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ORIGINAL ARTICLE

Identification of microRNAs responsive to shear loading in rat skin

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Abstract

Pressure injuries (PIs) are localised skin injuries that result from pressure with or without shear force. Shear force is more destructive than pressure in clinical settings. Therefore, determining the critical external forces is important for selecting the appropriate care to prevent PIs. To quantitatively distinguish pressure and shear loading with high specificity, we focused on microRNAs (miRs). This study aimed to identify the miRs that are distinguishable between pressure with and without shear loading in rat skin. Microarray analysis identified six candidate miRs from the comparisons among the pressure, shear, and unloaded groups. We analysed the expression levels of the candidate miRs in the process of PI development using real-time reverse transcriptase polymerase chain reaction. In the pressure and shear groups, miR-92b expressions at 6 hours after loading were 2.3 ± 1.3 and 2.9 ± 1.0, respectively, which were significantly higher than those in the control group ($P = .014$ and .004, respectively). miR-877 expression at 6 hours after loading was significantly increased only in the shear group (2.8 ± 0.9) compared with the control group ($P = .016$). These results indicate that miR-92b and miR-877 are promising biomarkers to determine for which external force healthcare professionals should intervene.

KEYWORDS

microRNAs, pressure ulcer

Key messages

- this study aimed to identify the miRs that are distinguishable between pressure with and without shear loading in rat skin.
- microarray profile was used to select candidate miRs responsive to pressure with and without shear loading.
- the expression levels of miR-92b in the pressure and shear group were significantly higher than that in the control group at 6 and 24 hours after

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loading, while the expression level of miR-877 was only significantly increased in the shear group at 6 hours after loading.
- this study indicated that miR-92b and miR-877 are promising biomarkers to determine for which external force healthcare professionals should intervene.

1 | INTRODUCTION

Pressure injuries (PIs), as known as pressure ulcers, are an ongoing public health issue that imposes considerable physical burdens on patients, increases the length of hospital stay and the risk of death, and therefore leads to substantial financial concerns.[1-3] The prevalence of PIs in Japan ranges from 0.77% to 2.81% (Survey of the Japanese Society of Pressure Ulcers),[4] which is relatively lower than in other countries, where the prevalence is between 11.8% and 13.9%.[5] However, considering the rapidly increasing older population in Japan, effective prevention strategies for PIs need to be established.

A PI is a localised injury to the skin and/or underlying tissue, usually over a bony prominence, which results from external forces.[6] The external forces include perpendicular force (pressure) and tangential force (shear force). External forces generate internal stresses (compressive, tensile, and shear stresses) within the tissues near bony prominences,[7] and they trigger localised ischemia, impaired lymphatic drainage, reperfusion injury, and cell deformation.[8] Shear force is more destructive than pressure as it distorts capillaries and occludes blood flow.[9,10] In a clinical setting, it is uncommon for a patient to experience pressure alone, and the combined forces of pressure and shear are more common, particularly during head-of-bed elevations.[11] In this situation, pressure combined with shear is applied on the buttocks, and the appropriate care for each external force is different. To decide the appropriate care for PI prevention, healthcare professionals need to identify the critical external forces for which they should intervene. However, identifying the intervention-required forces that cause patients to develop PIs is difficult. Therefore, quantitative evaluation of pressure and shear force is required for the accurate identification of critical forces in clinical settings.

Sensor systems can be used to measure the interface pressure and shear force.[12] A previous study indicated that pressure of more than 33 mmHg occludes the blood vessels, induces cell death in the skin and the underlying tissues, and eventually contributes to PI development.[13] However, a target value for shear force has not yet been determined. Furthermore, individual differences in tissue tolerance to mechanical loading exist,[14] possibly explaining why some patients still develop PIs despite the use of preventive interventions to measure external forces and redistribute pressure. Biochemical measurement of biomarkers, such as skin blotting[15] and Sebutape,[16] has been proposed to assess tissue status or viability due to mechanical loading. de Wert et al[16] reported that the interleukin-1α (IL-1α) to total protein ratio indicated the different tissue response to pressure with and without shear loading in the forearm. Though IL-1α can be measured quantitatively, it is broadly involved in the inflammatory response in the skin.[17] Therefore, it is necessary to identify other biomarkers which meet both requirements including the specificity to mechanical loading and the quantitative estimation of shear.

