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Genotoxicity evaluation of fullerene C_{60} nanoparticles in a comet assay using lung cells of intratracheally instilled rats

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ABSTRACT

The genotoxicity of fullerene C_{60} nanoparticles was evaluated *in vivo* with comet assays using the lung cells of rats given C_{60} nanoparticles. The C_{60} nanoparticles were intratracheally instilled as a single dose at 0.5 or 2.5 mg/kg or repeated dose at 0.1 or 0.5 mg/kg, once a week for 5 weeks, to male rats. The lungs were obtained 3 or 24 h after a single instillation and 3 h after repeated instillation. Inflammatory responses were observed in the lungs obtained 24 h after a single instillation at 2.5 mg/kg and repeated instillation at 0.5 mg/kg. Histopathological examinations revealed that C_{60} nanoparticles caused slight changes including hemorrhages in alveoli and the cellular infiltration of macrophages and neutrophils in alveoli. In comet assays using rat lung cells, no increase in % Tail DNA was found in any group given C_{60} nanoparticles. These findings indicate that C_{60} nanoparticles had no potential for DNA damage in comet assays using the lungs cells of rats given C_{60} even at doses causing inflammation.

1. Introduction

Nanomaterials are small-scale substances less than 100 nm in at least one dimension (ISO, 2008), which exhibit physical, chemical and/or biological characteristics associated with a nanostructure (Oberdörster et al., 2005a). Humans have been exposed to airborne nanoparticles throughout evolution, but exposure has increased dramatically because of anthropogenic factors including combustion engines, power plants, and other sources of thermodegradation (Oberdörster et al., 2005b). The rapidly developing field of nanotechnology, which is creating materials with size-dependent properties, is likely to become another source of exposure to nanosubstances. These nanosubstances have an increased surface area: mass ratio thereby greatly enhancing their chemical/catalytic reactivity compared to normal-sized forms of the same substance.

C_{60} fullerene is a remarkably stable compound consisting of 60 carbon atoms with a diameter of approximately 0.7 nm and a molecular weight of 720 g/mol, and 30 carbon double bounds are present in the structure, to which free radicals can easily be added (Aschberger et al., 2010). There are a variety of fullerene derivatives available, which stems from the number of carbon atoms used to generate fullerenes, diverse array of moieties that can be attached to the fullerene surface, and different processes utilized to render fullerenes water soluble (Johnston et al., 2010). Surface modifications are often used to make fullerenes dispersible in water, allowing their use in pharmaceutical applications or in cosmetics (Aschberger et al., 2010). Chemically-modified fullerenes are being developed for targeted drug delivery, molecular ball bearings acting as lubricants, and nanoscale chemical sponges (ENRHES, 2009). Widespread production and use have caused the release of increasing amounts of nanomaterials into the environment. Introduction of novel materials into industry requires safety evaluation as well as an understanding of the impact of the nanomaterials on human health, because the unique properties and size of nanomaterials may also result in unique health risks, which are not able to be predicted by the toxicological effects of larger substances of the same composition (Murray et al., 2009). Despite growing concern over the potential risk that nanomaterials pose, there is a lack of information on their potential toxicity. There is a knowledge gap between the increasing development and use of nanomaterials and the prediction of possible health risks. At present, knowledge on the toxicological effects of fullerenes is limited.

A key area governing the risk assessment of chemical substances for human health is genotoxicology due to the fact that classic genotoxic substances lead to carcinogenesis (Singh et al., 2009). Genotoxicity testing, and thus the evaluation of the carcinogenicity and mutagenicity of chemicals, is the most important

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part of the safety assessment of chemical compounds. Genotoxicity studies, including *in vitro* and *in vivo* assays, of fullerenes and their derivatives are available (Ema et al., 2011). Positive results were obtained from *in vitro* comet assays (Dhawan et al., 2006), gene mutation assays using bacteria (Sera et al., 1996) and transgenic mouse embryonic cells (Xu et al., 2009a), and micronucleus assays (Totsuka et al., 2009). Positive results were also reported on *in vivo* comet assays (Totsuka et al., 2009), oxidatively damaged DNA assays (Folkmann et al., 2009), and *gpt* gene mutation assays (Totsuka et al., 2009). Negative results were obtained using *in vitro* comet assays (Jacobsen et al., 2008), bacterial gene mutation assays (Babynin et al., 2002; Shinohara et al., 2009), and mammalian chromosomal aberration assays (Shinohara et al., 2009). Negative results were also reported on *in vivo* comet assays (Jacobsen et al., 2009) and micronucleus assays (Shinohara et al., 2009). As described, there are inconsistencies in the results of reports on the genotoxicity of fullerenes and their derivatives that make it very difficult to draw firm conclusions.

