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Metabolic, pathological, and genetic analyses of foals neonatal foals that died in Noma horses

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We evaluated metabolic abnormalities in six neonatal Noma foals (Nos. 54–57, 62, and 66) that died shortly after birth, using laboratory tests, pathological examinations, serum amino acid (AA) analyses, gas chromatography/mass spectrometry (GC/MS), and genetic analyses. Nonspecific clinical symptoms, such as poor suckling and weakness, were commonly observed at birth. Sepsis caused by various bacterial infections was detected in foal Nos. 54, 62, and 66, while a heart malformation was identified in foal No. 57. Laboratory tests showed high aspartate transaminase, lactate dehydrogenase, and creatine kinase levels and low globulin and glucose levels in dead foals. The AA and GC/MS analyses revealed elevated levels of ammonia, orotic acid, and uracil in foal Nos. 54 and 55, while citrulline, arginine, and ornithine levels were low or within normal ranges, suggesting accelerated pyrimidine synthesis and suppressed urea cycle activity. Foal No. 56 had high uric acid and tyrosine levels, hypoglycemia, and liver dysfunction, suggesting glycogen storage disease. In foal No. 57, hypertyrosinemia was suggested because of high phenylalanine and tyrosine levels. We conducted a sequencing analysis of the ornithine transcarbamylase, argininosuccinatelyase, argininosuccinate synthase 1, uridine monophosphate synthase, G6PC1, and G6PT1/SLC37A4 genes associated with metabolic disorders. However, no mutations were detected. In conclusion, although metabolic pathways abnormalities resembling certain hereditary metabolic disorders were observed in neonatal foals that died in Noma horses, no specific mutations were identified in candidate genes, making hereditary disorders less likely.

Keywords: *metabolic abnormality, neonatal death, Noma horse*

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Noma horses are Japanese native horses with small body sizes. Breeding of Noma horses began in 1978 with a small number of founders at a public ranch in Imabari City, Ehime, Japan. They were bred as a genetically closed population of less than 100 individuals [16]. Therefore, there may be a significant risk of hereditary disorders caused by inbreeding

depression within this population. Prevention hereditary disorders is crucial for the stable breeding and conservation of this breed. Recently, a considerable number of foals have been born, but a significant proportion of foals have been lost owing to abortion, stillbirth, or neonatal death. In this study, we focused on neonatal deaths and aimed to elucidate their biochemistry, pathology, etiology, and genetics.

Neonatal death in horses can have various causes, including premature birth, complications during delivery, infections and septicemia, congenital physical defects, and metabolic disorders [1, 3, 11, 14, 42]. However, the causes of neonatal death in Noma horses remain unknown. Amino acid (AA) and gas chromatography-mass spectrometry (GC/MS) analyses of urine, plasma, and serum have been used as mass screening tests for metabolic disorders in newborn human infants [28]. GC-MS has been used in horses to test for banned substances [21, 37], but there are few reports on its application in detecting metabolic disorders [30, 41]. Therefore, in this study, we aimed to screen for metabolic abnormalities in neonatal foals that died among Noma horses, using laboratory tests, AA and GC/MS analyses, and pathological examinations. We also investigated the nucleotide sequences of several genes potentially linked to metabolic disorders.

Materials and Methods

Animals

Between November 2021 and July 2023, 13 foals were born at the Noma horse ranch (Nomauma Highland Public Ranch, Imabari, Ehime, Japan). Of these foals, six successfully (normal foals), one was stillborn, and six died during the neonatal period (1 to 8 days after birth). Of these, the six normal foals and six dead neonatal foals were used in the present study (Table 1). No treatment could be administered to the foals in neonatal death foals, except for foal No. 56, which received rehydration therapy and chest compressions, and foal No. 57, which was administered colostrum and covered with a warming blanket. The experimental protocols were approved by the Animal Care and Use Committee of Okayama University of Science, Japan (approval number: 2018-37).

Blood sampling

Blood sampling was performed 0–4 days after birth. Blood was collected from the cervical vein of the horse with a vacuum blood collection tube (Venoject II vacuum blood collection tube, Terumo Corporation, Tokyo, Japan). After sampling, blood was chilled on ice, transported to the laboratory, and centrifuged, and the supernatants were separated promptly. Serum samples were stored at −70°C until determinations.

