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Original contribution

Detection of increased intracerebral lactate in a mouse model of Leigh syndrome using proton MR spectroscopy

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ABSTRACT

Purpose: To establish a brain proton magnetic resonance spectroscopy (^{1}H MRS) experimental system using a mouse model of Leigh syndrome for monitoring intracerebral lactate levels as a biomarker of mitochondrial disease progression.

Materials and methods: Brain ^{1}H MRS was performed in the *Ndufs4* homozygous knockout (KO) mice, a mouse model of Leigh syndrome, and control mice on a horizontal 7.0-T magnetic resonance imaging system at age 5–9 weeks. In a subset of KO mice, survival analysis was performed according to the median of the intracerebral lactate levels. In addition, in KO mice alive until 9 weeks of age, both ^{1}H MRS and T_2-weighted imaging (T_2WI) were longitudinally performed in the same individuals at 5, 7, and 9 weeks of age.

Results: Brain ^{1}H MRS demonstrated increased lactate levels in KO mice compared with control mice (6.4 ± 1.2 mM vs. 3.3 ± 0.8 mM, $p < 0.0001$). The increased intracerebral lactate levels were already observed at 5 weeks of age, while no obvious abnormal findings were detected in T_2WI. Notably, an increased lactate level of > 5.94 mM at week 5 was associated with a poor prognosis (median survival days: 24.5 vs. 42 days, log-rank $p = 0.03$). Longitudinal ^{1}H MRS experiments revealed temporal increase of intracerebral lactate levels, peaking at week 7 (mean change: 2.6 ± 0.7 mM, $p = 0.001$), followed by decrease at week 9 (mean change: −3.8 ± 2.5 mM, $p = 0.03$), along with further disease progression, with brain lesions being detected on T_2WI.

Conclusion: Using brain ^{1}H MRS, we demonstrated significant increase in intracerebral lactate levels in a mouse model of Leigh syndrome. Additionally, we demonstrated that intracerebral lactate is a useful biomarker of mitochondrial disease progression at stages preceding the development of brain lesions.

1. Introduction

Mitochondrial diseases are a group of disorders caused by mitochondrial dysfunction, owing to mutations either in the nuclear genome or mitochondrial deoxyribonucleic acid. A major role of the mitochondria is the energy production by oxidative phosphorylation, and therefore, mitochondrial dysfunction leads to depletion of adenosine triphosphate and redox imbalance [1–3]. Mitochondrial diseases typically present with symptoms of the brain and muscles, hence termed “mitochondrial encephalomyopathy,” because the cells of these tissues use more energy than other cells in the body. Mitochondria are so critical for cell function that mitochondrial diseases are life-threatening, progressive, and often associated with excess premature death [4–6]. Nevertheless, there are no disease-modifying treatments,

Abbreviations: GPC, glycerophosphocholine; KO, knockout; M, mol/kg wet weight; mM, mmol/kg wet weight; MR, magnetic resonance; MRI, magnetic resonance imaging; NAA, *N*-acetylaspartate; NAAG, *N*-acetylaspartylglutamate; PCh, phosphocholine; SD, standard deviation; T_2WI, T_2-weighted imaging; VAPOR, variable power radiofrequency pulses with optimized relaxation delays; WT, wild type; ^{1}H MRS, proton magnetic resonance spectroscopy

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and currently, the best management of mitochondrial diseases involves only symptomatic treatment [2,7,8]. Therefore, development of new drugs for mitochondrial diseases is strongly desired. For drug discovery research, monitoring disease activity in appropriate animal models is very important.