Two principle modes of genotoxic action can be considered for particles, referred to as primary and secondary genotoxicity (Greim et al., 2001; Schins, 2002; Schins and Knaapen, 2007). Primary genotoxicity is defined as genetic damage elicited by particles in the absence of inflammation, whereas secondary genotoxicity is a pathway of genetic damage resulting from oxidative DNA attack by reactive oxygen/nitrogen species (ROS/RNS), generated during particle-elicited inflammation (Greim et al., 2001; Schins, 2002; Schins and Knaapen, 2007). Clarification of the principle modes of genotoxic action is very important for risk assessment, because secondary genotoxicity is considered to involve a threshold. One of the major routes of exposure to fullerenes is inhalation, and the lungs area major target organ of fullerenes. Therefore, we conducted a genotoxicity study of C_{60} nanoparticles with the comet assay using the lung cells of rats intratracheally instilled with C_{60} nanoparticles, at doses that did and did not elicit pulmonary inflammation.

2. Materials and methods

The experiments were performed at the Biosafety Research Center, Foods, Drugs and Pesticides (BSRC, Iwata, Japan) in 2010–2011 according to Guidelines for Animal Experimentation (1987), the Law Concerning the Protection and Control of Animals (1973), and Standards Relating to the Care and Management of Experimental Animals (1980). The study was approved by the Institutional Animal Care and Use Committee of the BSRC and performed in accordance with the ethics criteria contained in the bylaws of the Committee of National Institute of Advanced Industrial Science and Technology (AIST).

2.1. Chemicals

Commercially available fullerene C_{60} nanoparticles (Nanom purple SU, refined by sublimation, purity >99.5%, Frontier Carbon Co., Ltd., Kitakyushu, Japan) were used throughout this study. The specific surface area of the purchased C_{60} before hand-grinding in agate mortar was 0.92 m^2/g, measured with the BET method (Brunauer et al., 1938). Ethyl methanesulfonate (EMS), Dulbecco's phosphate-buffered saline, regular melting agarose, and triton-X were obtained from Sigma–Aldrich Corporation (St. Louis, MO). Low melting agarose (Rockland, Inc., Troy, MI), ethylene diamine tetra acetic acid (EDTA) disodium salt (DOJINDO LABORATORIES, Kumamoto, Japan), Hanks' balanced salt solutions (Life Technologies Corporation, Carlsbad, CA), SYBR® Gold nucleic acid gel stain (Life Technologies Corporation), and TE buffer solution (pH 8.0) (Nippon Gene, Tokyo, Japan) were used. Polyoxyethylene sorbitan monooleate (Tween 80), dimethyl sulfoxide (DMSO), tris hydroxymethyl aminomethane, and sodium *N*-lauroyl sarcosinate were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

2.2. Particle preparation and characterization

Fullerene C_{60} nanoparticles were dispersed in distilled water containing 0.1% Tween 80. The preparation and characterization of the C_{60}nanoparticle suspension were reported by Morimoto et al. (2010). Briefly, bulk fullerene material was dispersed in distilled water containing 0.1% Tween 80 and milled in an agate mortar for 30 min. The milled fullerene material was suspended with 50-μm zirconium particles using a high-performance dispersion machine. The C_{60} nanoparticle suspension was separated by centrifugation at 8000*g* for 60 min. The concentration was determined by high-performance liquid chromatography. The mean diameter based on the volume and mass of fullerenes in the 0.1% Tween 80 aqueous solution was 33 nm.

