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順天堂醫事雜誌

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(938th issue)

JUNTENDO MEDICAL JOURNAL

順天堂醫事雑誌

The History of *Juntendo Medical Journal*

This *Juntendo Medical Journal* has been published under the Japanese name *Juntendo Igaku* (順天堂医学) from 1964 to 2012. However, the origin of *Juntendo Medical Journal* dates back to the oldest medical journal in Japan, *Juntendo Iji Zasshi* (順天堂醫事雑誌), which had been published between 1875 and 1877 (total of 8 issues). Between 1885 and 1886, Juntendo issued a limited release of a research journal titled *Houkoku* [*Juntendo Iji Kenkyukai*] (報告) for a total of 39 issues.

In 1887, *Juntendo Iji Kenkyukai Houkoku* (順天堂醫事研究會報告) was published with the government's approval and we used to regard this as the first issue of *Juntendo Medical Journal*. Since then, *Juntendo Medical Journal* has undergone a series of name changes: *Juntendo Iji Kenkyukai Zasshi* (順天堂醫事研究会雑誌), *Juntendo Igaku Zasshi* (順天堂医学雑誌), and *Juntendo Igaku* (順天堂医学).

Now in commemoration of the 175th anniversary of Juntendo University, starting with the first volume issued in 2013 (Volume 59 Number 1), we return to *Juntendo Medical Journal*'s original Japanese title in 1875-*Juntendo Iji Zasshi* (順天堂醫事雑誌). We also reconsidered the numbering of the journal and set the first issue in 1875 as the initial publication of *Juntendo Medical Journal*. The Volume-Number counting system and the English name *Juntendo Medical Journal* started in 1955 from the January 10 issue. Although this is not our intension, we will retain the Volume-Number counting system to avoid confusion. However, Volume 59 Number 1 will be the 882nd issue, reflecting the sum of all issues to date: 8 issues of *Juntendo Iji Zasshi* (順天堂醫事雑誌), 39 issues of *Houkoku* [*Juntendo Iji Kenkyukai*] (報告) (47 issues combined), and 834 issues from *Juntendo Iji Kenkyukai Houkoku* (順天堂醫事研究會報告) in 1887 to the present.

出典：小川秀興（OGAWA Hideoki, M.D., Ph.D.）：順天堂醫事雑誌（Juntendo Medical Journal）2013；59：6-10.

本誌は昭和39年（1964年）から平成24年（2012年）末まで『順天堂医学』として刊行されてきた．しかし，その起源は明治8年（1875年）から10年（1877年）にかけて発刊された日本最古の医学誌『順天堂醫事雑誌』（計8巻）にある．さらに明治18年（1885年）から19年（1886年）まで，会員限定配本として順天堂醫事研究會の雑誌『報告』（計39集）が発行されている．

その後『順天堂醫事研究會報告』が明治20年（1887年）に官許を受けて公刊されたので，順天堂ではこれを通刊1号としてきた．以来，『順天堂醫事研究会雑誌』，『順天堂医学雑誌』，『順天堂医学』と名称を変更して刊行されてきた．

今般，順天堂が創立175周年を迎える平成25年（2013年）の59巻1号を期して，本来の名称である『順天堂醫事雑誌』と復刻し，その起源である明治8年（1875年）第1巻をもって創刊号（通刊第1号）とすることとした．従来の巻号と欧文誌名は，昭和30年（1955年）1月10日発行のものを1巻1号としており，欧文誌名もこれより付け始めたもので不本意であるが，混乱を避けるためにこれらを継承する．ただし，通刊数は明治8年（1875年）から19年（1886年）にかけて刊行された『順天堂醫事雑誌』8巻分と順天堂醫事研究會の雑誌『報告』39集，計47巻分を通巻834号に加え，59巻1号を通刊882号とした．

出典：小川鼎三，酒井シヅ：順天堂医学　1980；26：414-418.
小川秀興：順天堂醫事雑誌　2013；59：6-10.

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Contents

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From the illustrator: Today I stopped by the shop that sells Vietnamese small goods; I visit there at least once a year. I purchased a unique wooden figure of a cat. The roughly carved object is colored in red, white, and gold. The form is simple; however, it is rather difficult to draw the figure.

Reviews

Juntendo Medical Journal
2022. 68(3), 200–207

Emergence of Carbapenem-resistant Clinical Isolates of *Providencia* Species

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Providencia is a genus of Gram-negative and non-spore forming bacteria belonging to the family *Morganellaceae*, which causes opportunistic infections in humans. Of the 10 *Providencia* species identified to date, three, *P. alcalifaciens*, *P. rettgeri* and *P. stuartii*, are clinically important. *P. alcalifaciens* causes diarrhea, including outbreaks arising from food-borne infections, and *P. stuartii* and *P. rettgeri* have been found to cause hospital acquired urinary tract infections. Four isolates of *P. rettgeri* and one isolate of *P. stuartii* were obtained from urine samples of five patients in Japan in 2018. All five isolates were highly resistant to carbapenems. Three isolates harbored $bla_{\text{IMP-70}}$, encoding a variant of IMP-1 metallo-β-lactamase, with two amino acid substitutions (Val67Phe and Phe87Val), one isolate harbored two copies of $bla_{\text{IMP-1}}$ and one isolate harbored $bla_{\text{IMP-11}}$. Expression of $bla_{\text{IMP-70}}$ conferred carbapenem resistance in *Escherichia coli*. Recombinant IMP-10, an IMP-1 variant with Val67Phe but without Phe87Val, had significant higher hydrolytic activities against meropenem than recombinant IMP-1, indicating that the Val67Phe amino acid substitution alters activities against meropenem in IMP-70. These results suggest that *Providencia* species. become more highly resistant to carbapenems by acquisition of two copies of $bla_{\text{IMP-1}}$ or by mutations in bla_{IMP} that result in amino acid substitutions, such as $bla_{\text{IMP-70}}$.

Key words: *Providencia rettgeri*, *Providencia stuartti*, metallo-β-lactamase

Taxonomy of the *Providencia* genus

Providencia, a genus of Gram-negative and non-spore forming bacteria, was originally assigned to the family *Enterobacteriaceae*, but has recently been assigned to the family *Morganellaceae*[1]. Species of *Providencia* genus have been isolated from many vertebrate and invertebrate animals, including humans and insects[2–4], and causes opportunistic infections in humans[5]. To date, 10 species belonging to the genus *Providencia* have been identified: *P. alcalifaciens*, *P. burhodogranariea*, *P. heimbachae*, *P. huaxiensis*, *P. rettgeri*, *P. rustigianii*, *P. sneebia*, *P. stuartii*, *P. thailandensis* and *P. vermicola*. Of these 10 species of *Providencia*, five, *P. alcalifaciens*, *P. friedericiana* (synonym of *P. rustigianii*), *P. rettgeri*, *P. stuartii* and *P. vermicola*, were isolated from humans, with three of these, *P. alcalifaciens*, *P. rettgeri* and *P. stuartii*, likely to be clinically important[5,6]. A phylogenetic tree based on whole genome sequences of the 10 *Providencia* species revealed that these species consist of three clusters, with the five species isolated from humans, being spread among these three clusters (Figure 1). These findings indicate that the genus *Providencia* does not have a subgenus associated with human infections. The five species were found to have specific genes associated with human infections, such as genes encoding adherence and invasion factors.

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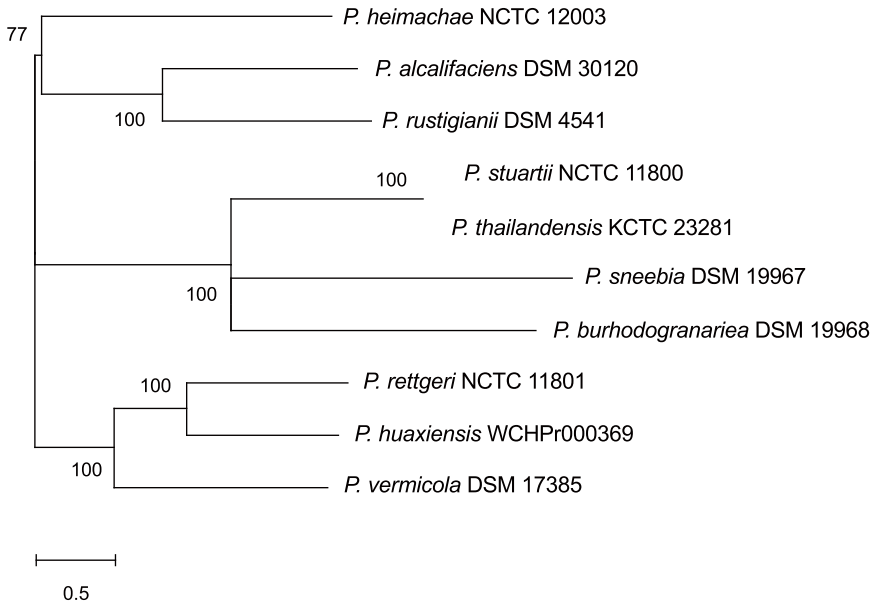

Figure 1 Maximum-likelihood (ML) tree based on single nucleotide polymorphisms (SNPs) in the core genome among contigs of strains, showing the relationships among type strains of the genus *Providencia*. Bootstrap values, expressed as percentages of 1,000 replications, are shown at the branching points when >50 %.

Providencia species as human pathogens

A study of the enteropathogenicity of *P. alcalifaciens* isolated from a child and two adults with diarrhea demonstrated that this species causes diarrhea in humans by invading the intestinal mucosal epithelium[7]. *P. alcalifaciens* was subsequently isolated from 2.1% of the stool specimens of diarrheal children younger than 5 years of age, indicating that this organism is significantly associated with diarrhea[8]. A large outbreak of food-borne infection caused by *P. alcalifaciens* occurred among children and teachers at two kindergartens and one high school in November 1996 in Fukui, Japan[9]. Specifically, of the 610 children and teachers who ate lunch cooked at a single catering facility, 270 showed symptoms of gastroenteritis[9]. Recent outbreaks of *P. alcalifaciens* have indicated that infection with this organism is a public health concern in both developing and developed countries[10]. Although epidemiological studies suggest that *P. alcalifaciens* causes diarrhea by invading the intestinal mucosa[10], the pathogenesis of *P. alcalifaciens* has not been established at the molecular level.

P. stuartii and *P. rettgeri* have been found to cause hospital acquired urinary tract infections[11] and have been shown to be the most common causes of urinary tract infections in hospitalized patients. In addition, *P. stuartii* and *P. rettgeri* have been found to cause pneumonia, meningitis, endocarditis, wound infections and bloodstream infections[11], and *P. stuartii* was found to cause invasive endocarditis[12] and neonatal sepsis[13]. *P. alcalifaciens*, *P. rettgeri* and *P. stuartii* were isolated from 17.6% of stool samples of patients with diarrhea at the Kansai airport quarantine station in 2002, with vomiting being especially frequent in patients infected with *P. rettgeri*, indicating that these three *Providencia* species cause travelers' diarrhea[5].

Emergence of carbapenem-resistant *Providencia* species

The emergence and spread of carbapenem-resistant Gram-negative pathogens have become serious public health problems worldwide[14]. Most of these carbapenem-resistant isolates produce metallo-β-lactamases (MBLs), including IMP-, NDM- and VIM-type MBLs[14], which confer high resistance against all β-lactams (penicillins, cephalosporines and carbapenems) except for monobactams[15]. Clinical isolates of carbapenem-resistant *P. rettgeri* producing IMP-1 MBL were first identified by laboratory-based surveillance in the Kinki region of Japan in 2000[16]. Clinical isolates of *P. stuartii* producing VIM-19 MBL were first identified in 2008 in Algeria[17]. To date, there have been 16

reports of carbapenem-resistant *P. rettgeri*, eight of carbapenem-resistant *P. stuartii* and one of carbapenem-resistant *P. vermicola* (Table 1). Most of these were clinical isolates, but one was obtained from a hospital environment and one from pet turtles (Table 1).

All of these isolates produced MBLs, with the majority of carbapenem-resistant *P. rettgeri* isolates producing IMP-type or NDM-type MBLs (Table 1). IMP-type MBLs were detected in isolates from Japan, Korea, and the United States, whereas NDM-type MBLs were detected in isolates worldwide. We obtained four clinical isolates of carbapenem-resistant *P. rettgeri*, which produced IMP-1, IMP-11 or IMP-70. One IMP-1 producing isolate was from Saitama, Japan, one IMP-11 producing isolate was from Kochi, Japan, and two IMP-70 producing isolates were from Osaka, Japan[18].

Most of the carbapenem-resistant *P. stuartii* isolates, obtained in Algeria, Greece and Korea, produced VIM-type MBLs. Carbapenem-resistant *P. stuartii* isolates producing NDM-type MBL-producing *P. stuartii* were obtained in Afghanistan and Peru, and we described an IMP-type MBL-producing *P. stuartii* from Japan[18]. Carbapenem-resistant *P. vermicola* isolates producing NDM-1 were isolated in the Congo.

Carbapenem-resistant clinical isolates of *Providencia* species in Japan

We obtained four clinical isolates of *P. rettgeri* and one clinical isolate of *P. stuartii* from the urine

Table 1 Reports of *P. rettgeri*, *P. stuartii* and *P. vermicola* producing MBL[a]

Species	Metallo-β-lactamase	Location of MBL-encoding gene	Inc type	Isolation source	Isolated year	Isolated country	Reference
P. rettgeri	IMP-1	–	–	–	2000	Japan	16)
	IMP-1	plasmid	–	sputum, blood	2002	Japan	25)
	IMP-1	chromosome		urine	2018	Japan	18)
	IMP-11	plasmid (84,930-bp)	IncT	urine	2018	Japan	18)
	IMP-27	plasmid (10,7365-bp)	IncQ	wound	2016	USA	26)
	IMP-27	–	–	pet turtles	2018	Korea	27)
	IMP-70	plasmid (204,791-bp)	IncA/C2	urine	2018	Japan	18)
	NDM-1	–	–	blood, rectum, pus	2008 2011	Israel	28)
	NDM-1	plasmid	–	sputum, pus	2012	Nepal	29)
	NDM-1	plasmid (178kb)	–	urine	2012	Mexico	30)
	NDM-1	plasmid (190kb)	IncA/C	urine	2014	China	31)
	NDM-1	–	–	–	2017	Bulgaria	32)
	NDM	–	–	wound	2013	Brazil	33)
	NDM-1, VIM-2	plasmid	–	urine	2015	Colombia	34)
	NDM-18	plasmid	–	effluent from a pediatric ward	2017	South Africa	35)
P. stuartii	IMP-70	plasmid (152,754-bp)	IncA/C	urine	2018	Japan	18)
	NDM-1	plasmid (178277kb)	IncA/C	blood	2012	Afghanistan	36)
	NDM-1	plasmid (18,480-bp)	IncA/C2	urine	2020	Peru	37)
	VIM-1	–	–	–	2011	Greece	38)
	VIM-1	plasmid (180kb)	IncA/C	rectum	2012	Greece	39)
	VIM-1	plasmid	–	–	2013	Greece	40)
	VIM-2	–	–	urine	2004	Korea	41)
	VIM-19	plasmid (180kb)	–	–	2008	Algeria	17)
P. vermicola	NDM-1	plasmid (151,684-bp)	–	blood	2017	Congo	42)

[a] Iwata S, Tada T, Hishinuma T, et al: Antimicrob Agents Chemother, 2020; 64.[18]
A dash (–) indicates there was no information about the location of MBL-encoding genes, Inc type and isolation source.

samples of five patients in Japan in 2018[18]. All five were multidrug-resistant, being resistant to aminoglycosides, carbapenems and fluoroquinolones[18]. These isolates harbored genes encoding aminoglycoside modifying enzymes, including *aac(6')-Ib4* and *aac(6')-Iae*, and MBL genes encoding carbapenemases; including IMP-1, IMP-11 and IMP-70 (Table 2). They also had three mutations with amino acid substitutions in *GyrA* and *ParC*, which were associated with quinolone resistance (Table 2). One isolate harbored aminoglycoside- and carbapenem-resistant genes on the chromosome, whereas the other four harbored these genes on plasmids.

Carbepenemase activities of IMP-1 MBL variants

All five *P. rettgeri* and *P. stuartii* clinical isolates were resistant to imipenem and meropenem, and three, two *P. rettgeri* isolates and one *P. stuartii* isolate, were highly resistant to both carbapenems, with minimum inhibitory concentrations (MICs) of 512 μg/ml (Table 3). These three highly carbapenem-resistant isolates harbored bla_{IMP-70}, whereas, of the other two, one harbored bla_{IMP-1} and the other harbored bla_{IMP-11}. IMP-70 is a variant of IMP-1 with two amino acid substitutions, Val67Phe and Phe87Val; IMP-10 is a variant of IMP-1 with one amino acid substitution, Val67Phe; and IMP-1(F87V) is a variant of IMP-1 with one amino acid substitution, Phe87Val. *E. coli* expressing bla_{IMP-1}, bla_{IMP-10}, $bla_{IMP-1(Phe87Val)}$, and bla_{IMP-70} showed significantly higher MICs for all carbapenems tested than a vector control (Table 4). The MICs for all carbapenems of the vector control ranged from ≤0.06 to 0.125. *E. coli* expressing bla_{IMP-70} showed higher MICs for doripenem and meropenem, but the same MICs for imipenem and panipenem, than *E. coli* expressing bla_{IMP-1}. *E. coli* expressing bla_{IMP-10} showed a significantly higher MIC for doripenem and an increased MIC for meropenem. Assessment of the carbepenemase activities of recombinant IMP-1, IMP-10, IMP-1(Phe87Val) and IMP-70 showed that IMP-10 had greater hydrolytic activities than IMP-1 against meropenem, with the k_{cat}/K_m values of IMP-70 and IMP-10 being 2.3- and 3.4-fold higher, respectively, than those of IMP-1 (Table 5). In contrast IMP-70 and IMP-1 showed similar carbapenemase activities against doripenem, imipenem and panipenem, and IMP-1(Phe87Val) showed similar or reduced carbapenemase activities against all carbapenems tested.

Table 2 Genetic characterization of carbapenem-resistant *Providencia* species isolates[a]

isolates	genome	size (bp)	antibiotic resistance genes		quinolone resistance genes	
			aminoglycosides	carbapenemase	*GyrA*	*ParC*
P. rettgeri BML2496	chromosome	4.65M	*aac(6')-Ib4*	bla_{IMP-1}	Ser83Ile Asp87Ala	Ser87Ile
P. rettgeri BML2526	chromosome	4.34M			Ser83Ile Asp87Glu	Ser87Ile
	plasmid	205K	*aac(6')-Iae*	bla_{IMP-70}		
P. rettgeri BML2531	chromosome	4.70M			Ser83Ile Asp87Glu	Ser87Ile
	plasmid	85K	*aac(6')-Il*	bla_{IMP-11}		
P. rettgeri BML2576	chromosome	4.35M			Ser83Ile Asp87Glu	Ser87Ile
	plasmid	205K	*aac(6')-Iae*	bla_{IMP-70}		
P. stuartii BML2537	chromosome	4.42M	*aac(2')-Ia*		Ser83Ile Asp87Glu	Ser87Arg
	plasmid	153K	*aac(6')-Iae*	bla_{IMP-70}		

[a] Iwata S, Tada T, Hishinuma T, et al: Antimicrob Agents Chemother, 2020; 64.[18]

Table 3 Drug susceptibility profiles of *Providencia* species clinical isolates

Antibiotic	MIC (μg/ml)				
	P. rettgeri				*P. stuartii*
	BML2496	BML2531	BML2526	BML2576	BML2537
Imipenem	16	32	512	512	>512
Meropenem	64	32	512	512	512

Table 4 Drug susceptibility profiles of *E. coli* expressing IMP-1, IMP-10, a variant of IMP-1 with an amino acid substitution (F87V) and IMP-70[a]

	MIC (μg/ml)				
antibiotic (s)	*E.coli* DH5α (pHSG398)	*E.coli* DH5α (pHSG398/IMP-1)	*E.coli* DH5α (pHSG398/IMP-10)[b]	*E.coli* DH5α (pHSG398/IMP-1 (F87V))	*E.coli* DH5α (pHSG398/IMP-70)
Doripenem	≤0.06	2	8	1	4
Imipenem	0.125	1	1	1	1
Meropenem	≤0.06	4	8	2	8
Panipenem	0.125	2	2	1	2

[a] Iwata S, Tada T, Hishinuma T, et al: Antimicrob Agents Chemother, 2020; 64.[18]
[b] IMP-10 and IMP-1 (V67F) amino acid arrays are the same

Table 5 Kinetic parameters of β-lactamases IMP-1, IMP-10, a variant of IMP-1 with an amino acid substitution (F87V) and IMP-70 with substrates[a]

	kcat/Km (μM-1·s-1)[b]			
Substrate	IMP-1	IMP-10	IMP-1 (F87V)	IMP-70
Doripenem	0.13	0.82	0.091	0.18
Imipenem	0.23	0.24	0.17	0.24
Meropenem	0.15	0.51	0.17	0.35
Panipenem	0.40	0.35	0.24	0.23

[a] Iwata S, Tada T, Hishinuma T, et al: Antimicrob Agents Chemother, 2020; 64.[18]
[b] *Km* and *k*cat were calculated as means ± SD from three independent experiments.

These results suggest that, in IMP-70, the Val67Phe amino acid substitution, but not the Phe87Val substitution, is important for the significantly increased carbapenemase activity against meropenem. The Val67 in IMP-1 is located at the end of "loop1", close to the active site consisting of amino acids residues 60 to 66 (Figure 2)[19]. Loop1 is a major determinant for the tight binding of substrates in the active site[19]. A Val67Phe amino acid substitution in IMP-43, a variant of IMP-7, has been reported to increase catalytic activities against imipenem and meropenem[20]. Amino acid substitutions at residue 67 in IMP-1 MBLs affect their hydrolytic activity against β-lactams[21]. Residue 67 was reported to be important for substrate binding in VIM-type MBLs[22]. Residue 87 plays a crucial role in the stability of VIM-2[23]. IMP-44, a variant of IMP-11 with two substitutions (Val67Phe and

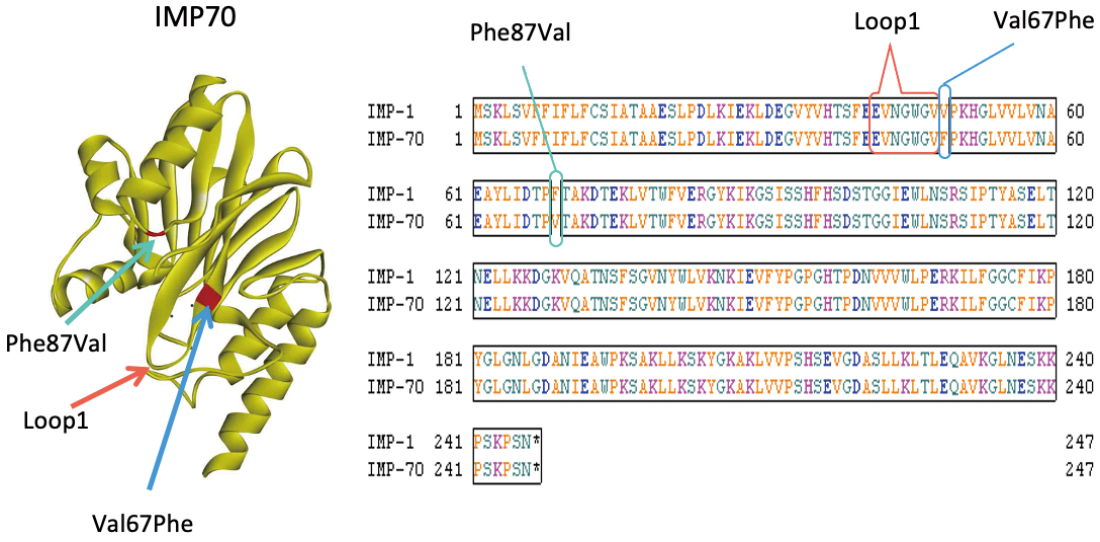

Figure 2 3D structure of IMP-70 MBL and amino acid sequences of IMP-1 and IMP-70 MBLs

Phe87Ser), had more efficient catalytic activities against carbapenems than those of IMP-11[24]. These results suggest that co-occurrence of two amino acid substitutions at these two positions increase the enzymatic activities of IMP-44, whereas the Phe87Val substitution did not affect the enzymatic activities of IMP-70. The substitution of Phe87 by a polar amino acid such as Ser, but not by a hydrophobic amino acid such as Val, may affect enzymatic activities.

Biological significance of two copies of *bla*$_{IMP-1}$ in tandem

One of the *P. rettgeri* isolates was found to harbor two copies of *bla*$_{IMP-1}$, in tandem on the chromosome, consisting of a repeat of the genetic structure *int1*Δ-*bla*$_{IMP-70}$-*qacE*Δ*1-sul1* (Figure 3). To confirm the presence of the two copies of *bla*$_{IMP-1}$, sequences were amplified by PCR using a primer set targeting the two copies. Amplification resulted in a 3.5-kbp PCR product as expected based on the whole-genome sequence, indicating that this isolate of *P. rettgeri* harbored two tandem copies of *bla*$_{IMP-1}$ on the chromosome. Western blotting analysis revealed that all five isolates tested produced IMP-type MBLs (Figure 4). Of these five isolates, the *P. rettgeri* isolates harboring two copies of *bla*$_{IMP-1}$ produced the largest quantities of IMP-type MBL (Figure 4), indicating these two copies of *bla*$_{IMP-1}$ produce high amounts of IMP-1 MBL.

Conclusions

The genus *Providencia*, belonging to the family *Morganellaceae*, consists of 10 species. Of these, three species, *P. alcalifaciens*, *P. rettgeri* and *P. stuartii*, are clinically important. *P. alcalifaciens* causes diarrhea by invading the intestinal mucosa, whereas *P. stuartii* and *P. rettgeri* have been found to cause hospital acquired urinary tract infections, as well as pneumonia, meningitis, endocarditis, wound infections, bloodstream infections, and travelers' diarrhea. Clinical isolates of carbapenem-resistant *P. rettgeri* producing IMP-1 MBL were first identified during laboratory-based surveillance in 2000 in Japan. To date, there have been 16 reports of carbapenem-resistant *P. rettgeri*, eight of carbapenem-resistant *P. stuartii* and one of carbapenem-resistant *P. vermicola*, with most of these being clinical isolates.

We recently obtained four *P. rettgeri* isolates and one *P. stuartii* isolate from urine samples of five patients. All five isolates were highly resistant to carbapenems. Three isolates harbored *bla*$_{IMP-70}$, encoding a variant of IMP-1 MBL with two amino acid substitutions, and one each harbored *bla*$_{IMP-1}$ and *bla*$_{IMP-11}$. Molecular analyses of these isolates strongly suggest that *Providencia* species become more highly resistant to carbapenems by acquisition of two copies of *bla*$_{IMP-1}$ or by mutations in *bla*$_{IMP}$ genes that result in amino acid substitutions, such as *bla*$_{IMP-70}$.

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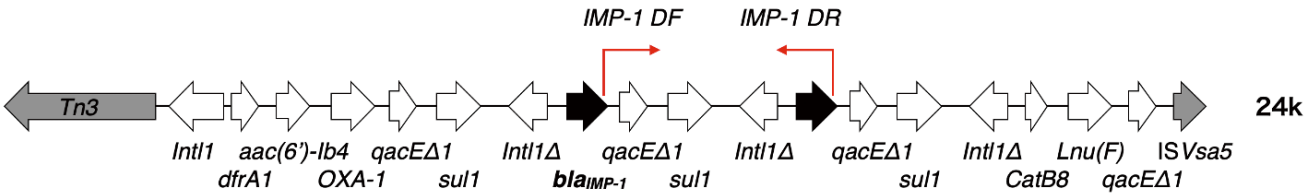

Figure 3 Genomic environments of *bla*$_{IMP-1}$ and *bla*$_{IMP-70}$ in clinical isolates of *P. rettgeri* and *P. stuartii*. Genes are represented as arrows, which indicate their transcription orientations and relative lengths. MBL genes, *tnp* genes, and truncated genes are shown as black arrows, gray arrows, and Δ, respectively. Label *orf1* represents a gene encoding a hypothetical protein, and *orf2* represents a gene encoding an ATP-binding protein. This figure is a modified version of FIG 1 in reference 18.
(Iwata S, Tada T, Hishinuma T, et al: Emergence of Carbapenem-Resistant *Providencia rettgeri* and. Antimicrob Agents Chemother, 2020; 64.)

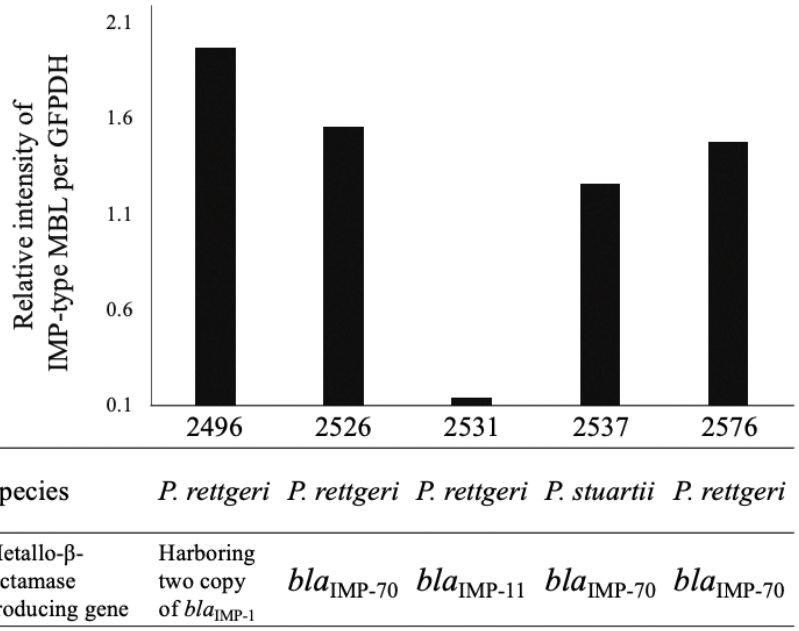

Figure 4 IMP-type MBL production in carbapenem-resistant clinical isolates of *P. rettgeri* and *P. stuartii*. Four clinical isolates of *P. rettgeri* (2496, 2526, 2531 and 2576) and one of *P. stuartii* (2537) were solubilized and subjected to western blot analysis using monoclonal antibodies against IMP-type MBL and GAPDH. The relative intensity of IMP-type MBL bands to GAPDH bands was calculated. This figure is a modified version of FIG 2 in reference 18. (Iwata S, Tada T, Hishinuma T, et al: Emergence of Carbapenem-Resistant *Providencia rettgeri* and. Antimicrob Agents Chemother, 2020; 64.)

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Author's contributions

SI collected and reviewed the presented data from previously published articles in medical journals, and drafted the manuscript. TT reviewed the data on drug-resistant genes. SO analyzed the data on biochemical experiments. TH supervised the data on drug-susceptibility profiles. MT constructed the phylogenetic tree and drafted the section of bacterial taxonomy. TT supervised this study.

Conflicts of interest statement

We have the following interests. Drs. Miho Ogawa and Masahiro Shimojima is employed by BMI Inc. There are no patents, products in development or marketed products to declare.

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Perspective

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Network Centrality Analysis Characterizes Brain Activity during Response Inhibition in Right Ventral Inferior Frontal Cortex

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Key words: inferior frontal gyrus, inferior frontal junction, boundary mapping

The right inferior frontal cortex (IFC) plays a critical role in response inhibition[1, 2]. It has also been demonstrated that the IFC is heterogeneous and that the ventral part of the IFC (vIFC) is more critical to inhibition of prepotent response tendency. Recent areal parcellation analyses based on resting-state functional connectivity have revealed that the right vIFC consists of multiple functional areas. Resting-state functional connectivity analyses have enabled parcellation of the cerebral cortex into functional areas based on their connectivity patterns[3–5]. Parcellation analyses have revealed multiple areas (parcels) in the vIFC[3–5], suggesting functional heterogeneity within the vIFC. In the present study, we characterized the parcellated areas (parcels) in the right vIFC using graph-theoretic analysis, which characterizes local connectivity properties of a brain network by referring to its global structure of functional connectivity. This abstract is based on a study first reported in Neuroscience[6].

Twenty right-handed subjects (10 men and 10 women, aged 26.6 ± 9.2 years (mean ± SD)) participated in the experiments. The experimental procedures were approved by the Institutional Review Board. Written informed consent was obtained from all subjects. Functional magnetic resonance imaging (fMRI) scans were acquired during resting state and during the performance of a stop-signal task[1, 7]. We used multi-band gradient-echo echo-planar sequences for functional images (TR = 1.0 sec, TE = 30 msec, flip angle = 62 deg, FOV = 192 × 192 mm^2, matrix size = 96 × 96, 78 contiguous slices, voxel size = 2.0 × 2.0 × 2.0 mm^3, multi-band factor = 6).

For the resting-state dataset, preprocessing was conducted mainly following the pipelines of Human Connectome Project. The parcellation analyses based on boundary mapping were applied to the cerebral cortical surface. For subsequent analyses, to avoid non-uniform signal to noise ratio caused by the different number of vertices in the parcels, we defined regions of interest (ROIs) of 40 vertices closest to the centroid of each parcel. When the parcel contained less than 40 vertices, the ROI

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included all the vertices in the parcel. Temporal correlations of preprocessed time-series between the ROIs were calculated as the strength of functional connectivity. A binary undirected network for each subject was defined using a proportional threshold (top 9%), and the betweenness centrality of each parcel was calculated[8-11]. For the task dataset, we contrasted Stop success and Go success trials to reveal the brain activation for response inhibition.

In total, 377 parcels were identified in the cortical surfaces. A ROI was defined for each parcel (Figure 1A). Figure 1B shows the centrality index across the ROIs for one representative subject. The subjects performed the stop-signal task in the scanner that comprised of Go trials and Stop trials (Figure 1C). Brain activation for response inhibition was observed in several regions including the right IFC (Figure 1D). Two parcels (ROI 1 and ROI 2) in the right vIFC were significantly activated (ROI 1, $t(19) = 5.42$, $p < 0.001$; ROI 2, $t(19) = 5.91$, $p < 0.001$). There was no significant difference in the task-related activity in the ROIs [$t(19) = -0.71$, $P = 0.49$]. ROI 1 was located more ventrally, whereas ROI 2 was located more dorsally in the vIFC (Figure 2A). The correlations between the centrality and the brain activity were calculated in the two ROIs. ROI 1 showed significant correlation ($r = 0.62$, $p = 0.003$) (Figure 2B), whereas ROI 2 did not ($r = -0.01$, $p = 0.97$) (Figure 2C). The difference in the correlation was also significant ($z = 2.15$, $p = 0.032$). For ROI 1, which was significantly correlated, the correlation between activation and SSRT was significant ($r = -0.50$, $p = 0.026$) (Figure 2D). However, the correlation was not significant between centrality and SSRT ($r = -0.01$, $p = 0.95$).