Nicotinamide adenine dinucleotide (reduced) dehydrogenase iron-sulfur protein 4 (*Ndufs4*) knockout (KO) mice have been established as a model for Leigh syndrome, the most common mitochondrial disease with childhood onset [5,9,10]. These mice manifest neurological symptoms, including growth retardation, ataxia, hypotonia, and lethargy, starting approximately 5 weeks after birth. In addition, brain magnetic resonance imaging (MRI) reveals bilateral and symmetrical lesions in the postlateral portion of the brainstem, developing approximately 8 weeks after birth [11]. The severe encephalopathy leads to death shortly after the development of brain lesions. Mitochondrial complex I activity of brain tissue is less than half that of control mice even before the development of brain lesion and the loss of complex I activity is independent of the age of the mouse [9,10]. In previous studies, *Ndufs4* KO mice were used to evaluate the effect of novel treatments for mitochondrial disease [11,12]. However, accurate evaluation of response to treatment is difficult because of the lack of objective markers reflecting disease activity. Brain lesions are objective compared with neurological symptoms; however, they develop only in the terminal stage, immediately prior to death [11,12]. Therefore, an early and reversible marker preceding the development of brain lesions is desired.

In the clinical setting, assessment of intracerebral lactate levels using proton magnetic resonance spectroscopy (^{1}H MRS) is the most widely used examination for mitochondrial diseases, and intracerebral lactate is expected to be a useful biomarker for evaluating mitochondrial disease activity [13–18]. As functionality of the tricarboxylic acid cycle is affected as a consequence of impaired oxidative phosphorylation, glycolysis is upregulated in compensation for failure of energy production, thereby leading to accumulation of lactate as an intermediate metabolite of glycolysis in patients with mitochondrial diseases [19,20]. In addition, intracerebral lactate level reflects energy deficiency in neurons, which have high energy consumption, and therefore, is reported to be a more sensitive biomarker than serum lactate [21–23]. However, there are no reports of evaluation of intracerebral lactate levels using brain ^{1}H MRS in animal models of mitochondrial disease in preclinical trials.

In the present study, for the first time, we evaluated intracerebral lactate levels in the *Ndufs4* KO mice using brain ^{1}H MRS, and demonstrated the association between intracerebral lactate levels and mitochondrial disease progression.

2. Methods

2.1. Animals

All animal procedures were approved by the Institutional Animal Care and Use Committee of Osaka University and National Cardiovascular and Cerebral Research Center. The animals were fed sterile food and water, housed in sterile cages, and placed in rooms with controlled temperature and humidity. Mice heterozygous for the deleted *Ndufs4* allele (Hetero mice) were obtained taka Jackson laboratories (Bar Harbor, ME, USA). In this study, we treated only homozygous *Ndufs4* KO mice as the model for Leigh syndrome (KO mice), and Hetero and WT mice as controls [9–11,24]. Animals were treated as previously reported [11]. Briefly, pups were weaned and genotyped at ~28 days after birth. All cages were provided with daily food, water, and hydrated gel. Food and gel were replaced three times per week, and cages were changed weekly.

Brain ^{1}H MRS was performed in KO (n = 25, 19 males) mice at 5–9 weeks of age and control mice (n = 15, 13 males) at 6–8 weeks of age. The KO mice were divided into two groups according to their age: middle-stage mice at 5 weeks of age (n = 9, 6 males) and late-stage mice at 6–9 weeks of age (n = 16, 13 males) [10]. Among control mice, Hetero (n = 8, 6 males) and WT (n = 7, 7 males) mice were also compared.

To examine whether intracerebral lactate levels reveal mitochondrial disease prognosis, survival analysis was performed in a subset of KO mice evaluated using ^{1}H MRS at 5 weeks of age. The KO (n = 8, 5 males) mice at 5 weeks of age were divided into groups according to the median of the intracerebral lactate levels. Cumulative mortality rate and median survival days were investigated in each group. In addition, in KO mice alive until 9 weeks of age (n = 5, 2 males), both ^{1}H MRS and brain MRI were longitudinally performed in the same individuals at 5, 7, and 9 weeks of age.

2.2. Brain MRI and ^{1}H MRS

Brain MRI and ^{1}H MRS were performed on a horizontal 7.0-T MR system (BioSpec 70/30 USR, Bruker Biospin, Ettlingen, Germany). All brain MRIs were performed with the mice under general anesthesia using low-dose isoflurane (KO mice, 1.0% for induction and 0.3–0.6% for maintenance; and control mice, 3.5% for induction and 1.5–2.5% for maintenance), as previously reported [11,25]. During MRI, mice were positioned on a dedicated stereotaxic frame with mouth and ear bars to prevent any movements during MR image acquisition. The temperature of the mice was maintained at 36.5 °C with regulated water flow, and continuously monitored using a physiological monitoring system (SA Instruments, Inc., Stony Brook, NY, USA).