2.3. Animals and treatment

MaleCrl:CD(SD) rats (7 weeks-old) were purchased from Charles River Laboratories, Japan, Inc. (Yokohama, Japan). Rats were maintained in an air-conditioned animal room at 20–26 °C with a relative humidity of 35–75%, a 12:12-h light/dark cycle, and ventilation with 12 air changes/h. Standard rodent pellet (CRF-1, Oriental Yeast Co., Ltd., Tokyo, Japan)and tap water were given *ad libitum*. After a 7-day acclimation, rats were subjected to treatment.

The C_{60} nanoparticles were intratracheally instilled at a dose of 0.5 or 2.5 mg/kg (single instillation groups), or 0.1 or 0.5 mg/kg once a week for 5 weeks (repeated instillation groups). In the single instillation groups, rats were sacrificed 3 or 24 h after the instillation. In the repeated instillation groups, they were sacrificed 3 h after the last instillation. At higher doses, eight rats were given C_{60} nanoparticles to secure five rats per group for comet assays. At lower doses, five rats per group for each time point were instilled. As a negative control, five rats were given Tween 80 at 1 mg/mL/kg by a single or repeated intratracheal instillation similar to the C_{60} nanoparticles. As a positive control, five rats were orally given a single dose of EMS at 500 mg/kg at 3 h before sacrifice. In five rats of each group, the left lobes of the lungs were used for histopathological examination and the right lobes were used for the comet assay.

Dosage levels were determined based on the results of a preliminary study in which male rats were given a single intratracheal instillation of C_{60} nanoparticles at 0.5 or 2.5 mg/kg. Rales were heard in one of the three rats at 0.5 and 2.5 mg/kg and one rat died immediately after the instillation at 2.5 mg/kg.

2.4. Histopathological examination

The left lobes of the lungs were fixed in 10% neutral buffered formalin for histopathological examination. Tissues were routinely processed, embedded in paraffin, sectioned at 4–6 μm, and stained with hematoxylin and eosin (HE).

2.5. Comet assay

The comet assay was conducted in accordance with the standard protocol "International Validation of the *In Vivo* Rodent Alkaline Comet Assay for the Detection of Genotoxic Carcinogens" issued by the Japanese Center for the Validation of Alternative Methods (JaCVAM). Briefly, the right lobes of the lungs were washed out with homogenizing buffer (Hanks' balanced salt solutions containing 25 mmol/L EDTA-2Na and 10% DMSO) and

homogenized in 5 mL of the homogenizing buffer using a Downs homogenizer. Cell suspensions were chilled on ice for about 5 min and centrifuged at 800 rpm for 5 min. After the supernatant was removed, the cells were re-suspended in homogenizing buffer. Ten microliters of the single cell suspension was mixed with 90 μL of 0.5% low-melting agarose gel, and 90 μL of the mixture was placed on a slide pre-coated with 1.0% agarose gel. Another 90 μL of low melting agarose was added. Two slides were prepared from each rat. The slides were transferred to lysing solution (2.5 mol/L NaCl, 100 mmol/L EDTA-2Na, 10 mmol/L, pH 10 Tris buffer, 10% DMSO and 1% Triton X-100) for at least one night at 4 °C in the dark. The slides were next covered with chilled electrophoresis buffer (pH >13) for 20 min to allow DNA to unwind. Electrophoresis was conducted at a constant voltage of 0.7 V/cm (25 V) (initial current: 300 mA) for 20 min. The slides were transferred into neutralization buffer and held for about 10 min. Subsequently, they were dehydrated with ethanol, and air-dried. The slides were stained with SYBR® Gold nucleic acid gel stain which was diluted 5000-fold with TE buffer solution. Images of DNA migration were examined using a fluorescence microscope (Olympus Corporation, Tokyo, Japan). The final magnification was 200×. The images were analyzed using a Comet assay analyzer (Comet Assay IV system, Perceptive Instruments Ltd., Suffolk, UK). The comet parameter to measure DNA damage in the cells was the percentage of DNA in the tail (% Tail DNA), because% Tail DNA could be considered meaningful and easy to conceptualize (Kumaravel and Jha, 2006). Images of 100 (50 × 2) cells per rat were analyzed. The mean of the % Tail DNA value (mean value of 100 cells) of each group was calculated.

2.6. Statistical analysis

Data for C_{60} nanoparticle-treated groups and negative and positive control groups were analyzed using Dunnett's multiple comparison tests. Data for the positive control was compared to that for the negative control with Aspin–Welch's *t* test.