In the present study, we focused on microRNAs (miRs) as biomarkers, as they exhibit highly quantitative traits under real-time reverse transcription polymerase chain reaction (RT-PCR),[18] and their specificity and sensitivity are superior to protein analysis.[19] miRs are small non-coding RNAs, which are relatively stable and perform a vital role in regulating gene expression by binding to target messenger RNAs (mRNAs), resulting in their degradation or inhibition of translation.[20] In addition, several miR studies demonstrated their potential as diagnostic, prognostic, or predictive biomarkers of disease, including cancer and wounds.[19,21] However, there are no studies that investigated the involvement of miRs in PI development. Therefore, we employed a high-throughput examination of miR expression profiles to explore candidate miRs that are distinguishable between pressure with and without shear loading in this study.

2 | METHODS

2.1 | Animals

A total of 105 6-month-old healthy male Sprague–Dawley rats were purchased from Japan SLC Inc. (Shizuoka, Japan). They were administered standard food and water ad libitum and housed in the animal facility at 23 ± 2°C under 55 ± 10% humidity and a 12-hours light/12-hours dark cycle (08:00–20:00).

The animal experimental protocols used in this study were approved by the Animal Research Committee of the University of Tokyo, and all animals were treated

according to guidelines established by the Japanese Association for Laboratory Animal Science (1987).

2.2 | Experimental design

To identify the miRs that were distinguishable between pressure with and without shear loading during PI development, we conducted the following three experiments.

Experiment 1: In order to determine the loading conditions for pressure with and without shear force required for Category I PI development, animals were randomly assigned to five different conditions: pressure with shear force for 1 and 2 hours and pressure without shear force for 1, 2, and 3 hours, which were decided through preliminary experiments. Three animals were included for histological analysis in each group, and based on the results of experiment 1, the loading conditions for experiments 2 and 3 were decided.

Experiment 2: In order to explore the candidate miRs that are responsive to pressure and shear force, animals were randomly assigned to three groups: (1) the shear group, in which pressure with shear force was applied, (2) the pressure group, in which pressure without shear force was applied, and (3) the control group, in which no forces were applied. In order to clarify the differences in miR expressions among the three groups, the loading conditions were set to exceed the conditions required for PI development. The miR profiles of the shear and pressure groups were compared with that of the control group.

Experiment 3: Animals were randomly assigned to the shear, pressure, and control groups. In the shear and pressure groups, the loading magnitude was the same, but the loading duration was shorter than that for experiment 1. These conditions mimicked the process of PI development. The expression levels of the candidate miRs at 0, 1, 6, 12, and 24 hours after loading were compared among the three groups.

2.3 | Loading procedure

Pressure was applied to rat skin with and without shear force based on a previous modified method[22] using the original device shown in Figure 1A. This loading device can apply pressure of between 1 and 10 kg at 1 kg intervals. The tip of the indenter is hemispherical in shape (ϕ2 cm, Figure 1B). The angle of the animal tray to the indenter was adjusted to 90° to generate pressure without shear force (Figure 1C) and 100° to generate pressure with shear force (Figure 1D). The rats were acclimatised for a week before the experiments. One day after shaving the trunk hair of rats, the loading site (ϕ2 cm) was marked at the right flank region. A stainless plate was inserted into the abdominal cavity through two sagittal incisions (3 cm long) and fixed to the animal tray under anaesthesia with isoflurane. After loading, the stainless plate was removed, the incisions were sutured, and a non-adhesive foam dressing (Hydrosite Plus, Smith & Nephew, Watford, UK) was

