in the serum and urine of young men and prepubertal children after the intake of cow's milk. Moreover, we investigated the influence of daily milk intake on the menstrual cycles of healthy women. If the milk contains high

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Received 23 July 2008; revised 22 April 2009; accepted 8 May 2009.

Table 1 Men: subject characteristics

	Height (cm)	Weight (kg)	Body surface (m^2)	Milk intake (mL)
1	177	73.0	1.90	1140
2	174	83.0	1.97	1182
3	161	66.0	1.69	1014
4	167	71.4	1.81	1086
5	162	59.0	1.62	972
6	175	60.0	1.73	1038
7	178	64.0	1.80	1080

concentrations of estrogens and progesterone, the timing of ovulation may be affected by successive milk intake.

Methods

The subjects included healthy young men aged 19–21 years (Table 1). All of the men drank a volume of 600 mL/m^2 (body surface) of milk within 10 min.

Seven prepubertal children were enrolled in the study (Table 2). Four of the seven children drank a volume of 600 mL/m^2 of milk, but two of them could only drink 61% and 73% of the milk volume. Another girl could not drink half of the volume. Six children except this girl took part in this study.

Five women who had regular menstruation were included in the study (Table 3). Four of the five women had menstrual cycles of 28 days, but one woman aged 36 years had a regular cycle of 36 days. Four of the women did not regularly drink cow's milk, and one woman had a cup of milk every morning.

The cow's milk used for the study was commercially available cow's milk containing more than 3.5% fat.

Procedure

Men

Intake of milk and dairy products was prohibited for 3 days prior to the study. All of the men drank 600 mL/m^2 of cow's milk within 10 min. Urine samples were collected 1 h before the milk intake and four times every hour after the intake. The volume of the urine samples was measured, and they were stored at −20°C for 2 days. Subsequently, urine concentrations of estrone (E1), E2, estriol (E3) and pregnanediol were measured. Urine excretion volume of these hormones every hour was calculated as follow; Urine excretion volume of hormone (ng or μg/h) = urine concentration of hormone (ng or μg/mL) × urine volume (mL/h). Urine data are expressed as excretion volume per hour. Serum

Table 3 Women: subject characteristics

	Age (years)	BMI	Menstrual cycle (days)
1	19	17.7	29–35
2	19	21.8	25–27
3	31	21.7	29–35
4	32	20.3	24–29
5	36	23.4	37–39

BMI, body mass index.

samples were obtained before and 15, 30, 45, 60, 90 and 120 min after the milk intake. Serum concentrations of E1, E2, luteinizing hormone (LH), follicle-stimulating hormone (FSH) and testosterone were measured. Hormones levels of urine and sera were measured at Special Reference Laboratory (Hachioji, Tokyo, Japan). Serum E1 levels were measured on radioimmunoassay (RIA), and E2, testosterone and progesterone, on electrochemiluminescence immunoassay. Serum LH and FSH levels were measured on chemiluminescence immunoassay. Urine estrogens (E1, E2, E3) were measured on RIA, and pregnanediol on gas chromatography–mass spectroscopy.

Prepubertal children

Intake of milk and dairy products was prohibited for 3 days prior to the study. Intake of milk volume was 600 mL/m^2, but two out of the six children could drink only 61% and 73% of the volume within 10 min, respectively. In children, serum samples were not obtained, and urine samples were collected in a method similar to that of the adults, and the concentration of E1, E2, E3 and pregnanediol was measured.

Women

In three successive menstrual cycles, basal body temperature was measured, and the day of ovulation was examined on measurement of urine LH concentrations using an ovulation checker from 17 days before the expected first day of the next menstruation cycle. All of the women drank 500 mL of cow's milk every night for 21 days beginning on the first day of the second menstruation. The day of ovulation in the second menstrual cycle during which milk was consumed was compared with that of the first and third menstrual cycles.

Statistical analysis

The data were analyzed using SPSS II (SPSS, Chicago, IL, USA). Non-parametric Wilcoxon signed rank tests were

Table 2 Prepubertal children: subject characteristics

	Age Years : Months	Sex	Height (cm)	Weight (kg)	Body surface (m^2)	Body surface-based milk volume (mL)	Total milk intake (mL) (%)
1	8 : 8	M	127.0	26.0	0.96	580	580 (100)
2	7 : 3	M	119.8	19.5	0.82	490	490 (100)
3	8 : 8	M	125.0	24.0	0.92	550	340 (62)
4	7 : 6	F	122.0	22.0	0.92	550	550 (100)
5	8 : 8	F	122.2	21.8	0.87	520	520 (100)
6	9 : 9	F	129.6	32.6	1.07	640	470 (73)
7	7 : 7	F	117.6	19.6	0.81	490	180 (37)

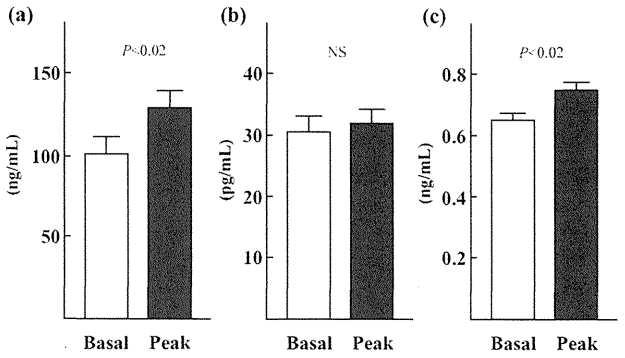

Fig. 1 Comparison between basal levels and peak levels of (a) serum estrone (E1), (b) estradiol (E2) and (c) progesterone before and after intake of cow milk in men (n = 7).

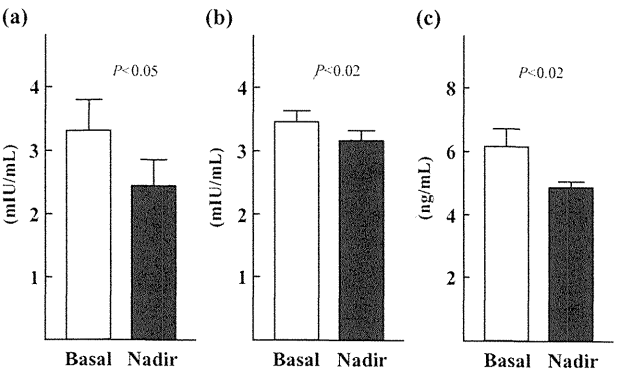

Fig. 2 Comparison between basal and nadir levels of (a) serum luteinizing hormone, (b) follicle-stimulating hormone and (c) testosterone before and after intake of cow milk in men (n = 7).

performed to examine the difference of hormone concentrations before and after the intake of milk. $P < 0.05$ was taken as significant.

Approval for the present study was obtained from the ethics committee of the University of Yamanashi School of Medicine.

