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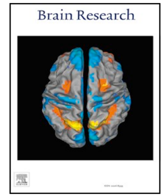

Research report

Light exercise without lactate elevation induces ischemic tolerance through the modulation of microRNA in the gerbil hippocampus

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HIGHLIGHTS

- Exercise without lactate elevation suppressed ischemia-induced CA1 neuron loss.
- Light exercise maintained short-term memory after ischemia.
- Expression of 20 miRNAs was dysregulated in ischemia but not after light exercise.
- Differentially expressed miRNAs involved in inflammation, metabolism, cell death.

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ABSTRACT

Previously we studied the possible neuroprotective effects of ischemia-resistant exercise in a gerbil model of transient whole-brain ischemia and evaluated the histology, expression of specific proteins, and brain function under different conditions. The present study investigated the neuroprotective effects of light exercise, without lactate elevation, in a gerbil model of ischemia/reperfusion injury. Transient whole-brain ischemia was induced by occlusion of the bilateral common carotid arteries for 5 min. A group of animals was subjected to treadmill exercise before ischemia induction. Hippocampal neuronal damage and miRNA expression, as well as behavioral deficits and plasma lactate levels, were evaluated. Light exercise suppressed hippocampal neuron loss and preserved short-term memory. Moreover, 14 miRNAs (mmu-miR-211-3p, -327, -451b, -711, -3070-3p, -3070–2-3p, -3097-5p, -3620-5p, -6240, -6916-5p, -6944-5p, 7083-5p, -7085-5p, and -7674-5p) were upregulated and 6 miRNAs (mmu-miR-148b-3p, -152-3p, -181c-5p, -299b-5p, -455-3p, and -664-3p) were downregulated due to ischemia. However, the expression of these miRNAs remained unchanged when animals performed light exercise before the ischemic event. Differentially expressed miRNAs regulate multiple biological processes such as inflammation, metabolism, and cell death. These findings suggest that light exercise reduces neuronal death and behavioral deficits after transient ischemia by regulating hippocampal miRNAs.

1. Introduction

MicroRNAs (miRNAs) are small single-stranded RNAs that do not encode proteins. Instead, it has become clear that miRNAs regulate the translation of target messenger RNAs. Although it has been thought that miRNAs may play an important role in mediating neuropathies in cerebral ischemia, the underlying mechanism has not been completely elucidated yet.

Abbreviations: AD, Alzheimer's disease; APP, amyloid precursor protein; CA1, Cornet d'Ammon 1; HE, hematoxylin-eosin; miRNAs, microRNAs; SSC, saline-sodium citrate; SDS, sodium dodecyl sulfate

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Previously, we studied ischemia-resistant exercise using a transient whole-brain ischemia model in gerbils and reported the possible neuroprotective effects of granulocyte colony-stimulating factor in the acute phase (Lu et al., 2014). In that study, we performed miRNA analysis and showed how ischemic tolerance exercise modulates the expression of previously reported miRNAs. It was first reported in 1994 that ischemic tolerance exercise prior to transient bilateral common carotid artery occlusion reduces the neuronal damage of the Ammon's horn and tends to preserve the number of neurons in the gerbil cerebral ischemia model (Stummer et al., 1994). Moreover, we previously examined the intensity of exercise and reported that exercise overload could damage the brain (Sumitani et al., 2002).

Therefore, in this study, we determined the expression of miRNAs after applying ischemic tolerance exercise and used serum lactic acid levels as a measure to control the intensity of exercise. Here, we report that exercise intervention induced ischemia tolerance in gerbils. We also found that the expression of hippocampal miRNAs was dysregulated upon ischemia. Some of these miRNAs are directly involved in the cleaning of neuronal cell death after ischemia via macrophages, while others protect against ischemia.

2. Results

The experimental procedure followed to study the effect of exercise on ischemic tolerance is outlined in Fig. 1.

2.1. Effect of exercise on ischemia-induced hippocampal neuronal death

Hematoxylin-eosin (HE) staining demonstrated the effect of exercise on hippocampal neuronal damage 72 h after global brain ischemia in gerbils (Fig. 2). The majority of pyramidal neurons within the Cornet d'Ammon 1 (CA1) area of the gerbils in the ischemia group were shrunken and darkly stained with minimal cytoplasm when compared to those of the sham group. In contrast, neurons within the same area in gerbils of the exercise plus ischemia group were well preserved and appeared normal (Fig. 2A). The significantly reduced hippocampal neuronal loss in the exercise plus ischemia group (Fig. 2B) demonstrates the beneficial effect of exercise ($F_{2,\ 21} = 27.477$, $p < 0.001$).

2.2. Effect of exercise on ischemia-induced behavioral deficits

Exercise also attenuated cerebral ischemia-induced behavioral deficits. As shown in Fig. 3A, short-term memory in the exercise plus ischemia group was significantly preserved compared with that of the ischemia group and was equivalent to that of the sham group ($F_{2,\ 19} = 14.861$, $p < 0.001$). The locomotor activity counts in each group did not vary significantly (Fig. 3B) ($F_{2,\ 19} = 2.385$, $p = 0.119$).

2.3. Lactate level and weight at sacrifice

The lactate level ($F_{2,\ 20} = 0.431$, $p = 0.656$) and weight ($F_{2,\ 21} = 0.508$, $p = 0.609$) of gerbils in each group at sacrifice were not significantly different (Fig. 4).

2.4. Identification of differentially expressed miRNA in the hippocampus

miRNA expression levels in the hippocampus of gerbils were compared. The custom microarray platform identified 14 miRNAs that were upregulated and 6 miRNAs that were downregulated in the hippocampus of gerbils in the ischemic group (Tables 1 and 2). As shown in Fig. 5, the upregulated miRNAs were: miR-211-3p (spot no. 1), miR-3070-2-3p (spot no. 2), miR-3070-3p (spot no. 3), miR-3097-5p (spot no. 4), miR-327 (spot no. 5), miR-3620-5p (spot no. 6), miR-451b (spot no. 7), miR-6240 (spot no. 8), miR-6916-5p (spot no. 9), miR-6944-5p (spot no. 10), miR-7083-5p (spot no. 11), miR-7085-5p (spot no. 12), miR-711 (spot no. 13), and miR-7674-5p (spot no. 14).