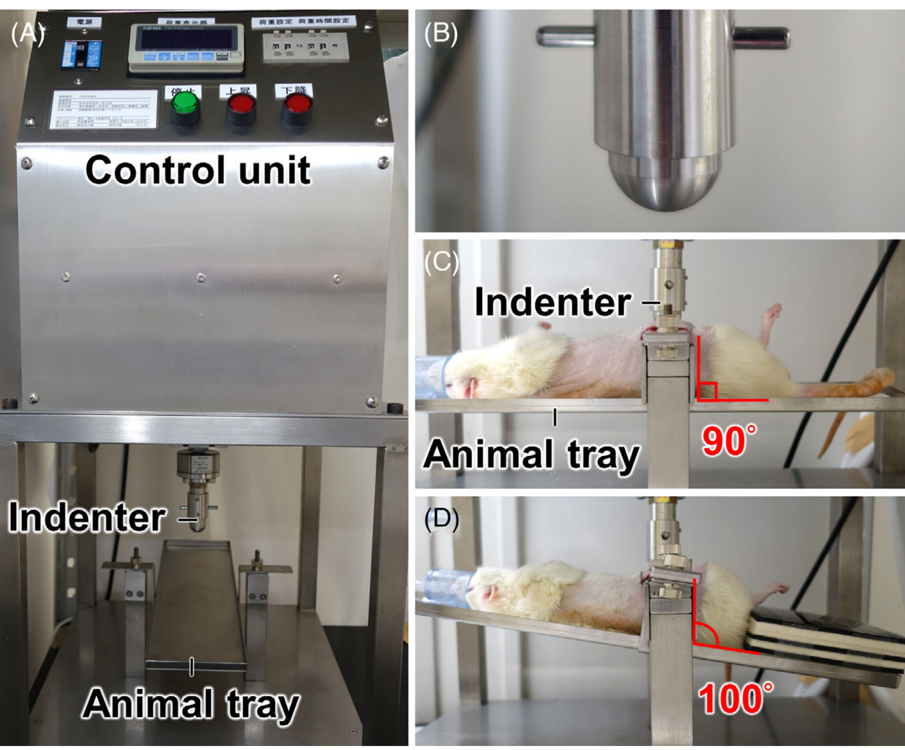

FIGURE 1 Pressure and shear loading to rat flank skin. A, Original loading device. B, The tip of the indenter. C, Pressure loading at 90°. D, Pressure with shear loading at 100°

applied with surgical tape to the marked skin (SKINERGATE™, Nichiban, Tokyo, Japan). Then, the trunk was fully covered with gauze.

The loading magnitude was 1 kg in experiments 1 and 3 and 10 kg in experiment 2. In experiment 1, the loading durations were 1 and 2 hours for applying pressure with shear loading (S1 and S2 groups) and 1, 2, and 3 hours for applying pressure without shear loading (P1, P2, and P3 groups). In experiments 2 and 3, an unloaded control was prepared, in which the stainless plate was inserted into the abdominal cavity and fixed to the animal tray at the same angle for the same duration as the other groups but without loading.

During the experimental period, dressings were changed daily and the macroscopic appearance of loaded skin was recorded using a digital camera (Sony Corporation, Tokyo, Japan). At the end of experimental period, animals were euthanized by a lethal dose of carbon dioxide under anaesthesia to collect samples from loaded skin. In experiment 1, tissue samples were harvested 1 hour after loading (n = 3 per group), and three additional animals were kept for 1 week after loading in the S2 and P3 groups to determine tardive deterioration such as deep tissue injury.[23] In experiment 2, three animals were included in each group. In experiment 3, tissues were collected at 0, 1, 6, 12, and 24 hours after loading. This experiment was repeated five times. Category I PIs were defined as areas of loaded skin with persistent redness that could be observed 1 hour after loading.