Anatomical T_2-weighted imaging (T_2WI) with rapid acquisition with refocused echoes sequence (repetition time/echo time = 4000/33 ms, rare factor: 8, field-of-view = 19.2 × 19.2 mm^2, image matrix size = 256 × 256, in-plane resolution = 75 × 75 μm^2, 10 slices with thickness = 500 μm, averaging = 4) in coronal orientation was used for accurate delineation of structures. Subsequently, the same high-resolution images were used for accurately positioning a voxel of 2 × 2 × 2 mm^3 in the center of the cerebrum, as shown in Fig. 1.

The homogeneity of the magnetic field was improved using fast automated shimming technique by mapping along projections sequence, and good shimming was reached in the voxel (between 6.8 and 10.8 Hz). The ^{1}H MRS acquisition was performed using a point resolved spectroscopy sequence (repetition time/echo time = 2500/20 ms combined with variable power radiofrequency pulses with optimized relaxation delays [VAPOR] water suppression, 4006-Hz spectral width, 2048 data points, transmitter offset of 4.7 ppm). Metabolite spectra were acquired using 256 repetitions with VAPOR and 32 repetitions without VAPOR for a total scan time of 12 min. Concentrations of metabolites, including lactate, myoinositol, glycerophosphocholine + phosphocholine (GPC + PCh), *N*-acetylaspartate + *N*-acetylaspartylglutamate (NAA + NAAG), creatine + phosphocreatine, and glutamate + glutamine were quantified using the water scaling method of LCModel (Stephen Provencher Inc., Okville, Ontario, Canada), which uses the unsuppressed water signal obtained from the same voxel as an internal reference for quantification (default water proton density of 35.9 M) [26].

2.3. Statistics

All data are presented as mean ± standard deviation. Statistical analyses were performed using JMP14 (SAS Institute Inc., Cary, NC, USA). Two-tailed unpaired or paired *t*-test was performed to compare the difference between two groups. Kaplan–Meier method was used for estimating cumulative mortality rate, and log-rank-test was used to assess the differences. A p value < 0.05 was considered statistically significant.

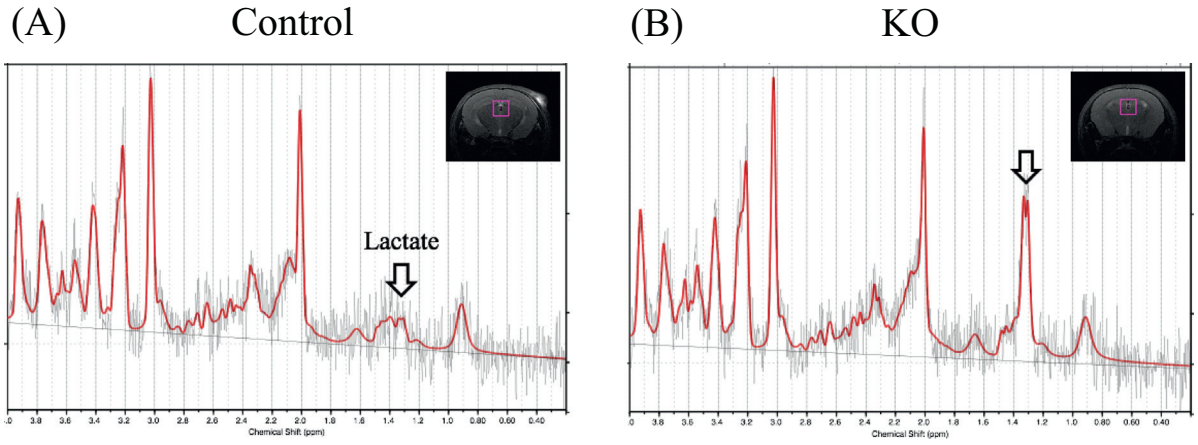

Fig. 1. Representative spectra of ^{1}H MRS. (A) Spectrum for control mice, with no significant lactate methyl peak (arrow). (B) Spectrum for KO mice, with an increased lactate methyl peak (arrow). The red curve represents the fitted spectrum, as derived from the analysis using LCModel. ^{1}H MRS: proton magnetic resonance spectroscopy, KO: knockout. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3. Results

