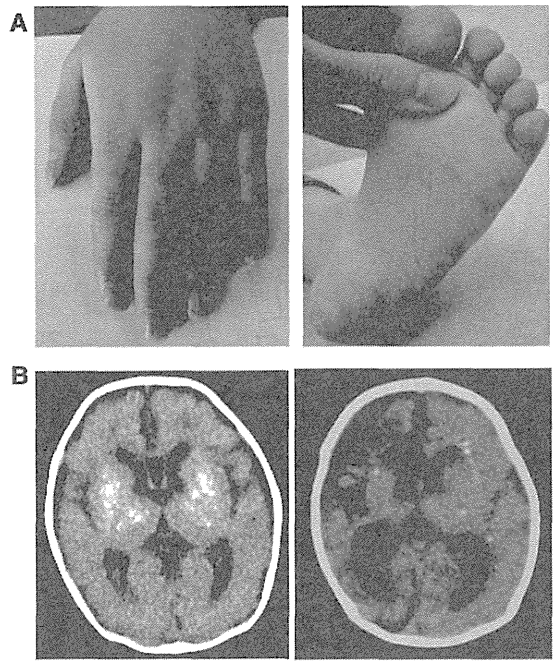
FIG. 3 Clinical presentations of Aicardi-Goutières syndrome patients



(**A**) Chilblain lesions observed in AGS patients. The left panel shows the hand of patient 1. The right panel shows the foot sole of patient 3. (**B**) Cranial calcifications on head CT scans. The left panel shows the head CT scan of patient 7 at the age of 4 months. The right panel shows head CT scan of patient 8 at the age of 3 months.

Laboratory findings

All of the AGS patients examined showed at least one CSF abnormality, including lymphocytosis, elevated IFN-α levels or elevated neopterin levels (Table 3). All three patients harbouring dominant-type *TREX1* mutations (patients 1–3) showed elevated serum IFN-α levels at the age of $\geqslant 7$ years.

Seven patients showed elevated levels of serum autoantibodies: ANA in four cases (patients 8–10 and 12), anti-ssDNA antibodies in four cases (patients 5, 7, 9 and 10), anti-dsDNA antibodies in patients 5 and 9, anti-RNP antibodies in patients 8 and 10, anti-DNA antibody in patient 8, anti-SS-A antibody in patient 10 and anti-LKM1 antibody in patient 11. All three patients harbouring *SAMHD1* mutations showed increases in multiple autoantibody types. Other immunological findings included hypergammaglobulinaemia in patients 9–11 and hypocomplementaemia in patients 9 and 11.

Abnormal liver function was observed in nine cases (patients 4–6, 8–11, 13 and 14). Hematological abnormalities included thrombocytopenia in four cases (patients 8, 9, 11, and 13) and anaemia in patients 8 and 14. Regarding endocrine complications, we found moderate hypothyroidism in patients 1 and 9 and diabetes mellitus in patients 8 and 10.

Imaging findings

CT scans revealed cranial calcifications in all 14 patients (Fig. 2B). Eleven patients (patients 1–7 and 9–12) had calcifications in the bilateral basal ganglia, and some had calcifications in the thalamus, periventricular lesions or cerebellar hemispheres. Eleven patients (patients 3–9 and 11–14) had white matter abnormalities. High-intensity signals were observed mainly in periventricular lesions, frontal lobes or temporal lobes on T2-weighted MRI scans. Brain atrophy was evident in all but patient 1; this patient showed only moderate developmental delay.

Discussion

Here we present the results of the first nationwide survey of AGS patients conducted to examine the clinical manifestations and genotype–phenotype correlations of this disease. *RNASEH2B* is reported to be the most common gene associated with AGS (50 of 127 pedigrees) [3]; however, this mutation was identified at a significantly lower rate in the Japanese cohort ($P < 0.05$). The majority of the AGS patients in the present study harboured mutations in *TREX1* and *SAMHD1*. Although our cohort was small, the results suggest that the frequency of AGS gene mutations is variable in different ethnic populations.

AGS is usually inherited in an autosomal recessive manner, and previous studies have shown that consanguineous pedigrees are often involved [2, 3]. However, there was no consanguinity among our patients. Interestingly, we identified two patients harbouring *de novo* dominant-type *TREX1* mutations; this is a higher proportion than previously reported [3], since such patients are reported very rarely [2, 10–12]. AGS patients usually show severe neurological disabilities and rarely have children. Thus pedigrees are unhelpful when identifying *de novo* dominant-type AGS; however, the presence of parental consanguinity would facilitate the diagnosis of the more common recessive-type AGS. Since similar *de novo* mutations are expected to occur among different ethnic origins, we speculate that there might be more AGS patients harbouring *de novo* dominant-type mutations in other countries, regardless of the presence or absence of consanguinity.

The neurological symptoms shown by our AGS cohort were similar to those reported in previous studies, and included developmental delay, seizures, microcephaly, dystonia and hypotonia [2, 3]. The cohort also comprised a high percentage of patients with severe developmental delay, although AGS cases that are neurologically milder were recently described [2]. All of the patients in the present cohort presented with the first symptoms of the disease by the age of 6 months. This could be because the cohort harboured few *RNASEH2B* mutations, which are associated with less severe neurological findings and later-onset presentation [2, 3].

AGS is highly associated with type I IFN-related autoimmunity [2, 16]. SLE shows many similarities to AGS, and previous reports describe two patients with

Table 3 Laboratory findings of Aicardi–Goutières syndrome patients

Patient	Genotype	CSF lymphocytosis	CSF elevated IFN-α	CSF elevated neopterin	Serum elevated IFN-α	Serum elevated autoantibody	Other clinical features
1	*TREX1* AD	n.d.	n.d.	n.d.	Yes (14 years)	None	Moderate hypothyroidism
2	*TREX1* AD	n.d.	n.d.	n.d.	Yes (13 years)	None	None
3	*TREX1* AD	Yes (1 years)	No (7 years)	No (7 years)	Yes (7 years)	None	None
4	*TREX1* AR	Yes (10 months)	Yes (2 years)	n.d.	n.d.	Unknown	Abnormal liver function
5	*TREX1* AR	Yes (23 days)	No (10 years)	n.d.	No (10 years)	Anti-ssDNA, anti-dsDNA	Abnormal liver function
6	*RNASEH2B* ?	Yes (8 months)	Yes (8 months)	n.d.	Yes (8 months)	None	Abnormal liver function
7	*RNASEH2A* AR	Yes (6 months)	Yes (6 months)	Yes (2 years)	No (6 months)	Anti-ssDNA	None
8	*SAMHD1* AR	Yes (2 months)	n.d.	n.d.	No (12 years)	ANA 1:20480, anti-DNA, anti-RNP	Thrombocytopenia, leucocytopenia, anaemia, abnormal liver function, diabetes mellitus
9	*SAMHD1* AR	Yes (9 months)	Yes (9 months)	n.d.	n.d.	ANA 1:640, anti-ssDNA, anti-dsDNA	Thrombocytopenia, abnormal liver function, hypocomplementaemia, hypergammaglobulinaemia, moderate hypothyroidism
10	*SAMHD1* AR	No (16 years)	n.d.	Yes (16 years)	n.d.	ANA 1:1280, anti-ssDNA, anti-RNP, anti-SS-A	Abnormal liver function, hypergammaglobulinaemia, diabetes mellitus
11	ND	No (11 months)	Yes (11 months)	n.d.	n.d.	Anti-LKM1	Thrombocytopenia, abnormal liver function, hypocomplementaemia, hypergammaglobulinaemia
12	ND	No (3 years)	No (3 years)	Yes (3 years)	n.d.	ANA 1:320	None
13	ND	n.d.	n.d.	n.d.	n.d.	Unknown	Thrombocytopenia, abnormal liver function
14	ND	Yes (4 days)	n.d.	n.d.	n.d.	None	Anaemia, abnormal liver function

AR: autosomal recessive; AD: autosomal dominant; ND: not detected; n.d.: not done. Patients 1–14 are the same as those listed in Table 2. The age at which the data were collected is shown in parentheses.

TABLE 4 Summary of Aicardi-Goutières syndrome patients with dominant-type *TREX1* mutations

Patient	Reference	Genotype	Mutation type	Ethnicity	Chilblain lesions	Developmental delay	Seizure	CSF elevated IFN-α	Serum elevated IFN-α
1	19	p.Asp18Asn	Het	Japanese	Yes	Moderate	FC only	n.d.	Yes (14 years)
2	—	p.His195Tyr	Het, *de novo*	Japanese	Yes	Severe	No	n.d.	Yes (13 years)
3	—	p.Asp200Asn	Mos, *de novo*	Japanese	Yes	Severe	No	No (7 years)	Yes (7 years)
—	10	p.Asp200Asn	Het, *de novo*	Scottish	Yes	Severe	No	Yes (3 years)	Undescribed
—	2	p.Asp200His	Het, *de novo*	German	Yes	Undescribed	Undescribed	Undescribed	Undescribed
—	11	p.Asp18Asn	Het, *de novo*	Undescribed	Yes	Relatively mild	No	Yes (14 years)	Yes (14 years)
—	12	p.Asp18His	Het, *de novo*	Undescribed	Yes	Severe	Undescribed	Yes (4 months)	Undescribed

Het: heterozygous; Mos: mosaic; FC: febrile convulsion; n.d.: not done. The age at which the data were collected is shown in parentheses.

molecularly proven AGS that were also diagnosed with SLE, one harbouring a *SAMHD1* mutation and the other a *TREX1* mutation [2, 17]. In the present study, the first AGS patient complicated with SS, which is also known as a type I IFN-related disease [31], was identified, in addition to two AGS patients diagnosed with SLE. All three patients harboured *SAMHD1* mutations and tested positive for multiple autoantibodies. These findings suggest that, of all the genes associated with AGS, *SAMHD1* mutations may be most closely associated with autoimmunity. Further studies of this association might shed light on the pathophysiology of AGS and autoimmune diseases.

All five patients harbouring *TREX1* mutations had chilblain lesions, a frequency significantly greater than that observed for the rest of the cohort (2/9 patients; $P < 0.05$). However, no previous studies have reported that chilblain lesions are more common in AGS patients harbouring *TREX1* mutations than in those harbouring other gene mutations. Thus we paid attention to the three patients harbouring dominant-type *TREX1* mutations. The clinical features of seven AGS patients with dominant-type *TREX1* mutations, who consist of the three reported herein and four additional cases reported in the literature, are presented in Table 4 [2, 10–12]. Notably, all seven cases had chilblain lesions, which is a significantly higher proportion than that observed in AGS patients as a whole (43% of 123 patients; $P < 0.01$) [3]. Besides AGS, heterozygous *TREX1* mutations are also associated with FCL, which presents with skin symptoms alone [10, 18, 19, 32]. Since dominant-type *TREX1* mutations are more likely to cause chilblains, it would be interesting to examine the differences in the underlying molecular mechanisms.

Although IFN-α levels in the CSF of AGS patients usually normalize during the first few years [3], serum IFN-α levels have not been studied extensively. We found that AGS patients harbouring dominant-type *TREX1* mutations tended to show persistent increases in serum IFN-α levels, even after they became older (Table 4). Previous reports have shown that patients receiving type I IFN present with vasculitic lesions that are similar to the chilblain lesions seen in AGS patients [33, 34]. Therefore we speculate that the chilblain lesions observed in AGS patients harbouring dominant-type *TREX1* mutations are related to persistently high serum IFN-α levels. Since the data on serum IFN-α levels of AGS patients are limited, further studies of more AGS cases from different ethnic backgrounds are needed to confirm the relationship between chilblains and IFN-α levels.