In the ventral parcel in the vIFC, the correlation between centrality and brain activity during

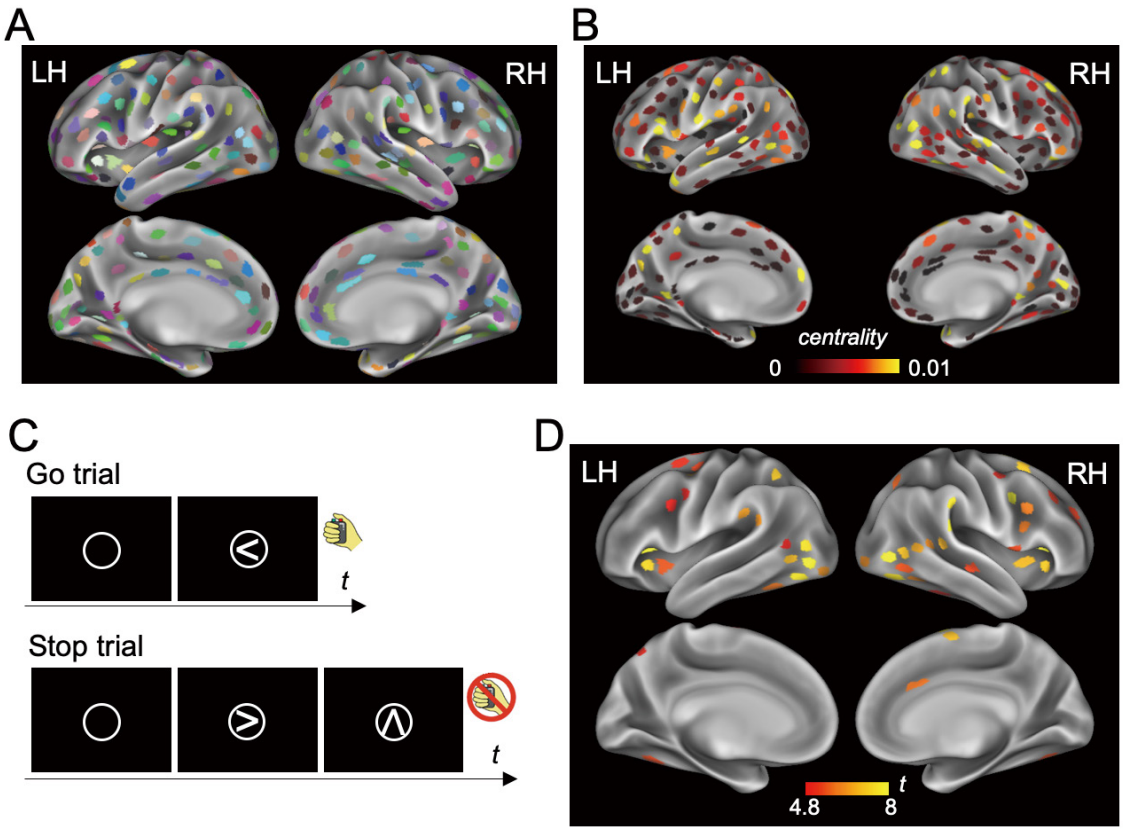

Figure 1 Centrality map and activation in stop signal task. **A**. ROIs defined using areal parcellation. In total, 377 ROIs were defined (192 in left hemisphere and 185 in right hemisphere). The colors are randomly selected. **B**. Centrality map on ROIs of one representative subject. **C**. Stop signal task. A circle was presented at the center of the screen as a warning. In Go trials, a left- or right-pointing arrow was presented inside the circle. The participants were instructed to press a button indicating the corresponding side. In Stop trials, an arrow was first presented inside the circle, similar to Go trials. Then, the arrow was changed to an up-pointing arrow. The participants were required to withhold the response. **D**. Activated ROIs in the vIFC (Stop success minus Go success). LH, Left hemisphere; RH, Right hemisphere. Modified from Fujimoto et al.[6].

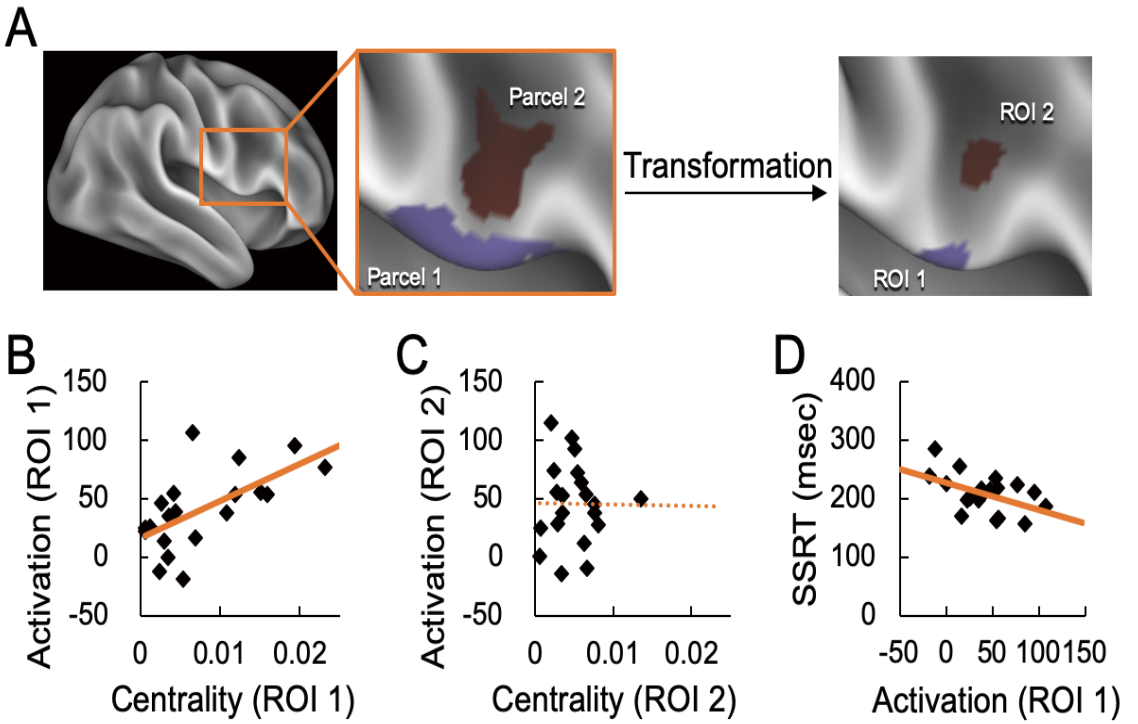

Figure 2 Relations among behavior, task activation, and network centrality. **A**. Target parcel ROIs in the IFC. **B**. Correlation between the centrality indices and the brain activity (Stop success minus Go success) in ROI 1. They are significantly correlated ($r = 0.62$, $p = 0.003$). **C**. Correlation between centrality indices and brain activity in ROI 2. They are not significantly correlated ($r = -0.01$, $p = 0.97$). **D**. Correlation between brain activity and SSRT in ROI 1. They are significantly correlated ($r = -0.50$, $p = 0.026$). Modified from Fujimoto et al.[6].

response inhibition was significant. Whereas the correlation between brain activity and SSRT was also significant, the correlation between the centrality and SSRT was not significant in the parcel. These results suggest that the ventral part of right vIFC is involved in stopping behavior and plays a critical role in the brain network for response inhibition.

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Author Contributions

UF, AO, and SK designed research. AO, TO, MT, AS, NH, KK, and SA performed research. UF, AO, SK, and TO summarized data and wrote the paper.

Conflicts of interest statement

The authors declare that there are no conflicts of interest.

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Health Topics for Tokyoites

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The Role of Footcare Nurses in Foot Lesions Treatment

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Footcare awareness and practice are limited in Japan, which is attributable to unavailability of specialized podiatry services, in contrast to the Western healthcare system. Japan does not have national educational courses in podiatry and footcare, and daily foot care is not routinely practiced owing to the cultural background. Moreover, medical insurance covers only diabetic footcare, which contributes to the limited popularity of footcare in Japan. Footcare in Japan is provided by qualified nurses (foot care nurses) who are certified by various organizations and societies. Footcare nurses render the following services: (a) Provision of professional footcare after evaluation and patient education for foot self-care. (b) Multidisciplinary coordination between the footcare team. Owing to lack of podiatry services in Japan, a multidisciplinary therapeutic approach to foot lesions is necessary. The footcare nurse coordinates communication of patient information across team members and interdepartmental referrals for effective multidisciplinary therapy. (c) Patient education to improve awareness of footcare. Footcare is not currently widely established as a component of medical and nursing care and patient welfare, and greater awareness regarding its role is necessary. The importance of footcare to maintain healthy walking needs to be emphasized among individuals with foot lesions. In view of the high life expectancy and rapid population aging in Japan, maintaining a healthy gait is essential to improve healthy life expectancy, and foot care nurses can play an active role in the future.

Key words: footcare, footcare nurse, foot lesions, evaluation, footcare team

1. Introduction

The term "footcare" was introduced in Japan only recently. In 2003, the Ministry of Health, Labor and Welfare added a "project for toe and nail care" to its long-term care prevention and community support program, and efforts were initiated to promote footcare in the domain of long-term care. The project aimed to reduce the risk of falls secondary to nail disorders through promotion of footcare among elderly individuals, their families, and care workers[1]. In 2008, a new fee was included in the medical reimbursement system for the management of diabetic foot complications, which led to the implementation of footcare services in many hospitals. This fee included a medical fee for footcare rendered to patients with diabetes at high risk of foot lesions, and many medical centers/hospitals that were unable to provide footcare owing to lack of funds (medical fees for the service) established outpatient footcare clinics. However, the fees for management of diabetic complications can be calculated only for patients who meet the following criteria: (1) a history of foot ulcers and toe or lower limb amputation, (2) diagnosis of arteriosclerosis obliterans, or (3) diagnosis of diabetic neuropathy. Moreover, foot care services such as edema care, skin care, and nail clipping are not reimbursed and are often unprofitable with regard to revenue generation. In this context, many patients with non diabetic foot conditions including rheumatic deformities, hallux valgus, flat foot, foot

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paralysis associated with cerebrovascular disease, and ingrown toenails do not receive appropriate footcare at hospitals. Thus, since the start of the management of diabetic foot complications fee in 2008, foot care has become widely known in the field of diabetes treatment, but it is still not recognized in other fields.

Several individuals in Japan are unaware that their feet need care. Owing to the Japanese cultural milieu, footcare is not considered an important routine activity, in contrast to practices followed in Western countries. The shoe culture was established in Japan only over the past 100 years (compared with approximately 1,000 years ago in Europe and the United States); zori (sandals) and geta (wooden clogs) was the traditional footwear among the previous generations of the Japanese. Therefore, podiatry is not widely established as a medical speciality[2]. Furthermore, culturally, footwear is considered a fashion accessory; shoes are often changed to match clothing, such as zori to match kimonos and shoes to match clothes. Usually, loose-fitting shoes such as zori and geta, which do not require the use of hands and are comfortable for the feet are preferred[3]. Therefore, appropriate selection and correct use of shoes may not receive much attention, and shoe-induced chafing is treated as a common issue and calluses, chicken eye deformity, and ingrown toenails are ignored and remain untreated.

The lack of podiatrists and footcare specialists is an important contributor to the low levels of awareness regarding footcare in Japan. Podiatry is a separate and specialized field integral to medical practice in Europe and the United States. Podiatrists treat foot lesions with drug or surgical therapy similar to the role of dentists in Japan. Medizinischer Fusspfleger, which refers to a specialist in footcare and Shoe Meister, which refers to a specialist in shoes are national qualifications in this field in Germany. The Germans routinely visit a podiatrist for foot concerns, or visit a footcare salon on a daily basis and buy shoes under the guidance of a specialist. In contrast, in Japan, footcare is provided only by outpatient footcare clinics at some hospitals for patients with diabetes and at private footcare salons for cosmetic purposes.

The aging rate was 28.1% in Japan in 2018, and the mean life expectancy was 87 years for women and 81 years for men, which indicates significantly high longevity[4]. Interestingly, that year, 6.44 million individuals were certified as requiring nursing care or support under long-term care insurance[5], and despite the long life expectancy, several Japanese are dependent on continuous medical and nursing care, and the gap between life expectancy and healthy life expectancy is concerning. It is important to maintain activities of daily living (ADL) and walking ability to extend healthy life expectancy[6]. Maintenance of walking ability requires protection of foot health, which forms the primary focus of footcare. In essence, footcare plays a key role in preventive medicine and is indispensable to the rapidly aging Japanese society characterized by high longevity.

Juntendo University Hospital established the "Podiatry Center" as the first podiatry center in a university hospital in 2019, for comprehensive footcare services for all foot lesions regardless of the disease. The author is in charge of the outpatient footcare at this center. In this section, I will describe the role of footcare nurses in foot care, which is currently not well established in Japanese society.

2. Footcare nurses in Japan

As mentioned earlier, national qualifications for footcare specialists are not established in Japan; however, nurses with domain-specific qualifications recognized by various organizations and academic societies function as footcare nurses. Certifications for diabetic foot lesion specialization include the Certified Diabetic Nurse Practitioner certification provided by the Japan Nurses Association and the Japan Diabetes Care Instructor certification provided by the Japan Diabetes Care Instructor Certification Organization. Qualifications for specialization in foot ulcers and skin care include the Certified Nurse in Wound, Ostomy and Continence Nursing certification provided by the Japan Nurses Association. The Elastic Stocking and Compression Therapy Conductor certification provided by the Japanese Society of Phlebology, the Lymphedema Therapist certification provided by the Japanese Association for Lymphedema Therapy, and the Vascular Technician certification provided by the Vascular Technician Certification Organization are credentials for foot lesions of the

vascular system. Qualifications that provide a wide range of knowledge regarding overall footcare include footcare instructors certified by the Japanese Society of Footcare and Podiatry and certification provided by the same society. Each of these qualifications trains professionals in a specific area of expertise; therefore, footcare nurses acquire multiple qualifications to provide comprehensive footcare and to compensate for the lack of knowledge and skills.

Qualified footcare nurses are engaged in foot care outpatient clinics at medical centers, dialysis clinics, and nursing homes[7,8]. However, not all qualified footcare nurses are engaged in footcare; there is a shortage of trained footcare nurses and medical centers and care facilities that provide foot care in Japan[9].

3. Role of footcare nurses

Footcare nurses provide the following services: (1) professional footcare, (2) management of the footcare team and, (3) patient education to improve awareness regarding the importance of footcare.

Implementation of footcare based on technical expertise and skills is important and includes not only direct patient care but also self-care and rehabilitation guidance and adjustment of the treatment environment.

In the absence of specialized podiatry services in Japan, a multidisciplinary team-based approach that includes a footcare team is necessary for management of foot disorders. Compared with other professionals, footcare nurses have more direct patient interaction and can better understand patients' backgrounds and possible difficulties associated with treatment. Therefore, the footcare nurse is best suited for coordination with the multidisciplinary team to facilitate patient referrals for specialized treatment[10].

Despite the increasing need for footcare, medical, nursing, and welfare personnel in Japan are not particularly familiar with footcare services. Education of personnel involved in footcare is essential to provide appropriate footcare to patients with foot disorders. Moreover, the expression "footcare" is not widely recognized in Japanese society, and individuals with foot lesions tend to neglect these issues. Footcare nurses play a key role in education of medical, nursing, and welfare professionals regarding the importance of timely attention to foot lesions to maintain walking ability and for improvement of overall social awareness.

4. Evaluation and implementationof foot care

In this section, we will discuss evaluation necessary for the implementation of footcare and the contents of a footcare program.

4-1 Evaluation

4-1-1 Foot Evaluation

In foot care, proper evaluation of the foot is very important. This chapter describes each item of foot evaluation.

4-1-1-1 Lower extremity blood flow disorders

Patients with lifestyle-related diseases such as diabetes mellitus, dyslipidemia, hypertension, hyperuricemia, chronic kidney disease, obesity, and smoking should be considered high-risk patients for arteriosclerosis obliterans[11]. During the early stages of lower extremity blood flow disorders, patients may experience only a sense of coldness; however, disease progression invariably manifests as intermittent claudication, and many patients seek medical attention for evaluation of this symptom. However, intermittent claudication also occurs in patients with lumbar spinal canal stenosis. Therefore, it is important to remember that patients with blood flow disorders may visit an orthopedic surgeon, and accurate diagnosis may be delayed. Patients with impaired blood flow through the lower extremities are susceptible to ulceration secondary to skin erosion and often develop intractable ulcers even after minor skin injuries. Additionally, severe lower extremity edema can lead to gangrene.

Physical examination should include measurement of skin temperature and color tone, palpation of the popliteal, posterior tibial, and dorsalis pedis, evaluation of hair loss, subjective symptoms, and the presence and extent of erosions and ulcers. Notably, friction may result in interdigital ulcers; therefore, careful foot inspection is necessary to ensure that no area is overlooked. Such evaluation can be performed merely through observation and palpation and does not require sophisticated equipment; such examinations can be performed by home healthcare and nursing home personnel. Measurement of blood flow using the ankle-bra-

chial index, skin perfusion pressure, and magnetic resonance angiography, as well as cardiology consultation become necessary in cases of suspected or confirmed ischemia or ulcers.

4-1-1-2 Neurological disorders

Diabetic neuropathy, the most important among all peripheral neuropathies that affect the lower extremities is characterized by symmetrical appearance of the distal extremities[12]. Sensory neuropathy results in decreased sensation of warmth and pain, which may delay detection of shoe-induced chafing and cold burns, with consequent serious foot lesions, which may even necessitate lower extremity amputation. Motor neuropathy causes muscle atrophy in the foot and joint contractures secondary to accumulation of advanced-glycation end products, which results in deformities such as hammertoes and crooked toes. Disruption of the arch structure leads to flat and open feet (Figure 1), with greater susceptibility to callus formation (Figure 2). Progressive autonomic neuropathy results in reduced sweating, which negatively affects the excretory function of the skin and causes dryness that predisposes patients to infection. Sympathetic neuropathy causes opening of arteriovenous shunts and consequently accelerated bone resorption and small fatigue fractures, which result in a characteristic Charcot foot deformity (Figure 3). Therefore, patients with diabetic neuropathy are at a high risk of multiple concomitant foot lesions.

In addition to diabetes mellitus, trauma, osteoarthritis, spinal disease, cranial neuropathies, prolonged alcohol consumption, and aging contribute to neuropathy. Similar to diabetic neuropathy, alcoholic neuropathy tends to show a distal and symmetric presentation; however, careful history taking and details regarding the patient's lifestyle can distinguish between these conditions[13].

Evaluation of subjective symptoms, muscle atrophy, and the degree of deformity, if any, is important in addition to objective tests including the touch test, testing for vibration sense, and Achilles tendon reflex. It is also important to confirm shoe comfort and fit in patients with neuropathy because owing to sensory loss, patients are unable to determine the proper fit and tend to wear shoes that are too large or small.

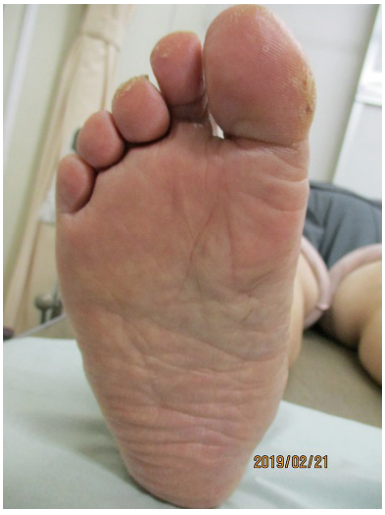

Figure 1 flat and open foot

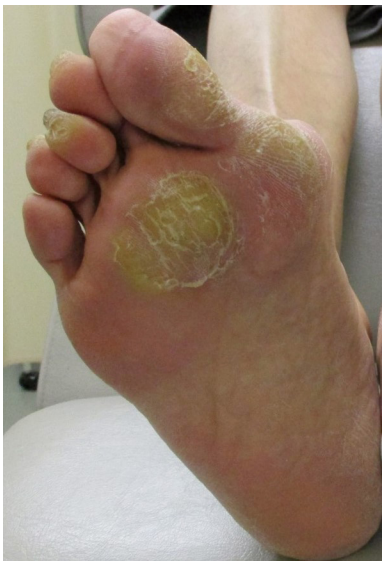

Figure 2 callus

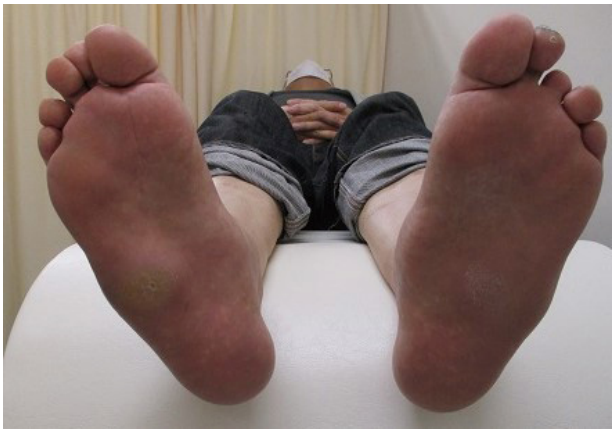

Figure 3 Charcot foot

4-1-1-3 Edema

Lower extremity edema can be broadly categorized into generalized and localized types. Localized edema is mainly associated with foot lesions and includes lymphedema, venous edema, and disuse edema[14]. In recent years, an increasing number of patients tend to present with disuse edema due to loss of muscle strength and reduced ADL[15]. Medical treatment is prioritized in patients with systemic edema; however, lower extremity edema requires appropriate management under the guidance of a specialized department.

Edema-induced skin thinning causes dryness, a decline in the natural skin barrier function, and itchiness, which trigger scratching and predispose patients to infection after even minor wounds, resulting in cellulitis. Prolonged untreated edema can lead to fibrosis of the skin and loss of flexibility, which may result in joint contractures and motor dysfunction. Additionally, worsening edema can lead to intractable ulcers secondary to leakage of water that cannot be stored under the skin. Venous edema caused by varicose veins or impaired venous return typically manifests at the skin around the ankle joint, which shows pigmentation, leading to blistering, erosions, and stasis ulcers. Stasis ulcers produce a large amount of effusion and are painful and significantly negatively affect patients' quality of life (QOL)[16]. Use of elastic stockings is encouraged for management of lymphedema to avoid enlargement of the lower extremities and gait difficulties, which negatively affect QOL and ADLs[17]. The degree of lower extremity edema varies with the duration of lower extremity weakness and the extent of activity. The foot size tends to fluctuate within the day, which can cause shoe-induced chafing, bedsores, and ingrown toenails. Furthermore, the increased weight of the lower extremity secondary to edema can cause gait difficulties and predispose the patient to falls.

Evaluation should be performed to determine the cause of the edema (systemic vs. localized). Screening for heart failure, liver and renal disease, hypothyroidism, and anemia is necessary to confirm systemic edema. Following exclusion of systemic edema, the cause of edema should be investigated using ultrasonography to measure venous return and, if necessary, lymphatic scintigraphy. Edematous feet may be scarred and show continuous subcutaneous fluid leakage. Even minor wounds may lead to cellulitis and should be closely monitored. Careful evaluation is necessary to determine patients' range of motion and gait disturbances, if any, caused by skin fibrosis.

4-1-1-4 Deformities

In addition to trauma- or fracture-induced deformities, foot deformities may be associated with osteoarthritis, rheumatoid arthritis, diabetic neuropathy, other peripheral neuropathies, aging, and use of inappropriate footwear. Foot deformities can lead to abnormal load balance during walking and cause calluses and clavus.

Metatarsalgia, an inward deformity of the first metatarsal bone is more commonly observed in women and is associated with rheumatoid arthritis, genetic factors, flat feet, weakness of the foot muscles, and inappropriate footwear[18]. Progressive metatarsalgia is characterized by severely deformed and overlapping toes and causes difficulty with wearing shoes and walking. Hallux valgus, which is often mistaken for hallux valgus. Hallux valgus is characterized by callus formation on the dorsal aspect of the metatarsophalangeal joint of the first toe. Bunions tend to develop on the lateral aspect of the foot owing to footwear-induced friction, and a callus or clavus is often observed on the lateral aspect of the fifth toe. Hammertoe or claw-toe deformity is associated with diabetic neuropathy or inappropriate footwear, which results in calluses and clavus involving the proximal interphalangeal joint and tip of toes.

A flatfoot deformity is commonly observed in children and elderly individuals. Disruption of the arch structure, which causes a flatfoot deformity, is primarily caused by foot muscle weakness, with consequent plantar tendonitis and plantar fasciitis, which result in chronic pain and impaired walking ability[19, 20].

Charcot foot is characteristically associated with diabetic neuropathy (Figure 3) and occurs secondary to the accumulation of small fatigue fractures in the foot[21]. Patients with diabetic Charcot foot are considered to have developed peripheral neuropathy, which clinically presents with reduced warmth and pain sensation, and ulcers resulting from calluses and shoe abrasions are often detected late and tend to become serious.

Evaluation includes inspection and palpation to confirm bony deformities, muscle and tendon atrophy, and joint range of motion. The patient's load balance is evaluated in the erect position, and the extent of the deformity is confirmed radiographically. Callosities, clavus, and evidence of worn shoe soles are important indicators of load imbalance.

4-1-1-5 Skin lesions

Evidence of skin dryness, desquamation, and maceration of the interdigital spaces is associated with a high index of clinical suspicion for tinea pedis. Tinea pedis is a common skin lesion that affects approximately 40% of the adult population[22] and is associated with hyperkeratosis, greater susceptibility to infection owing to skin dryness, aggravation of calluses, and fissuring. Lower extremity ischemia presents with reddish-purple discoloration and coldness of the skin over the ischemic areas. Alopecia and atrophy or loss of nails also indicate lower extremity ischemia. Patients who receive long-term steroid therapy show atrophic and fragile skin, and even mild irritation can cause ulcers.

Nail abnormalities include ingrown and thickened toenails, which are usually associated with inappropriately designed footwear, an incorrect fit or manner of wearing shoes, the patient's gait, and toe deformities such as bunions, and onychomycosis. Nail abnormalities affect walking balance and predispose patients to falls[23].

Evaluation includes observation of skin thinning and fibrosis secondary to edema, skin atrophy secondary to chronic steroid medication, ischemic skin changes, redness secondary to deformities, evidence of calluses and clavus, and nail deformities. Microscopic examination should be performed for prompt diagnosis and treatment in cases of suspected toenail onychomycosis.

4-1-2 Evaluation of general condition

4-1-2-1 Factors that affect skin lesions

Patients who receive chronic steroid therapy often present with atrophic fragile skin that is vulnerable to injury even with mild irritation. Generalized or localized edema leads to skin thinning and easy scarring, which predispose to cellulitis. Dryness caused by diabetic neuropathy, edema, and aging is associated with itchiness, and scratching predisposes to infection secondary to impaired barrier function.

4-1-2-2 Factors associated with reduced body defense mechanisms

Diseases associated with immune dysfunction, hyperglycemia, low nutrition, peripheral circulatory disorders, and aging affect the body's defense mechanism; even minor wounds may get severely infected, healing is delayed and the skin is predisposed to ulcer formation and aggravation. Specifically, patients with diabetes and persistent hyperglycemia (blood glucose levels ≥250 mg/dL) show reduced ability to phagocytose neutrophils with greater susceptibility to infection.

4-1-2-3 Factors associated with walking ability

Patients with cranial neuropathies, spinal disease, and osteoarthritis are susceptible to falls secondary to abnormal gait balance. Anemia and medication use (antipsychotics and anticancer drugs, among others) can also cause unsteadiness and increase the risk of falls. Patients with impaired lower extremity blood flow may develop intermittent claudication with difficulty in walking long distances. Gait imbalance secondary to a variety of factors may cause pain and joint deformity due to the load on the affected as well as the unaffected side.

4-1-2-4 Factors that affect foot self-care

Daily self-care is a fundamental principle of effective foot care; however, several factors prevent foot self-care in patients. For example, hemiplegia due to cerebral infarction, motor dysfunction due to spinal disease or limited range of motion of joints, decreased visual acuity, decreased manual dexterity, decreased cognitive ability, and decreased adherence are known obstacles to self-care. Additionally, housing conditions without bathrooms, financial difficulties, and lack of support affect foot self-care. Patients tend to harbor misconceptions about foot-care. For example, patients often treat calluses using scissors or clip nails sensitively, without direct visualization, which increases the risk of ulcer formation.

4-1-3 Footwear evaluation

Careful scrutiny of footwear is often sufficient in patients with foot lesions; shoe width, size, shape, material, sole, heel, laces, and design should be

closely examined. In addition to the shoes worn when visiting the outpatient clinic, it is necessary to examine the shoes that the patient uses frequently and also work footwear to confirm the effects of footwear on foot lesions[24].

Calluses and clavus on the feet unaccompanied by significant deformities are often indicative of footwear-induced lesions, which may be associated with an inappropriate size, high heels, and thin soles of shoes, as well as shoes with many designs and stitches at the back of the foot. Ingrown toenails, hallux valgus, and little toe varus are often caused by narrow-toed shoes, inappropriate shoe sizes, and high heels.

In addition to the type of footwear, the manner in which it is worn can result in foot lesions; shoes with loosely tied laces or those in which the foot is not well secured result in back and forth slippage of the foot and instability. Therefore, shoe-induced chafing, calluses, and clavus tend to occur more frequently, and toes are pushed into the shoe tip during the kicking motion of walking, which results in ingrown toenails.

Patients with diabetic neuropathy experience sensory loss and therefore prefer tighter shoes owing to the sensation that their footwear does not fit correctly. Poorly fitting footwear can cause shoe chafing; therefore, patients with diabetic neuropathy should undergo careful footwear evaluation[25].

4-2. Foot care

Footcare includes care of lesions such as calluses and nail treatment, as well as foot self-care guidance. Footcare should be prioritized based on the results of evaluation, and issues that require urgent attention should receive immediate care. Care is selected within the scope of self-care that can be implemented by the patient and family, and the method of implementation is taught. Patients and their families who cannot perform self-care should be referred to nursing care and welfare services. In this section, the specific content of foot care will be explained.

4-2-1 Nail care

Nail clipping, trimming of thickened nails, and correction of ingrown nails are components of nail care. Usually, square-off nail trimming is recommended; the tip of the nail blade is cut straight and only the nail corners are rounded using a file (Figure 4). However, nails should be trimmed carefully to avoid skin injury considering the patient's foot shape in those with toe deformities including bunions. Periungual inflammation may occur secondary to the accumulation of plaque around the nail in patients with thickened or ingrown nails[26]. Taping and cotton packing may be useful for symptomatic treatment of ingrown nails and ingrown toenails; however, angle correction is necessary for pain or wounds. Ingrown nails and ingrown toenails often develop secondary to the shoe design and manner in which they are worn; therefore, it is necessary to provide guidance regarding the appropriate method of wearing shoes to prevent recurrence.

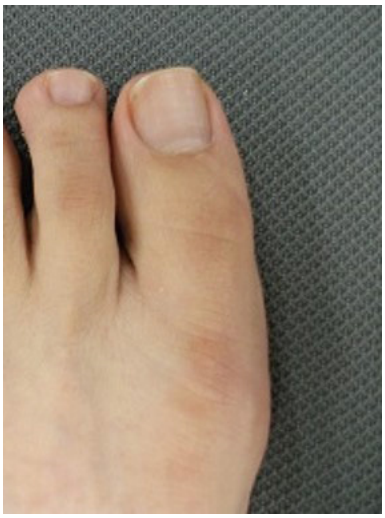

Figure 4 square-off

4-2-2 Care of calluses and clavus deformities

Hardened, painful, bleeding calluses and clavus should be trimmed, and patients should be educated regarding factors that predispose to callus formation, such as foot deformities, shoe design, manner of wearing shoes, and an individual's gait. The patient should also be instructed regarding daily cleansing and moisturizing care, because dryness causes friction and aggravates calluses. Alignment correction and decompression using plantar orthoses are effective for patients with calluses and clavus deformities. It is important to coordinate with prosthetists to improve these conditions. Patients who do not improve with outdoor foot orthoses alone require prostheses for indoor use as well . Taping

and foot rehabilitation are also effective to prevent progression of foot deformities.

4-2-3 Edema care

Compression therapy is effective for localized lower extremity edema; however, the degree, method (elastic stockings vs. bandages), and extent of compression should be considered on a patient-by-patient basis. Appropriate use of compression therapy depends on the patient's motor and cognitive function; therefore, it is important to select a feasible method. Inappropriate compression therapy can worsen edema or cause pressure ulcers[27]; therefore, it is important to perform compression therapy with the patient or family's consent having confirmed that it can be performed appropriately. An edematous foot is prone to cellulitis; therefore, patients should be instructed regarding skin care, including cleansing and moisturizing. Lifestyle guidance including exercise therapy and weight loss may be necessary in patients with disuse- or obesity-induced edema. Manual lymphatic drainage massage is effective for lymphedema and venous stasis edema; a nurse trained in the appropriate technique performs the massage and the patient is accordingly instructed regarding the technique.

4-2-4 Care of diabetic foot lesions

Footcare for patients with diabetes commences with evaluation of diabetic peripheral neuropathy. Owing to the progressive nature of this condition, it is difficult for patients to monitor changes in their feet in a timely manner. Therefore, patients with diabetes should have basic knowledge of preventive footcare from the early stage of diagnosis. Diabetic footcare does not include only management of peripheral neuropathy but also involves the treatment of several overlapping concerns such as impaired blood flow due to arteriosclerosis obliterans and reduced self-care ability due to retinopathy. It is important to establish a rapport between medical professionals and patients for timely advice regarding protection of their feet[28].

Progression of diabetic foot lesions is associated with glycemic control; persistent hyperglycemia is known to cause infection. Therefore, diabetic footcare must necessarily include close monitoring of blood glucose control and progression of complications other than foot lesions and the overall treatment of diabetes in addition to that of foot lesions.