Measurement methods

Laboratory tests were performed on the day of blood collection at the Biomedical Research and Service Center of Okayama University of Science. Blood and biochemical tests were conducted using a blood cell counter (Nihon Kohden Ltd., Tokyo, Japan) and an autoanalyzer (3100, Hitachi High-Tech Science Corp., Tokyo, Japan), respectively. Additionally, 40 AAs and related substances were analyzed using high-performance liquid chromatography employing the post-column ninhydrin method (L-8900 High-Speed Amino Acid Analyzer; Hitachi High-Tech Science Corp., Tokyo, Japan) at a commercial clinical laboratory center (Shikoku Chuken Inc., Matsuyama, Japan), as described previously [15]. Furthermore, 112 metabolites, including organic acids, AAs, carbohydrates, nucleobases, and neurotransmitters, in serum samples were simultaneously analyzed using GC/MS at a specialized laboratory (MILS International, Yokohama, Japan). The sample preparation procedure for the GC/MS analysis was

Table 1. Neonatal foals used in the present study

Group	Horse No.	Sex	Date of birth	Date of death	Age at death (days)	Age of blood sampling (days)
Normal	58	Female	2023/2/9	-	-	4
	59	Male	2023/3/6	-	-	1
	61	Female	2023/4/14	-	-	0
	63	Male	2023/5/4	-	-	0
	64	Female	2023/5/25	-	-	0
	65	Male	2023/6/9	-	-	0
Neonatal death	54	Male	2021/11/21	2021/11/22	1	1
	55	Female	2021/12/24	2021/12/26	2	1
	56	Male	2022/3/8	2022/3/9	1	0
	57	Female	2023/2/9	2023/2/11	2	1
	62	Male	2023/5/3	2023/5/5	2	0
	66	Male	2023/7/2	2023/7/10	8	0

described in a previous report [25]. The treated samples were analyzed using a GC/MS system (JMS-Q1000GC, JEOL Ltd., Tokyo) equipped with an ultra-alloy capillary column (Frontier Laboratories, Fukushima, Japan). Peak identification and metabolite analysis were performed using MILS International's original routine metabolomic data analysis software.

The six neonatal foals that died were autopsied at the Faculty of Veterinary Medicine, Okayama University of Science. The heart, spleen, liver, thymus, lymph nodes, and the respiratory, digestive, and genitourinary tracts were sampled, fixed in 10% neutral buffered formalin, routinely processed, embedded in paraffin, sectioned at 3 μm, and stained with hematoxylin and eosin stain. The histopathological lesions of foal Nos. 54, 62, and 66, which observed to contain bacteria, were additionally subjected to Gram staining. The liver of foal No. 54, lungs of foal No. 62, and cerebral fluid of foal No. 66 were subjected to bacterial examinations at a commercial clinical laboratory center, similar to the AA analysis (Shikoku Chuken Inc., Matsuyama, Japan). Furthermore, the livers of foal Nos. 54 and 62 and the paraffin-embedded brain of foal No. 66 were subjected to polymerase chain reaction targeting the bacterial 16S ribosomal RNA gene, according to the Japanese Pharmacopoeia [19].

Sequencing of candidate genes

Genomic DNA of the six neonatal foals that died was extracted from blood samples using phenol-chloroform extraction. We used 10 genomic DNA samples from the normal Noma horses as controls. The entire coding sequences of the equine genes for ornithine transcarbamylase (OTC, XM_001488750.6), argininosuccinatelyase (ASL; XM_023655541.1), argininosuccinate synthase 1 (ASS1; XM_001499340.6), uridine monophosphate synthase (UMPS; XM_001500039.5), glucose-6-phosphatase 1 (G8PC1; XM_001492705.3), and glucose-6-phosphate transporter 1/solute carrier family 37 member 4 (G6PT1/SLC27A4; XM_014741189.2) were amplified from genomic DNA of the foals, and their nucleotide sequences were determined via PCR-direct sequencing using 52 primer pairs (primer sequences are available upon request). PCR amplification was carried out in a reaction mixture containing 10 ng of genomic DNA and 1 UK OD FX Taq DNA polymerase (Toyobo Co., Ltd., Osaka, Japan), with 35 cycles of amplification. Each cycle consisted of denaturation at 94°C for 30 sec, annealing at 56 to 60°C for 30 sec, and extension at 72°C for 60 sec. The nucleotide sequences of the genes were determined using the dideoxy chain termination method after purification with ExoSAP-IT (Thermo Fisher Scientific, Waltham, MA, USA).

Data analysis

The data are expressed as the mean and standard deviation (SD) values for normal-foals and as individual results for neonatal foals that died. Abnormalities in laboratory test results were determined based on the mean and SD values for normal foals, as well as the reference values for horses [3, 4, 14]. However, no established reference values exist for serum AA concentrations and the substances analyzed using GC/MS in neonatal foals or Noma horses. There has been a report of a metabolic disorder in humans in which values exceeding three times or 1/3 the normal levels were considered abnormal [26]. In the present study, values five times or more or less than 1/5 the normal values were considered abnormal to ensure certainty.