The dimeric protein, TREX1, is a major component of the 3′–5′ exonucleases in mammalian cells and functions to eliminate ssDNA and degrade nicked genomic DNA [6, 35, 36]. A previous study showed that *TREX1* mutations causing dominant-type AGS are localized to Asp18 and Asp200, which are highly conserved Mg^{2+}-coordinating aspartate residues required for catalytic function (Table 4) [37]. The heterozygous mutations p.Asp18Asn, p.Asp200Asn and p.Asp200His cause loss of function and exert dominant negative effects on the wild type [38, 39]. We identified a novel heterozygous *TREX1* mutation, p.His195Tyr, in our cohort. The exonuclease assays revealed that TREX1$^{p.His195Tyr}$ showed defective enzymatic activity, similar to TREX1$^{p.Asp18Asn}$ and TREX1$^{p.Asp200Asn}$. Although we did not generate heterodimers to prove the dominant negative effect, we identified a third TREX1 residue, His195, which causes AGS in a dominant-type manner when mutated.

We also identified the first AGS patient harbouring a somatic mosaicism for a *TREX1* mutation, suggesting that mutated cells could cause AGS, even when co-existing with normal cells. This mosaicism is consistent with the hypothesis that IFN-α released from non-hematopoietic cells acts in a paracrine fashion and plays a vital role in the pathogenesis of AGS [40]. The finding that the

frequency of the mosaicism was similar in every cell lineage or tissue tested suggests that neural cells would also show a similar frequency. From a diagnostic point of view, clinical AGS patients showing an even lower rate of mosaicism in *de novo* dominant-type *TREX1* mutations might be missed by conventional direct sequencing, like some reported cases of cryopyrin-associated periodic syndrome [22, 23, 41, 42].

In conclusion, the present nationwide survey identified more sporadic AGS cases harbouring *de novo* dominant-type *TREX1* mutations than expected. By exploring the genotype–phenotype correlations, we also observed a strong association between dominant-type *TREX1* mutations and chilblain lesions, as well as between *SAMHD1* mutations and autoimmunity. These findings need to be confirmed in AGS patients from different ethnic backgrounds. Nonetheless, these findings emphasize that rheumatologists need to pay attention to possible sporadic AGS cases that present with neurological disorders and autoimmune manifestations, even from non-consanguineous families.

Rheumatology key messages

- A strong association between dominant-type *TREX1* mutations and chilblain lesions was observed in Aicardi–Goutières syndrome patients.
- Special attention should be paid to Aicardi–Goutières syndrome patients with *de novo* dominant-type *TREX1* mutations.

Acknowledgements

We are grateful to Y. Takaoka (Kyoto University, Kyoto, Japan) for technical assistance and to F. Perrino and C. Orebaugh (Wake Forest School of Medicine, Winston-Salem, NC, USA) for helpful suggestions regarding the ssDNA assay.

Funding: This work was supported by grants from the Health Labour Sciences Research Grant for Research on Intractable Diseases from the Ministry of Health, Labour and Welfare of Japan (H22-nanchi-ippan-123, H23-nanchi-ippan-096) and the Ministry of Education, Culture, Sports, Science and Technology of Japan (24390263).

Disclosure statement: The authors have declared no conflicts of interest.

References

1 Chahwan C, Chahwan R. Aicardi-Goutieres syndrome: from patients to genes and beyond. Clin Genet 2012;81:413–20.

2 Ramantani G, Kohlhase J, Hertzberg C *et al*. Expanding the phenotypic spectrum of lupus erythematosus in Aicardi-Goutières syndrome. Arthritis Rheum 2010;62:1469–77.

3 Rice G, Patrick T, Parmar R *et al*. Clinical and molecular phenotype of Aicardi-Goutieres syndrome. Am J Hum Genet 2007;81:713–25.

4 Orcesi S, La Piana R, Fazzi E. Aicardi-Goutieres syndrome. Br Med Bull 2009;89:183–201.

5 Blau N, Bonafé L, Krägeloh-Mann I *et al*. Cerebrospinal fluid pterins and folates in Aicardi-Goutières syndrome: a new phenotype. Neurology 2003;61:642–7.

6 Crow YJ, Hayward BE, Parmar R *et al*. Mutations in the gene encoding the 3′-5′ DNA exonuclease TREX1 cause Aicardi-Goutières syndrome at the AGS1 locus. Nat Genet 2006;38:917–20.

7 Crow YJ, Leitch A, Hayward BE *et al*. Mutations in genes encoding ribonuclease H2 subunits cause Aicardi-Goutières syndrome and mimic congenital viral brain infection. Nat Genet 2006;38:910–6.

8 Rice GI, Bond J, Asipu A *et al*. Mutations involved in Aicardi-Goutières syndrome implicate SAMHD1 as regulator of the innate immune response. Nat Genet 2009;41:829–32.

9 Rice GI, Kasher PR, Forte GM *et al*. Mutations in ADAR1 cause Aicardi-Goutières syndrome associated with a type I interferon signature. Nat Genet 2012;44:1243–8.

10 Rice G, Newman WG, Dean J *et al*. Heterozygous mutations in TREX1 cause familial chilblain lupus and dominant Aicardi-Goutieres syndrome. Am J Hum Genet 2007;80:811–5.

11 Haaxma CA, Crow YJ, van Steensel MA *et al*. A de novo p.Asp18Asn mutation in TREX1 in a patient with Aicardi-Goutières syndrome. Am J Med Genet A 2010;152A:2612–7.

12 Tüngler V, Silver RM, Walkenhorst H *et al*. Inherited or de novo mutation affecting aspartate 18 of TREX1 results in either familial chilblain lupus or Aicardi-Goutières syndrome. Br J Dermatol 2012;167:212–4.

13 Crow YJ. Type I interferonopathies: a novel set of inborn errors of immunity. Ann N Y Acad Sci 2011;1238:91–8.

14 Obermoser G, Pascual V. The interferon-alpha signature of systemic lupus erythematosus. Lupus 2010;19:1012–9.

15 Lee-Kirsch MA, Gong M, Chowdhury D *et al*. Mutations in the gene encoding the 3′-5′ DNA exonuclease TREX1 are associated with systemic lupus erythematosus. Nat Genet 2007;39:1065–7.

16 Cheng MH, Anderson MS. Monogenic autoimmunity. Annu Rev Immunol 2012;30:393–427.

17 Ramantani G, Häusler M, Niggemann P *et al*. Aicardi-Goutières syndrome and systemic lupus erythematosus (SLE) in a 12-year-old boy with SAMHD1 mutations. J Child Neurol 2011;26:1425–8.

18 Lee-Kirsch MA, Gong M, Schulz H *et al*. Familial chilblain lupus, a monogenic form of cutaneous lupus erythematosus, maps to chromosome 3p. Am J Hum Genet 2006;79:731–7.

19 Abe J, Izawa K, Nishikomori R *et al*. Heterozygous TREX1 p.Asp18Asn mutation can cause variable neurological symptoms in a family with Aicardi-Goutieres syndrome/familial chilblain lupus. Rheumatology 2013;52:406–8.

20 Shintaku H, Asada M, Sawada Y. Diagnosis and treatment of 6-pyruvoyl-tetrahydropterin synthase deficiency. Brain Dev 2000;22(Suppl 1):S118–21.

21 Nishikomori R, Akutagawa H, Maruyama K *et al.* X-linked ectodermal dysplasia and immunodeficiency caused by reversion mosaicism of NEMO reveals a critical role for NEMO in human T-cell development and/or survival. Blood 2004;103:4565–72.

22 Izawa K, Hijikata A, Tanaka N *et al.* Detection of base substitution-type somatic mosaicism of the NLRP3 gene with >99.9% statistical confidence by massively parallel sequencing. DNA Res 2012;19:143–52.

23 Tanaka N, Izawa K, Saito MK *et al.* High incidence of NLRP3 somatic mosaicism in patients with chronic infantile neurologic, cutaneous, articular syndrome: results of an international multicenter collaborative study. Arthritis Rheum 2011;63:3625–32.

24 Orebaugh CD, Fye JM, Harvey S *et al.* The TREX1 exonuclease R114H mutation in Aicardi-Goutières syndrome and lupus reveals dimeric structure requirements for DNA degradation activity. J Biol Chem 2011;286:40246–54.

25 Brucet M, Querol-Audí J, Serra M *et al.* Structure of the dimeric exonuclease TREX1 in complex with DNA displays a proline-rich binding site for WW Domains. J Biol Chem 2007;282:14547–57.

26 Brucet M, Querol-Audí J, Bertlik K *et al.* Structural and biochemical studies of TREX1 inhibition by metals. Identification of a new active histidine conserved in DEDDh exonucleases. Protein Sci 2008;17:2059–69.

27 Bailey SL, Harvey S, Perrino FW *et al.* Defects in DNA degradation revealed in crystal structures of TREX1 exonuclease mutations linked to autoimmune disease. DNA Repair (Amst) 2012;11:65–73.

28 Kavanagh D, Spitzer D, Kothari PH *et al.* New roles for the major human 3′-5′ exonuclease TREX1 in human disease. Cell Cycle 2008;7:1718–25.

29 Tan EM, Cohen AS, Fries JF *et al.* The 1982 revised criteria for the classification of systemic lupus erythematosus. Arthritis Rheum 1982;25:1271–7.

30 Miyawaki S. Revised Japan criteria for Sjögren syndrome. Ryumachi 2000;40:48–53.

31 Yao Y, Liu Z, Jallal B *et al.* Type I interferons in Sjögren's syndrome. Autoimmun Rev 2013;12:558–66.

32 Lee-Kirsch MA, Chowdhury D, Harvey S *et al.* A mutation in TREX1 that impairs susceptibility to granzyme A-mediated cell death underlies familial chilblain lupus. J Mol Med (Berl) 2007;85:531–7.

33 Bachmeyer C, Farge D, Gluckman E *et al.* Raynaud's phenomenon and digital necrosis induced by interferon-alpha. Br J Dermatol 1996;135:481–3.

34 Campo-Voegeli A, Estrach T, Marti RM *et al.* Acrocyanosis induced by interferon alpha(2a). Dermatology 1998;196:361–3.

35 Stetson DB, Ko JS, Heidmann T *et al.* Trex1 prevents cell-intrinsic initiation of autoimmunity. Cell 2008;134:587–98.

36 Chowdhury D, Beresford PJ, Zhu P *et al.* The exonuclease TREX1 is in the SET complex and acts in concert with NM23-H1 to degrade DNA during granzyme A-mediated cell death. Mol Cell 2006;23:133–42.

37 de Silva U, Choudhury S, Bailey SL *et al.* The crystal structure of TREX1 explains the 3′ nucleotide specificity and reveals a polyproline II helix for protein partnering. J Biol Chem 2007;282:10537–43.

38 Lehtinen DA, Harvey S, Mulcahy MJ *et al.* The TREX1 double-stranded DNA degradation activity is defective in dominant mutations associated with autoimmune disease. J Biol Chem 2008;283:31649–56.

39 Fye JM, Orebaugh CD, Coffin SR *et al.* Dominant mutations of the TREX1 exonuclease gene in lupus and Aicardi-Goutieres syndrome. J Biol Chem 2011;286:32373–82.

40 Gall A, Treuting P, Elkon KB *et al.* Autoimmunity initiates in nonhematopoietic cells and progresses via lymphocytes in an interferon-dependent autoimmune disease. Immunity 2012;36:120–31.

41 Saito M, Fujisawa A, Nishikomori R *et al.* Somatic mosaicism of CIAS1 in a patient with chronic infantile neurologic, cutaneous, articular syndrome. Arthritis Rheum 2005;52:3579–85.

42 Saito M, Nishikomori R, Kambe N *et al.* Disease-associated CIAS1 mutations induce monocyte death, revealing low-level mosaicism in mutation-negative cryopyrin-associated periodic syndrome patients. Blood 2008;111:2132–41.

