



## Establishment of repeated liver biopsy technique in experimental mice

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### ABSTRACT

Biopsy is a commonly used method for determining pathological diagnoses by directly using human tissues and cells. Biopsies are widely used to determine disease progression and treatment efficacy. Although organs and tissues are usually obtained by sacrifice during animal experiments, it is theoretically possible to use the same biopsy techniques in humans. In the present study, we examined the feasibility of performing four repeated liver biopsies in a spontaneous metabolic syndrome mouse model. Even though a small number of mice died accidentally, most mice were able to undergo four liver biopsies without significant adverse events. We also performed three liver biopsies in mouse liver tumor carcinogen models at 4, 8, and 12 weeks of age. In addition to the sample collected at 16 weeks of age during sacrifice, we successfully collected four liver samples from the same mice at different stages of disease progression. The application of this liver biopsy technique might make it possible for direct evaluation of pathological conditions in the same individual over time, thereby reducing the number of experimental animals.

### 1. Introduction

Biopsy techniques have advanced to the use of endoscopes to collect living tissue from human organisms and are widely used in actual pathological diagnosis. In addition to providing precise histopathological images and being able to detect various proteins and genes at the time of collection, biopsied tissues also can be used as a valuable tool for assessing disease progression and assessing the effectiveness of treatment by comparing multiple samples. This multiplex analysis allows the identification of temporal changes in tissue components and molecular markers, leading to a more complete understanding of the disease course and response to treatment. Consequently, biopsy techniques have become an essential component in the diagnosis and management of various diseases [1].

Single liver biopsy procedure to collect samples from the same individual before treatment in mice has been used in a relatively

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large number of publications [2–6]. Recently, a paper was published that presents a video guide to the technique of mouse liver biopsy [7]. In brief, liver tissue is observed grossly after opening the abdomen, with a portion of the liver tissue harvested and sutured to allow recovery and regaining normal function, which allows comparison of the biopsy material and histology at the time of sacrifice. As liver tissue is a rapidly regenerating organ, it is thought that the collection of a small amount of tissue can minimize any of the harmful effects. Furthermore, it has been confirmed in past experiments involving surgical manipulation that a direct approach to the intra-abdominal organs in mice is almost free from side effects such as peritonitis, provided a specific clean environment is maintained. Thus, one-time liver biopsy techniques are generally well established, however there are few reports that have performed multiple liver biopsies [8,9]. If a simple technique could be established to perform repeated liver biopsies, it would greatly contribute to reducing the number of individuals used in experiments.

The current study conducted a detailed investigation for the purpose of establishing a protocol that would enable more frequent liver biopsies. If this technique could be used to collect tissues from the same individual repeatedly at different points, this could potentially make it possible to examine different stages of the disease, such as before, just before, after, and after the treatment of a tumor. As a result, by being able to follow the tissue changes and protein and/or molecular changes over time, this would likely lead to a reduction in the number of analyses and the experimental animals required for a study. Thus, this study investigated whether performing three and four liver biopsies would be possible in the same animal when using Tsumura-Suzuki obese diabetes (TSOD) mice, a mouse model of spontaneous metabolic syndrome [10–14], and DIAR (ddY from the Institute for Animal Reproduction)-nSTZ mice, a model that exhibits a rapid onset of hepatocellular carcinoma (HCC) [15–17].

## 2. Materials and methods

**Animals:** TSOD mice are inbred strains of mice established in Japan by selecting obese and hyperglycemic individuals from the ddY strain, a closed colony, and breeding them for more than 30 generations, and the strains are maintained at the Institute for Animal Reproduction (Ibaraki, Japan). DIAR mice are a ddY strain maintained by the Institute for Animal Reproduction. All mice used in this study were in good health and had been confirmed to be free of specific pathogens. The rearing environment is 18–26 °C, the humidity is 30–70%, the light is 12 h/day (lights on at 7:00, lights off at 19:00). Temperature and humidity were recorded daily and stored as raw data. Three mice are placed in polycarbonate cages for mice (182 W x 260D x 128H mm) (Nippon Crea Co., Ltd.) with wooden chips (Japan SLC Co., Ltd.) as nesting material during the rearing period. The animals are fed ad libitum with autoclaved (sterilized at 121 °C for 15 min and dried for 5 min) solid feed MF for mice (Oriental Yeast Co., Ltd.). Water was supplied in 200 mL polycarbonate water bottles, and 0.025% sodium hypochlorite (Turcuron, Tsurumi Soda, Ltd., Japan) was added to the water for home use, which was also fed ad libitum. Individuals and cages are identified by ear numbers using the ear-punch method. Labels indicating the test number, strain name, generation, date of birth, and sex will be affixed to the cages.