4-2-5 Care of footwear

It is well known that in addition to shoes with high heels and pointed toes, those with many seams and designs can cause foot lesions. Knowledge regarding selection of appropriate shoes and the correct methods of wearing them is limited among the Japanese; therefore, it is necessary to discuss these shoe-related footcare issues with patients. Footwear is a means of self-expression and individuality. Moreover, specific types of shoes may be designated by school or an individual's occupation; therefore, it may not be possible to change shoes from the viewpoint of "appropriate shoes for feet." Therefore, it is necessary to provide shoes suitable for an individual and also offer education regarding the appropriate method of wearing shoes until the patient is convinced.

Correction of foot alignment is the fundamental principle of treatment of foot lesions. Creation of shoe insoles effectively relieves pain, treats calluses and clavus, and prevents progression of deformities. The Japanese medical insurance system provides coverage for orthotics once every 18 months.

5. Management of the foot care team

The footcare team includes orthopedics, plastic surgery, vascular surgery, cardiology, dermatology, diabetology, nephrology, rehabilitation, and anesthesiology (pain clinic), although these departments vary depending on the size of the medical institution. Nurses, clinical laboratory technicians, medical staff including physical therapists, exercise therapists, dietitians, pharmacists, and other prosthetists are also members of the footcare team. Therefore, establishment of a medical system comparable with podiatry services available in Europe and the United States warrants a multidisciplinary approach to improve the quality and efficiency of medical care. Compared with other professionals, footcare nurses have greater interaction with patients and are therefore better suited to understand patients' viewpoints and background. Footcare nurses provide footcare to patients across all stages and are capable of understanding a patient's condition closely, as the treatment phase shifts from the acute to the rehabilitation and subsequently the home treatment phase. There-

fore, footcare nurses play an important role in multidisciplinary coordination, such as for communication of patient information across team members and interdepartmental treatment transfers. They also collaborate with those involved in nursing care insurance and home medical care to support patients' home care needs, to manage footcare within the range that can be implemented in the home environment, and to promote team medical care that includes those outside the hospital.

Therefore, several medical and nursing personnel from and outside hospitals participate in the footcare team. The footcare nurse plays a key role as a team coordinator, to support the transfer of treatment across various departments and professionals and for effective team functioning to ensure that patients receive the best and prompt medical care.

6. Role of foot care nurses in education and awareness activities

Western podiatry service are not available in Japan; therefore, the approach to foot lesions differs across medical centers/hospitals, which results in non-standardized medical care available to patients[29]. Reportedly, lower extremity amputation is preferred over revascularization in patients with severely injured ischemic extremities, patients with deformed feet or nail abnormalities may remain untreated, and patients may develop pressure ulcers secondary to the use of thromboprophylactic elastic stockings[27]. Currently, it is necessary to improve awareness among the Japanese involved in medical and nursing care and welfare in the field of limb salvage and footcare.

To address these concerns, the Japanese Society of Footcare and Podiatry has designated February 10 as Footcare Day and is actively involved in improving awareness regarding this important issue. Additionally, the society certifies footcare instructors who "aim to improve the footcare abilities (knowledge and skills) of patients and care providers and play a leading role in each field"[30, 31]. Most footcare instructors are nurses who provide care in hospitals, as home health care aids, and home nursing care and also educate medical personnel, as well as patients and their families. Footcare nurses serve as models for footcare practitioners with their specialized knowledge and skills and are responsible for fostering footcare teams through their activities.

The establishment of an "additional fee for guidance and management of peripheral arterial disease of the lower extremities" in 2016 has focused attention on early identification of high-risk patients with lower extremity blood flow disorders[32]. This medical fee enables referrals of high-risk patients with foot lesions to specialized hospitals. Footcare for ischemic extremities is not widely available in Japan currently; therefore, footcare nurses need to provide education to collaborating medical centers to ensure that the medical staff at the referral centers can continue patient care.

In Japan, owing to the cultural background, routine footcare is not widely recognized; therefore, public education is necessary to ensure that care of feet and their disorders are not neglected. It is important to improve awareness regarding the importance of footcare in the general population because even individuals without access to medical care include high-risk patients with foot lesions. In view of the increasing life expectancy and longevity trends in Japan, fall prevention and steps to prevent bedriddenness among the elderly population are important. Foot health also plays an important role in the national policy of extending healthy life expectancy. Optimal footcare can help individuals to regain their foot health, and those who receive footcare can serve as models of healthy longevity to improve awareness regarding footcare across the general population.

7. Conclusion

In this section, we discuss foot lesions associated with the maintenance of ambulation and the role of footcare nurses in the treatment of these disorders. Footcare is the mainstay of treatment for foot lesions and is necessary for all aspects of life, ranging from prevention to treatment and thereafter. In an aging society, the need for footcare will continue to increase. Therefore, there is an increasing need to train footcare nurses to play an active role in establishing a culture in which footcare is performed on a daily basis as an essential component of an individual's health care, similar to the practice followed in Western countries.

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Author contributions

YT contributed to the conception, drafting the paper, and preparing the figure.

Conflict of interest statement

The Author declare that there are no conflicts of interest.

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Original Articles

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Comparing Accuracy of the Final Height Prediction Models for Elite Football Players and Developing a New Model

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Objective: This study aimed to assess the accuracy of previously developed height prediction models in male Japanese football players and create new height prediction models.
Materials: The participants were elite academy male football players. We collected current height, parent's height, calendar age and bone age in 6th grade of primary school and obtained actual final height at 20 to 28 years old.
Methods: We compared the accuracy of two conventional models for predicting final height. These used current height, calendar age and either bone age (Model 1) or parental height (Model 2). We then developed a new model to optimize the coefficients of Model 1 (Model 3). The final model added parental height to Model 3 and optimized the coefficients (Model 4).
Results: Prediction accuracy was higher for Model 2 ($R = 0.52$, $P < 0.001$) than Model 1 ($p = 0.33$, $P < 0.001$). The equation of Model 3 was final height = 0.63229313×actual measured height−8.2541327×calendar age−2.3009853×bone age (TW2)+206.627184. The R-square was 0.49 ($P < 0.0001$). The equation of Model 4 was final height = 0.32156081×actual measured height−4.6652063×calendar age+0.41903909×father's height+0.34952508×mother's height−0.740469×bone age (TW2)+62.1007751. The R-square was 0.61 ($P < 0.0001$).
Conclusions: In the two previous conventional models, a formula using parental height had better predictive accuracy. We developed a new height prediction model using current height, calendar age, father's and mother's height and bone age.

Key words: height prediction, bone age, tanner whitehouse 2, football

Introduction

In some competitive sports, efforts have been made to predict future height. There have been various reports of positive correlations between height and athletic performance[1–3]. Assessing athletic potential based on skeletal development, such as predicting future height, is therefore considered an important factor in estimating athletic talent.

Current methods of predicting final height from a single time point include models estimated with bone age and parents' height. Bone age, which measures biological bone maturity, is estimated in three ways: the numerical method[4], the qualitative

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method[5], and the measurement method[6]. The numerical method counts the number of ossification centers on the hand. The qualitative method compares the shape of the ossification centers on hands to a standard chart, and the measurement method measures the area and aspect ratio of the ossification centers on the hand. For the Japanese population, Murata et al. standardized the Tanner Whitehouse 2 (TW2) method, a qualitative method[7].

The Growth Potential method[8] and the Bayley-Pinneau method[9] have been used to predict final height using bone age, and a prediction formula for Japanese children was developed by Matsuoka et al. in 1994.[10, 11]. As an alternative, Ogata et al. developed the target height method, which calculates the predicted height of Japanese children based on their parents' height rather than using bone age[12].

In clinical practice, bone age is usually used for differential diagnosis or follow-up of growth disorders in children. Its use to predict the future height of athletes might therefore be challenging, because it requires radiography, and not many athletes have access to this. Estimates based on parental height are therefore often used. Previous studies have suggested several models to predict future height, but it is not known which formula is accurate and whether the model fits athletes. This study therefore aimed to compare the accuracy of the prediction formulas, and to develop a new prediction formula for athletes.

Materials and Methods

This study was approved by the ethical committee of Juntendo University (No.2021-12) and was carried out from June to October 2021. Consent forms were sent to potential participants and we enrolled any who agreed to participate and provided written consent. The participants were elite academy football players who belonged to a football club linked to the Japan Football Association. They were all dormitory lives and had a similar lifestyle of eating, sleeping and practicing for 6 years.

The participants had all belonged to the football club for six years from the age of 12 to 18 (from 2006 to 2019). We collected data in October of their 6th grade of primary school (11 to 12 years old) with agreement of their parents (the fiscal year starts in April and ends in March in Japan) and then at the age of 20 to 28. In 6th grade of primary school, we obtained participants' height, weight, parents' height, and hand x-rays. X-rays were taken at a nearby clinic. At the age of 20-28, we obtained participants' current height, weight and age. Height measured after 20 years old was defined as final height and was self-reported by participants to the investigator. We excluded those who were less than 20 years old in 2021.

Estimated bone age

We used the TW2 method[5] to calculate bone age. This has three types of evaluation methods: RUS (radius, ulna and short bone), carpal and 20Bone. The RUS method evaluates 13 nuclei; the carpal method evaluates seven nuclei (carpal bone); and the 20bone method evaluates 20 nuclei (both RUS and carpal). We used the RUS method, which was reported to have a high correlation with final height[11], to calculate the bone age.

Models predicting future height

First, we compared two conventional models. The first model (Model 1) was reported by Matsuoka et al. in 1994[11]. The formula was developed using actual measured height, calendar age and bone age, and the equation was $Y = a \times$ actual measured height $+ b \times$ calendar age $+ c \times$ bone age $+ d$. In the model, a, b, c and d change depending on the calendar age. In this study, for those with a calendar age of 11.5 to 12 years, we used $Y = 0.639 \times$ actual measured height $- 9.221 \times$ calendar age $- 3.567 \times$ bone age $+ 230.39$. We used $Y = 0.557 \times$ actual measured height $+ 7.809 \times$ calendar age $- 3.413 \times$ bone age $+ 36.161$ for those with a calendar age of 12 to 12.5 years, and $Y = 0.288 \times$ actual measured height $+ 6.475 \times$ calendar age $- 1.825 \times$ bone age $+ 36.161$ for those aged 12.5 to 13 years. Actual measured heights at 6th grade of primary school were measured on the day of the hand X-ray. We measured the left hand[12] unless it had previously been broken. The second model (Model 2) was the target height method developed by Ogata et al. in 1990[13]. The formula uses parents' height and the equation was (father's height + mother's height + 13)/2.

After assessing Models 1 and 2, we created new height prediction models, Models 3 and 4. We conducted a multiple regression analysis using the

same variables as Model 1 and developed a new equation by changing coefficients, to give Model 3. We then developed another prediction model with different variables (Model 4). To develop this final model, we used regression analyses, added or removed variables and calculated the accuracy. The final Model 4 included actual measured height, calendar age, father's height, mother's height and bone age.

Statistical analysis

Multiple regression analysis used the statistical software JMP® pro version 16. The significance level of the test was set at 5%.

Results

Participants

Of the 128 male players who belonged to the football club from 2006 to 2019, 53 men agreed to participate and were included in this study. The demographic characteristics of the participants are shown in Table 1. The mean (SD) bone age calculated by the TW2 method was 12.2 (1.2). The mean difference (SD) between bone age and calendar age was 0.89 (0.75) years, and the mean difference between final height and measured height in 6th grade of primary school was 22.9 (8.2).

Difference between predicted height and final height

We assessed the accuracy of the conventional models by correlation analysis. For Model 1, the mean (SD) of the predicted heights was 173.2 (4.5) and the actual final height was 172.1 (6.8). The R-square was 0.33 ($P < 0.0001$) (Figure 1). For Model 2, the mean (SD) of the predicted heights was 172.4 (4.3) and the actual final height was 172.1 (6.8). The R-square was 0.52 ($P < 0.0001$) (Figure 2).

Table 1 Characteristic of the study participants (N = 53)

	Mean (SD)
Data in 6th grade of primary school	
Calendar Age (years)	12.2 (0.3)
Bone age (years)	12.2 (1.2)
Height (cm)	149.2 (9.7)
Predicted height of Model 1 (cm)	173.2 (4.5)
Data in adults (20–28years old)	
Age (years)	23.9 (2.8)
Actual final height (cm)	172.1 (6.8)
Father's height (cm)	173.5 (5.4)
Mother's height (cm)	158.3 (5.3)

New prediction models

Model 2 was more accurate than Model 1. Model 1 was accurate at a final height of around 170–175cm. However, the difference was more prominent below 170cm or above 175cm. The difference between the predicted height and actual final height was linearly distributed, and the R-square was 0.56 ($P < 0.0001$) (Figure 3). The equation of Model 3 was final height = 0.63229313 × actual measured height − 8.2541327 × calendar age − 2.3009853 × bone age (TW2) + 206.627184. The R-square was 0.49 ($P < 0.0001$) (Figure 4).

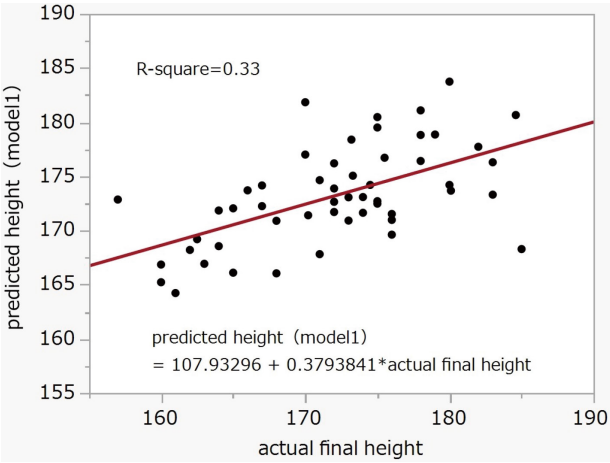

Figure 1 Correlation between final and predicted height (Model 1)
R-square = 0.33, $P < 0.0001$

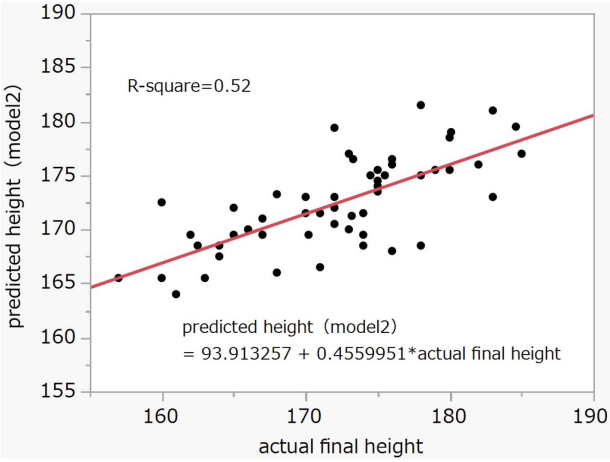

Figure 2 Correlation between final and predicted height (Model 2)
R-square = 0.52, $P < 0.0001$

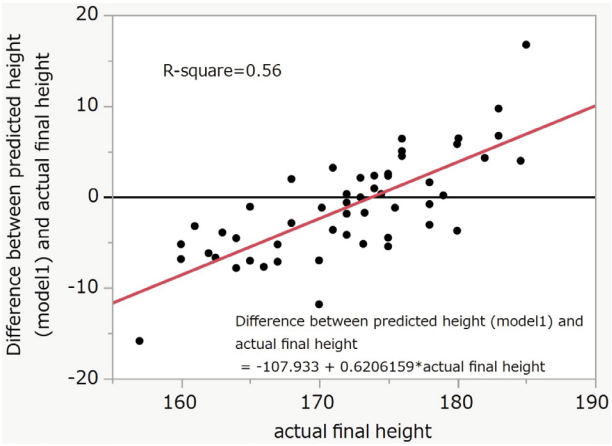

Figure 3 Difference between final and predicted height (Model 1)
R-square = 0.56, P < 0.0001

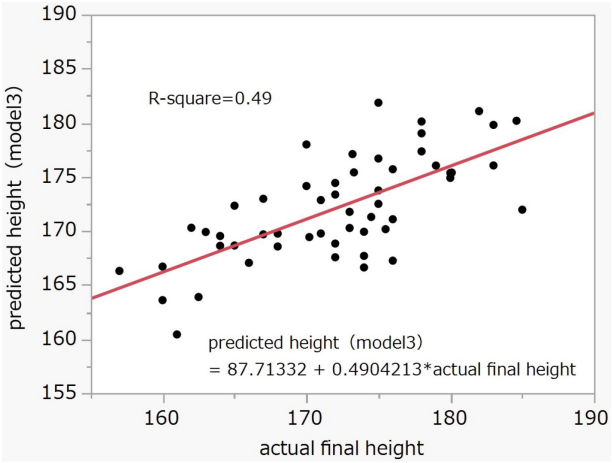

Figure 4 Correlation between final and predicted height (Model 3)
R-square = 0.49, P < 0.0001

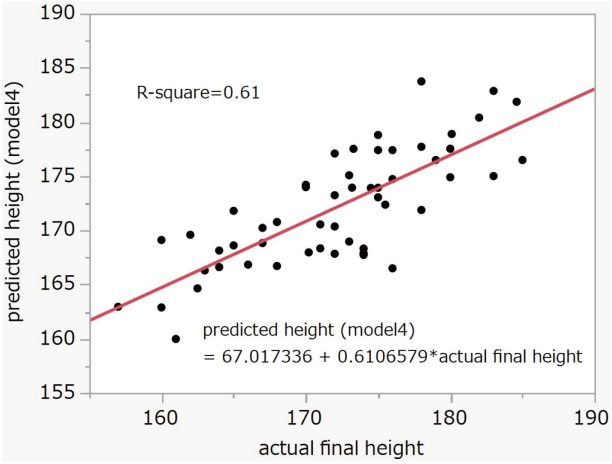

Figure 5 Correlation between final and predicted height (Model 4)
R-square = 0.61, P < 0.0001

Finally, we added the height of the parents as a parameter and as a result, it showed higher accuracy than model 2 (model 4). The equation was final height = 0.32156081 × actual measured height − 4.6652063 × calendar age + 0.41903909 × father's height + 0.34952508 × mother's height − 0.740469 × bone age (TW2) + 62.1007751. The R-square was 0.61 (P < 0.0001) (Figure 5).

Discussion

In this study, we assessed the accuracy of the two conventional models of final height prediction for Japanese young people, in a group of football players. One model uses bone age, calendar age, and actual measured height as variables and the other uses the parents' height. We demonstrated that the model using parents' height was more accurate (R-square was 0.52 compared with the bone age model developed by Matsuoka et al.). In the bone age model developed by Matsuoka et al., accuracy was good for a final height of between 170 to 175 cm, but less accurate outside this range. The discrepancy was distributed linearly, and we therefore optimized the coefficients with the same four variables. The R-square became 0.49, but the accuracy was still lower than using the parents' heights (R-square = 0.52). In a new model with bone age added as well as parental height, the R-square became 0.61. As a result, a new predictive model by changing the coefficients of the bone age model developed by Matsuoka et al. and adding the height of the parents as variables demonstrated better accuracy. According to our data, the prediction model that did not use bone age was less accurate than our newly developed model. However, we think that the parental height model developed by Ogata et al. has sufficient accuracy and could be used in athletes without radiation exposure.

This study aimed to develop prediction models at the 6th grade of primary school. Although several models for predicting final height using bone age have been reported[14–16], the accuracy varies. Models with low prediction accuracy uses bone age at a single point, while those with high accuracy use changes in height and bone age over a period. As individuals' growth speed differs[17,18], taking into account the growth process may improve accuracy. However, previous reports showed that considering growth curves did not have good accu-

racy. Therefore, how we consider the growth process is needed to be investigated. We should note that data of body weight was obtained in the study; however, it was not helpful to predict future height. Therefore we did not use the variable.

This study had several limitations. First, we enrolled 53 participants to compare the accuracy of traditional models and develop new models, but we did not calculate a required sample size. However, all the data in the study have a low p-value of less than .05, and type 2 errors therefore should not be a problem. Second, this new model may not fit non-elite athletes. In this study, the mean final height was 172.1cm, which is slightly taller than the mean of all Japanese men[19]. The mean parental heights in this study were also greater than the population mean. The height of athletes may have been taken into account, as well as their playing skills, in deciding on elite status. This may have introduced selection bias, and may make this model suitable only for a limited group of people. Third, we did not assess whether this model fits future athletes because we used all data to develop a new model. Another data set is needed to validate these models.

In conclusion, we showed that a conventional model of future height prediction using parents' height was more accurate than those using bone age, and we developed a new and more accurate height prediction model using actual measured height, calendar age, father's height, mother's height and bone age.

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Authors' contributions

Conception and design of the study: TA, MN, TM, MY

Research data collection: TA, MD, HI, TT

Analysis and interpretation of data: TA, MN

Writing the paper: TA, MN

Approval of final manuscript: All authors

Conflicts of interest statement

The authors have no conflict of interest to disclose.

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Original Articles

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Effects of Drinking Oxygenated Water on Blood Oxygen Saturation During Exercise Under Normobaric Hypoxic Conditions: A Randomized Placebo-controlled Single-blinded Trial

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Objectives: This study aimed to investigate the effects of drinking oxygenated water on oxygen saturation during exercise under normobaric hypoxic conditions.

Materials: A randomized placebo-controlled single-blinded trial was performed. Twenty-two healthy adults (16 men and 6 women), with a mean age (standard deviation) of 22.4 (2.73) years, participated in the study. The participants were randomly assigned to one of two groups: an OX group (drinking oxygenated mineral water) and a control group (drinking normal mineral water). Both groups performed walking exercises under normobaric hypoxic conditions. Blood oxygen saturation (SpO_2), pulse rate (PR), and walking distance were measured during exercise.

Results: SpO_2 decreased and PR increased during exercise in both groups. The decrease in SpO_2 was smaller and the increase in PR was greater in the OX group compared with those in the control group. No significant difference was found in walking distance between the two groups.

Conclusions: Drinking oxygenated water before exercise may inhibit SpO_2 reduction under normobaric hypoxic conditions.

Key words: water, oxygen, hypoxia, randomized controlled trial

Introduction

Enhancement of athletic ability is of interest not only for athletes, but for anyone engaged in playing sports. In recent years, to exploit the beneficial effects of oxygen on athletic performance, it has been proposed that drinking water containing high concentrations of dissolved oxygen may have the potential to improve athletic performance. A previous study demonstrated that high concentrations of dissolved oxygen in water (up to 40 times that of typical drinking water) improved athletic performance[1)]. In addition, oxygenated water is marketed as a drink that can replenish water and oxygen, with claimed benefits including aiding recovery from fatigue after exercise and increasing concentration. However, scientific evidence regarding the effects of high-concentration oxygenated water remains controversial[2–8)]. Because excessive enhancement of athletic performance can be detrimental to an athlete's health and interfere with fair competition, well-designed scientific experiments are needed.

Under normal conditions, the partial pressure of oxygen is known to decrease with high-intensity training[9, 10)]. In a study investigating exercise under normobaric hypoxic conditions, peripheral blood

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oxygen saturation (SpO_2) was reported to be significantly decreased with bicycle training in healthy adults[11]. On the basis of these findings, we hypothesized that the intake of high-concentration oxygenated water before and during exercise could prevent SpO_2 reduction during exercise in a hypoxic environment.

Therefore, the current study aimed to evaluate changes in blood oxygen saturation during exercise under hypoxic conditions after consumption of high-concentration oxygenated water in normal adults.

Materials and Methods

This randomized placebo-controlled single-blinded trial was performed from July to August 2021. The current study was approved by the institutional human ethics committee (2021-77) at Juntendo University School of Health and Sports Science, and was in compliance with the Declaration of Helsinki and existing legal regulations. Written consent was obtained from all participants before their enrollment. The inclusion criteria were as follows: healthy men or women aged > 20 years. The exclusion criteria were as follows: body temperature > 37.5 °C; SpO_2 < 95%; systolic blood pressure > 145 mmHg or diastolic blood pressure < 90 mmHg; history of heart or pulmonary disease; and other comorbidities, disorders and diseases that could affect the results. We recruited 22 participants, who were randomly assigned to either the control group (drinking normal mineral water) or the OX group (drinking oxygenated mineral water with high oxygen concentration). Participants were blinded from group allocation. Age, sex, body height, body weight, smoking history, and sports habits were collected as demographic data.

Intervention

A bottle of normal mineral water (control group) or oxygenated mineral water (OX group) was given to participants before the experiment began. An OXMAX ($Wells O_2$ Inc, Tokyo, Japan) oxygen capsule was used to make the oxygenated water. The same commercially available bottled mineral water at room temperature was used for the experiments in both groups. The experiment was conducted at the High-Alti Training Studio (High Altitude Management Co., Ltd, Tokyo, Japan), where a normobaric hypoxic environment (oxygen partial pressure of 15.2%, equivalent to 2600 m above sea level) was available. During the experiment, participants were asked to walk for 30 min on a self-propelled treadmill (Speedboard ProXL, Speed Fit, USA) under normobaric hypoxic conditions, and during exercise, SpO_2 and pulse rate (PR) were measured every 2 s using a pulse oximeter ($Ring O_2$, Viatom Technology Co. Ltd). Exercise intensity was set at 11-13 on the subjective fatigue Borg scale[12], and the score was recorded every 3 min during exercise. The participants were instructed to drink 200 mL of water 10 min before walking, 100 mL 6 min before walking, 100 mL 1 min before walking, and 100 mL 10 min after starting walking. Participants entered the hypoxic room 5 min before the exercise and left the room after 30 min of walking exercise. After the exercise period, participants rested for 10 min in a normoxic environment (Figure 1).

Statistical analysis

Before the experiments, the average SpO_2 and PR values under the normal environment were used as baseline values. These were analyzed every 5 min. The mean values for each phase were compared between the two groups. In addition, the walking distance displayed on the treadmill monitor at the end of the exercise period was recorded and compared between the two groups. We used two-way analyses of variance for comparing the mean quantitative variable changes. A p-value less than 0.05 was considered to indicate statistical significance. Statistical analyses were performed using GraphPad Prism 9 (GraphPad Software Inc., La Jolla, CA, USA).

Results

Sixteen men and six women participated in this study. The mean age (standard deviation; SD) was 22.4 (2.73) years. The mean height (SD) was 167.1 cm (8.0) and mean weight (SD) was 63.5 kg (15.3), as shown in Table 1. Of the 22 participants, 3 were smokers. The participants, on average, exercised for 2.2 (2.15) days per week. The resting SpO_2 values (95% confidence interval [CI]) in both the control and OX groups were 97.2% (1.49) and 95.0% (3.98), respectively, and no significant difference was observed (P = 0.13).

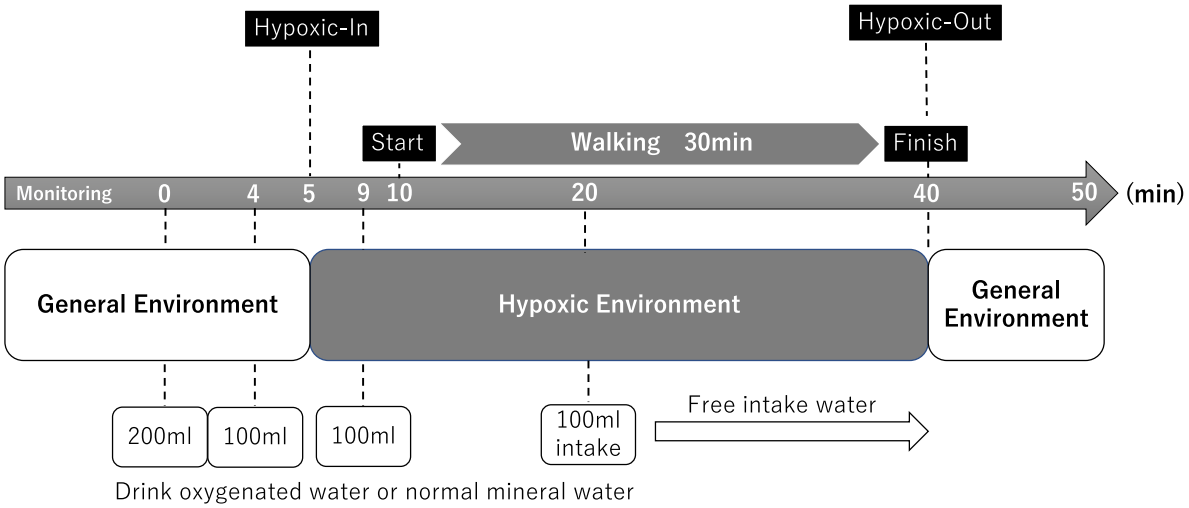

Figure 1 Flow of the experiments
Intake: drinking oxygenated water or normal mineral water

Table 1 Demographic characteristics of participants

	Control (SD) n=11	Oxgen Water (SD) n=11	P-value
Sex (Male/Fmale)	8/3	8/3	
Age (Y)	22.7 (2.53)	22.1 (3.02)	0.60
Height (cm)	167.7 (8.94)	166.6 (7.36)	0.76
Weight (kg)	66.7 (18.77)	60.4 (10.85)	0.34
Smoker	3/11	0/11	
Exercise habits (days/week)	1.5 (2.34)	2.9 (1.81)	0.14
SpO_2 (%)	97.2 (1.49)	95.0 (3.98)	0.13
Pulse rate (bpm)	85.1 (10.65)	79.7 (18.47)	0.41

The walking distances during exercise in the OX (SD) and control groups (SD) were 3.5 (0.3) km and 3.39 (0.27) km, respectively, demonstrating no significant difference ($P = 0.32$). During walking, the Borg scores (SD) in the OX and control groups were 12.0 (0.7) and 11.5 (0.8), respectively, showing no significant difference ($P = 0.14$).

At 10–40 min, SpO_2 reduction was observed in both groups compared with the baseline values ($P < 0.05$). Differences between the two groups were observed at 15–40 min (Figure 2). Regarding PR, the baseline values of the control and OX groups (95% CI) were 85.1 (10.7) and 79.7 (18.5) bpm, respectively. During exercise, the PR values at 10–40 min in both groups were increased compared with the baseline values. Furthermore, at 10–35 min, the PR values in the OX group were increased compared with those in the control group ($P < 0.05$), similar to SpO_2 (Figure 3).

Discussion

In this study, we compared temporal changes in SpO_2 and PR during exercise under hypoxic conditions after consumption of oxygenated mineral water or normal mineral water. The results demonstrated a decrease in SpO_2 and an increase in PR during walking exercise in both groups under hypoxic conditions. Moreover, the decrease in SpO_2 was smaller and the increase in PR was greater in the group that drank oxygenated water compared with the group that drank normal mineral water.

A decrease in arterial partial oxygen pressure (PaO_2) and arterial oxygen saturation (SaO_2) caused by exercise is known as exercise-induced arterial hypoxemia (EIAH)[13]. EIAH is defined as a decrease in PaO_2 of ≥ 10 mmHg or a decrease in SaO_2 of ≥ 3% compared with these values at rest. SpO_2 and transcutaneous measurement of SaO_2 are

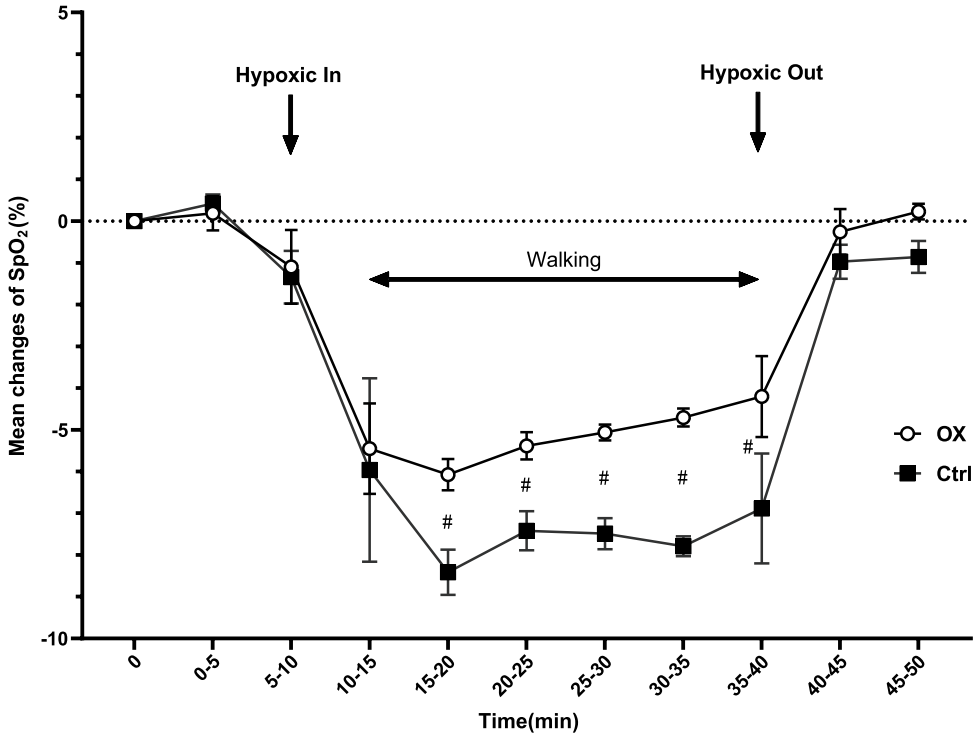

Figure 2 Changes in SpO_2 during 30 min walking exercise
Error bar, 95% confidence interval. #, P < 0.05
OX: OX group (drinking oxygenated mineral water with high oxygen concentration)
Ctrl: Control group (drinking normal mineral water)

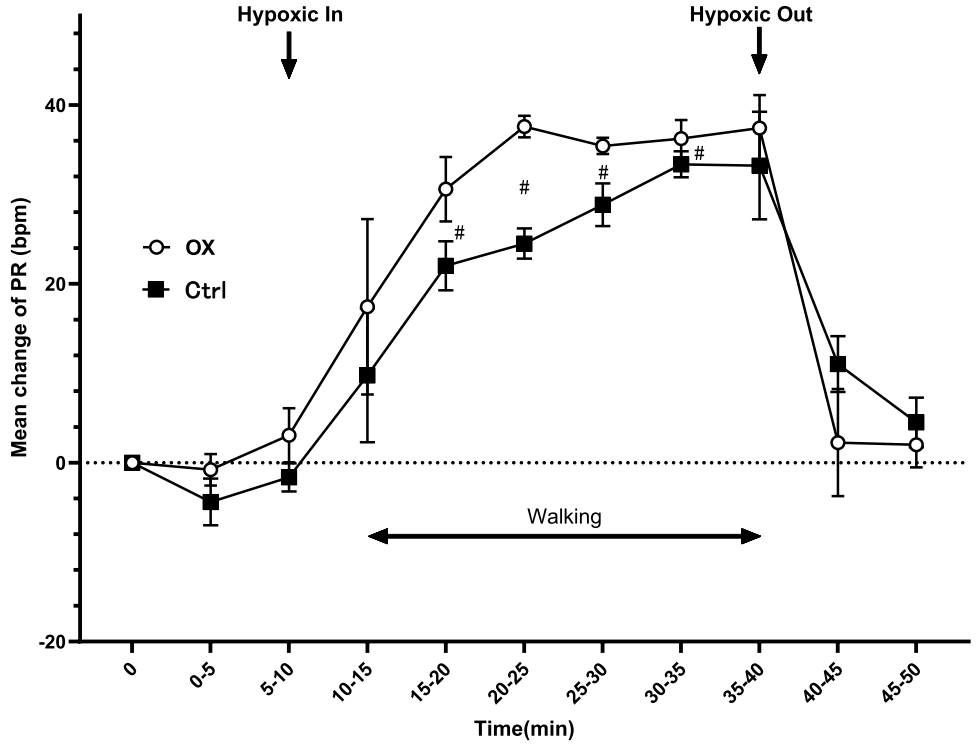

Figure 3 Changes in pulse rate (PR) during 30-min walking exercise
Error bar, 95% confidence interval. #, P < 0.05
PR: pulse rate
OX: OX group (drinking oxygenated mineral water with high oxygen concentration)
Ctrl: Control group (drinking normal mineral water)

almost identical[14]. In this study, SpO_2 was decreased by ≥ 3% in both groups, indicating that the participants exhibited EIAH. A previous study reported that maximal performance capacity is impaired in highly trained cyclists working under 87% SaO_2, but not under a milder desaturation level of 90%[15]. Arterial desaturation occurs in healthy, highly trained endurance athletes during heavy exercise, and the level of arterial desaturation is inversely related to maximal oxygen consumption (VO_2max)[10]. Although drinking oxygenated water did not protect against EIAH in the present study, SpO_2 was maintained at 90%. Given that exhaustion caused by EIAH shortens the exercise time and decreased SaO_2 affects muscle fatigue[16], drinking oxygenated water may protect athletes from performance decreases.