Epilepsy & Behavior Case Reports 2 (2014) 37–39

Contents lists available at ScienceDirect

Epilepsy & Behavior Case Reports

journal homepage: www.elsevier.com/locate/ebcr

Case Report

Clinical characteristics of epileptic seizures in a case of dihydropteridine reductase deficiency

Mahoko Furujo [a,*], Masako Kinoshita [b], Yozo Ichiba [c], Anne Romstad [d], Haruo Shintaku [e], Toshihide Kubo [a]

[a] Department of Pediatrics, Okayama Medical Center, National Hospital Organization, Okayama, Japan
[b] Department of Neurology, Utano National Hospital, National Hospital Organization, Kyoto, Japan
[c] Department of Health, Okayama Institute of Health Foundation, Okayama, Japan
[d] Department of Inherited Metabolic Diseases and Molecular Genetics, the John F. Kennedy Institute, Glostrup, Denmark
[e] Department of Pediatrics, Osaka City University Graduate School of Medicine, Osaka, Japan

ARTICLE INFO

Article history:
Received 15 January 2014
Accepted 16 January 2014
Available online 13 March 2014

Keywords:
Dihydropteridine reductase deficiency
Epileptic seizures
Levodopa
5-Hydroxytryptophan
Levetiracetam

ABSTRACT

We assessed the clinical characteristics and efficacy of neurotransmitters and levetiracetam in a patient with hyperphenylalaninemia due to dihydropteridine reductase (DHPR) deficiency who developed epileptic seizures. A boy with DHPR deficiency, who had been successfully treated with tetrahydrobiopterin (BH_4), levodopa, and 5-hydroxytryptophan (5-HTP) since he was 2 months old, started having monthly episodes of blurred vision, loss of consciousness, and falls at the age of 12 years. He was taking BH_4 510 mg/day, levodopa 670 mg/day, 5-HTP 670 mg/day, and entacapone 300 mg/day. We evaluated the seizure semiology, EEG findings, and efficacy of levodopa, 5-HTP, and levetiracetam (LEV). His seizures were comprised of an abrupt loss of awareness and eye deviation to the right. Interictal EEG showed slightly slow posterior-dominant rhythm in 7–8 Hz; intermittent, irregular slowing in the bilateral parieto-occipital region; and multiregional independent spikes in bilateral hemispheres. Ictal EEG showed a seizure pattern starting at the left temporal region. Brain MRI showed diffuse signal increase of deep white matter on T2-weighted and FLAIR images. Dosage increase of levodopa to 1340 mg/day, of 5-HTP to 1500 mg/day, or of both did not suppress seizures. Levetiracetam 2000 mg/day markedly reduced seizures without any adverse events. Patients with DHPR deficiency can develop epileptic seizures of partial onset which can be successfully and safely treated with LEV.

1. Introduction

Dihydropteridine reductase (DHPR) deficiency is a rare inherited disorder which affects the metabolism of tetrahydrobiopterin (BH_4), causing hyperphenylalaninemia and neurotransmitter deficiency [1]. In Japan, about 1 per 2 million newborns is diagnosed with BH_4 deficiency, and about 10% of them have DHPR deficiency [2]. In an international survey, DHPR deficiency is the second most common form of BH_4 deficiency (34.7%) [3]. Dihydropteridine reductase deficiency in patients is highly associated with epileptic seizures that are frequently severe [3,4]. However, the clinical characteristics of their seizures, EEG findings, and treatment strategies have not been fully established.

Here, we assessed the clinical characteristics and efficacy of neurotransmitters and levetiracetam (LEV) to epileptic seizures in a patient with DHPR deficiency who had been successfully treated with BH_4, levodopa, and 5-hydroxytryptophan (5-HTP). Part of this manuscript was presented at the 30th International Epilepsy Congress, 2013, and appeared in an abstract form [5].

2. Case presentation

A boy, the second child of nonconsanguineous parents, was born at 38 weeks of gestation with a weight of 3.1 kg. At 4 days, phototherapy was done for jaundice. He was found to have hyperphenylalaninemia on a routine Guthrie test for newborn screening. His older brother is healthy. His serum phenylalanine (Phe) level ranged between 492 and 756 μmol/l (normal range: 38–91 μmol/l [4]). He was diagnosed as having DHPR deficiency based on a BH_4 loading test; the measurement of urinary, serum, and CSF pterins; the DHPR activity in dried blood spots; and the presence of neurotransmitter metabolites in the urine and CSF. Details of these diagnostic tests were described in our previous paper [6]. Gene analysis revealed a compound heterozygous mutation of the *QDPR* gene (G18C/S59X, both are new mutations). At 2 months, treatment was started with BH_4 15–20 mg/kg/day, levodopa 15 mg/kg/day, and 5-HTP 15 mg/kg/day, as previously described [4]. The dosage of BH_4 was adjusted to keep his serum Phe concentration less than 480 μmol/l without dietary restriction of Phe [6]. Entacapone 300 mg/day was added at the age of 10 years. With this therapy, his

* Corresponding author at: Department of Pediatrics, Okayama Medical Center, National Hospital Organization, 1711-1 Tamasu, Kitaku, Okayama 701-1192, Japan. Tel.: +81 86 294 9911; fax: +81 86 294 9255.
E-mail address: fuhkun@okayama3.hosp.go.jp (M. Furujo).

http://dx.doi.org/10.1016/j.ebcr.2014.01.007

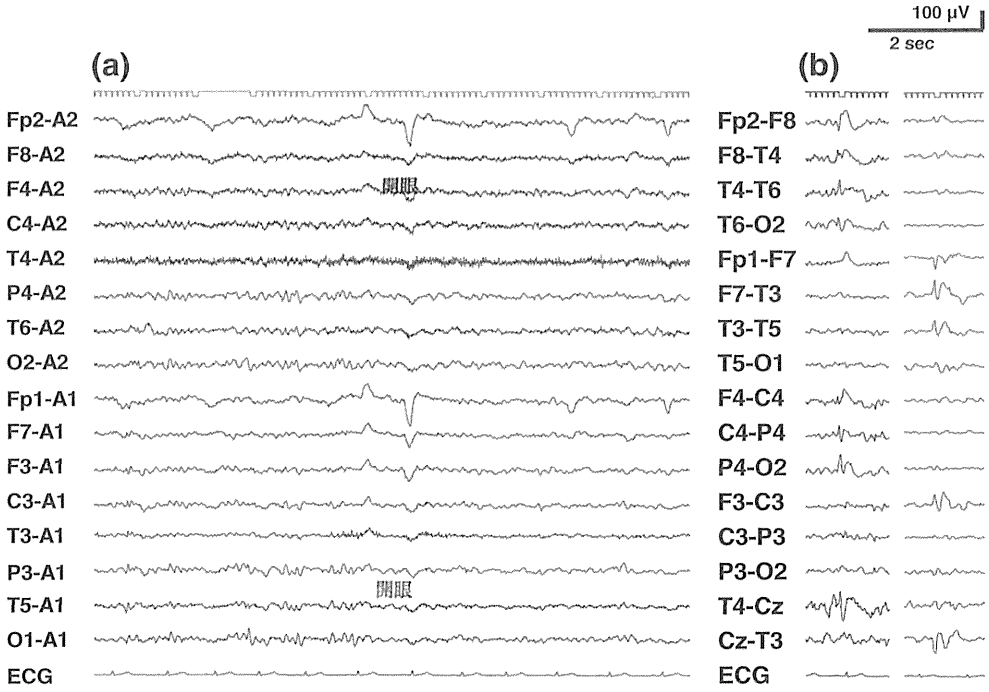


Fig. 1. Interictal EEG. (a) Slightly slow posterior dominant rhythm in 7–8 Hz and intermittent irregular slow waves in the bilateral parieto-occipital regions. (b) Multiregional independent spikes in bilateral hemispheres.

physical development is within normal limits. Folic acid was not administered because its level in his serum – 3.4–5.9 ng/ml (normal range: 3.6–12.9 ng/ml) – is of sufficient amount. He attended an ordinary elementary school, though he showed mild mental retardation (IQ of around 60).

However, he started having monthly episodes of blurred vision, loss of consciousness, and falls at the age of 12 years. At that time, he was taking BH_4 510 mg/day, levodopa 670 mg/day, 5-HTP 670 mg/day, and entacapone 300 mg/day. Interictal EEG showed slightly slow posterior-dominant rhythm in 7–8 Hz; intermittent irregular slow waves in

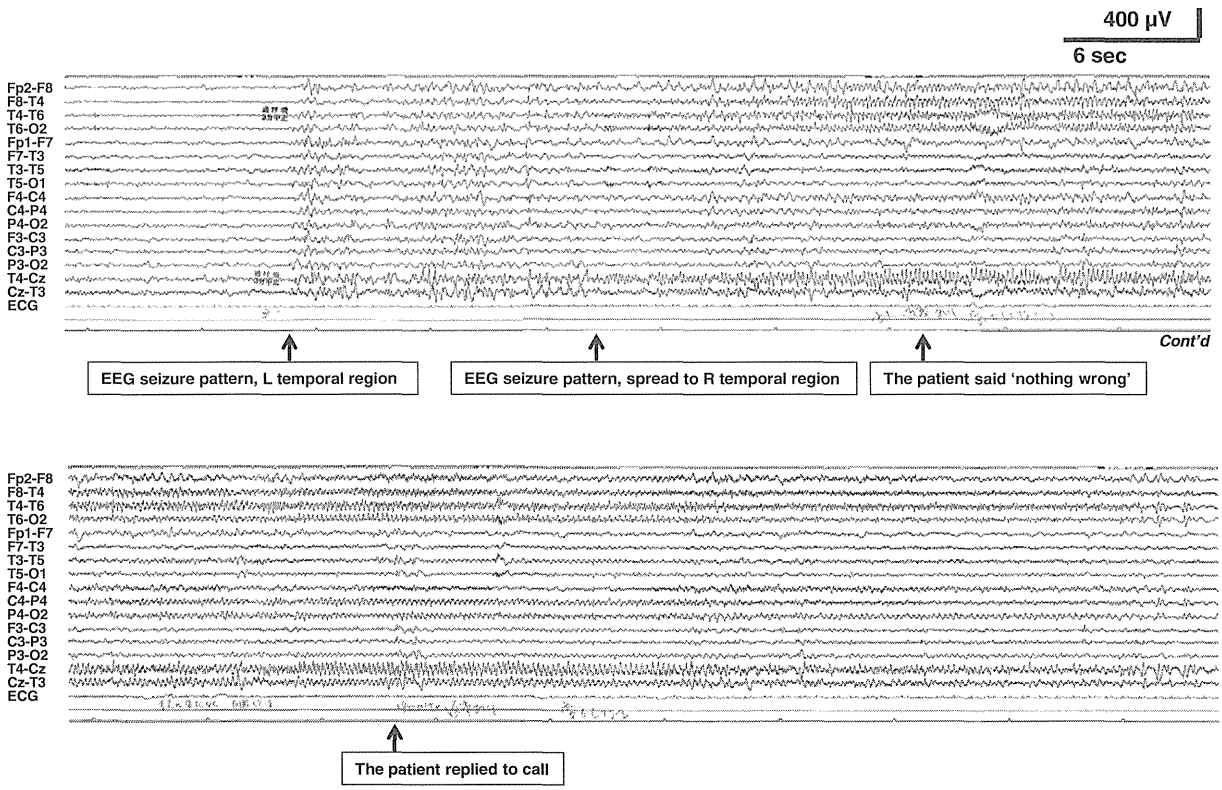


Fig. 2. Ictal EEG. An electroencephalographic seizure pattern starting at the left temporal region and then a rhythmic burst of fast activities starting at the right temporal region. The patient was conscious in this particular event, but the same pattern of EEG was observed while he exhibited consciousness disturbance in other complex partial seizures.

