

markers, such as mRNA levels of macrophage inflammatory protein-2 (*Mip-2*), interleukin-6 (*Il-6*), and macrophage/monocyte chemoattractant protein-1 (*Mcp-1*), in lung tissue were elevated (Jacobsen et al., 2009). Although comparing the results between previous studies and the present study is difficult because of differences in experimental conditions, including test substances, endpoints, cells, animal species or strain, and doses, the findings of Jacobsen et al. (2009) may support the present finding that C₆₀ did not cause DNA damage in the lungs even at doses generating pulmonary inflammation.

A wide range of nanomaterials have been shown to create ROS both *in vitro* and *in vivo* (Allion et al., 2009; Azad et al., 2009; Møller et al., 2010). Although current understanding of fullerene toxicity must recognize that limitation in some initial techniques have led to unintentional erroneous reports of nC₆₀ ROS generation and toxicity (Henry et al., 2011), the main molecular mechanism of nanotoxicity is considered to be the induction of oxidative stress resulting from the generation of ROS. Fullerenes and their derivatives generated ROS in various experimental conditions (Sera et al., 1996; Kamat et al., 2000; Sayes et al., 2004, 2005), but their potential for the generation of ROS is weak and lower than that of silica, titanium dioxide, and single-walled carbon nanotubes (Jacobsen et al., 2008; Folkmann et al., 2009; Møller et al., 2010). The generation of ROS in turn provokes inflammatory responses (Azad et al., 2009; Nielsen et al., 2008).

As for pulmonary inflammation due to C₆₀ and its derivatives, Sayes et al. (2007) noted that C₆₀ nanoparticles and a water-soluble derivative, C₆₀(OH)₂₄, caused little or no toxic effects in male Crl:(SD)IGS BR rats given a single intratracheal instillation at 0.2–3.0 mg/kg. Minimum toxicity was observed in the histopathology of the lungs, hematology and serum chemistry, and BAL fluid (BALF) parameters in male Fischer rats that inhaled C₆₀ nanoparticles at 2.35 mg/m³ for 3 h a day, for 10 consecutive days using a nose-only exposure (Baker et al., 2008). Examinations of lung histopathology and BALF parameters revealed that no strong potential effects of fullerenes for the development of neutrophil inflammation were induced in male Wistar rats given well-dispersed C₆₀ by a single intratracheal instillation at 0.1 to 1 mg/rat or inhalation for 6 h a day for 5 days a week (Morimoto et al., 2010). Park et al. (2010) reported that C₆₀ nanoparticles caused inflammatory responses including increased levels of inflammatory cytokines in BALF and cell infiltration in lung tissue of male ICR mice after a single intratracheal instillation at 0.5–2.0 mg/kg. These inflammatory responses are thought to be slight because the degree of histopathological change was very slight to slight. However, moderate to severe inflammatory changes in the lungs were noted in male SD rats exposed for three days by intratracheally instillation to water-soluble polyhydroxylated fullerenes [C₆₀(OH)_x, X = 22, 24] at 5 or 10 mg/rat, but not at 1 mg/rat (Xu et al., 2009b). These doses were extremely high, approximately equivalent to 25–50 mg/kg. These observations indicate that C₆₀ nanoparticles, except for high doses of water-soluble fullerene derivatives, have no or very weak potential to cause inflammation. Consideration of these findings suggests that the lack of DNA damage is, at least in part, attributable to the low potential of C₆₀ nanoparticles for the generation of ROS and development of inflammatory responses.

In conclusion, the present findings showed that C₆₀ nanoparticles did not induce DNA damage in the lung cells of rats intratracheally instilled with C₆₀ nanoparticles even at doses that elicited inflammatory responses. These findings suggest that C₆₀ nanoparticles have no potential for genotoxicity *in vivo*.

Conflict of interest statement

The authors declare there are no conflicts of interest.

Acknowledgments

This study was conducted under the “Evaluating Risks Associated with Manufactured Nanomaterials” Project (P06041) funded by the New Energy and Industrial Technology Development Organization (NEDO), Japan.

References

- Allion, K.L., Xie, Y., El-Gendy, N., Berkland, C.L., Forrest, M.L., 2009. Effects of nanomaterial physicochemical properties on *in vivo* toxicity. *Adv. Drug Deliv. Rev.* 61, 457–466.
- Aoshima, H., Yamana, S., Nakamura, S., Mashino, T., 2010. Biological safety of water-soluble fullerenes evaluated using tests for genotoxicity, phototoxicity, and prooxidant activity. *J. Toxicol. Sci.* 35, 401–409.
- Aschberger, K., Johnston, H.J., Stone, V., Aiken, R.J., Tran, C.L., Hankin, S.M., Peters, S.A.K., Christensen, F.M., 2010. Review of fullerene toxicity and exposure-appraisal of a human health risk assessment, based on open literature. *Regul. Toxicol. Pharmacol.* 58, 455–473.
- Azad, N., Rojanasakul, Y., Vallyathan, V., 2009. Inflammation and lung cancer: roles of reactive oxygen/nitrogen species. *J. Toxicol. Environ. Health Part B* 11, 1–15.
- Babynin, E.V., Nuretdinov, I.A., Cusbkaya, V.P., Barabanshchikov, B.I., 2002. Study of mutagenic activity of fullerene and some of its derivatives using his⁺-reversions of *Salmonella typhimurium* as an example. *Russian J. Genet.* 38, 453–457.
- Baker, G.L., Gupta, A., Clark, M.L., Valenzuela, B.R., Staska, L.M., Harbo, S.J., Pierce, J.T., Dill, J.A., 2008. Inhalation toxicity and lung toxicokinetics of C₆₀ fullerene nanoparticles and microparticles. *Toxicol. Sci.* 101, 122–131.
- Brunauer, S., Emmett, P.H., Teller, E., 1938. Absorption of gasses in multimolecular layers. *J. Am. Chem. Soc.* 60, 309–319.
- Dhawan, A., Taurozzi, J.S., Pandey, A.K., Shan, W., Miller, S.M., Hashsham, S.A., Tarabara, V.V., 2006. Stable colloidal dispersions of C₆₀ fullerenes in water: evidence for genotoxicity. *Environ. Sci. Technol.* 40, 7394–7401.
- Ema, M., Kobayashi, N., Naya, M., Nakanishi, J., 2011. Genotoxicity evaluation of fullerenes and their derivatives. *Jpn. J. Environ. Toxicol.* 14, 69–80 (in Japanese).
- ENRHES (Engineered Nanoparticles: Review of Health and Environmental Safety). 2009. Available from: <<http://ihpc.jrc.ec.europa.eu/whats-new/enrhres-final-report>> (cited May 18, 2011).
- Folkmann, J.K., Risom, L.R., Jacobsen, N.R., Wallin, H., Loft, S., Møller, P., 2009. Oxidatively damaged DNA in rats exposed by oral gavage to C₆₀ fullerenes and single-walled carbon nanotubes. *Environ. Health Perspect.* 117, 703–708.
- Greim, H., Borm, P.J.A., Schins, R.P.F., Donaldson, K., Driscoll, K.E., Hartwig, A., Krumpel, E., Overdörster, G., Speit, G., 2001. Toxicity of fibers and particles. Report of the workshop held in Munich, Germany. *Inhal. Toxicol.* 13, 101–119.
- Henry, T.B., Peterson, E.J., Compton, R.N., 2011. Aqueous fullerene aggregates (nC₆₀) generate minimal reactive oxygen species and are of low toxicity in fish: a revision of previous reports. *Curr. Opin. Biotechnol.* 22, 533–537.
- ISO (International Organization for Standardization), 2008. ISO/TS 27687. Nanotechnologies-terminology and definitions for nanoobjects–nanoparticle, nanofiber and nanoplate.
- Jacobsen, N.R., Pojana, G., White, P., Møller, P., Cohn, C.A., Korsholm, K.S., Vogel, U., Marcomini, A., Loft, S., Wallin, H., 2008. Genotoxicity, cytotoxicity, and reactive oxygen species induced by single-walled carbon nanotubes and C₆₀ fullerenes in the FE1-Muta™ mouse lung epithelial cells. *Environ. Mol. Mutagen.* 49, 476–487.
- Jacobsen, N.R., Møller, P., Jensen, K.A., Vogel, U., Lsfefoged, O., Loft, S., Wallin, H., 2009. Lung inflammation and genotoxicity following pulmonary exposure to nanoparticles in ApoE^{-/-} mice. *Part. Fibre Toxicol.* 6, 2. doi:10.1186/1743-8977-6-2.
- Johnston, H.J., Hutchison, G.R., Christensen, F.M., Aschberger, K., Stone, V., 2010. The biological mechanisms and physicochemical characteristics responsible for driving fullerene toxicity. *Toxicol. Sci.* 114, 162–182.
- Kamat, J.P., Devasagayam, T.P.A., Priyadarsini, K.L., Mohan, H., 2000. Reactive oxygen species mediated membrane damage induced by fullerene derivatives and its possible biological implications. *Toxicology* 155, 55–61.
- Kumaravel, T.S., Jha, A.N., 2006. Reliable comet assay measurements for detecting DNA damage induced by ionizing radiation and chemicals. *Mutat. Res.* 605, 7–16.
- Mori, T., Takada, H., Ito, S., Matsubayashi, K., Miwa, N., Sawaguchi, T., 2006. Preclinical studies of fullerene upon acute oral administration and evaluation for no mutagenesis. *Toxicology* 225, 48–54.
- Møller, P., Jacobsen, N.R., Folkmann, A.K., Danielsen, P.H., Mikkelson, L., Hemmingsen, J.G., Vesterdal, L.K., Forchhammer, L., Wallin, H., Loft, S., 2010. Role of oxidative damage in toxicity of particles. *Free Rad. Res.* 44, 1–46.
- Morimoto, Y., Hirohashi, M., Ogami, A., Oyabu, T., Myojo, T., Nishi, K., Kodaya, C., Todoroki, M., Yamamoto, M., Murakami, M., Shimada, M., Wang, W.N., Yamamoto, K., Fujita, K., Endoh, S., Uchida, K., Shinohara, N., Nakanishi, J., Tanaka, I., 2010. Inflammogenic effect of well-characterized fullerenes in inhalation and intratracheal instillation studies. *Part. Fibre Toxicol.* 7, 4. doi:10.1186/1743-8977-7-4.
- Murray, A.R., Kisin, E., Leonard, S.S., Young, S.H., Kommineni, C., Kagan, V.E., Castronova, V., Shevedova, A.A., 2009. Oxidative stress and inflammatory