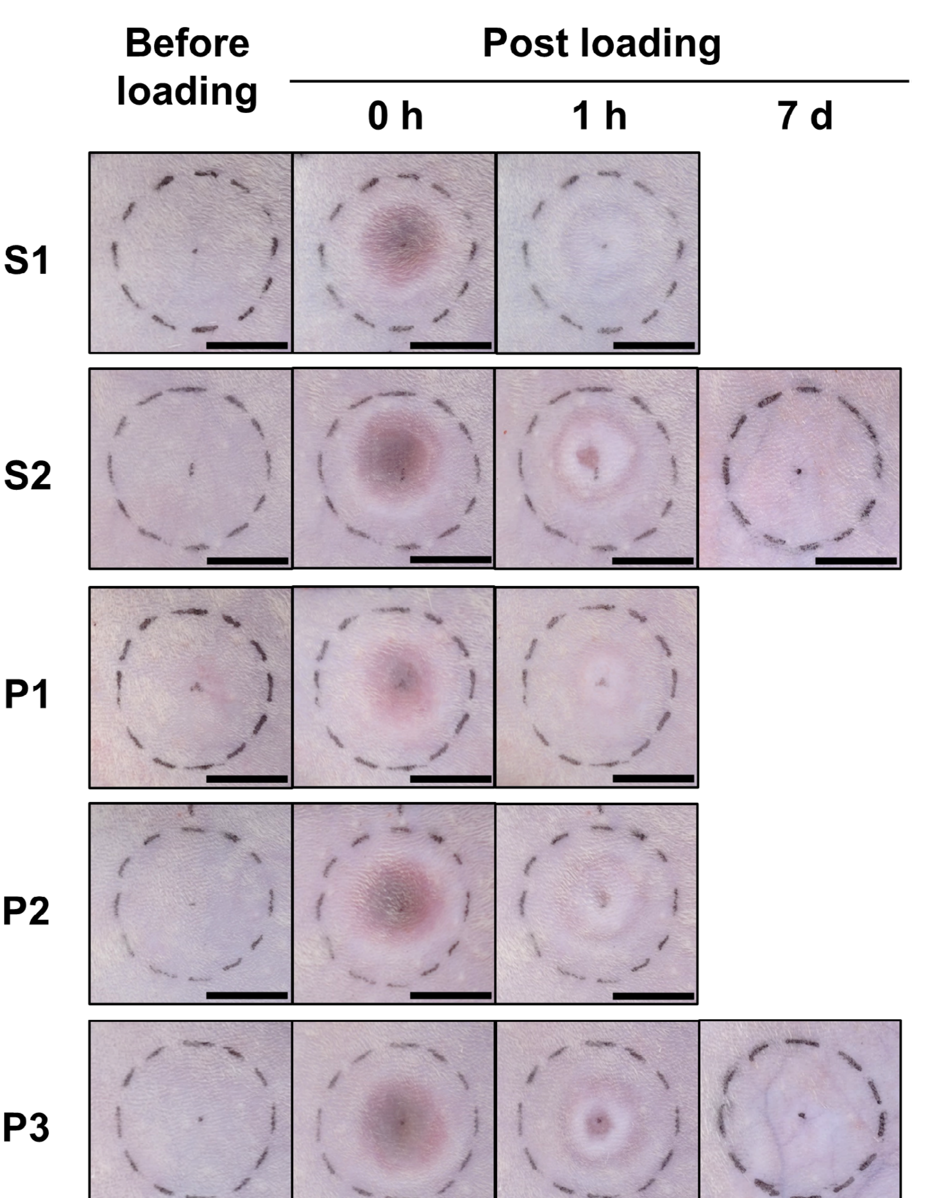

FIGURE 2 Representative images of macroscopic findings of compressed skin before and after loading in experiment 1. Pressure with and without shear force was loaded at 100° and 90° in the rat flank skin for 1–2 and 1–3 hours, respectively. Macroscopic appearance was recorded just before loading, 0, 1 hour, and 7 days after loading. Development of Category I pressure injury was judged at 1 hour after loading. S1, 1-hour pressure with shear loading group; S2, 2-hour pressure with shear loading group; P1, 1-hour pressure without shear loading group; P2, 2-hour pressure without shear loading group; P3, 3-hour pressure without shear loading group. Scale bar = 1 cm

2.4 | Histological analyses

All harvested tissues from experiment 1 were fixed overnight in 10% neutral buffered formalin at room temperature, dehydrated in a series of the ethanol and xylene substitute, G-Nox (Genostaff, Tokyo, Japan), and embedded in paraffin. The paraffin-embedded tissues were sectioned at 3 μm thicknesses for haematoxylin and eosin (HE) and Masson's trichrome (MT) staining. The sections were observed using a light microscope (BX41; Olympus Corporation, Tokyo, Japan) and photographed using an inverted microscope (BZ-X710, Keyence, Osaka, Japan).

2.5 | Microarray profiles of miRs

miRs were extracted using a Purelink™ microRNA Isolation Kit (ThermoFisher Scientific, Waltham, MA). Ten microliters of individual samples were mixed into a pooled sample in each group. miR profiling of three pooled samples was outsourced to Filgen, Inc. (Nagoya, Japan). In brief, 336 ng of miR was biotinized using a FlashTag™ Biotin HSR RNA Labeling Kit (ThermoFisher Scientific) and then reacted with a GeneChip® microRNA 4.0 Array (ThermoFisher Scientific), which included probes for 490 rat precursor miRs (pre-miRs) and 728 mature miRs,