Results

Men

Serum basal and peak concentrations of E1, E2 and progesterone during examination of the milk intake are shown in Figure 1. Serum E1 concentration was significantly increased and peaked 30–60 min after the intake of milk (mean ± SE, before and peak: 102.3 ± 10.3 pg/mL and 128.9 ± 11.8 pg/mL, $P < 0.02$). Serum E2 concentration was unchanged during the 2 h examination (before and peak: 31 ± 4 pg/mL and 32 ± 4 pg/mL, NS). Serum progesterone concentration significantly increased and peaked 30–60 min after the intake of milk (mean ± SE, before and peak: 0.66 ± 0.08 ng/mL and 0.75 ± 0.10 ng/mL, $P < 0.02$).

Serum basal and nadir concentrations of LH, FSH and testosterone before and after milk intake are shown in Figure 2. Serum LH and FSH concentration gradually decreased in six out of seven men, and reached a nadir 60–120 min after the intake of milk (before and lowest point: LH, 3.29 ± 0.49 mIU/mL and 2.48 ± 0.43 mIU/mL, $P < 0.05$; FSH, 3.43 ± 0.17 mIU/mL and 3.19 ± 0.15 mIU/mL, $P < 0.02$). Serum testosterone concentrations decreased considerably 120 min after intake in all subjects (before and lowest point: 6.04 ± 0.38 ng/mL and 4.94 ± 0.13 ng/mL, $P < 0.02$).

As shown in Figure 3, the volume per hour of urinary excretion of E1, E2, E3 and pregnanediol significantly increased after the intake of milk in all subjects ($P < 0.02$). Urine E1 excretion increased 1 h after intake, and reached a peak 4 h after intake in five out of seven men. Urine E2 excretion increased 1 h after the intake in six out of seven men. Peak excretion of E2 in urine was detected after 1 h in three subjects, and after 4 h in another three subjects. Urine E3 excretion also increased after 1 h in six out of seven men, and reached peak levels after 4 h in five men. Urine preganediol excretion peaked after 1 h in two out of seven men, and after 4 h in another four men.

Prepubertal children

Changes in urinary excretion volumes of E1, E2, E3 and pregnanediol are shown in Figure 4. Urinary excretion patterns were similar among these four hormones. Peak excretion volume of

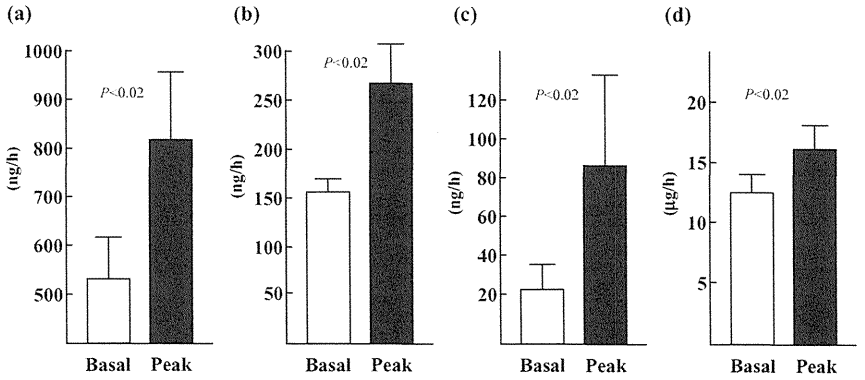

Fig. 3 Comparison between basal excretion volumes (basal) and maximum excretion volumes (peak) of (a) urine estrone, (b) estradiol, (c) estriol and (d) pregnanediol before and after intake of cow milk in men (n = 7).

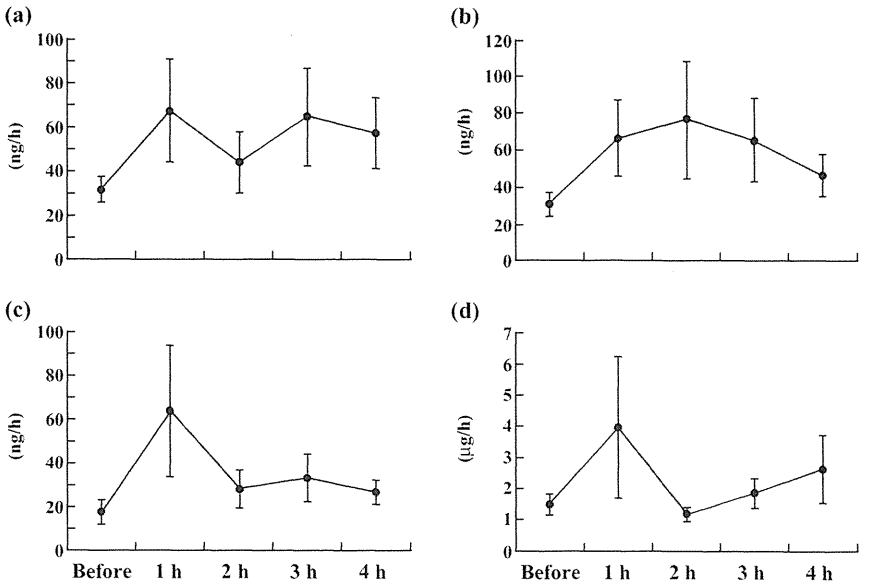

Fig. 4 Changes in mean urine excretion volume of (a) estrone, (b) estriol, (c) estradiol and (d) pregnanediol after intake of cow milk in prepubertal children (mean ± SE, $n = 6$).

the hormones was detected at 1 h in three children, at 3 h in three children, and at 4 h in one child. The net increase in volume of urine excretion of E2 at 4 h after the intake of milk ranged from 39 to 109 ng. Urinary basal and peak excretion volumes of E1, E2, E3 and pregnanediol before and after milk intake are shown in Figure 5. Urinary excretion of these hormones significantly increased after intake ($P < 0.02$).

Women

In four out of five women, ovulation occurred during milk intake in the second menstrual cycle, and the timing of ovulation was similar among the three menstrual cycles. In these four women, the third menstruation and ovulation occurred regularly. In one woman, however, aged 36 years who had a menstrual cycle of 37–39 days, ovulation did not occur during the intake of milk. She ovulated 7 days after stopping milk intake.