- response in derma toxicity of single-walled carbon nanotubes. *Toxicology* 257, 161–171.
- Nielsen, G.D., Roursgaard, M., Jensen, K.A., Poulsen, S.S., Larsen, S.T., 2008. In vitro biology and toxicology of fullerenes and their derivatives. *Basic Clin. Pharmacol. Toxicol.* 103, 197–208.
- Oberdörster, G., Maynard, A., Donaldson, K., Castranova, V., Fitzpatrick, J., Ausman, K., Carter, J., Karn, B., Kreyling, W., Lai, D., Olin, S., Monterio-Riviere, N., Warheit, D., Yang, H., 2005a. Principles for characterizing the potential human health effects from exposure to nanoparticles: elements of a screening strategy. Part *Fibre Toxicol.* 2, 8. doi:10.1186/1743-8977-2-8.
- Oberdörster, G., Oberdörster, E., Oberdörster, J., 2005b. Nanotoxicology: an emerging discipline evolution from studies of ultrafine particles. *Environ. Health Perspect.* 113, 823–839.
- Park, E.J., Kim, H., Kim, Y., Yi, J., Choi, K., Park, K., 2010. Carbon fullerenes (C₆₀s) can induce inflammatory responses in the lung of mice. *Toxicol. Appl. Pharmacol.* 244, 226–233.
- Sayes, C.M., Fortner, J.D., Guo, W., Lyon, D., Boyd, A.M., Ausman, K.D., Tao, Y.J., Sitharaman, B., Wilson, L.J., Hughes, J.B., West, J.L., Colvin, V., 2004. The differential cytotoxicity of water-soluble fullerenes. *Nano Lett.* 4, 1881–1887.
- Sayes, C.M., Gobin, A.M., Ausman, K.D., Mendez, J., West, J.L., Colvin, V.L., 2005. Nano-C₆₀ cytotoxicity is due to lipid peroxidation. *Biomaterials* 26, 7587–7595.
- Sayes, C.M., Marchione, A.A., Reed, K.L., Warheit, D.B., 2007. Comparative pulmonary toxicity assessments of C₆₀ water suspensions in rats: few differences in fullerene toxicity in vivo contrast to in vitro profiles. *Nano Lett.* 7, 2399–2406.
- Schins, R.P.F., 2002. Mechanisms of genotoxicity of particles and fibers. *Inhal. Toxicol.* 14, 57–78.
- Schins, R.P.F., Knaapen, A.M., 2007. Genotoxicity of poorly soluble particles. *Inhal. Toxicol.* 19 (Suppl. 1), 189–198.
- Sera, N., Tokiwa, H., Miyata, N., 1996. Mutagenicity of the fullerene C₆₀-generated singlet oxygen dependent formation of lipid peroxides. *Carcinogenesis* 17, 2163–2169.
- Shinohara, N., Matsumoto, K., Endoh, S., Maru, J., Nakanishi, J., 2009. In vitro and in vivo genotoxicity tests on fullerene C₆₀ nanoparticles. *Toxicol. Lett.* 191, 289–296.
- Singh, N., Manshian, B., Jenkins, G.L.S., Griffiths, S.M., Williams, P.M., Maffei, T.G.G., Wright, C.J., Doak, S.H., 2009. NanoGenotoxicity: the DNA damaging potential of engineered nanomaterials. *Biomaterials* 30, 3891–3914.
- Totsuka, Y., Higuchi, T., Imai, T., Nishikawa, A., Nohmi, T., Kato, T., Masuda, S., Kinae, N., Hiyoshi, K., Oga, S., Kawanishi, M., Yagi, T., Ichinose, T., Fukumori, N., Watanabe, M., Sugimura, T., Wakabayashi, K., 2009. Genotoxicity of nano/microparticles in *in vitro* micronuclei, *in vivo* comet and mutation assay system. *Part. Fibre Toxicol.* 6, 23. doi:10.1186/1743-8977-6-23.
- Xu, A., Chai, Y., Nohmi, T., Hei, T.K., 2009a. Genotoxic response to titanium dioxide nanoparticles and fullerene in *gpt* delta transgenic MEF cells. *Part. Fibre Toxicol.* 6, 3. doi:10.1186/1743-8977-6-3.
- Xu, J.Y., Han, K., Li, S.X., Cheng, J.S., Xu, G.T., Li, W.X., Li, Q.N., 2009b. Pulmonary responses to polyhydroxylated fullerenols, C₆₀(OH)_x. *J. Appl. Toxicol.* 29, 578–584.



Evaluation of Transplacental Treatment for Fetal Congenital Bradyarrhythmia

– Nationwide Survey in Japan –

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Background: There are few large studies of fetal congenital bradyarrhythmia. The aim of the present study was to investigate the effects and risks of transplacental treatment for this condition.

Methods and Results: Using questionnaires, 128 cases of fetal bradyarrhythmia were identified at 52 Japanese institutions from 2002 to 2008. Of the 128 fetuses, 90 had structurally normal hearts. Among these 90 fetuses, 61 had complete atrioventricular block (CAVB), 16 had second-degree AVB, 8 had sinus bradycardia, and 5 had other conditions. The 61 CAVB fetuses were divided into those who did (n=38) and those who did not (n=23) receive transplacental medication. Monotherapy with β -sympathomimetics, steroid monotherapy, and combination therapy with these agents was given in 11, 5 and 22 cases, respectively. Beta-sympathomimetics improved bradycardia ($P<0.001$), but no medication could significantly improve the survival rate. Fetal hydrops was associated with a 14-fold increased risk of perinatal death ($P=0.001$), and myocardial dysfunction was a significant risk factor for poor prognosis ($P=0.034$). Many adverse effects were observed with steroid treatment, with fetal growth restriction increasing significantly after >10 weeks on steroids ($P=0.043$).

Conclusions: Treatment with β -sympathomimetics improved bradycardia, but survival rate did not differ significantly in fetuses with and without transplacental medication. It is recommended that steroid use should be limited to <10 weeks to avoid maternal and fetal adverse effects, especially fetal growth restriction and oligohydramnios. (*Circ J* 2012; **76**: 469–476)

Key Words: Anti-Ro/SSA antibody; Congenital atrioventricular block; Pregnancy; Steroids; Transplacental treatment

Fetal congenital bradyarrhythmia is an uncommon but life-threatening disease, especially in the case of complete atrioventricular block (CAVB), which has a poor prognosis because of fetal hydrops, endocardial fibroelastosis and late-onset dilated cardiomyopathy.^{1–9} Predominantly untreated CAVB has a significant mortality rate of 14–34%, while congenital CAVB is irreversible and requires a pacemaker in approximately 66% of cases after birth.¹⁰ The asso-

ciation of CAVB with maternal anti-Ro/Sjögren's syndrome A (SSA) antibodies is well established, but the trigger for the maternal antibody interaction with the fetal Ro particle is unknown in some cases of antibody-exposed babies.^{2,7–9,11,12}

There is limited evidence for the clinical efficacy of transplacental treatment of congenital AVB.^{13–19} Steroids and i.v. immunoglobulins are given as anti-inflammatory treatment, while β -sympathomimetics are used for fetal pacing.²⁰ A recent

Received September 8, 2011; revised manuscript received October 27, 2011; accepted November 1, 2011; released online December 23, 2011 Time for primary review: 18 days

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ISSN-1346-9843 doi:10.1253/circj.CJ-11-1020

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Table 1. Baseline Characteristics of CAVB Fetuses

	Medication group (n=38)	No medication group (n=23)	P value
Maternal anti-SSA antibodies	29 (76.3)	11 (47.8)	<0.05 [‡]
Gestational age at diagnosis (weeks)	24±3.2	28±5.7	<0.005 [†]
Fetal heart rate at diagnosis (beats/min)	58±7.9	63±14.7	NS [†]
Fetal hydrops	16 (42.1)	6 (26.1)	NS [‡]
Fetal myocardial dysfunction	13 (34.2)	7 (30.4)	NS [‡]
Gestational age at initiation of therapy (weeks)	26±3.6	—	
Fetal heart rate at initiation of therapy (beats/min)	56±8.4	—	
Gestational age at delivery (weeks)	34±4.0	35±4.5	NS [†]
Birth weight (g)	2,120±620	2,528±653	<0.001 [†]
Delivery mode			
Vaginal	8	7	NS [‡]
Cesarean section	30	16	NS [‡]
Permanent pacemaker implantation	14 (46.7)	6 (35.3)	NS [‡]
Neonatal survival	30 (78.9)	17 (73.9)	NS [‡]

Data given as mean±SD or n (%). P<0.05, significant difference.

[†]Student's t-test; [‡]chi-square test and Fisher's exact test.

CAVB, complete atrioventricular block; SSA, Sjögren's syndrome A.

cohort study found an improved survival rate of >90% with initiation of maternal high-dose dexamethasone at the time of CAVB detection, and maintenance of this drug during pregnancy with use of β -sympathomimetics to keep fetal heart rates at >55 beats/min.^{9,21} It was also suggested that prolonged use of dexamethasone might render fetuses with congenital CAVB less likely to develop the additional manifestations of myocarditis, cardiomyopathy, and hydrops fetalis, thus improving the overall outcome. Use of steroids, however, is controversial because of the potential risks for the fetus, including problems with neurological development, growth retardation, and oligohydramnios.^{22–25}

Few large studies of fetal congenital bradyarrhythmia have been performed in Japan. The aims of the present study were to determine the features of fetal congenital bradyarrhythmia in Japan, and to examine the effects and risks of transplacental treatment for this condition.

Methods

Subjects

Data were collected using questionnaires sent to Departments of Perinatology and Pediatric Cardiology at 750 institutions in Japan over 7 years (2002–2008). The response rate was 60.7% (455 institutions). Fetal bradyarrhythmia was defined as ventricular heart rate <100 beats/min at the time of diagnosis.⁴ The following perinatal data were also collected: gestational age at diagnosis and delivery, presence or absence of a congenital heart defect (CHD), type of bradyarrhythmia, method of diagnosis, presence or absence of maternal autoantibodies such as anti-Ro/SSA antibodies, presence or absence of fetal hydrops, presence or absence of fetal myocardial dysfunction, fetal ventricular and atrial heart rate at presentation, prenatal treatment, mode of delivery, and outcome. Adverse effects related to prenatal treatment were also evaluated.

Statistical Analysis

Statistical analysis was performed using STATA 11.1 (Stata-Corp, College Station, TX, USA) and JMP 9 (SAS Institute, Cary, NC, USA). Data are presented as mean±SD or number of patients and were analyzed using Student's t-test. Categorical variables were evaluated on chi-square test and Fisher's

exact test. Logistic regression was used to adjust for baseline variables known to be associated with fetal ventricular heart rate, fetal hydrops, fetal myocardial dysfunction, and maternal anti-Ro/SSA antibody. Time to fetal or neonatal death was analyzed using the Kaplan-Meier method with a log-rank test and a Cox proportional hazard model. P<0.05 was considered significant.

Results

Baseline Characteristics

A total of 128 cases were registered from 52 institutions during 7 years (2002–2008). All cases of fetal bradyarrhythmia were diagnosed during fetal life using echocardiography. In 8 cases, magnetocardiography was performed due to fetal bradyarrhythmia and family history of long QT syndrome (LQTS). Of the 128 fetuses, 38 (29.7%) had CHD, 15 had left atrial isomerism, 1 had right atrial isomerism, 5 had atrioventricular septal defect, 4 had corrected transposition of the great arteries, 4 had pulmonary stenosis, and 9 had other conditions. Patent ductus arteriosus and atrial septal defect were categorized as an absence of CHD. Ninety fetuses (70.3%) had a structurally normal heart, of whom 61 had CAVB, 16 had second-degree AVB, 8 had sinus bradycardia, 3 had sick sinus syndrome. Nine LQTS cases occurred in combination with another condition.

CAVB

Of the 61 fetuses with a structurally normal heart and CAVB (Table 1), 38 received transplacental medication. No fetus showed improvement of heart block. Monotherapy with β -sympathomimetics was given in 11 cases, steroids were given in 5 cases, and combination therapy with these agents was used in 22 cases. No transplacental medication was given in 23 cases. Ritodrine hydrochloride was used as the β -sympathomimetic agent. Steroids tended to be used in fetuses that were positive for maternal anti-Ro/SSA antibody throughout pregnancy, but the chosen steroid differed among institutions. Maternal i.v. immunoglobulin was not used. After birth, a pacemaker was implanted based on the Japanese guidelines of syncope, ventricular heart rate <50 beats/min, decreased cardiac function, LQTS, and a sudden pause longer than 2–3-fold the regular ventricular heart rate.

Table 2. Factors in Improvement of Bradycardia

	OR	95%CI	P value
β -sympathomimetics	49.02	5.18–464.02	<0.005
Steroids	1.32	0.24–7.20	0.745
β -sympathomimetics+steroids	725,448.8	0	0.996
Fetal heart rate	1	0.93–1.08	0.924
Fetal hydrops	0.41	0.07–2.39	0.319
Fetal myocardial dysfunction	1.14	0.20–6.60	0.883
Maternal anti-Ro/SSA antibodies	0.22	0.04–1.36	0.105

P<0.05, significant difference.

Logistic regression was used to adjust for baseline variables known to be associated with fetal ventricular heart rate, fetal hydrops, fetal myocardial dysfunction, and maternal anti-Ro/SSA antibody.

OR, odds ratio; CI, confidence interval; SSA, Sjögren's syndrome A.

Table 3. Factors in Fetal or Neonatal Death

	HR	95%CI	P value
β -sympathomimetics	1.16	0.37–3.63	0.792
Steroids	0.56	0.20–1.58	0.273
Fetal heart rate	0.98	0.92–1.05	0.546
Fetal hydrops	13.84	3.12–61.44	0.001
Fetal myocardial dysfunction	2.44	0.71–8.40	0.157
Maternal anti-Ro/SSA antibodies	1.07	0.33–3.47	0.906

P<0.05, significant difference.

Logistic regression was used to adjust for baseline variables known to be associated with fetal ventricular heart rate, fetal hydrops, fetal myocardial dysfunction, and maternal anti-Ro/SSA antibody.

HR, hazard ratio; CI, confidence interval; SSA, Sjögren's syndrome A.