All institutional and national guidelines for the care and use of laboratory animals were followed. Furthermore, this study was performed following the animal experiment guidelines specified in the Institute for Animal Reproduction (Ibaraki, JAPAN), (Permission number: IarAW No. 27–8 and Iar AW No. 3–30), which strictly adhering to the rules of guidance on animal research ethics from the International Association of Veterinary Editors' Consensus Author Guidelines on Animal Ethics and Welfare. This article does not involve any studies with human subjects.

**Surgical Procedures and liver biopsy:** The following is our modified biopsy method based on previous literatures [5,8].

### 1. Anesthetize the mouse:

We administered a three-in-one anesthetic mixture (medetomidine hydrochloride 0.03 mg/mL + midazolam 0.4 mg/mL + butorphanol 0.5 mg/mL) at a dose of 0.1 mL per 10 g of mouse subcutaneously on the back of the neck. Adequate anesthetic depth was determined by Ref. [1] checking for the disappearance of the reflex to return to the supine position and [2] checking for loss of reflex to stimulation.

### 2. Trim the hair on the chest and abdomen:

After shaving the surgical site, the area was washed with a 70% isopropyl alcohol-osban diluent.

### 3. Make a skin incision along the center line and visually check the liver:

The animal was placed in the dorsal recumbent position with its head up, and an approximately 3 mm or 10 mm midline incision was made in the skin above the liver slightly to the right below the xiphoid process.

### 4. Insert a biopsy needle and collect the liver:

We checked the liver under direct vision, harvested tissue fragments with an 18G biopsy needle, and collected tissue in a tube containing preservation solution.

### 5. Confirm hemostasis, apply penicillin, and then suture the incision:

After the biopsy, we placed a cotton ball over the cut and applied pressure for hemostasis. After confirming that the bleeding had stopped, penicillin was applied. The abdominal muscles and skin were then sutured with Nylon suture thread USP No. 4-0.

6. Postoperative management:

A three-in-one anesthetic mixture antagonist (atipamezole hydrochloride 0.03 mg/mL) was administered subcutaneously at 0.1 mL per 10 g of mouse to induce arousal. We kept the cage warm with a heater at 23 °C until the animal woke up.

**Euthansia and Tissue Harvest:** After euthanasia by an overdose of pentobarbital anesthesia (ip), organs are weighed on an electronic balance ( The weight of each was determined using an electronic balance (AUY220: Shimadzu Corporation). The harvested tissues were immediately observed for appearance, photographed, and immersed in 10% neutral buffered formalin.

**Three-group comparison experiment with four-times liver biopsies over a long period of time: a study using TSOD mice:** Fifteen male TSOD mice were divided into three groups of five mice each. Group A was set as the control for the liver biopsy group, with a 3 mm skin incision and peritoneal incision followed by suturing without performing a liver biopsy. Groups B and C were the test groups where liver biopsy was performed after the skin incision and peritoneal incision, respectively. In these experiments, 3 mm and 10 mm skin and peritoneal incisions were performed in Groups B and C, respectively. The first liver biopsy was performed at 16 weeks, the second at 20 weeks, the third at 32 weeks, and the fourth at 49 weeks using an 18 G needle (Fig. 1). All mice were sacrificed at 50 weeks, with the liver weight measured along with a check for the presence of liver tumors and adhesions to the peritoneum. Body weights were measured every four weeks throughout the experimental period.

**Comparison experiment between no-biopsy group and three-times liver biopsies in a short period of time: a study using DIAR-nSTZ mice:** Eight male DIAR-nSTZ mice were divided into two groups. Group A (n = 5) was set as the control for the liver biopsy group, without liver biopsy, the skin incision was 10 mm and the peritoneal incision was sutured after the incision. Groups B (n = 3) was the liver biopsy group which was performed after the skin incision and peritoneal incision, respectively. The first liver biopsy was performed at 4 weeks, the second at 8 weeks, the third at 12 weeks using an 18 G needle. All mice were sacrificed at 16 weeks, with the liver weight measured along with a check for the presence of liver tumors and adhesions to the peritoneum. Body weights were measured once a week throughout the experimental period.