Discussion

Toxicological and epidemiological studies have indicated that E2 could be categorized as a carcinogen.[14] Milk is considered to be a rich source of estrogens. Indeed, E2 concentration is higher in mammary drainage than in the peripheral circulation in high-yielding cows.[12] Pregnant cows are under the control of relatively high levels of estrogens, and milk produced from pregnant cows contains correspondingly high concentrations of estrogens. Estrogen concentration in milk has been measured since the 1970s, mainly as an indicator of pregnancy.[15,16] Concentration of E1 sulfate increases from 30 pg/mL in non-pregnant cows to

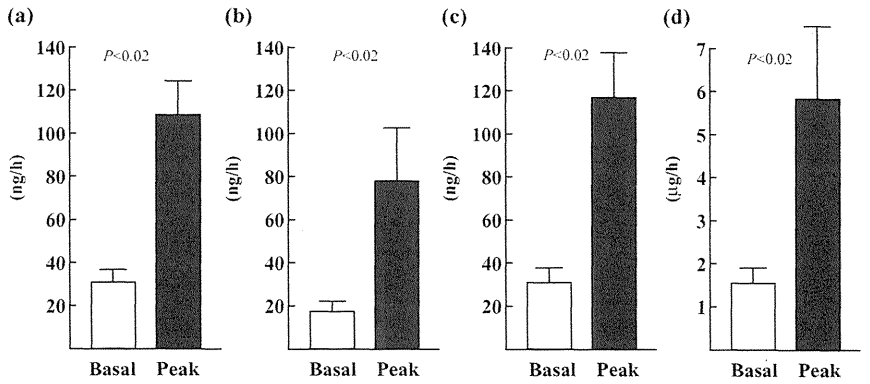

Fig. 5 Comparison between basal excretion volumes (basal) and maximum excretion volumes (peak) of (a) urine estrone, (b) estradiol, (c) estriol and (d) pregnanediol before and after intake of cow milk in prepubertal children ($n = 6$).

151 pg/mL in pregnant cows at 40–60 days of gestation, and to a maximum level of 1000 pg/mL in cows at 220 days of gestation.[16] Recently, according to Malekinejad *et al.*, the concentration of estrogens in cow's milk under various conditions was measured using liquid chromatography–tandem mass spectrometry after enzymatic deconjugation.[4] Their results indicated that processed milk with 3.5% fat contains high concentrations of E1 and E2. Qin *et al.* also reported high concentrations of estrogen in pregnant cow's milk.[5] Moreover, milk and milk products contain high amounts of progesterone, which accumulate with increasing milk-fat content.[3]

In the present study in men, concentrations of serum E1 and progesterone increased after intake of cow's milk, concentrations of serum LH, FSH and testosterone significantly decreased 2 h after intake, and the volume of urine excretion of E1, E2 and E3 significantly increased. These results suggest that estrogens in milk were absorbed, and gonadotropin secretion was suppressed, followed by a decrease in testosterone secretion. The activation of a negative feedback mechanism due to exogenous estrogens in cow's milk indicates that men are affected by intake of commercial cow's milk. Because the main estrogen in milk is E1, and E2 concentration is relatively low compared to E1, serum E2 levels do not change for 2 h after the intake, but instead urine excretion of E2 significantly increases, suggesting that the conversion from E1 to E2 progresses slowly.

In prepubertal children, excretion volumes of estrogens and pregnanediol significantly increased 1–3 h after intake. The net increase of E2 excretion from the basal level (E2 in urine before the intake) was 39–109 ng/4 h in the present study.

In prepubertal boys, serum E2 level measured on ultrasensitive recombinant cell bioassay is 0.08 ± 0.2 pg/mL.[10] Based on these data, the E2 production rate is 40 ng/day in prepubertal boys.[17] The E2 in urine may be equal to or more than the daily E2 production rate in prepubertal boys. Reliable data on the daily production rate of E2 are still lacking in prepubertal children. Andersson and Skakkebaek reported that the conventional E2 production rate, according to the JECFA 1988 report, was presumably highly overestimated.[17] Sheehan tested the hypothesis that no threshold exists when estradiol acts through the same mechanism as an active endogenous estrogen, and he found evidence that contradicted the threshold assumption and low-dose safety.[18] Premature thelarche, gynecomastia, and pubertal growth spurt occur at very low or undetectable serum E2 levels, suggesting that prepubertal children are highly sensitive to estrogens.[19] Serum E2 level (0.6 ± 0.6 pg/mL) in prepubertal girls was significantly greater than the level (0.08 ± 0.2 pg/mL) in prepubertal boys.[10] Although this gender difference is extremely small in absolute figures, the higher level of E2 in girls may explain their earlier pubertal onset and growth spurt. Growth-promoting effects of very low doses of estrogen (25 ng/kg per day of ethinylestradiol) have also been observed in Turner syndrome.[20] Even small changes in serum E2 concentrations within the extremely low prepubertal range may, therefore, have significant biological implications. The present data on men and children indicate that the intake of estrogens from 600 mL/m^2 of cow's milk may correspond to the daily estrogen production rate in prepubertal boys, and height growth and sexual maturation of prepubertal children could be affected by normal intake of cow's milk.

Recent surveys on the timing of pubertal onset show an alarming trend of earlier sexual maturation in girls.[21] Anderson *et al.* reported a drop of approximately 2.5 months in the average age of menarche between 1963–1970 and 1988–1994 in US girls, and referred the relationship of earlier menarche to increased body mass index (BMI).[22] Several reports concerning puberty in girls suggest a positive correlation between the timing of breast development or menarche and that of increases in BMI.[23–25] Because adipose tissue is a source of estrogens, the cause of earlier sexual maturation may not only be a change in nutritional status, but also an increase in estrogen secretion from adipose tissue. Exposure to exogenous estrogens through intake of commercial milk produced from pregnant cows has spread around the world since the 1970s. We think that the intake of pregnant cow's milk is one of the major causes of early sexual maturation in prepubertal children.

The menstrual cycle in women is controlled by relatively high levels of E2 and progesterone. Commercial milk produced from pregnant cows contains not only estrogens but also progesterone.[3] The prolonged intake of estrogen and progesterone compounds may affect the timing of ovulation. We examined the effect of the intake of cow's milk for 21 consecutive days from the start of the last menstruation to ovulation. The timing of ovulation in four of five women was not affected by the intake of milk. These four women were healthy young women, but the fifth woman suffered from oligomenorrhea of a 37–39 day cycle. Her ovulation occurred 7 days after the discontinuation of milk. These results suggest that ovulation in women with subclinical hypogonadism might be affected by an abundant intake of milk, although normal menstrual cycles are not influenced.

Since 1985, daily intake of cow's milk has been extensively recommended, especially to prepubertal children in Japan. The average age of menarche in girls living in metropolitan Tokyo occurred at 12 years 5 months in 1987, and 12 years 3 months in 1993.[26] These findings and the present data indicate that the intake of cow's milk may cause earlier sexual maturation. The relationship between estrogens in pregnant cow's milk and sexual maturation in children must be acknowledged as an important theme.