Results

Table 2 presents the clinical records and pathological findings. The mean birth weight of normal neonatal foals was 14.9 ± 3.0 kg. Foal No. 56 was underweighted, while

Table 2. Clinical records and autopsy findings

No.	Birth weight (kg)	Colostrum intake	Clinical records	Autopsy and histopathological findings
54	14	+	Stopped suckling and weakness on the 1st day after birth	Suppurative inflammation in the liver and kidney, sepsis (*Actinobacillus equuli*)
55	15	+	Dysstasia and weakness on the 2nd day after birth	Aspiration dyspnea (probably due to amniotic fluid)
56	9.7	+	Small body size, dysstasia, and poor suckling	Fragility
57	12.7	+	Dyspnea and slight cyanosis	Heart malformation (circulatory disturbance)
62	11.7	+	Loss of energy on the 1st day after birth, death the day after	Intestinal intussusception, focal necrosis with bacterial infection in the lung and liver, sepsis (*Escherichia coli*)
66	16.1	+	Good health at birth Died of weakness on the 8th day	Bacterial meningitis and sepsis caused by *Enterobacter* sp.

The mean birth weight was 14.9 ± 3.0 kg in the six normal neonatal foals.

the other five foals had birth weights within the normal range. It was confirmed by their caregivers that all foals had received colostrum at birth. Nonspecific symptoms, such as poor or absent suckling and weakness, were observed in all foals that died, except foal No. 57. Foal No. 57 exhibited signs of circulatory disturbance, including dyspnea and slight cyanosis. Autopsy and histopathological examination revealed sepsis caused by infection with various bacteria in three foals (Nos. 54, 62, and 66). Foal No. 55 had aspiration dyspnea, while foal No. 57 had heart malformation. The major lesions in the sepsis cases consisted of infectious suppurative inflammation in the liver and kidneys in foal No. 54; focal necrosis with bacterial infection in the lungs and liver, along with mild to moderate multifocal hepatic vacuolar degeneration, in foal No. 62; and severe bacterial suppurative meningitis with hemorrhage as well as mild multifocal hepatic necrosis with inflammatory cell aggregation in foal No. 66. We detected *Actinobacillus equuli* in the liver of foal No. 54, *Escherichia coli* in the lungs of foal No. 62, and *Enterobacter* sp. in the cerebrospinal fluid of foal No. 66 through bacterial isolation. Specific bacterial genes were also detected in the livers of foal Nos. 54 and 62 and in the paraffin-embedded brain of foal No. 66. The heart of foal No. 57 exhibited an atrial septal defect, discontinuity between the right atrium and right ventricle with no valve at the tricuspid orifice, narrowing of the lumen near the pulmonary artery valve, two semilunar valves in the pulmonary artery valve, a patent ductus arteriosus, and a ventricular septal defect [24]. The major histopathological findings in the livers of the foals, excluding the sepsis cases, included congestion and mild diffuse hepatic vacuolar degeneration in foal No. 55, moderate to severe diffuse hepatic vacuolar degeneration with dilation of the space of Disse in foal No. 56, and moderate congestion in foal No. 57.

Table 3 presents the results of the laboratory tests. WBC counts were low in two neonatal death foals (Nos. 54 and 55), slightly increased in two foals (Nos. 56 and 57), and normal in two foals (Nos. 62 and 66). The RBC, hemoglobin, and hematocrit levels were increased. One foal (No. 54) had a low platelet count, but no breeding tendency was observed. High levels of aspartate transaminase (AST) in Nos. 54, 56, and 62, lactate dehydrogenase (LDH) in Nos. 54, 56, and 57, and creatine kinase (CK) in Nos. 56 and 57 were observed in foals with neonatal deaths. Alanine aminotransferase (ALT), alkaline phosphatase (ALP), and γ-glutamyl transpeptidase (GGT) levels were normal in all the foals that died. Globulin and glucose levels were low in all foals, except for foal No. 66. Bilirubin levels tended to be high in foals that died. Moderately high values of total bile acids were observed in foal Nos. 54 and 57. Hyperammonemia was detected in foal Nos. 54, 55, and 57. In foal No. 56, the serum ammonia concentration could