**Tissue preparation:** Formalin-fixed, paraffin-embedded specimens were prepared in conventional methods, and 5 μm thin-section specimens were prepared by microtome, which was followed by hematoxylin and eosin (HE) staining.

**Statistical analysis:** Differences between groups were analyzed using one-way analysis of variance and Student’s t-test, respectively. P-values <0.05 was considered statistically significant. Statistical analyses was performed using IBM SPSS statistics software, version 29.0 (IBM Co., Somers, NY) on a computer for Windows.

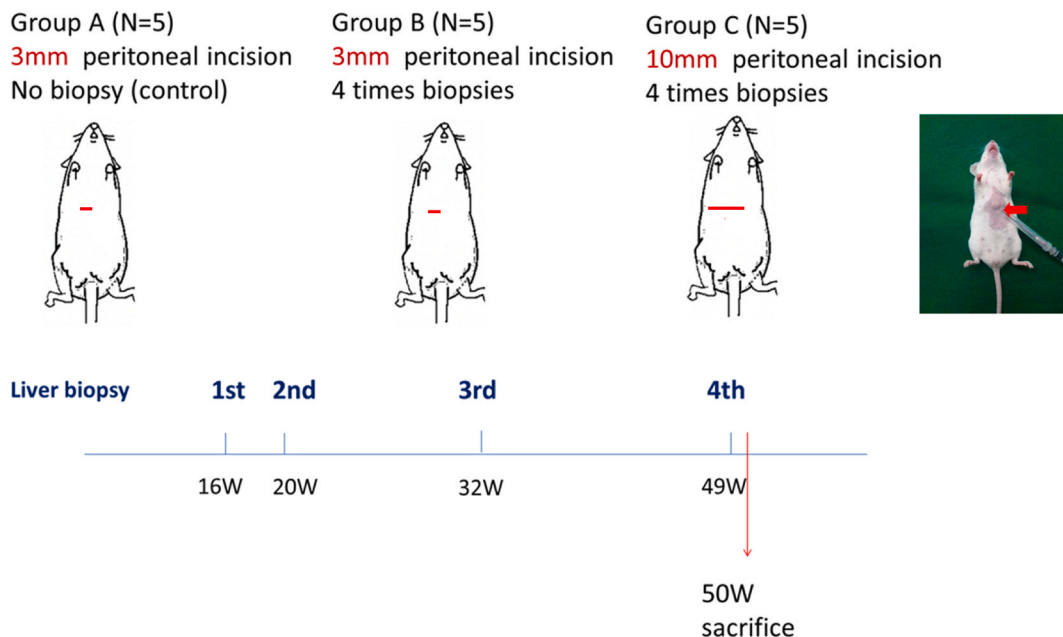


Fig. 1. Description of the experimental groups and timing of the four liver biopsies.

### 3. Results

#### 3.1. Results of preliminary experiments

Each biopsy sample had enough volume to perform a histological evaluation (Fig. 2A). Although at one week after the liver biopsy, the liver showed no peritoneal adhesions or morphological changes, there was a tissue defect observed at the puncture site (Fig. 2B and C). HE staining revealed that the hepatic capsule surrounding the tissue defect was mildly fibrotic and thickened with a collection of lymphocytes and pigmented macrophages. The hepatocytes surrounding the tissue defect showed only regeneration, and there was no atypia or pyogenic inflammation observed (Fig. 2D and E). A small venous thrombus was also observed in a part of the hepatic capsule adjacent to the tissue defect (Fig. 2F).

Results of the three-group comparison experiment using TSOD mice.