3. Results

3.1. Single instillation (autopsy at 3 h after instillation)

No changes were observed in clinical signs and body weights of rats given C_{60} nanoparticles at 0.5 and 2.5 mg/kg. At autopsy, brown-patches on the lungs were found in three rats at 0.5 mg/kg and in all rats at 2.5 mg/kg, whereas no brown-patches were noted in the rats given Tween 80 or EMS.

Images of histopathological changes in the lungs are presented in Fig. 1. The degree of all these changes was slight. The histopathological examinations revealed the focal accumulation of macrophages in one rat of the Tween 80-treated control group, the focal accumulation of macrophages in one rat at 0.5 mg/kg, and hemorrhage in one rat at 2.5 mg/kg.

The results of comet assays using the lung cells of rats given C_{60} nanoparticles are shown in Table 1. The average value of percent Tail DNA in the lung cells was 2.42 in the Tween 60-treated control group, and 2.09 and 3.07 in the group instilled with C_{60} nanoparticles at 0.5 and 2.5 mg/kg, respectively. There was no significant difference in % Tail DNA between the Tween 80-treated control and C_{60} nanoparticle-treated groups. The average % Tail DNA value was 16.98 in the EMS-treated positive control group, significantly higher than that in the Tween 80-treated control group.

3.2. Single instillation (autopsy at 24 h after instillation)

There was no change in clinical signs and body weights of rats given C_{60} nanoparticles at 0.5 and 2.5 mg/kg. At autopsy, a single brown-patch was noted in the lungs of one rat given Tween 80. A single brown-patch on the lungs was observed in two rats at 0.5 mg/kg, and multiple brown-patches on the lungs were found in all rats at 2.5 mg/kg.

Images of histopathological changes in the lungs are shown in Fig. 1. The degree of all these changes was slight. The focal accumulation of macrophages and hemorrhage in the alveoli in one rat

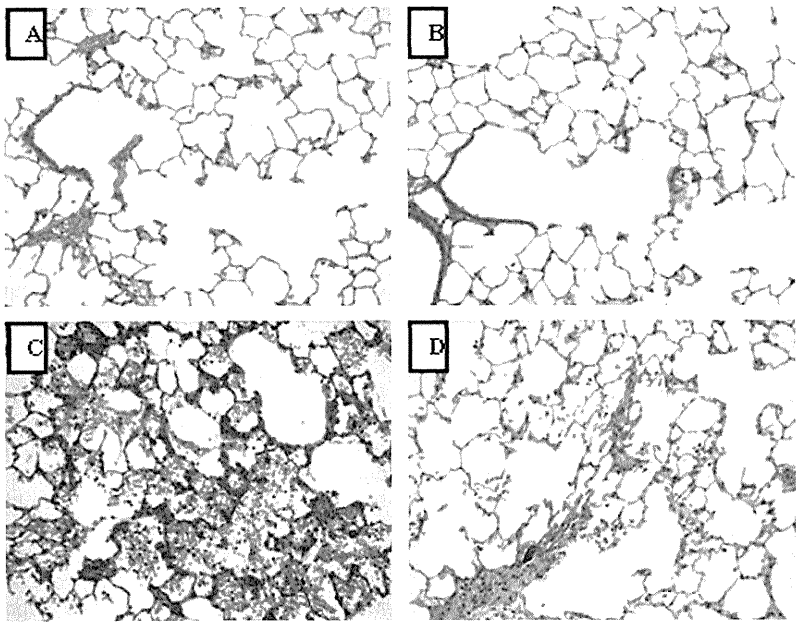

Fig. 1. HE staining of lung histopathology sections (magnification, 20×). (A) negative control: no significant lesions, (B) a single intratracheal instillation at 2.5 mg/kg autopsied 3 after the instillation): no significant lesions, (C) a single intratracheal instillation at 2.5 mg/kg (autopsied 24 h after the instillation): hemorrhage, infiltration of alveolar macrophages and neutrophils in the alveolus, thickening of the alveolar wall, and deposition of the test substances in alveolar macrophages, (D) repeated intratracheal instillation for 5 weeks at 0.5 mg/kg/week (autopsied 3 h after the last instillation): hemorrhage, infiltration of alveolar macrophages, and deposition of the test substances in alveolar macroophages.