2.5. Quantification of miR-181c-5p and miR-455-3p

The expression levels of miR-181c-5p and miR-455-3p were determined through reverse transcription-quantitative polymerase chain reaction (RT-qPCR) to validate the miRNA array data. The mean $2^{-\Delta\Delta Cq}$ ± standard deviation of miR-181c-5p expression was 1.16 ± 0.36 and 1.55 ± 0.66 for the exercise plus ischemia group and ischemia group, respectively (Fig. 6A). Similarly, for miR-455-3p, these values were 1.41 ± 0.63 and 1.71 ± 0.36 for the exercise plus ischemia group and ischemia group, respectively (Fig. 6B). We did not observe any significant differences between the levels of these miRNAs in the exercise plus ischemia group and ischemia group.

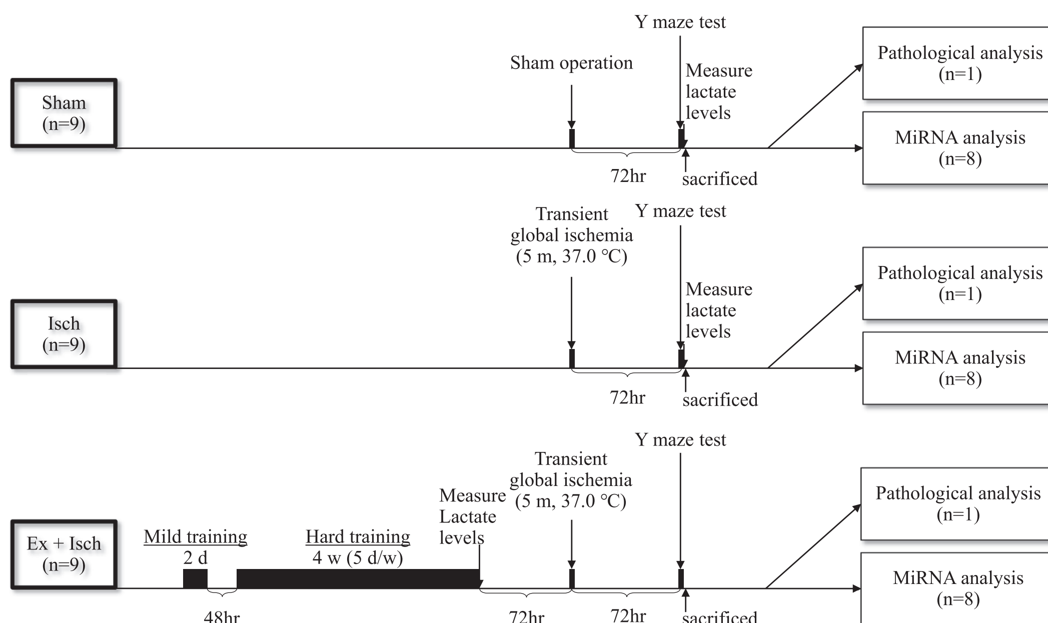

Fig. 1. Schematic showing the timeline of the animal experiments. The arrows and rectangle indicate the timing of training or lactic acid measurement, transient global ischemia, Y maze test, and sacrifice.

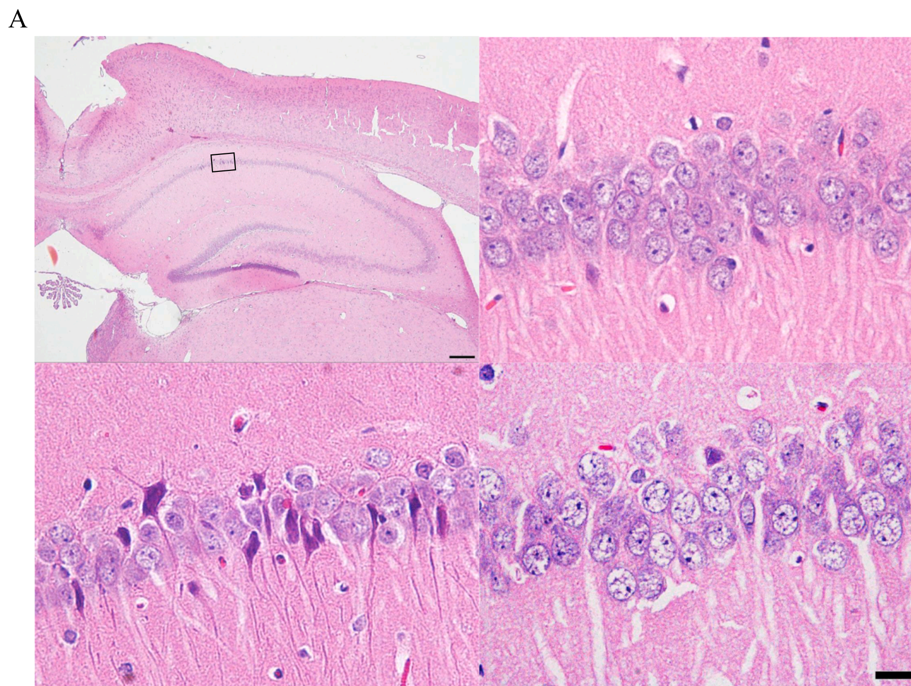

Fig. 2. (A) HE staining of the ischemic gerbil hippocampal CA1 region at low magnification (upper left panel; scale bar, 200 μm). Representative sections of the gerbil hippocampal CA1 region in the sham (upper right panel; the same magnification as the lower right panel), ischemia (lower left panel; the same magnification as the lower right panel), and exercise plus ischemia (lower right panel; scale bar, 20 μm) groups. (B) Viable neurons per mm of the hippocampal CA1 region of gerbils in the sham operation, ischemia, and exercise plus ischemia groups.