Environments with low SpO_2 include high-altitude situations, such as in-flight or alpine environments. SpO_2 decreases during exercise in a hypoxic environment and results in a lack of oxygen supply to tissues, leading to illness or fatigue[17]. Reports on mountaineering exercise show that SpO_2 decreases and PR increases, particularly when physical conditions are not good. Understanding the hypoxic state of the body and managing and assessing its physical condition are effective for preventing acute mountain sickness[14, 18]. Reducing the decrease in SpO_2 by drinking oxygenated water may prevent acute mountain sickness, prolong walking time, and increase walking speed. Similarly, in in-flight environments (altitude of 40,000 ft), the oxygen concentration is approximately 16%, and SpO_2 is reported to decrease in individuals with respiratory dysfunction[19]. Even in healthy individuals, SpO_2 decreases significantly in in-flight environments[20]. Therefore, drinking oxygenated water before or during flight may suppress SpO_2 reduction and help maintain physical condition.

Oxygen is mainly absorbed into the body by inhalation. However, a previous study in pigs demonstrated that administering oxygenated water into the stomach increased SaO_2. These results suggest that the administration of oxygenated water could be an alternative route of oxygen absorption[21]. In the present study, oxygenated water with an oxygen concentration of 110 ppm (110 mg O_2/L) was used, but the speed of absorption into the body was unclear. In a previous study[22], different concentrations of oxygenated water (40, 80, and 150 mg O_2/L) were intraperitoneally injected in rabbits, and the authors demonstrated that different oxygen concentrations have different absorption speeds. Optimizing oxygen levels in water could potentially lead to better outcomes, and further research on this topic is warranted.

Our data demonstrated that PR was higher in the OX group compared with the control group. We hypothesized that SpO_2 decreases in hypoxic environments, as demonstrated in a previous study[23]. A previous study involving a walking exercise experiment reported that pulse oximeter measurements in the hand may provide false readings because of body movement, and this effect is more likely to be seen in the measurement of PR compared with that of SpO_2[24]. Alternatively, it is possible that our findings were caused by differences in the physical abilities of the participants. Although participants were randomly assigned and their baseline demographic data were similar, some of the participants in the OX group may have had inferior physical abilities. However, we monitored exercise intensity using the Borg scale[12, 25], and walking distances did not differ between groups. Although it is unclear why participants in the OX group had higher PR, it is important to be aware of this phenomenon to identify individuals who might experience harmful effects of increased PR.

One strength of the current study is the experimental design involving a randomized placebo-controlled single-blinded trial, which would be expected to reduce the effects of bias. However, this study also involved several limitations. First, the sample size was small. Although a small sample size can result in type 2 error, it is unlikely that this error occurred in the current study because the differences between the groups were statistically significant. Second, the participant selection method may have affected the results. We enrolled young adults aged 20–29 years. Although the changes in younger and older populations were not investigated, changes in SpO_2 and PR may be more prominent in older participants. Third, the effects of oxygenated water under normal conditions remain unclear. In conclusion, the current study demonstrated that SpO_2 was decreased and PR was increased under normobaric hypoxic conditions. Moreover, consumption of oxygenated water

containing 110 ppm oxygen during walking exercise under normobaric hypoxic conditions suppressed SpO_2 reduction.

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Author contributions

MN and SN analyzed and interpreted the data of the participants in this study. All authors read and approved the final manuscript.

Conflicts of interest statement

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Original Articles

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Protective Effects of Hydrogen-rich Water Intake on Renal Injury in Neonatal Rats with High Oxygen Loading

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Objectives: This study aimed to investigate the protective effects of hydrogen-rich water (HW) intake on renal injury in neonatal rats with high oxygen loading.

Materials: We used pregnant and newborn Sprague-Dawley rats.

Methods: Four groups were set up, with mother and newborn rats immediately after delivery as one group: RA-PW (room air and purified water), RA-HW (room air and HW), O_2-PW (80% oxygen and purified water), and O_2-HW (80% oxygen and HW). The newborn rats were maintained in either a normoxic (room air, 21% oxygen) or controlled hyperoxic (80% oxygen) environment from birth. Then, HW (O_2-HW and RA-HW groups) or PW (O_2-PW and RA-PW groups) was administered to parents of each group.

Results: The number of immature glomeruli significantly increased in the O_2-PW group (exposed to hyperoxia). Conversely, the O_2-HW group had significantly fewer immature glomeruli than O_2-PW group. In the RT-PCR analysis of kidney tissue, α-SMA, TGF-β, and TNF-α levels were significantly higher in the O_2-PW group than in the RA-PW group and significantly lower in the O_2-HW group than in the O_2-PW group.

Conclusions: HW intake can potentially reduce oxidative stress and prevent renal injury in neonates with high oxygen loading.

Key words: nephrogenesis, immature glomeruli, neonatal hyperoxia, molecular hydrogen

Introduction

Advancement in perinatal care has improved the survival rate of premature and low-birth-weight infants, but the risk of developing organ dysfunction remains (Development Origins of Health and Disease [DOHaD] hypothesis)[1)]. Kidney injury (glomerular filtration rate decrease/proteinuria), hypertension, and renal failure reportedly occur in preterm and low-birth-weight infants[2)]. Oftentimes, preterm neonates are born while their renal system is still developing because normally, nephrogenesis is not completed until 34–36 gestational weeks[3)]. Thus, renal development continues after birth in these infants. However, the glomerular abnormalities and reduced glomerular formation,

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as well as increased proportion of immature glomeruli, are observed in this population. Thus, postnatal nephrogenesis may be potentially impaired[3,4]. Several children and adults born prematurely suffer from hypertension[5,6], reduced kidney size[7,8], and impaired renal function[9,10], highlighting the long-term consequences of preterm birth on renal health. The cause of impaired renal development after preterm birth and the mechanisms through which it may predispose adults to renal disease are still largely unknown; exposure to oxygen in the extrauterine environment may be a contributing factor[11-13]. At birth, infants are exposed to oxygen concentrations far exceeding the intrauterine levels[14], causing oxidative stress[15,16], which they are particularly susceptible to because they have low antioxidant levels[17,18]. Macrophage infiltration, reactive oxygen species (ROS) activity, and renin-angiotensin system activation are essential in renal injury pathogenesis[19]. Oxidative stress is a common pathway leading to chronic renal damage, with damage to cells, tissues, and organs caused by ROS[20]. It has been implicated in numerous commonly occurring preterm birth-related conditions, including retinopathy of prematurity (ROP)[21]. High oxygen load increases oxidative stress in rats, resulting in glomerular developmental disorders[22].

Alternatively, hydrogen reduces oxidative stress, effectively protecting the tissue from injury. Hydrogen selectively decreases the production of hydroxyl radical and peroxynitrite, which are two of the most cytotoxic ROS, and protects against oxidative stress[23]. In rats, hydrogen-rich water (HW) and hydrogen saline significantly attenuate renal ischemia-reperfusion injury and reduce the serum levels of 8-OHdG (a biomarker of oxidative DNA damage)[24,25]. Several nephrology studies have also demonstrated the efficacy of hydrogen.

In this study, we aimed to investigate the effect of hydrogen on oxidative stress and glomerular developmental disorders in newborn rats with a high-concentration oxygen load.

Materials and Methods

Animals

The Juntendo University Animal Care Facility (Tokyo, Japan) approved all of our study procedures. Female Sprague-Dawley rats at gestational day 19 were purchased from Nihon SLC, Co., Ltd. (Shizuoka, Japan) and housed in individual cages in the same room at 24°C-25°C, with a relative humidity of 60% under a 12-hour/12-hour light/dark cycle and free access to food and water, at the Juntendo University Animal Care Facility. Pups were born naturally at term and maintained in either 80% oxygen (a mixture of medical-grade 100% oxygen and room air [RA]; Oxycycler ProOx 110; Biospherix, Lacona, NY, USA) or RA from postnatal day (PD) 0 to PD 12. The approval number obtained from the Juntendo University Animal Care Facility is 2020176.

Experimental groups

Pregnant Sprague-Dawley rats were divided into four groups. From birth to PD 12 , two groups were bred in 80% oxygen, and the other two were in RA. HW (O_2-HW and RA-HW groups) or purified water (PW; O_2-PW and RA-PW groups) was administered to parents of each group after birth. To administer water and molecular hydrogen to newborn rats, there is a method of injecting water and molecular hydrogen directly into the stomach with a gastric catheter. However, this method had a problem in continuing the experiment for small rats. Therefore, with a view to future clinical application, we selected a method in which water and molecular hydrogen were orally administered to the mother rat and the effect was judged in the newborn rat raised by the mother rat's breast milk. The pups were sacrificed at PD 19, and their kidneys were excised for tissue histologic examinations and biochemical assays. The weight of kidneys, number of glomeruli and immature glomeruli, and levels of other markers were compared and validated.

Histopathological and immunohistochemical analyses

The harvested kidneys were fixed in 10% formalin, embedded in paraffin, and sliced into sections starting from the central region of the kidney across the full coronal plane. The slides containing the sliced sections were stained with hematoxylin and eosin and examined by optical microscopy. For each section, three microphotographs from the anterior, posterior, and mediolateral regions of the kidney, each including the full thickness of the cortex, were obtained using a digital

camera (DS-L3; Nikon, Tokyo, Japan) at 400× magnification. To determine any changes in the number of nephrons, we counted the glomeruli and immature glomeruli in the whole section starting from the central region of the kidney across the full coronal plane in P19 pups. Mature glomeruli were defined as glomeruli with loose tuft structure, lobulation, and patent capillary loops lined with typical podocytes, whereas immature glomeruli referred to glomeruli with at least half of the circumference of capillary loops was densely lined with dark cuboidal epithelial cells. The lumen of the loops is generally narrow, with no tuft lobulation.

Reverse transcription-polymerase chain reaction (RT-PCR)

The expression levels of α-SMA, TGF-β, and TNF-α in the renal cortex were determined by real-time RT-PCR using the TaqMan system according to the manufacturer's protocol. The samples were homogenized with a buffer RLT added and rotated twice at 3000 rpm for 30 seconds using a rotor-stator homogenizer. TaqMan probe-based quantitative RT-PCR was conducted using cDNA synthesized from kidney biopsy. RNA was prepared using the High-Capacity cDNA Reverse Transcription Kit and analyzed using the default protocols of the 7500 Fast Real-Time PCR System (Life Technologies). Using the Standard Curve Method, we normalized each gene expression to GAPDH gene expression. Furthermore, primers and probes for α-SMA ACTA (Rn01759928_g1), TGF-β (Rn00572010_m1), and TNF-α (Rn99999017_m1) were prepared using TaqMan Gene Expression Assays.

Statistical analysis

Data are expressed as mean ± standard deviation. We used one-way analysis of variance to determine the differences between groups and the Bonferroni method for post hoc multiple comparisons. All statistical data were analyzed using SPSS Statistics software, version 17.0 (SPSS Inc., Chicago, IL, USA), and a P value of < 0.05 was considered statistically significant.

Results

Effect of hyperoxia on glomeruli

Hyperoxia exposure did not change the body weight, kidney weight, and the number of glomeruli per section. However, the number of immature glomeruli in the O_2-PW group significantly increased (Figure 1a). The average number of immature glomeruli in the RA-PW and O_2-PW

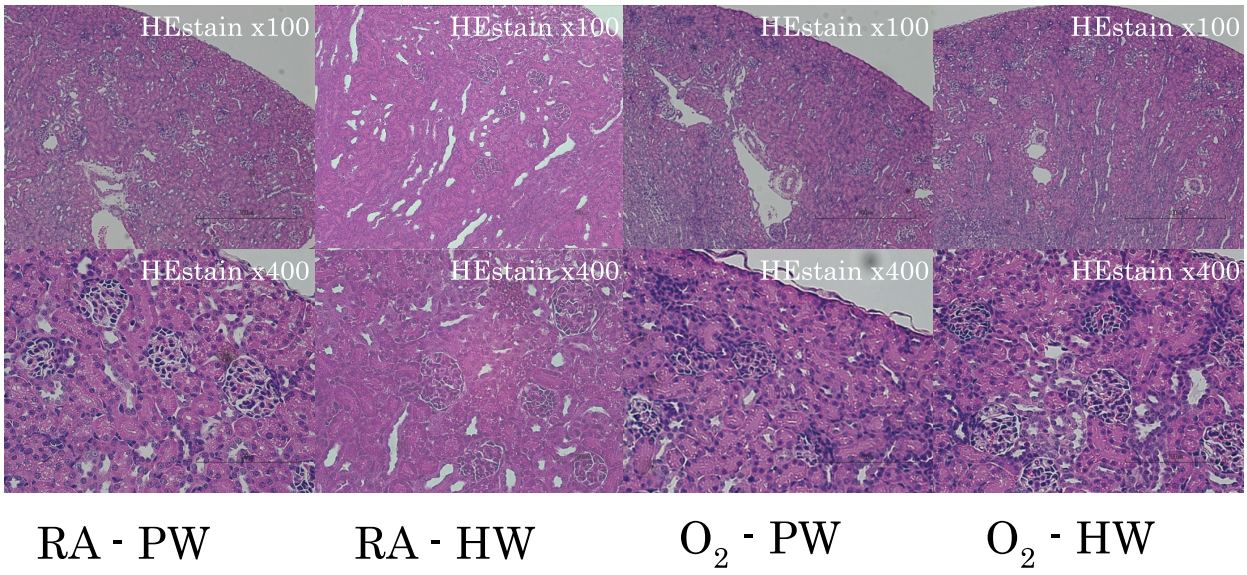

Figure 1a Histological images of the kidneys of newborn rats
In the O_2-PW group, the number of immature glomeruli significantly increased (Hematoxylin and eosin staining of 3μm-thick kidney sections; 100× and 400× magnification).
Abbreviations: RA-PW, the group reared in room air with purified water; RA-HW, the group reared in room air with hydrogen-rich water; O_2-PW, the group reared in 80% oxygen with purified water; O_2-HW, the group reared in 80% oxygen with hydrogen-rich water.

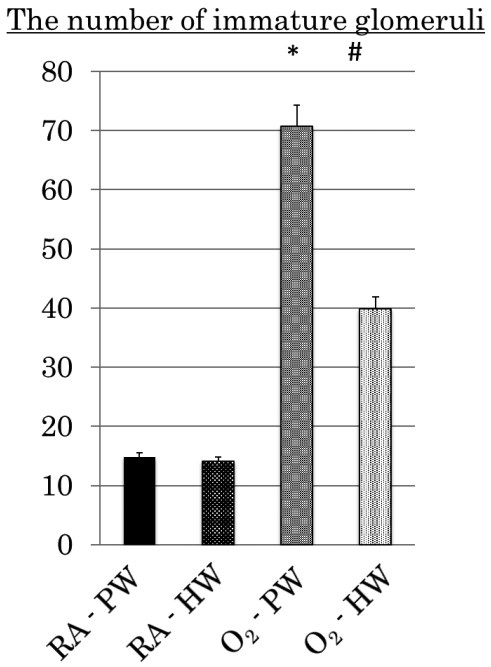

Figure 1b The number of immature glomeruli of newborn rats
Hydrogen-rich water (HW) administration decreased the number of immature glomeruli.

groups was 14.8 ± 1.62 and 70.8 ± 3.30, respectively. Conversely, the O_2-HW group (39.9 ± 2.38) had significantly fewer immature glomeruli (Figure 1b).

Effect of HW on α-SMA, TGF-β, and TNF-α mRNA expression in hyperoxic rats

The RT-PCR analysis of kidney tissue revealed characteristic findings in the O_2-PW group. The O_2-PW group had significantly higher α-SMA, TGF-β, and TNF-α levels than the RA-PW group (α-SMA: 1.89±0.44 vs. 0.86 ± 0.07; $P < 0.05$; Figure 2-A) (TGF-β: 1.28 ± 0.24 vs. 0.70 ± 0.07; $P < 0.05$; Figure 2-B) (TNF-α: 1.83 ± 0.60 vs. 0.67 ± 0.08; $P < 0.05$; Figure 2-C). The O_2-HW group also showed characteristic findings. The expression of α-SMA, TGF-β, and TNF-α was significantly suppressed in the O_2-HW group than in the O_2-PW group (α-SMA: 0.79 ± 0.08 vs. 1.89 ± 0.44; $P < 0.05$; Figure 2-A) (TGF-β: 0.74 ± 0.08 vs. 1.28 ± 0.24; $P < 0.05$; Figure 2-B) (TNF-α: 0.91 ± 0.05 vs. 1.83 ± 0.60; $P < 0.05$; Figure 2-C).

Discussion

This study demonstrated that molecular hydrogen administration suppressed the generation of immature glomeruli caused by high-concentration oxygen administration. Yzydorczyk et al.[12] showed that nephron endowment was reduced by 25% in 25- to 35-week-old rats exposed to hyperoxia during postnatal nephrogenesis (80% oxygen from P3 to P10). Although the glomerular number decreased,

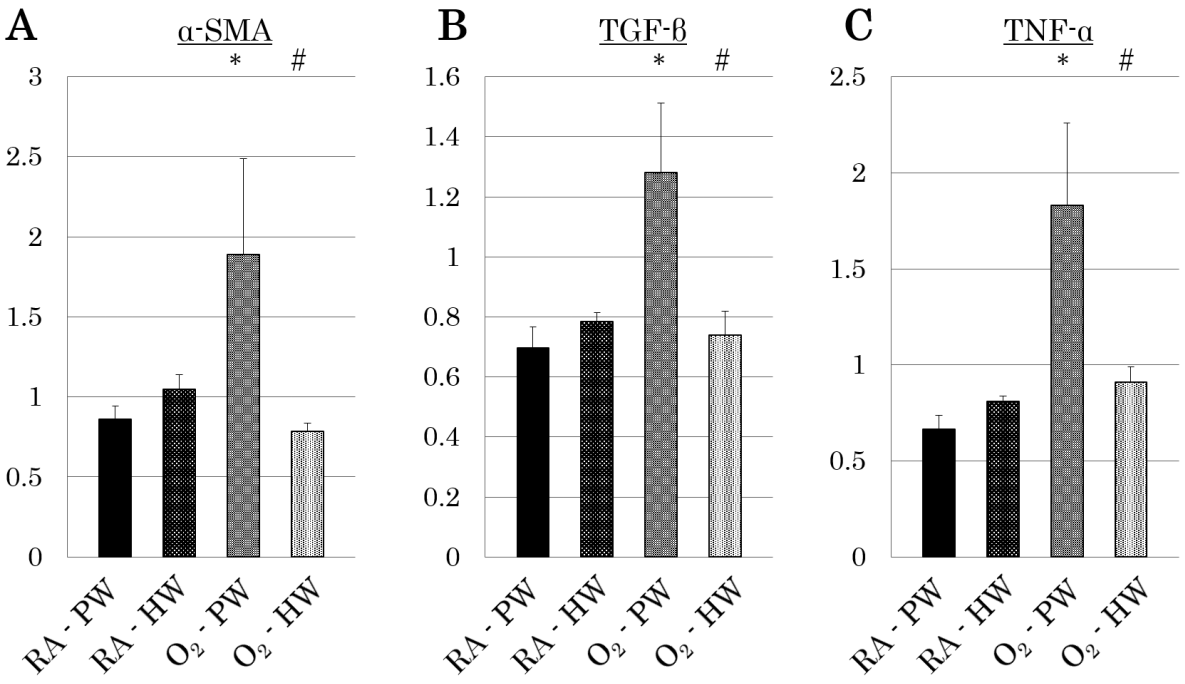

Figure 2 RT-PCR analysis of renal specimens
In RT-PCR analysis, hyperoxia exposure led to the increase of α-SMA (A), TGF-β (B), and TNF-α (C) expression, and hydrogen-rich water (HW) administration decreased their expression.

they vaguely addressed whether hyperoxic exposure in the neonatal period directly impaired nephrogenesis because the end points were assessed in adult rats. Popescu et al.[26] reported that P5 rat pups exposed to hyperoxia showed a decreased glomerular size during postnatal nephrogenesis (80% oxygen from P3 to P10), whereas the P10 rat pups showed no change in glomerular count. Hyperoxia exposure in mice during postnatal nephrogenesis (65% oxygen from P0 to P7) did not cause overt adverse effects on renal development[27], but the glomeruli were enlarged in early adulthood. Nakagawa et al.[20] also reported that hyperoxia exposure suppresses glomerular development. Regarding the association between oxidative stress and immature glomeruli, the proportion of immature glomeruli increases in preterm birth[28]. In our study, high oxygen levels significantly increased the number of immature glomeruli.

With regard to the relationship between oxidative stress and hydrogen, hydrogen selectively scavenges harmful ROS, such as hydroxyl radical and peroxynitrite; thus, nucleic acid oxidation and lipid peroxidation are mitigated, and the cells or tissues are protected against oxidant stress and apoptotic injury[23]. Moreover, hydrogen increases the activity of antioxidant enzymes, including superoxide dismutase and catalase, and inhibits TNF-α and interleukin (IL)-6, thereby suppressing inflammation[29]. In addition to directly neutralizing highly reactive oxidants, hydrogen indirectly reduces oxidative stress by regulating the expression of various genes. In the study of Nakashima et al., hydrogen decreased apoptosis and nephrotoxicity in rat models of cisplatin nephrotoxicity[30]. Moreover, by reducing oxidative stress and suppressing the activation of inflammatory signaling pathways and cytokine production, HW prevented chronic allograft nephropathy in a kidney transplantation model; thus, the allograft function and overall survival improved[31]. Therefore, HW can decrease oxidative stress and prevent damages in the kidneys.

Our study revealed that through hyperoxia exposure, the number of immature glomeruli can be significantly increased, but HW can significantly suppress such increase. The abovementioned studies suggest that molecular hydrogen suppresses active oxygen and is related to the suppression of renal development disorder. This study is the first to report that molecular hydrogen suppresses the production of immature glomeruli caused by high oxygen load.

In the RT-PCR analysis of kidney tissue, the levels of α-SMA, TGF-β, and TNF-α were significantly higher in the O_2-PW group than in the RA-PW group. In contrast, they were significantly suppressed in the O_2-HW group compared with those in the O_2-PW group.

TGF-β induces fibrosis by promoting α-SMA expression and enhancing collagen fibronectin secretion. It also causes glomerular capillaries in rat renal development[32]. Our study suggested that oxygen administration impaired the formation of glomerular capillaries and was suppressed by molecular hydrogen administration.

Angiotensin II elevation resulting from renal damage and oxidative stress promotes TNF-α production[33]. The intravenous administration of TNF-α causes damage to glomerular endothelial cells and the glomerular endothelial surface layer[34]. Our study suggested that TNF-α increased by oxidative stress damaged the glomerular endothelium but was suppressed by molecular hydrogen administration.

Most hydrophilic antioxidants cannot penetrate biomembranes and remain on the membrane surface, whereas molecular hydrogen administration can be distributed rapidly into lipids and cytosol and has no cytotoxicity, even at high concentrations[35]. A study reported that inhalation of molecular hydrogen gas did not affect physiological parameters such as pH and blood electrolytes and had no adverse effects[36]. In addition, oral HW intake is a useful route for molecular hydrogen administration because it is portable, safe, and does not alter the taste, smell, or pH of foods, drinks and drugs. In our study, no adverse events were observed in rats.

Our findings demonstrate the protective effect of HW intake on renal impairment. Therefore, HW may delay dialysis or kidney transplantation in children with renal impairment.

In conclusion, hyperoxia exposure during nephrogenesis causes renal impairment, whereas HW reduces oxidative stress and suppresses renal impairment.

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Author Contributions

MS, AE, HM, AM, MN analyzed and interpreted the data regarding the newborn rats. AM, YG, TH, KS, YM, NN, SF, YO and TS prepared or revised for important intellectual content. AE was a major contributor in writing the manuscript. All authors read and approved the final manuscript.

Conflicts of interest statement

The authors declare that there are no conflicts of interest.

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Original Articles

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Development of the Japanese Version of the Self-Endangering Work Behavior (J-SEWB) Scale

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Objective: The concept of self-endangering work behavior (SEWB) was recently proposed to describe problematic behaviors to cope with heavy workloads and self-management. Although SEWB may enable workers to achieve immediate goals, it risks health and long-term work capacity. In this study, we developed a Japanese version of the SEWB (J-SEWB) scale, which was originally in German, and verified its validity and reliability.

Materials: The original SEWB scale consisted of 21 items, constituting five subscales: "Intensification of working hours," "Prolongation/extension of working hours," "Refraining from recovery/leisure activities," "Working despite illness," and "Use of stimulating substances." We translated the scale into Japanese, then checked the wording using back-translation.

Methods: The J-SEWB scale and questions for working conditions and sociodemographic variables was administered via an online survey with 600 participants registered with an internet survey company in Japan. Cronbach's α coefficients were calculated for each subscale to assess internal consistency. Construct validity was examined using principal factor analysis with equamax rotation. An analysis of variance evaluated the relationships of J-SEWB scores with working conditions and sociodemographic variables.

Results: Cronbach's α coefficients ranged from 0.846 to 0.964 for five subscales, and 0.957 for all 21 items (total J-SEWB score) in 600 participants. The factor analysis identified five factors, classifying 21 items into corresponding subscales. Total J-SEWB scores were significantly higher for flexible work as well as longer working hours.

Conclusions: The J-SEWB scale appears to be an effective tool for assessing SEWB in Japanese employees, with satisfactory reliability and construct validity.

Key words: self-endangering work behavior, overwork, presenteeism, flexible work style

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Introduction

In Japan, the needs of workers are diversifying[1] and the government is promoting flexible work styles, such as telework, side jobs and freelance work, establishing new work styles and promoting regional revitalization through remote work[2]. The proportion of remote workers is increasing, and remote work is estimated to account for more than 20% of the working population in Japan[3]. The demand for such flexible working styles is increasing worldwide, and companies are giving workers more control over their daily work based on self-management and self-discipline[4,5]. While these changes provide opportunities for personal growth and coordination between work and personal life, they also require the need for self-management, and can lead to problematic behaviors to cope with heavy workloads[6–8]. This coping behavior is referred to as self-endangering work behavior (SEWB), which refers to work behaviors that may help achieve immediate goals while simultaneously creating risks for employee health and long-term work capacity[6–8].

The concept of SEWB is a combination of several maladaptive coping styles that have so far been studied separately, such as extension of working hours, intensification of working hours, sickness presenteeism, faking, substance abuse to recuperate, substance abuse to perform, reduction of quality, and bypassing safety standards[6–8] A central predictor for the occurrence of SEWB is increased results-oriented management in companies (indirect control instead of command-and-control)[9,10]. In workplaces using this type of management, employees are given freedom to make decisions, but at the same time they are responsible for achieving demanding goals. Knecht et al.[11] reported that SEWB contributed to the link between work burden and exhaustion in 607 workers under indirect control. Baeriswyl et al.[12] confirmed these results in a sample of 560 teachers, reporting that extension of working hours partially mediated the effect of workload on emotional exhaustion. Steidelmüller et al.[13] reported that the frequency of presenteeism (working despite illness) increased with the number of hours of teleworking per week among 25,465 respondents in the 6th wave of the European Working Conditions Survey 2015.

To quantitatively evaluate the SEWB, some of the authors of the present study[6,8] created a self-administered questionnaire called the SEWB scale, in German, consisting of 21 items with a 5-step Likert scale, which included five subscales: "Intensification of working hours," "Prolongation/extension of working hours," "Refraining from recovery/leisure activities," "Working despite illness," and "Use of stimulating substances." Using this scale, the authors found that emotional exhaustion and psychosomatic complaints of workers were increased by SEWB (excluding "Extension of working hours") in 485 professionals, including engineers, architects, computer engineers, advertisers, and lawyers as well as scientists. The study showed the relationship between stressors and exhaustion is partially mediated by SEWB. Another study[14] reported that SEWB may moderate the relationship between alleged challenge stressors such as time pressure, irritation and work engagement, increasing the strain effect and reducing the challenge effect of time pressure.

In the current study, we developed a Japanese version of the SEWB scale (J-SEWB) to enable further research in Japanese employees regarding work behavior and health status, as the population of employees engaging in flexible work is rapidly increasing. Understanding the processes by which flexible work arrangements can lead to health impairments via maladaptive coping behaviors such as SEWB may help to better achieve the benefits of flexible work without the negative health impacts in Japan.

Materials and Methods

Self-Endangering Work Behavior (SEWB) scale

The SEWB scale was developed as a self-administered questionnaire consisting of 21 items, including five subscales: "Intensification of working hours," "Prolongation/extension of working hours," "Refraining from recovery/leisure activities," "Working despite illness," and "Use of stimulating substances." The five subscales contain 3, 4, 6, 5, and 3 items, respectively. All of the self-endangering items were scored on a five-point Likert scale that ranged from 1 (rarely/never) to 5 (very often). Respondents were asked to report the frequencies of various behaviors, such as working despite illness. The scores of the items were totaled

and used as the subscale score. The total score of the five subscale scores was taken as the total SEWB score. The process of selecting questionnaire items and validation as well as English translation of 21 items was reported previously by some of the authors of the present study[6, 8].

Translation

First, the German version of the SEWB scale was translated into Japanese by one of the authors (YK). Next, a bilingual (German and Japanese) author (FN), who had not read the original items, conducted back-translation into German. The German author of this study (JD) examined the quality of translated versions, having compared them with the original German version. Based on this author's evaluation, corrections were made for words, meanings, and item content by the authors (KY, YK, and FN) with the assistance of a Japanese employee in JD's laboratory. The final items of the J-SEWB scale are listed in the Appendix.

Survey questionnaire and protocol

The questionnaire used in the current study consisted of the J-SEWB scale and questions regarding sociodemographic variables, such as age, gender, job, work conditions, and annual income. The internet survey was outsourced to a research company (hamon Inc, Yokohama, Japan). Of the 1,052,566 registered individuals, 4,057 full-time employees aged 20 to 64 years who worked 30 hours or more a week were randomly selected (2,399 men and 1,658 women). These respondents were asked to answer an online questionnaire from September 8th, 2021, adjusting the number of responses by age group to be similar to the result of Labor Force Survey in Japan (2020)[15]. The survey was discontinued when the total number of answers reached 600; this sample size was the maximum that the research budget allowed, and exceeded the size (300 or more) that would give stable results in factor analysis[16]. Other than listed here, there were no conditions to include or exclude study subjects.

Statistical analysis

The Cronbach's α coefficient was calculated for each subscale to assess the internal consistency. Construct validity was examined using principal factor analysis with equamax rotation. Relationships among J-SEWB scores and sociodemographic variables were examined using t-test, χ^2 test, or analysis of variance. All of the statistical analyses were conducted using IBM SPSS version 26.0.

Ethical issue

This study was conducted after approval by the International University of Health and Welfare Research Ethics Committee (21-Ig-13, May 19, 2021). Participants agreed to participate voluntarily in the survey under a contract with the research company and were anonymous to us; we did not get informed consent from each.

Results

Sociodemographic characteristics of 600 participants by gender are shown in Table 1. The proportions of participants in each age group were 18.3%, 21.2%, 27.3%, 24.1%, and 9% for the 20–29, 30–39, 40–49, 50–59 and 60–64 years age groups, respectively. Participants were mainly office workers (76.2%) and public officials (10.8%) with a smaller proportion of professionals and faculty/researchers (5.7%). Working hours per week were 30–49 hours for most of participants (86.3%), whereas a small proportion (13.7%) reported working 50 hours or more per week. Approximately 30% of participants worked under a non-fixed (flexible) working hours system, whereas the remaining 70% worked under a fixed working hours system. Half of the participants earned 4 million Yen or more per year.

Table 2 shows scores and Cronbach's α coefficients for the J-SEWB scale. Cronbach's α coefficients were 0.846 to 0.946 for five subscales, and were 0.958 (men), 0.951 (women), and 0.957 (combined) for total SEWB scores. Average scores on the total SEWB and five subscales in men were higher than those in women. These differences were statistically significant (t-test, $p < 0.05$) except for "Intensification of working hours" and "Use of stimulating substances" ($p > 0.05$).