3.1. Difference in intracerebral lactate levels between KO and control mice

Brain ^{1}H MRS revealed a significant lactate methyl peak at 1.3 ppm in all KO mice. Representative spectra are shown in Fig. 1. In KO mice, the lactate levels were significantly higher than those in control mice (6.4 ± 1.2 mM vs. 3.3 ± 0.8 mM, $p < 0.0001$), as shown in Fig. 2. More specifically, among control mice, there were no significant differences between Hetero and WT mice (3.3 ± 1.0 mM vs. 3.3 ± 0.7 mM, $p = 0.93$). On the other hand, in KO mice, it is noteworthy that the increased intracerebral lactate levels were already observed at 5 weeks of age. Contrary to expectations, there were no significant differences between middle- and late-stage KO mice (6.0 ± 1.4 mM vs. 6.6 ± 1.1 mM, $p = 0.29$).

3.2. Differences in levels of other metabolites between KO and control mice

In KO mice, GPC + PCh levels were slightly lower than those in control mice (1.6 ± 0.3 mM vs. 1.8 ± 0.2 mM, $p = 0.02$), and levels of other metabolites were not statistically significantly different between KO and control mice, as shown in Fig. 2.

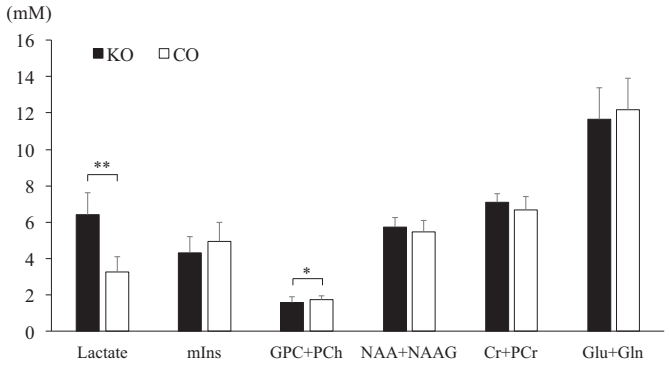

Fig. 2. The intracerebral metabolite levels in KO and control mice, quantified using LCModel. In KO (n = 25, 19 males) mice, the lactate levels were significantly higher than those in control (n = 15, 13 males) mice (6.4 ± 1.2 mM vs. 3.3 ± 0.8 mM, $p < 0.0001$) and GPC + PCh levels were slightly lower than those in control mice (1.6 ± 0.3 mM vs. 1.8 ± 0.2 mM, $p = 0.02$). Levels of other metabolites were not significantly different between KO and control mice. Values are presented as mean ± SD. Two-tailed unpaired *t*-test, *: $p < 0.05$, **: $p < 0.0001$. KO: knockout, CO: control, mIns: myo-inositol, GPC: glycerophosphocholine, PCh: phosphocholine, NAA: *N*-acetylaspartate, NAAG: *N*-acetylaspartylglutamate, Cr: creatine, PCr: phosphocreatine, Glu: glutamate, Gln: glutamine, SD: standard deviation.

3.3. Prognosis of KO mice according to intracerebral lactate levels

To verify whether intracerebral lactate levels reveal the prognosis of mitochondrial disease, survival analysis was performed. Eight KO mice were divided into two groups according to the median of the intracerebral lactate levels at 5 weeks of age: high lactate, > 5.94 mM (n = 4, 4 males); and low lactate, < 5.94 mM (n = 4, 1 male). The Kaplan–Meier curves for the mortality rate according to intracerebral lactate levels are shown in Fig. 3. The high intracerebral lactate group had a shorter median survival than the low intracerebral lactate group (24.5 vs. 42 days), and high intracerebral lactate levels at 5 weeks of age were associated with a poorer prognosis (log-rank $p = 0.03$).