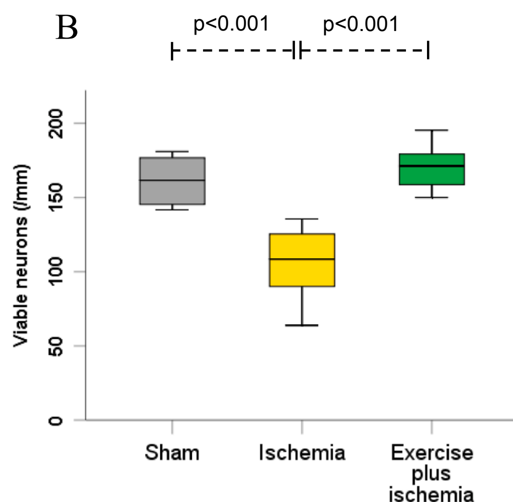

Fig. 2. (*continued*)

3. Discussion

The present study showed that light exercise contributes to the acquisition of ischemic tolerance and maintenance of short-term memory under ischemic conditions. It was proven that functional and histologic ischemic tolerance could be induced by light exercise load without an increase in lactate levels. Furthermore, we identified miRNAs that were dysregulated in the hippocampus of gerbils subjected to ischemic conditions and showed that their expression was maintained in those previously subjected to light exercise. Fourteen miRNAs were upregulated and 6 miRNAs were downregulated in the hippocampus of gerbils in the ischemia group. These miRNAs have various functions in short-term memory, such as an acutely impaired mechanism and a slowly impaired mechanism. Thus, these miRNAs may serve as effective biomarkers for ischemia.

3.1. *Light exercise with non-elevated lactate levels preserved short-term memory*

An apparent decline in short-term memory was observed in the ischemia group; however, the exercise plus ischemia group showed scores similar to that of the sham group, and the cognitive function was maintained. Histological analysis showed that the number of neurons in CA1 of gerbils in the exercise plus ischemia group was also conserved. In a previous report, locomotor activity counts were increased in the ischemia group compared to those of the exercise plus ischemia and sham groups (Liu et al., 2014), but no significant difference was found. The reason for this discrepancy is that, although in the previous report the CA1 neurons were reduced to one-seventh in the ischemic group compared to the sham group, in the present study, these neurons were only reduced to two-thirds. The difference in the ischemic intensity was probably caused by differences in the experimental environment and procedure. Although the exercise method adopted in this study was established based on a lactate threshold on rats (Takahashi et al., 2012), our results confirmed that the exercise load did not increase lactic acid levels in the exercise plus ischemia group (Fig. 4). Altogether, these findings proved that functional and histologic ischemic tolerance could be induced by light exercise load under stable lactic acid levels.

3.2. *miRNA in the hippocampus*

3.2.1. *Correlation between the function of differentially expressed miRNA and ischemia*

Various functions have been reported for each miRNA that was found to be differently expressed in the ischemia group (Table 3 and 4). Regarding the miRNAs that were upregulated under ischemia, in particular, miR-327, it is possible that, in this study, neuronal damage was reduced by exercise according to the mechanism described in a report that showed an exercise-induced reduction of myocardial damage (Ji et al., 2018). In another report on the role of miR-711 in cardiomyocyte apoptosis, it was shown that the upregulation of miR-711 might induce

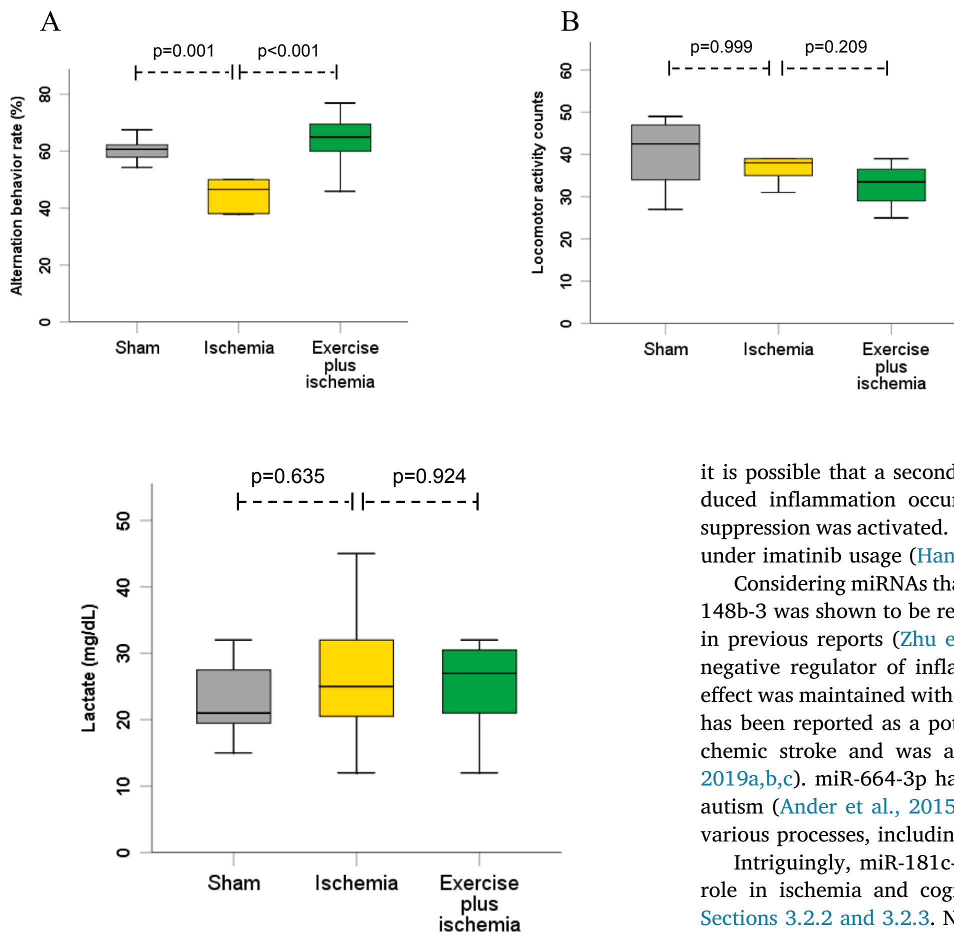

Fig. 3. Behavior assessed by (A) the alternation behavior rate and (B) locomotor activity counts with the Y maze test task in the sham, ischemia, and exercise plus ischemia groups. (A) n = 8 per group; $F_{2,\ 19}$ = 14.861, p < 0.001; p = 0.001 the sham group vs. the ischemia group; p = 0.559 the sham group vs. the exercise plus ischemia group; p < 0.001 the ischemia group vs. the exercise plus ischemia group. (B) n = 8 per group; $F_{2,\ 19}$ = 2.385, p = 0.119.