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Journal of Pediatric Endocrinology & Metabolism, 23, 153-158 (2010)

Late-Onset Circulatory Dysfunction After Thyroid Hormone Treatment in an Extremely Low Birth Weight Infant

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ABSTRACT

Late-onset circulatory dysfunction (LCD) is a phenomenon specific to premature infants and is characterized by sudden onset of hyponatremia, hypotension, oliguria and non-physiological weight gain, without an obvious cause, in premature infants after stabilization of circulation and respiration. The cause of LCD is not clear, but adrenal insufficiency in premature infants is a severe syndrome because steroid replacement therapy is often essential to treat the symptoms. We report a rare case of a premature infant who developed an LCD crisis the day after thyroxine replacement therapy. The female infant was born at 25 weeks of gestational age, weighing 672 g, and appeared to have hypothyroidism, with free T4 of 0.19 ng/dl and elevated TSH levels of 26.3 μIU/ml at Day 14. She developed an LCD crisis the day after starting thyroxine treatment. She received steroid replacement therapy for 4 weeks and her adrenal function progressively recovered. She also needed thyroxine supplementation for 13 weeks, which maintained her thyroid function as euthyroid. Because she exhibited cortisol insufficiency and thyroid hormone insufficiency, the antecedent thyroid hormone replacement may be responsible for the onset of LCD. We must consider monitoring adrenal function when starting thyroxine therapy in premature infants with hypothyroxinemia.

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KEY WORDS

late-onset circulatory dysfunction, hypothyroidism, extremely low birth weight infant, adrenal insufficiency

INTRODUCTION

Premature infants often have poor pituitary function and hormone synthesis, and often develop relative hormone insufficiency. In Japan, a number of premature infants with late-onset circulatory dysfunction (LCD; or late-onset circulatory collapse) have been reported[1]. This syndrome is classified as adrenal insufficiency of prematurity (AOP) when steroids need to be administered to overcome impaired adrenal function. LCD is usually characterized by sudden onset of hyponatremia, hypotension, oliguria, and non-physiological weight gain, without an obvious cause, in infants after stabilization of circulation and respiration. Some LCD cases are considered to show relative adrenal insufficiency because volume expanders (physiological saline or plasma albumin agents) and inotropic agents are often ineffective, whereas steroid replacement therapy is usually effective[2]. On the other hand, hypothyroxinemia is often reported in premature infants[3], and many trials of thyroxine replacement therapy (predominantly levothyroxine) have been reported[4]. However, it is unclear whether thyroxine replacement is effective in terms of neurodevelopmental outcome in premature infants. Some cases of premature infants who developed LCD after receiving thyroxine treatment for hypothyroxinemia have recently been experienced in Japanese neonatal intensive care units (NICU). These cases have not been reported yet, and the relationship between thyroxine therapy and LCD

is unclear. Therefore, we report here an extremely low birth weight infant who developed LCD after thyroxine treatment.

PATIENT REPORT

A female infant was born at the gestational age of 25 weeks and 1 day, with a birth weight of 672 g and height of 31.5 cm. She was the first child of healthy parents of Brazilian nationality. Her mother was admitted to our hospital due to premature rupture of the membranes, and her laboratory data were WBC 13,000/μl and CRP 0.56 mg/dl. Therefore, continuation of pregnancy was difficult and an emergency Cesarean section was performed. Antenatal steroids were not administered because of the emergency delivery. The infant had a 1-min Apgar score of 5 and a 5-min score of 7. Therefore, she was intubated while in the delivery room for positive pressure ventilation and she was then admitted to our NICU.

The clinical course of the emergency period is shown in Figure 1. She was treated with intratracheal surfactant due to respiratory distress syndrome; antibiotics, gamma-globulin and granulocyte-colony stimulating factor due to infection; and inotropic agent (dopamine) and indomethacin because of the presence of patent ductus arteriosus. After stabilizing her respiratory and circulatory status, breast-milk feeding was started at day 5 and her body weight increased slightly.

Fourteen days after birth, her laboratory examination revealed hypothyroidism; her serum free T3 concentration was 1.65 pg/ml, free T4 was 0.19 ng/dl and TSH was 26.3 μIU/ml. Therefore, thyroxine therapy (5.0 μg/day) was commenced. The next day (Day 15), she suddenly developed hyponatremia (serum Na: 129 mEq/l), hypotension (blood pressure: 45/24 mm Hg; <80% of the mean for the previous day), oliguria (urine volume: 0.9 ml/kg/h), and non-physiological weight gain with severe edema. We performed blood examinations, culture and ultrasonography; sepsis, PDA and intraventricular hemorrhage (IVH) were excluded as possible causes of hypotension. Thyroid function on Day 14 and emergency data on Day 15 are shown in Table 1. Because her serum cortisol concentration was very low (2.0 μg/dl), relative adrenal insufficiency was suspected.

Based on these findings, we diagnosed LCD and started steroid therapy (hydrocortisone, 1 mg/dose, twice per day), and continued thyroxine therapy. Her blood pressure and urine volume improved within 24 hours, she was able to continue milk feeding, and showed weight gain. The clinical course after steroid therapy is shown in Figure 2. Her steroid therapy was continued for 40 days after the first administration. Thyroxine therapy was set to 5.0 μg/day between Days 15 and 116. On Day 116, her body weight had increased to 1,700 g and thyroxine was discontinued. Her thyroid hormone status was maintained as euthyroid and, 1 month later, her TSH, free T3 and free T4 levels were 2.58 μIU/ml, 3.68 pg/ml and 1.28 ng/dl, respectively. On day 160, we performed a CRH loading test to assess her adrenal function, and the results are shown in Figure 3. The CRH loading test revealed normal adrenal function, although a TRH administration test was not performed at that time. The patient was discharged on Day 165 weighing 3,586 g and continued treatment at an outpatient department. At this time, she had chronic lung disease (CLD) and mild deafness, without periventricular leukomalacia (a major complication associated with LCD).

DISCUSSION

Here, we have reported a rare case of an extremely low birth weight infant who had cortisol insufficiency and thyroid hormone insufficiency. We considered that it would be dangerous to treat hypothyroidism in a premature infant, and it was difficult to assess the association between LCD and thyroxine therapy.

The underlying pathogenesis of LCD is obscure although it seems to represent adrenal dysfunction because intravenous steroids are effective in some cases. Relative adrenal insufficiency occurs when an infant's cortisol response is inadequate for the degree of illness or stress[5]. The diagnostic criteria for LCD are now considered to include the sudden development of hypotension or oliguria requiring treatment, without obvious cause, in preterm infants with