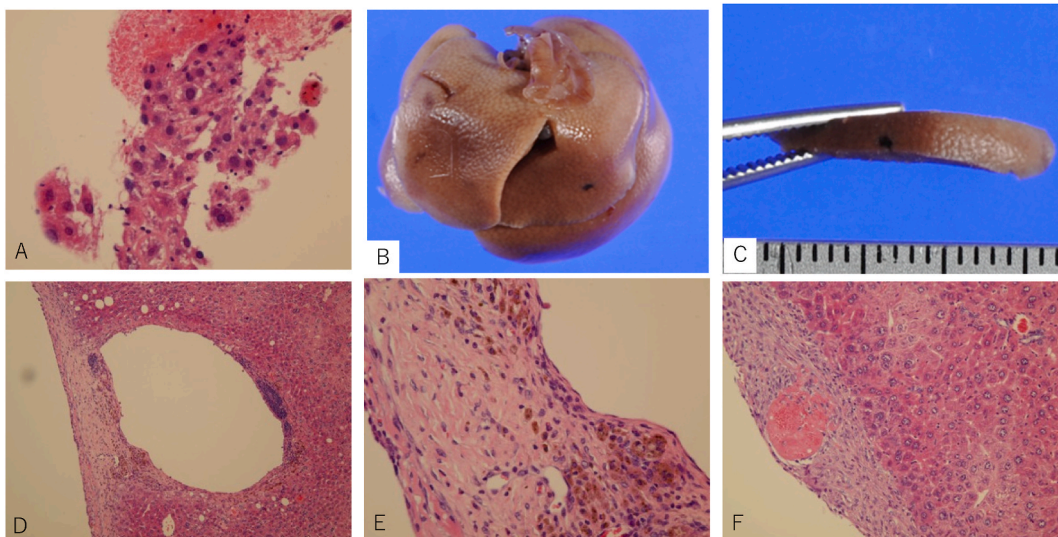
In Groups B and C, one mouse died in each group after biopsy at 20 weeks of age (Table 1). Since TSOD mice rarely die at the age of 20 weeks without treatment, we cannot deny the possibility that these deaths were caused by the biopsy operation. However, because of the strong postmortem changes at the time of discovery, an autopsy could not be performed, and thus, the correlation between biopsy and death remains unclear.

The remaining mice in Groups B and C did not show significant differences in appearance or activity as compared to Group A throughout the experiment. Analysis of only the surviving mice indicated that there were no significant differences in body weight among the three groups when sacrificed at 50 weeks of age (Table 1). In addition, there were also no significant changes between the three groups for the body weight of all mice when evaluated every four weeks (Fig. 3). Although mild adhesions between the liver and peritoneum were observed in one out of the four surviving mice in Groups B and C, this was not observed in the other mice. There were no other noticeable changes in appearance. Due to the large differences in the liver weight depending on the presence or absence of liver tumors, liver weight was excluded from the comparative study among the three groups (Table 1).

The excised liver showed only slight surface irregularities. All of the collected biopsy specimens were about  $1 \times 3$  mm in size. In all cases, changes in the liver parenchyma (presence of microvesicular lipid droplets, inflammatory cells and perivenular fibrosis) were clearly observed (Fig. 4). In Groups B and C, although two out of four animals in each group had grossly visible tumors on the surface of the liver at the time of sacrifice, none were visible at the time of biopsy (Table 1).

Results of the comparison experiment using DIAR-nSTZ mice.

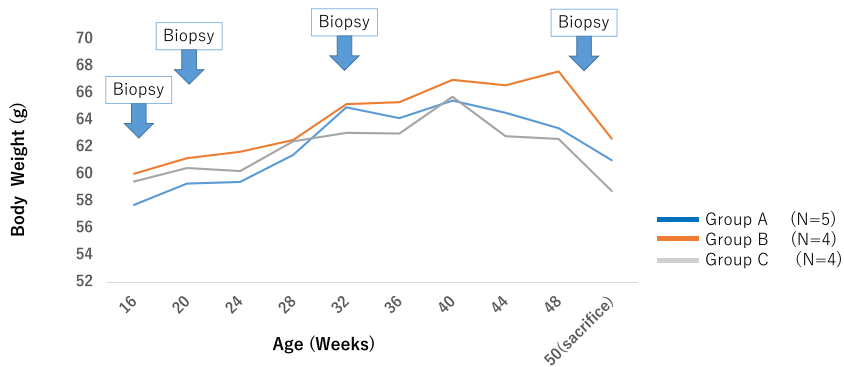
In Groups A and B, no mice died before sacrifice at 16 weeks of age (Table 2). After the liver biopsy, Group B experienced a slight decrease in body weight, but recovered quickly, and there was no significant difference in body weight between Group A and Group B at any age week, as shown in Fig. 5. At the time of sacrifice at 16 weeks, the liver weight was  $2.25 \pm 0.21$  g in Group A and  $2.30 \pm 0.4$  g in Group B, with no significant difference between the two groups. Both large and small liver tumors were observed in both Group A and Group B, but since no tumors were visible at the time of liver biopsy, all the liver samples taken were considered as background livers.