Table 3 shows factor loadings for 21 items. A scree plot is drawn in the Figure 1. Five factors were extracted by factor analysis, classifying 21 items into their corresponding subscales, except that item 7, "I work more than 10 hours a day without being directed to do so," which was most heavily loaded on "Prolongation/extension of working

Table 1 Sociodemographic characteristics of 600 participants (329 men and 271 women)

	Men	Women	Total
Age (years):			
20–29	57	53	110
30–39	70	57	127
40–49	91	73	164
50–59	79	66	145
60–64	32	22	54
Job:			
Public official (office work)	23	23	46
Public official (technical)	18	1	19
Office worker (office work)	79	140	219
Office worker (technical)	78	20	98
Office worker (sales)	25	12	37
Office worker (other)	65	38	103
Professionals (e.g., doctors, lawyers)	12	16	28
Faculty / Researcher	2	4	6
Working hours/week:			
30–39	83	122	205
40–49	180	133	313
50–59	49	13	62
60 or more	17	3	20
Working hour system:			
Fixed	212	197	409
Non-fixed:			
Variable	30	32	62
Flextime	57	28	85
Exemption	22	9	31
Advanced professional type	2	0	2
Others	6	5	11
Annual income in 2020 (1,000 Yen):			
less than 2,000	13	36	49
2,000–3,999	95	156	251
4,000–7,999	171	73	244
8,000–11,999	39	5	44
12,000 or more	11	1	12

hours" in the original German scale, exhibited the highest loading on Factor 2 ("Refraining from recovery/leisure activities").

Table 4 shows the sociodemographic variables that were significantly related to the total J-SEWB scores in a two-way analysis of variance. The total J-SEWB scores were significantly higher for flexible work and longer working hours. The proportion of participants working with flexible hours significantly increased as working hours prolonged.

Discussion

The J-SEWB scale showed good reliability, as indicated by Cronbach's α coefficients of 0.846 or above for all five subscales and the total scores. The factor analysis identified five factors, classi-

Table 2 Scores and Cronbach's alpha coefficients of the J-SEWB scale in 600 participants

	Average	SD	Min	Max	Cronbach's alpha
Men:					
Intensification of working hours	7.2	2.8	3	15	0.891
Prolongation/extension of working hours	8.7	3.7	4	20	0.848
Refraining from recovery/leisure activities	12.3	5.2	6	30	0.934
Working despite illness	9.3	4.9	5	25	0.953
Use of stimulating substances	6.5	3.1	3	15	0.955
Total SEWB	43.9	16.3	21	105	0.958
Women:					
Intensification of working hours	6.8	3.1	3	15	0.933
Prolongation/extension of working hours	7.3	3.5	4	20	0.834
Refraining from recovery/leisure activities	9.9	4.6	6	27	0.933
Working despite illness	8.0	4.1	5	25	0.939
Use of stimulating substances	6.1	3.4	3	15	0.972
Total SEWB	38.0	15.0	21	92	0.951
Total:					
Intensification of working hours	7.0	2.9	3	15	0.912
Prolongation/extension of working hours	8.0	3.7	4	20	0.846
Refraining from recovery/leisure activities	11.2	5.1	6	30	0.937
Working despite illness	8.7	4.6	5	25	0.949
Use of stimulating substances	6.3	3.2	3	15	0.964
Total SEWB	41.2	16.0	21	105	0.957

fying 21 items into their corresponding subscales with one exception, indicating that the construct validity was satisfactory. Furthermore, the total J-SEWB scores were significantly higher with flexible work and longer working hours; the proportion of participants working with flexible hours significantly increased as working hours prolonged. This suggests that the J-SEWB scale reflected overwork, possibly related to flexible work conditions. Thus, the J-SEWB scale appears to be an effective tool for assessing SEWB related to autonomy and self-management in Japanese employees. It should be emphasized that these results were obtained from subjects with the same age structure as the labor force in whole Japan[15].

Among four items of "Prolongation/extension of working hours," item 7, "I work more than 10 hours a day without being directed to do so," was most heavily loaded on "Refraining from recovery/leisure activities" in the present study. A positive response to item 7 indicates that employees work for a long time, while the other three items (No. 4–6) indicate a tendency to work even in leisure time. The results for item 7 may indicate that work interferes with leisure time and causes recreation to be abandoned.

The total SEWB and subscale scores in men were significantly higher than those in women, except for the two subscales, "Intensification of working hours" and "Use of stimulating substances." The gender differences in the total SEWB scores were still significant after controlling for the effects of working hours as well as the working hours system (fixed or non-fixed) in a two-way analysis of variance. Although the reasons for this gender difference were not investigated in the current study, this finding could potentially be related to the working environment of female workers in Japanese companies. In Japan, the proportion of managerial positions occupied by women was only

Table 3 Factor loadings on 21 items of the J-SEWB (principal factor analysis with equamax rotation)

Subscales	Items	Factor 1	Factor 2	Factor 3	Factor 4	Factor 5
IW	1	.089	.123	.157	**.790**	.187
	2	.156	.183	.198	**.825**	.212
	3	.272	.201	.194	**.795**	.202
PW	4	.205	.155	.167	.266	**.733**
	5	.168	.204	.133	.171	**.848**
	6	.203	.455	.195	.255	**.514**
	7	.160	**.523**	.184	.369	.306
RR	8	.260	**.563**	.229	.240	.441
	9	.225	**.576**	.151	.384	.230
	10	.287	**.682**	.214	.273	.325
	11	.323	**.657**	.211	.251	.333
	12	.354	**.699**	.217	.191	.314
	13	.370	**.656**	.217	.196	.353
WI	14	**.715**	.250	.199	.260	.243
	15	**.676**	.339	.252	.161	.277
	16	**.747**	.337	.227	.158	.206
	17	**.833**	.214	.210	.217	.237
	18	**.811**	.164	.209	.255	.249
US	19	.191	.142	**.853**	.173	.171
	20	.147	.115	**.927**	.153	.129
	21	.135	.119	**.926**	.165	.113

Subscales:
IW = Intensification of working hours
PW = Prolongation/extension of working hours
RR = Refraining from recovery/leisure activities
WI = Working despite illness
US = Use of stimulating substances

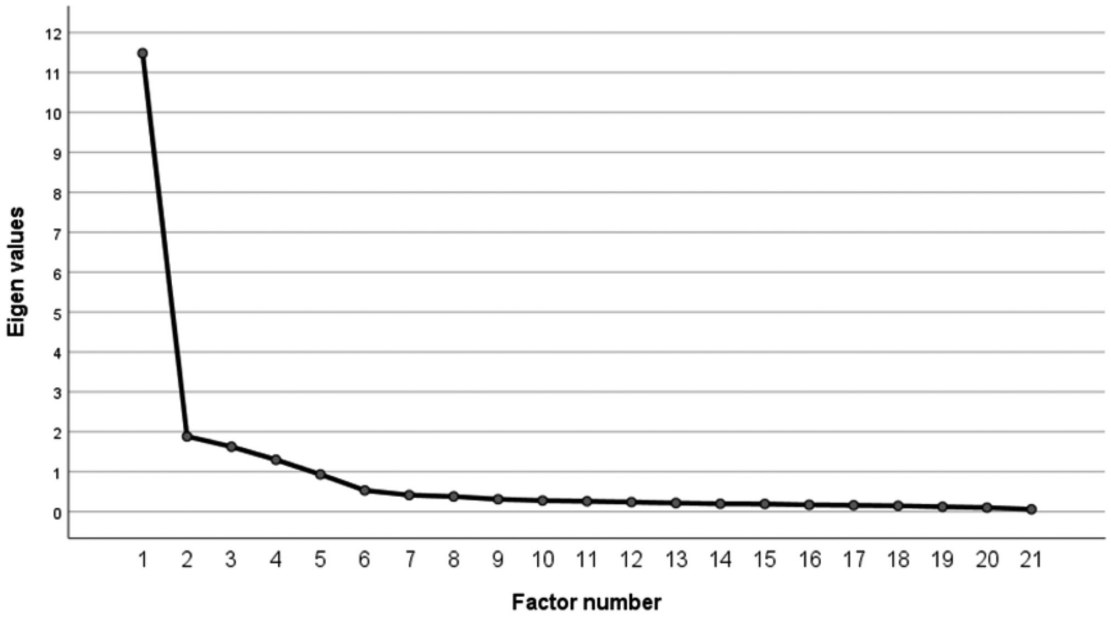

Figure 1 Scree plot of principal factor analysis on 21 items of the J-SEWB in 600 participants (before rotation)

Table 4 Sociodemographic variables significantly related to J-SEWB scores in 600 participants: Two-way analysis of variance

	J-SEWB scores		F-values[a]	
	Mean	SD	Variable	Gender
Working hour system:			11.55*	18.88**
Fixed	39.6	16.0		
Non-fixed	44.8	15.6		
Working hours/week[b]:			6.94**	13.20**
30–39	39.2	16.0		
40–49	40.6	15.4		
50–59	46.6	15.3		
60 or more	56.0	17.9		

*$p < 0.01$, **$p < 0.001$

[a] Two-way analysis of variance using gender and each variable as factors (main effects).

[b] Among participants who worked 40 hours or less, 40–49 hours, 50–59 hours, and 60 or more hours a week, 56 (27.3%), 98 (31.3%), 28 (45.2%), and 9 (45.0%) were working under a flexible working hour system ($\chi^2 = 8.640$, $p < 0.05$).

14.8% in 2019, which is much lower than that in many other countries[17]. The lower degree of flexibility at work might have been underlying the lower SEWB scores in women in the present study.

SEWB is an active coping behavior that has adverse health effects for workers, although it is conventionally believed that active coping behaviors mitigate the psychosomatic effects of stress in contrast to avoidance coping behaviors[7,8]. SEWB might resemble psychological states such as work engagement and overcommitment, in terms of overwork. However, SEWB is a specific observable behavior that may mediate the effects of overload on health impairment[7,8]. Thus, in addition to the occupational stressors and psychosocial modifiers related to the health effects on employees, which have been extensively studied in Japan[18–21], it may be valuable for future studies to examine SEWB as a coping strategy for flexible working styles. Such studies will also verify the concurrent validity of J-SEWB scale by examining its relationships to the existing scales[18–21], which could not be done due to insufficient preparation in the present study.

The increased incidence of SEWB in the workplace may indicate that the design of flexible forms of work needs to be improved. In such cases, determinants of SEWB in relation to occupational stressors and modifiers, including gender, should be investigated to reveal how to improve them. The J-SEWB scale can be used to assess determinants as well as the effectiveness of interventions and may be helpful in health management of workers. A previous study proposed that long working hours may have a cultural background that is specific to Japan, such as an emphasis on signals that show commitment/loyalty to the company and one's efforts for others, rather than results/achievements, groupism, hierarchical relationships, and workload unrelated to core business[22]. An international comparative study on the SEWB that considers differences in workplace culture in future may provide valuable insight.

Conclusions

The J-SEWB scale appears to be an effective tool for assessing SEWB related to autonomy and self-management in Japanese employees, with satisfactory reliability and construct validity.

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Author contributions

KY contributed to all aspects of the study,

including research design, data collection and analysis, and writing the manuscript. JD and AN designed the study with KY.

JD, ND, AK provided the original German version of SEWB scale. The translation was performed by YK, FN, JD, and KY. All of the authors participated in writing the manuscript.

Conflicts of interest statement

The authors declare that there are no conflicts of interest.

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Appendix　Subscales and items of the J-SEWB scale

以下の記述は、仕事上のプレッシャーやストレス、問題の増加に対処するために、労働者がとる反応について述べたものです。あなたが仕事上のプレッシャーやストレス、問題の増加に直面したときに、どのくらいの頻度でこうした反応を示すかをお答えください。

仕事の強化（Intensification of working hours）

1. 負担だと感じるペースで働く。
2. 長期的には維持できないペースで働く。
3. 自分には良くないと分かっているペースで働く。

余暇に及ぶ仕事と待機姿勢（Prolongation/extension of working hours）

4. 余暇の時も自分の上司、同僚、顧客から連絡を受けられるようにする。
5. 余暇の時も仕事の電話を受けられるようにする。
6. 余暇（終業後、休暇、週末、祝祭日）でも（通常に加えて）働く。
7. 指示されなくても、1日10時間以上働く。

レクリエーションの断念（Refraining from recovery/leisure activities）

8. 余暇の活動を取りやめて、その代わりに働く。
9. 仕事のために、十分な睡眠を断念する。
10. 仕事のために、レクリエーション活動（たとえば、趣味、社会活動や文化活動）を断念する。
11. 仕事のために、プライベートの約束（夕食、スポーツ、友人と会うことなど）を取りやめる。
12. 仕事のために、家族とのレクリエーション（たとえば、夕飯や誕生日祝い）を断念する。
13. 仕事のために、レジャー（たとえば、ウォーキングやスポーツ）による癒しを断念する。

疾病就業（Working despite illness）

14. 病気でも職場に行く。
15. 医者に思いとどまるよう忠告されていても働く。
16. 重い病気の症状（たとえば、痛み、悪寒、発熱）があっても働く。
17. 病気でも、勤務時間・シフトいっぱい働く。
18. 病気であるにもかかわらず、無理をして仕事に行く。

パフォーマンス向上のために何かを摂取（Use of stimulating substances）

純粋に楽しむ目的を超えて何か（たとえば、カフェイン、ニコチン、アルコール、薬、その他）を摂取することにより、

19. 仕事のパフォーマンスを上げる。
20. 仕事をうまくやりぬく。
21. 仕事をかたづける。

回答（5段階リッカート尺度）

1＝まったくない／非常にまれである　　2＝まれである
3＝ときどきある　　4＝よくある　　5＝非常によくある

Original Articles

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Effects of Trace Elements on Anthropometric Characteristics of Children: Cobalt and Childhood Body Mass Index

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Objectives: There are many reports on the effects of trace elements on human anthropometric characteristics. Among these elements, cobalt has consistently shown an inverse relationship with obesity risk. In the present study, we aimed to investigate the relationship between urinary levels of trace elements, focusing on cobalt, and childhood obesity, as indicated by the body mass index (BMI) in early adolescents, focusing on the participants' gender.

Design: A cross-sectional study was conducted in the Tokyo Teen Cohort study. Based on urinary samples, we obtained the anthropometric characteristics (weight and height) and potential covariates associated with childhood BMI for 1542 children (mean age=12 years; 860 boys and 682 girls).

Methods: Concentrations of urinary cobalt and 17 other trace elements were measured using inductively coupled plasma-mass spectrometry or inductively coupled plasma-atomic emission spectrometry.

Results: Pearson's correlation coefficient revealed an inverse relationship between the log of cobalt concentrations in the urine and the BMI for the boys ($r=-0.125$, $p<0.001$) and girls ($r=-0.082$, $p=0.033$). Multivariate analysis, adjusted for various covariates, reconfirmed the correlation between urine cobalt and the childhood BMI, only in the boys (beta=−0.14, $p<0.001$).

Conclusions: Among the 18 elements measured in the children's urine, cobalt may exhibit sufficient potency to decrease the risk of childhood obesity, particularly in boys. Future studies are required to clearly determine the magnitude of the effect and the underlying mechanism(s).

Key words: cobalt, trace elements, gender, childhood obesity, body mass index

Introduction

Over the past three decades, the frequency of childhood obesity has been increasing, leading to a worrisome epidemic worldwide[1)]. Currently, it is estimated that more than 38 million children under the age of 5 years and 340 million children/adolescents aged 5–19 years are overweight or obese[2)].

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Owing to the risk of adulthood obesity[3], cardiometabolic mortality, and morbidity[4], childhood obesity is an important public health challenge[5,6]. Moreover, the Harvard Growth Study (1992) found that overweight adolescents had an increased risk of morbidity and mortality from coronary artery disease in the future, regardless of their adulthood weight[7].

Childhood obesity is a complex problem with a multifactorial etiology, including environmental, genetic, and ecological factors[8,9]. For example, excess calorie intake in children may be an intrinsic consequence of unhealthy eating habits. This may include insufficient intake of necessary nutrients or excessive consumption of toxic substances. Additionally, trace elements may contribute to obesity by influencing metabolism. Many previous studies have examined trace element exposure as a risk factor for childhood obesity, and have reported inverse associations between cobalt concentrations in various biological samples and obesity risk (BMI) in children[10–15] and adults[15–17]. In addition, an experimental study demonstrated a difference in lipid profile (TG, HDL, and LDL) and body weight between mice exposed to cobalt and those that were not[18]. However, such relationships between cobalt and glucose and lipid metabolism have not yet been revealed in humans[19].

Previous reports have suggested that the effects of trace metals may vary according to the child's gender, such as lowering of birth weight in male newborns due to elevation of arsenic or lead concentrations in maternal blood[20,21] and increase in the body weight of adult female participants due to higher hair cadmium levels[22]. Additionally, cobalt absorption and/or excretion can be influenced by gender, as serum and urine cobalt concentrations are higher in women than in men[23]. Similarly, studies in France[24] and Taiwan[25] have reported higher urinary cobalt (UCo) concentrations in women than in men. Furthermore, regarding the gender differences in the lipid profile after the onset of puberty, it would be important to assess the relationship between cobalt and childhood obesity in early adolescent boys and girls.

In the present study, we aimed to measure 18 trace elements in urine samples from children to assess their relationship with childhood BMI, mostly focusing on cobalt. Because gender plays an important role in cobalt biokinetics and affects children's anthropometric characteristics, we additionally compared cobalt concentrations, BMI, and their correlations between boys and girls. To our best knowledge, this study is the first to investigate the relationship between UCo and childhood BMI in early adolescents, focusing on the participants' gender.

Materials and methods

In the present retrospective, cross-sectional study, data and urinary samples were obtained from the Tokyo Teen Cohort (TTC)[26]. The TTC is a birth cohort study conducted by the Tokyo Metropolitan Institute of Medical Science for investigating children's physiological and psychological development, including self-regulation and personalized values on adolescents and their primary parents (usually mothers). In this community-based survey, participants were recruited randomly from three municipalities in the Tokyo metropolitan area using the resident registry. Self-report questionnaires and interviews were conducted using 3171 children (10 years old at the baseline survey). In phase two of the study, the participants were aged 12 years when the data were collected for the current study. In this phase, urine samples were collected from 1582 children and stored at −80 °C until the metal analyses.

We extracted data on body weight and height from the TTC dataset, as well as known potential covariates associated with childhood BMI, such as age (month), birth weight (g)[27], sleep duration (weighted average of weekday and weekend sleep hours per night)[28–30], parents' BMI[31–33], parental smoking[34], household income, and parents' education[35]. Forty participants' data were excluded because of missing information regarding the children's height and/or weight. Finally, we included 1542 children, 860 boys (55.8 %) and 682 girls (44.2 %), for statistical analysis (Figure 1).

Analysis of urine sample

Concentrations of trace elements in children's urine samples were measured by inductively coupled plasma-mass spectrometry (ICP-MS)[13,36] or inductively coupled plasma-atomic emission spectrometry (ICP-AES), as previously reported[37]. ICP-MS (Agilent 8800, Agilent Technologies, California, USA) was used for determining Li, V, Cr,

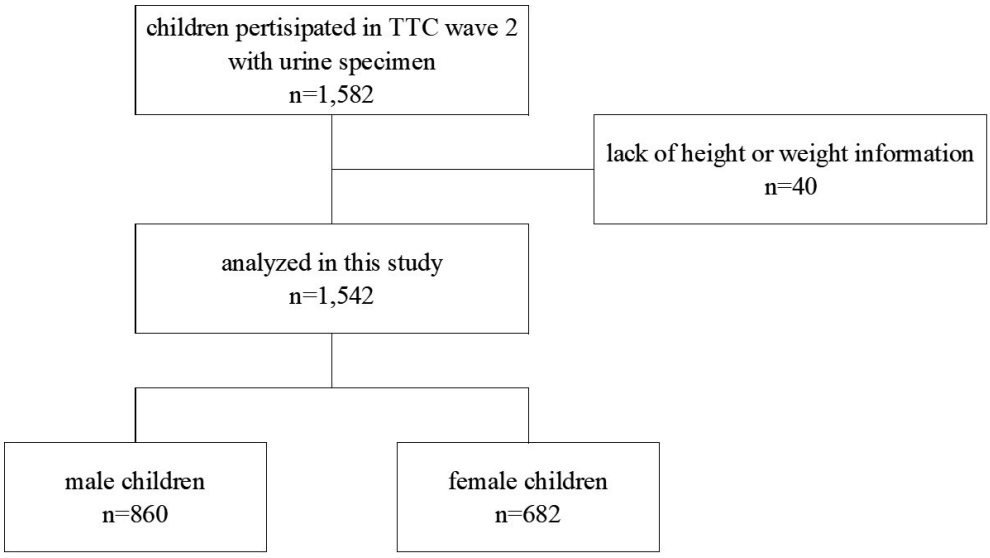

Figure 1 Study flow chart

Co, Ni, Cu, Zn, As, Se, Sr, Mo, Cd, Ba, and Tl, and ICP-AES (Optima 2100, PerkinElmer, Massachusetts, USA) was used for measuring Na, Ca, Mg, and K. For the measurement preparation, urine samples were melted at room temperature and mixed with 0.5 % HNO_3 with 5-fold dilution in ICP-MS and analyzed by the multi-element standard solution XSTC-13 (SPEX CertiPrep, New Jersey, USA) as the external standard solution. For ICP-AES, the urine samples were diluted 10-fold with 0.5 % HNO_3 and analyzed using XSTC-2A (SPEX CertiPrep, New Jersey, USA) as the external standard solution. Measurements were repeated three times, and the average of the three measurements was used for statistical analyses. For instrument calibration throughout the measurements, at least 10 % of the analyses were external standards, and 5 % were blank (pure water).

For statistical analysis, the values under the limit of detection (LOD) were substituted with half the LOD. To correct for variations in urine dilution, the concentration of every trace element was expressed as a ratio to urinary creatinine concentration. To reduce the influence of outliers and normalize the right-skewed distribution, we used the natural logarithm of the urinary concentration of trace elements in the statistical analysis. Among the 18 trace elements, we focused on cobalt, since several studies showed an inverse association of this element with childhood obesity (Table 1).

Statistical analysis

Student's t-test (for continuous variables), Fisher's exact test (for categorical variables), or Mann-Whitney's U-test (ordinal scale) were used for comparisons between the two groups. Pearson's correlation coefficient was used to analyze the relationships between UCo and BMI. Multiple linear regression analysis was performed for assessing the relationships between UCo and urinary concentrations of the other 17 trace elements and BMI, controlling for possible confounding variables. All covariates were included using the forced-entry method. Analyses of all models were gender-stratified. The variance inflation factor (VIF) was employed for checking the multicollinearity problem among the variables. We used Bonferroni's correction to correct multiple comparisons, such as repeating the statistical tests 36 times (18 measured trace elements for both boys and girls), with a p-value<0.001 (0.05/36), which was considered to indicate a statistically significant difference. All statistical analyses were conducted using IBM Statistical Package for Social Sciences (SPSS), version 27.0 (IBM Corp., New York, USA).

Results

The mean BMI of the children was 17.9 kg/m^2 and very close between boys and girls (17.8 and 17.9 kg/m^2, respectively). The mean log UCo was −0.295 μg/g, and there was no significant difference between boys and girls (mean=−0.308 and

Table 1 Previous studies on relationship between trace elements, in different biological samples, and anthropometric characteristics

Country	Poland			USA	Turkey	Iran	Six countries from Europe	USA		USA	Russia
Age	6–17 y			6–19 y	Children with obesity: 10.59±2.90 y; healthy control: 10.71±2.07 y	20 mo to 3 y	6–11 y	Adolescents	Adults	All (women only)	All
Outcome	Obesity			Weight	BMI	Weight	BMI	BMI		BMI	Weight
Sample	Blood	Plasma	Urine	Urine	Serum	Hair	Urine	Urine		Toenails	Hair
Co	(−)	(−)	N/A	(−)	(−)	(−)	(−)	(−)	N/A	(−)	(−)
Li											N/A
Be											N/A
B											N/A
Mg										N/A	(−)
Al											(+)
Si											N/A
V					(−)					N/A	N/A
Cr										N/A	N/A
Mn	N/A	N/A	(+)							N/A	(−)
Fe	(−)	(−)	(−)							N/A	N/A
Ni	(+)	N/A	N/A							N/A	(−)
Cu	(−)	(−)	N/A				(+)			N/A	(−)
Zn	(−)	(−)	(−)			N/A				N/A	(−)
As										N/A	(+)
Se										N/A	N/A
Mo				(−)		N/A	(−)	N/A	N/A		
Cd	(+)	N/A	(+)	(−)	N/A	N/A		N/A	(−)	N/A	N/A
Sn										N/A	N/A
Sb				N/A		N/A		N/A	N/A	N/A	
I											(−)
Cs				N/A			(+)	N/A	(−)		
Ba				(+)	N/A			(+)	(+)		
W				N/A				N/A	N/A		
Hg										N/A	N/A
Tl				N/A				N/A	(+)		
Pb				(−)	N/A	N/A		(−)	(−)	N/A	N/A
Reference	10)			11)	12)	13)	14)	15)		16)	17)

(+): positive relationship, (−); negative relationship, N/A: no relationship

−0.279 μg/g, respectively). The Student's t-test showed a significant difference in mean birth weight between boys and girls (mean±SD=3062±412 and 2995±401 g, respectively, p=0.002). The statistical analysis did not indicate any significant differences in the other characteristics between boys and girls (Table 2).

Pearson's correlation coefficient revealed a weak inverse correlation between log UCo and BMI in boys (r=−0.125, p<0.001) and girls (r=−0.082, p=0.033) (Figure 2). Multiple linear regression analysis showed an inverse correlation between log

Table 2 Comparison of continuous and categorical variables between boys and girls

Characteristics[a]	Total (n=1542)	Boys (n=860)	Girls (n=682)	p-value[b]
Log UCo	−0.295±0.317	−0.308±0.323	−0.279±0.308	0.075
Age (months)	145.6±3.6	145.6±3.5	145.5±3.7	0.745
Height (cm)	150.1±7.0	149.8±7.4	150.4±6.5	0.092
Weight (kg)	40.4±7.5	40.2±7.7	40.8±7.1	0.118
BMI (kg/m^2)	17.9±2.4	17.8±2.4	17.9±2.4	0.243
Birthweight (g)	3032.2±408.2	3061.6±411.9	2995.2±400.8	0.002
Average sleep duration (hour)	8.8±0.7	8.8±0.7	8.8±0.7	0.357
Father's BMI	23.6±3.2	23.4±3.2	23.7±3.2	0.068
Mother's BMI	21.0±2.7	20.9±2.7	21.1±2.8	0.448
Parental smoking				0.821
Yes	460 (29.8)	256 (29.8)	204 (29.9)	
No	1006 (65.2)	552 (64.2)	454 (66.6)	
Unknown (%)	76 (4.9)	52 (6.0)	24 (3.5)	
Annual household income (10000 yen)				0.383
0–299	69 (4.5)	42 (4.9)	27 (4.0)	
300–599	349 (22.6)	205 (23.8)	144 (21.1)	
600–999	577 (37.4)	304 (35.3)	273 (40.0)	
1000+	528 (34.2)	295 (34.3)	233 (34.2)	
Unknown	19 (1.2)	14 (1.6)	5 (0.7)	
Father's education				0.811
High school or less	262 (17.0)	136 (15.8)	126 (18.5)	
2-year college	218 (14.1)	129 (15.0)	89 (13.0)	
4-year university	821 (53.2)	450 (52.3)	371 (54.4)	
Graduate university	176 (11.4)	98 (11.4)	78 (11.4)	
Unknown	65 (4.2)	47 (5.5)	18 (2.6)	
Mother's education				0.075
High school or less	248 (16.1)	140 (16.3)	108 (15.8)	
2-year college	687 (44.6)	402 (46.7)	285 (41.8)	
4-year university	544 (35.3)	284 (33.0)	260 (38.1)	
Graduate university	54 (3.5)	28 (3.3)	26 (3.8)	
Unknown	9 (0.6)	6 (0.7)	3 (0.4)	

[a] Data presented as mean ± SD or number (percentage)
[b] Comparison between boys and girls
Student's t-test was used for continuous variables, Fisher's exact probability test was used for categorical variables, Mann-Whitney's U-test was used for ordinal scale

UCo and BMI in boys after adjustment for confounding factors (beta=−0.14, p<0.001) (Table 3). The VIF did not demonstrate a multicollinearity problem among the predictor variables (VIF<2.0). In addition, the statistical analysis failed to indicate any significant relationship between the BMI and the urine levels of the other trace element (Table 4).

Discussion

This study's findings showed that increasing UCo levels were associated with a decrease in BMI in boys. This association was confirmed after adjustment for several covariates, including genetic, behavioral, and environmental factors, in the multivariate analysis. Similarly, many previous studies

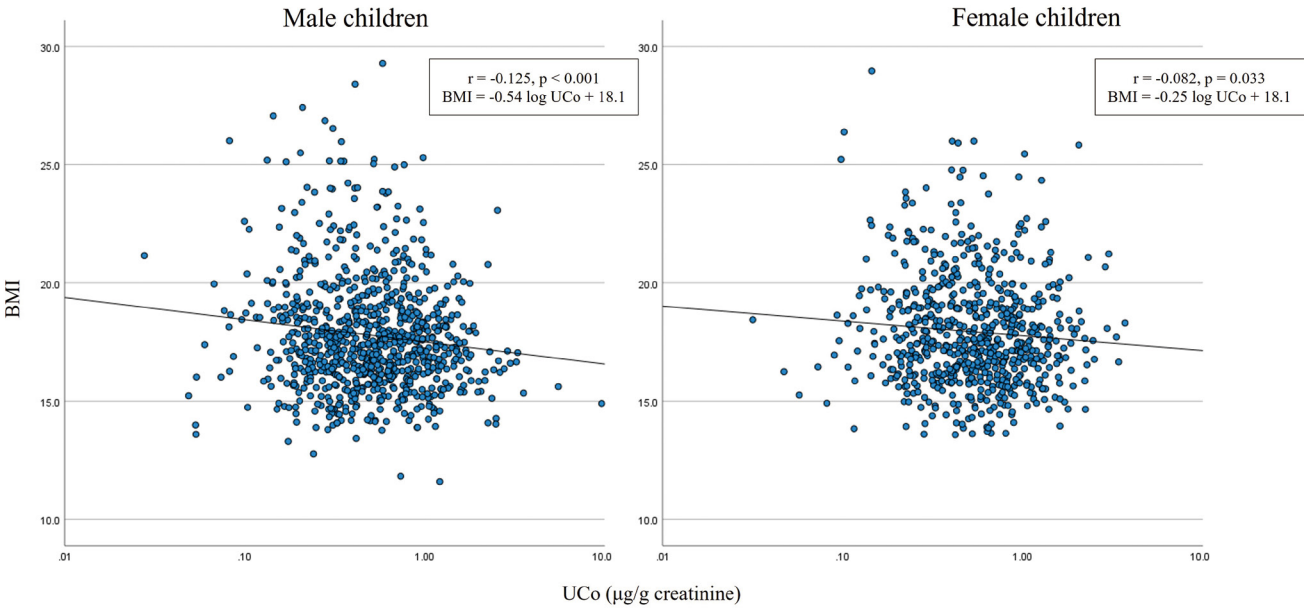

Figure 2 Pearson correlation coefficients between urinary cobalt concentration (UCo) and BMI in 860 boys and 682 girls

Table 3 Relationships of log UCo and other variables to BMI: Multiple linear regression analysis by the forced-entry method

	Boys (adjusted R^2=0.137)		Girls (adjusted R^2=0.096)	
	Beta[a]	p-value	Beta[a]	p-value
Log UCo	−0.14	<0.001	−0.06	0.106
Age (months)	0.06	0.075	0.10	0.011
Birthweight (g)	0.07	0.033	0.06	0.140
Average sleep duration (hours)	−0.14	<0.001	−0.05	0.185
Father's BMI	0.18	<0.001	0.14	<0.001
Mother's BMI	0.19	<0.001	0.20	<0.001
Parental smoking (Yes=1, No=0)	0.09	0.013	0.02	0.586
Father's education (years)	0.02	0.685	−0.07	0.094
Mother's education (years)	0.04	0.291	0.04	0.350
Annual household income	0.01	0.784	0.07	0.122

[a] Standardized partial regression coefficient

have shown an inverse association between cobalt and obesity/overweight rates, regardless of age, gender, and type of the biological samples. Among them, five studies assessed only children[10-14], one study had no age limitation[15], and two studies assessed adults[16, 17].

Consistent with the present study, Padilla (2010)[15], Shao (2017)[11], and Vrijheid (2020)[14] reported an inverse association between UCo levels and childhood BMI and weight. Błażewicz (2013)[10] showed lower plasma and blood cobalt concentrations in children with obesity than in those without obesity; however, the study failed to demonstrate the same results for UCo. Similarly, Vigeh (2017) and Skalnaya (2018) reported higher levels of hair cobalt in children and adults with low body weight than in those with normal weight[13, 17]. In adult women and children, an inverse correlation has been reported between cobalt levels in the toenail[16] and serum[12], respectively, and BMI. In addition, experimental studies (mice and rats) have shown that blood/urine/serum cobalt produce the same effects on animal weight/BMI[18, 38]. Thus, the findings of the current study and previous epidemiological/exper-

Table 4 Relationships of 18 trace elements to BMI

Log urinary concentration[a]	Boys		Girls	
	Beta[b]	p-value	Beta[b]	p-value
Li	−0.08	0.029	−0.04	0.328
Na	−0.06	0.093	−0.00	0.910
Mg	−0.08	0.020	−0.06	0.138
K	−0.07	0.061	0.01	0.848
Ca	−0.02	0.501	0.08	0.046
V	−0.03	0.348	−0.03	0.418
Cr	−0.02	0.495	−0.03	0.525
Co	−0.14	<0.001	−0.06	0.106
Ni	−0.08	0.019	−0.07	0.095
Cu	−0.06	0.079	−0.06	0.126
Zn	−0.06	0.079	−0.08	0.051
As	−0.05	0.126	0.00	0.926
Se	0.02	0.501	0.05	0.233
Sr	−0.04	0.227	0.02	0.536
Mo	−0.05	0.133	−0.09	0.018
Cd	−0.02	0.649	−0.05	0.208
Ba	−0.05	0.170	−0.01	0.785
Tl	−0.03	0.453	0.01	0.900

[a] Adjusted for age, birthweight, average sleep duration, father's BMI, mother's BMI, parental smoking, annual household income, father's education, and mother's education, by multiple linear regression analysis using the forced-entry method
[b] Standardized partial regression coefficient

imental studies suggest that cobalt may reduce the risk of obesity.