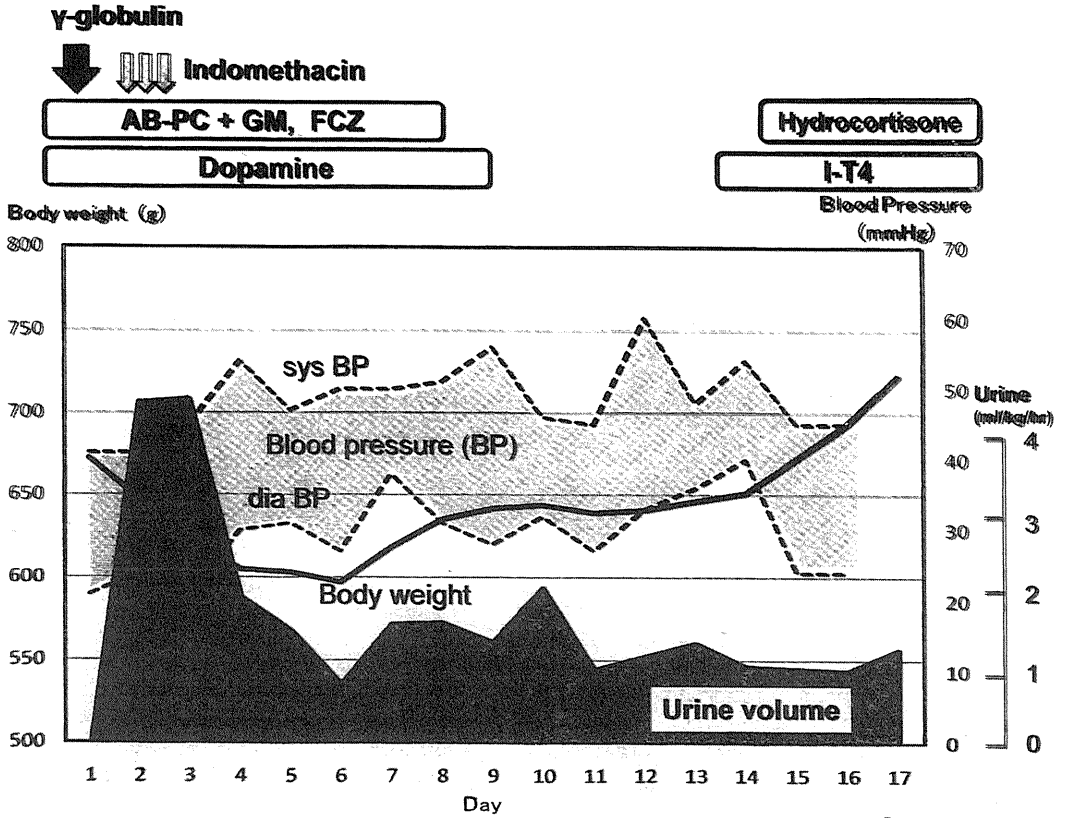
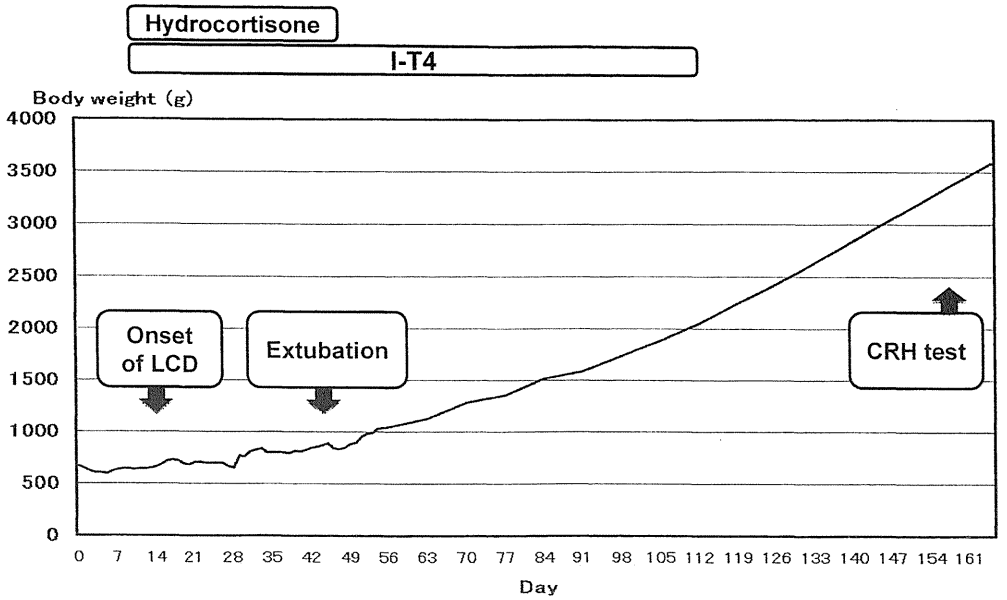


Fig. 1: Clinical course of the emergency period (Days 0–17 after birth). The upper broken line represents systolic blood pressure and the lower broken line represents diastolic blood pressure. The solid line represents body weight. The lower area represents urine volume.

TABLE 1

Laboratory findings at onset of LCD (Day 15 after birth)

Blood cell count		**Biochemistry**		**Endocrinology**	
WBC	14000 /μl	CK	424 IU/l	ACTH	51.9 pg/ml
RBC	329×10⁴/μl	BUN	17.5 mg/dl	Cortisol	2.0 μg/dl
Hb	12.1 g/dl	Crtn	2.55 mg/dl	17-OHP	9.4 ng/ml
Ht	35.5 %	Na	129 mEq/l		
Plt	29.6×10³/μl	K	6.4 mEq/l	**Thyroid function**	
Blood Gas Analysis		Cl	98 mEq/l	(day 14)	
pH	7.362	Glucose	75 mg/dl	TSH	26.3 μIU/ml
pCO_2	46.5 mmHg	CRP	0 mg/dl	free-T_3	1.65 ng/ml
pO_2	32.2 mmHg	Lactate	12 mmol/l	free-T_4	0.19 pg/ml
HCO_3^-	25.8 mmol/l	**Urinalysis**			
BE	0.6 mmol/l (V-line)	Na	45.4 mEq/l		
		FENa	5.6		



Day	14	25	42	116	147
TSH (μIU/ml)	26.36	2.56	0.67	3.38	2.58
fT3 (ng/ml)	1.65	1.41	1.89	2.68	3.68
fT4 (pg/ml)	0.19	0.33	0.96	0.91	1.28

Fig. 2: Clinical course of body weight and thyroid function during hospitalization (Days 0-165 after birth). The solid line represents body weight.