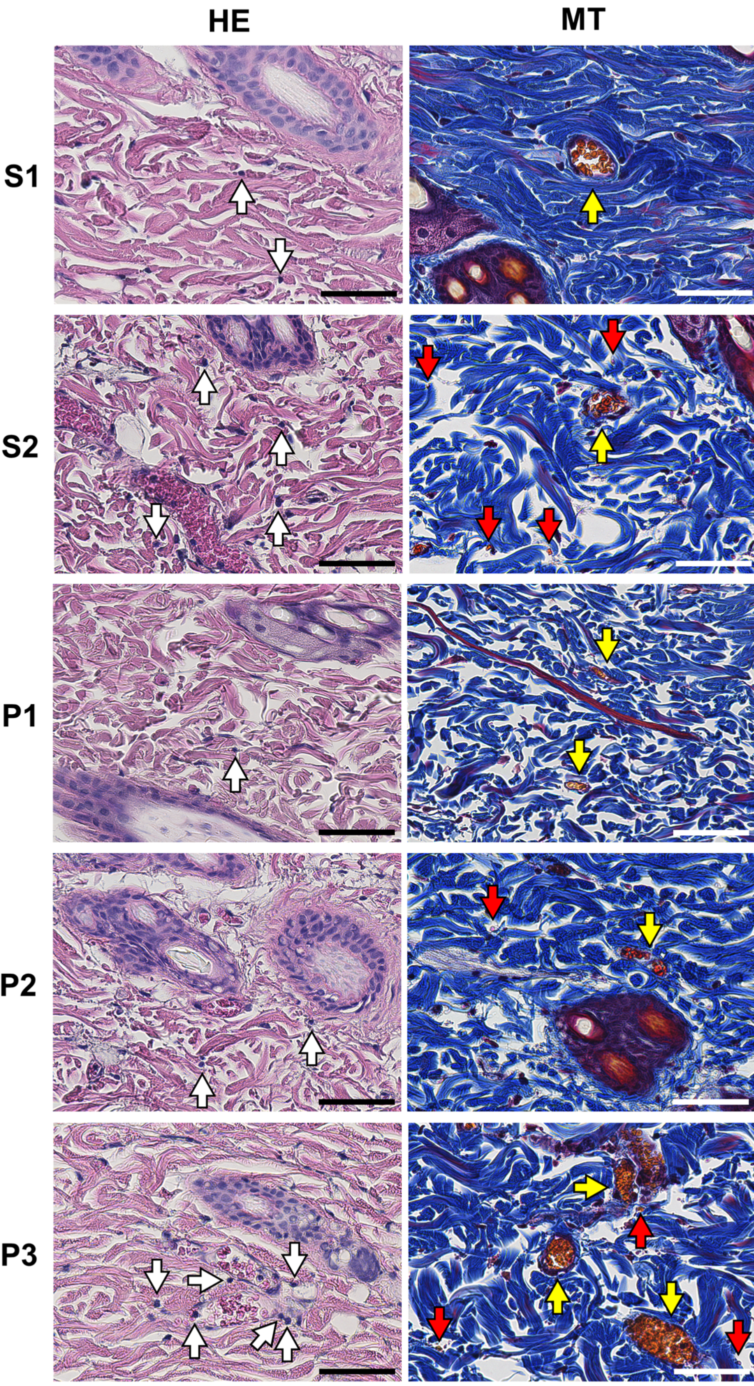

FIGURE 3 Representative images of haematoxylin and eosin (HE) and Masson's trichrome (MT) staining of the compressed skin. S1: 1-hour pressure with shear loading group; S2: 2-hour pressure with shear loading group; P1: 1-hour pressure without shear loading group; P2: 2-hour pressure without shear loading group; P3: 3-hour pressure without shear loading group; White arrows: infiltration of inflammatory cells; Yellow arrows: thrombosis; Red arrows: bleeding. Scale bar = 50 μm