Table 4. Factors in Development of Fetal Hydrops

	OR	95%CI	P value
β -sympathomimetics	2	0.35–11.50	0.439
Steroids	0.27	0.04–1.97	0.198
Fetal heart rate	1.01	0.94–1.08	0.813
Fetal myocardial dysfunction	5.71	1.14–28.62	0.034
Maternal anti-Ro/SSA antibodies	0.71	0.13–3.90	0.698

P<0.05, significant difference.

Logistic regression was used to adjust for baseline variables known to be associated with fetal ventricular heart rate, fetal myocardial dysfunction, and maternal anti-Ro/SSA antibody.

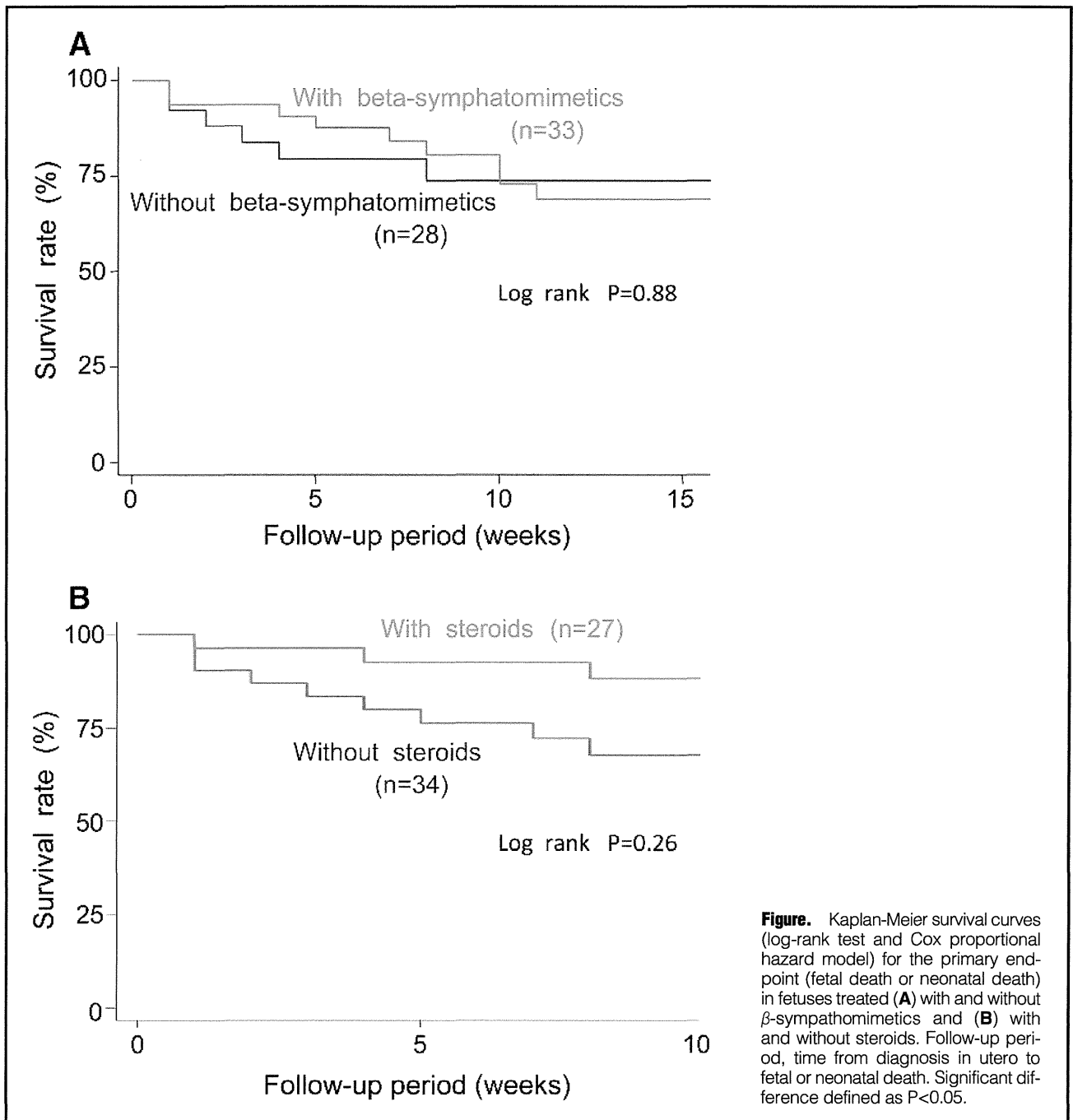
OR, odds ratio; CI, confidence interval; SSA, Sjögren's syndrome A.

The anti-Ro/SSA antibody-positive rate was significantly higher in fetuses treated with transplacental medication compared to those who did not receive this medication (76.3% vs. 47.8%; $P=0.031$). Gestational age at diagnosis was significantly lower in those receiving transplacental medication (24.0 weeks vs. 28.3 weeks; $P=0.003$). Fetal ventricular heart rate at diagnosis did not differ between the 2 groups, but the ventricular heart rate was significantly lower in fetuses treated with transplacental medication (56 beats/min vs. 63 beats/min; $P=0.034$). Birth weight was also significantly lower in fetuses treated with transplacental medication (2,120 g vs. 2,528 g; $P=0.006$). Gestational age at delivery, neonatal survival rate, and pacemaker implantation rate did not differ between the 2 groups.

Multivariate analysis was performed with adjustment for baseline variables with a known association with fetal ventricular heart rate, fetal hydrops, fetal myocardial dysfunction, and the presence of maternal anti-Ro/SSA antibodies (Tables 2–4). In this analysis, β -sympathomimetic treatment was significantly associated with improved bradycardia (odds ratio [OR], 49.02; 95% confidence interval [CI]: 5.18–464.02; $P<0.001$),

whereas steroids were ineffective, and no evidence of a synergistic effect was obtained. The presence of maternal anti-Ro/SSA antibodies may inhibit improvement of bradycardia, but this effect was not significant (OR, 0.22; 95%CI: 0.04–1.36; $P=0.105$). Drug therapy had no significant effect on survival. Fetal ventricular heart rate and the presence of maternal anti-Ro/SSA antibodies also had no influence on prognosis, but fetal hydrops was associated with a 14-fold increased risk of perinatal death (hazard ratio [HR], 13.84; 95%CI: 3.12–61.44; $P=0.001$).

Kaplan-Meier survival curves are shown in Figure. The primary endpoint was intrauterine death or neonatal death. Beta-sympathomimetic treatment was not associated with improved prognosis. Steroid also did not improve the prognosis (HR, 0.56; 95%CI: 0.20–1.58; $P=0.273$). Fetal myocardial dysfunction was a significant risk factor for fetal hydrops (OR, 5.71; 95%CI: 1.14–28.62; $P=0.034$). Fetal ventricular heart rate and the presence of maternal anti-Ro/SSA antibodies were not associated with fetal hydrops. Beta-sympathomimetic treatment did not inhibit development of fetal hydrops. Steroids tended to inhibit fetal hydrops, but again this effect was not



statistically significant (OR, 0.27; 95%CI: 0.04–1.97; $P=0.198$). Drug therapy had no significant effect on improvement of fetal myocardial dysfunction.

Second-Degree AVB With Bradycardia

Of the 90 fetuses with a structurally normal heart, second-degree AVB was present in 16 cases (Table 5). Transplacental medication was given in 8 of these cases: β -sympathomimetic monotherapy in 4, steroids in 3, and a combination of these therapies in 1. In the 8 medication cases, fetal ventricular heart rate at diagnosis was significantly lower than that in the non-medication cases (70 beats/min vs. 79 beats/min; $P=0.017$). No other clinical characteristics differed significantly between the 2 groups. Of the 8 medicated fetuses, 3 developed CAVB,

3 maintained second-degree AVB, 1 improved to first-degree AVB, and 1 had no AVB at the time of delivery. Of the 8 non-medicated fetuses, 2 developed CAVB, 3 maintained second-degree AVB, and 3 had no AVB at the time of delivery. Survival rate did not differ between the groups (87.5%).

Adverse Effects of Transplacental Treatment

Treatment-related adverse events were examined in the 63 fetuses with a structurally normal heart and no fetal hydrops (Table 6). Steroids were given in 23 cases, drugs other than steroids were given in 10 cases, and no treatment was given in 30 cases. Gestational age at delivery did not differ among these 3 groups. In the steroid group, birth weight was significantly lower than in the non-treatment group (2,201 g vs.

Table 5. Second-Degree AVB Fetus Baseline Characteristics vs. Medication

	Medication (n=8)	No medication (n=8)	P value
Maternal anti-Ro/SSA antibodies	4	3	NS [†]
Gestational age at diagnosis (weeks)	28±4.3	26±5.0	NS [†]
Fetal heart rate at diagnosis (beats/min)	70±9.0	79±10.4	<0.05 [†]
Fetal hydrops	2	2	NS [†]
Fetal myocardial dysfunction	3	2	NS [†]
Gestational age at initiation of therapy (weeks)	29±4.8	—	
Fetal heart rate at initiation of therapy (beats/min)	70±10.0	—	
Gestational age at delivery (weeks)	35±3.8	37±2.1	NS [†]
Birth weight (g)	2,207±688	2,533±544	NS [†]
Delivery mode			
Vaginal	2	5	NS [†]
Cesarean section	6	3	NS [†]
Degree of AVB at delivery			
Complete	3	2	NS [†]
Second	3	3	NS [†]
First	1	0	NS [†]
None	1	3	NS [†]
Neonatal survival	7 (87.5)	7 (87.5)	NS [†]

Data given as mean±SD or n (%).

P<0.05, significant difference. [†]Wilcoxon test; [†]chi-square test and Fisher's exact test.

AVB, atrioventricular block; SSA, Sjögren's syndrome A.

Table 6. Baseline Characteristics vs. Treatment Type[†]

	Steroid treatment (n=23)	Non-steroid treatment (n=10)	No treatment (n=30)
Treatment (weeks)	8.8±4.4	5.6±3.2	—
Gestational age at delivery (weeks)	36±2.6	35.8±2.6	36.8±3.0
Birth weight (g)	2,201±525*	2,413±552	2,713±512*
Fetal arrhythmia: CAVB	21	6	23
Fetal arrhythmia: Second-degree AVB	1	2	5
Maternal diabetes	1 (4.3)	0	0
Fetal growth restriction	6 (26.1)	0	2 (6.7)
Fetal oligohydramnios	2 (8.7)	0	0
Neonatal adrenal insufficiency	1 (4.3)	0	0

Data given as mean±SD or n (%).

[†]For fetuses without fetal hydrops and with a structurally normal heart. *P<0.05 (Student's t-test).

CAVB, complete atrioventricular block; AVB, atrioventricular block.

Table 7. Baseline Characteristics vs. Length of Steroid Treatment

	<10 weeks (n=12)	≥10 weeks (n=11)	P value
Treatment (weeks)	5.4±2.7	12.5±2.5	<0.01 [†]
Gestational age at delivery (weeks)	35±3.2	36±1.7	NS [†]
Birth weight (g)	2,184±569	2,218±503	NS [†]
Maternal diabetes	0	1 (9.1)	NS [†]
Fetal growth restriction	1 (8.3)	5 (45.5)	<0.05 [†]
Fetal oligohydramnios	0	2 (18.2)	NS [†]
Neonatal adrenal insufficiency	0	1 (9.1)	NS [†]

Data given as mean±SD or n (%). P<0.05, significant difference.

[†]Student's t-test; [†]chi-square test and Fisher's exact test.

2,713 g; P=0.001) and fetal growth restriction was close to being significantly higher than in the non-steroid (26.1% vs. 0%; P=0.050) and non-treatment (26.1% vs. 6.7%; P=0.074) groups. Adverse effects that might have been attributable to the use of steroids included development of oligohydramnios

in 8.7% of cases, maternal diabetes in 4.3%, and neonatal adrenal insufficiency in 4.3%. All these adverse effects were observed in cases of steroid use >10 weeks (Table 7). In particular, fetal growth restriction increased significantly after steroid use >10 weeks (45.5% vs. 8.3%; P=0.043).