Although many previous studies ignored the bioavailability of trace elements and their effects according to the subjects' gender, the present study examined UCo levels and stratified the effects by the participants' gender. There was no significant difference in the UCo levels between boys and girls in the present study. However, previous studies have reported higher levels of UCo in women than in men[23–25]. This difference may be induced by the greater iron demand in women. Cobalt and iron may share a common intestinal uptake mechanism[39]; thus, iron deficiency (a common problem in young women) increases cobalt absorption and urinary excretion in animals[40] and humans[41]. Since the participants of the present study were in early adolescence (56 % of the girls did not experience menarche), the difference in UCo between girls and boys could not be detected at their ages.

Pearson's correlation analysis revealed an inverse correlation between log UCo and BMI in both boys and girls. When we adjusted for confounding factors, in the multiple linear regression analysis, a significant correlation was demonstrated only in the boys. These gender-related findings suggest that UCo is a protective factor against childhood obesity, predominantly in boys. The molecular or biochemical mechanisms underlying the reduction of BMI by cobalt have not been clearly understood. Tascilar (2011) found a correlation between plasma cobalt and the insulin resistance index (HOMA-IR), which suggests that cobalt acts as a regulator of glycogen depot by suppressing glucagon signaling and its effect on body weight[12]. In rats, cobalt administration was reported to result in decreased blood glucose levels, regulated glucose tolerance, and reduced body weight[38]. In addition, cobalt can decrease obesity risk by altering lipid metabolism, such as increasing leptin; the magnitude of the effect varies according to gender. For instance, leptin levels in women are higher after the onset of puberty[42, 43]. Similarly, cobalt increases

plasma HDL cholesterol and decreases LDL cholesterol, free fatty acids, and triglycerides[18]. Although cobalt may reduce childhood BMI by influencing lipid metabolism, we did not determine the level of leptin in the participants of the present study. Another possible underlying mechanism might be iron metabolism, as iron plays an important role during rapid growth periods, such as adolescence. A recent study reported lower iron concentrations in children and adolescents who are overweight and a 50 % incidence of iron-deficiency anemia in individuals with a BMI above the 97th percentile[44]. Cobalt may influence iron metabolism, consequently increasing obesity risk by increasing the hemoglobin, hematocrit, and red blood cell counts in men[45]. Thus, cobalt may influence BMI by changing the metabolism of glucose, lipids, and iron differently in men and women.

In the present study, with a relatively large sample size, we considered several potentially confounding factors. However, the limitations of this study need to be addressed. First, the cross-sectional design of the present study may not draw conclusions regarding the causal relationship between UCo and childhood BMI. The generalizability of the results might be limited owing to the study design. Second, child anthropometric characteristics (i.e., weight and height) develop over several months to years; thus, a single UCo measurement may not reflect cumulative concentrations or exposure levels at an earlier life. Third, urinary excretion of cobalt is multiphasic, with a rapid increase in hours and a peak of elimination at 24 h following exposure[46]. In other words, the current study obtained the level of cobalt exposure via measurement of one spot-urine sample. It would be better to collect a 24 h urine sample for cobalt measurements. Finally, some considerable confounding factors associated with weight and BMI, such as the participants' diet and physical activities, were not adjusted for in the current study because information on these was limited in the TTC dataset.

In summary, among the 18 measured trace elements in the present study, only cobalt showed a significant inverse relationship with BMI in Japanese boys. Thus, cobalt may have sufficient potency to decrease the risk of obesity in children. Future epidemiological and experimental studies may need to clarify the magnitude of the effect and underlying mechanism(s).

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Author contributions

Conception and design, JT and TM. Formal analysis and drafting of the article, JT. Critical revision for important intellectual content, KY, SY, SA, AN, MHH, and KK. Measurement of trace elements' concentration, TM. All authors read and approved the final manuscript.

Conflicts of interest statement

The authors declare that they have no conflicts of interest.

Ethics approval

The present study was conducted after approval was received from the ethics committees of the Tokyo Metropolitan Institute of Medical Science (approval no. 14-08) and Juntendo University (approval no. 2016092).

Consent to participate

Written informed consent was obtained from each participant and the participant's primary parent before participation, as part of the Tokyo Teen Cohort study.

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Original Articles

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Exploring the Objective Signs of Imminent Suicide Risk in Psychiatric In-patients

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Objective: This study retrospectively explores the objective signs of imminent suicide risk in psychiatric in-patients.
Design: The study analysed the diagnostic and nursing records of a psychiatric hospital that covered the last 14 days before the suicide attempts of 18 people, who, between March 2008 and July 2019, were found to have died by suicide during their hospital stay.
Methods: Three professionals used a fishbone diagram to separately identify the factors that led to each person's suicide, the objective signs that indicated imminent suicide risk, possible preventive strategies, and other observations. They compared their findings and used the KJ method (Kawakita Jiro Method) to categorise the items on which they all agreed.
Results: Objective signs of imminent suicide risk were condensed into five categories: 'signs emanating from the patient', 'signs gleaned through engagement', 'signs from response to treatment', 'signs associated with reports from the family', and 'signs inferred from multiple sources of information'. Five categories describing issues with the way in which the hospital staff handled information were extracted, namely 'omission in diagnostic records during admission', 'omission in conference records', 'communication lapse during transfer', 'need for integrated information', and 'systemic issues'.
Conclusions: The findings offer insights on assessing suicide risk and preventing suicide.
Key words: suicide, in-patient, psychiatric hospital, objective signs

Introduction

Psychiatric hospitals play an important role in suicide prevention by protecting and treating people who present a risk of suicide. However, suicides still occur among psychiatric in-patients, with an estimated suicide rate of 147 per 100,000 patients[1]. Aside from preventing any possibility of treatment, suicides inflict severe trauma on the patients' families and the people around them, and potentially expose the hospital to litigation. It is therefore essential to prevent suicides among psychiatric in-patients. Although factors causing suicide have been investigated for a long time, the results derived so far present limited sensitivity and low positive predictive value of the combinations of risk factors, suggesting that suicide risk models have limited use in clinical settings[2–4]. For example, Neuner et al. (2008) identified four risk factors of suicide among in-patients, including resistance to psychopharmacological treatment and past suicide attempts, but these could explain

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only 10% of the variance[5], and a study by Powell et al. (2000) similarly found that predictors correctly identified a mere 2% of suicide patients[6]. Chronic risk factors such as past suicide attempts and mental illness have been considered ill-suited by practitioners from the perspective of clinical effectiveness, as such risk factors cannot assess suicide risks that fluctuate over time. Recently, however, studies that have highlighted the value of proximal, near-term risk factors have been gaining attention. For example, Rudd et al. (2006) proposed a set of warning signs of suicide risk in the near term—in time frames of minutes, hours, and days[7]. Yaseen et al. (2019) proposed diagnostic criteria for predicting imminent suicide risk, namely the suicide crisis syndrome (SCS)[8]. However, one issue is that the warning signs do not necessarily arise only immediately before death by suicide[9]. One issue with the SCS is that it predicts post-discharge suicidal behaviour only in a limited number of cases[8].

Other authors have suggested that as interventions based on clinical risk assessments are insufficient in preventing suicides among psychiatric in-patients, it is necessary to focus on a mix of other strategies, such as providing a safe environment and observing patients effectively[10–12]. Menon (2013) argued that suicide risk should be assessed in three areas, namely risk factors, warning signs (current mental state), and protective factors[4]. However, he did not clarify the warning signs that are particular to psychiatric in-patients. Clinical inquiries, structured interviews, and self-assessment questionnaires have limited value in assessing suicide risk, given that some patients have attempted suicide despite denying having suicidal ideation[13, 14] and in cases where the patient has trouble communicating or shows a decline in comprehension, or has limited trust in healthcare professionals. It is possible to obtain information through alternative means, such as by having ward staff monitor the in-patients' behaviour or chat with them. Thus, it should be possible to improve the precision of risk assessments by looking out for specific objective signs that indicate imminent suicide risk in the in-patients' behaviour in the ward, instead of relying on diagnostic records alone. We conducted a retrospective study of the objective signs of imminent suicide risk among psychiatric in-patients as drawn from a combination of diagnostic and nursing records (nurses' recorded observations of patient behaviour in the ward).

Materials and Methods

Sample

We analysed the diagnostic and nursing records of 18 in-patients (6 male, 12 female) maintained by a psychiatric hospital. These people were found to have died by suicide during their hospital stay, between March 2008 and July 2019 (11 years and 4 months). In each case, the surviving family members did not object to their data being used in this study. The sample included cases in which the patient died by suicide during temporary leave and in which the patient was rushed to hospital after a suicide attempt and subsequently died in the hospital. It may be unclear in some cases as to whether the death really was by suicide or accident (the person is no longer around to confirm this). Accordingly, in this study, we defined suicide as an event in which the person committed an act that resulted in their death, irrespective of whether or not they anticipated this fatal outcome[15]. We considered utterances, behaviours, attitudes, and mental states observed over a period of 14 days before the patient's fatal suicide attempt as signs of imminent suicide risks.

Preparing the Data to be Analysed

In Resource A, we compiled the patients' basic information, which comprised diagnostic records made at time of admission, their diagnosis, period of hospitalisation, and history of suicide attempts, psychiatric symptoms, suicide in their families, and diseases. In Resource B, we arranged the diagnostic and nursing records into a time series for a period of 14 days before the fatal suicide attempt. In both Resources A and B, all data were anonymised.

Analysis 1

From Resources A and B, we aggregated the cases in which the patient had attempted suicide in the past; the patient's problem list at the time of admission mentioned suicidal behaviour or ideation; and the patient showed signs of suicide in the 14 days before the fatal suicide attempt, including suicidal utterances, self-injury, and unsuccessful suicide attempts.

Analysis 2

Analysis 2 was conducted by three professionals from the field of psychiatric care ('professionals'), namely a doctor, a nurse, and a clinical psychologist, each of whom had at least 10 years of experience in clinical psychiatric care, including ward duty. To ensure objectivity, none of the professionals had engaged in any way with the cases in our sample. The three professionals were provided with Resources A and B in advance and were asked to analyse three sets of items using a fishbone diagram (a diagram for analysing the potential causes of an outcome, which is often used to analyse the causes of accidents). The three sets of items were: factors that led to each person's suicide (C), imminent suicide signs (D), and preventive strategies or other observations (E). The professionals conducted this analysis separately and then came together to compare and consolidate their findings for C, D, and E. The items they all agreed upon (F) were set aside for further analysis.

Analysis 3

Analysis 3 focused on F, as derived through analysis 2. The imminent signs and risk factors were labelled/encoded and organised by content using the KJ Method (where data are sorted into groups that present relationships of affinity, opposition, and causality in order to generate a hypothesis). We prioritized reducing omission of behaviours that could be signs, and as such included two behaviours that the three professionals had not agreed upon but had all noted, and one sign that, although not noted by the professionals, was evidently a sign of imminent suicide risk.

Analysis 4

We considered F in terms of the human factor, such as cases where the staff failed to record important patient information. We labelled/encoded items describing the handling of patient information by the hospital staff and categorised them using the KJ Method.

Ethical Considerations

This study was approved by the psychiatric hospital in question (approval number: 2) and the ethics committee of the Graduate School of Health and Sports Science at Juntendo University (approval number: 31-74).

Results

Patient Characteristics

Table 1 shows the patients' characteristics based on Resource A: sex, age, the diagnosis stated in the medical records, the retrospective diagnosis given by the three professionals in Analysis 2, and the period of hospitalisation.

Rates of suicidal ideation, self-injury, and suicide attempt (Analysis 1)

Of the 18 cases analysed, 11 (61.1%) had a problem-list entry of attempted suicide or suicidal ideation at the time of admission, and 11 (61.1%) had a past suicide attempt more than two weeks before the suicide attempt that led to death (or before hospitalisation if the hospitalisation period was less than fourteen days). Further, in the two weeks preceding their death (or during the hospitalisation period if the hospitalisation period was less than fourteen days), 7 (38.9%) had made suicidal utterances, 2 (11.1%) had self-harmed, and 3 (16.7%) had attempted suicide unsuccessfully, while 11 (61.1%) had had no suicidal utterances or suicide-related behavior.

Objective Signs of Imminent Suicide Risk (Analysis 3)

Table 2 shows the major categories, sub-categories, and sub-sub-categories of the signs indicating imminent suicide risk, along with examples. In the following, the major categories are denoted by ' ', sub-categories by [ ], sub-sub-categories by < >. Our analysis yielded five major categories. The first one, namely 'signs emanating from the patient', describes signs that the patient expresses spontaneously, without any engagement from the hospital staff. The second, namely 'signs gleaned through engagement', describes signs that the hospital staff glean through their engagement with the patient. The third, namely 'signs from response to treatment', describes signs that the patient exhibits in response to medical intervention. The fourth, that is, 'signs associated with reports from the family', describes signs reported by the patient's family. The fifth, namely 'signs inferred from multiple sources of information', describes signs that are inferred from an integrated analysis of diagnostic

Table 1 Patients' characteristics

Id	Sex[a)]	Age	Diagnosis in medical records	Diagnosis by analysis 2	Period of hospitalisation (day)
1	F	21	Depressive Disorder	Possibility of Personality Disorder	2
2	M	21	Schizophrenia		20
3	F	27	Borderline Personality Disorder		40
4	M	33	Adjustment Disorder, Narcissistic Personality Disorder		104
5	F	34	Dissociative Disorder	(Complex-PTSD or other trauma-related conditions)	30
6	F	40	PTSD, Bipolar II Disorder	Not PTSD, but Acute Stress Disorder?	1
7	F	43	Schizophrenia		916
8	F	48	Schizophrenia		2416
9	M	53	Schizoaffective Disorder		99
10	F	54	Bipolar II Disorder		17
11	F	54	Depressive Disorder		40
12	F	55	Depressive Disorder		61
13	M	56	Bipolar I Disorder		17
14	M	63	Recurrent Depressive Disorder		3
15	M	67	Depressive Disorder (suspected of having Dementia with Lewy bodies)		147
16	F	69	Depressive Disorder (suspected of having Front-temporal or another form of Dementia during the intake interview)	Possibility of Bipolar Disorder (The principal doctor described 'elevated mood' and 'unusual talkativeness' among other things)	3
17	F	69	Depressive Disorder		79
18	F	79	Schizophrenia	Not Schizophrenia, but Depressive Disorder? (The principal doctor had prescribed medication for depression)	7001

a) M: Male, F: Female

and nursing records along with other information on the patient.

The first major category contained two sub-categories, namely [behaviour] and [symptoms]. [Behaviour] contained six sub-sub-categories, namely <suicide attempt>, <self-injury>, <morbid behaviour> (such as reciting sutras), <accesses means of suicide>, <desire to leave> (escape attempts, hesitating to stay overnight), and <reticence> (among patients who had been complaining of suicidal ideation). The second sub-category, [symptoms], contained three sub-sub-categories, namely <sudden deterioration> (in schizophrenia), <symptoms aggravated by discharge anxiety>, and <unstable symptoms> (intra-day fluctuations in depressive disorder).

The second major category contained two sub-categories, namely [verbal information] and [nonverbal information]. [Verbal information] contained five sub-sub-categories. The first three described explicit verbal communication from the patient: <talks about suicidal ideation>, <talks about suicidal ideation due to proactive communication>, and <declares suicidal intention>. The fourth sub-sub-category is <complains of loneliness>. The final one is <communication difficulties>, which implies a possible deterioration in symptoms. The other sub-category, [nonverbal information], contained five sub-sub-categories. Four of these described behaviours and attitudes, namely <evasiveness>, <poor treatment motivation>, <resists treatment>, and <fails to understand the importance of treatment>. The fifth sub-sub-category, <reluctant expressions>, described the patient's feelings of reluctance toward safety restrictions on objects that patients could use to

Table 2 Objective signs of imminent suicide risk (Analysis 3)

Major category	Sub-category	Sub-sub-category	Example
Signs emanating from the patient	Behaviour	Suicide attempt	Twelve days before the event, the patient attempted suicide by swallowing rat poison.
		Self-injury	On the day before the event, the patient hurt their right wrist with their fingernail.
		Morbid behaviour	The patient repeatedly recited Buddhist sutras beginning five days before their suicide.
		Accesses means of suicide	The patient tried to open a window in the closed ward.
		Desire to leave	On the day of the event, the patient tried to escape from the closed ward.
			During the patient's last several days, they repeatedly applied for temporary leave, only to cancel it.
		Reticence	The patient discussed their suicidal ideation in the past, but never mentioned a desire to die in their last four days.
	Symptoms	Sudden deterioration	The patient's schizophrenic symptoms suddenly deteriorated.
		Symptoms aggravated by discharge anxiety	The patient had no social relationships. Ten days prior to their death, the patient discussed their anxiety about the upcoming discharge. Subsequently, the patient experienced an escalation in anxiety, dread, and dissociation.
		Unstable symptoms	The patient experienced intra-day fluctuations in depressive disorder (cycling between gleeful and gloomy utterances).
Signs gleaned through engagement	Verbal information	Talks about suicidal ideation	The patient repeatedly told a nurse that they wanted to die because they could not bear the hallucinations and other psychiatric symptoms, and because of the self-disgust they felt from when the symptoms worsened.
		Talks about suicidal ideation due to proactive communication	The patient booked a period of leave, but on the day before the event, they expressed second thoughts. Noticing that the patient had not left their room since then, the nurse entered the room. The patient remained silent and pensive for 15 minutes, before declaring that they had something to say. The patient then admitted that they had reserved the period of leave out of a desire to die.
		Declares suicidal intention	On the day of the event, the patient, without returning to the ward, made a silent phone call to the hospital. The staff returned the call and the patient answered. When asked for their location, the patient said that they did not want to give it and that they no longer needed any food as they were about to die. [a)]
		Complains of loneliness	The patient complained of loneliness and a sense of not fitting in anywhere. The patient also mentioned poor marital relationships.
		Communication difficulties	The patient experienced a sudden deterioration in schizophrenic symptoms. An entry in the nursing record made on the day before the event reads thus: 'The patient is making no sense'. It also means that the patient's condition had become worse.
	Nonverbal information	Reluctant expressions	On the day of the event, the patient was taken to a seclusion room and told that her bra would need to be removed. On hearing this, the patient gave a grudging grimace.
		Evasiveness	The patient tried to hide when they were called by the principal doctor, and drew back during temperature tests. [b)]
		Poor treatment motivation	The patient had no desire to get better.
		Resists treatment	Lacking awareness of the fact that they were ill, the patient denied experiencing psychiatric symptoms or suicidal ideation and repeatedly refused treatment, while demanding to be discharged.
		Fails to understand the importance of treatment	The patient failed to recognise that they were admitted because they had attempted suicide, and that they had failed to comprehend the importance of treatment.

Signs from response to treatment	Response to learning of diagnosis		The patient was informed of their diagnosis four days before the event. The patient struggled to come to terms with the diagnosis, saying that they were in a state of shock and wanted to find a way to ease it.
	Response to clinical environment	Response to admission	The patient had never been admitted to a psychiatric hospital before and experienced anxiety and dread in the new environment.
		Response to ward transfer	Deemed a suicide risk, the patient was transferred to a closed ward. Although this was necessary, the new environment caused the patient's psychotic symptoms to worsen.
	Response to dosage change		The patient's dose was decreased because of side-effects. Consequently, the patient experienced anxiety and irritability, symptoms that were suppressed by the medicine or withdrawal symptoms.
	Unnatural clinical response		Following the first session of ECT after resuming therapy, the patient exhibited lively behaviour that was incongruous with their behaviour theretofore. For example, after drinking something, the patient gleefully declared that the drink was delicious. They also purchased a sports drink and consumed 90% of a curry. [b]
Signs associated with reports from the family	Reports from the family	Report on suicidal ideation	The family informed the staff that the patient had thought about suicide.
		Information provided during the meeting	On the day of the event, the family informed the staff that the patient was crying after their meeting. A nurse visited the patient's room, and the patient lamented that their hopes of discharge had been dashed.
	Family report not discussed		The family informed the staff about the patient's suicidal ideation. However, the staff neglected to discuss this with the patient.
Signs inferred from multiple sources of information	Attitude inferred from integrated information	Tries to please the principal doctor	What the patient talked about and how they behaved varied between the principal doctor and nurses. In the presence of the principal doctor, the patient tried to give the impression that they were getting better. In contrast, with the nurses, the patient complained of anxiety and anxiety-induced somatic conditions.
	Situational inferences	Isolation	The family said that they would not allow the patient to return to the family home after discharge.

a) Behaviour not noted by the three professionals during Analysis 2 (we subsequently added them)
b) Behaviour that was focused on during Analysis 2 but for which the three professionals' interpretations were different

kill themselves.

The third major category, 'signs from response to treatment', contained four sub-categories. [Response to learning of diagnosis] described cases in which the patient had just learned of their diagnosis and struggled to come to terms with it; [response to clinical environment] described responses to being admitted to hospital or being transferred to a closed ward; [response to dosage change] described symptoms associated with a reduction in the dosage following an adverse reaction. The fourth sub-category, [unnatural clinical response], described a case in which a patient who had discontinued electroconvulsive therapy (ECT) exhibited, upon resuming ECT, animated behaviour that was incongruous with their behaviour theretofore.

The fourth category, 'signs associated with reports from the family', contained two sub-categories. The first, [reports from the family], included <report on suicidal ideation> and <information provided during the meeting (with the patient's family)>. The second, [family report not discussed], described cases in which, despite being informed by the family of the patient's suicidal ideation, the staff neglected to discuss it with the patient.

The fifth category, 'signs inferred from multiple sources of information', contained two sub-categories: [attitude inferred from integrated information] and [situational inferences]. The former contained the single sub-sub-category <tries to please the principal doctor>, which described cases in which the attitude only became apparent once the staff had integrated diagnostic and nursing records, and the patient behaved as though he/she was getting better only in the presence of the doctor, in order to meet the doctor's expectations. The latter contained the single sub-sub-category

<isolation>, which described cases in which the patient was assumed to be isolated given that the family refused to let the patient live with them after being discharged.

Issues in the Manner in which the Hospital Staff Handle Information (Analysis 4)

Table 3 presents the major categories and sub-categories drawn from Analysis 4, along with examples. Our analysis yielded five major categories: 'omission in diagnostic records during admission', 'omission in conference records', 'communica-

Table 3 Issues in the way in which the hospital staff handle information (Analysis 4)

Major category	Sub-category	Example
Omission in diagnostic records during admission	Omission in diagnostic records and treatment policy	The diagnostic record made for admission omitted the diagnosis and treatment policy.
	Omission in patient information during readmission	The patient was readmitted just four days after being discharged. The diagnosis record for readmission omitted any opinion on the recurrent symptoms and suicidal ideation. It also omitted the treatment plan. The diagnostic record for the original admission was in a separate document.
	Abridging report on suicide risk ↓ Factors influencing ward staff's judgements and behaviours	In the record for admission, the doctor mentioned suicidal ideation despite the patient denying it. However, the record contained no opinion on the occurrence of suicidal ideation. While delivering the medication, the nurse noticed that the patient was missing. The nurse knocked on the lavatory door and there was no response. The nurse finished delivering medication to the other patients and then looked for the missing patient, whom they discovered hanging in the lavatory. The nurse may have elected to leave the area (instead of searching for the patient immediately) because the severity of the suicide risk was not fully appreciated.
Omission in conference records	Omission in treatment policy	The conference records omitted the treatment plan.
Communication lapse during transfer	Hard to relay complex information such as the principal doctor's decision-making process	The patient had been transferred from a seclusion room in an open ward to the general area of the closed ward for their protection following a suicide attempt. While bathing alone, the patient attempted suicide by swallowing shampoo and conditioner. The staff in the destination ward may have assumed that the patient no longer needed to be secluded because their psychiatric condition had improved, and they may have got this impression because the staff failed to share the intent of the principal doctor's orders.
	Omission in transfer application records	Nine days before the event, the patient tried to hang themselves. Seven days before the event, the patient, in a seclusion room, banged their head against the wall and toilet out of a desire to kill themselves. One day before the event, the patient was transferred. The principal doctor's transfer application did not mention the suicide attempts.
Need for integrated information		Despite the nursing records having plenty of information on patient complaints and symptoms, the patient, during medical screenings, was judged as stable. Accordingly, the form of hospitalisation was changed and the patient was given more freedom. Decisions should be based on the information in both the nursing and diagnostic records.
Systemic issues	Hard to view records	It is hard to share the records among the staff, as they comprised a single paper document.
	Hard to access past information	The patient died by suicide by swallowing detergent after their delusions worsened. This mirrored a previous suicide attempt (in which the patient had attempted suicide by swallowing detergent after their delusions worsened). An entry in the diagnostic record made nine days before the event mentioned the worsening delusions, and an entry in the nursing record mentioned that the patient had bought detergent. However, due to turnover in ward staff over time, it may have been difficult for them to piece the disparate information together and identify the suicidal pattern.

tion lapse during transfer', 'need for integrated information', and 'systemic issues'.

In the first category ('omission in diagnostic records during admission'), the following three sub-categories pertained to the same patient: [omission in patient information during readmission], [abridging report on suicide risk], and [factors influencing the ward staff's judgements and behaviours]. The patient in question had been readmitted soon after being discharged, which may explain why the staff decided to cut corners and abridge the diagnostic records. Although the patient had evidently been readmitted because of severe suicidal ideation, there was just a brief, concise record stating that the patient had denied having such an ideation. On the second day after readmission, the ward received a report from the family referring to the patient's suicidal ideation. However, the staff never discussed it with the patient. On the third day, the patient was absent from their room when the nurse came to deliver their medication. The nurse did not search for the patient right away; she first finished delivering medication to the other patients. Eventually, the nurse searched for the absconding patient and found them inside the ward lavatory. It was suicide by hanging. During Analysis 2, the professionals suggested that the omission of the diagnostic record may have prevented the hospital staff from appreciating the severity of the suicide risk, and that this complacency may explain why the nurse had delayed the initial response to the patient's absence.

One of the sub-categories of 'communication lapse during transfer' was [hard to relay complex information such as the principal doctor's decision-making process]. This sub-category pertained to a patient who had been transferred from a seclusion room in an open ward to the general area of the closed ward for the patient's own protection following a suicide attempt. While bathing alone, the patient died by suicide by swallowing a substance. During Analysis 2, the professionals suggested that the staff at the closed ward, in whose care the patient had been transferred, may have mistakenly assumed that the patient no longer needed to be in seclusion because their symptoms had improved.

Discussion

Assessing the suicide risk, which is non-constant and fluctuates over time, is essential in enabling timely intervention, and is therefore a top clinical priority. Of the cases we analysed, 61.1% of the patients died by suicide despite the absence of any verbalised references to suicidal ideation or exhibitions of suicidal behaviour in the 14 days leading up to the event. It is therefore crucial to look for signs of suicide other than explicit suicidal ideation statements or behaviour.

In this study, we retrospectively analysed the signs of imminent suicide risk that were present in psychiatric patients during the 14 days before their fatal suicide attempt, using diagnostic and nursing records pertaining to that period. In Table 2, we listed the items that describe signs. However, not all these items imply imminent suicide risk in themselves. Some of the items can be more accurately described as the lack of protective factors rather than imminent suicide risk. Three notable examples are the major category 'signs from response to treatment', the sub-category [symptoms] (in 'signs emanating from the patient'), and the sub-category [attitude inferred from integrated information] (in 'signs inferred from multiple sources of information'). These items imply that the person's psychiatric condition has failed to improve sufficiently or has deteriorated, that the person is mentally unstable, or has a personality pathology. They do not directly imply imminent suicide risk to the extent that certain other items, such as <suicide attempt> or <accesses means of suicide>, do, and they are apt to occur in non-suicidal patients, too. Thus, to assess whether an item implies suicide risk, one must consider the specific details and severity of the case in question. Auditory hallucinations, for example, are more likely to imply suicide risk if they comprise suicide-related commands[16]. All items under [nonverbal information], such as <evasiveness> and <poor treatment motivation>, describe, in our opinion, behaviours and attitudes that suggest the absence of protective factors. That is, inasmuch as the behaviours and attitudes that these items describe indicate a lack of readiness for treatment, they could be treated as evidence of the lack of the kind of protective factors discussed by Britton et al. (2020), which include a readiness to

start finding value in one's life and to participate in one's own treatment[17].

The items that indicate the signs of imminent suicide risk in Table 2 represent the perspectives of the staff rather than those of the patients. The five major categories our analysis yielded reveal that suicide risk information can be obtained from 'signs emanating from the patient', 'signs gleaned through engagement', 'signs from response to treatment' (signs that occur as a result of medical intervention), 'signs associated with reports from the family', and 'signs inferred from multiple sources of information' (denoting the need to base decisions on multiple kinds of information). In psychiatric wards, not only the principal doctor and nurse in charge, but also staff from different professions are stationed to provide care collectively. The perspectives extracted in our analysis can be particularly useful to staff other than those who are immediately in charge of patients. To such staff, the perspectives provide observational insights on the patients and can serve as criteria in deciding on the kinds of information to include (and excluding) while reporting to medical teams.

In-patient suicide is an example of a sentinel event—a sudden and unanticipated event or complication in a healthcare setting that results in death or serious injury. We used the fishbone diagram, which is an example of a sequential accident model. Other models for analysing accidents extend their focus to epidemiological and systemic accident models[18]. There is a drive for suicide in patient, so such issues with the handling of information by staff should be seen less as a factor that causes a suicide event and more as a factor pertaining to the defence barriers in an epidemiological accident model. Such models envisage effective communication among healthcare workers as a defence against accidents. Defences must be as solid as possible to prevent accidents. We extracted five issues pertaining to how hospital staff handle information: 'omission in diagnostic records during admission', 'omission in conference records', 'communication lapse during transfer', 'need for integrated information', and 'systemic issues'. Information may be omitted during admission, conferences, or ward transfers because the staff are busy or have a defensive mental state, or the hospital is understaffed. To address these issues, it may be useful to build a system that makes omissions impossible, such as by standardising the reporting format. The staff may be particularly tempted to omit or abridge information in the case of readmission. They may do so out of complacency, believing that the facts on the readmitted patient are already well known. The fact that a patient is being readmitted because of the lack of improvement in their condition may create a sense of helplessness among the staff, which may, in turn, dull their alertness to the suicide risk. Thus, hospitals must impress upon the staff the importance of recording suicide risk information during readmission. We found that communication lapses occur during ward transfers because it is [hard to relay complex information such as the principal doctor's decision-making process]. It is difficult to explicate the principal doctor's often inscrutable thinking to the staff at the destination ward. For example, the doctor may give the staff seemingly contradictory instructions to transfer a patient from a seclusion room in an open ward to a general area in a closed ward, i.e. to increase or decrease the level of protection. It is therefore essential to develop strategies to convey this information. For example, when the staff in the destination ward prepares a nursing plan for the transferee, the principal doctor and staff from the original ward can participate in the process. The 'need for integrated information' implies the need for collating information from different staff members and evaluating multiple sources of information in an integrated manner. This task poses a major challenge in that it is subject to human factors such as how busy one is and what their career and abilities are. Possible strategies toward addressing 'systemic issues' include introducing an electronic medical record and displaying information on past suicidal behaviour patterns in places where the staff can easily see it.

This study has four limitations. First, the results have limited transferability given the small size and peculiarity of the sample. Second, our analysis was unable to cover factors that were not included in the records. Third, as the cases were retrospectively analysed by parties who were not involved, the analysis could not cover the perspectives of psycho-dynamics and group dynamics between each case and staff and the participant observation of the staff. Fourth, another aspect that was not

covered in the analysis are factors that were related to the hospital setting during the period in question. Examples include the conditions of in-patients, staff numbers and transfers, and relationships among the staff and between the frontline and other staff in the hierarchy of the organisation. Addressing these limitations in future research can provide insights on the signs of imminent suicide risk among psychiatric in-patients.

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Author contributions

HS, IU, NS, and TF designed this study. HS, IU, CS, SS, and KY carried out the analyses. HS summarised the result and wrote the manuscript with the support and guidance from IU, NS, and TO. IU, NS, and TF supervised the project. All authors read and approved this manuscript.

Conflicts of interest statement

The authors declare that there are no conflicts of interest.

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Original Articles

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Evaluating Small Intestinal Motility in a Rat Model of Adolescent Irritable Bowel Syndrome

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Objectives: The correlation between altered small intestinal motility and irritable bowel syndrome is not well evaluated. This study aimed to assess the small intestinal and colonic transits in an adolescent irritable bowel syndrome rat model with restraint stress and determine the role of small intestinal motility in the irritable bowel syndrome pathophysiology.
Materials: Restraint stress was utilized to prepare adolescent irritable bowel syndrome rat models that were evaluated for clinical signs, including stool frequency and diarrhea. The small intestinal motility and transit rate were also evaluated.
Methods: The amounts of mRNA encoding corticotropin-releasing hormone, mast cell, and serotonin (5-Hydroxytryptamine) receptor 3a were quantified using real-time polymerase chain reaction; the 5-Hydroxytryptamine expression was evaluated using immunostaining.
Results: Restraint stress significantly increased the number of fecal pellet outputs, stool water content, and small intestinal motility in the adolescent irritable bowel syndrome rat models. There was no difference in real-time polymerase chain reaction results; however, immunostaining analysis revealed that 5-Hydroxytryptamine expression in the small intestine was significantly increased in the adolescent irritable bowel syndrome rat models.
Conclusions: In the rat model of adolescent irritable bowel syndrome with restraint stress, we observed an increase in small intestinal and colonic motility. In the small intestine, enhanced 5-Hydroxytryptamine secretion in the distal portion may be involved in increasing the small intestinal motility. Although the present study focused on 5-Hydroxytryptamine, further investigation of other factors that regulate intestinal peristalsis may lead to the establishment of more effective treatment methods for adolescent irritable bowel syndrome.

Key words: adolescent, enterochromaffin cells, gastrointestinal motility, irritable bowel syndrome, serotonin 3 receptor

Introduction

Irritable bowel syndrome (IBS) is a common functional gastrointestinal disorder characterized by the symptoms of abdominal pain and altered bowel habits, such as diarrhea or constipation, in the absence of any organic diseases. Although the pathophysiology of this disease remains uncertain, sufficient compelling evidence has accumulated to indicate that IBS is a multifactorial syndrome resulting from interactions among gastrointestinal motility, visceral hypersensitivity, intestinal inflammation, gastrointestinal infection, fecal flora alterations, bacterial overgrowth, food sensitivity, genetic factors, and psychosocial dysfunction[1]. The prevalence of IBS is high in children, with a global prevalence of 2–20%, and IBS has a significant impact on daily activities, school life, friendship and health related quality of life of affected children[2, 3]. Therefore, the Rome IV criteria established diagnostic criteria for IBS in children and adolescents[4].