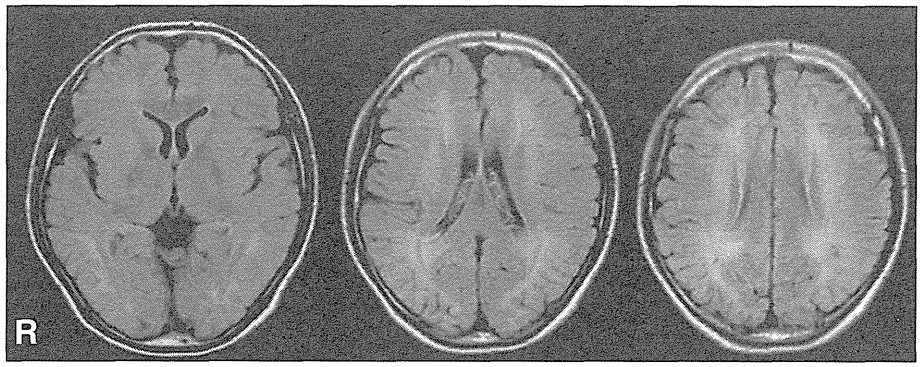


Fig. 3. Brain MRI, FLAIR images at the age of 12 years. Note diffuse signal increase of deep white matter.

the bilateral parieto-occipital regions and multiregional independent spikes in bilateral hemispheres (Fig. 1). During EEG recordings, he had habitual seizures which comprised an abrupt loss of awareness and eye deviation to the right without convulsion. Ictal EEG showed an electrographic seizure pattern starting at the left temporal region and then a burst of fast activities spreading to the other side (Fig. 2). Brain MRI showed diffuse signal increase of deep white matter on T2-weighted and FLAIR images (Fig. 3). Dosage increase of levodopa to 1340 mg/day, of 5-HTP to 1500 mg/day, or of both did not suppress his seizures. Levetiracetam 2000 mg/day markedly reduced seizures without any adverse events.

3. Discussion

The present male patient with DHPR deficiency, who had been treated with supplementation of neurotransmitters since he was 2 months old, developed epileptic seizures at the age of 12 years. By EEG evaluation of ictal and interictal epochs, we could document that his epileptic seizures were complex partial seizures originating from the left temporal region, and he also has diffuse and multiregional abnormalities. We also demonstrated that antiepileptic drugs such as LEV should be introduced in order to suppress his seizures; on the other hand, dosage increase of neurotransmitter supplementation was not effective.

A previous paper described that 6 out of 10 patients with DHPR deficiency, whose onset of neurotransmitter treatment was at 6 months of age or older, developed severe epileptic seizures [4]. The present case, where the treatment was started when the patient was 2 months old, suggests that neurotransmitter supplementation should be started earlier than 2 months to prevent the development of seizures. In literature describing severe seizures or convulsions in patients with DHPR deficiency, seizure types are myoclonic seizures [7] and seemingly generalized tonic–clonic seizures, considering that their EEG showed generalized hypsarrhythmic activity or diffuse sharp wave activity [4,8,9]. In contrast, our patient showed complex partial seizures. This may also suggest that the timing of treatment administration may affect the clinical seizure types.

Phenobarbital, clonazepam, and sodium valproate (VPA) have been used to treat severe seizures in patients with DHPR deficiency [4,8,9]. However, antiepileptic drugs can lead to adverse events; VPA, in particular, may lead to extrapyramidal tract signs that can worsen the symptoms in patients with DHPR deficiency and in patients with other metabolic disorders with dopamine depletion. Our experience with this patient suggests that LEV can be used in DHPR deficiency safely.

4. Conclusion

Patients with DHPR deficiency can develop epileptic seizures of partial onset, which can be successfully and safely treated with LEV. Further studies are needed to clarify the mechanism of epileptogenesis and the therapeutic strategies in patients with DHPR deficiency.

Conflict of interest

The authors declare that they have no conflict of interets.

Acknowledgment

This study was supported in part by Health and Labour Sciences Research Grants for Research on Rare and Intractable Diseases.

References

[1] Shintaku H. Disorders of tetrahydrobiopterin metabolism and their treatment. Curr Drug Metab 2002;3:123–31.
[2] Shintaku H, Ohwada M. Long-term follow-up of tetrahydrobiopterin therapy in patients with tetrahydrobiopterin deficiency in Japan. Brain Dev 2013;35:406–10.
[3] Opladen T, Hoffmann GF, Blau N. An international survey of patients with tetrahydrobiopterin deficiencies presenting with hyperphenylalaninaemia. J Inherit Metab Dis 2012;35:963–73.
[4] Jäggi L, Zurflüh MR, Schuler A, Ponzone A, Porta F, Fiori L, et al. Outcome and long-term follow-up of 36 patients with tetrahydrobiopterin deficiency. Mol Genet Metab 2008;93:295–305.
[5] Furujo M, Kinoshita M, Shintaku H, Kubo T. Clinical characteristics of epileptic seizures in a case of dihydropteridine reductase deficiency. Epilepsia 2013;54(s3):234.
[6] Furujo M, Ichiba Y, Sintaku H, Asada M. A case of dihydropteridine reductase deficiency. Pteridines 2000;11:126–8.
[7] Cotton RG, Jennings I, Bracco G, Ponzone A, Guardamagna O. Tetrahydrobiopterin non-responsiveness in dihydropteridine reductase deficiency is associated with the presence of mutant protein. J Inherit Metab Dis 1986;9:239–43.
[8] Longhi R, Valsasina R, Buttè C, Paccanelli S, Riva E, Giovannini M. Cranial computerized tomography in dihydropteridine reductase deficiency. J Inherit Metab Dis 1985;8:109–12.
[9] Longhi R, Riva E, Valsasina R, Paccanelli S, Giovannini M. Phenylketonuria due to dihydropteridine reductase deficiency: presentation of two cases. J Inherit Metab Dis 1985;8(s2):97–8.

Risk Factors for the Recurrence of the Congenital Diaphragmatic Hernia—Report from the Long-Term Follow-Up Study of Japanese CDH Study Group

Kouji Nagata[1] Noriaki Usui[2] Keita Terui[3] Hajime Takayasu[4] Keiji Goishi[5] Masahiro Hayakawa[6] Yuko Tazuke[7] Akiko Yokoi[8] Hiroomi Okuyama[9] Tomoaki Taguchi[1]

[1] Department of Pediatric Surgery, Kyushu University, Fukuoka, Japan
[2] Department of Pediatric Surgery, Osaka University Graduate School of Medicine, Suita, Osaka, Japan
[3] Department of Pediatric Surgery, Graduate School of Medicine, Chiba University, Chiba, Japan
[4] Department of Pediatric Surgery, Faculty of Medicine, University of Tsukuba, Tsukuba, Japan
[5] Division of Neonatology, Department of Maternal and Perinatal Medicine, National Center for Child Health and Development, Tokyo, Japan
[6] Division of Neonatology, Center for Maternal-Neonatal Care, Nagoya University Hospital, Nagoya, Japan
[7] Department of Pediatric Surgery, Osaka Medical Center and Research Institute for Maternal and Child Health, Osaka, Japan
[8] Department of Pediatric Surgery, Kobe Children's Hospital, Kobe, Japan
[9] Department of Pediatric Surgery, Hyogo College of Medicine, Nishinomiya, Japan

Address for correspondence Kouji Nagata, MD, Department of Pediatric Surgery, Graduate School of Medical Sciences, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan (e-mail: koujin@pedsurg.med.kyushu-u.ac.jp).

Eur J Pediatr Surg 2015;25:9–14.

Abstract

Aim of the Study Few follow-up studies focused on the recurrence regarding the postoperative course of congenital diaphragmatic hernia (CDH) survivors. The aim of this study was to report on risk factor for CDH patients who had the recurrence during the follow-up.

Materials and Methods A multicenter retrospective survey was conducted on neonates diagnosed to have CDH between January 2006 and December 2010. Follow-up survey was conducted between September 2013 and October 2013 (ethical approval: No. 25–222). Nine institutions agreed to participate in this survey. Out of 228, 182 (79.8%) patients were alive and 180 patients were included in this study. Two patients were excluded because the defect had not repaired at the primary operation. The patients were divided into the recurrence group ($n = 21$) and the nonrecurrence group ($n = 159$). Postnatal and postoperative variables were compared between these two groups. Baseline variables which showed significance in univariate analysis were entered into multiple logistic regression analysis for analyzing the recurrence. A value of

Keywords
- congenital diaphragmatic hernia
- long-term complication
- recurrence

received
May 22, 2014
accepted after revision
August 21, 2014
published online
December 19, 2014

DOI http://dx.doi.org/10.1055/s-0034-1395486.
ISSN 0939-7248.

$p < 0.05$ was considered to be statistically significant by using the JMP software program (version 9; SAS Institute, Inc, Cary, North Carolina, United States).

Main Results Out of 180, 21 (11.7%) CDH neonates had the recurrence during the course of the follow-up. Five (2.8%) patients had the recurrence before primary discharge and 16 (8.9%) patients had the recurrence after discharge. Univariate analysis showed that liver herniation (crude odds ratio [OR], 7.4; 95% confidence interval [CI], 2.73–23.68), defect size C and D, proposed by the CDH Study Group (crude OR, 7.09; 95% CI, 2.73–19.99) and patch repair (crude OR, 5.00; 95% CI, 1.91–14.70) were risk factors. Multivariate logistic regression analysis showed liver herniation (adjusted OR, 3.96; 95% CI, 1.01–16.92) was the risk factor for the recurrence.

Conclusion A wide spectrum of the disease severity and the rarity of the disease mask the risk of the recurrence for CDH patients. This study showed the only factor to predict the recurrence was the liver herniation. These data will be helpful for providing information for the long-term follow-up of the CDH patients.

Introduction

Recent advances in the prenatal and postnatal congenital diaphragmatic hernia (CDH) treatment strategy, there have been increased improvement in outcome.[1,2] Therefore, much of the clinical interests have shifted to the long-term outcomes of CDH survivors, since as the survivors can suffer from the long-term morbidities, including recurrence, pulmonary disease, gastrointestinal tract disease, hearing loss, impairment in growth and development, and orthopedic deformities.[3]

The reported incidence of recurrent diaphragmatic hernia varies from 5 to 65%.[3–5] The recurrence of the diaphragmatic hernia sometimes causes gastrointestinal tract disorders and respiratory disorders, which will severely affect the quality of life in survivors. Recent articles recommend the chronological follow-up protocol to monitor the recurrence and other complications to be early diagnosed and cured when occurred.[6]

The rarity of CDH makes conducting well-designed clinical studies extremely difficult because no single institution can accrue sufficient patients to reach meaningful conclusions. Therefore, the long-term follow-up study for CDH has based on retrospective studies in a single center experience or from multiple centers using different treatment strategies.[7]

In Japan, the cohort study had started from the multicenter retrospective cohort study for an antenatally diagnosed CDH, and then, performed a nationwide survey to understand the current treatment and the actual outcome during the period from 2006 to 2010.[8] Thereafter in 2013, a long-term follow-up study had performed among the dedicated institutions to better understand the associated long-term complications of CDH survivors. The purpose of this study was to analyze the risk factors for the recurrence among CDH neonates as part of the long-term follow-up study.

Materials and Methods

Patient Selection

This retrospective survey was approved by the ethics committees of the nine representative institutions, including the Graduate School of Medical Sciences, Kyushu University; National Center for Child Health and Development; Nagoya University Hospital; Osaka Medical Center and Research Institute for Maternal and Child Health; Kobe Children's Hospital; Faculty of Medicine, University of Tsukuba; Graduate School of Medical Sciences, Chiba University; Hyogo College of Medicine; and Graduate School of Medicine, Osaka University (representative institutional review board approval no. 25–222, Graduate School of Medical Sciences, Kyushu University).

A nationwide retrospective cohort study was conducted on CDH neonates diagnosed to have CDH between January 2006 and December 2010, and a total of 674 CDH neonates diagnosed during the 5-year period. Subsequent long-term follow-up study was conducted among the Japanese CDH Study Group and finally nine institutions which had 228 CDH neonates in the previous study consented to participate. According to this recurrence survey, 180 patients who had repaired the defect at the primary operation and the survived at least 3 years were included. Two right CDH patients who had not repaired the defect at the primary operation were excluded in this survey. One had to stop the operation because of the decrease in blood pressure during the course of correcting the liver position, while the other had been despaired the operation because of the ipsilateral lung defect.