LQTS

Of the 90 fetuses with a structurally normal heart, 9 (10.0%) were diagnosed with LQTS, including 4 diagnosed on electrocardiography after birth and 5 diagnosed on magnetocardiography during fetal life. The background of the LQTS fetuses included a family history of LQTS (n=2), maternal anti-Ro/SSA antibody (n=2), fetal hydrops (n=3), myocardial dysfunction (n=2), CAVB (n=6), second-degree AVB with bradycardia (n=1), and sinus bradycardia (n=2). In 4 of the 9 cases of LQTS, emergency cesarean section was performed because of fetal ventricular tachycardia/torsades de pointes (VT/TdP) at 33–36 weeks of gestation. In 2 of the 9 cases, fetal hydrops caused neonate death.

Discussion

This is the first large-scale study to investigate the effects and risks of transplacental treatment for fetal congenital bradyarrhythmia in Japan. The results indicate that fetal hydrops is associated with a 14-fold increased risk of perinatal death, and that fetal myocardial dysfunction is a significant risk factor for fetal hydrops. Fetal ventricular heart rate and the presence of maternal anti-Ro/SSA antibodies were not associated with neonatal prognosis. Beta-sympathomimetics improved bradycardia, but survival rate did not differ significantly with regard to transplacental medication. Maternal and fetal adverse effects were observed in cases of steroid use. In particular, fetal growth restriction increased significantly after steroid use >10 weeks.

Evaluation of Anti-Ro/SSA Antibodies

Ro/SSA is one of the major immunogenic ribonucleoproteins, and antibodies against these proteins are found in a number of connective diseases, especially in Sjögren's syndrome (SS) and systemic lupus erythematosus (SLE). Anti-Ro/SSA antibodies are detected in 60–90% of SS cases and in 30–50% of SLE cases.^{26,27} Interestingly, these antibodies are relatively common and are detected in 1–2% of randomly tested pregnant women.²⁸ Currently, the outcome of anti-Ro/SSA-positive pregnancies is very good when prospectively followed by multidisciplinary teams with experience in this field.¹ Transplacental passage of anti-Ro/SSA antibodies from mother to fetus, however, is associated with a risk of development of neonatal lupus erythematosus (NLE).^{2,11,12} NLE is an uncommon but life-threatening disease of the fetus and neonate, with important cardiac complications of CAVB, sinus bradycardia, QTc interval prolongation, endocardial fibroelastosis, and late-onset dilated cardiomyopathy.^{3–5} Congenital CAVB develops in 1–5% of anti-Ro/SSA antibody-positive pregnancies, typically between 18 and 24 weeks of gestational age. Predominantly untreated CAVB has a mortality rate of 14–34%,^{1–9} consistent with the untreated CAVB mortality rate of 26% in the current study.

The association of NLE with maternal anti-Ro/SSA antibodies is well established, but the trigger of the maternal antibody interaction with the fetal Ro particle is unclear in some antibody-exposed babies. The percentage of maternal anti-Ro/SSA antibody-positive fetuses with CAVB diagnosed in utero is unknown. Brucato et al and Jaeggi et al found maternal anti-Ro/SSA antibodies in 92% of 37 CAVB cases,^{7,9} whereas in the present study maternal anti-Ro/SSA antibodies were detected in only 66% of 61 CAVB fetuses with a structurally normal heart. Jaeggi et al also reported that CAVB occurred in 5% of prospectively screened pregnancies with anti-Ro/SSA ELISA levels >100 U/ml, but did not occur in pregnancies with levels <50 U/ml.⁶ Approximately two-thirds of anti-Ro/SSA antibody-positive mothers had low anti-Ro/SSA lev-

els and probably little risk of development of fetal cardiac NLE.⁸ It is unclear why the anti-Ro/SSA-positive rate in the present study was lower than in other reports. It is unlikely to be due to the sensitivity of the laboratory methods, but it is possible that other undetectable antibodies associated with congenital AVB are present in the Japanese population. Brucato et al and Lopes et al found similar mortality rates in the anti-Ro/SSA-positive and -negative groups,^{7,8} and in the present multivariate analysis anti-Ro/SSA antibodies were not associated with prognosis.

Benefits and Risks of Transplacental Treatment

Congenital AVB is a progressively developing disease that evolves through 2 fundamental phases: an early phase characterized by the occurrence of still reversible AV conduction abnormalities (first- or second-degree AVB) and a final phase in which development of irreversible damage of the conduction system leads to the appearance of CAVB.²⁹ The specific pathogenetic mechanisms involved in the 2 phases have not been clarified, but there are 2 main theories. The first is based on an inflammatory-driven injury elicited by interaction between anti-Ro/SSA antibodies and specific antigens expressed in the conduction tissue of the fetal heart (inflammatory theory). The second theory involves electrophysiologic interference of anti-Ro/SSA antibodies with heart conduction (electrophysiological theory).²⁰ Consistent with these respective theories, steroids and i.v. immunoglobulins are used for anti-inflammatory treatment, while β -sympathomimetics are given for fetal pacing.

Several studies have found that a ventricular heart rate <55 beats/min is a risk factor for fetal and neonatal death,^{4,14} and have recommended transplacental treatment with β -sympathomimetics to increase the heart rate. Jaeggi et al and Maeno et al, however, found that fetuses with CAVB without CHD and with a ventricular heart rate of <55 beats/min were not at risk.^{30,31} In the present study, fetal ventricular heart rate did not influence fetal hydrops and prognosis, but treatment with a β -sympathomimetic agent was significantly associated with improved bradycardia.

To date, evidence of clinical efficacy of transplacental treatment has been limited to cases of congenital AVB.^{13–19} Jaeggi et al reported a significant improvement in the outcome of fetal CAVB simultaneously with the introduction of routine perinatal treatment guidelines in 1997.⁹ Hutter et al obtained an improved survival rate of >90% by initiation of maternal high-dose dexamethasone at the time of CAVB detection and maintenance of this dose during pregnancy, with addition of β -sympathomimetics to keep the fetal heart rate above 55 beats/min.²¹ It was also suggested that prolonged use of dexamethasone might render a fetus with congenital CAVB less likely to develop additional manifestations of cardiac NLE such as myocarditis, cardiomyopathy, and hydrops fetalis, thus improving the overall outcome. The present findings suggest that use of steroids might render the affected fetus less likely to develop fetal hydrops, but that the neonatal survival rate improved only to 79%. The reason for the relatively bad prognosis in the present study may have been the difference in the rate of fetal hydrops compared to the Hutter et al study (42% vs. 10%). Undetectable autoantibodies or virus infection may be related to the increased rate of fetal hydrops in the Japanese population. Furthermore, Hutter et al initiated maternal high-dose dexamethasone at the time of CAVB diagnosis, at a mean gestational age of 24 weeks. The mean age of diagnosis was similar in the present study, but mean gestational age at which steroids were started was 26 weeks. In addition, the percentage of steroids used in transplacental treatment was

lower in the present patients (71% vs. 95%). These findings suggest that sufficient steroid dose at an early stage is very important to prevent fetal hydrops and to improve prognosis.

Use of steroids is controversial because of the potential risks for the fetus and mother, including problems with fetal growth restriction, oligohydramnios, and neurological development. Animal models suggest that repeated antenatal steroid doses can interfere with the growth and development of the immature brain, and human studies suggest that antenatal and postnatal dexamethasone may negatively affect a child's neuropsychological development.²²⁻²⁴ In contrast, Brucato et al found no negative effects on neuropsychological development and intelligence in a cohort of preschool- and school-age children with CAVB who had been prenatally exposed to maternal anti-Ro antibodies and prolonged dexamethasone treatment.²⁵ The association of fetal growth restriction and oligohydramnios with antenatal steroids is well established, but the amount and length of steroid treatment that can be used safely is unclear. We note that development of fetal growth restriction and oligohydramnios are dose-related complications of steroids. Consequently, we recommend limiting steroid use to <10 weeks to avoid maternal and fetal adverse effects.

Prevention of Progression to Congenital CAVB

There are many case reports describing prevention of congenital CAVB, and first- or second-degree AVB is also relatively common and often normalizes spontaneously before or soon after delivery.³² Recent prospective studies suggest that steroids and i.v. immunoglobulins are not beneficial for preventing progression to congenital AVB.^{33,34} Similarly, the present study found a lack of superiority of transplacental treatment for second-degree AVB with bradyarrhythmia.

LQTS

Recent evidence has shown that anti-Ro/SSA antibodies are associated with prolongation of the QTc interval.³⁵ Although the exact arrhythmogenic mechanisms have not been clarified, anti-Ro/SSA antibodies may trigger rhythm disturbances through inhibition of cross-reactions with several cardiac ionic channels, including calcium channels and the hERG potassium channel.^{36,37} Beta-sympathomimetics may trigger life-threatening arrhythmia such as VT/TdP in patients with LQTS, and therefore use of these drugs should be avoided in fetuses with QTc interval prolongation.^{38,39} In the present study, in 4 of the 9 LQTS cases, emergency cesarean section was performed because of fetal VT/TdP at 33–36 weeks of gestational age. Oka et al also recently described atrioventricular block-induced TdP.⁴⁰ With this background, we recommend avoidance of β -sympathomimetics in a fetus with a heart rate >55 beats/min. Furthermore, assessment of QTc interval prolongation on magnetocardiography may be required to evaluate the risk of fetal congenital bradyarrhythmia.

Study Limitations

There were several limitations in the present study due to retrospective data selection bias and the relatively small sample size. The nature of a multicenter retrospective observational study using a questionnaire is such that the clinical data obtained vary among cases, so treatment bias may exist. Only ritodrine hydrochloride was used as β -sympathomimetic treatment, but was given in cases involving fetal heart rate >55 beats/min at some institutions, while dexamethasone, betamethasone and prednisolone were used as steroids at different doses among institutions. The follow-up period after birth was insufficient to permit analysis of long-term morbidity and mortality, and

this prevented evaluation of potential long-term benefits and risks of transplacental medication. Finally, the sample size might have been too small to detect the effects of steroids on fetal congenital bradyarrhythmia. The steroid effect may become significant in a study with a higher number of cases.

Guidelines are required for transplacental treatment of fetal congenital bradyarrhythmia and follow-up after birth. We expect to analyze long-term outcome of fetal congenital bradyarrhythmia in a future study. Further large prospective studies are also needed to establish the most appropriate treatment strategies in Japan.

Conclusion

Beta-sympathomimetics improved bradycardia, but survival rate did not differ significantly in fetuses treated with and without transplacental medication. We recommend limiting steroid use to <10 weeks to avoid maternal and fetal adverse effects, with fetal growth restriction and oligohydramnios being of particular concern.

Acknowledgments

This work was supported by a grant from The Ministry of Health, Labor and Welfare of Japan (Health and Labour Science Research Grants for Clinical Research for New Medicine).

Disclosures

Sources of financial support: The Ministry of Health, Labor and Welfare of Japan (Health and Labour Science Research Grants for Clinical Research for New Medicine). None of the authors have a conflict of interest to disclose.

References

1. Brucato A, Cimaz R, Caporaili R, Ramoni V, Vuyon J. Pregnancy outcome in patients with autoimmune diseases and anti-Ro/SSA antibodies. *Clin Rev Allergy Immunol* 2011; **40**: 27–41.
2. Silverman ED, Buyon J, Laxer RM, Hamilton R, Bini P, Chu JL, et al. Autoantibody response to the Ro/La particle may predict outcome in neonatal lupus erythematosus. *Clin Exp Immunol* 1995; **100**: 499–505.
3. Buyon JP, Ben-Chetrit E, Karp S, Roubey RA, Pompeo L, Reeves WH, et al. Acquired congenital heart block: Pattern of maternal antibody response to biochemically defined antigens of the SSA/Ro-SSB/La system in neonatal lupus. *J Clin Invest* 1989; **84**: 627–634.
4. Schmidt KG, Ulmer HE, Silverman NH, Kleinman CS, Copel JA. Perinatal outcome of fetal complete atrioventricular block: A multicenter experience. *J Am Coll Cardiol* 1991; **17**: 1360–1366.
5. Ichikawa R, Sumitomo N, Komori A, Abe Y, Nakamura T, Fukuhara J, et al. The follow-up evaluation of electrocardiogram and arrhythmias in children with fulminant myocarditis. *Circ J* 2011; **75**: 932–938.
6. Jaeggi ET, Laskin CA, Hamilton RM, Kingdom J, Silverman ED. The importance of the level of maternal anti-Ro/SSA antibodies as a prognostic marker of the development of cardiac neonatal lupus erythematosus: A prospective study of 186 antibody-exposed fetuses and infants. *J Am Coll Cardiol* 2010; **55**: 2778–2784.
7. Brucato A, Grava C, Bortolati M, Ikeda K, Milanese O, Cimaz R, et al. Congenital heart block not associated with anti-Ro/La antibodies: Comparison with anti-Ro/La-positive cases. *J Rheumatol* 2009; **36**: 1744–1748.
8. Lopes LM, Tavares GM, Damiano AP, Lopes MA, Aiello VD, Schultz R, et al. Perinatal outcome of fetal atrioventricular block: One-hundred-sixteen cases from a single institution. *Circulation* 2008; **118**: 1268–1275.
9. Jaeggi ET, Fouron JC, Silverman ED, Ryan G, Smallhorn J, Hornberger LK. Transplacental fetal treatment improves the outcome of prenatally diagnosed complete atrioventricular block without structural heart disease. *Circulation* 2004; **110**: 1542–1548.
10. Buyon JP, Hiebert R, Copel J, Craft J, Friedman D, Katholi M, et al. Autoimmune-associated congenital heart block: Demographics, mortality, morbidity and recurrence rates obtained from a national neonatal lupus registry. *J Am Coll Cardiol* 1998; **31**: 1658–1666.