3.4. Temporal changes in intracerebral lactate levels in KO mice

To verify whether intracerebral lactate levels reflect mitochondrial disease progression, both ^{1}H MRS and brain T_2WI were longitudinally performed. Temporal changes in intracerebral lactate levels and the corresponding presence/abundance of brain lesions are shown in Table 1 and Fig. 4. Longitudinal ^{1}H MRS revealed significant increase in intracerebral lactate levels from 5 to 7 weeks of age (mean change:

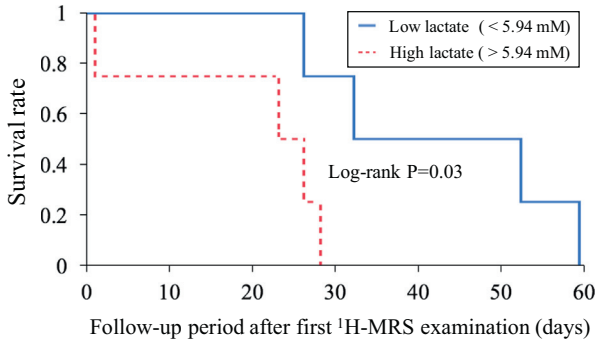

Fig. 3. Kaplan–Meier curves for the cumulative survival rate in KO mice according to intracerebral lactate levels at 5 weeks of age. The KO mice were divided into two groups according to the median of the intracerebral lactate levels at 5 weeks of age: high lactate, > 5.94 mM (n = 4, 4 males); and low lactate, < 5.94 mM (n = 4, 1 male). High intracerebral lactate levels were associated with a poor prognosis (median survival days after first ^{1}H MRS study: 24.5 vs. 42 days, log-rank $p = 0.03$). ^{1}H MRS: proton magnetic resonance spectroscopy, KO: knockout.

Table 1
Chronological changes of intracerebral metabolites levels and the presence of brain lesions in KO mice.

	5 weeks (n = 5)	7 weeks (n = 5)	9 weeks (n = 5)
Brain lesions	0 (0)	0 (0)	5 (100)
Lactate (mM)	5.4 ± 0.6	8.0 ± 0.7[*]	4.3 ± 2.2[†]
mIns (mM)	4.2 ± 0.5	4.8 ± 0.6	4.7 ± 0.7
GPC + PCh (mM)	1.7 ± 0.3	1.7 ± 0.3	1.4 ± 0.4
NAA + NAAG (mM)	5.5 ± 0.5	5.7 ± 0.4	5.6 ± 1.1
Cr + PCr (mM)	7.1 ± 0.6	6.9 ± 0.4	6.9 ± 0.7
Glu + Gln (mM)	12.1 ± 0.9	11.4 ± 0.9	10.9 ± 1.1

Values are n (%) or mean ± SD. Two-tailed paired *t*-test, [*]:$p < 0.01$ vs. 5 weeks. [†]:$p < 0.05$ vs. 7 weeks. KO: knock out, mIns: myoinositol, GPC: glycerophosphocholine, PCh: phosphocholine, NAA: *N*-acetylaspartate, NAAG: *N*-acetylaspartylglutamate, Cr: creatine, PCr: phosphocreatine, Glu: glutamate, Gln: glutamine, SD: standard deviation.

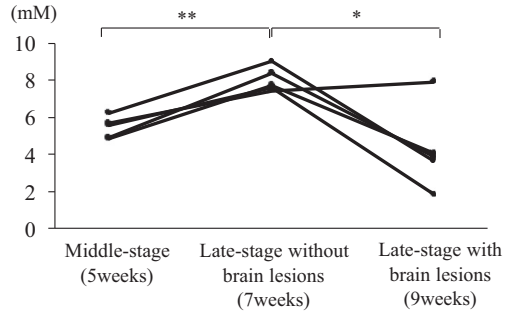

Fig. 4. Temporal changes in intracerebral lactate levels in KO mice. Intracerebral lactate levels increased from middle-stage (at 5 weeks of age) to late-stage (at 7 weeks of age) without brain lesions (mean change: 2.6 ± 0.7 mM, p = 0.001). However, in contrast, intracerebral lactate levels were significantly decreased with the development of brain lesions (mean change: −3.8 ± 2.5 mM, p = 0.03). Two-tailed paired *t*-test, *: $p < 0.05$, **: $p < 0.01$. KO: knockout.