Data Collection

The postnatal variables, including the presence of a prenatal diagnosis, birth location, sex, the presence of the associated anomalies, the side of the defect, the use of inhaled nitric oxide (iNO), and extracorporeal membrane oxygenation (ECMO), gestational age at delivery and birth weight, Apgar score at 1 minute, and the lowest oxygenation index (OI)

Table 1 Postnatal characteristics, comparing the Rec and non-Rec

Variables	Rec ($n = 21$)	Non-Rec ($n = 159$)	p value
Prenatal diagnosis (%)	21 (100)	134 (84.3)	0.048
Inborn (%)	21 (100)	134 (86.5)	0.048
Male gender (%)	7 (33.3)	91 (57.2)	0.06
Non-isolated CDH (%)	3 (14.3)	9 (5.7)	0.151
Right side of hernia (%)	2 (9.5)	11 (6.9)	0.651
iNO (%)	20 (95.2)	102 (64.2)	0.001
ECMO (%)	1 (4.8)	9 (5.7)	0.863
Continuous variables			
Gestational age (d), mean ± SD	261.8 ± 18.4	264.8 ± 11.0	0.279
Birth weight (d), mean ± SD	2,533.7 ± 601.5	2,703.3 ± 453.7	0.124
Apgar score at 1 minute, median (interquartile range)	5 (2–7)	5 (3–7)	0.358
Lowest OI, median (interquartile range)	4.8 (3.3–6.4)	3.8 (2.8–5.9)	0.906

Abbreviations: ECMO, extracorporeal membrane oxygenation; iNO, inhaled nitric oxide; non-Rec, nonrecurrence group; OI, oxygenation index; Rec, recurrence group; SD, standard deviation.

within 24 hours after birth were reviewed. An isolated CDH was defined as the CDH without any associated life-threatening or chromosomal anomalies.[8]

The perioperative variables were also examined. The presence of liver herniation, the type of diaphragmatic closure, and the defect size were also reviewed. Liver herniation was defined as CDH patients whose liver had herniated into the thoracic cavity during the operation. The defect size was determined according to the CDH Study Group's criteria, as previously reported in the literature.[8–10]

Statistical Analysis

The patients were divided into the recurrence group (Rec) ($n = 21$) and nonrecurrence group (Non-Rec) ($n = 159$). Postnatal and intra- or postoperative variables were compared between these two groups. The frequencies and percentages were used to describe the categorical data. The χ^2 test and Fisher exact test were used for the analysis of the categorical data. The mean and standard deviation or median and interquartile range were used to describe continuous variables. Student t-test and an analysis of variance were used to compare continuous variables. Baseline variables which showed significance in univariate analysis were entered into multiple logistic regression analysis for analyzing the recurrence. Kaplan–Meier analysis and Cox proportional hazards regression were also used for the recurrence analyses. The statistical analyses were performed with the JMP software program (version 9; SAS Institute, Inc, Cary, North Carolina, United States). A value of $p < 0.05$ was considered to indicate a statistically significant difference.

Results

Patient Characteristics and the Comparison between Recurrence Group and Nonrecurrence Group

Out of 228, 182 (79.8%) patients were alive and 180 patients were included in this study. A total of 11.6% ($n = 21/180$) CDH patients had a recurrence during the course of the follow-up. Out of 21, 20 CDH patients had reoperations and 1 patient have not operated during the follow-up period. Five patients had a primary recurrence before the primary discharge. Three patients had a second recurrence and all of them had repaired by using an abdominal muscle flap procedure.

The postnatal characteristics of the CDH neonates are shown in ►Table 1, with comparison between the Rec and Non-Rec. There were significant differences in the prenatal diagnosis rate and the inborn rate (Rec vs. Non-Rec = 100 vs. 84.3%, $p = 0.048$ and 100 vs. 86.5%, $p = 0.048$, respectively). There were no significant differences between two groups, according to the rate of the male gender, nonisolated CDH, right side hernia, and the use of ECMO. The use of ECMO was only 4.8% ($n = 1/21$) in Rec and 5.7% ($n = 9/159$) in Non-Rec. There were no differences in the gestational age, birth weight, Apgar score at 1 minute, and the lowest OI. Other variable which revealed significance was the iNO. These advanced therapies are recognized as the useful instruments for the persistent pulmonary hypertension, although their indications were not standardized in participants.

The perioperative characteristics of the CDH neonates are shown in ►Table 2. A total of 76.2% ($n = 16/21$) had liver herniation in Rec and 30.2% ($n = 48/159$) in Non-Rec. Patch repair was also significantly correlated to the recurrence with 22.1% ($n = 15/21$) in patch repair group, although 5.4% ($n = 6/112$) in direct repair group. Defect size was also correlated to the recurrence with 28.6% ($n = 14/49$) had larger defect (C and D) and 5.3% ($n = 7/131$) had smaller defect (A and B). Liver herniation, patch repair, and the defect size were valuables which were significantly associated with the recurrence. As would be expected, the patients who had a large defect are likely to involve the liver in thoracic cavity and require the patch during the operation. Another significant variable was the age at operation. The severity of disease might affect the timing of operation; however, there also was

Table 2 Preoperative characteristics, comparing the Rec and Non-Rec

Variables	Rec ($n = 21$)	Non-Rec ($n = 159$)	p value
Liver herniation (%)	16 (76.2)	48 (30.2)	0.0001
Patch repair at the primary operation			
Direct repair, $n = 112$ (%)	6 (5.4)	106 (94.6)	0.001
Patch repair, $n = 68$ (%)	15 (22.1)	53 (77.9)	
Defect size			
A and B, $n = 131$ (%)	7 (5.3)	124 (94.7)	< 0.001
C and D, $n = 49$ (%)	14 (28.6)	35 (71.4)	
Continuous variables			
Age at repair (h), median (interquartile range)	101 (54.5–145.5)	50 (24–77)	0.025
Length of stay (d), mean ± SD	86.2 ± 53.7	84.1 ± 123.8	0.939
Follow-up period (d), mean ± SD	1,645.8 ± 538.4	1,682.4 ± 594.5	0.787

Abbreviations: Non-Rec, nonrecurrence group; Rec, recurrence group; SD, standard deviation.

no definite standardization among participants. Also, the technical and material aspects were also excluded because of the absence of the standardized protocol. Actually, nine out of seven institutions, including 139 patients used the PTFE patch (GORE-TEX Soft Tissue Patch; W. L. Gore & Associates, Inc., Arizona, United States), whereas one institution, including 27 patients used polyester patch (Sauvage Filamentous Fabric; C.R. Bard, Inc., New Jersey, United States) and the other institution, including 14 patients used polypropylene and PTFE composix mesh (COMPOSIX EX Mesh; C.R. Bard, Inc.). There were neither significant differences in the length of hospital stay nor the follow-up period between these groups.

Analysis of Risk Factors and Prediction for Recurrence

A multivariate analysis was performed for all baseline variables which were significant in the univariate analysis for recurrence. Baseline variables were considered as the presence of the prenatal diagnosis, inborn, male gender, nonisolated CDH, right side hernia, gestational age, birth weight, Apgar score at 1 minute, the lowest OI, liver herniation, patch repair, defect size, and the length of stay. Other variables were not included in the multivariate analysis because the postnatal treatment strategy was not standardized among the cooperative institutions.

Univariate analysis in ►Table 3 showed that liver herniation (crude odds ratio [OR], 7.4; 95% confidence interval [CI], 2.73–23.68), defect size C and D (crude OR, 7.09; 95% CI, 2.73–19.99), and patch repair (crude OR, 5.00; 95% CI, 1.91–14.70) were significant risk factors. Multivariate logistic regression analysis in ►Table 4 showed liver herniation (adjusted OR, 3.96; 95% CI, 1.01–16.92) was the only significant risk factor for the recurrence. Defect size C and D (adjusted OR, 3.79; 95% CI, 0.86–23.12) and patch repair (adjusted OR, 1.30; 95% CI, 0.24–8.83) had not showed significance. The Kaplan–Meier analysis associated with the recurrence in ►Fig. 1 showed the recurrence rate after 6 months, 1 , and 2 years of the primary operation. The each recurrence rate was higher in liver herniation group, compared with the nonliver herniation group (liver herniation vs. nonliver herniation = 6 months: 15.7 vs. 1.7%, 1 year: 18.9 vs. 4.4%, and 2 years: 25.4 vs. 4.4%). Cox proportional hazards regression analyses showed significance in liver herniation (hazard ratio, 3.66; 95% CI, 1.03–14.42; $p = 0.045$).

Discussion

As the survival rate for patients with CDH have increased during the past decades with the advent of "gentle ventilation" and specific strategy and care, clinicians has led to focus on the frequency and importance of postoperative morbidities.[1,3,6] Significant morbidities such as the recurrence, respiratory diseases, neurocognitive delay, gastrointestinal disorders, hearing loss, poor growth, chest deformity, and the complications associated with congenital anomalies continue

Table 3 Univariate analysis for the recurrence

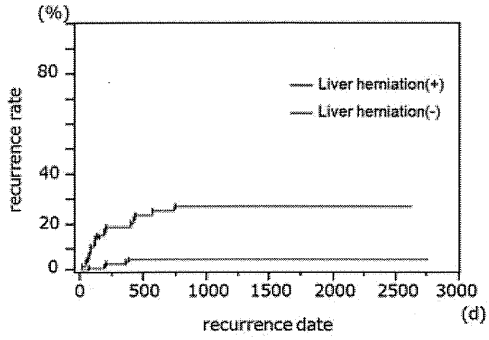
Variables	Crude odds ratio	95% CI	p value
Liver herniation	7.4	2.73–23.68	0.0002
Defect size C and D	7.09	2.73–19.99	< 0.001
Patch repair	5.00	1.91–14.70	0.0017

Abbreviation: CI, confidence interval.

Table 4 Multiple logistic regression analysis for the recurrence

Variables	Adjusted odds ratio	95% CI	p value
Liver herniation	3.96	1.01–16.92	0.0483
Defect size C and D	3.79	0.86–23.12	0.0796
Patch repair	1.30	0.24–8.83	0.7666

Abbreviation: CI, confidence interval.



Recurrence rates	Liver herniation (+) (n=64)	Liver herniation (-) (n=116)
6 months after the operation	15.7%	1.7%
1 year after the operation	18.9%	4.4%
2 years after the operation	25.4%	4.4%

Fig. 1 The Kaplan–Meier analysis for the recurrence ($n = 21$). Cox proportional hazards regression analyses showed significance in liver herniation (hazard ratio, 3.66; 95% confidence interval, 1.03–14.42; $p = 0.045$).

to affect the quality of life of many infants with CDH beyond the neonatal period.[1,3] Recurrence is one of the most insidious complication during the follow-up. Reoperation will be considered when the patients revealed herniation of the stomach or intestinal loops, or progressing elevation of the diaphragm over time. However, few systemic reviews of the CDH patients with recurrence exist, and therefore, risk factors for predicting the recurrence remains controversial.[4–6,11,12] Most of the literatures associated with the recurrence were small case series or the insufficient follow-up period with lack of important data.[4–6,11,12] In this survey, 180 CDH survivors with more than 3-year follow-up period were collected, these numbers and periods might be favorable.

The previously reported risk factors for the recurrence were larger defect, prosthetic patch, surgeon's skills or experiences, ECMO, and minimally invasive surgery (MIS).[4–6,11–13] The risk factors for the recurrence might be composed of two kinds of aspects: one is the severity of the disease, and the other is the technical issue. Likewise, in this study, baseline variables and univariate analysis showed the significant differences in liver herniation, larger defect, and patch repair. These three factors were confounding factors, which will certainly represent the severity of the disease among CDH patients. Finally, multiple logistic regression analysis showed the significance in liver herniation. This result showed the correlation between diaphragmatic defects; otherwise, the disease severity might be strongly associated with the recurrence. In addition, the chronological recurrence rates between liver herniation group and nonliver herniation group were primarily sated as follows: the liver herniation group became plateau to 25.4% at 2 years after the primary operation, and nonliver herniation group become plateau to 4.4% at 1 year after the primary operation. This statistical analysis would be practical and helpful for the parental counseling and the prognostic prediction.

In this study, no one was treated by MIS, 4 were treated via a transthoracic approach, and 176 were treated via a transabdominal approach. The surgical approach for CDH varies regionally and the technical problems might exist, especially, in the field of MIS. Although, a meta-analysis showed a statistically higher recurrence rate in the thoracoscopic group,[13] the actual risk and benefit of MIS might need more time to conclude because the MIS for CDH repair was still not yet to be established at present.