Table 3. Laboratory test results

Variable	Unit	Normal foal (n=6)		Neonatal death foal					
		Mean	SD	54	55	56	57	62	66
WBC	$10^2/\mu l$	83	16	6	10	135	124	109	63
RBC	$10^4/\mu l$	1,204	78	1,372	1,351	1,207	1,135	1,111	1,213
Hemoglobin	g/d*l*	15.8	0.59	16.4	15.6	15.1	17.0	14.0	17.4
Hematocrit	%	47.7	1.16	55.2	53.9	50.5	50.1	42.5	52.8
Platelet	$10^4/\mu l$	24.5	5.2	3.5	10.9	21.9	28.3	34.7	20.0
Aspartate transaminase	U/*l*	172	57	416	217	1,303	276	403	113
Alanine transaminase	U/*l*	8.50	4.51	19	13	ND	17	25	9
Alkaline phosphatase	U/*l*	5,776	1,823	2,434	4,810	7,250	6,683	4,680	4,370
Glutamyltransferase	U/*l*	25.0	8.9	31	29	30	28	23	15
Lactate dehydrogenase	U/*l*	450	204	1,071	549	1,715	851	531	369
Creatine kinase	U/*l*	250	158	759	519	7,127	1,865	753	208
Blood urea nitrogen	mg/d*l*	9.1	3.8	17.6	17.8	13.1	14.0	8.1	14.9
Creatinine	mg/d*l*	1.30	0.51	1.71	1.69	1.13	0.92	1.40	1.15
Total protein	g/d*l*	3.94	0.61	3.1	3.9	3.7	4.2	3.6	4.0
Albumin	g/d*l*	3.03	0.32	2.4	3.5	3.1	3.6	3.1	3.5
Globulin	g/d*l*	0.92	0.38	0.7	0.4	0.6	0.6	0.5	0.5
Glucose	mg/d*l*	98	57	4	11	11	44	38	140
Bilirubin	mg/d*l*	3.3	0.8	7.7	6.7	5.6	5.0	4.3	5.8
Triglyceride	mg/d*l*	47	39	86	362	52	32	15	132
Cholesterol	mg/d*l*	176	55	165	229	338	278	269	153
Total bile acid	mM/*l*	21.1	5.3	49.9	23.4	27.8	64.4	15.4	27.6
Ammonia	mg/d*l*	49	15	271	218	ND	134	41	62

Underlining: abnormal value. ND: not determined.

not be determined through standard laboratory testing, but the serum AA analysis indicated a normal ammonia level. Although not shown, neonatal foals that died had normal concentrations of calcium, inorganic phosphorus, magnesium, iron, sodium, potassium, and chloride.

Metabolic abnormalities were examined for each metabolic pathway using laboratory test data, AA profiles, and GC/MS analyses. First, we focused on foals with high levels of orotic acid. Table 4 shows the pyrimidine biosynthesis, ammonia transport, and urea cycle pathways in the normal neonatal foals and three neonatal death foals. These three foals (Nos. 54, 55, and 62) had high serum orotic acid signal levels that were more than five times those of normal neonatal foals. In particular, foal No. 55 had an extremely high signal level, which was 171,771 times higher than that of the normal foals. Uracil, a component of the pyrimidine biosynthesis pathway, was also high in two of these neonatal death foals (Nos. 54 and 55). In the ammonia transport pathway, high alanine, glutamate, and ammonia levels were detected in foal Nos. 54 and 55, but not in foal No. 62. Substances in the urea cycle (citrulline, arginine, and ornithine) were normal or slightly low in all three of these foals. Among the substances surrounding the urea cycle, fumarate was higher in foal Nos. 54 and 55, and aspartate and urea were elevated in foal No. 55. The urea cycle did not appear to be functional in foal Nos. 54 and 55. In addition, the orotic acid level was high in foal No. 62, but the levels of substances in the ammonia transport pathway, those of substances in the urea cycle, and that of uracil were normal.

Foal No. 56 showed high uric acid and tyrosine levels as well as hypoglycemia (Table 5), which are common features of glycogen storage diseases. Table 6 shows the metabolic profiles of foal Nos. 57 and 66. In foal No. 57, phenylalanine and tyrosine levels tended to be high, but the levels of fumaric acid and acetoacetic acid (metabolites of tyrosine) and those of 4-hydroxyphenylpyruvate, 4-hydroxyphenyllactic acid, and 4-hydroxyphenylacetic acid (the biomarkers used for the metabolic analysis of hypertyrosinemia) [22, 31, 32] were not high. Foal No. 66 showed no significant abnormalities in its metabolic profile.

Based on the data from the AA and GC/MS analyses, we focused on genes encoding enzymes involved in the urea cycle, orotic acid metabolism, and glycogen storage diseases, namely *OTC*, *ASL*, *ASS1*, *UMPS*, *G6PC1*, and *G6PT1/SLC27A4*, to investigate whether mutations in these genes are responsible for the metabolic disorders associated with the neonatal deaths in Noma horses. We performed PCR-direct sequencing of the coding regions and exon/intron boundaries of these six genes and compared the sequences with the reference horse genome sequence (EquCab3.0). As shown in Table 7, we found a synonymous variant in *ASS1*, a synonymous and intronic variant

Table 4. Measured values of substances in the pyrimidine biosynthesis, ammonia transport, and urea cycle pathways in the normal neonatal foals and foal Nos. 54, 55, and 62 that died neonatally