Table 1
Effects of C_{60} nanoparticles on % Tail DNA in lung cells following a single or repeated intratracheal instillation in rats.

Treatments	Groups (compounds)	No. of rats	No. of cells analyzed/rat	% Tail DNA[a]
A single intratracheal instillation (autopsy at 3 h after instillation)	Negative control (Tween 80)[b]	5	100	2.42 ± 0.76
	C_{60} (0.5 mg/kg)	5	100	2.09 ± 0.62
	C_{60} (2.5 mg/kg)	5	100	3.07 ± 0.93
	Positive control (EMS)[c]	5	100	16.98 ± 7.08*
A single intratracheal instillation (autopsy at 24 h after instillation)	Negative control (Tween 80)[b]	5	100	3.13 ± 0.76
	C_{60} (0.5 mg/kg)	5	100	2.53 ± 0.62
	C_{60} (2.5 mg/kg)	5	100	3.07 ± 0.93
Repeated intratracheal instillation (autopsy at 3 h after instillation)	Negative control (Tween 80)[b]	5	100	4.65 ± 1.56
	C_{60} (0.1 mg/kg)	5	100	6.80 ± 1.76
	C_{60} (0.5 mg/kg)	5	100	5.08 ± 0.83
	Positive control (EMS)[c]	5	100	16.66 ± 1.94*

[a] Values are given as the mean ± S.D.
[b] Tween 80 was intratracheally instilled at 1 mg/mL/kg.
[c] EMS (ethyl methanesulfinate) was orally administered at 10 mL/kg.
* Significantly different from the negative control group ($p < 0.05$).

each were noted in the Tween 80-treated control group. The focal accumulation of macrophages in the alveoli in two rats was observed at 0.5 mg/kg. Multifocal hemorrhages in the alveoli in four rats, deposition of the test substances in macrophages of the alveoli and cellular infiltration of neutrophils and macrophages in the alveoli in five rats, thickening of the alveolar wall in two rats, and acute pneumonia with focal deposition of the hematoidin crystals in one rat were found at 2.5 mg/kg.

The results of comet assays using the lung cells of rats given C_{60} nanoparticles are summarized in Table 1. The average % Tail DNA value was 3.13 in the Tween 80-treated control group, and 2.53 and 3.07 in the group given C_{60} nanoparticles at 0.5 and 2.5 mg/kg, respectively. There was no significant difference in % Tail DNA between the Tween 80-treated control and C_{60} nanoparticle-treated groups.

3.3. Repeated instillation (autopsy at 3 h after instillation)

No difference was found in clinical signs and body weights between the Tween 80-treated control group and groups given C_{60} nanoparticles at 0.1 and 0.5 mg/kg. At autopsy, no brown-patches were found in the lungs of rats given C_{60} nanoparticles at 0.1 mg/kg or Tween 80. Multiple brown-patches on the lungs were observed in all rats given C_{60} at 0.5 mg/kg.

Images of histopathological changes in the lungs are shown in Fig. 1. The degree of all these changes was slight. No histopathological changes in the lungs were noted in rats of the Tween 80-teated control group. Acute focal pneumonia in two rats and focal hemorrhages in the alveoli in one rat were observed at 0.1 mg/kg. The focal accumulation of macrophages and hemorrhage in the alveoli in one rat each and focal or multifocal deposition of the test substances in the macrophages in the alveoli and cellular infiltration of the macrophages in the alveoli in five rats were found at 2.5 mg/kg.

The results of comet assays of the lung cells are also summarized in Table 1. The average value for % Tail DNA was 4.65 in the Tween 80-treated control group, and 6.80 and 5.08 in the group instilled with C_{60} nanoparticles at 0.5 and 2.5 mg/kg, respectively. There was no significant difference in % Tail DNA between the Tween 80-treated control and C_{60} nanoparticle-treated groups. The average value was 16.66 in the EMS-treated positive control group, significantly higher than in the C_{60}-treated groups.