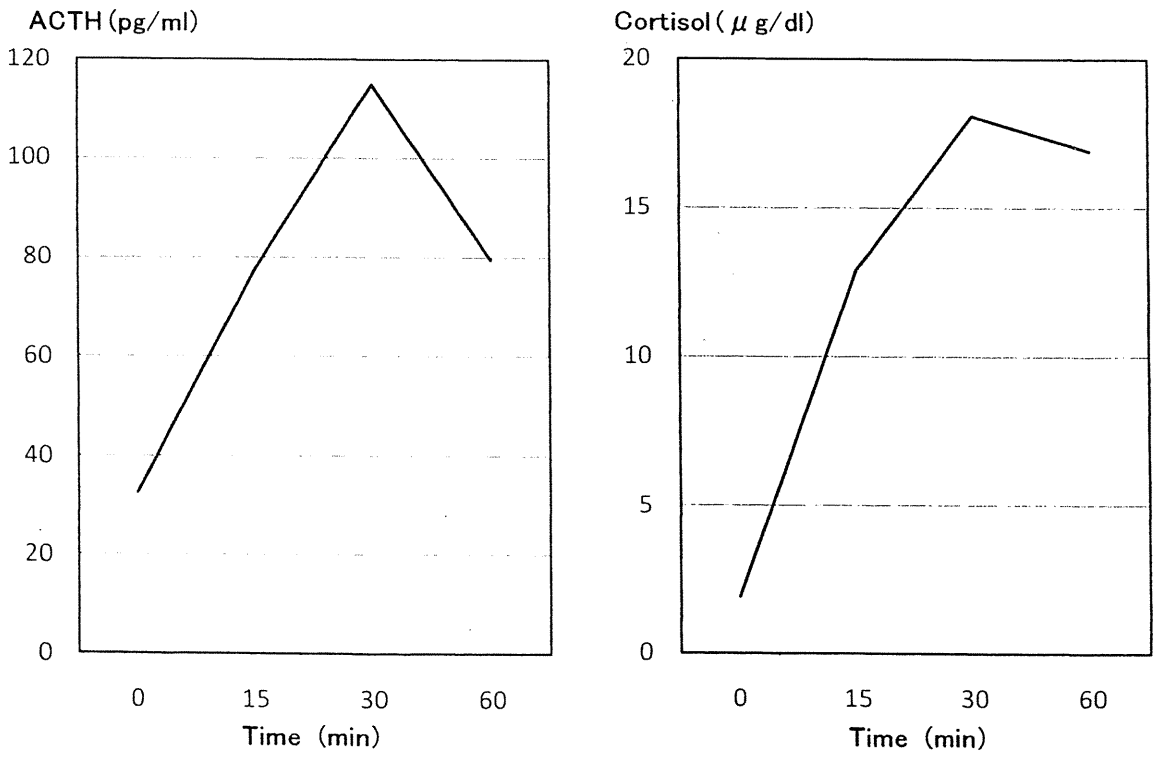


Fig. 3: Result of the CRH loading test on Day 160 after birth. CRH load: 1 μg/kg iv.

stable circulatory and respiratory conditions for several days. Hypotension is defined as a blood pressure <80% of the mean value before the episode, and oliguria is defined as at least one of the following: (1) passed less than 50% of the urine volume before the episode over 8 hours; (2) passed <1 ml/kg/h over 8 hours; or (3) anuria lasting 4 hours[6]. We believe our patient met these criteria, but steroid therapy was not completely successful because she developed steroid dependence and steroid replacement therapy could not be withdrawn for 40 days, while the mean steroid therapy period reported is within 8 days. The concomitant thyroxine therapy likely affected her steroid dependence. At the onset of LCD, her serum cortisol was low (2.0 μg/dl), which was considered to be due to adrenal immaturity, which was considered to be insufficient to maintain homeostasis during acute stress or clinical syndromes such as hypotension. Thus, the best therapeutic plan was considered to be to maintain observation until her adrenal function had matured. In fact, her adrenal function, as determined by the CRH stimulation test on Day 160, was nearly normal.

In preterm infants, thyroxine (T4) and triiodothyronine (T3) levels are lower than those in term infants, and thyroid function in premature infants is characterized by decreased serum free T4 and TSH levels during the first 2-4 postnatal weeks of life, a condition known as transient hypothyroxinemia of prematurity[7]. On the other hand, hypothyroxinemia with elevated TSH levels is suspected to represent congenital hypothyroidism and the administration of thyroid hormone should be started immediately[8]. Thyroid function in the present infant on Day 14 revealed hypothyroidism with elevated TSH levels; therefore, we did not hesitate to commence thyroid hormone replacement. However, she was successfully withdrawn from thyroid hormone replacement, and we believe this case represents transient hypothyroidism.

In Japan, some cases of LCD after thyroxine therapy have been experienced. The incidence of LCD is 11.6% in extremely low birth weight infants (birth weight below 1,000 g), and 9.3% of all cases of LCD occurred after thyroxine therapy[9]. However, it is unclear whether thyroid hormone affects the onset of LCD, and there are no clear reports describing such events in the literature. Another report suggested that some cases of LCD may occur in premature infants with hypothyroxinemia and elevated TSH[10]. Therefore, thyroxine therapy should be started carefully in infants with immature adrenal function. In patients with complex hypopituitarism, glucocorticoids should be administered before starting thyroid hormone replacement, because thyroid hormone administration in hypothyroid patients increases the requirement for glucocorticoids during stress[11]. Our patient exhibited adrenal insufficiency and low cortisol production and, because thyroid hormone activates cortisol metabolism, she was unable to compensate for hypotension and was dependent on steroids for one month. Therefore, assessment of adrenal function (e.g., serum cortisol concentration) is important when starting thyroxine therapy. Further studies are needed to clarify the pathophysiology of LCD and the association between LCD and hypothyroidism.

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Biosci. Biotechnol. Biochem., **74** (7), 1466–1469, 2010

Note

Expression of the Centrosomal Colon Cancer Autoantigen Gene during Spermatogenesis in the Maturing Rat Testis

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Received January 18, 2010; Accepted April 8, 2010; Online Publication, July 7, 2010
[doi:10.1271/bbb.100044]

We analyzed the gene and protein expression of serologically defined colon cancer antigen 8. Gene expression was upregulated in the maturing rat testis, and was localized to the spermatocytes. Protein was detected in the spermatids and at the sites of mRNA expression. Specific expression of colon cancer antigen 8 was observed in the maturing rat testis.

Key words: centrosomal colon cancer autoantigen protein; immunohistochemistry; *in situ* hybridization; serologically defined colon cancer antigen 8; spermatogenesis

Spermatogenesis is a complex process during which many genes are systematically expressed. Male reproductive cells are arranged in the seminiferous tubules from the basement membrane as spermatogonia, spermatocytes, and spermatids. Spermatogonia undergo mitosis to form primary spermatocytes, which undergo meiosis to form spermatids. We have screened the genes expressed during this process using differential display (DD). The genes classified and characterized so far include (i) molecular motor proteins that transfer cytoplasmic cellular substances (*e.g.*, kinesin);[1)] (ii) cytoplasmic regulators of membrane trafficking (*e.g.*, RabGAP/TBC);[2)] (iii) molecular chaperones (*e.g.*, the small heat shock protein Hsp20, which is a member of the αB crystallin-related protein family);[3)] (iv) the sperm flagellum-movement associated protein ODF1 and an adenylate kinase domain-containing protein that catalyzes ATP synthesis;[4)] and (v) tumor suppressor gene products in relation to Ha-ras (*e.g.*, hrasls5).[5)]

In addition to these genes, we identified a novel class of gene that was specifically expressed during spermatogenesis. This gene product was identified as serologically defined colon cancer antigen 8 (SDCCAG8) or centrosomal colon cancer autoantigen protein (CCCAP),[6)] and its C-terminal portion was identified as serologically defined human colon cancer autoantigen (NY-CO-8). These cancer autoantigens were initially screened by serological analysis of a recombinant cDNA expression library (SEREX) derived from human tumors. During this process, a range of antigens (NY-CO-1 to 48) was screened.[7)] The *Sdccag8* gene product was determined to be a component of the centrosome. It possessed a coiled-coil domain in its C-terminus. Coiled-coil domains are found in many proteins (*e.g.*, structural proteins, motor proteins, and transcription factors) that interact with other proteins to perform specific functions. In the case of the bacteriophage Mnt repressor, dimerization of two anti-parallel coiled-coil structures is required to bind DNA and regulate gene expression controlling the switch between the lysogenic and lytic growth cycles of bacteria.[8)] Mouse CCCAP has also been determined to be capable of homo-oligomerization using the yeast 2-hybrid system.[6)] These observations suggest that this gene product has certain specific functions related to spermatogenesis.