2.6 ± 0.7 mM, p = 0.001). However, in contrast, intracerebral lactate levels were significantly decreased from 7 to 9 weeks of age (mean change: −3.8 ± 2.5 mM, p = 0.03). Longitudinal T_2WI revealed no obvious abnormal findings in KO mice at 5 and 7 weeks of age, and only in KO mice at 9 weeks of age, bilateral and symmetrical lesions were detected in the postlateral portion of the brainstem. Representative T_2W images are shown in Fig. 5.

3.5. Temporal changes in levels of other metabolites in KO mice

Temporal changes in levels of metabolites other than lactate are shown in Table 1. There were no significant changes in the levels of other metabolites with disease progression.

4. Discussion

This is the first study to monitor disease progression in a mouse model of Leigh syndrome using ^{1}H MRS. We detected increased intracerebral lactate levels even in juvenile mice, before the development of brain lesions, and demonstrated that high intracerebral lactate levels are associated with a poor prognosis, as shown by a study in humans [14]. We also demonstrated that the intracerebral lactate levels increased with mitochondrial disease progression until the development of brain lesions and then decreased with further disease progression to stages at which brain lesions were detected using T_2WI.

4.1. The utility of ^{1}H MRS in translational research

In preclinical as well as clinical settings, ^{1}H MRS is a promising evaluation method. Even in small animals, ^{1}H MRS is an established method and can evaluate various organs, including the brain, non-invasively and repeatedly [27–29]. Evaluation of energy metabolism using in vitro assay is not accurate because the concentration of energy metabolites at the time of measurement is strongly affected by the tissue preparation procedure. Compared with in vitro assay, ^{1}H MRS can be performed in vivo, and therefore, can precisely measure the concentration of energy metabolites. In addition, ^{1}H MRS can sensitively detect early changes in cellular metabolism preceding organic changes [27,28,30]. For example, in the present experimental system, increased lactate levels were observed even in juvenile mice without morphological change. Therefore, ^{1}H MRS is expected to be a highly sensitive evaluation method, and intracerebral lactate is considered an early biomarker of mitochondrial disease compared with the brain lesions detected using T_2WI. Finally, the greatest benefit is that ^{1}H MRS is available in both humans and small animals, which is especially significant for translational research.

4.2. The utility of intracerebral lactate as a biomarker of mitochondrial disease progression

There are several serum biomarkers of mitochondrial diseases [20,22,24,31,32]. However, compared with other serum biomarkers, intracerebral lactate is expected to be more useful. In patients with mitochondrial disease, a significant intracerebral lactate methyl peak is observed, and the association between intracerebral lactate levels and mitochondrial disease severity has been discussed in many studies [13–18]. In particular, symptomatic patients have higher lactate levels than asymptomatic patients [13,14,16], and patients with higher intracerebral lactate have a poor prognosis [14], as reported in the present study. However, there were few studies on the use of ^{1}H MRS for monitoring mitochondrial disease progression at multiple time points because patients with mitochondrial disease are rare and exhibit a prolonged disease course; in addition, MRS is not yet widely prevalent in clinical practice. Therefore, the present study is useful for