The limitation of this study was the restriction in retrospective multicenter cohort study design. In this study, nine cooperative institutions do not have the standardized protocol, and hence, the indications for treatment were lack of consensus. Therefore, the indication for iNO and the timing of operation were different, in which these factors had to be excluded from the univariate analysis in consideration for bias. Technical and material aspects, such as the cone-shaped Dualmesh (W. L. Gore & Associates, Inc., Arizona, United States) patch, or the PTFE/Marlex composite graft were not also examined; nevertheless, recent literature showed significances in recurrence rate.[4,6] These technical and material factors should be standardized when we plan to perform the prospective study in future.

Two-thirds of CDH patients with recurrence were reported to be asymptomatic at the time of diagnosis.[6] Late diagnosis of recurrence sometimes leads patients to the risk of bowel obstruction, respiratory failure, and the other serious sequelae. Recent CDH follow-up protocol recommends the periodic plain chest X-ray and the other additional complete examinations when the recurrence are to be suspected.[5,6] Ideally, establishing an international, multicenter long-term follow-up registry will be favorable to better understand the incidence patterns of morbidity in CDH patients.

A wide spectrum of the disease severity and the rarity of the disease mask the risk of the recurrence for CDH patients. This study showed the only factor to predict the recurrence was the liver herniation. These data will be helpful for providing information for the long-term follow-up of the CDH patients.

Conflict of Interest
None.

References

1 Bagolan P, Morini F. Long-term follow up of infants with congenital diaphragmatic hernia. Semin Pediatr Surg 2007;16(2):134–144
2 Masumoto K, Teshiba R, Esumi G, et al. Improvement in the outcome of patients with antenatally diagnosed congenital diaphragmatic hernia using gentle ventilation and circulatory stabilization. Pediatr Surg Int 2009;25(6):487–492
3 Lally KP, Engle W; American Academy of Pediatrics Section on Surgery; American Academy of Pediatrics Committee on Fetus and Newborn. Postdischarge follow-up of infants with congenital diaphragmatic hernia. Pediatrics 2008;121(3):627–632
4 Tsai J, Sulkowski J, Adzick NS, Hedrick HL, Flake AW. Patch repair for congenital diaphragmatic hernia: is it really a problem? J Pediatr Surg 2012;47(4):637–641

5 Jawaid WB, Qasem E, Jones MO, Shaw NJ, Losty PD. Outcomes following prosthetic patch repair in newborns with congenital diaphragmatic hernia. Br J Surg 2013;100(13):1833–1837
6 Jancelewicz T, Chiang M, Oliveira C, Chiu PP. Late surgical outcomes among congenital diaphragmatic hernia (CDH) patients: why long-term follow-up with surgeons is recommended. J Pediatr Surg 2013;48(5):935–941
7 Tsao K, Lally KP. The congenital diaphragmatic hernia study group: a voluntary international registry. Semin Pediatr Surg 2008;17(2): 90–97
8 Nagata K, Usui N, Kanamori Y, et al. The current profile and outcome of congenital diaphragmatic hernia: a nationwide survey in Japan. J Pediatr Surg 2013;48(4):738–744
9 Usui N, Nagata K, Hayakawa M, et al. Pneumothoraces as a fatal complication of congenital diaphragmatic hernia in the era of gentle ventilation. Eur J Pediatr Surg 2014;24(1):31–38
10 Lally KP, Lasky RE, Lally PA, et al; Congenital Diaphragmatic Hernia Study Group. Standardized reporting for congenital diaphragmatic hernia—an international consensus. J Pediatr Surg 2013;48(12): 2408–2415
11 Romao RL, Nasr A, Chiu PP, Langer JC. What is the best prosthetic material for patch repair of congenital diaphragmatic hernia? Comparison and meta-analysis of porcine small intestinal submucosa and polytetrafluoroethylene. J Pediatr Surg 2012;47(8): 1496–1500
12 Gander JW, Fisher JC, Gross ER, et al. Early recurrence of congenital diaphragmatic hernia is higher after thoracoscopic than open repair: a single institutional study. J Pediatr Surg 2011;46(7): 1303–1308
13 Lansdale N, Alam S, Losty PD, Jesudason EC. Neonatal endosurgical congenital diaphragmatic hernia repair: a systematic review and meta-analysis. Ann Surg 2010;252(1):20–26

Original Paper

Developmental Neuroscience

Dev Neurosci
DOI: 10.1159/000368396

Received: November 26, 2013
Accepted after revision: September 12, 2014
Published online: February 17, 2015

Administration of Umbilical Cord Blood Cells Transiently Decreased Hypoxic-Ischemic Brain Injury in Neonatal Rats

Tetsuo Hattori[a, b] Yoshiaki Sato[a] Taiki Kondo[a] Yuko Ichinohashi[a] Yuichiro Sugiyama[a] Michiro Yamamoto[c] Tomomi Kotani[d] Hitoshi Hirata[c] Akihiro Hirakawa[e] Satoshi Suzuki[e] Masahiro Tsuji[f] Tomoaki Ikeda[g] Keiko Nakanishi[h] Seiji Kojima[b] Klas Blomgren[i] Masahiro Hayakawa[a]

[a]Division of Neonatology, Center for Maternal-Neonatal Care, Nagoya University Hospital, Departments of [b]Pediatrics, [c]Hand Surgery and [d]Obstetrics and Gynecology, and [e]Center for Advanced Medicine and Clinical Research, Nagoya University Graduate School of Medicine, Nagoya, [f]Department of Regenerative Medicine and Tissue Engineering, National Cerebral and Cardiovascular Center Research Institute, Osaka, [g]Department of Obstetrics and Gynecology, Mie University, Mie, and [h]Department of Perinatology, Institute for Developmental Research, Aichi Human Service Center, Aichi, Japan; [i]Karolinska Institute, Department of Women's and Children's Health, Karolinska University Hospital, Stockholm, Sweden

Key Words
Neuroprotection · Oxidative stress · Cell therapy · Asphyxia

Abstract
This study aimed to investigate whether the administration of mononuclear cells derived from human umbilical cord blood cells (UCBCs) could ameliorate hypoxic-ischemic brain injury in a neonatal rat model. The left carotid arteries of 7-day-old rats were ligated, and the rats were then exposed to 8% oxygen for 60 min. Mononuclear cells derived from UCBCs using the Ficoll-Hypaque technique were injected intraperitoneally 6 h after the insult (1.0×10^7 cells). Twenty-four hours after the insult, the number of cells positive for the oxidative stress markers 4-hydroxy-2-nonenal and nitrotyrosine, in the dentate gyrus of the hippocampus in the UCBC-treated group, decreased by 36 and 42%, respectively, compared with those in the control group. In addition, the number of cells positive for the apoptosis markers active caspase-3 and apoptosis-inducing factor decreased by 53 and 58%, respectively. The number of activated microglia (ED1-positive cells) was 51% lower in the UCBC group compared with the control group. In a gait analysis performed 2 weeks after the insult, there were no significant differences among the sham-operated, control and UCBC groups. An active avoidance test using a shuttle box the following week also revealed no significant differences among the groups. Neither the volumes of the hippocampi, corpus callosum and cortices nor the numbers of neurons in the hippocampus were different between the UCBC and control groups. In summary, a single intraperitoneal injection of UCBC-derived mononuclear cells 6 h after an ischemic insult was associated with a transient reduction in numbers of apoptosis and oxidative stress marker-positive cells, but it did not induce long-term morphological or functional protection. Repeated administration or a combination treatment may be required to achieve sustained protection.

Tetsuo Hattori and Yoshiaki Sato contributed equally to this work.

KARGER 125

E-Mail karger@karger.com
www.karger.com/dne

0378–5866/15/0000–0000$39.50/0

Yoshiaki Sato, MD, PhD
Division of Neonatology, Center for Maternal-Neonatal Care
Nagoya University Hospital
65 Tsurumai-cho, Showa-ku, Nagoya 466-8560 (Japan)
E-Mail yoshiaki@med.nagoya-u.ac.jp

Introduction

Perinatal hypoxia-ischemia (HI) remains an important cause of neonatal death and permanent neurological deficits [1]. Notwithstanding the developments made in perinatal medicine, perinatal HI occurs in 1.3–1.7/1,000 live births; its incidence is high even in developed countries [2]. Many survivors of perinatal HI experience long-term neurological disabilities and impairments resulting in major socioeconomic burdens. At present, there are no effective treatments for HI-induced brain damage, except for brain hypothermia [3], which is not effective in severe cases [4, 5]. Therefore, it is of the utmost importance to develop a novel and effective therapy against perinatal HI-induced brain injury.

Stem cell therapy is expected to be used in the treatment of many central nervous system diseases in the future. Various kinds of stem cells are possible sources of cell therapy for future clinical applications [6, 7]. We recently demonstrated that intracerebroventricular injection of neural stem/progenitor cells together with chondroitinase ABC – which digests glycosaminoglycan chains on chondroitin sulfate proteoglycans – significantly decreased the degree of cerebral infarction after perinatal HI injury in a rat model [8]. However, ethical concerns hinder the use of postmortem human brains as a source of neural stem/progenitor cells in future clinical applications. Furthermore, intracerebral administration is an invasive procedure, and the injected cells themselves may lead to gliotic changes in the host brain [9], thereby necessitating more detailed examinations to ensure the safety of the procedure.

Umbilical cord blood cells (UCBCs) are a promising source of stem cell therapy. They are readily available and can be used for autologous transplantations. Thus, many ethical considerations can be avoided. Furthermore, UCBCs can be administered intravenously [10] and cross the blood-brain barrier [11]. Meier et al. [12] first reported the treatment effects of UCBCs in the amelioration of HI-induced brain damage in a neonatal rat model; moreover, several recent studies reported favorable effects of UCBCs [13–18]. However, the mechanisms underlying the favorable effects remain to be fully elucidated. In the present study, we administered UCBCs to HI rats to investigate their effects and the underlying mechanisms.

Materials and Methods

Animals

All animal experimental protocols in the present study were approved by the Institutional Review Board of Nagoya University School of Medicine (Nagoya, Aichi Prefecture, Japan; permit No.: 23181-2011 and 24337-2012). Wistar/ST rat pups were obtained from Japan SLC Inc. (Shizuoka, Japan) and maintained under a 12-hour light/dark cycle (8.00 a.m. to 8.00 p.m.) with ad libitum access to food and water. The animal room and experimental space were always maintained at 23°C.

UCBC Preparation

Human UCBCs were donated by women who delivered at Nagoya University Hospital. Written, informed consent was obtained from the donors and their spouses, and this experimental protocol using human cells was reviewed and approved by the local ethics committee of our hospital (permit No.: 794). The donors underwent normal delivery or elective cesarean section because of previous cesarean section, breech position or cephalopelvic disproportion. The donors and infants had no major perinatal complications; all were singleton pregnancies of more than 36 weeks of gestational age.

Umbilical cord blood was collected immediately after placental delivery in bags containing citrate phosphate dextrose (CBC-20; Nipro Corporation, Osaka, Japan). Mononuclear cells were isolated using the Ficoll-Hypaque technique, suspended in Roswell Park Memorial Institute (RPMI) 1640 medium (Life Technologies, Carlsbad, Calif., USA) at a concentration of 1×10^7 cells/ml, and cryopreserved in liquid nitrogen with an equal amount of a cryoprotectant (CP-1; Kyokuto Pharmaceutical Industrial Co. Ltd., Tokyo, Japan). CP-1 is a mixture of dimethylsulfoxide and hydroxyethyl starch, which makes it possible to preserve stem cells in a frozen state. Immediately before administration, the cells were thawed to 37°C, washed 3 times with phosphate-buffered saline (PBS) and resuspended in 0.3 ml of RPMI 1640 medium.