Fig. 4. The lactate level was measured at sacrifice in the sham and the ischemia groups. In the exercise plus ischemia group, blood samples were collected after the last exercise ($F_{2,\ 20}$ = 0.431, p = 0.656).

apoptosis against cerebral ischemic cell death via calnexin or NF-κB (Zhang and Yu, 2017). The exercise plus ischemia group kept the same level regulation of miR-711 as the sham group. Moreover, the upregulation of miR-211-3p and miR-3620-5p was shown to enhance the phagocytic ability of macrophages that collect necrotic cells via interferon-ϒ (Lai et al., 2018; Tiwari et al., 2017). It has also been reported that the inhibition of miR-7674-5p expression increases interleukin-10, an anti-inflammatory factor (Huck et al., 2017). In our study, miR-7674-5p upregulation was observed only in the ischemia group. Hence, it is possible that a secondary cytoprotective response to ischemia-induced inflammation occurred and that compensatory inflammation suppression was activated. As for miR-6240, its effect was reported only under imatinib usage (Hanousková et al., 2019).

Considering miRNAs that were downregulated under ischemia, miR-148b-3 was shown to be reduced in the serum in acute ischemic stroke in previous reports (Zhu et al., 2019). It has also been reported as a negative regulator of inflammatory genes, and its anti-inflammatory effect was maintained without downregulation by exercise. miR-152-3p has been reported as a potential target for neuroprotection during ischemic stroke and was also maintained by exercise (Zhang et al., 2019a,b,c). miR-664-3p has been only reported to be associated with autism (Ander et al., 2015). It is possible that exercise contributes to various processes, including the mechanisms described so far.

Intriguingly, miR-181c-5p and miR-455-3p are reported to have a role in ischemia and cognition, and therefore will be discussed in Sections 3.2.2 and 3.2.3. Nevertheless, no detailed reports were found on ischemia and miR-299b-5p, miR-451b, miR-3070-3p, miR-3070–2-3p, miR-3097-5p, miR-6916-5p, miR-6944-5p, miR-7083-5p, and miR-7085-5p.

3.2.2. miR-181c-5p

Hippocampal pyramidal neurons are vulnerable to ischemic injury, especially in CA1. This region undergoes delayed neuronal death, often reported as apoptosis with DNA fragmentation (Miyamoto et al., 2003). There has been no previous report showing the relationship between exercise load and miR-181-5p in the hippocampus, although some reports have related this miRNA to ischemic load. In a previous study with a slightly different experimental procedure (three-times induction of whole cerebral ischemia by clipping of the bilateral common carotid arteries for 2 min at 2-d intervals), the signal strength of mmu-miR-

Table 1
The custom microarray platform identified 14 miRNAs with significant differences that were upregulated in the hippocampus of the ischemic group.

mmu-miR	Sham group	Ischemia group	Exercise plus ischemia group	p-value (sham vs. ischemia group)	p-value (ischemia vs. exercise plus ischemia group)
211-3p	1.93 ± 0.0664	2.10 ± 0.195	1.90 ± 0.0423	0.035	0.014
327	0.935 ± 0.138	1.17 ± 0.195	0.995 ± 0.109	0.014	0.04
451b	1.41 ± 0.144	1.63 ± 0.231	1.43 ± 0.0792	0.036	0.035
711	1.95 ± 0.235	2.19 ± 0.136	1.93 ± 0.242	0.024	0.019
3070-3p	0.786 ± 0.156	1.00 ± 0.200	0.802 ± 0.155	0.031	0.044
3070–2-3p	0.958 ± 0.0791	1.13 ± 0.178	0.946 ± 0.0633	0.029	0.018
3097-5p	0.635 ± 0.127	0.806 ± 0.176	0.592 ± 0.108	0.042	0.011
3620-5p	1.937 ± 0.143	2.13 ± 0.172	1.95 ± 0.126	0.03	0.034
6240	2.70 ± 0.232	2.93 ± 0.0950	2.60 ± 0.290	0.02	0.008
6916-5p	1.79 ± 0.0804	2.03 ± 0.254	1.79 ± 0.116	0.021	0.028
6944-5p	2.27 ± 0.213	2.48 ± 0.174	2.24 ± 0.200	0.049	0.021
7083-5p	1.63 ± 0.140	1.83 ± 0.160	1.64 ± 0.118	0.019	0.016
7085-5p	1.99 ± 0.143	2.17 ± 0.166	2.02 ± 0.110	0.037	0.042
7674-5p	1.32 ± 0.130	1.58 ± 0.239	1.39 ± 0.0675	0.017	0.044

Signal intensity (log10) in the sham, ischemia, and exercise plus ischemia groups are indicated.

Table 2
The custom microarray platform identified 6 miRNAs with significant differences that were downregulated in the hippocampus of the ischemic group.

mmu-miR	Sham group	Ischemia group	Exercise plus ischemia group	p-value (Sham vs. ischemia group)	p-value (Ischemia vs. exercise plus ischemia group)
148b-3p	1.66 ± 0.195	1.43 ± 0.172	1.84 ± 0.279	0.025	0.004
152-3p	1.49 ± 0.0840	1.29 ± 0.186	1.57 ± 0.0831	0.012	0.002
181c-5p	2.40 ± 0.125	2.11 ± 0.242	2.41 ± 0.160	0.011	0.012
299b-5p	0.619 ± 0.102	0.411 ± 0.139	0.723 ± 0.346	0.004	0.033
455-3p	1.36 ± 0.193	1.19 ± 0.0915	1.42 ± 0.160	0.036	0.003
664-3p	2.23 ± 0.161	2.05 ± 0.126	2.25 ± 0.127	0.027	0.007

Signal intensity (log10) in the sham operation, ischemia, and exercise plus ischemia groups are indicated.