11. Lee LA, Bias WB, Arnett FC Jr, Huff JC, Noris DA, Harmon C, et al. Immunogenetics of the neonatal lupus syndrome. *Ann Intern Med* 1983; **99**: 592–596.
12. Watson RM, Lane AT, Barnett NK, Bias WB, Arnett FC, Provost TT. Neonatal lupus erythematosus: A clinical, serological and immunogenetic study with review of the literature. *Medicine* 1984; **63**: 362–378.
13. Bierman FZ, Baxi L, Jaffe I, Driscoll J. Fetal hydrops and congenital complete heart block: Response to maternal steroid therapy. *J Pediatr* 1988; **112**: 646–648.
14. Carreira PE, Gutierrez-Larraya F, Gomez-Reino JJ. Successful intra-uterine therapy with dexamethasone for fetal myocarditis and heart block in a woman with systemic lupus erythematosus. *J Rheumatol* 1993; **20**: 1204–1207.
15. Saleeb S, Copel J, Friedman D, Buyon JP. Comparison of treatment with fluorinated glucocorticoids to the natural history of autoantibody associated congenital heart block. *Arthritis Rheum* 1999; **42**: 2335–2345.
16. Groves AMM, Allan LD, Rosenthal E. Therapeutic trial of sympathomimetics in three cases of complete heart block in the fetus. *Circulation* 1995; **92**: 3394–3396.
17. Harris JP, Alexson CG, Manning JA, Thompson HO. Medical therapy for the hydropic fetus with congenital complete atrioventricular block. *Am J Perinatol* 1993; **10**: 217–219.
18. Copel JA, Buyon JP, Kleinman CS. Successful in utero therapy of fetal heart block. *Am J Obstet Gynecol* 1995; **173**: 1384–1390.
19. Cuneo BF, Zhao H, Strasburger JF, Ovadia M, Huhta JC, Wakai RT. Atrial and ventricular rate response and patterns of heart rate acceleration during maternal-fetal terbutaline treatment of fetal complete heart block. *Am J Cardiol* 2007; **100**: 661–665.
20. Lazzarini PE, Capecchi PL, Laghi Pasini F. Anti-Ro/SSA antibodies and cardiac arrhythmias in the adult: Facts and hypotheses. *Scand J Immunol* 2010; **72**: 213–222.
21. Hutter D, Silverman ED, Jaeggi ET. The benefits of transplacental treatment of Isolated congenital complete heart block associated with maternal anti-Ro/SSA antibodies: A review. *Scand J Immunol* 2010; **72**: 235–241.
22. French NP, Hagan R, Evans SF, Godfrey M, Newnham JP. Repeated antenatal corticosteroids: Size at birth and subsequent development. *Am J Obstet Gynecol* 1999; **180**: 114–121.
23. Abbasi S, Hirsch D, Davis J, Tolosa J, Stouffer N, Debbs R, et al. Effect of single versus multiple courses of antenatal corticosteroids on maternal and neonatal outcome. *Am J Obstet Gynecol* 2000; **182**: 1243–1249.
24. Spinillo A, Viazzo F, Colleoni R, Chiara A, Cerbo RA, Fazzi E, et al. Two-year infant neurodevelopmental outcome after single or multiple antenatal courses of corticosteroids to prevent complications of prematurity. *Am J Obstet Gynecol* 2004; **191**: 217–224.
25. Brucato A, Astori MG, Cimaz R, Villa P, Li Destri M, Chimini L, et al. Normal neuropsychological development in children with congenital complete heart block who may or may not be exposed to high-dose dexamethasone in utero. *Ann Rheum Dis* 2006; **65**: 1422–1426.
26. Franceschini F, Cavazzana I. Anti-Ro/SSA and La/SSB antibodies. *Autoimmunity* 2005; **38**: 55–63.
27. Routsias JG, Tzioufas AG. Sjögren's syndrome: Study of autoantigens and autoantibodies. *Clin Rev Allergy Immunol* 2007; **32**: 238–251.
28. Taylor PV, Taylor KF, Norman A, Griffiths S, Scott JS. Prevalence of maternal Ro (SS-A) and La (SS-B) autoantibodies in relation to congenital heart block. *Br J Rheumatol* 1988; **27**: 128–132.
29. Sonesson SE, Salomonsson S, Jacobsson LA, Bremme K, Wahren-Herlenius M. Signs of first-degree heart block occur in one-third of fetuses of pregnant women with anti-SSA/Ro 52-kd antibodies. *Arthritis Rheum* 2004; **50**: 1253–1261.
30. Jaeggi ET, Hamilton RM, Silverman ED, Zamora SA, Hornberger LK. Outcome of children with fetal, neonatal or childhood diagnosis of isolated congenital atrioventricular block. *J Am Coll Cardiol* 2002; **39**: 130–137.
31. Maeno Y, Himeno W, Saito A, Hiraishi S, Hirose O, Ikuma M, et al. Clinical course of fetal congenital atrioventricular block in the Japanese population: A multicentre experience. *Heart* 2005; **91**: 1075–1079.
32. Rein AJ, Mevorach D, Perles Z, Gavri S, Nadjari M, Nir A, et al. Early diagnosis and treatment of atrioventricular block in the fetus exposed to maternal anti-SSA/Ro -SSB/La antibodies: A prospective, observational, fetal kinetocardiogram-based study. *Circulation* 2009; **119**: 1867–1872.
33. Friedman DM, Kim MY, Copel JA, Davis C, Phoon CK, Glickstein JS, et al. Utility of cardiac monitoring in fetuses at risk for congenital heart block: The PR Interval and Dexamethasone Evaluation (PRIDE) prospective study. *Circulation* 2008; **117**: 485–493.
34. Friedman DM, Llanos C, Izmirly PM, Brock B, Byron J, Copel JA, et al. Evaluation of fetuses in a study of intravenous immunoglobulin as preventive therapy for congenital heart block: Results of a multi-center, prospective, open-label clinical trial. *Arthritis Rheum* 2010; **62**: 1138–1146.
35. Lazzarini PE, Acampa M, Guideri F, Capecchi PL, Campanella V, Morozzi G, et al. Prolongation of the corrected QT interval in adult patients with anti-Ro/SSA-positive connective tissue diseases. *Arthritis Rheum* 2004; **50**: 1248–1252.
36. Ravens U, Cerbai E. Role of potassium currents in cardiac arrhythmias. *Europace* 2008; **10**: 1133–1137.
37. Nakamura K, Katayama Y, Kusano KF, Haraoka K, Tani Y, Nagase S, et al. Anti-KCNH2 antibody-induced long QT syndrome: Novel acquired form of long QT syndrome. *J Am Coll Cardiol* 2007; **50**: 1808–1809.
38. Cuneo BF, Zhao H, Strasburger JF, Ovadia M, Huhta JC, Wakai RT, et al. Atrial and ventricular rate response and patterns of heart rate acceleration during maternal-fetal terbutaline treatment of fetal complete heart block. *Am J Cardiol* 2007; **100**: 661–665.
39. Nishizaki M, Hiraoka M. Gene mutations associated with atrioventricular block complicated by long QT syndrome. *Circ J* 2010; **74**: 2546–2547.
40. Oka Y, Itoh H, Ding WG, Shimizu W, Makiyama T, Ohno S, et al. Atrioventricular block-induced Torsades de Pointes with clinical and molecular backgrounds similar to congenital long QT syndrome. *Circ J* 2010; **74**: 2562–2571.

ORIGINAL ARTICLE

Historical control data on prenatal developmental toxicity studies in rabbits

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A Study Group for Historical Control Data on Prenatal Developmental Toxicity Studies in Rabbits

ABSTRACT Historical control data on rabbit prenatal developmental toxicity studies, performed between 1994–2010, were obtained from 20 laboratories, including 11 pharmaceutical and chemical companies and nine contract laboratories, in Japan. In this paper, data were incorporated from a laboratory if the information was based on 10 studies or more. Japanese White rabbits and New Zealand White rabbits were used for prenatal developmental toxicity studies. The data included maternal reproductive findings at terminal cesarean sections and fetal findings including spontaneous incidences of morphological alterations. No noticeable differences between strains or laboratories were observed in the maternal reproductive and fetal developmental data. The inter-laboratory variations in the incidences of fetal external, visceral, and skeletal alterations seem to be due to differences in the selection of observation parameters, observation criteria, and classification of the findings, and terminology of fetal alterations.

Key Words: background data, developmental toxicity, fetal malformation, historical control data, rabbit, reproductive parameters

INTRODUCTION

The rabbit is one of the most commonly used animal models for standardized developmental toxicity testing of pharmaceuticals, crop protection compounds, and industrial chemicals (Kimmel and Price 1990; Foote and Carney 2000; Barrow 2009; Wise et al. 2009). Rabbits offer several advantages in developmental toxicity studies: they have been shown to be a useful indicator of teratogenic potential; induced ovulation can be used to produce a uniform starting and termination time for experiments because rabbits do not ovulate spontaneously; the variability caused by the use of semen from more than one male can be eliminated by artificial insemination; and the relatively large size of the rabbit fetus, as compared to the mouse or rat fetus, permits a more precise gross examination (Gibson et al. 1996). Other reasons for using rabbits as experimental animals are that sufficient numbers can be obtained and historical control data are available in the testing laboratories.

It is important to have comprehensive historical control data available because a comparison of data from study controls with historical control data may be beneficial to evaluations of toxicity. Historical control data on reproductive and developmental toxicity studies are required for adequate interpretation of the experimental results and evaluation for reproductive and developmental toxicity. Historical control data may help to distinguish treatment-induced changes from spontaneous occurring background changes specific to the species/strains. Previously, historical control data on prenatal developmental toxicity studies are available for New Zealand White (NZW) rabbits (Palmer 1968; Kimmel and Price 1990; Feussner et al. 1992), Himalayan rabbits (Viertel and Trieb 2003), and Dutch Belted rabbits (Wise et al. 2009). In Japan, historical control data on reproductive and developmental toxicity studies in experimental animals including rabbits were reported by Kameyama et al. (1980) (data from 13 laboratories), Morita et al. (1987) (Japanese Pharmaceutical Manufacturer's Association (JPMA) survey, data between 1980 and 1985 from 52 laboratories), and Nakatsuka et al. (1997) (JPMA survey, data between 1986 and 1993 from 71 laboratories).

However, no historical control data have been published on reproductive and developmental toxicity studies over the last decade in Japan. Subtle changes may occur with time due to genetic alterations in the strain or stock of the species used and changes in environmental conditions both in the breeding colony of the supplier and in the laboratory (Kimmel and Price 1990). It is important to examine changes in data over time within the historical control data and to compare study control data with recent as well as cumulative historical control data. Therefore, recent historical control data for rabbits between 1994 and 2010 were collected and organized in the present paper.

MATERIALS AND METHODS

Participating pharmaceutical and chemical companies and contract laboratories are shown in Table 1. Data were obtained from 20 laboratories, including 11 pharmaceutical and chemical companies and nine contract laboratories, in Japan. Data regarding terminal cesarean sections, and fetal external, visceral, and skeletal anomalies in rabbits were collected from prenatal developmental toxicity studies conducted from 1994 through 2010. Data were incorporated from a laboratory if the information was based on 10 studies or more. Data from range-finding studies that utilized a small number

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Received January 25, 2012; revised and accepted February 19, 2012.