Since the function of the *Sdccag8* gene product is largely unknown in relation to spermatogenesis, we determined its mRNA and protein expression in maturing rat tissues.

Gene expression in 7-week-old rat testes was compared with the expression observed in 3-week-old rat testes by DD. We identified several differentially regulated gene fragments and analyzed the expression of rat *Sdccag8* (accession no. NM_177929). Gene expression was determined by Northern blotting. Five μg of RNA was electrophoresed in a formaldehyde-containing agarose gel, and blotted onto a Hybond N^+ membrane (GE Healthcare, Buckinghamshire, UK). The 582-bp PCR product containing the 5′ non-coding and coding cDNA fragments of *Sdccag8* was used as a probe in the Northern blot experiments. Probe DNA was labeled using a random primer [α-^{32}P] dCTP labeling system (GE Healthcare). The membrane was rehybridized with rat β-actin cDNA as an internal control for the amount and integrity of RNA. Gene expression in the testis was determined by *in situ* hybridization (ISH), as previously reported,[4,5)] except for the following modifications: Riboprobes were synthesized using the T7 promoter attached to a cDNA fragment from the +308 to the +733 region of the gene as a template using DIG RNA Labeling kit (Roche, Basel, Switzerland). The

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Abbreviations: CCCAP, centrosomal colon cancer autoantigen protein; CT antigens, cancer/testis antigens; DD, differential display; ISH, *in situ* hybridization; SDCCAG8, serologically defined colon cancer antigen 8; SEREX, serological analysis of recombinant cDNA expression library

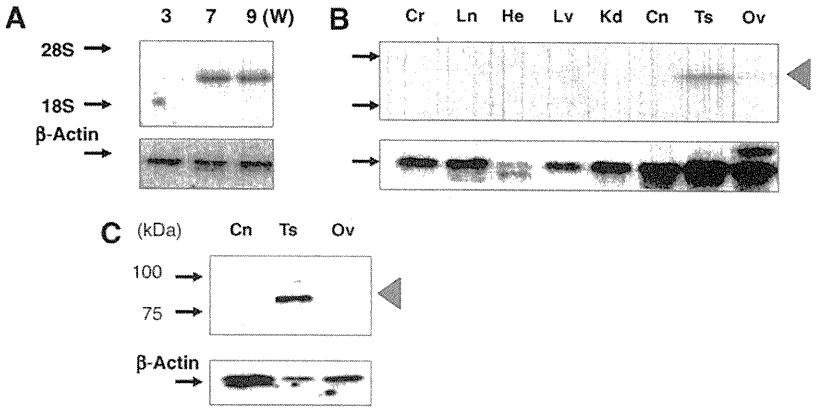

Fig. 1. *Sdccag8* Transcript and SDCCAG8 Protein Expression.

A, *Sdccag8* expression was analyzed in stage 3-, 7-, and 9-week (W)-old rat testis; B, Expression in the organs of 9-week-old rats (Cr, cerebrum; Ln, lung; He, heart; Lv, liver; Kd, kidney; Cn, colon; Ts, testis; Ov, ovary). Five μg of RNA was loaded in each lane of a formaldehyde-containing gel. The positions of ribosomal RNA are indicated by arrows on the left, as 28S (28S rRNA) and 18S (18S rRNA). The *Sdccag8* mRNA signal position is indicated by an arrowhead on the right. To confirm the integrity and quantity of the RNA, the rehybridized signal of rat β-actin mRNA is indicated at the bottom. C, SDCCAG8 protein expression of 9-week-old rats. Three μg of protein (Cn, colon; Ts, testis; Ov, ovary) was loaded in each lane on the polyacrylamide gel. The positions of the protein standards are indicated on the left by arrows. The SDCCAG8 signal position is indicated on the right as an arrowhead. The blotted signal detected using an anti β-actin antibody as an internal control is indicated at the bottom.

probe was hybridized to 5-μm sections of 8-week-old rat testis, and the signal was developed using the DIG Nucleic Acid Detection kit (Roche) with methyl-green as a counterstain, following the manufacturer's protocol.

Protein expression was determined by Western blotting. Two synthetic oligo-peptides, C + SSLAEAQ-ERETSAYK and DQLRAQLPSMPQSDC, were used to immunize rabbits, and sera were collected as a poly-clonal antibody by a custom antibody producing service (Operon Biotechnologies, Tokyo). Rat tissue proteins were prepared and purified using Sample Grinding kit (GE Healthcare). We electrophoresed 3 μg of each protein on a sodium dodecyl sulphate containing 7.5% polyacrylamide gel, and transferred it to a Hybond C membrane (GE Healthcare). The membrane was hybridized with the antisera (1:1,000) and incubated with HRP-conjugated goat anti-rabbit IgG (1:5,000; Cosmo Bio, Tokyo) as the secondary antibody. Bound antibody was detected using Immunoblot Western Chemiluminescent HRP Substrate (Millipore, Tokyo). As a control, rabbit polyclonal anti-β-actin (NT) (Cosmo Bio) was used as the primary antibody (1:1,000). Protein expression in the testis was determined by immunohistochemistry. The sections used in ISH were hybridized with the same primary antisera (1:500) used in Western blotting, and the signals were detected using Histofine DAB Substrate kit (Nichirei, Tokyo) following the manufacturer's protocol.

We screened genes that were upregulated during spermatogenesis using DD and focused on one candidate gene, *Sdccag8*. The gene information on *Sdccag8* was deposited in a DNA database as the CCCAP coding gene under accession no. NM_177929. Using Northern blot analysis, we determined that *Sdccag8* was expressed at a low level in the 3-week-old rat testis and the levels had increased at 7 weeks, and that level of expression was maintained until 9 weeks (Fig. 1A). Expression was specific to the testis. No expression was detected in the other organs examined, including the colon (Fig. 1B).