HI Insult and UCBC Administration

HI brain damage was produced using postnatal day 7 (P7) rats according to the method of Rice et al. [19] with slight modifications. Each pup was anesthetized using isoflurane inhalation and the left carotid artery was subsequently doubly ligated and incised between the ligatures. After a 1-hour rest with a dam, the pups were exposed to a hypoxic environment of 8% O_2 at 37°C for 60 min, after which they were returned to the dam in the animal room maintained at 23°C. Six hours later, the pups in the treatment group (UCBC group) were injected intraperitoneally with mononuclear cells derived from UCBCs (1×10^7 cells/0.3 ml). A control group underwent ligation of the left carotid artery and hypoxia in the same manner, but received an equivalent volume of RPMI medium alone. The sham group underwent neither left carotid artery ligation nor hypoxia.

Histological and Immunohistochemical Procedures

Histological and immunohistochemical procedures were performed as previously described [20] with minor modifications. Briefly, rats were deeply anesthetized and intracardially perfusion-fixed with 0.9% NaCl followed by 4% paraformaldehyde in PBS. The brains were removed and immersion-fixed in the same solution at 4°C for 24 h, dehydrated with a graded series of ethanol and xylene, embedded in paraffin and cut into 5-μm-thick coronal sections. After deparaffinization and rehydration, antigen retrieval was performed by heating the sections for 10 min in 10 mM citrate buffer (pH 6.0). Nonspecific binding was blocked with 3% donkey serum in PBS. Then, sections were incubated overnight at 4°C with rabbit anti-active caspase-3 (product No. 559565; dilution 1:200; BD Pharmingen, Franklin Lakes, N.J.,

USA), goat anti-apoptosis-inducing factor (AIF; product No. SC-9416; dilution 1:100; Santa Cruz Biotechnology, Dallas, Tex., USA), rabbit anti-4-hydroxy-2-nonenal (4-HNE; product No. HNE11-S; dilution 1:400; Alpha Diagnostic International, San Antonio, Tex., USA), rabbit antinitrotyrosine (product No. A-21285; dilution 1:200; Life Technologies; control samples are shown in the online suppl. fig. 1; for all online suppl. material, see www.karger.com/doi/10.1159/000368396), mouse anti-ED1 (activated microglia marker; product No. MAB1435; dilution 1:300; Merck Millipore), goat anti-neuronal nuclei (NeuN) antibody (product No. MAB377; dilution 1:400; Merck Millipore) or rat anti-myelin basic protein antibody (product No. MAB386; dilution 1:200; Merck Millipore) in PBS with 0.1% Triton. The sections were subsequently incubated with the appropriate biotinylated secondary antibodies (Vector Laboratories, Burlingame, Calif., USA) for 1 h at room temperature. Endogenous peroxidase activity was blocked with 3% H_2O_2 in PBS for 10 min and then avidin-biotin-peroxidase complex (Vectastain ABC Elite kit; Vector Laboratories), followed by peroxidase detection for 10 min (0.12 mg/ml 3,3′-diaminobenzidine, 0.01% H_2O_2 and 0.04% $NiCl_2$).

Cell Counting for Acute Injury Markers

Cells positive for active caspase-3, nuclear AIF, 4-HNE, nitrotyrosine and ED1 were counted throughout the hippocampal granule cell layer (GCL). In the section at the hippocampal level, which is approximately –3.3 to –3.6 mm posterior to the bregma in the adult rat brain [21], the GCL was outlined under low magnification (×100), and the area was measured. Further, the positive cells were counted under high magnification (×400) using Stereo Investigator version 10 stereology software (MicroBrightField Europe EK, Magdeburg, Germany) and expressed as densities.

Behavioral Tests

Gait Analysis

To evaluate hemiparesis, the CatWalk® quantitative gait analysis system (Noldus Information Technology, Wageningen, the Netherlands) was used for gait assessment following experimental stroke [22]. Briefly, we had the experimental rats run across a glass walkway transversely and recorded the runs using a camera positioned below. If an animal failed to complete a run within 5 s, walked backwards or reared during the run, it was made to repeat the process. Each rat ran 3 times, and the average was calculated. The experiment was performed in the dark; the glass walkway was illuminated with beams of light, thereby allowing the animals' paws to reflect light as they touched the glass floor. An observer labeled each paw on the recorded video to calculate paw-related parameters. The gait-related parameters measured using the CatWalk system were the following: run duration, time duration of entire run; print area, area of paw print; stride length, distance the paw traveled from one step to the next; swing speed, the stride length divided by the duration of one stride length; mean intensity, the mean intensity of each paw in the run (the intensity is proportional to the load on the paw); duty cycle, percentage of time the paw accounted for the total step cycle of that paw.

Active Avoidance Test

The active avoidance test was performed according to the methods of Ichinohashi et al. [23] using the same equipment. For 4 consecutive days, each rat underwent 20 sessions of the active avoidance test each day. The test was conducted in an automated shuttle box (Med Associates Inc., St. Albans, Vt., USA) subdivided into 2 compartments with independently electrified stainless steel bars as a floor. One session consisted of a buzzer tone and stimulation with light (conditioning stimulus), and an electric shock (unconditioned stimulus). The conditioning stimulus was continued for 5 s, as was the subsequent unconditioned stimulus with a positive half-wave constant current of 0.5 mA. When the conditioning stimulus occurred, the rat had to escape to the other side of the shuttle box to turn it off and avoid the unconditioned one. The average interval time between each trial was 30 s (range, 10–90 s). The parameters were analyzed using the MED-PC IV program (Med Associates Inc.). Each day, we evaluated the avoidance proportion, i.e. the number of sessions in which the rat successfully avoided the unconditioned stimulus.

Volume Measurement and Stereological Cell Counting

After the behavior tests, the rats were deeply anesthetized, intracardially perfusion fixed, and had their brains removed. To evaluate the whole cortex, corpus callosum and hippocampus, every 150th section from the whole cerebrum and corpus callosum (typically 17 sections) and every 75th section from the hippocampus level (typically 6 sections) were stained using goat anti-NeuN antibody for cortex and hippocampus, or anti-myelin basic protein antibody for the corpus callosum. The bilateral cortex, hippocampus (NeuN-positive areas) and corpus callosum of each section were outlined, and the areas of each were measured using Stereo Investigator. The volumes of the bilateral cortex and hippocampus were calculated from the NeuN-positive areas according to the Cavalieri principle using the following formula: $V = \Sigma A \times P \times T$, where V = the total volume, ΣA = the sum of area measurements, P = the inverse of the sampling fraction, and T = the section thickness [20]. The numbers of NeuN-positive cells were counted in the whole GCL, including the subgranular zone, of the hippocampus using unbiased stereological counting techniques. After outlining the borders of the GCL, the computer program overlays the outlined area with a grid system of counting frames. Cells within these frames as well as those touching 2 out of 4 predetermined sides of the frames were counted.

Statistical Analysis

All data are presented as mean ± standard error of the mean. A 2-sample Student's t test was used to compare the two groups in figure 2. The Steel-Dwass test as nonparametric multiple comparison procedure was used to compare the three groups in figures 3 and 4. A p value <0.05 was considered statistically significant.

Results

Impact of UCBCs on Expression of Acute Injury Markers after HI

P7 rats were subjected to HI, and 6 h later, they were administered cryopreserved, thawed and washed mononuclear cells isolated from the UCBCs or vehicle. We examined various acute injury biomarkers 24 h after HI: apoptosis markers (active caspase-3 and AIF), oxidative

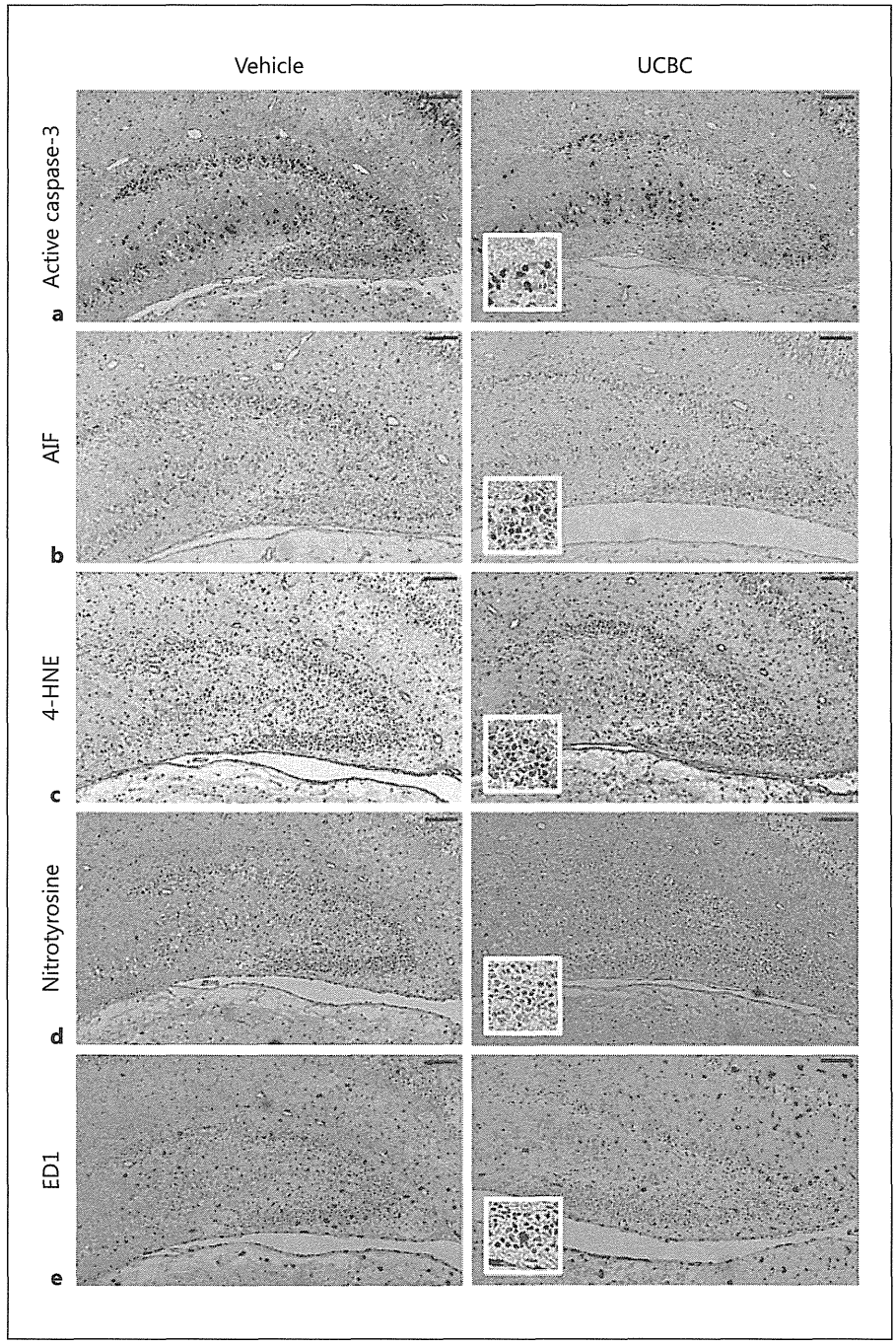


Fig. 1. Photomicrographs of the hippocampus stained for acute injury markers. Representative photomicrographs of the dentate gyrus of the hippocampus 24 h after HI insult. The sections from vehicle-treated rats (vehicle) and UCBC-treated rats (UCBC) were stained for active caspase-3 (**a**), AIF (**b**), 4-HNE (**c**), nitrotyrosine (**d**) and ED1 (**e**). Bar = 100 μm. Insets show higher magnifications.

stress markers (4-HNE and nitrotyrosine) and an activated microglia marker (ED1). Photomicrographs of representative hippocampi are shown in figure 1. The number of the apoptosis marker-positive cells in the ipsilateral GCL significantly decreased in the UCBC group compared with those in the control group (activated caspase-3, 53%, and AIF, 58%; fig. 2a, b; $p < 0.05$ and $p < 0.01$, respectively). The numbers of oxidative stress marker-positive cells also decreased in the UCBC group compared with the control group (4-HNE, 36%, and nitrotyrosine, 42%; fig. 2c, d; $p < 0.05$). Moreover, the number of ED1-positive cells was 51% lower in the UCBC group compared with the control group (fig. 2e; $p < 0.05$).