Table 1 Participating laboratories and researchers

Laboratory #	Laboratory	Researcher
01	Astellas Pharma Inc., Drug Safety Research Laboratories	Seiki Matsuo Hiroko Noyori
02	Biosafety Research Center, Foods, Drugs and Pesticides (BSRC)	Keiichi Itoh Ryota Tanaka
03	Bozo Research Center Inc.	Yuzo Asano Yasumoto Mizoguchi
04	Daiichi Sankyo Co., Ltd., Medicinal Safety Research Laboratories	Ken-ichi Noritake Kazuhiro Shimomura
05	Dainippon Sumitomo Pharma Co., Ltd., Safety Research Laboratories	Kazuhiro Chihara Hiroshi Inada
06	Eisai Co., Ltd., Tsukuba Drug Safety/Sunplanet Co., Ltd., Preclinical Safety Research Laboratories	Maki Maeda Hiroshi Mineshima
07	Ina Research Inc.	Hiroaki Hara Tatsuya Shimizu
08	Institute of Environmental Toxicology, Toxicology Division	Hiroaki Aoyama Hitoshi Hojo
09	Kissei Pharmaceutical Co., Ltd.	Ikuro Takakura Ryohei Yokoi
10	Mitsubishi Chemical Medience Corporation, Kashima Laboratory	Nobuhito Hoshino Ikuo Matsuura
11	Mitsubishi Chemical Medience Corporation, Kumamoto Laboratory	Hiroyuki Izumi Takafumi Ohta
12	Nihon Bioresearch Inc.	Katsumi Endoh Yoji Miwa
13	Ono Pharmaceutical Co., Ltd., Safety Research Laboratories, Fukui Research Institute	Hidenori Miyata Harutaka Oku
14	Otsuka Pharmaceutical Co., Ltd., Tokushima Research Institute	Kei Shiozawa Tohru Uesugi
15	Safety Research Institute for Chemical Compounds Co., Ltd.	Sakiko Fujii Kaoru Yabe
16	Shin Nippon Biomedical Laboratories (SNBL) Ltd., Drug Safety Research Laboratories	Akihiro Arima Ayumi Inoue
17	Shionogi & Co., Ltd.	Nao Nakano Atsuko Hishikawa
18	Sumitomo Chemical Co., Ltd., Environmental Health Science Laboratory	Hashihiro Higuchi Yoshinori Hosokawa
19	Taiho Pharmaceutical Co., Ltd.	Hanako Nishizawa
20	Takeda Pharmaceutical Co. Ltd., Drug Safety Research Laboratories	Kiyoshi Matsumoto Toshiaki Yamauchi
	Chiba Institute of Science, Faculty of Risk and Crisis Management	Masao Horimoto
	National Institute of Advanced Industrial Science and Technology (AIST), Research Institute of Science for Safety and Sustainability	Makoto Ema Masato Naya

of does per group were also included in this data set. Data were summarized separately during 1994 to 2000 and 2001 to 2010.

The day of copulation was designated as gestational day (GD) 0. The category of fetal mortality included early resorptions and late fetal deaths. As for fetal alterations, incidence data are based on the

number of alterations observed in each category as a percentage of the total number of live fetuses examined. If more than one alteration was observed in a fetus, each was reported individually. The incidence of fetuses with malformations was expressed as proportion of number of fetuses with malformations to number of fetuses

examined. The terminology used for fetal external, internal, and skeletal alterations was principally based on Horimoto et al. (1998) and Makris et al. (2009).

RESULTS AND DISCUSSION

For Japanese White (JW) rabbits, data from between 1994–2000 were obtained from eight laboratories and incorporated from four laboratories and data from between 2001–2010 were obtained from 11 laboratories and incorporated from six laboratories. For NZW rabbits, data from between 1994–2001 were obtained from 12 laboratories and incorporated from eight laboratories and data from between 2001–2010 were obtained from 15 laboratories and incorporated from 11 laboratories. Kbl:JW and Kbl:NZW were used in all laboratories that performed 10 studies or more (Kitayama Labes Co., Ltd, Ina, Japan).

Mating and cesarean section data

Mating and cesarean section data during 1994–2000 and 2001–2010 in JW rabbits are shown in Table 2, and data for NZW rabbits between 1994–2000 and 2001–2010 are presented in Tables 3 and 4, respectively. To prepare pregnant rabbits, an artificial insemination technique was used in three of 12 laboratories between 1994 and 2000 and in five of 17 laboratories between 2001 and 2010. In JW rabbits, the average pregnancy rate was 92.7 to 94.3% between 1994 and 2000 and 92.9 to 95.0% between 2001 and 2010. In NZW rabbits, the average pregnancy rate ranged from 87.0 to 95.1% in 1994–2000 and from 87.2 to 93.7% in 2001–2010. The average abortion rate was 0 to 0.41% for 1994–2000 and 0 to 1.6% for 2001–2010 in JW rabbits, and 0.6 to 9.0% for 1994–2000 and 0 to 5.5% for 2001–2010 in NZW rabbits. In 12 laboratories, cesarean section was performed in does on GD 28, and in five laboratories, cesarean section was performed in does on GD 29. The average number of corpora lutea was 10.2 to 10.5 during 1994–2000 and 9.3 to 10.9 during 2001–2010 in JW rabbits, and 9.9 to 11.4 during 1994–2000 and 8.8 to 11.3 during 2001–2010 in NZW rabbits. The average number of implantations was 8.2 to 9.0 for 1994–2000 and 8.3 to 8.6 for 2001–2010 in JW rabbits, and 7.6 to 9.1 for 1994–2000 and 7.8 to 8.8 for 2001–2010 in NZW rabbits. The average number of live fetuses was 7.6 to 8.3 for 1994–2000 and 7.6 to 7.9 for 2001–2010 in JW rabbits, and 7.3 to 8.5 for 1994–2000 and 7.3 to 8.2 for 2001–2010 in NZW rabbits. The average fetal mortality ranged from 8.0 to 10.2% in 1994–2000 and 5.3 to 10.3% in 2001–2010 in JW rabbits, and from 5.3 to 9.7% in 1994–2000 and 4.9 to 8.2% in 2001–2010 in NZW rabbits. One laboratory determined fetal body weight with males and females combined, and the remaining laboratories evaluated fetal body weight for each sex separately. There were no noticeable strain differences in the reproductive parameters. Between the two intervals evaluated (1994–2000 and 2001–2010), there was no noticeable variation in the reproductive parameters of JW and NZW rabbits. The values of reproductive parameters were not clearly different from those in previous surveys in Japan (Kameyama et al. 1980; Morita et al. 1987; Nakatsuka et al. 1997). The average numbers of implantations and live fetuses were slightly higher than those of NZW rabbits reported by Kimmel and Price (1990) and Feussner et al. (1992) and of Himalayan rabbits reported by Viertel and Trieb (2003).

External anomalies

Tables S1 and S2 show data on external anomalies in JW rabbits during 1994–2000 and during 2001–2010, respectively. The data

for NZW rabbits in 1994–2000 and 2001–2010 are presented in Tables S3 and S4, respectively. In JW rabbits, the average total incidence of external malformations ranged from 0.14 to 0.70% during 1994–2000 and from 0.09 to 0.72% during 2001–2010 in JW rabbits. In NZW rabbits, the average was 0 to 1.74% in 1994–2000 and 0.08 to 0.73% in 2001–2010. These incidences were comparable to those reported previously (Kameyama et al. 1980; Morita et al. 1987; Kimmel and Price 1990; Nakatsuka et al. 1997). The common anomalies in this survey were observed in the limb, paw, digit, and tail. These anomalies were also reported previously and the incidences of these anomalies were comparable to those in previous reports (Kameyama et al. 1980; Morita et al. 1987; Kimmel and Price 1990; Nakatsuka et al. 1997). No noticeable variation with time or no noticeable difference between strains was observed in types of external anomalies.

Visceral anomalies

Data on visceral anomalies in JW rabbits during 1994–2000 and during 2001–2010 is presented in Tables S5 and S6, respectively. The data for NZW rabbits during 1994–2000 and during 2001–2010 are presented in Tables S7 and S8, respectively. In JW rabbits, the average total incidence of visceral malformations was 0.88 to 2.36% during 1994–2000 and 0.37 to 3.71% during 2001–2010. In NZW rabbits, the average ranged from 1.19 to 20.10% in 1994–2000 and from 0.41 to 20.85% in 2001–2010. These incidences were comparable to those reported previously (Morita et al. 1987; Nakatsuka et al. 1997). No noticeable variation with time or no noticeable difference between strains was observed in types of visceral anomalies. Large variations were noted in the incidences of visceral malformations among laboratories. These phenomena seem to be due to differences in the classification of visceral anomalies among laboratories. Visceral anomalies such as thymic cord and some anomalies of the vessels were classified as malformations in some laboratories, but as variations in other laboratories.

Skeletal anomalies

Tables S9 and S10 show data on skeletal anomalies in JW rabbits during 1994–2000 and during 2001–2010, respectively. The data for NZW rabbits during 1994–2000 and during 2001–2010 are presented in Tables S11 and S12, respectively. In JW rabbits, the average total incidence of skeletal malformations ranged from 1.68 to 2.53% in 1994–2000 and from 1.10 to 2.65% in 2001–2010. In NZW rabbits, it ranged from 0.31 to 4.31% during 1994–2000 and 1.22 to 9.69% during 2001–2010. These incidences were comparable to those reported previously (Kameyama et al. 1980; Morita et al. 1987; Kimmel and Price 1990; Nakatsuka et al. 1997). Anomalies in the vertebral column, sternbrae, and ribs were commonly observed. Between the two intervals evaluated (1994–2000 and 2001–2010), there was no noticeable variation and no noticeable difference between strains in types of skeletal anomalies. Skeletal variations are shown at the website of The Japanese Teratology Society (<http://jts.umin.jp/supplements.html>).

CONCLUSION

Historical control data on rabbit prenatal developmental toxicity studies, which were performed from 1994 to 2010, were obtained from 20 laboratories in Japan. In this paper, data were incorporated from a laboratory if the information was based on 10 studies or more. The stability of maternal reproductive and fetal developmental data was shown by the similarity of the data among laboratories. The inter-laboratory variations in the incidences of fetal external,

Table 2 Mating and cesarean section data from Japanese White (JW) rabbits during 1994–2000 and 2001–2010

Laboratory #	17	10	12	20	16	12	08	15	10	07
Strain	Kbl:JW	Kbl:JW	Kbl:JW	Kbl:JW	Kbl:JW	Kbl:JW	Kbl:JW	Kbl:JW	Kbl:JW	Kbl:JW
Year	1994–2000	1994–2000	1996–2000	1994–2000	2001–2010	2001–2009	2001–2010	2001–2010	2001–2010	2001–2010
Treatment	V	V	V	V	V	V	V	V	V	V
No. does	178	256	241	263	649	449	325	121	192	487
No. studies	17	15	15	14	51	28	13	13	10	25
No. does/study (Min-Max)	4–20	12–20	6–20	17–20	4–20	5–20	25	4–24	18–23	16–25
Mating	NM	AI	NM	NM	NM	NM	AI	NM	AI	NM
Pregnancy rate (%)†	94.3 (80.0–100)	93.1 (80.0–100)	92.7 (80.0–100)	93.9 (85.0–100)	93.7 (71.4–100)	94.1 (83.3–100)	92.3 (88.0–100)	94.9 (66.7–100)	95.0 (90.0–100)	94.5 (90.0–100)
Abortion rate (%)†	0	0	0.41 (0–7.1)	0.36 (0–5.0)	0.6 (0–14.3)	0.4 (0–16.7)	0.9 (0–4.0)	1.0 (0–8.3)	0	1.6 (0–10.0)
Day of cesarean section	28	28	28	28	28	28	28	28	28	29
No. corpora lutea†	10.4 (9.2–11.6)	10.4 (9.2–11.2)	10.5 (9.6–11.8)	10.2 (9.0–11.3)	9.3 (7.5–11.0)	9.4 (8.4–11.6)	10.5 (9.7–11.7)	10.2 (8.3–11.6)	10.7 (9.5–12.1)	10.9 (8.3–12.5)
No. implantations†	8.9 (7.2–9.4)	8.5 (7.1–10.0)	9.0 (7.9–11.0)	8.2 (6.7–9.3)	8.3 (5.8–10.3)	8.3 (5.4–9.6)	8.4 (7.7–9.1)	8.6 (7.1–10.0)	8.6 (7.3–9.5)	8.6 (7.5–9.9)
No. live fetuses†	8.2 (7.2–9.4)	7.8 (6.6–9.0)	8.3 (6.8–11.0)	7.6 (5.9–8.5)	7.8 (5.5–10.0)	7.7 (5.2–9.2)	7.5 (6.8–8.3)	7.8 (5.7–9.1)	7.9 (6.7–8.8)	7.9 (6.8–9.1)
Fetal mortality (%)†	9.2 (2.0–15.0)	10.2 (4.2–15.7)	8.0 (0–22.9)	8.4 (4.5–22.1)	5.3 (0–16.0)	6.6 (1.3–12.9)	10.1 (6.3–16.4)	10.3 (4.4–20.1)	8.1 (4.2–12.3)	7.9 (4.1–13.1)
Body weight (g)†										
All fetuses	35.8 (31.4–39.6)				38.0 (34.8–41.0)					
Male	36.1 (31.8–38.9)	40.2 (35.3–45.7)	37.8 (32.6–39.1)	38.6 (37.0–40.4)	38.4 (35.3–41.9)	38.2 (34.9–40.3)	38.6 (36.5–40.4)	38.3 (34.2–43.0)	37.7 (35.6–41.6)	44.8 (40.8–47.9)
Female	35.1 (30.7–39.0)	39.1 (35.3–44.1)	37.1 (33.5–39.0)	37.8 (34.9–39.3)	37.7 (33.0–40.6)	37.2 (32.5–40.2)	37.5 (35.5–39.4)	37.3 (31.6–41.4)	37.2 (35.2–39.7)	43.8 (41.8–45.9)