Pathway	Variable	Analysis method	Unit	Normal foal (n=6)		Neonatal death foal					
						No. 54		No. 55		No. 62	
				Mean	SD	Measurements	Ratio	Measurements	Ratio	Measurements	Ratio
Pyrimidine biosynthetic	Orotate	GC/MS	Signal level	0.0005	0.0008	0.0038	(8.27)	79.72	(171,771)	0.0034	(7.39)
	Uracil	GC/MS	Signal level	0.0002	0.0002	0.0073	(45.3)	5.42	(33,814)	0	(0.00)
Ammonia transport	Alanine	AA	nM/m*l*	321	74	843	(2.63)	636	(1.98)	381	(1.19)
	Alanine transaminase	BT	U/*l*	9.3	4.6	19.0	(2.05)	13	(1.40)	25	(2.69)
	Glutamate	GC/MS	Signal level	0.016	0.014	0.118	(7.48)	0.704	(44.4)	0.020	(1.25)
	Ammonia	AA	nM/m*l*	68	17	215	(3.18)	161	(2.38)	41	(0.61)
	Glutamine	AA	nM/m*l*	584	188	965	(1.65)	850	(1.46)	609	(1.04)
Urea cycle	Citrulline	AA	nM/m*l*	99.1	52.3	151.0	(1.52)	43.9	(0.44)	75.2	(0.76)
	Arginine	AA	nM/m*l*	132	58	109	(0.82)	125	(0.95)	87	(0.66)
	Ornithine	AA	nM/m*l*	56.1	34.7	85.6	(1.53)	25.9	(0.46)	27.7	(0.49)
	Fumarate	GC/MS	Signal level	0.004	0.001	0.011	(3.23)	4.04	(120)	0.008	(2.27)
	Aspartate	GC/MS	Signal level	0.394	0.255	0.379	(0.96)	5.83	(14.8)	0.207	(0.53)
	Urea	GC/MS	Signal level	0.0061	0.0050	0.006	(1.03)	0.607	(100)	0.003	(0.52)
	Aspartate transaminase	BT	U/*l*	211	116	416	(1.98)	218	(1.03)	403	(1.91)

Data are expressed as mean and standard deviation values for the normal neonatal foals and as measured values and ratios of the mean values of the normal foals (in parentheses) for foals that died neonatally. AA, amino acid; BT, biochemical test; GC/MS, gas chromatography/mass spectrometry; AST, aspartate transaminase.

Table 5. Measured values of glucose-6-phosphatase-related substances in the normal neonates and foal No. 56 that died neonatally

Pathway	Variable	Analysis method	Unit	Normal foal (n=6)		Neonatal dead foal No. 56	
				Mean	SD	Measurements	Ratio
Glucose-6-phosphatase-related	Alanine	AA	nM/m*l*	321	74	951	(2.97)
	Uric acid	GC/MS	Signal level	0.0001	0.0001	0.060	(1.088)
	Tyrosine-1	GC/MS	Signal level	0.523	0.377	0.150	(0.29)
	Tyrosine-2	GC/MS	Signal level	0.003	0.004	0.081	(24.3)
	Pyruvic acid	GC/MS	Signal level	0.065	0.011	0.111	(1.72)
	Lactic acid	GC/MS	Signal level	8.88	5.77	7.95	(0.9)
	Glucose	BT	mg/d*l*	103	54	11	(0.11)
	AST	BT	U/*l*	211	116	1,303	(6.19)

Data are expressed as mean and standard deviation values for the normal neonatal foals and as measurements and ratios of the mean values of the normal foals (in parentheses) for the foals that died neonatally. AA, amino acid; BT, biochemical test; GC/MS, gas chromatography/mass spectrometry; AST, aspartate transaminase.

Table 6. Measured values of phenylalanine-tyrosine metabolism pathway in the normal neonates and foal Nos. 57 and 66

Pathway	Variable	Analysis method	Unit	Normal foal (n=6)		Neonatal death foal No. 57		No. 66	
				Mean	SD	Measurements	(Ratio)	Measurements	(Ratio)
Phenylalanine-tyrosine metabolism	Phenylalanine 1	GC/MS	Signal level	0.19	0.09	0.55	(2.90)	0.24	(1.26)
	Phenylalanine 2	GC/MS	Signal level	0.05	0.03	0.28	(5.12)	0.07	(1.35)
	Tyrosine	AA	nM/m*l*	116	64	504	(4.35)	116	(1.00)
	Fumaric acid	GC/MS	Signal level	0.004	0.001	0.002	(0.63)	0.003	(0.90)
	Acetoacetic acid	GC/MS	Signal level	0.029	0.023	0.022	(0.76)	0.031	(1.06)
	4-hydroxyphenyl-pyruvate	GC/MS	Signal level	0.0001	0.0003	0	(0)	0	(0)
	4-hydroxyphenyl-lactic acid	GC/MS	Signal level	0.075	0.037	0.235	(3.14)	0.1	(1.33)
	4-hydroxyphenyl-acetic acid	GC/MS	Signal level	0.0023	0.003	0.001	(0.27)	0	(0)
Cori cycle	Glucose	BT	mg/d*l*	103	54	44	(0.76)	140	(1.36)
	Lactate	GC/MS	Signal level	8.9	5.8	20.6	(2.3)	34.9	(3.9)
Glucose-alanine cycle	Alanine	AA	nM/m*l*	321	74	1,409	(4.39)	268	(0.84)