Colonic transit disorders may contribute to symp-

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toms among patients with IBS; in fact, transit and contractile abnormalities have been observed in a subset of patients with IBS. The primary alteration of mucosal absorption and secretion has been suggested as non-contributory to the frequent stools with diarrhea in patients with IBS[5].

Several previous studies have shown a relationship between the accelerated transit of colonic contents with IBS and diarrhea. In animal models, previous studies have shown that stress loading increases stool frequency and causes diarrhea[6]. Mönnikes et al. reported a significant acceleration of colonic transit in rats with restraint stress compared to that in non-restrained rats[7].

Furthermore, visceral sensation hypersensitivity from the autonomic nervous system associated with the brain-gut interaction is thought to be the pathophysiology of IBS[8]. Corticotropin-releasing hormone (CRH) is an important factor in explaining the pathophysiology of brain-gut interactions. CRH stimulates the pituitary adrenocorticotropic hormone and increases intestinal motility in the human body[9]. Recently, there has been a growing interest in serotonin (5-Hydroxytryptamine; 5-HT) because of its possible involvement in IBS. 5-HT released by enterochromaffin cells (EC cells) within the mucosa via intraluminal distension or irritation stimulates 5-HT3 receptors located on the primary afferent neurons of both splanchnic and vagal fibers, thereby modulating a sensory response[10].

Although increased stool water in diarrhea is thought to result from diminished contact time of the luminal contents with the colonic mucosa, it is well known that approximately 80% of ingested water is absorbed from the small intestinal mucosa[11]. Therefore, small intestine dysfunction induces diarrhea in many diseases; however, the correlation between diarrhea and motility alteration in the small intestine among patients with IBS, especially childhood and adolescent age, has not been well evaluated. In this study, we aimed to assess the small intestinal transit in adolescent IBS rat models with restraint stress and to determine the role of small intestinal motility in the pathophysiology of IBS.

Materials and Methods

Animal models

The experiments were performed using adolescent male Sprague-Dawley rats aged 5-6 weeks as adolescent, weighing 160-250 g and were housed in cages in a standardized environment with a temperature of 24 °C, relative humidity of 55 % ± 15 %, and a 12-hour/12-hour light-dark cycle for a day. We chose male rats because we wanted to avoid female hormonal effects. They were allowed to access food and water freely. The animal care and experimental protocols were approved by the Institutional Review Board of Juntendo University (No. 310185).

The animals were randomly assigned to two groups: a restraint group and a control group. The restraint group was isolated in the individual compartments of stress cages (Natsume Seisakusho Co. Ltd. Tokyo, Japan; KN-325-C-3) for 1 hour before dissection. Rats in the control group assumed an hour of isolation in the cages without restriction. The rats in both groups did not have free access to food and water during isolation.

All methods were performed in accordance with relevant guidelines and regulations, and this study was conducted in compliance with the ARRIVE guidelines.

Fecal pellet output and water contents

The number of fecal pellet outputs during the one-hour isolation was counted. The stool first excreted during isolation was collected, as well as the stool located in the most distal side of the gastrointestinal tract after isolation was collected for comparison. These fecal pellets were stored at a temperature of -80 °C and were freeze-dried overnight after weighing. The freeze-dried fecal weight was measured on the next day to calculate the water content (%), which was as follows: (fecal weight before drying - fecal weight after drying) / fecal weight before drying × 100 %.

Small intestinal transit

We examined the intestinal propulsion of powdered carbon to evaluate small intestinal motility under restraint stress. The rats received 0.5 mL of a powdered carbon suspension in saline per 100 g of their weight (5 % W/V) intragastrically through an oral sonde (Primetech Co. Ltd. Tokyo, Japan; FTP-15-78-50). After administration, the rats in the restraint group were immediately exposed to stress, as described above. Rats in

both groups underwent an autopsy 1 hour after intragastric administration of the powdered carbon suspension. The process was shown in the figure (Figure 1). When the abdominal wall was opened during dissection, the intestinal tract was exposed. At that time, the intestines that had been exposed to carbon suspension were stained black and could be observed visually. The small intestines were collected, and their entire lengths placed naturally were measured. We also measured the length of the small intestine containing the carbon marker. The small intestinal transit rate (%) was calculated as follows: (the length of the small intestine containing the marker / total small intestinal length) × 100 %.

Real-time polymerase chain reaction (PCR)

The amount of mRNA encoding CRH, mast cells, and 5-Hydroxytryptamine Receptor 3A (5-HTR3a) was quantified using real-time PCR. Full-thickness segments of the small intestine and proximal and distal colonic tissue samples were preserved in RNA stabilization solution and stored at −80 °C until use. Each tissue sample was homogenized in Tri Reagents (Tomy, Japan; MS100) to extract total RNA according to the manufacturer's instructions (Applied Biosystems). Real-time PCR was performed using the 7500 Fast Real-Time PCR system (Applied Biosystems). The expression of each gene was normalized to the expression of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) using the standard curve method. TaqMan was used for analysis using primers for CRH (Assay number Rn01462137_m1), mast cells (Assay number Rn04342812_g1), and 5-HTR3a (Assay number Rn00667026_m1). The results were compared between the restraint and control groups for both the small intestine and colon. Furthermore, the proximal and distal segments of the small intestine were also compared.

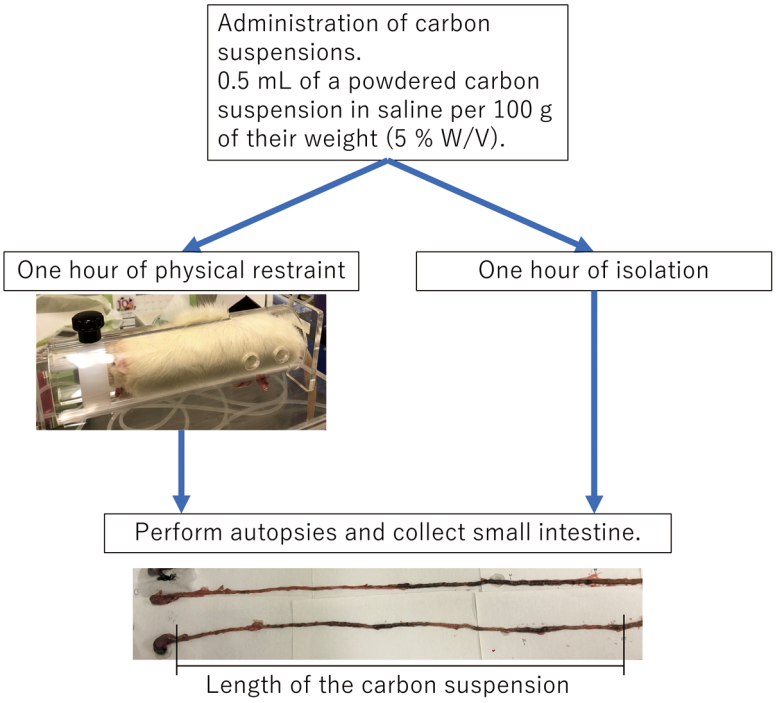

Figure 1 The process from administration of the carbon suspension to dissection was shown. After administration of the carbon suspension, the restraint group was restrained for one hour and the control group was isolated for one hour. After one hour of restraint and isolation, the animals were dissected. In this figure, I presented a picture of a rat in physical restraint. And also presented a picture of the removed small intestine. This picture was taken after the carbon suspension was administered. As shown, the area where the carbon had passed through was stained black, and this length was measured.

Immunohistochemical analysis

Small intestine and colon tissues were dissected from rats and fixed in 4 % paraformaldehyde in 100-mM phosphate buffer at room temperature for 24 hours. Serial sections (4-μm thick) were prepared from formalin-fixed paraffin-embedded tissue sections of the small intestine and colon. For 5-HT detection, cells were incubated with 5HT (1: 10; Thermo Fisher Scientific, Rockford, USA) and then stained using the iVIEW™ DAB Detection Kit (Ventana) and Hematoxylin Counterstain II (Ventana). For 5-HTR3a detection, paraffin sections were heat-treated in Cell Conditioning Solution (Ventana Medical Systems) for 5-HTR3a, incubated with normal horse serum (Vector), rabbit anti-rat 5-HTR3a (Abcam), HRP-polymer-conjugated horse anti-rabbit IgG (Vector), and then stained using *ultra*View™ Universal DAB Detection Kit (Ventana) and Hematoxylin Counterstain II (Ventana). All the stains mentioned above were used according to the manufacturer's protocol. An automated immunostainer (BenchMark; Ventana) was used to stain both 5-HT and 5-HTR3a. For each specimen, the number of 5-HT-positive cells was counted in six randomly selected fields per section using a KS400 Image Analyzer System (Zeiss). The data were expressed as the average number of positive cells per 400 μm2 of the mucosa.

Similar to real-time PCR, the restraint and control groups were compared for both the small intestine and colon; distal and proximal comparisons were also made in the small intestine.

Statistical methods

All experimental data are presented as the mean ± standard deviation. All statistical analyses were performed using EZR[12] (Saitama Medical Center, Jichi Medical University, Saitama, Japan). More precisely, EZR is a modified version of R commander (version 2.7-0, 2021) designed to add statistical functions frequently used in biostatistics. As appropriate, the t-test was used for comparisons between the two groups. Differences between means at a level of $p < 0.05$ were defined as statistically significant.

Data Accessibility

The data that support the findings of this study are available from Juntendo University, but restrictions apply to the availability of these data, as they were used under license for the current study and are not publicly available. However, data are available from the authors upon reasonable request and with the permission of Juntendo University.

Results

Fecal pellet output and water content

We counted the fecal pellet outputs of 12 restraint rats and 12 control rats. The mean number of fecal pellets was 6.9 ± 1.7 and 0.8 ± 0.8 in the restraint and control groups, respectively, showing that restraint stress significantly increased the number of fecal pellet outputs ($p < 0.001$, Figure 2A). We also calculated the water content of fecal pellets in six restraint rats and six control rats. The mean water content of the first stool during isolation was 75.5 % ± 7.5 % and 74.7 % ± 5.4 % in the restraint and control groups, respectively, with no significant difference in primary stools between the two groups during isolation ($p = 0.80$). On the other hand, the mean water content of the fecal pellets after isolation was significantly different between the restraint rats and control rats, at 94.8 % ± 4.2 % and 79.1 % ± 6.0 %, respectively ($p < 0.001$, Figure 2B). This result indicates that restraint stress led to increased water content in the stool and induced diarrhea in the stress models.

Small intestinal transit

Nine restraint rats and 16 control rats were compared for small intestinal motility. The mean length of the small intestine was not significantly different between the restraint and control groups (115.3 ± 3.8 cm vs 113.3 ± 9.2 cm; $p = 0.63$) (Figure 2C). The mean small intestinal transit rate was 77.9 % ± 7.4 % in the restraint group and 69.5 % ± 8.9 % in the control group, with the restraint group showing a significant increase in the small intestinal transit rate ($p < 0.03$) (Figure 2D).

Real-time PCR

Six rats each in the restraint and control groups were compared for the expression of CRH, mast cells, and 5-HTR3a in the small intestine and colon using real-time PCR. The transcripts encoding these were normalized to those encoding GAPDH. We found that the mRNA expression was not significantly different between the restraint and control groups (Table 1). Examining the segment

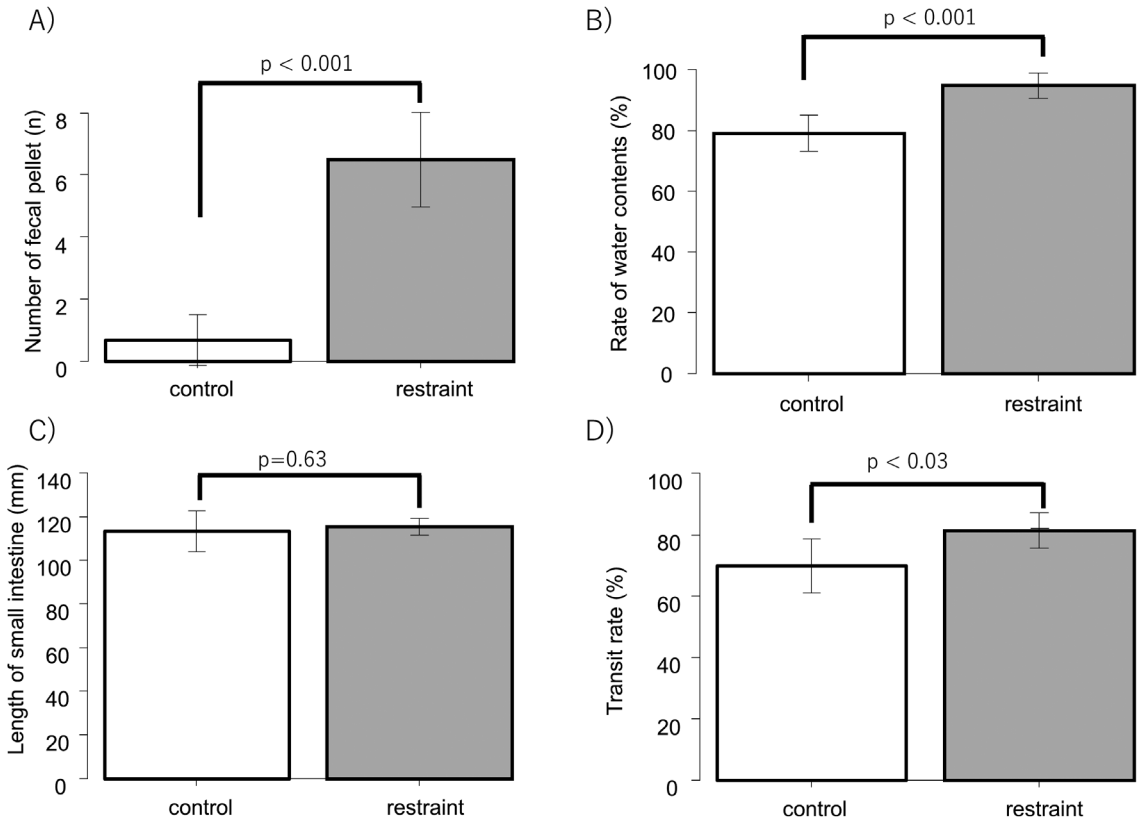

Figure 2 A: The number of fecal pellet outputs during 1 hour of isolation was counted. The restraint (n = 12) and control (n = 12) groups were compared. B: Comparison of the rate of fecal water content in the restraint (n=6) and control (n=6) groups. The first fecal pellet collected during isolation and after isolation was compared ((fecal weight before drying − fecal weight after drying) / fecal weight before drying (%)). C: The length of the small intestine was compared between the restraint (n = 9) and control (n = 16) groups. D: The transit rate of the small intestine was compared between the restraint (n = 9) and control (n = 16) groups. The small intestinal transit rate was calculated as the length of the small intestine containing the marker / total small intestinal length × 100%.

Table 1 Amount of mRNA encoding CRH, mast cell, and 5-HT receptor 3a mRNA expression in the small colon and colon as quantified by real-time PCR

		CRH	Mast cell	5-HTR3a
Small intestine	Restraint group	−	1.50±1.50	0.03±0.02
	Control group	−	2.36±2.42	0.04±0.03
	P value	−	0.408	0.336
Colon	Restraint group	−	1.28±1.00	1.94±0.93
	Control group	−	1.37±0.96	1.25±0.68
	P value	−	0.81	0.05

Values are mean ± standard deviation. There was no difference in CRH, 5-HT receptor and mast cell subunits between the restraint and control groups.
CRH, corticotropin-releasing hormone; PCR, polymerase chain reaction; 5-HT, 5-Hydroxytryptamine; 5-HTR3a, 5-Hydroxytryptamine Receptor 3A

of the small intestine, 5-HTR3a expression was significantly increased at the distal portion compared to that at the proximal section in both the restraint and control groups (restraint: p = 0.009; control: p = 0.01) (Figure 3A, B). Moreover, in the distal small intestine, there was no significant difference in 5-HTR3a expression between the restraint and control groups (0.06 ± 0.03 vs. 0.04 ± 0.01: p = 0.16) (Figure 3C).

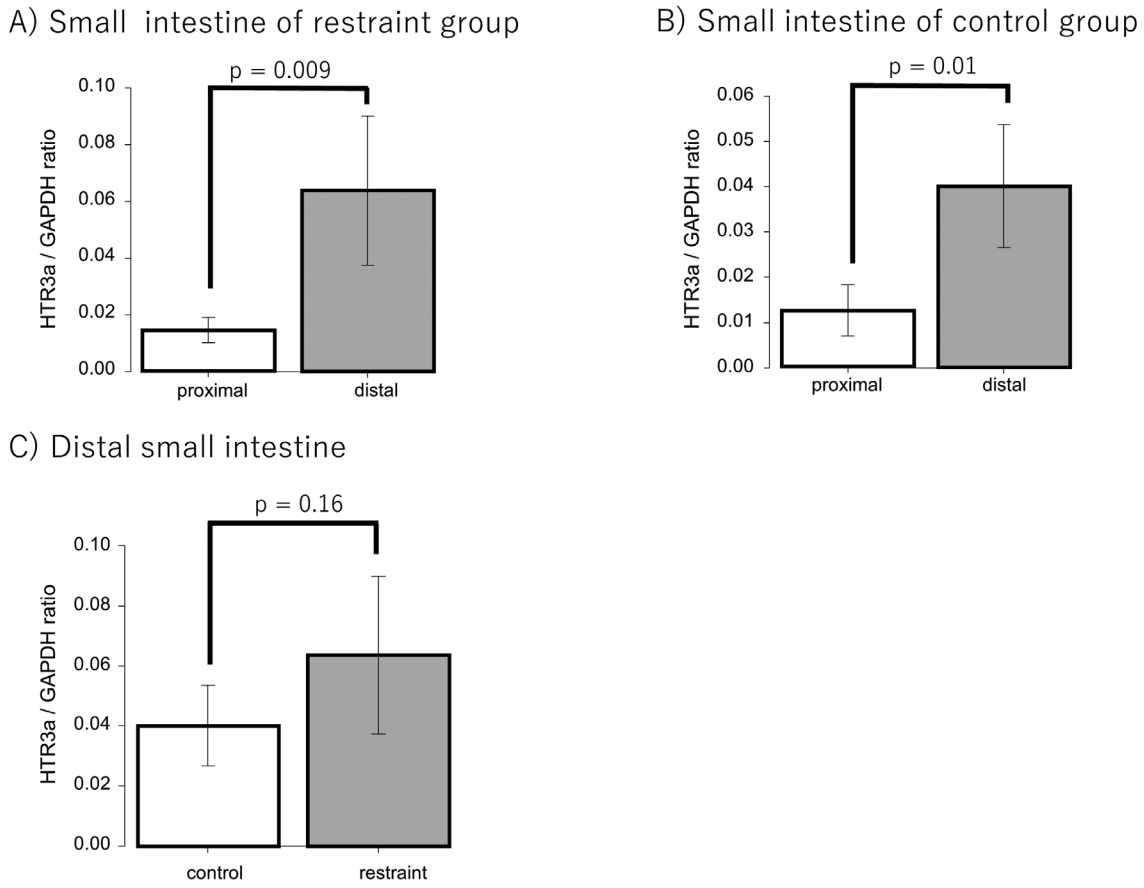

Figure 3 A: The expression of 5-Hydroxytryptamine Receptor 3A (5-HTR3a) in the proximal and distal small intestine was compared in the restraint group. B: The expression of 5-HTR3a in the proximal and distal small intestine was compared in the control group. C: The expression of 5-HTR3a was compared between the restraint and control groups only in the distal small intestine.

Immunohistochemical analysis

Immunostaining of 5-HT and 5-HTR3a was performed using tissues from the small intestine and colon. Six rats each from the restraint and control groups were used for comparison. Since 5-HT is secreted by EC cells, the 5-HT antibody staining of the small intestine and colon tissues was used to stain the EC cells. Positive cells in the control and restraint groups were counted and compared. As a result, EC cell expression tended to increase in the restraint group compared to that in the control group in both the small intestine and colon (Figure 4A, B). The number of positive cells was significantly higher in the restraint group than in the control group in the small intestine instead of the colon (small intestine: $p = 0.004$; colon: $p = 0.3$) (Figure 5). When the number of EC cells in the small intestine was compared between the proximal and distal small intestine, no significant difference was observed between the restraint and control groups (restraint group: $p = 0.95$; control group: $p = 0.48$) (Figure 6A, B). In the distal small intestine, the number of EC cells significantly increased in the restraint group compared to that in the control group (1.33 ± 0.52 vs. 0.53 ± 0.18, respectively; $p = 0.012$) (Figure 7A). In the proximal small intestine, there was no significant difference in the number of EC cells between the restraint and control groups (1.36 ± 0.82 vs. 0.67 ± 0.37, respectively; $p = 0.13$) (Figure 7B). Moreover, there was no significant difference in 5-HTR3a between the control and restraint groups in both the small intestine and colon (Figure 8).

Discussion

Animal models of bowel motility dysfunction associated with stress induced by cold, acoustics, ether, cold restraint, and wrap restraint have been previously reported. For example, Murakami et al.[13] intravenously administered CRH to induce stress

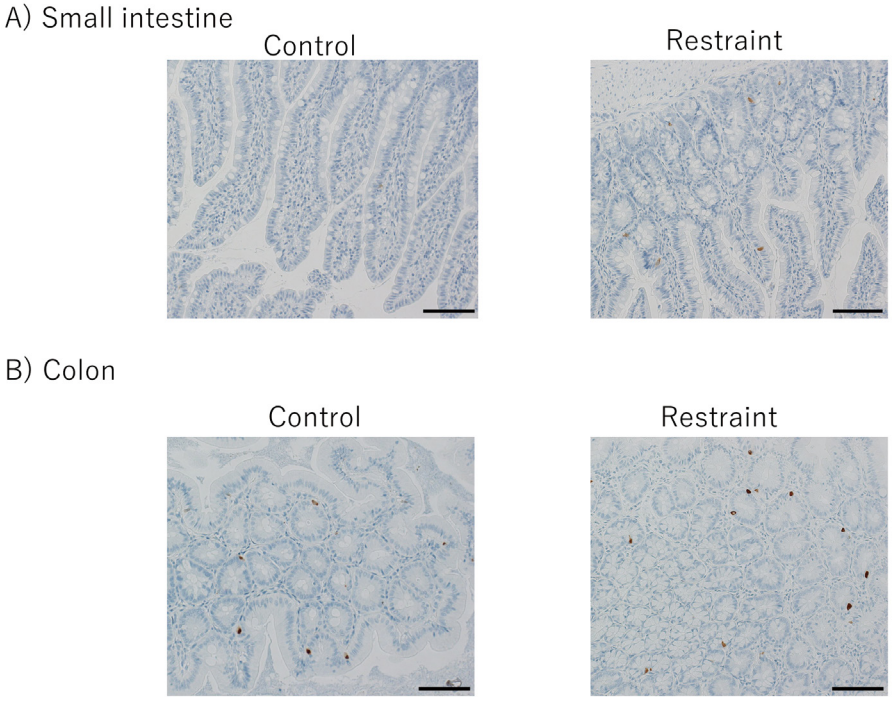

Figure 4 Enterochromaffin cell (EC cell) expression tended to increase in the restraint group compared to that in the control group in both the small intestine and colon. Bar = 100 μm. A: Photographs of the small intestine tissue. The positive cells (EC cells) were detected. The number of positive cells between the restraint and control groups was compared. B: Photographs of the colon tissue. The number of positive cells between the restraint and control groups was compared.

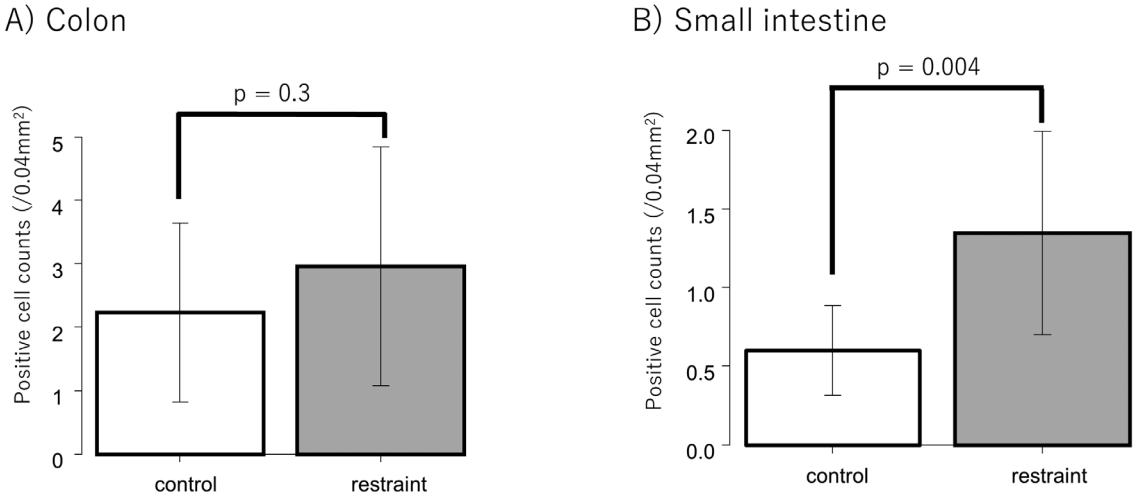

Figure 5 Comparison of the number of positive cells in the restraint (n = 6) and control (n = 6) groups and expression of 5-Hydroxytryptamine (5-HT) in the small intestine and colon. 5-HT is secreted from EC cells. EC cells that were stained and positive were counted. The number of EC cells in the small intestine increased in the restraint group, but not in the colon. A: The counted positive cells in the colon were compared between the restraint and control groups. B: The counted positive cells in the small intestine were compared between the restraint and control groups.

in rats. On the other hand, Bradesi et al.[14] used water-avoidance stress. Furthermore, restraint stress has been used in rat stress models. The number of defecations in rats reportedly increase by restraining the body[6]. In the present study, adolescent rats subjected to restraint alone at room temperature showed an increase in the fecal pellet output without the formation of gastrointestinal

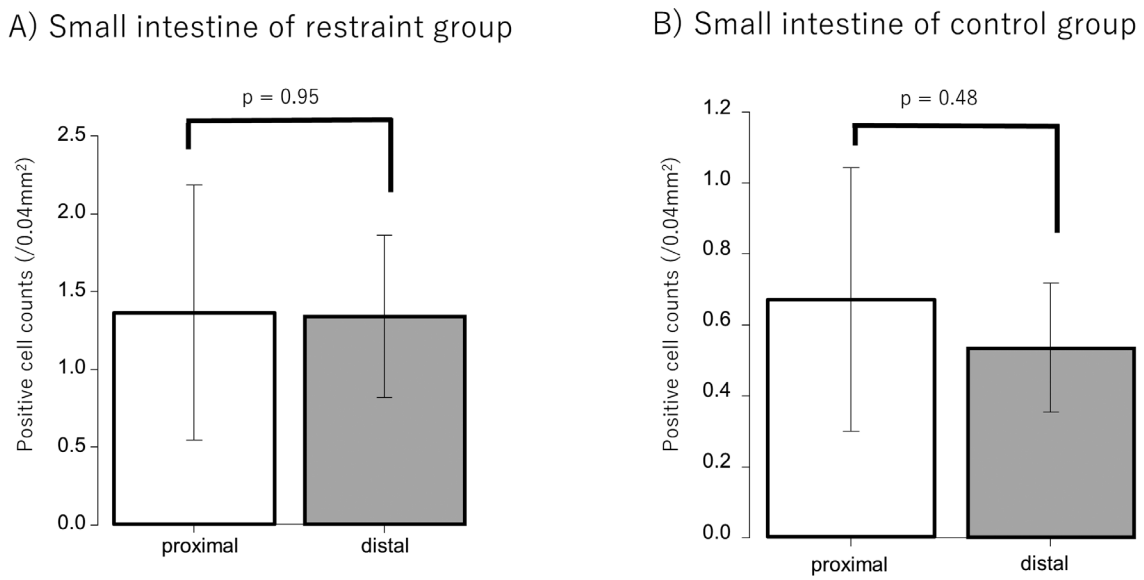

Figure 6 A: Comparison of enterochromaffin cell (EC cell) expression between the proximal small intestine and the distal small intestine in the restraint group. B: Comparison of EC cell expression between the proximal small intestine and the distal small intestine in the control group.

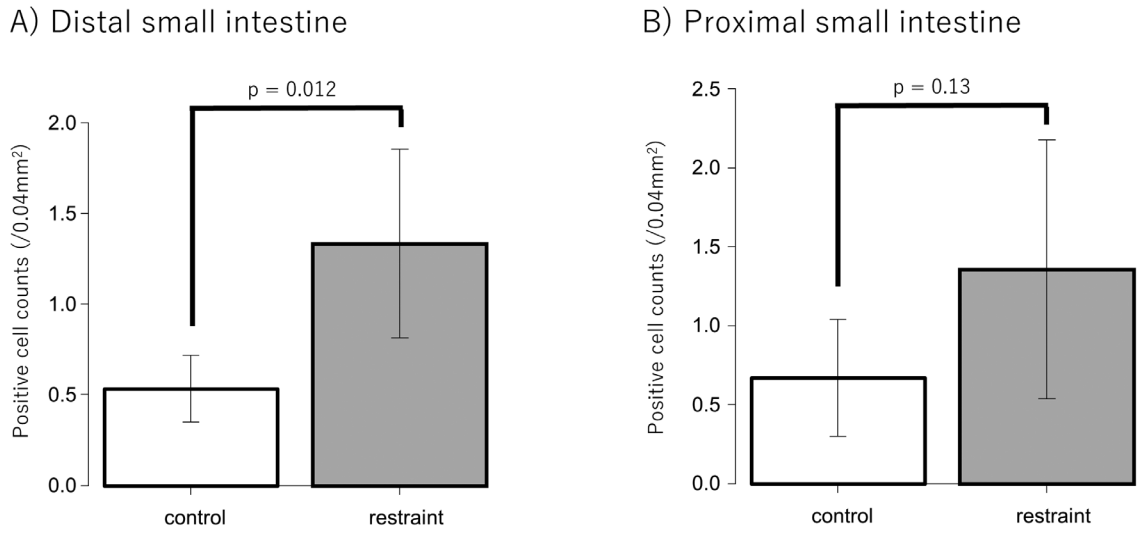

Figure 7 A: The number of Enterochromaffin cells (EC cells) in the distal small intestine was compared between the restraint and the control groups. B: The number of EC cells in the proximal small intestine was compared between the restraint and control groups.

lesions. We chose restraint stress to prepare IBS rat models because this stress method is simple and provides equivalent stress for each rat. In humans, IBS is diagnosed based on the clinical symptoms. Symptoms are very important to confirm IBS even in the animal models created in our experiment. In the present study, rats showed an increased fecal pellet output and diarrhea as clinical signs. Although we could not evaluate visceral hypersensitivity as no increased CRH release was observed, it was possible to create IBS rat models. There are three types of IBS: diarrheal, constipated, and mixed. The present study is a rat model of IBS with diarrhea.

Currently, most studies on IBS focus on visceral hypersensitivity and the brain-gut interaction. Therefore, it is important to understand gastrointestinal motility during IBS treatment. There are some reports about colon motility in IBS[15-17]. However, Hardy et al.[18] reported that when comparisons were made between diarrhea-type and constipation-type IBS, it was observed that

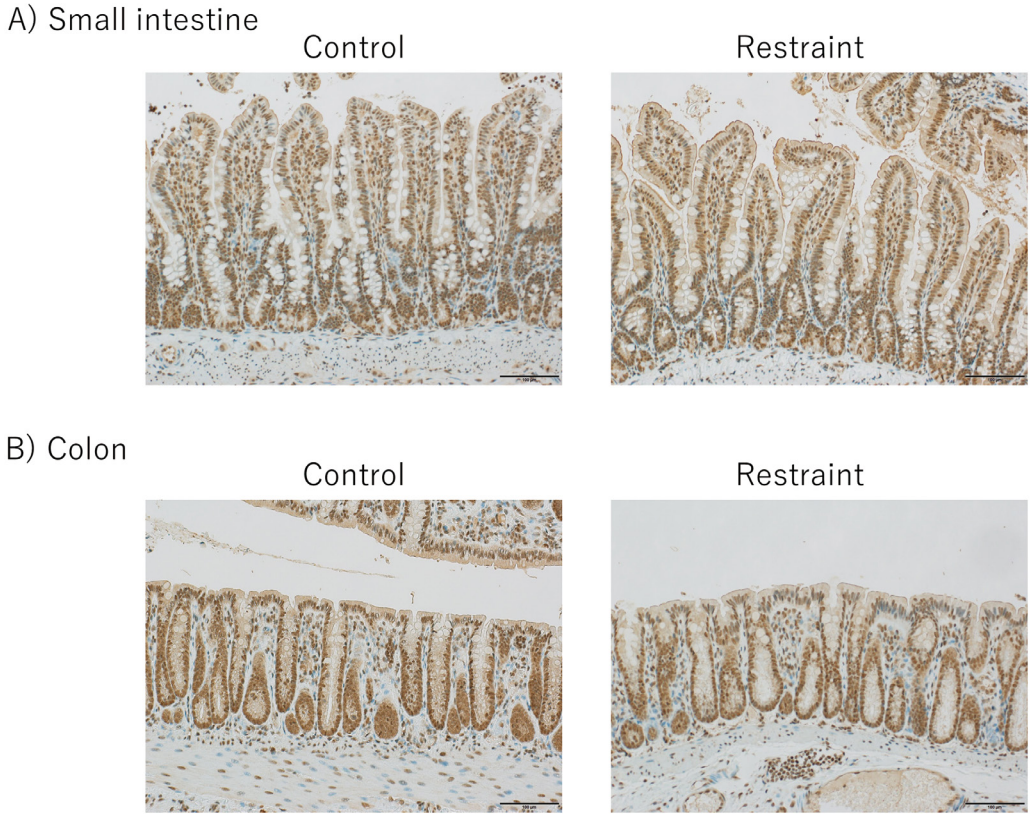

Figure 8 The expression of 5-Hydroxytryptamine Receptor 3A (5-HTR3a) in the small intestine and colon. Bar = 100 μm. A: Photographs of the small intestine tissue. The expression of 5-HTR3a between the restraint and control groups was compared. B: Photographs of the colon tissue. The expression of 5-HTR3a between the restraint and control groups was compared.

the colonic transit time in diarrhea-type IBS was shorter, whereas no difference in transit time was observed in the small intestine. Therefore, there was no influence on the small intestine movement in IBS. However, in the present study, small intestinal motility was significantly elevated, as observed in the small intestinal transit rate. Furthermore, colon motility was increased, as observed in the fecal pellet output and water content examination. Our IBS rat model had a shortened transit time in the entire intestine. In general, it is known that water is absorbed in the large intestine. However, in reality, 80% of the water we ingest is absorbed in the small intestine. Therefore, it is suggested that increased peristalsis of the small intestine may play a significant role in the appearance of diarrhea symptoms.