4. Discussion

In this study, we determined the genotoxicity of fullerene C_{60} nanoparticles in comet assays using the lung cells of rats given C_{60} nanoparticles by intratracheal instillation. Inflammatory changes in the lungs were found 24 h after a single instillation and 3 h after the repeated instillation of C_{60} nanoparticles at high dose. In the lungs obtained 3 h after a single instillation at both doses and 24 h after a single instillation at low dose, and after the repeated instillation at low dose, focal hemorrhage and the accumulation of macrophages in the alveolus and pneumonia were observed. These changes are not thought to be inflammatory responses due to the instillation of C_{60} nanoparticles because they were very slight, also found in the Tween80-treated control groups, and commonly observed in the background control data. These findings indicate that a single instillation at 2.5 mg/kg and repeated instillation at 0.5 mg/kg caused pulmonary inflammation in rats. The most important finding of the present study is that C_{60} nanoparticles did not produce DNA damage in the lungs of rats given C_{60} even at doses causing inflammatory changes. This evidence indicates that C_{60} nanoparticles have no potential for genotoxicity *in vivo*.

Several genotoxicity studies of fullerenes and their derivatives have been performed using lung cells. *In vitro* chromosomal aberration assays were conducted using Chinese hamster lung cells and negative results were reported for C_{60} (Shinohara et al., 2009), a mixture of C_{60} and C_{70} (Mori et al., 2006), and a mixture of water-soluble polyvynylpyrroidone (PVP)-enwrapped C_{60} and C_{70} (Aoshima et al., 2010). In studies *in vitro*, positive results for pure C_{60} were obtained in micronucleus assays using human lung cancer cells (A549) (Totsuka et al., 2009) and comet assays using FE1-MML epithelial cells established from the lungs of a transgenic mouse (Jacobsen et al., 2008). The level of 8-oxoG was increased in the lung tissue of female Fischer rats after a single intragastric administration of pure C_{60} nanoparticles at 0.64 mg/kg (Folkmann et al., 2009). Pure C_{60} increased the frequency of *gtp* mutations and inflammatory changes in the lung tissue of male transgenic *gtp* delta mice intratracheally instilled a single or multiple dose of C_{60} at 0.2 mg/mouse (Totsuka et al., 2009). They also noted that DNA tail moment was increased in the lungs of male C57BL/6J mice given a single intratracheal instillation of C_{60} at 0.2 mg/mouse, but not at 0.05 mg/mouse (Totsuka et al., 2009). As described by the authors, the doses of C_{60} nanoparticles (approximately equivalent to 1.5 and 6 mg/kg) were extremely high compared with human exposure in the workplace. Discrepancy in the findings between this study and our study could be explained by the difference in endpoint, animal strain, and dose of C_{60} used in these experiments. Meanwhile, Jacobsen et al. (2009) reported that pure C_{60} did not significantly increase the values of % Tail DNA in broncho-alveolar lavage (BAL) cells of female apolipoprotein E knockout mice ($ApoE^{-/-}$), a model that may closer resemble humans with elevated cholesterol levels, given a single dose of C_{60} by intratracheal instillation at 0.054 mg/mouse. In these mice, inflammatory

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 C_{60} 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 recognized that limitation in some initial techniques have led to unintentional erroneous reports of nC_{60} 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 C_{60} and its derivatives, Sayes et al. (2007) noted that C_{60} nanoparticles and a water-soluble derivative, $C_{60}(OH)_{24}$, 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 C_{60} nanoparticles at 2.35 mg/m^3 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 C_{60} by a single intratracheal instilation 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 C_{60} 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 intratrachel 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 intratacheally instillation to water-soluble polyhydroxylated fullerenes [$C_{60}(OH)_XX$ = 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 C_{60} 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 C_{60} nanoparticles for the generation of ROS and development of inflammatory responses.

In conclusion, the present findings showed that C_{60} nanoparticles did not induce DNA damage in the lung cells of rats intratracheally instilled with C_{60} nanoparticles even at doses that elicited inflammatory responses. These findings suggest thatC_{60} nanoparticles have no potential for genotoxicity *in vivo*.

Conflict of interest statement

The authors declare there are no conflicts of interest.

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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.

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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

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, sternebrae, 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 Kbl:NZW	14 Kbl:NZW	12 Kbl:NZW	09 Kbl:NZW	18 Kbl:NZW	19 Kbl:NZW	02 Kbl:NZW	13 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).
Al, 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.