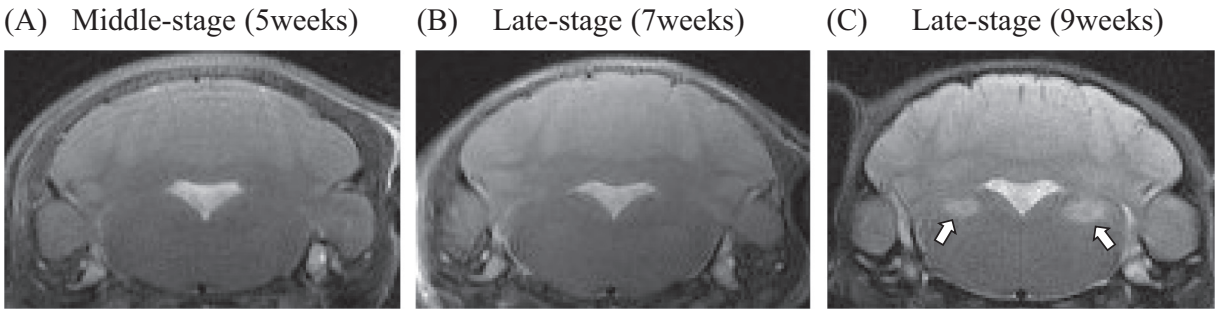

Fig. 5. Representative T_2W image of the KO mouse brain. (A) An image of middle-stage (at 5 weeks of age) KO mice. (B) An image of late-stage (at 7 weeks of age) KO mice. (C) An image of late-stage (at 9 weeks of age) KO mice, showing hyperintense lesions located in the postlateral portion of the brainstem (arrows). T_2W: T_2-weighted, KO: knockout.

understanding the association between mitochondrial disease progression and intracerebral lactate levels.

Our results revealed that intracerebral lactate levels reflect mitochondrial disease progression before the development of brain lesions. However, in contrast, with the development of brain lesions, intracerebral lactate levels were significantly decreased. In this mouse model of Leigh syndrome, neuronal death and consequent glial reactivity are major causes of mitochondrial encephalopathy, as demonstrated using immunohistochemical analysis [10]. We consider that significant neuronal loss leads to decreased energy demand, resulting in decreased intracerebral lactate levels. According to a previous report using a rat cerebral infarction model [33], intracerebral lactate levels are increased in the acute stage. However, with the progressive neuronal loss, intracerebral lactate levels decrease and finally normalize in the chronic stage, which is compatible with our explanation.

4.3. Levels of other metabolites in a mouse model of Leigh syndrome

According to a few case reports regarding Leigh syndrome in humans, NAA + NAAG levels were decreased in brain lesions [34–36], and GPC + PCh levels were slightly increased [35,36]; however there are no reports systemically evaluating intracerebral metabolites other than lactate in Leigh syndrome. In the present study, unlike previous reports, NAA + NAAG levels were not significantly different and GPC + PCh levels were slightly decreased in a mouse model of Leigh syndrome. In addition, these metabolite levels did not significantly change with disease progression. The reason NAA + NAAG levels did not decrease despite progressive neuronal loss is probably that the ^{1}H MRS spectra were obtained from the center of the cerebrum, i.e., a different area from that exhibiting brain lesions (the brainstem). Nevertheless, in the present evaluation, only lactate levels changed dramatically with disease progression, and therefore, was considered an essential metabolite for mitochondrial diseases.

4.4. Limitations

There are some limitations to our study. Firstly, we did not compare intracerebral lactate levels with the levels of other serum biomarkers, and thus, we could not conclusively establish intracerebral lactate as the most useful biomarker using the present experimental system. However, in experiments involving mice, repeatedly collecting blood sample is invasive and generally difficult. On the other hand, ^{1}H MRS can be used noninvasively and repeatedly with high sensitivity.

Secondly, our study lacks the assessment of response to therapeutic interventions. This is because there are no established curative treatments for mitochondrial diseases. Considering the pathological physiology, improvement of oxidative phosphorylation leads to normal function of tricarboxylic acid cycle and should decrease the levels of lactate, as an intermediate metabolite of glycolysis. To clarify whether intracerebral lactate levels are influenced by therapeutic interventions, further research using novel drugs to improve oxidative phosphorylation is certainly needed.

5. Conclusion

In the present study, we demonstrated significant increase of intracerebral lactate levels in a mouse model of Leigh syndrome using brain ^{1}H MRS. In addition, we demonstrated that intracerebral lactate, measured using ^{1}H MRS, is a useful biomarker of mitochondrial disease progression preceding the development of brain lesions. This novel experimental system can be of great use for drug discovery research in the future.

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