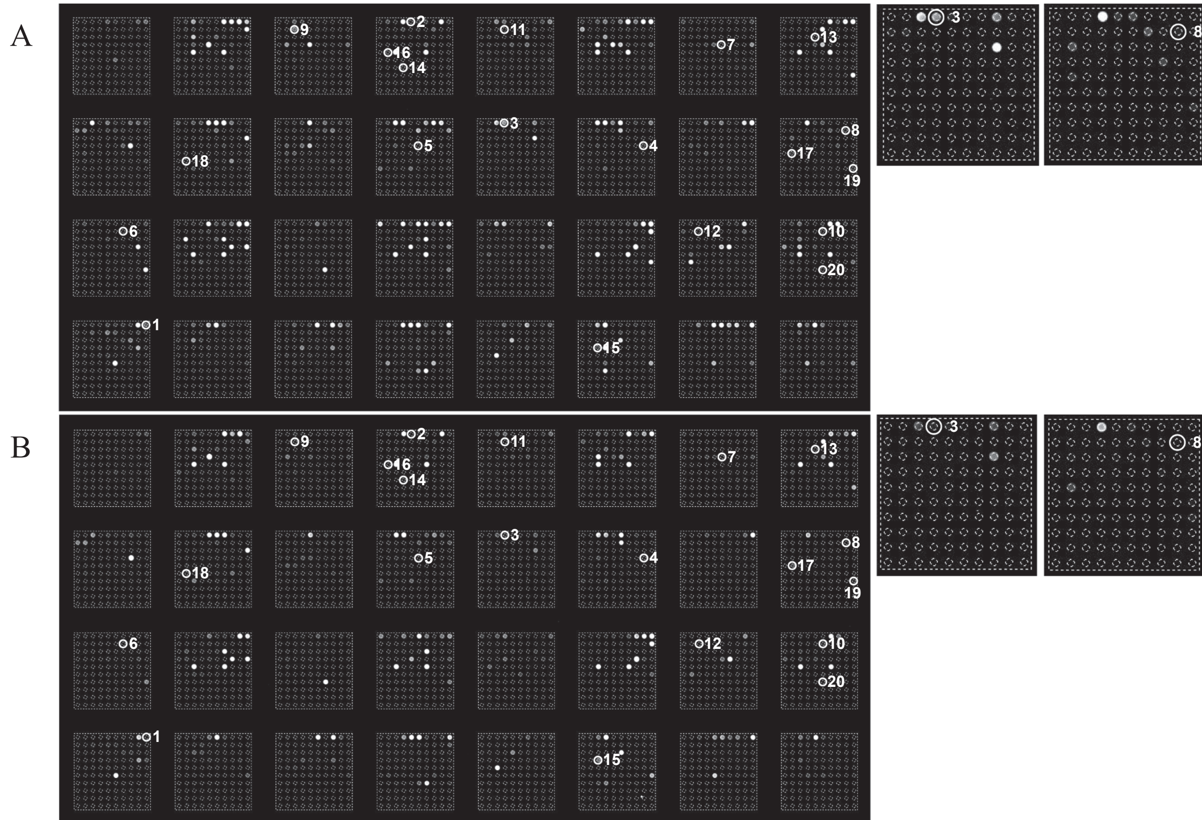

Fig. 5. miRNA expression in (A) the exercise plus ischemia and (B) the ischemia groups analyzed by miRNA chip. Spot numbers 1 to 20 are presented: 1, miR-148b-3p; 2, miR-152-3p; 3, miR-181c-5p; 4, miR-211-3p; 5, miR-299b-5p; 6, miR-327; 7, miR-451b; 8, miR-455-3p; 9, miR-664-3p; 10, miR-711; 11, miR-3070-2-3p; 12, miR-3070-3p; 13, miR-3097-5p; 14, miR-3620-5p; 15, miR-6240; 16, miR-6916-5p; 17, miR-6944-5p; 18, miR-7083-5p; 19, miR-7085-5p; 20, miR-7674-5p.

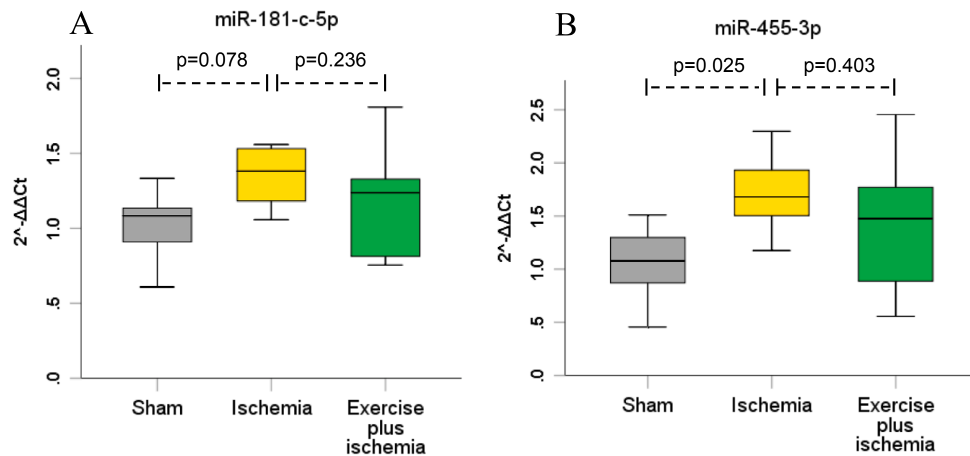

Fig. 6. Expression levels of miR-181c-5p and miR-455-3p in the hippocampus of gerbils in the sham, ischemia, and exercise plus ischemia groups. Expression levels of (A) miR-181c-5p ($F_{2, 21}$ = 2.832, p = 0.081) and (B) miR-455-3p ($F_{2, 21}$ = 4.053, p = 0.032) were compared between each group.