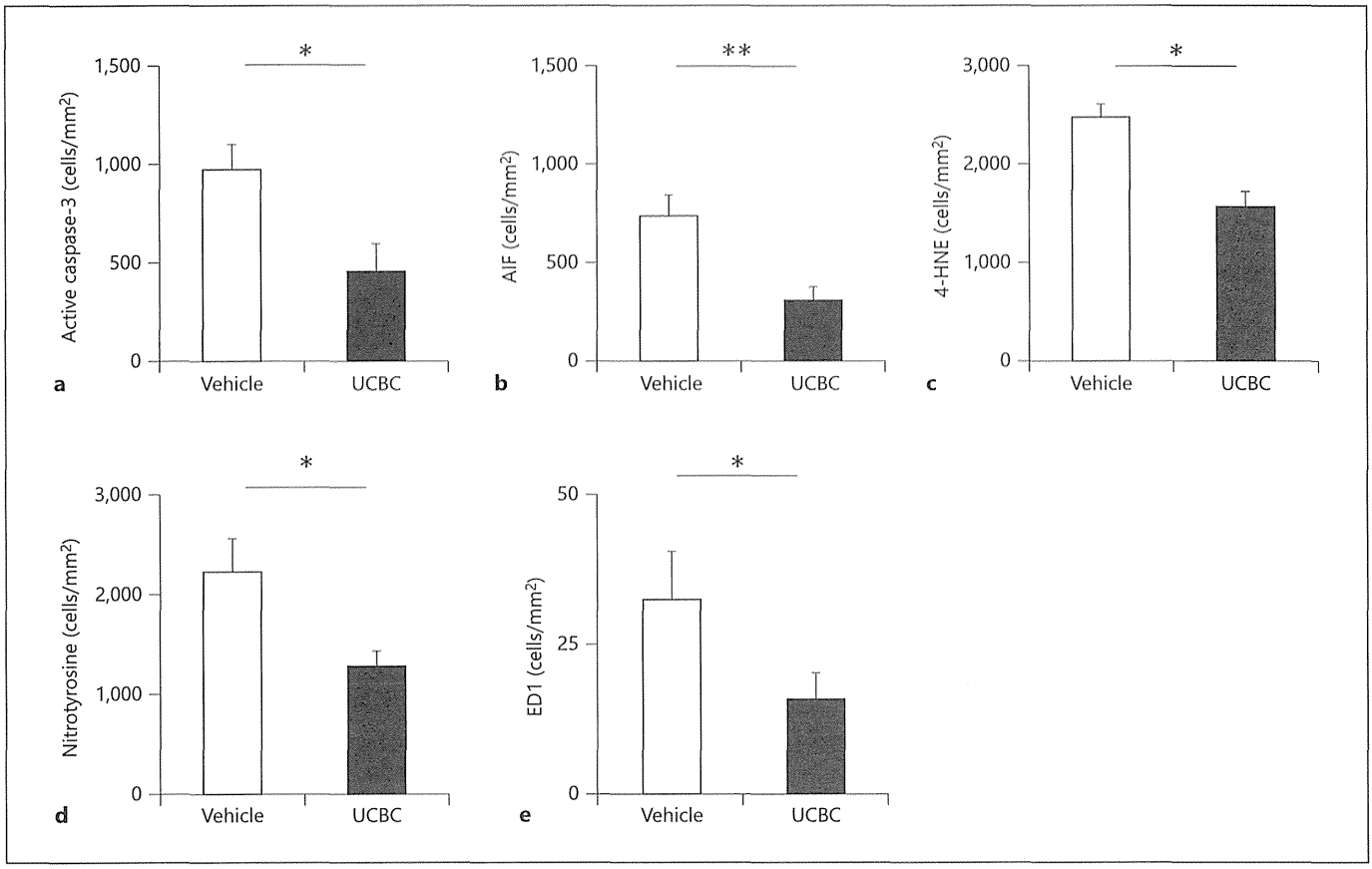


Fig. 2. Impact of UCBCs on apoptosis, oxidative stress and activation of microglia. The numbers of various marker-positive cells in figure 1 were counted. The numbers of active caspase-3-positive (**a**) and AIF-positive cells (**b**) in the ipsilateral GCL were significantly lower in the UCBC group (n = 11) than in the vehicle group (n = 12). The numbers of both 4-HNE- (**c**) and nitrotyrosine-positive cells (**d**) were also significantly lower in the UCBC group. The number of ED1-positive cells (**e**) was significantly decreased in the UCBC group. Data are presented as means ± standard error of the mean. * $p < 0.05$, ** $p < 0.01$.

Impact of UCBCs on Behavior after HI

Gait Analysis

To evaluate the effect of HI on motor function and of UCBC administration on HI-induced motor deficits, gait analysis was performed 14 days after the insult (P21) using the CatWalk system. There were no significant differences in run duration, right front paw (RF) print area, RF swing speed, RF/left front paw (LF) ratio of mean intensity, RF duty cycle or RF stride length among the three groups (sham-operated, control and UCBC groups; fig. 3).

Active Avoidance Test

Further, to evaluate the effects of UCBCs on HI-induced learning impairments, an active avoidance test was performed 21–24 days after the insult (P28–31). The mean avoidance proportion of each group was calculated for each consecutive day (fig. 4). The avoidance rates increased with time in all groups; however, there were no significant differences among the three groups for each day.

Impact of UCBCs on Histological Changes after HI

Finally, to assess the absolute tissue loss after HI, the volumes of the cortex, corpus callosum and hippocampus of both hemispheres were evaluated. After the behavioral tests, sections throughout the whole cerebrum were evaluated.

Representative photomicrographs stained for NeuN are shown in figure 5a and b. In both groups, there was apparent unilateral atrophy with partial collapse. We evaluated the volumes of the cortex (fig. 5c), corpus callosum (fig. 5d) or hippocampus (fig. 5e), but found no significant differences in the volumes between the groups.

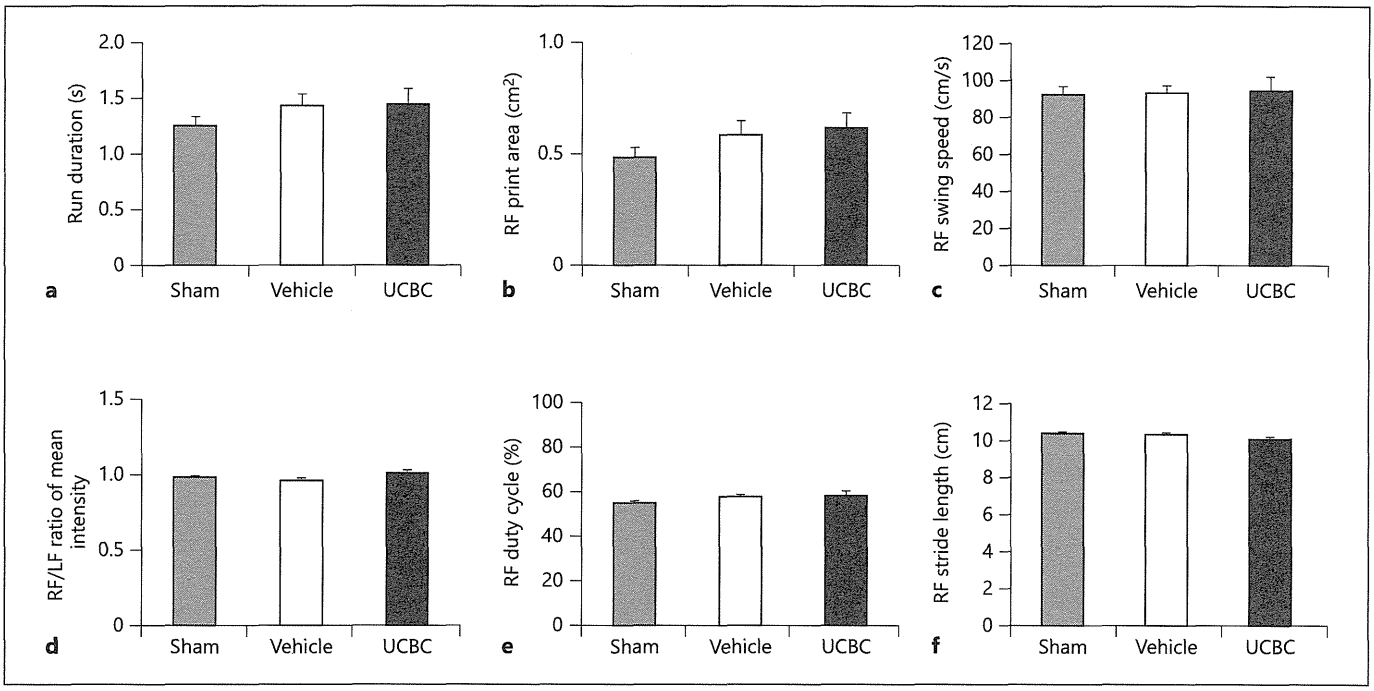


Fig. 3. Gait analysis. Gait analysis was performed 2 weeks after the insult. Each parameter was compared among the groups. There was no significant difference in run duration (**a**), RF print area (**b**), RF swing speed (**c**), RF/LF ratio of mean intensity (**d**), RF duty cycle (**e**) or RF stride length (**f**) among the three groups (sham-operated, n = 6; vehicle, n = 8; UCBC, n = 6).

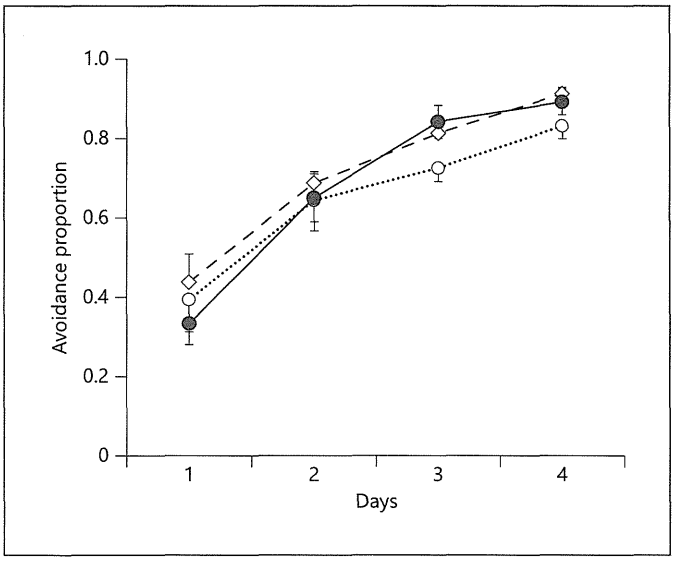


Fig. 4. Active avoidance test. The active avoidance was performed 21–24 days after insult (P28–31). Each rat underwent 20 sessions every day, and the mean avoidance proportion was calculated for each group. The avoidance proportions increased with time in all groups. There was no significant difference in avoidance proportions among the groups on any day. Open squares and dashed line: sham-operated, n = 4; open circles and dotted line: vehicle, n = 8; closed circles and solid line: UCBC, n = 6.

Therefore, we examined whether there was a difference in the number of neurons between the groups, even though the volume reductions were equivalent. The numbers of NeuN-positive neurons in the hippocampus were counted using stereological principles (Stereo Investigator, MicroBrightField) but they were not significantly different between the groups in the hippocampus of either the ipsilateral or contralateral hemisphere (fig. 5f).

Discussion

In the present study, we demonstrated that intraperitoneal UCBC administration caused antiapoptotic and antioxidative effects 24 h after the insults; however, we failed to demonstrate a prolonged reduction of neurological damage. We administered UCBCs in the early phase (6 h after HI). The optimal timing of administration is one of the most critical points to establish a new cell therapy. In regenerative medicine using stem cells, grafting in the early postinjury phase is generally not recommended. In neural stem/progenitor cell transplantation, early grafting in the acute phase (i.e. 24 h after insult), during which inflammatory chemical mediator and