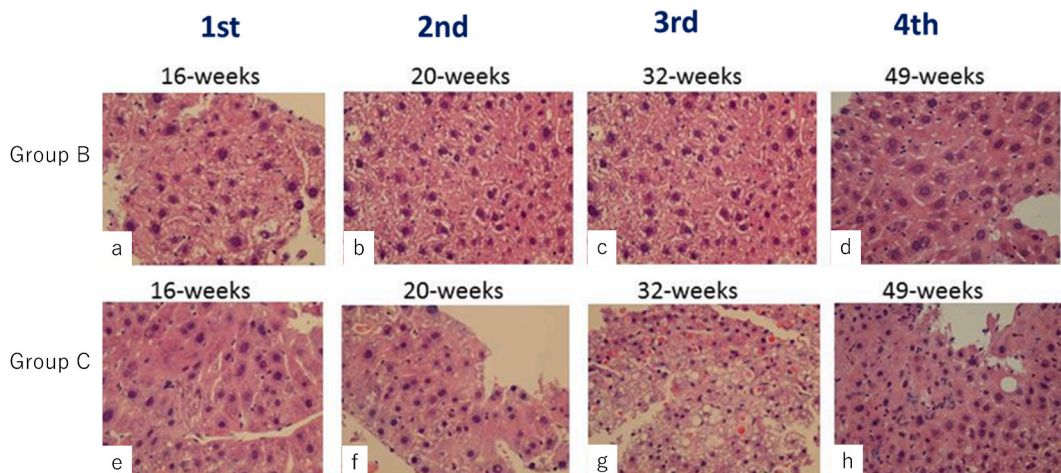
**Fig. 2.** Histological images of the liver biopsy (A) and the liver images (macroscopic; B, C and microscopic D-F) at one week after biopsy. Liver parenchyma was observed in the biopsy sample (A). Although resected whole liver showed no peritoneal adhesions or morphological changes, there was a tissue defect noted for the puncture site (B,C). HE staining showed the hepatic capsule surrounding the tissue defect was mildly fibrotic and thickened with a collection of lymphocytes and pigmented macrophages. The hepatocytes surrounding the tissue defect showed only regeneration, and there was no atypia or pyogenic inflammation observed (D,E). A small venous thrombus was also observed in part of the hepatic capsule that was adjacent to the tissue defect (F).

**Table 1**  
Status of each individual at autopsy of TSOD-mice.

	No.	Body weight (g)	Liver weight (g)	Live tumor	Peritoneal adhesion
Group A (control)	A1	59.4	2158	-	-
	A2	57.2	2472	+	-
	A3	63.8	3060	+	-
	A4	60.5	2088	-	-
	A5	64	2748	-	-
Group B (3 mm)	B1	60.5	4085	+	-
	B2	63.6	2355	-	-
	B3	60.8	2196	-	+
	B4	died at 20 weeks			
	B5	65.6	4768	+	-
Group C (10 mm)	C1	52.5	2213	+	-
	C2	60.5	2440	-	-
	C3	died at 20 weeks			
	C4	59.9	2333		+
	C5	62.1	2263	+	-



**Fig. 3.** Changes in the body weight of the three groups of TSOD mice that were measured every 4 weeks. There were no significant differences observed between any of the groups.

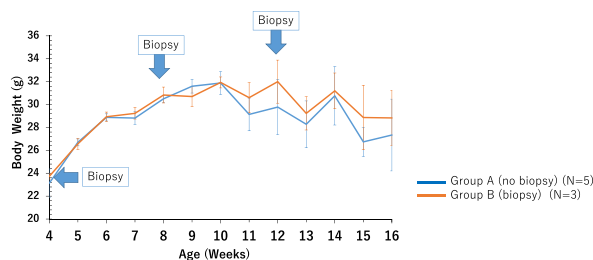


**Fig. 4.** Changes in liver biopsy images (1st biopsy (a,e), 2nd biopsy (b,f), 3rd biopsy (c,g), and 4th biops (d,h)) observed over time for one representative individual in Groups B (a–d) and Group C (e–h). Results indicated that it was possible to adequately evaluate the presence or absence of fatty degeneration of hepatocytes, morphology, mononuclear cell infiltration, and fibrosis.