†Values are given as mean (min-max).

AI, artificial insemination; NM, natural mating; V, vehicle-treated.

Table 3 Mating and cesarean section data from New Zealand White (NZW) rabbits during 1994–2000

Strain	03	14	12	09	18	19	02	13
	Kbl:NZW	Kbl:NZW	Kbl:NZW	Kbl:NZW	Kbl:NZW	Kbl:NZW	Kbl:NZW	Kbl:NZW
Year	1994–2000	1994–2000	1996–2000	1995–2000	1994–2000	1994–1998	1994–2000	1994–1999
Treatment	V	U/V	V	V	V	U/V	V	V
No. does	563	349	260	154	96	127	248	188
No. studies	35	30	13	12	11	11	22	15
No. does/study (Min-Max)	10–21	4–19	6–20	4–20	3–18	5–11	4–22	4–22
Mating	NM	NM	NM	NM	AI	NM/AI	NM	NM
Pregnancy rate (%)†	87.0 (75.0–100)	89.2 (65.0–100)	92.6 (85.0–100)	95.1 (80.0–100)	89.7 (60.0–100)	93.2 (75.0–100)	94.2 (80.0–100)	90.0 (80.0–100)
Abortion rate (%)†	1.6 (0–11.8)	1.4 (0–16.7)	1.2 (0–5.0)	3.3 (0–25.0)	2.1 (0–11.1)	3.3 (0–18.2)	0.6 (0–9.5)	9.0 (0–28.6)
Day of cesarean section	28	28	28	28	28	28	29	29
No. corpora lutea†	9.9 (9.1–11.3)	10.3 (8.5–12.4)	10.2 (8.8–11.4)	10.4 (9.0–11.5)	11.4 (8.7–13.6)	9.9 (8.2–10.9)	10.1 (8.7–11.7)	10.5 (9.6–12.3)
No. implantations†	8.5 (7.6–9.9)	8.9 (6.4–10.9)	9.1 (7.3–10.2)	8.6 (6.5–10.0)	7.8 (5.3–10.2)	7.6 (5.2–9.3)	8.9 (6.7–10.3)	8.6 (6.8–10.3)
No. live fetuses†	8.0 (6.9–9.4)	8.4 (5.6–10.8)	8.5 (6.9–9.9)	7.7 (4.0–9.8)	7.4 (5.0–9.6)	7.3 (5.0–9.0)	8.3 (6.7–10.0)	8.0 (5.9–10.3)
Fetal mortality (%)†	6.4 (2.0–18.9)	5.8 (0–13.9)	6.4 (1.5–9.4)	9.7 (2.5–25.0)	5.8 (0–6.1)	5.3 (0–24.0)	6.1 (0–16.3)	6.4 (0–13.7)
Body weight (g)†								
All fetuses				35.8 (32.1–40.1)				40.5 (33.6–44.6)
Male	36.0 (31.7–39.1)	34.0 (30.6–37.8)	35.1 (33.6–38.9)	36.2 (32.7–39.7)	38.7 (35.9–44.7)	36.1 (32.7–40.9)	40.5 (36.5–43.5)	40.8 (34.2–44.1)
Female	34.8 (30.9–39.1)	33.0 (29.8–36.5)	34.1 (32.4–37.4)	35.3 (31.5–40.5)	37.0 (32.6–40.6)	34.9 (31.8–37.2)	39.2 (37.1–41.5)	40.0 (33.1–44.0)

†Values are given as mean (min-max).

AI, artificial insemination; NM, natural mating; V, vehicle-treated.

Table 4 Mating and cesarean section data from New Zealand White (NZW) rabbits during 2001–2010

Laboratory #	16	03	14	12	10	18	04	11	07	06	02
Strain	Kbl:NZW	Kbl:NZW	Kbl:NZW	Kbl:NZW	Kbl:NZW	Kbl:NZW	Kbl:NZW	Kbl:NZW	Kbl:NZW	Kbl:NZW	Kbl:NZW
Year	2002–2010	2001–2010	2001–2010	2001–2009	2001–2010	2001–2009	2001–2010	2001–2010	2001–2010	2001–2010	2001–2006
Treatment	V	V	U/V	V	V	V	V	V	V	V	V
No. does	796	693	298	249	346	150	299	578	505	258	170
No. studies	68	37	25	20	19	17	16	31	27	25	13
No. does/study (Min-Max)	5–22	9–22	4–19	4–20	16–21	3–23	16–21	13–20	15–25	3–19	4–22
Mating	NM	NM	NM	NM	AI	AI	NM	NM	NM	AI	NM
Pregnancy rate (%)†	93.7 (83.3–100)	87.9 (72.7–100)	87.2 (57.1–100)	91.9 (80.0–100)	90.6 (76.0–100)	92.1 (71.4–100)	91.6 (81.0–100)	92.5 (80.0–100)	92.3 (85.0–98.8)	92.4 (75.0–100)	88.5 (80.0–100)
Abortion rate (%)†	0.5 (0–16.7)	2.2 (0–18.8)	0.7 (0–5.6)	0	1.4 (0–5.9)	5.5 (0–25.0)	1.3 (0–11.1)	0.35 (0–5.9)	1.0 (0–11.1)	2.4 (0–28.6)	2.4 (0–16.7)
Day of cesarean section	28	28	28	28	28	28	28	29	29	29	29
No. corpora lutea†	9.2 (8.0–10.7)	10.0 (9.1–10.9)	9.8 (8.7–10.8)	9.5 (7.6–10.6)	10.5 (9.2–11.4)	11.3 (9.2–13.0)	9.9 (9.1–10.6)	8.8 (8.2–10.3)	10.7 (9.6–12.1)	9.2 (7.0–11.3)	9.0 (8.3–11.1)
No. implantations†	8.3 (6.5–10.5)	8.6 (7.1–9.6)	8.6 (7.1–10.0)	8.5 (6.6–9.8)	8.7 (7.7–9.7)	8.0 (5.3–9.2)	8.8 (7.8–9.7)	7.9 (6.3–9.0)	8.8 (7.4–9.8)	7.8 (4.3–10.0)	7.9 (7.8–9.4)
No. live fetuses†	7.9 (5.8–9.8)	8.1 (6.9–6.1)	8.1 (6.6–9.7)	8.0 (6.2–9.2)	8.0 (7.2–9.0)	7.5 (4.7–8.8)	8.2 (7.3–8.9)	7.3 (5.3–8.6)	8.1 (6.2–9.3)	7.3 (3.5–9.0)	7.4 (7.2–8.8)
Fetal mortality (%)†	5.1 (0–14.2)	6.6 (2.0–15.2)	7.1 (0–18.4)	4.9 (1.9–10.7)	7.9 (2.1–15.9)	7.6 (0–14.8)	6.4 (2.4–11.6)	7.4 (0.76–16.0)	8.2 (1.4–20.5)	5.8 (0–15.5)	5.3 (3.3–11.2)
Body weight (g)†											
All fetuses	34.5 (30.7–37.8)									42.2 (36.2–52.3)	
Male	35.0 (31.0–38.8)	34.8 (33.0–36.6)	33.8 (30.5–36.0)	34.3 (32.3–38.5)	35.7 (33.2–37.9)	36.8 (32.6–45.1)	37.0 (35.2–39.4)	41.0 (38.4–44.2)	40.3 (38.0–42.4)		38.1 (38.5–46.0)
Female	33.9 (29.8–37.4)	33.6 (31.2–35.5)	33.1 (30.8–35.2)	33.3 (32.5–38.3)	34.4 (32.0–37.0)	35.7 (31.8–45.9)	35.9 (33.9–38.6)	39.6 (36.7–42.7)	39.0 (36.7–40.8)		37.3 (37.5–45.7)

†Values are given as mean (min-max).

AI, artificial insemination; NM, natural mating; V, vehicle-treated.

visceral, and skeletal alterations seem to be due to differences in the selection of observation parameters, observation criteria, classification, and terminology of the fetal alterations. For further interpretation of the data, evaluation of the toxicity, and its assessment for human health, it is necessary to harmonize the classification and terminology of fetal alterations.

ACKNOWLEDGMENT

This work was partially supported by Health and Labour Sciences Research Grants (Research on Regulatory Science of Pharmaceuticals and Medical Devices: H23-Kenkou-005) from the Ministry of Health, Labour and Welfare of Japan.

REFERENCES

- Barrow PC. 2009. Reproductive toxicity testing for pharmaceuticals under ICH. *Reprod Toxicol* 28:172–179.
- Feussner EL, Lightkep GE, Hennesy RA, Hoerman AM, Christian MS. 1992. A decade of rabbit fertility data: study of historical control animals. *Teratology* 46:349–365.
- Foote RH, Carney EW. 2000. The rabbit as a model for reproductive and developmental toxicity studies. *Reprod Toxicol* 14:477–493.
- Gibson JP, Staples RE, Newberne JW. 1996. Use of the rabbit in teratology studies. *Toxicol Appl Pharmacol* 9:398–408.
- Horimoto M, Ariyuki F, Daidohji S et al. 1998. Terminology of developmental abnormalities in common laboratory mammals. *Congenit Anom Kyoto* 38:153–237.
- Kameyama Y, Tanimura T, Yasuda M. 1980. Spontaneous malformations in laboratory animals – Photographic atlas and reference data. *Congenit Anom Kyoto* 20:25–106.
- Kimmel CA, Price CJ. 1990. Developmental toxicity studies. In: Arnold DL, Grice HC, Krewski DR, eds. *Handbook of In Vivo Toxicity Testing*. San Diego, CA: Academic Press, pp. 271–299.
- Makris S, Solomon HM, Clark R et al. 2009. Terminology of developmental abnormalities in common laboratory mammals (version 2). *Congenit Anom Kyoto* 49:123–246.
- Morita H, Ariyuki F, Inomata N et al. 1987. Spontaneous malformations in laboratory animals: frequency of external, internal and skeletal malformations in rats, rabbits and mice. *Congenit Anom Kyoto* 27:147–206.
- Nakatsuka T, Hoimoto M, Ito M, Matsubara Y, Akaike M, Ariyuki F. 1997. Japan Pharmaceutical Manufacturers Association (JPMA) survey on background control data of developmental and reproductive toxicity studies in rats, rabbits and mice. *Congenit Anom Kyoto* 37:47–138.
- Palmer AK. 1968. Spontaneous malformations of New Zealand White Rabbit: the background to safety evaluation test. *Lab Anim Sci* 21:195–206.
- Viertel B, Trieb G. 2003. The Himalayan rabbit (*Oryctolagus cuniculus* L.): spontaneous incidences of endpoints from prenatal developmental toxicity studies. *Lab Anim* 37:19–36.
- Wise LD, Bushmann J, Feuston MH et al. 2009. Embryo-fetal developmental toxicity study design for pharmaceuticals. *Birth Defects Res B* 86:418–428.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Table S1 External anomalies in Japanese White (JW) rabbits during 1994–2000.