Table 3
IL, interleukin; N.D., no data; NF-κB p65, nuclear factor kappa B subunit p65; NLRP3, NLR family member pyrin domain containing 3. The miRNAs presented in this table were upregulated by ischemia and maintained by exercise.

mmu-miR	Regulation	Function/effect	Cell/tissue type	References
211-3p	Up	Inhibitory effects of lipopolysaccharide	Human skin fibroblasts	Wang and Wang, 2019
	Up	Enhancement of interferon-γ expression	Human myeloid leukemia K562 cells	Lai et al., 2018
327	Down	Alleviation of ischemia/reperfusion-induced myocardial damage via RP105	Rat myocardium and plasma	Yang et al., 2018
	Up	Upregulation of fibrosis-associated gene expression	Rat myocardium	Ji et al., 2018
	Down	Reduction of development of cardiac fibrosis and ventricular dysfunction Mitigation of angiotensin II-induced differentiation	Mouse myocardium	
	Up	Diminishment of activation of cardiac fibroblasts via integrin β3 targeting	Mouse myocardium	
	Down	Targeting of growth factors in the stromal-vascular fraction under conditions that promote white adipose tissue browning	Mouse adipose tissues	Fischer et al., 2017
	Down	Chitooligosaccharide infiltration of macrophages induced by CCL2 expression	Mouse peripheral neuron	Zhao et al., 2017
451b	N.D.			
711	Down	Oxygen-induced retinopathy	Mouse retina	Zhang et al., 2019a,b,c
	Down	Angiopoietin-1-mediated neuronal cell death	Mouse brain	Sabirzhanov et al., 2018
	Down	Downregulation of NLRP3 via adiponectin	Mouse muscle	Boursereau et al., 2018
	Up	Cardiomyocyte apoptosis via calnexin	Rat myocardium	Zhao et al., 2018
	Up	Promotion of NF-κB p65 expression in ischemia–reperfusion and acceleration of NF-κB p65 transport into the nucleus	Rat myocardium	Zhang and Yu, 2017
	Up	Downregulation of NLRP3	Human muscle	Boursereau et al., 2017
	Up	Inhibition of the v-akt murine thymoma viral oncogene homolog 1 pathway	Mouse brain	Sabirzhanov et al., 2016
3070-3p	N.D.			
3070–2-3p	N.D.			
3097-5p	N.D.			
3620-5p	Up	Cellular iron ion homeostasis, response to hypoxia, and zinc ion binding (macrophage effector functions)	*Leishmania donovani* (AG83)	Tiwari et al., 2017
	Up	G-quadruplexes	Human	Tan et al., 2016
6240	N.D.	(Only report using imatinib)		Hanousková et al., 2019
6916-5p	N.D.			
6944-5p	N.D.			
7083-5p	N.D.			
7085-5p	N.D.			
7674-5p	Down	Increase of anti-inflammatory factor IL-10 secretion	Mouse	Huck et al., 2017

181c-5p in CA1 was reported to have higher expression through 1 day to 7 days (Wu et al., 2015). miR-181 was identified as a regulator of several heat shock protein 70 family members, and an increased level of miR-181a is associated with a decrease in endoplasmic reticulum glucose-regulated protein 78, which is related to the cerebral ischemic core. However, a decreased level of miR-181a in the penumbra is associated with cell survival (Ouyang et al., 2012). Thus, depending on the degree of ischemia, miR-181 can regulate either upregulation or downregulation. It is considered that the change in neuronal cell death is more remarkable by targeting the CA1 region (Sun et al., 2015). In this study, miR-181c-5p was downregulated in the ischemia group due to ischemic load. Moreover, in this study, we did not target CA1 specifically, but rather the Ammon angle. This indicates that the element of penumbra is stronger than that of CA1 neuronal cell death, and it may be that changes in miRNA expression in the penumbra were reflected in the results (Table 2). In any case, the exercise plus ischemia group maintained the same miRNA expression as the sham group; thus, light exercise may have inhibited the downregulation of miR-181c-5p, resulting in ischemic tolerance.

Furthermore, inhibition of miR-181c and upregulation of tripartite motif-containing 2 (TRIM2) were found to be associated with decreased dendritic branching and dendritic spine density of hippocampal neurons in the bilateral common carotid artery occlusion rat model (Fang et al., 2017). In addition, in recent years, miR-181c-5p was also reported to be an anti-angiogenic miRNA (Hourigan et al., 2018). With several functions, including those described above, it is possible that this miRNA is involved in the acquisition of ischemia tolerance upon light exercise.

3.2.3. miR-455-3p

We also focused on miRNAs associated with amyloid precursor protein (APP) as factors related to short-term memory. It has been reported that the function of miR-455-3p is increased in the brain of APP transgenic mice and also in Alzheimer's disease (AD) (Kumar et al., 2017). Furthermore, the same authors showed the protective effects of miR-455-3p on APP regulation, amyloid-beta levels, mitochondrial biogenesis and dynamics, synaptic activities, and cell viability or apoptosis (Kumar et al., 2019). They concluded that miR-455-3p regulates APP processing and protects against mutant APP-induced mitochondrial and synaptic abnormalities in AD. In our study, downregulation of miR-455-3p was observed after ischemia, suggesting that APP processing dysfunctions might have occurred, which in turn might have induced the accumulation of amyloid. In addition, as the expression of miR-455-3p was maintained by exercise (Table 2), the accumulation of amyloid might be prevented. Similar to miR-181c-5p, the exercise plus ischemia group maintained the same miR-455-3p expression as the sham group, suggesting that light exercise may have inhibited the downregulation of miR-455-3p, inducing ischemic tolerance. Furthermore, if the accumulation of amyloid was prevented, light exercise not only has the potential to develop ischemia resistance but could also prevent AD.

In conclusion, light exercise with non-elevated lactate levels was shown to induce ischemic tolerance. Neurons were preserved in CA1, and short-term memory was retained. Although several mechanisms may lead to this effect, it was shown that ischemic tolerance induced by light exercise is associated with miRNA expression level in the hippocampus. This study shows that light exercise may be protective against short-term memory decline due to an acutely impaired mechanism and a slowly impaired mechanism such as amyloid accumulation. Nonetheless, some limitations of our study need to be addressed. For instance, the sample size was small, and no validation of the miRNA expression level was performed. In future investigations, a higher number of gerbils should be included, and individual miRNA expression levels should be determined by RT-qPCR. In addition, the present study analyzed hippocampal miRNAs but not serum and cerebrospinal fluid miRNAs; the latter may be more appropriate for clinical applications as it is easier to collect those samples when dealing with humans.