In this study, 5-HT, 5-HTR3a, and mast cells were evaluated in the colon and small intestine as factors that regulate the movement of the intestinal tract. Most 5-HT are synthesized and stored in EC cells, and 5-HT receptors are located throughout the intestinal tract. 5-HTR3 antagonists reportedly reduce visceral hypersensitivity and pain in patients with IBS[19]. Therefore, 5-HT and 5-HTR3 play important roles in the gastrointestinal tract motility. However, their expression in the small intestine remains unclear. In our study, the motility of the small intestine in the restraint group as adolescent IBS model rats was significantly enhanced. Therefore, an increase in 5-HT and 5-HTR3 levels in the small intestine was expected. In the restraint group, EC cell expression in the small intestine was significantly increased, but not 5-HTR3a. This result suggests that 5-HT strongly affects the enhancement of small intestinal motility under acute stress. When the increase in EC cell expression in the small intestine among rats in the restraint group was evaluated with respect to the segment of the small intestine, we observed that the expression was significantly increased in the distal small intestine compared to that in the proximal small intestine. Furthermore, real-time PCR did not show a stress-related increase

in 5-HTR3a in the small intestine; however, the distal small intestine expressed more 5-HTR3a than the proximal small intestine in both the restraint and control groups. Therefore, the distal small intestine may be mainly involved in small intestinal motility. The high expression of EC cells in the small intestine may result in increased intestinal motility and may be involved in developing diarrheal symptoms in IBS. This suggests that the small intestine is more susceptible to stress than the colon. In our study, real-time PCR showed no significant difference in mast cells between the restraint and control groups. However, there are some studies on an increased number of mast cells in patients with IBS. Chadwick et al.[20] reported that the number of mast cells increased in the colonic mucosa of patients with IBS. We found that 5HTR3a was not significantly increased by stress loading, but its expression was significantly higher in the distal small intestine. In addition, 5HT was increased by stress loading, but the increase was more prominent in the small intestine than in the colon. Furthermore, when compared within the small intestine, the increase in the distal small intestine was significantly greater than that in the proximal. Therefore, we believe that the increase of 5HT in the distal small intestine, where the expression of 5HTR3a is originally high, is strongly involved in the enhancement of small intestinal peristalsis. In the present study, we used stress loading, so the involvement of 5HT is likely to be large, but we believe that the site of high expression of 5HTR3a is also important. Weston et al.[21] reported that the number of these cells increased in the ileal mucosa of patients with IBS. It is possible that the reason the mast cells did not increase in our study was because the stress introduced was acute and not chronic. However, it remains unclear whether stress and IBS are correlated with an increase in the number of mast cells. The number of mast cells can also be increased by inflammation. Based on these facts, it can be reported that the adolescent IBS rat model created in our study had no inflammation and that the IBS rat model could be accurately created.

Nevertheless, our study had some limitations. First, in the creation of adolescent IBS model rats, the stress was acute, not chronic. While it might have been better if we chose chronic stress because IBS is a chronic syndrome, there was the possibility of ulcer formation due to chronic stress, which we wanted to avoid. We decided that chronic stress causing intestinal ulceration should not be a part of the evaluation, since this study dealt with functional intestinal disorders. Second, in the present study, only diarrhea-type IBS model rats were included. The pathogenesis of IBS includes diarrhea, constipation, and mixed types. Therefore, it is suggested that the results may differ depending on the pathology. Third, we were unable to evaluate increased CRH release. We tried to evaluate the increase in CRH using RT-PCR, but it could not be detected. Therefore, we determined whether the adolescent IBS rat model could be created based on clinical symptoms. Fourth, 5-HTR3a was evaluated using immunostaining; however, the difference in expression levels could not be quantified. RT-PCR did not show a significant increase in 5-HTR3a expression in both the small intestine and colon. If the expression level had been quantified using immunostaining, the evaluation would be more accurate. In the present study, the evaluation focused on 5-HT. However, there are other factors known to be involved in intestinal motility. We would like to evaluate these factors in future studies.

IBS model rats could be created by applying restraint stress. We found that the small intestine was prominently involved in enhancing intestinal peristalsis, which causes diarrhea. In particular, the distal small intestine of adolescent IBS model rats may be significantly involved in enhancing small intestine movement due to acute stress loading.

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Author contributions

T.K., M.S., T.I. and K.S. formulated the ideas, research goals and aims, and development or design of methodology; M.S., T.I., T.K. and Y.O. curated data and conducted formal analysis; N.A., R.K., K.H. and K.J. conducted the research and investigation process, specifically performing the experiments or collecting data/evidence; T.S. was entrusted with the leadership responsibility for the research activity planning and execution, including providing mentorship to the core team; M.S., T.K. and Y.O. created and/or presented the published work, specifically writing the initial draft; T.I. and T.K. performed critical review, commentary, or revision. All authors read and approved the final manuscript.

Conflicts of interest statement

The Authors declares that there are no conflicts of interest.

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Case Reports

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Detection of *Acinetobacter Baumannii* and *Staphylococcus Capitis* in Bile from Two Patients with Chronic Xanthogranulomatous Cholecystitis: The Impact of Metagenomic Analysis

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Background: *Escherichia coli* is thought to cause xanthogranulomatous cholecystitis (XGC). However, it is unclear whether other pathogens are associated with the cause and progression of XGC.

Case presentation: Patient 1 was a 55-year-old man with a previous surgical history of right lung cancer. He presented with abdominal pain and was diagnosed with acute cholecystitis. He underwent endoscopic nasogallbladder drainage (ENGBD), antimicrobial therapy, and endoscopic sphincterotomy (EST). He underwent cholecystectomy on day 59. The patient was pathologically diagnosed with chronic phase XGC. *Acinetobacter baumannii* was isolated from the bile sample during the operation. Patient 2 was a 58-year-old man with no previous medical history. He presented with abdominal pain and was diagnosed with acute cholecystitis. He underwent endoscopic retrograde biliary drainage (ERGBD) and antimicrobial therapy. His symptoms improved, but acute cholecystitis became exacerbated on day 53. The patient was treated with antimicrobial therapy. He underwent cholecystectomy on day 88. The patient was pathologically diagnosed with focal acute inflammatory phase XGC. *Staphylococcus capitis* was isolated from the bile during the operation. This study describes two patients with XGC, one infected with *A. baumannii* and the other with *S. capitis*, in their gallbladders, which was identified by bacterial culture. Metagenomic analysis revealed that the genera *Acinetobacter* and *Staphylococcus* predominated and that other genera, including *Delftia* and *Anaerobacillus*, were also present, suggesting that these bacteria play a significant role in the pathological changes associated with XGC.

Conclusions: This is the first report of *A. baumannii* and *S. capitis* infections in patients with XGC.

Key words: xanthogranulomatous cholecystitis, *Acinetobacter baumannii*, *Staphylococcus capitis*, metagenomic analysis

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Introduction

Xanthogranulomatous cholecystitis (XGC) is characterized histologically by the accumulation of numerous foamy macrophages in the gallbladder, resulting in thickening of the gallbladder wall[1]. Moreover, XGC is frequently diagnosed as gallbladder carcinoma. *Escherichia coli* antigens have been detected immunohistologically in XGC lesions[2], and *E. coli* was reported to be involved in the pathogenesis of XGC, with scavenger receptor class A and CXCL16-CXCR interactions[3]. These results suggested that *E. coli* infections of the gallbladder play an important role in the onset and/or early stages of XGC. However, it is unclear whether other pathogens are associated with the cause and progression of XGC.

This study describes the isolation of two other species of bacteria, *Acinetobacter baumannii* and *Staphylococcus capitis*, from the bile samples of two patients with XGC. Metagenomic analysis of the microbiota in bile samples confirmed that these pathogens, as well as other microorganisms, were present in bile.

Case report

Two patients (P1 and P2) underwent cholecystectomy for suspected chronic cholecystitis in July 2018 at the Department of Hepato-biliary Pancreatic Surgery, Juntendo University Hospital. Written informed consent was obtained from these patients before surgery. This study was conducted according to the principles of the Declaration of Helsinki, and approved by the Juntendo University ethics committee (JHS 18-060 Juntendo University Hospital Independent Ethics Committee). The clinical characteristics of these two patients are summarized in Table 1. Chronic cholecystitis in these patients was diagnosed as XGC by histopathological examination of the gallbladder samples.

Patient 1 was a 55-year-old Japanese man with a previous surgical history of right lung cancer seven years earlier, and was admitted to an intensive care unit (ICU) with a mechanical ventilator. He presented with abdominal pain and was diagnosed with acute cholecystitis. He underwent endo-

Table 1 Clinical presentations and results of bacterial culture

Patient	P1	P2
CHARACTERISTICS OF PATIENTS		
Age Sex	55M	58M
Past medical history	Right lung cancer Af	NA
Cause of acute cholecystitis	Cholesterol stones	Cholesterol stones
LABORATORY DATA		
White blood cell count(cells/mm3)	15500	9600
C-reactive protein (mg/dL)	21	26
CEA(ng/ml) (< 5.0) / CA19-9(U/ml) (< 37.0)	3.1 / 11	1.9 / 13
PREOPERATIVE CHARACTERISTICS		
Time from onset to operation (days)	59	88
Drainage	ENGBD EST	ERGBD
Antimicrobial agents	CMZ MEPM	CMZ PIPC/TAZ CTRX LVFX
Discharge (Postoperative day)	7	5
TG 18 SEVERITY CLASSIFICATION		
Mild/Moderate/Severe	Moderate	Moderate
Blood culture	Negative	Negative
Bacterial profile of bile culture	Acinetobacter baumanii	Staphylococcus capitis
OPERATION		
Operation	Laparotomy cholecystectomy	Laparoscopic cholecystectomy

※NA, not applicable; Af, Atrial fibrillation; ERGBD, Endoscopic retrograde gallbladder drainage; ENGBD, Endoscopic nasogallbladder drainage; EST, Endoscopic sphincterotomy; CMZ, cefmetazole; PIPC/TAZ, Piperacillin/Tazobactam; CTRX, Ceftriaxone; LVFX, Levofloxacin; MEPM, Meropenem

scopic nasogallbladder drainage (ENGBD) on the first day and was treated with 3 g/day cefmetazole (CMZ) for three days. The bile sample obtained during ENGBD was negative for bacterial culture, but his AST/ALT was elevated. Endoscopic sphincterotomy (EST) was performed on the third day, and he was treated with 1.5 g/day meropenem (MEPM) for three days, followed by 3 g/day CMZ for three days. His symptoms disappeared and the patient was not administered any antimicrobial agents after day 10. He underwent cholecystectomy on day 59. Gross examination of the resected gallbladder showed the hemorrhagic mucosa with marked wall thickness (Figure 1A). Microscopic examination showed diffuse infiltration of foam cells along with multinucleated giant cells, lymphocytes, and cholesterol deposit (Figure 1B, C). No bacterial colonies or neutrophilic reactions were evident histologically. The patient was pathologically diagnosed with chronic phase XGC. *A. baumannii* was isolated from the bile sample obtained from Patient 1 during the operation. This isolate was susceptible to all drugs tested (Table 2). Metagenomic analysis of the bile sample from Patient 1 showed bacterial DNA derived from seven genera, with the genus *Acinetobacter* being predominant. Filtering of the data sets to include OTUs present in > 0.5% of the samples revealed bacterial DNA from four phyla, *Actinobacteria* (0.8%), *Bacteroidetes* (1%), *Firmicutes* (10%) and *Proteobacteria* (88%) (Figure 2).

Patient 2 was a 58-year-old Japanese man with no previous medical history. He presented with abdominal pain and was diagnosed with acute cholecystitis. He underwent endoscopic retrograde biliary drainage (ERGBD) on the first day, and was treated with 6 g/day CMZ for two days, followed by 18 g/day piperacillin-tazobactam (PIPC/TAZ) for five days. His symptoms improved, but acute cholecystitis became exacerbated on day 53. The patient was treated with 1 g/day ceftriaxone (CTRX) for one day, followed by 0.5 g/day levofloxacin (LVFX) for seven days. His symptoms disappeared and the patient was not administered any antimicrobial agents after day 60. We adjusted the waiting period for a month to improve the inflammation, and he underwent cholecystectomy on day 88. Gross examination of the resected gall-

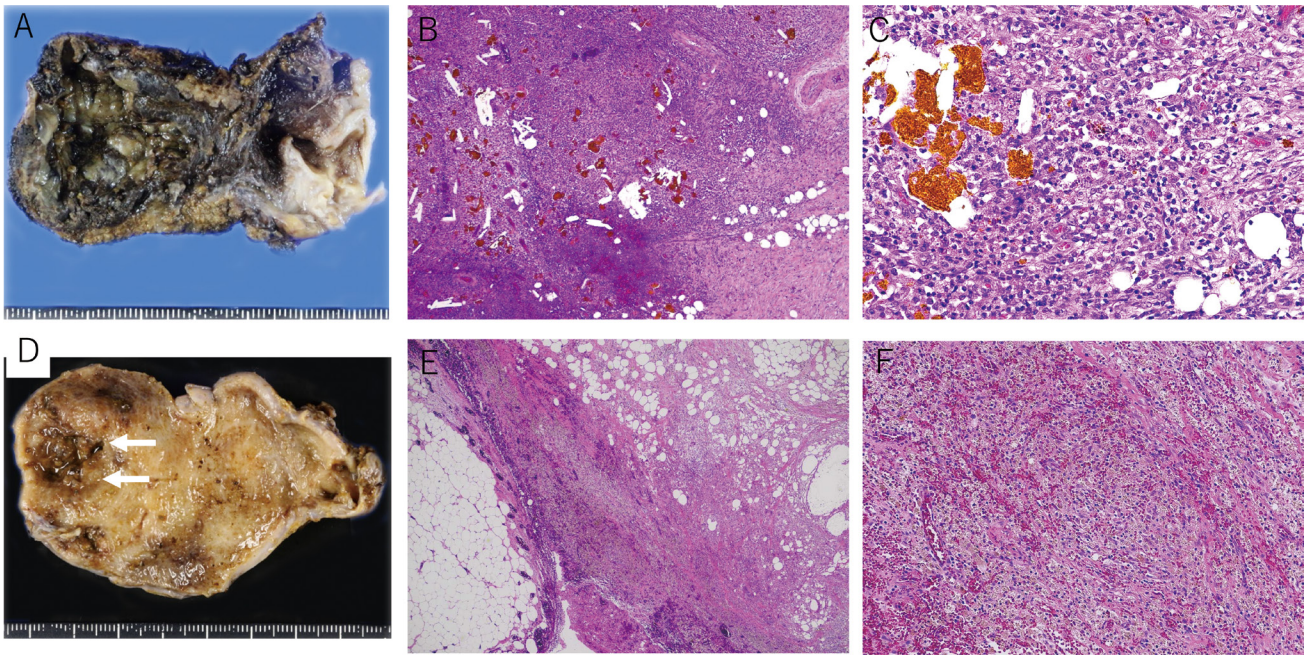

Figure 1 Pathological view of the 2 cases of gallbladder with xanthogranulomatous cholecystitis
1A-C: Patient 1. A: Grossly, a thickened black gallbladder is seen. B and C: Microscopically, diffuse infiltration of foam cells and lymphocytes are observed along with bile and cholesterol deposits.
1D-F. Patient 2. D. Grossly, a thickened gallbladder with coarse mucosa at the fundus (Arrows) is seen. E and F: Microscopically, diffuse infiltration of foam cells with bile pigment is seen.

Table 2 MIC and susceptibility of isolated bacteria to antibiotics

Drug susceptibility profiles of *A. baumannii*

Drugs	MICs in mg/L
ABK	1
AMK	4
AZT	16
CAZ	2
IPM	0.25
MPM	0.25
CIP	0.125
CST	0.5
PIP	16

*ABK, arbekacin; AMK, amikacin; AZT, aztreonam; CAZ, ceftazidime; IPM, imipenem; MPM, meropenem; CIP, ciprofloxacin; CST, colistin; PIP, piperacillin

Drug susceptibility profiles of *S. capitis*

Drugs*	MICs in mg/L
ABK	2
ABPC	64
CAZ	256
CEZ	128
EM	>2048
GM	64
LVFX	512
OXA	1024
PCG	64
TEIC	0.25
VCM	1

*ABK, arbekacin; ABPC, aminobenzylpenicillin; CAZ, ceftazidime; CEZ, cefazolin; EM, erythromycin; GM, gentamicin; LVFX, levofloxacin; OXA, oxacillin; PCG, penicillin G; TEIC, teicoplanin; VCM, vancomycin

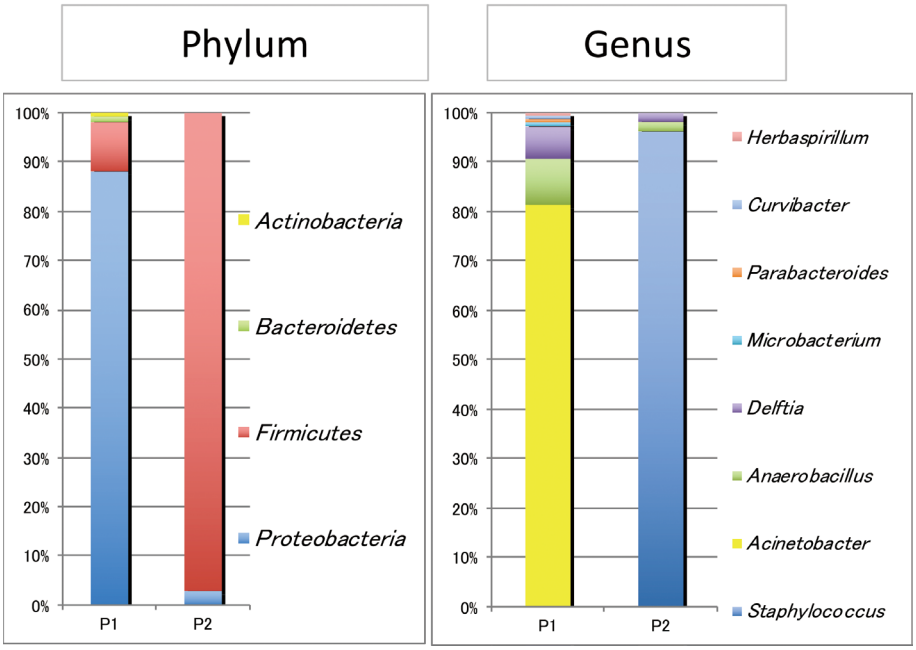

Figure 2 Relative abundance of major bacteria in bile

bladder showed that rough and coarse mucosa at the fundus with marked wall thickness and microscopic examination showed infiltration of bile containing foam cells along with lymphocytes (Figure 1D–F). Gram staining showed no evident bacterial colonies, although some neutrophilic reactions were observed, histologically. The patient was pathologically diagnosed with focal acute inflammatory phase XGC. *S. capitis* was isolated from the bile sample obtained from Patient 2 during the operation, with this isolate being resistant to ABPC, CAZ, EM, GM, LVFX, OXA, and PCG, but susceptible to ABK, TEIC, and VCM (Table 2). Metagenomic analysis of the bile sample from Patient 2 showed bacterial DNA derived from four genera, with the genus *Staphylococcus* being predominant. Filtering of the data sets revealed bacterial DNA from two phyla, *Firmicutes* (97%)

and *Proteobacteria* (3%) (Figure 2).

Laboratory procedure

During cholecystectomy, bile samples were obtained under sterile conditions from the fundus of the gallbladder using an 18G needle and 20 ml syringe. A portion of each was immediately frozen at −80℃ for metagenomic analysis, and the remaining bile samples were sent to the bacteriologic laboratory for culture. Samples were inoculated onto 5% sheep blood trypticase soy agar (Nissui Pharmaceutical, Japan), Drigalski agar (Nissui Pharmaceutical) and Anaero Columbia agar RS (Becton Dickinson and Company, Japan), and incubated in an atmosphere containing 10% CO_2 at 35℃ for 5 days. The isolates were identified using the MALDI biotyper (Bruker Co., Ltd., Japan) over a score of 1.700. The minimum inhibitory concentrations (MICs) of antimicrobial agents were determined using the microdilution method, according to the guidelines of the Clinical & Laboratory Standards Institute (CLSI, M100-S25). The antimicrobial agents tested included amikacin (AMK), aminobenzylpenicillin (ABPC), arbekacin (ABK), aztreonam (AZT), cefazolin (CEZ), ceftazidime (CAZ), ciprofloxacin (CIP), colistin (CST), erythromycin (EM), gentamicin (GM), imipenem (IPM), levofloxacin (LVFX), meropenem (MEPM), oxacillin (OXA), penicillin (PCG), piperacillin (PIP), teicoplanin (TEIC) and vancomycin (VCM). Genomic DNA of bacterial isolates was extracted using DNeasy Blood & Tissue kits (QIAGEN, Tokyo, Japan). Genomic libraries were prepared using Nextera XT DNA kits (Illumina, San Diego, CA). Paired-end sequencing was performed using MiSeq Reagent Kit v3 (600-cycles). Quality trimming, filtering and assembly of the obtained sequence reads were performed using CLC Genomics Workbench v11 (QIAGEN, Hilden, Germany). The assembled genome sequence data were searched for genes associated with drug resistance, using the ABRicate program (https://github.com/tseemann/abricate) and data from the National Center for Biotechnology Information (NCBI), the Comprehensive Antibiotic Resistance Database (CARD), and the ResFinder databases.

DNA was extracted from bile samples using DNeasy PowerSoil Kits (QIAGEN). Each library was prepared in accordance with "Illumina 16S Metagenomic Sequencing Library Preparation Guide" with a primer set (27Fmod: 5′- AGR GTT TGA TCM TGG CTC AG -3′ and 338R: 5′- TGC CTC CCG TAG GAG T -3′) targeting the V1–V2 region of the 16S rRNA gene. The 251 bp paired end sequencing of the amplicon was performed on a MiSeq. The obtained paired end sequences were merged using PEAR (http://sco.h-its.org/exelixis/web/software/pear/). Subsequently, 30,000 reads per sample were randomly sampled using seqtk (https://github.com/lh3/seqtk) for taxonomic assignment. These sampled sequences were clustered into operational taxonomic units (OTU) defined as 97% similarity using UCLUST version 1.2.22q. Representative sequences for each OTU were classified taxonomically using RDP Classifier version 2.2 with the Greengenes database (gg_13_8).

The isolate harbored 10 genes associated with drug resistance, including *AAC(6′)-Ie-APH(2″)-Ia, ant(9)-Ia, blaI, blaR1, blaZ, ermA, mecA, mecI, mecR1, and tetM.*

Discussion

Because *E. coli* is the organism most frequently isolated from bile samples of patients with cholecystitis, it has been regarded as a cause of this condition. Other microorganisms isolated from the bile samples of patients with cholecystitis include *Enterobacter, Enterococcus, Klebsiella, Streptococcus*, and *Pseudomonas* spp.[4]. These pathogens are thought to enter the gallbladder from the duodenum in a retrograde manner, although there is other possibility that they enter the gall bladder via the portal vein through the hepatic sinusoids and space of Disse[5]. Gallbladder stones play an important role in the pathological conditions observed in patients with cholecystitis.

Pathologically, XGC is characterized by thickening of the gallbladder wall, mimicking advanced gallbladder carcinoma[1]. Although these pathological changes are thought to be due to intense acute or chronic inflammation, the pathogenesis of XGC remains unclear. XGC is often associated with gallstones. Gallstones cause ulceration of the gallbladder mucosa, rupture of Rokintansky-Aschoff sinuses, and eventually xanthogranulomatous changes[6].

Next-generation DNA sequencing has enabled analysis of the microbiota in the biliary tracts of patients with various diseases, including bacterial

infection associated with acute cholecystitis[7], chronic cholecystitis[8], and XGC. This study found that *A. baumannii* and *S. capitis* were present in the bile samples of two patients with XGC, suggesting that bacteria other than *E. coli* contribute to pathological changes in XGC. To our knowledge, no previous study has reported that other species of bacteria, including *A. baumannii* and *S. capitis*, contribute to XGC. Two types of nephritis pathologically similar to XGC, xanthogranulomatous pyelonephritis and urinary malakoplakia, are caused by *Enterobacteriaceae*, including *E. coli*[9, 10]. Histologic examination of gallbladder samples showed localized accumulation of abundant foamy macrophages[1, 9, 10], with these pathological changes induced by *E. coli* infection[2, 3].

A. baumannii is a Gram-negative, opportunistic pathogen that can survive on solid and dry surfaces for up to 5 months. *A. baumannii* can grow over wide ranges of temperature and pH and forms biofilms on abiotic substrates[11]. *A. baumannii* may have been present in bile when Patient 1 was admitted to the ICU.

S. capitis is a coagulase-negative staphylococcus with documented potential for both human diseases and nosocomial spread[12]. *S. capitis*, which causes prosthetic valve endocarditis and joint infection[12], is mainly distributed on the head (primary ears and forehead), arms and occasionally the legs[13]. The *S. capitis* isolate obtained from the Patient 2 was multidrug-resistant, suggesting that this patient must have been infected with this pathogen during in-hospital treatment with antibiotics. In addition, the procedures used during ERCP may have been responsible for the entry of this pathogen into the biliary tract.

To our knowledge, this is the first report of *A. baumannii* and *S. capitis* infections in patients with XGC. *Delftia* and *Anaerobacillus*, as well as other several genera, may play a role in the histopathological changes involved in progression from chronic cholecystitis to XGC. However, it is unclear whether these pathogens directly contribute to the pathological change of XGC. Further studies are needed to analyze the bacteriology and metagenome involved in gallbladder diseases. Metagenomic analysis is a useful tool for detection of pathogens in chronic cholecystitis such as XGC.

Declarations

Ethics approval and consent to participate

This study was conducted according to the principles of the Declaration of Helsinki, and approved by the Juntendo University ethics committee (JHS 18-060 Juntendo University Hospital Independent Ethics Committee).

Consent for publication

Written informed consent was obtained from these patients before surgery for publication of this case report.

Availability of data and materials

All sequence data of the 16S rRNA sequences for metagenome analysis and two isolates in this study were deposited in the DDBJ/GenBank/EMBL database under accession number DRA007947, DRA007974 and DRA007975, respectively.

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Author contributions

MM and AS treated the patient. MT, TK, and AN assessed the bacteriological analysis. DM, SN, and TI assessed the microbial analysis. YF performed pathological evaluation. MM, SW, and TK drafted the manuscript and responsible for study design. All authors read and approved the final manuscript.

Conflicts of interest statement

The authors declare that they have no competing interests.

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Aging Biology in Health and Disease

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Diagnostics and Therapeutics of Intractable Diseases

〈Original Articles〉

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〈Reviews〉

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Format

The first page of each manuscript should contain: Title, Authors' full names, Affiliations, Key words, Running Title, and the name and full address (including telephone number, facsimile number, and e-mail address) of the corresponding author. Manuscripts should be divided into the following sections and presented in this order: Introduction; Materials and Methods; Results; Discussion; Acknowledgements; Funding; Authors' contributions; Conflicting interest statement; and References.

Manuscripts should be arranged in the following order: 1. Title page; 2. Abstract and keywords; 3. main text; 4. Acknowledgements, Funding, Author Contributions, Conflict of interest statements; 5. tables together with any accompanying legends; 6. figure legends; 7. other as required. Each of the numbered items should begin on a separate page.

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SI or SI-derived units should be used. More information on SI units is available at the Bureau International des Poids et Mesures (BIPM) website (https://www.bipm.org/en/about-us/).

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The second (and, if necessary, the third) page of the manuscript should contain only the abstract (maximum 250 words). The abstract must be fully comprehensible without reference to the text. Abstracts should be divided into sections as follows:

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2. Materials (or "Design")
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5. Conclusions

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Acknowledgments

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major contributor in writing the manuscript. All authors read and approved the final manuscript."

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All manuscripts must include a "Conflicts of Interest statement" in line with the 'Author competing interests and conflicts of interest' section above. If no conflicts exist, please state that "The Author(s) declare(s) that there are no conflicts of interest".

References

References, including those given in tables and figure legends, should be numbered sequentially in the order they appear in the text and listed in numerical order at the end of the manuscript under the heading "References". In the text, citations should be indicated as superscript numbers with an end parenthesis character following each citation number. Three or more consecutive citations should be indicated as a range using a hyphen, e.g. "3)-5)". Journal titles should be abbreviated as shown in Index Medicus and List of Journals Indexed. When there are six or fewer authors, all should be listed; when there are seven or more, include only the first three and add "et al." Please note the following examples.

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1) You WC, Blot WJ, Li JY, et al: Precancerous gastric lesions in a population at high risk of stomach cancer. Cancer Res, 1993; 53: 1317-1321.

Book

2) Matsumoto A, Arai Y: Hypothalamus. In: Matsumoto A, Ishii S, eds. Atlas of Endocrine Organs. Berlin: Springer-Verlag, 1992: 25-38.

Tables

Tables with suitable titles and numbered with Arabic numerals should be placed at the end of the text on separate sheets (one table per page). They should be understandable without referring to the text. Column headings should be kept as brief as possible, with units for numerical information included in parentheses. Footnotes should be labeled a), b), c), etc. and typed on the same page as the table they refer to.

Figures

Figures should also be submitted online as separate files. They should be numbered in order of appearance with Arabic numerals (e. g. Fig. 1, Fig. 2). Author(s) must pay printing costs for color photographs. Electron micrographs should contain a scale. Individual figures may not exceed the size of a Journal page. Graphs or drawings containing typewritten characters are unacceptable. Numbers, letters and symbols must be large enough to be legible after reduction. In principle, figures should be suitable for publication, and jpg digital files preferred. Each figure must have an accompanying legend, which should be understandable without reference to the text. All figure legends are to be double spaced, and should be collected together as text page(s), rather than being attached to their respective figures.

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Summarize briefly the important points of the submitted work including a brief description of the study to be submitted, that it is an original study presenting novel work, that it has not been previously submitted to or accepted by any other journal, that is has been approved by all authors, and explain whether any author has a conflict of interest.

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Call for feature article proposals

To introduce the latest medical findings, Juntendo Medical Journal features a specific focus area for each issue. We would like to request all our readers to address any suggestions or proposals for suitable focus areas to our editorial office.

編 集 後 記

1875年に発刊された、現在まで続く日本最古の医学誌『順天堂醫事雑誌』として、伝統ある本学術雑誌の編集委員会に、2021年6月より保健医療学部からも編集委員メンバーとして参加させていただいています。

保健医療学部は、2019年4月に新たな学部として、本郷・お茶の水キャンパスに誕生しました。本学部では、「仁」の精神に基づき、豊かな人間性と専門的知識･実践実技力を兼ね備え、多岐にわたるフィールドで活躍できる理学療法士と診療放射線技師を養成することを目的に、理学療法学科と診療放射線学科の2つの学科があります。今年度に完成年度を迎え、それぞれの学科で約480名（計960名）の学生が在籍しています。本学部の1期生である4年生は、7月初旬頃まで臨床実習に励み、その後、卒業研究と国家試験に向けて邁進していきます。

卒業研究では、各ゼミナールで研究テーマを決定し、倫理委員会承認後に研究データを取得し、卒業論文を作成します。先輩もいないため手探りで進めている状況ですが、ゼミ教員の指導を受けながら、社会に貢献できる成果が報告できるように研究計画を練っています。この過程において、英語論文とは縁遠かった学生も次第に英語論文を読むようになっています。卒業研究の経験を通して、将来的に、理学療法士や診療放射線技師として、順天堂醫事雑誌に掲載される論文のように、質の高い研究と論文作成ができるようになることを期待しています。

順天堂醫事雑誌をご覧いただいている先生方には、理学療法や臨床放射線領域に関わる論文が掲載された際には、ぜひご一読いただけますと幸いです。

山口智史
保健医療学部理学療法学科

イラスト作者より

年に一度は行くヴェトナムの小物を売る店に行って来ました。今日は変ったオブジェ風、木製の猫の置物を買いました。粗削りの上に赤、白、金色で彩色していて、単純なフォルムですが、かえって描くのが難しかったです。（宮道明子）

順天堂醫事雑誌の記事については既に明治8年の創刊号から電子化されており，J-STAGE（科学技術情報発信・流通総合システム）の電子ジャーナル公開システムにおいて閲覧することができます．順天堂医学会のホームページからもご覧いただけますので，ご活用頂ければ幸いです（https://www.juntendo.ac.jp/journal/）．

特集の企画募集

「順天堂醫事雑誌」では，医学界の最新知識を紹介するために，特集として総説を毎号に掲載しています．
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順天堂医学会 会員の皆様

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順天堂医学会短期海外留学時助成金給付制度

順天堂医学会では短期海外留学時助成金給付制度を開始いたしました。

1. 要件

下記すべての要件を満たす者

(1) 順天堂大学（大学院を含む）の学生で 1 か月以上 12 か月未満の海外留学をする者
(2) 留学先の研究機関または財団などからの援助がない者
(3) 医学会の正会員として 1 年以上の経歴を有し、医学会費を完納している者

2. 申請書類

(1) 順天堂医学会短期海外留学時助成金申込書
(2) 所属長の推薦書
(3) 申請者の主な研究テーマ・研究業績
(4) 留学受け入れ機関の指導者からの推薦状

3. 助成金の給付金額

留学期間	助成金額
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4 か月以上 7 か月未満	20 万円
7 か月以上 12 か月未満	30 万円

4. 申請スケジュール（年 2 回）

申請期限	助成決定時期
6 月末	8 月
12 月末	2 月

5. 選考機関：順天堂医学会短期海外留学時助成金選考委員会

6. 助成後の義務

(1) 帰国後直近の順天堂医学会学術集会において研究成果の発表および、その内容を「順天堂醫事雑誌」に報告する。
(2) 帰国後は、順天堂大学またはその関連機関に原則として 3 年以上勤務する。

7. 本件の照会先

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以上

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