Data are expressed as mean and standard deviation values for the normal neonatal foals and measurements and ratios of the mean values of the normal foals (in parentheses) for the foals that died neonatally. AA, amino acid; BT, biochemical test; GC/MS, gas chromatography/mass spectrometry. In foal No. 57, the glucose concentration increased to 120 mg/d*l* on the next day.

in the *UMPS* genes, a missense variant in *G6PC1*, and two synonymous and one missense variant in *G6PT1/SLC37A4*. Among the seven variants detected in the present study, six are common variants that have already been recorded in the variant database (European Variation Archive), while a missense mutation in *G6PT1/SLC37A4* was a newly identified variant. As synonymous and intronic variants are unlikely to affect the activities of the encoded enzymes, we further investigated the effects of the remaining two missense variants of *G6PC1* (Chr11:20280287 A>C) and *G6PT1/SLC37A4* (Chr7:27379237 C>T), which cause AA substitutions of leucine with arginine at the 89th codon (L89R) and arginine with glutamine at the 445th codon (R445Q), respectively. To determine whether these variants and their genotypes were specific to foal No. 56 in the neonatal death, we genotyped these variants in 10 healthy normal Noma horses. As shown in Table 8, the R/R genotype of *G6PC1 L89R* and R/Q genotype of *G6PT1/SLC37A4 R445Q* were observed in the normal Noma horses and were identical to those in foal No. 56. We evaluated the impact of these AA substitutions on the function of these enzymes using the PolyPhen-2 software to predict the functional effect of the AA substitutions in the protein. The results indicated that L89R and R445Q were predicted to be benign AA substitutions with scores of 0.002 and 0.004, respectively. These findings strongly suggest that neither L89R nor R445Q cause neonatal death in Noma horses.

Table 7. Variants of the candidate genes

Gene	Posiiton of variant	Exon	Variant ID	Ref/Alt	Function class	Genotype	Foal
OTC	No variant						
ASL	No variant						
ASS1	Chr25: 33853800	6	rs3432856809	C>T	Synonymous	Heterozygous	54
UMPS	Chr19: 37492521	1	rs397209516	A>T	Synonymous	Heterozygous	54, 56
	Chr19: 37489030	Intron 2	rs3429545509	delC	Intronic variant	Heterozygous	56
G6PC1	Chr11: 20280287	2	rs394198060	A>C	Missense (L89R)	Homozygous (R/R)	56
G6PT1/SLC37A4	Chr7: 27382638	2	rs397209516	G>A	Synonymous	Heterozygous	56
	Chr7: 27380033	7	rs3429545509	C>G	Synonymous	Homozygous	56
	Chr7: 27379237	9		C>T	Missense (R445Q)	Heterozygous (R/Q)	56

Table 8. Genotype of G6PC1 and G6PT1/SLC37A4 in normal horse

Gene	Variant	Genotype		
G6PC1	L89R	L/L	L/R	R/R
		0	1	9
G6PT1/SLC37A4	R445Q	R/R	R/Q	Q/Q
		4	6	0

Discussion

In the pedigree records of the Noma horse population, no specific common ancestor was identified for the neonatal dead foals within the past three generations. However, as mentioned in the Introduction, Noma horses have been bred as a genetically closed population originating from a small number of ancestors, making them highly inbred [16]. Therefore, there is a considerable possibility that the neonatal deaths in the Noma horses were caused by recessive genetic defects inherited from common ancestors.

Pathological and bacterial examinations revealed that three of the foals (Nos. 54, 62, and 66) were diagnosed with sepsis caused by *Actinobacillus equuli*, *Escherichia coli*, and *Enterobacter* spp., respectively, suggesting neonatal sepsis caused by opportunistic infections. All six foals had low serum globulin concentrations, with two foals (Nos. 54 and 55) showing low WBC counts. The intake of colostrum was confirmed by caregivers, but its absorption from the gastrointestinal tract and effectiveness were unknown. Weak resistance to infection may have been a problem in these three foals. Although foal No. 55 experienced respiratory failure due to aspiration and the low birthweight of foal No. 56 suggested that it might have been immature owing to his low birth weight, the causes of these abnormalities remain unknown. Foal No. 57 may have died from severe circulatory disturbance and hypoxemia due to heart malformation (tricuspid atresia with ventricular and atrial septal defects). The details of this foal have been previously reported [24]. Specific causes could not be detected in the autopsy findings of the six foals that died neonatally.