Table 4
AD, Alzheimer's disease; APP, amyloid precursor protein; IL, interleukin; COX, cyclooxygenase. The miRNAs presented in this table were downregulated by ischemia and maintained by exercise.

mmu-miR	Regulation	Function/effect	Cell/tissue type	References
148b-3p	Down	Contribution to exaggerated inflammation	Human IgA vasculitis-affected skin	Jurčić et al., 2019
	Down	Lnc H19 is a negative regulator and participate in hypoxia stress via positively regulating Nicotinamide adenine dinucleotide phosphate oxidases and negatively regulating endothelial nitric oxide synthase signaling.	Human hepatic sinusoidal endothelial cells	Zhu et al., 2019
	Up	Blood-based biomarkers for diagnosing ischemic stroke	Human blood	Cheng et al., 2018
	Up	Regulation of p53 signaling by targeting Sirtuin 7 and contribution to the regulation of hypoxia/reoxygenation-induced cardiomyocyte apoptosis	Mouse myocardium	Sun et al., 2018a,b
	Down	Blood-based biomarkers for diagnosing amyotrophic lateral sclerosis	Human blood	Liguori et al., 2018
152-3p	Up	Protection of neurons from oxygen-glucose-deprivation/reoxygenation-induced apoptosis and reactive oxygen species production by reinforcing nuclear factor erythroid 2-related factor 2/antioxidant response element signaling through targeting and inhibiting postsynaptic density protein-93	Mouse brain	Zhang et al., 2019a,b,c
	Up	Oxygen-induced retinopathy	Rat retina	Desjarlais et al., 2019
181c-5p	Up	Exacerbation of hypoxia/reoxygenation-induced cardiomyocyte injury and apoptosis via targeting protein tyrosine phosphatase nonreceptor type 4	Rat cardiomyocyte-derived cell line H9C2	Ge et al., 2019
	Up	Inhibition of IL-1A	Rat colon tissues	Ji et al., 2019
	Up	Potential biomarker for AD	Human blood	Siedlecki-Wullich et al., 2019
	Up	Suppression of hypoxia-inducible factor-1α accumulation and glycolysis-associated enzyme expression	Human breast cancer cell lines MCF-7 and MDA-MB-231	Lee et al., 2019
	Up	Inhibition of IL-6-induced apoptosis in beta cells	Rat insulinoma cell line INS-1	Oh et al., 2019
	Up	Downregulation of cyclin B1 expression, cell cycle arrest, and inhibition of osteoblast proliferation	Mouse osteoblasts	Sun et al., 2019
	Down	Correlation with mild traumatic brain injury	Rat whisker hair follicles	Zhang et al., 2019a,b,c
	Up	Inhibition of endothelial vascular network formation	Human cardiac microvascular endothelial cells (HCMECs and PromoCell)	Hourigan et al., 2018
	Down	Promotion of tubule formation in vitro		
	Up	Suppression of ataxin-3 expression by binding to the 3′UTR	Human spinocerebellar ataxia type 3 lymphoblastoid cells	Krauss et al., 2019
	Down	Increase of tumor necrosis factor-α expression	Human pituitary adenoma samples	Zhu et al., 2018
	Down	Blood-based biomarkers for diagnosing AD	Human blood	Fransquet and Ryan, 2018
	Up	Decrease of COX1 expression	Human muscle	Silver et al., 2018
	Up	Impairment of mitochondrial function by dysregulating the formation of complex IV	Rat myocardium	
	Up	Reduction of BRICK1 subunit of SCAR/WAVE actin-nucleating complex protein expression	Human T cell kit	Lim et al., 2018
	Down	Promotion of high-glucose-induced dysfunction by regulating leukemia inhibitory factor	Human blood	Shen et al., 2018
	Up	Downregulation of a death domain-containing member of the TNF receptor superfamily receptor expression	Human mesenchymal stem cells	Kawano et al., 2018
	Up	Promotion of T helper 17 cell differentiation	Mouse brain and spinal cord	Zhang et al., 2018a,b
299b-5p	N.D.			
455-3p	Up	APP processing	Mouse neuroblastoma cell line N2a	Kumar et al., 2019
		Protection against mutant APP-induced mitochondrial and synaptic abnormalities		
	Up	Suppression of heat shock factor 1 expression	Mouse liver	Wei et al., 2019
	Up	Symptomatic Human Immunodeficiency Virus-associated distal sensory polyneuropathy	Human blood	Asahchop et al., 2018
	Up	Repression of Rho-associated coiled coil-containing protein kinase 2 expression	Human liver	Wu et al., 2018
	Up	Activation of the canonical Phosphoinositide 3-kinase/Akt (known as Serine/threonine kinase) signaling pathway through the reduction of its methylation levels	Human articular cartilage	Sun et al., 2018a,b
	Up	Expression in AD	Human brain	Kumar and Reddy, 2018
	Up	Reinforcement of the miR-455-3p/Matrix metalloproteinase-3 regulatory pathway	Human blood	Tenorio et al., 2018
	Up	Negative regulation of serine/threonine kinase 17B	Human liver	Lan et al., 2018
	Up	Restraining of iron overload-induced oxidative stress	Mouse osteoblastic cell line MC3T3-E1	Zhang et al., 2018a,b
	Down	Biomarkers for chronic obstructive pulmonary disease	Human lung	Ong et al., 2017
	Up	Suppression of adenosine monophosphate-activated protein kinase subunit beta 2	Human liver	Lin et al., 2018
	Down	Vascular remodeling	Rat brain	Pearson-Leary et al., 2017
	Up	Induction of a skeletal muscle hypertrophic phenotype	Mouse muscle	Jung et al., 2017
	Up	Regulation of endothelial nitric oxide synthase protein stability	Human umbilical vein endothelial cells	Li et al., 2017
664-3p	Up	Expression in autism spectrum disorders	Human brain	Ander et al., 2015

4. Experimental procedure

4.1. Animals

A total of 27 adult male Mongolian gerbils (Kyudo, Saga, Japan) with an average body weight of 64.2 ± 4.3 g were used for this study. Animal protocols were approved by the Animal Committee of Kagawa University Faculty of Medicine. Gerbils were randomly divided into three groups (nine gerbils per group) – the ischemia group, the exercise plus ischemia group, and the sham group. miRNA evaluation was performed on eight animals from each group. Histological evaluation was performed on one animal from each group.