**Table 2**  
Status of each individual at autopsy of DIAR-nSTZ mice.

	No.	Body weight (g)	Liver weight (g)	Live tumor	Peritoneal adhesion
Group A (control)	A1	29.9	2.4287	+	–
	A2	26.1	1.9389	+	–
	A3	25.9	2.1752	+	–
	A4	28.3	2.1999	+	–
	A5	27.4	2.5321	+	–
Group B (10 mm)	B1	27.0	1.9712	+	–
	B2	26.6	2.0672	+	–
	B3	31.9	2.8719	+	–



**Fig. 5.** Changes in the body weight of the two groups of DIAR-nSTZ mice that were measured every one weeks. There were no significant differences observed between the groups. Data are presented as the mean  $\pm$  SD. There is no significant differences between the two groups.

#### 4. Discussion

Preliminary experiments showed that one week after liver biopsy, the liver was in the process of absorbing local bleeding and replacing it with regenerated hepatocytes. This three-group comparison study using TSOD mice found no significant adverse events, such as weight loss or decreased activity in the surviving mice in Groups B and C who underwent liver biopsy. In the study using TSOD mice, 80% of the mice in Groups B and C were able to undergo four liver biopsies, with the results considered to be satisfactory with regard to the benefits of frequent liver biopsies. Although the number of samples tested was small, no deaths were observed in DIAR-nSTZ mice after three liver biopsies. Reducing accidental deaths to zero is still challenging, however additional treatments, such as hemostatic agent application after liver biopsy, is expected to help improve the success rate.

In contrast, from a histological observation point, results indicated that the present conditions will need to be improved upon. The TSOD mouse used in this study is a spontaneous metabolic syndrome model characterized by the appearance of mild non-alcoholic steatohepatitis in the liver, with the appearance of liver tumors in old age. HE-stained specimens were prepared from liver biopsy materials, and although the hepatocyte properties were well observed in all cases, the changes in the portal region were not thoroughly analyzed. While the sample volume obtained in our current study was sufficient for molecular biological analysis, the sampling size will need to be increased for a definitive histological analysis. Although the use of larger biopsy needles is the first suggested change, there are concerns about damaging large blood vessels when using these needles. Thus, when making histological observations, it would be better to obtain biopsies from two or three different sites using the same biopsy needle as was utilized in our study. Another weakness of the present technique is that the liver can only be partially visualized. In the present study, while liver tumors were found in four out of the eight surviving animals in Groups B and C of TSOD mice and three out of the three animals in Group B of DIAR-nSTZ mice, these could not be confirmed at the time of the biopsy. Furthermore, while a 10 mm incision allowed us to see some of the lobes of the liver, it was impossible to observe the entire liver. Therefore, in order to establish tumor-targeted biopsy techniques, it is essential to confirm the presence of a tumor at the time of biopsy. Tumors can be identified by pulling the liver out as far as possible through the incision in order to increase the visible area, or by extensively palpating the liver surface with a biopsy needle to look for prominences.

#### 5. Conclusions

We successfully performed three or four liver biopsies in mice through a 10 mm skin incision and a peritoneal incision. By applying this method, it might be possible to monitor the course of both liver disease and the treatment effect over time in a single mouse. The next step will be to reduce the number of deadly events and to ensure a sufficient sampling volume. If we can establish the best conditions under which liver tissue can be consistently collected over time, this will not only make a significant contribution to the analysis of pathological conditions, but will also help to reduce the number of experimental animals.

## Author contribution statement

Wenhua Shao: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Mayuko Ichimura-Shimizu: Performed the experiments; Wrote the paper.

Hirohisa Ogawa: Shengjian Jin: Performed the experiments.

Mitsuko Sutoh: Satoko Nakamura: Miki Onodera: Horosuke Tawara: Shunji Toyohara: Performed the experiments; Contributed reagents, materials, analysis tools or data.

Ryoji Hokao: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data.

Yasusei Kudo: Takeshi Oya: Analyzed and interpreted the data.

Koichi Tsuneyama: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

## Data availability statement

Plans to register with the Tokushima University Institutional Repository, but has not yet been registered.

## Additional information

No additional information is available for this paper.

## Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

KOICHI TSUNEYAMA reports financial support was provided by JSPS-KAKENHI. KOICHI TSUNEYAMA reports a relationship with JSPS-KAKENHI that includes: funding grants.

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