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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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ORIGINAL ARTICLE
Pediatric Cardiology and Adult Congenital Heart Disease

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 association 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

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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 (StataCorp, 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

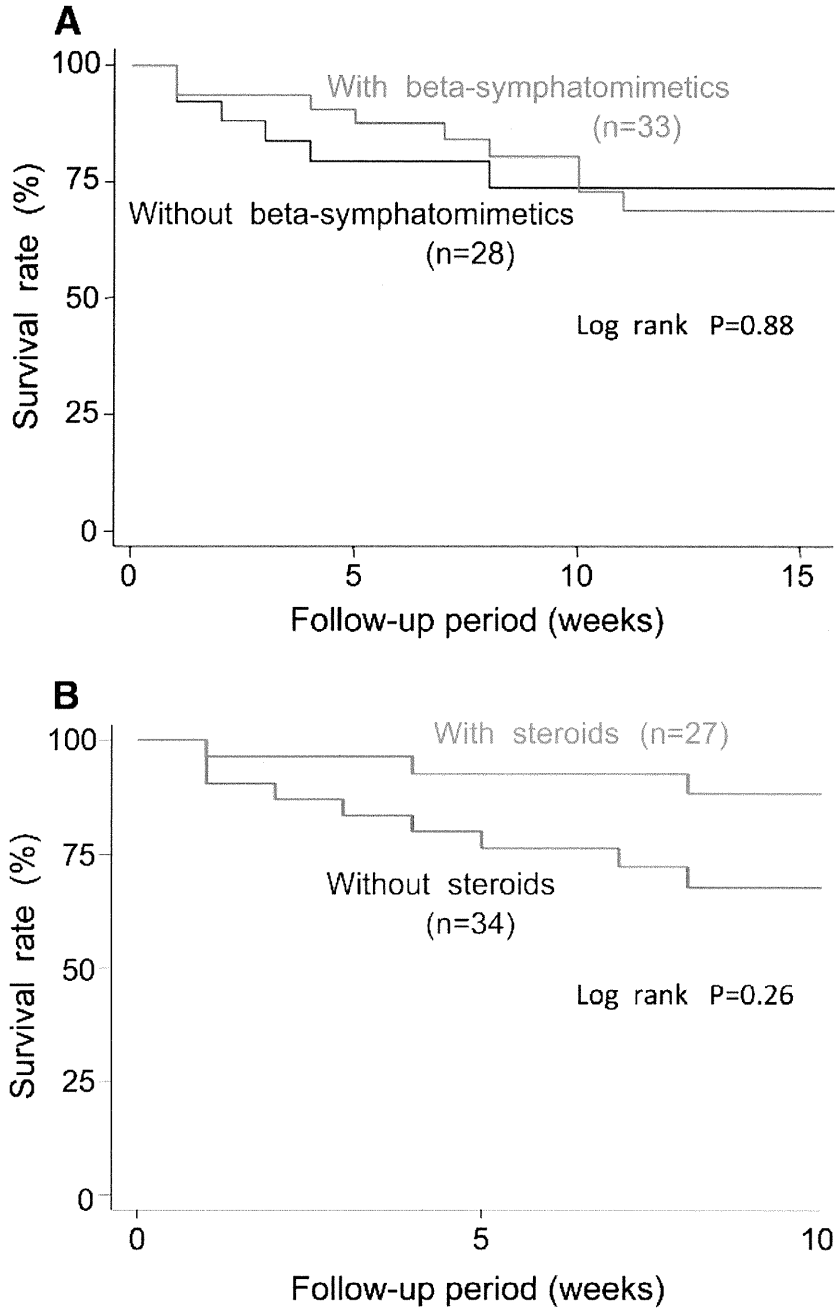

Figure. Kaplan-Meier survival curves (log-rank test and Cox proportional hazard model) for the primary endpoint (fetal death or neonatal death) in fetuses treated (**A**) with and without β-sympathomimetics and (**B**) with and without steroids. Follow-up period, time from diagnosis in utero to fetal or neonatal death. Significant difference defined as P<0.05.

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 arrythmia: CAVB	21	6	23
Fetal arrythmia: 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 levels 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.

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Disclosures

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