4.2. Exercise and ischemia

All gerbils were allowed free access to food and water. The gerbils in the exercise intervention group were trained to run for 30 min at 15 m/min, 5% slope on a treadmill (MK-680, Muromachi Kikai, Tokyo, Japan), 5 d/wk for 4 wk. Before the exercise intervention, as a formal treadmill training, gerbils were acclimatized to run for 15 min at 5 m/min, 0% slope for 2 d. Initially, electrical shocks (1.0 mA) were applied to stimulate animals to run forward. Subsequently, they ran without electrical stimulation. After adaptive running sessions, the gerbils started the formal training. After the training, gerbils were anesthetized with sodium pentobarbital (30 mg/kg intraperitoneal administration), after which transient global ischemia was induced for 5 min by occlusion of the bilateral common carotid arteries with micro-aneurysm clips (Sugita Clip, Mizuho, Nagoya, Japan). During the surgery, rectal temperature was maintained at 37.0 ℃ using a feedback-controlled heating pad (CMA, Stockholm, Sweden) to prevent hypothermia. After a short-term memory test, which is described later in *4.3.*, gerbils were sacrificed. The ischemia group underwent the same surgical procedure and evaluation during the same period but did not receive the exercise training. The sham group underwent the same surgical procedure except for the occlusion of the carotid arteries, during the same period and did not receive the exercise training. Before sacrifice, blood was collected to measure lactate levels. After sacrifice, brains were removed and processed to measure miRNA expression in the hippocampus.

4.3. Behavioral test

Seventy-two hours after ischemia induction, a Y maze test was conducted. The gerbils were let in the maze for 8 min, and the arms into which they entered were recorded in order. The number of times the animal entered each arm within the measured time (total number of arms entered) and the number of combinations that included three different arms entered in succession (number of alternations) were examined, and the alternation behavior rate (%), which was used as an index of short-term memory, was calculated using the formula, alternation behavior rate % = number of alternations/(total number of arms entered–2) × 100).

4.4. RNA isolation

Total RNA was extracted from the brain samples using a miRNeasy Mini Kit (Qiagen, Inc., Valencia, CA, USA) following the manufacturer's instructions. The integrity of RNA was determined using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Inc., Waltham, MA, USA). The quality of total RNA was determined using RNA Nano 6000 chips and an Agilent 2100 Bioanalyzer (Agilent Technologies, Inc., Santa Clara, CA, USA) according to the manufacturer's protocol. Briefly, total RNA from all brain samples was heated at 70 °C for 2 min and incubated on ice for 5 min. Subsequently, samples (1 μL) were loaded into each lane of the RNA Nano 6000 chips, and the bands of 18S and 28S ribosomal RNA in the gel were detected using the Agilent 2100 Bioanalyzer. The RNA samples were stored at −80 °C.

4.5. miRNA arrays

Total RNA was labeled with Hy3 dye using a miRCURY LNA microRNA Array Hi-Power labeling kit (Exiqon A/S, Vedbæk, Denmark). Total RNA (2 μg) was incubated with a spike of 30 min at 37 °C and then at 95 °C for 5 min. Hy3 dye and Hi-Power labeling enzyme were then added to each sample. The enzyme was heat-inactivated at 16 °C for 1 h and at 65 °C for 15 min, protected from light. The samples were loaded onto the arrays by capillary force using 3D-Gene miRNA oligo chips (version 17; Toray Industries, Inc., Tokyo, Japan). The chips enabled the examination of the expression of 679 miRNAs printed in duplicate spots. The arrays were incubated at 32 °C for 16 h, briefly washed in a 30-°C washing buffer solution [0.5 × saline-sodium citrate (SSC), 0.1% sodium dodecyl sulfate (SDS)], rinsed in washing buffer solution (0.2 × SSC, 0.1% SDS), and then washed again in another buffer solution (0.05 × SSC), according to the manufacturer's instructions (Toray Industries, Inc.). The arrays were centrifuged for 1 min at 600 × *g* and room temperature to dry, followed by immediate scanning using a 3D-Gene 3000 miRNA microarray scanner (Toray Industries, Inc.). The relative expression level of each miRNA was calculated by comparing the average signal intensities of the valid spots with their mean value throughout the microarray experiments, following normalization to their adjusted median values.

4.6. Quantification of miRNA

Isolation of RNA was performed using a miRNeasy Mini Kit and adding spike in control sno-RNA-202 (Qiagen, Inc.), according to the manufacturer's protocol. cDNA was individually synthesized for each target miRNA using the miRNA Reverse Transcription Kit (Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. The detection of miRNA expression was performed by RT-qPCR, using TaqMan miRNA Assays and TaqMan Universal Master MixII (Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. Thermocycling conditions were as follows: initial denaturation at 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s, and 60 °C for 60 s. The relative expression level of miRNAs was calculated using the comparative Cq method (Livak and Schmittgen, 2001) and normalized to sno-RNA-202 expression. Experiments were performed in triplicate.

4.7. Measurement of lactate

Serum lactate levels were measured using Lactate Pro2 LT-1730 (ARKRAY, Inc. Kyoto, Japan). Blood was collected from the tail of the gerbils with a 30 G puncture needle at sacrifice in the ischemia and sham operation groups. In the exercise plus ischemia group, blood samples were collected after the last exercise.

4.8. Pathological analysis

Paraffin-embedded tissue sections with a thickness of 6 μm were cut in the coronal plane, and the number of nerve cells per unit area of the bilateral hippocampus (4 splits centering on lateral CA1) was counted after HE staining. Neurons whose normal nuclei could be identified were counted. This was conducted to confirm the induction of ischemia.

4.9. Statistical analysis

Statistical analyses were performed using SPSS (ver. 13.0; IBM, Armonk, NY, USA). All tests were considered statistically significant when $p < 0.05$. The significance of differences in the alternation behavior rate, locomotor activity data, and lactate levels were assessed with one-way ANOVA followed by Tukey's post-hoc test.

CRediT authorship contribution statement

Tadayuki Takata: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Data curation, Writing - original draft, Visualization. **Wakako Nonaka:** Methodology. **Hisakazu Iwama:** Formal analysis, Visualization. **Hideki Kobara:** Supervision. **Kazushi Deguchi:** Supervision. **Hisashi Masugata:** Supervision. **Tetsuo Touge:** Supervision. **Osamu Miyamoto:** Validation. **Takehiro Nakamura:** Validation. **Toshifumi Itano:** Validation, Project administration. **Tsutomu Masaki:** Resources, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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