The laboratory test results of normal neonatal Noma horses were similar to those of Western horses [3]. Noma horses had slightly higher alkaline phosphatase and total cholesterol levels and slightly lower blood urea nitrogen, total protein, and glucose levels than Western horses. However, these differences were not very substantial; therefore, we used the laboratory data of normal neonatal Noma horses as reference values. In some of the foals that died neonatally, liver injury was not evident, as their GGT and albumin levels were normal. However, their liver function may have still been impaired, given their elevated AST, LDH, and bilirubin levels, as well as their results of metabolic analysis. However, the high levels of AST and LDH in foal No. 54, which was confirmed to have had septicemia with suppurative liver inflammation, may have been associated with liver dysfunction. Low glucose concentrations could have been due to insufficient colostrum intake or poor intestinal absorption, even if colostrum was ingested, suggesting adverse effects on multiple metabolic pathways.

A summary of the results of the AA, GC/MS, and sequencing analyses associated with the suggested metabolic diseases in foal Nos. 54, 55, and 56 is shown in Table 9. Figure 1 shows a flowchart of the pyrimidine biosynthesis, ammonia transport, and urea cycle pathways observed in foal Nos. 54, 55, and 62. Hyperammonemia can result from various congenital and acquired conditions and may also occur as part of other disorders that involve various metabolic abnormalities [5, 12]. Ammonia levels increase when the liver is unable to metabolize ammonia because of an enzymatic defect or hepatocellular damage. Congenital disorders involving enzymatic defects include urea cycle defects, organic acidemia, congenital lactic acidosis, fatty acid oxidation defects, and dibasic AA deficiency. Severe perinatal asphyxia can induce transient hyperammonemia in newborns [2]. The AA and GC/MS analyses detected high levels of ammonia, orotic acid, and uracil in the sera of neonatal foal No. 54, which died from septicemia, and foal

Table 9. Summary of the results of laboratory tests and sequencing analyses that were associated with the suggested metabolic diseases in foal Nos. 54, 55, and 56

No.	Pathology	Laboratory test results	Expected disorders	Results of candidate-genesequence
54	Septicaemia	GC/MS: high ammonia, orotic acid AA: high uracil level	Suppression of the urea cycle and acceleration of the pyrimidine synthesis pathway	*OTC*: no variant *ASL*: no variant *ASS1*: no mutation *UMPS*: no mutation
55	Aspiration dyspnea	GC/MS: high ammonia, orotic acid AA: high uracil level	Suppression of the urea cycle and acceleration of the pyrimidine synthesis pathway	*OTC*: no variant *ASL*: no variant *ASS1*: no mutation *UMPS*: no mutation
56	Fragility	High phenylalanine and tyrosine, uric acid and tyrosine levels	Hypertyrosinemia	*UMPS*: no mutation
		High uric acid and tyrosine levels, and hypoglycemia Liver disfunction by histopathology	Glycogen storage disease type I	*G6PC1*: no mutation *G6PT1/SLC37A4*: no mutation Histopatholgical findings: no glygcogen storage in liver and kidney