Table S2 External anomalies in Japanese White (JW) rabbits during 2001–2010.

Table S3 External anomalies in New Zealand White (NZW) rabbits during 1994–2000.

Table S4 External anomalies in New Zealand White (NZW) rabbits during 2001–2010.

Table S5 Visceral anomalies in Japanese White (JW) rabbits during 1994–2000.

Table S6 Visceral anomalies in Japanese White (JW) rabbits during 2001–2010.

Table S7 Visceral anomalies in New Zealand White (NZW) rabbits during 1994–2000.

Table S8 Visceral anomalies in New Zealand White (NZW) rabbits during 2001–2010.

Table S9 Skeletal anomalies in Japanese White (JW) rabbits during 1994–2000.

Table S10 Skeletal anomalies in Japanese White (JW) rabbits during 2001–2010.

Table S11 Skeletal anomalies in New Zealand White (NZW) rabbits during 1994–2000.

Table S12 Skeletal anomalies in New Zealand White (NZW) rabbits during 2001–2010.

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First-trimester serum folate levels and subsequent risk of abortion and preterm birth among Japanese women with singleton pregnancies

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Received: 2 March 2012 / Accepted: 24 July 2012 / Published online: 9 August 2012
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Abstract

Objectives To determine whether a low serum folate level during the first trimester predicts subsequent late abortion, preterm birth, or fetal growth restriction (FGR).

Study design A prospective cohort study involving 5,075 women whose serum folate levels were measured during the first trimester. The participants were informed of their serum folate levels.

Results The pregnancy duration, birthweight, rate of late abortion/preterm birth, and the rate of FGR did not differ significantly among the four groups classified according to folate status. The mean serum folate levels did not differ among quartiles classified according to the gestational week at the time of delivery. Nineteen of the 20 women with folate deficiency gave birth at term to infants with a birthweight of 3.132 ± 321 g; only one infant had FGR.

Conclusion Low serum folate levels during the first trimester were not associated with the risk of late abortion, preterm birth, or FGR.

Keywords Abortion · Nutrition · Preterm birth · Serum folate

Introduction

Adequate folate status is important not only during the periconceptional period, but also throughout pregnancy to achieve a satisfactory pregnancy outcome [1]. Since unambiguous evidence has shown that folic acid protects against both the first occurrence and the recurrence of neural tube defects [2, 3], expert committees worldwide have issued folic acid recommendations. In 2000, the Japanese government also issued a recommendation that all women planning to conceive consume a daily supplement containing 400 µg of folic acid to reduce the risk of neural tube defects during pregnancy.

Because folate is essential for DNA and RNA biosynthesis, an increased demand for folate exists during pregnancy because of the cellular proliferation occurring in the placenta and fetus. Indeed, the amount of folate breakdown products in the urine doubles during the second trimester and returns to the baseline level postpartum, suggesting an extra demand for dietary folate during pregnancy [4]. Since the calculated total fetal and placental folate content is 800 µg at term [5], the increased demand for folate may be due not only to fetal transfer, but also to the accelerated breakdown of the vitamin through folate's participation in DNA synthesis [6]. This hypothesis suggests a possible relation between the folate status in the mother and fetal growth. Indeed, the effects of folate on increases in birthweight have been observed with some consistency among poorly nourished women [7, 8].

Folate is also required for homocysteine metabolism. Folate is the major determinant of homocysteine

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concentrations, with well-established evidence that supplementation with folic acid has a marked homocysteine-lowering effect [9]. Folic acid supplementation during pregnancy decreases the plasma total homocysteine level [10]. Thus, the serum homocysteine level can be used as a marker of folate status. Hyperhomocysteinemia is associated with pregnancy complications and an adverse pregnancy outcome, including miscarriage [11], placental abruption [12], and severe preeclampsia [13]. Since all these complications may shorten the length of gestation, the preterm birth rate may be higher among women with folate deficiency than among those with a normal folate status.

Indeed, according to the results of a previous study [14], women with serum folate levels <16.3 ng/mL (<37.3 nmol/L) during the second trimester have a higher risk of preterm birth than those with folate levels ≥ 16.3 ng/mL; however, no information regarding frequency of preeclampsia or placental abruption has been provided.

Whether the folate status during the first trimester is associated with the risk of preterm birth or inadequate fetal growth remains unknown. Accordingly, we conducted this prospective cohort study to examine whether the first-trimester serum folate level is associated with preterm birth or fetal growth.

Methods

This study was performed after receiving the approval of our institutional review board and obtaining informed consent from all the participants. The study participants were native Japanese pregnant women recruited from an ongoing birth cohort: the Hokkaido study on environment and children's health. The cohort was started in February 2003 and recruitment is ongoing; the details of the study have been described previously elsewhere [15]. All pregnant indigenous Japanese women who were booked to receive antenatal care at any of 37 participating health facilities in Hokkaido were considered eligible for the cohort study. This report analyzed data from 5,075 participants who were recruited during their first trimester between February 2003 and March 2006. Data on the demographic characteristics were obtained from baseline self-administered questionnaires at the time of recruitment and the infant's hospital birth records. We were able to collect information including maternal age, number of prior births, body height, prepregnancy body weight, folate supplementation, smoking, alcohol ingestion, annual income, gestational week at delivery and infant birthweight. Small for gestational age (SGA) was defined as a birthweight below the tenth percentile for gestational age for Japanese infants [16].

Serum folate concentrations were assayed in the blood specimens obtained during the first trimester [gestational

week (GW) of $5^{-0/7}$ to $13^{-6/7}$] using an automated competitive protein-binding chemiluminescent enzyme immunoassay (CLEIA) technique according to the assay manufacturer's instructions; the measurements were performed by a commercial laboratory (SRL Inc., Tokyo, Japan). Analyses were conducted in batches because of the continuous recruitment of the study participants. The laboratory personnel were blinded to the folate supplement status of the participants whose biological specimens were assayed. Each participant's physician informed the participant of her serum folate level, approximately 3 weeks after the blood sample collection.

Statistical analyses were performed using the JMP8 statistical software package (SAS, Cary, NC). Data are reported as mean \pm SD, unless otherwise stated. The Mann–Whitney *U* and the Kruskal–Wallis tests were used when comparing two or three groups, respectively. Differences in the means were tested using the Tukey–Kramer HSD (honestly significant difference) test between each group, and categorical variables were compared using Pearson's Chi-squared test. A *p* value of less than 0.05 was considered to indicate statistical significance.

Results

Among the 5,075 women analyzed in the present study, the mean serum folate level determined at GW 10.7 ± 1.5 was 18.4 ± 21.6 nmol/L (Table 1). Forty-eight (1.0 %) and 220 (4.3 %) women experienced a late abortion between GW $14^{-0/7}$ and $21^{-6/7}$ and preterm birth between GW $22^{-0/7}$ and $36^{-6/7}$, respectively. The intake of folate supplements significantly increased the serum folate levels (Table 1) and the number of women with higher serum folate levels (Fig. 1). Serum folate levels (nmol/L) were 16.9 ± 13.8 in 4,499 women with no folate supplementation, 29.2 ± 59.2 in 389 women who began to take folate supplementation after conception, and 36.0 ± 19.0 in 129 women who initiated folate supplementation before conception (Table 1). Serum folate levels increased with advancing maternal age (Table 1; Fig. 2). In addition, the prepregnancy maternal body mass index, use of infertility treatment, alcohol ingestion, and annual income were associated with serum folate levels. Women with smoking habit had a significantly lower serum folate level than non-smokers. Multiple linear regression analysis was performed with serum folate concentration as the dependent variable and maternal age, prepregnancy BMI, infertility treatment, folate supplementation, smoking, alcohol intake, and annual income as independent variables, with the result that only folate supplementation was a statistically significant variable ($p = 0.0004$). None of the maternal

characteristics were significantly associated with the rate of late abortion or preterm birth (Table 1).

The effect of folate status during the first trimester on the subsequent preterm birth and fetal development was examined. The rate of late abortion/preterm birth, the mean gestational week at delivery, the mean birthweight, the SGA rate, or the female-to-male ratio did not differ significantly among quartiles divided according to folate status (Table 2). Unexpectedly, the mean serum folate

level was significantly higher among women who gave birth at a GW of 28–36 than among women who gave birth at term (Table 3). The outcomes of the 20 women with folate deficiency (<6.8 nmol/L) were investigated individually. One woman miscarried at GW 20, but the remaining 19 women gave birth to infants with a birthweight of $3,132 \pm 321$ g (mean \pm SD) at term (GW 39.5 ± 1.1). Only one of them gave birth to an SGA infant weighing 2,550 g at GW 38.

Table 1 Relationships between demographic characteristics, serum folate levels, late abortion, and preterm birth

	Serum folate level (<i>n</i>) (nmol/L)	<i>p</i> value ^a	Late abortion (%)	<i>p</i> value (χ^2)	Preterm delivery (%)	<i>p</i> value (χ^2)
Maternal age (years)						
≤ 19	46 (13.2 \pm 4.0)	<0.0001	0 (0.0)	0.0825	2 (4.4)	0.3626
20–29	2,370 (17.2 \pm 8.1) ^b		18 (0.8)		90 (3.8)	
30–39	2,565 (19.6 \pm 29.2)		27 (1.1)		124 (4.8)	
≥ 40	94 (19.5 \pm 10.7)		3 (3.2)		4 (4.3)	
Prepregnancy BMI (kg/m²)						
<18.5	899 (17.8 \pm 11.0)	0.0019	9 (1.0)	0.6611	42 (4.7)	0.1617
18.5–24.9	3,528 (18.8 \pm 25.0)		31 (0.9)		140 (4.0)	
≥ 25.0	550 (17.3 \pm 8.2)		7 (1.3)		37 (5.6)	
Parity						
0	1,075 (18.2 \pm 9.4)	0.4237	8 (0.7)	0.1587	46 (4.3)	0.8739
≥ 1	2,570 (18.3 \pm 24.2)		33 (1.3)		107 (4.2)	
Infertility treatment						
No	4,871 (18.3 \pm 21.9)	0.0006	45 (0.9)	0.3367	208 (4.3)	0.4556
Yes	185 (20.8 \pm 11.1)		3 (1.6)		10 (5.4)	
Folate supplementation						
No	4,499 (16.9 \pm 13.8)	<0.0001	43 (1.0)	0.9888	194 (4.3)	0.4694
After conception	389 (29.2 \pm 59.2) ^c		4 (1.0)		19 (4.9)	
Before conception	129 (36.0 \pm 19.0) ^d		1 (0.8)		5 (3.9)	
Smoking						
No	2,207 (19.5 \pm 26.3)	<0.0001	18 (0.8)	0.3691	99 (4.5)	0.6460
Yes	2,820 (17.7 \pm 17.2)		30 (1.1)		119 (4.2)	
Alcohol ingestion						
No	1,866 (17.8 \pm 16.9)	0.0060	18 (1.0)	0.9512	92 (5.0)	0.0971
Yes	3,167 (18.8 \pm 18.0)		30 (1.0)		125 (4.0)	
Annual income (USD)						
$<36,585$	889 (17.2 \pm 9.7)	<0.0001	11 (1.2)	0.0817	33 (3.7)	0.7985
36, 586–60, 974	1,927 (18.4 \pm 20.1)		11 (0.6)		87 (4.5)	
60, 975–97, 560	1,092 (19.7 \pm 35.5)		16 (1.5)		47 (4.3)	
$>97,561$	323 (20.2 \pm 9.7)		4 (1.2)		13 (4.0)	
Overall	5,075 (18.4 \pm 21.6)		48 (1.0)		220 (4.3)	

Exchange rate: 1 USD/82.00 Japanese Yen

USD united states dollar, BMI body mass index

^a The Mann–Whitney *U* and the Kruskal–Wallis tests were used when comparing the means in two or three groups, respectively

^b *p* = 0.0005 versus age 30–39 years

^c *p* = 0.0089 versus no folate supplementation

^d *p* < 0.0001 versus no folate supplementation and folate supplementation after conception