The protein expression level of SDCCAG8 was determined by Western blotting. Antisera were raised by immunizing rabbits with two synthesized oligo-peptides designed to a rat-specific region of the protein, excluding the coiled-coil domain. We analyzed the expression of SDCCAG8 in the testis, ovary, and colon, and detected a testis-specific band for SDCCAG8 of approximately 80 kDa (Fig. 1C). Since the mouse CCCAP protein is reported to be 83 kDa in size,[6] we concluded that the antisera raised from the two synthesized oligo-peptides can be used to detect rat SDCCAG8, and we used the antisera in a subsequent immunohistochemical experiment.

Since the expression of *Sdccag8* was specific to the testis and increased during maturation, we determined its expression in the testis using ISH on 8-week-old rat testis sections. We observed the individual stages of the seminiferous tubules. Gene expression was widespread in the spermatocytes (Fig. 2).

Protein expression in the testis sections was determined by immunohistochemistry using the antisera that were used for Western blotting. Signals were detected in round, elongated spermatids, as well as in the spermatocytes (Fig. 3), in agreement with the observations from ISH. These results confirm that the SDCCAG8 translated in spermatocytes is stably maintained in spermatids, and has specific functions during the meiotic process and the subsequent morphological changes in sperm cells.

SDCCAG8 has been identified as CCCAP in mice and humans. It contains a typical C-terminal coiled-coil domain, and its molecular size in the mouse was predicted to be 83 kDa. Its C-terminal region is identical to NY-CO-8, a colon cancer autoantigen. CCCAP is a component of the centrosome and it can homo-oligomerize. Expression of murine *CCCAP* and of human *NY-CO-8* has been reported to be low but ubiquitous in organs examined, although relatively high expression was observed in the human testis using an *NY-CO-8* probe in Northern blotting.[6,7] In agreement

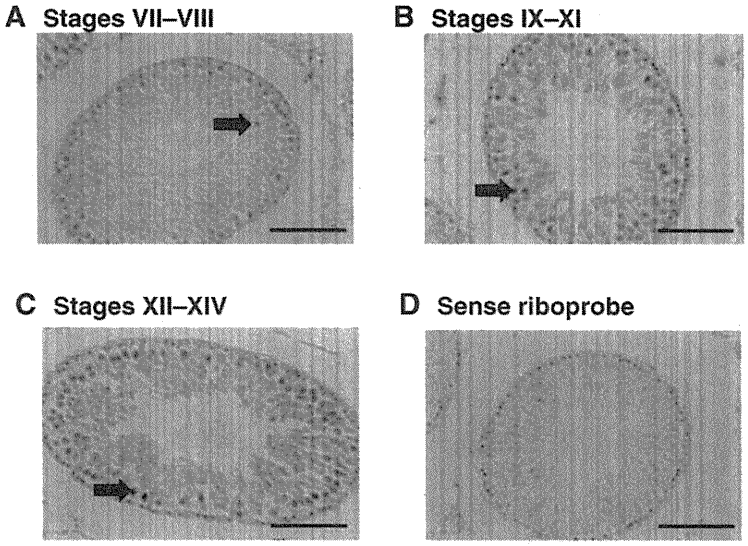

Fig. 2. Cellular Localization of the Rat *Sdccag8* Transcript in 8-Week-Old Rat Testis.
In situ hybridization of the *Sdccag8* probe at various stages in 5-μm-thick sections of the seminiferous tubules of 8-week-old rat testis (A) to (C), and a sense riboprobe as a negative control (D). The tubules were expected to be (A) stages VII to VIII, (B) stages IX to XI, and (C) stages XII to XIV. Signals (purple) in the cytoplasm of the spermatocytes (arrows) can be observed. Cells were counterstained with methyl-green (green). The scale bar represents 100 μm.

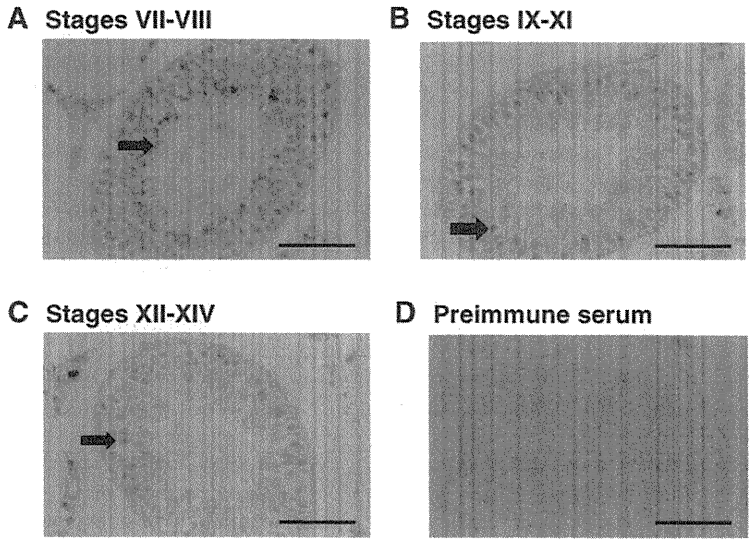

Fig. 3. Cellular Localization of Rat SDCCAG8 Protein in 8-Week-Old Rat Testis.
Immunohistochemistry using rat SDCCAG8 antisera at various stages on 5-μm-thick sections of the seminiferous tubules of 8-week-old rat testis (A) to (C), and using pre-immune serum as a negative control (D). Tubules were expected to be (A) stages VII to VIII, (B) stages IX to XI, and (C) stages XII to XIV. Signals (brown) in the cytoplasm of the spermatocytes (arrows in B and C), and spermatids (arrow in A) can be observed. The scale bar represents 100 μm.

with this observation, we observed high, specific expression of rat *Sdccag8* in the maturing testis. Hence, we predict a specific function of SDCCAG8 in the maturing rat testis, in addition to its centrosome-associated function.

More than 100 cancer/testis (CT) antigen genes have been identified.[9,10] Their expression is restricted to normal adult testicular germ cells, and various types of cancer cells. These candidates, including NY-CO-8, have been analyzed using SEREX.[7] Gene expression is regulated by the abundance of methylation in the promoter sequences of the genes, including CT antigen, such as mouse maelstrom[11] and human threonine protease genes.[12] In particular, the roles of CT antigens in cancer cells and testicular germ cells remain largely unknown. Nevertheless, CT antigens can be used not only as a diagnostic tool in cancer treatment, but also as promising target molecules in cancer immunotherapy.

The importance of the gene expression of *Sdccag8* in relation to meiosis and spermatid differentiation should be determined in future work, since the finding that the CT antigen gene has a role in the development of spermatocytes and spermatids is novel.

Acknowledgments

This work was supported in part by a grant from the Education and Research Improvement and Promotion Program 2009 from the President of Tottori University. We also thank Mr. Makoto Kita, Research Center for Bioscience and Technology, Tottori University, for support with radioisotopes.

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