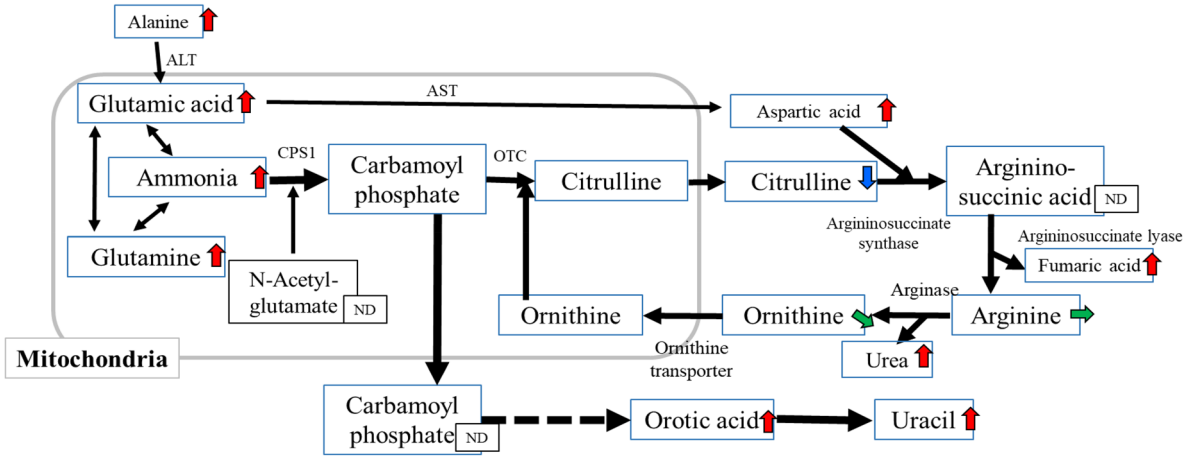

Fig. 1. Flowchart of the pyrimidine biosynthesis, ammonia transport, and urea cycle pathways observed in foal Nos. 54, 55, and 62. Upward, downward, and sideways arrows indicate increase, decrease, and within normal range, respectively. ALT, alanine transaminase; AST, aspartate transaminase; CPS1, carbamoyl-phosphate synthetase 1; OTC, ornithine transcarbamylase; ND, not determined.

No. 55, which died from apparent from aspiration dyspnea. Although these findings represent only part of a series of metabolic pathways, they can be inferred to be the result of urea cycle suppression [12] and acceleration of the de novo pyrimidine synthesis pathway [18, 29, 43]. These metabolic abnormalities suggest a condition in which the urea cycle is insufficiently functional, indicating a restricted disruption in the nitrogen metabolic pathway. Therefore, we sequenced the *OTC*, *ASL*, and *ASS1* genes, which encode enzymes involved in the urea cycle, as candidate genes for metabolic disorders [10, 35, 40], along with the *UMPS* gene, as elevated blood orotic acid levels have been observed in cattle with *UMPS* deficiency [34, 36]. However, no mutations affecting enzyme activity were detected in these genes. Orotic acidemia and hyperammonemia may be associated with severe trauma [20], pregnancy, liver disorders [6, 9, 17], or cancer [39]. Metabolic abnormalities observed near death may also be associated with elevated serum orotic acid levels. In addition, a previous report indicated that horses have lower hepatic OTC activity than cattle, sheep, and rabbits [33]. Therefore, hyperammonemia and orotic acidemia associated with urea cycle disturbance might be more likely to occur due to liver dysfunction.

Foal No. 56, which died due to fragility and showed high uric acid and tyrosine levels, hypoglycemia, and liver dysfunction in laboratory tests and histopathology, was suspected to have had glycogen storage disease type I, a glycolytic disorder [38]. Thus, we sequenced the *G6PC1*

and *G6PT1/SLC37A4* genes, which are known to be causative genes for human glycogen storage disease type I, but could not find any mutations in them. In this foal, the uric acid and tyrosine levels were not very high, and histological examination showed no glycogen storage in the liver or kidney, suggesting a lower possibility of glycogen storage disease. In foal No. 57, which had heart malformations, the phenylalanine and tyrosine levels were high, suggesting hypertyrosinemia. However, metabolites of tyrosine and disorder biomarkers were not elevated, making it uncertain whether the foal had hypertyrosinemia. In the six foals that died neonatally, the results of the serum AA and GC/MS analyses suggested metabolic disorders, except in the case of one foal (No. 66). However, we did not find any mutations in related genes that could be the cause of these disorders. These results indicate that, while the Noma horse population is highly inbred, the metabolic abnormalities observed in foals that die neonatally are unlikely to be caused by specific hereditary disorders. At present, the causes of neonatal death vary among individual foals and include bacterial or viral infections and other environmental factors. However, it is important to note that genetic factors that could not be identified in this study may contribute to the neonatal deaths in Noma horses. For example, there may still be a possibility that mutations in *OTC*, *ASL*, *ASS1*, *UMPS*, *G6PC1*, and *G6PT1/SLC37A4* are responsible for the neonatal deaths. While no mutations were found in the coding regions of these genes, mutations could exist in non-coding regions, such as the promoter and enhancer regions. Additionally, many hereditary disorders are caused by large insertions, deletions, or rearrangements near genes, which cannot be detected through Sanger sequencing of the candidate genes themselves. In addition to *OTC*, *ASL*, *ASS1*, *G6PC1*, and *G6PT1/SLC37A4*, mutations in arginase 1 (*ARG1*), carbamoyl phosphate synthetase I (*CPS1*), and ornithine transporter 2 (*ORNT1/SLC25A15*) have been reported to be involved in human urea cycle disorders [7, 13, 23], and mutations in the alpha subunit of muscle phosphorylase kinase and alpha-1,4-glucosidase genes are associated with glycogen storage diseases [8, 27]. Therefore, the involvement of these genes in the neonatal deaths of Noma horses cannot be excluded. While the cause of neonatal death in Noma horses could not be clarified in this study, determining whether genetic factors contribute to these deaths is essential for future breeding programs aimed at conserving the Noma horse population. More comprehensive genetic analyses, such as whole genome sequencing using next-generation sequencing, may be required for this purpose.

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