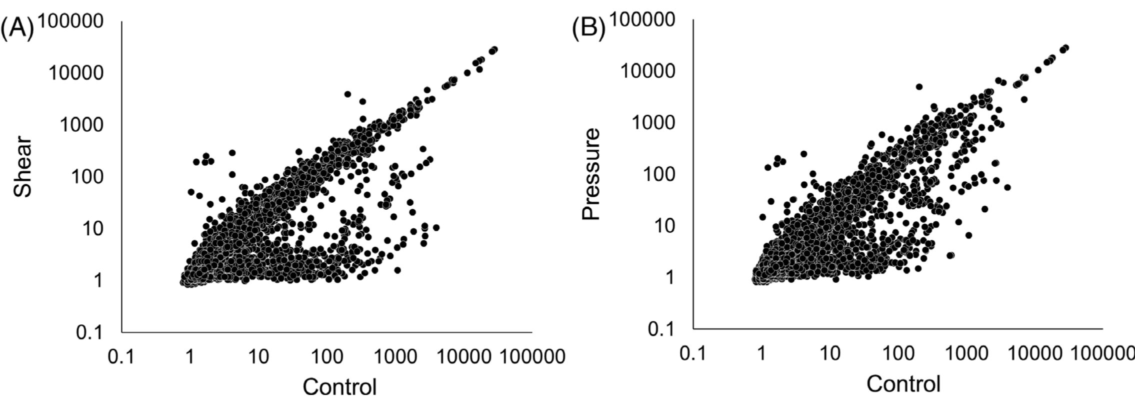

FIGURE 4 Scatter plot of microRNA expression in comparisons, A, between the control and shear, and B, between the control and pressure groups

TABLE 1 Expression levels of the candidate microRNAs to distinguish pressure and shear loading in microarray analysis

miRs	Pressure	Shear
miR-25-5p	0.68	2.13
miR-92b-5p	2.91	0.97
miR-328b	Absent	4.62
miR-503-5p	Absent	3.25
miR-652-5p	4.73	Absent
miR-877	0.76	3.06

at 48°C for 18 hours. After washing, the array was scanned using a GeneChip Scanner 3000 7G (ThermoFisher Scientific).

The results were qualitatively and quantitatively evaluated. In the qualitative evaluation, miRs that were detected only in the pressure or shear groups were chosen (pressure- or shear-specific miRs). In the quantitative evaluation, the miRs whose expression levels were more than two times higher or less than 0.5 times lower in the pressure or shear groups compared with the control group were selected. miRs with no commercially available specific primer and probe set for real-time RT-PCR were excluded.

TABLE 2 Detection frequency of microRNAs in real-time reverse transcriptase polymerase chain reaction examination

		Treatment			
miRs	Time (h)	C	P	S	*P*-value
miR-328b	0	3/5	2/5	5/5	.392
	1	4/5	5/5	5/5	.875
	6	3/4	4/4	4/4	.873
	12	3/5	4/4	5/5	.579
	24	3/5	5/5	5/5	.523
miR-503	0	5/5	4/5	5/5	.875
	1	4/5	5/5	5/5	.875
	6	3/4	4/4	4/4	.901
	12	4/5	4/4	5/5	.862
	24	4/5	5/5	5/5	.875
miR-652	0	4/5	3/5	3/5	.963
	1	2/5	5/5	5/5	.179
	6	3/4	4/4	4/4	.873
	12	3/5	3/4	5/5	.672
	24	2/5	5/5	5/5	.179

Note: Data were analysed using Fisher's exact test.
Abbreviations: C, control group; P, pressure group; S, shear group.

2.6 | Real-time RT-PCR

In experiment 3, the precursor form of the miR was also incorporated in the detection if their mature form was selected from experiment 2.

The expression levels of the candidate miRs were individually measured by real-time RT-PCR. The complementary DNA (cDNA) was synthesised using a High-Capacity cDNA Reverse Transcription Kit (ThermoFisher Scientific) and random or target-specific primers. The target cDNA was amplified using an Mx3000P QPCR System (Agilent Technologies, Santa Clara, CA) and TaqMan® MicroRNA Assays (ThermoFisher Scientific). The thermal conditions for the real-time RT-PCR were as follows: 50°C for 2 minutes, 95°C for 10 minutes, followed by 50 cycles at 95°C for 15 seconds, and 60°C for 1 minute. All samples were tested in triplicate. The relative expression levels of the target miRs were normalised to U6 expression and compared among the groups using the ΔΔ cycle threshold (*Ct*) method based on the following formulas:

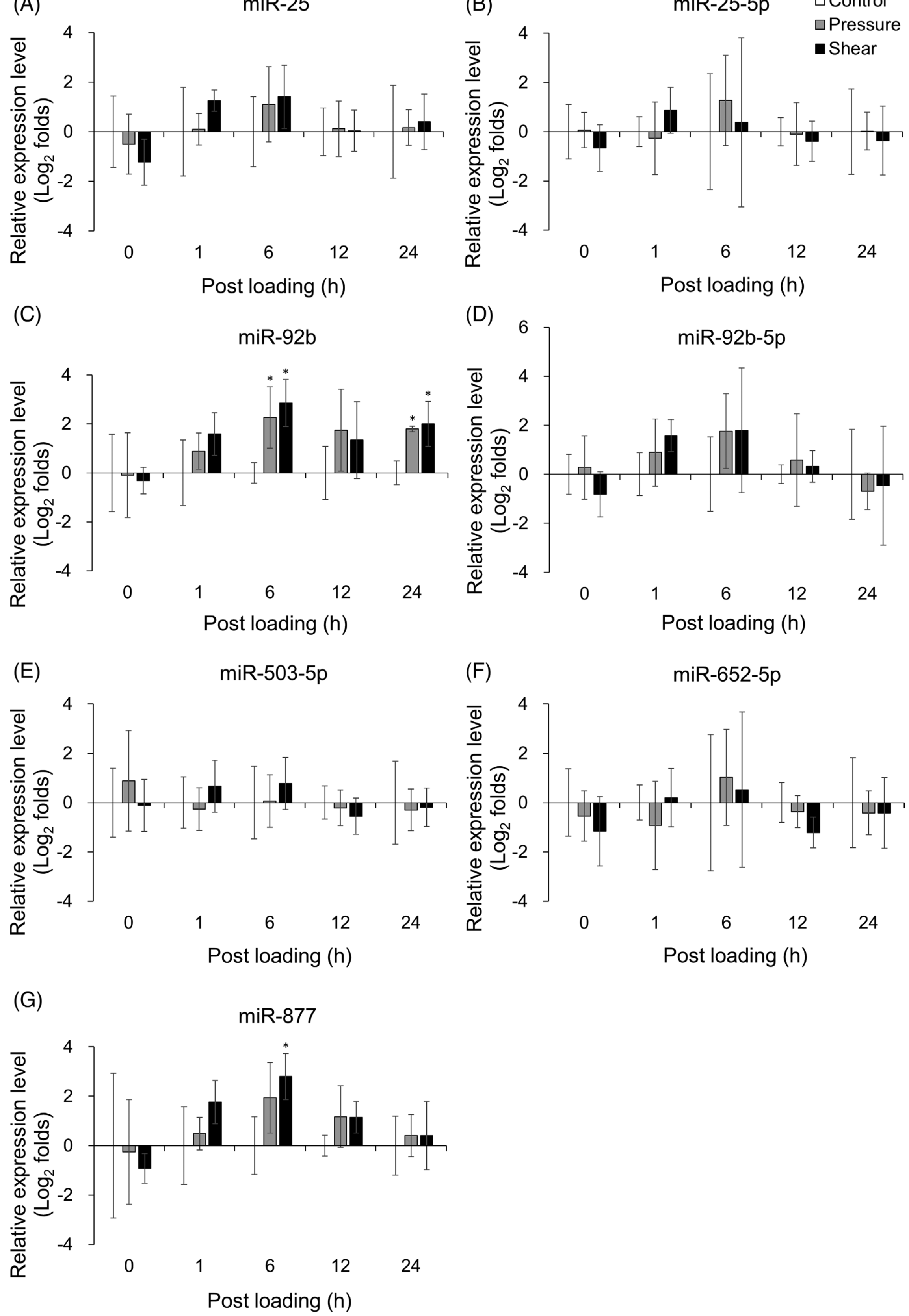

FIGURE 5 Relative expression levels of A, miR-25; B, miR-25-5p; C, miR-92b; D, miR-92b-5p; E, miR-503-5p; F, miR-652-5p; and G, miR-877. White bar: control group; grey bar: pressure group; black bar: shear group. Multiple comparisons were performed by analysis of variance followed by post-hoc Dunnett's test compared with the control group. Data were presented as mean ± SD. $^*P < .05$

$$\Delta Ct = \left(Ct_{\text{target gene}} - Ct_{\text{U6}}\right)$$

$$\Delta\Delta Ct = \Delta Ct - \text{Average of } \Delta Ct_{\text{control}}$$

$$\text{Relative expression level} = 2^{(-\Delta\Delta Ct)}$$

The $-\Delta\Delta Ct$ values which indicated relative expression levels ($\log_2$ folds) were used for statistical analysis. Samples with *Ct* values for U6 higher than 27 were excluded from the analysis.

2.7 | Statistical analysis

All data were presented as the mean ± SD. For comparisons of continuous variables, we used analysis of variance followed by post-hoc Dunnett's test. Categorical data were analysed using Fisher's exact test. A *P*-value of less than .05 was considered statistically significant. All statistical analyses were performed using Stata/SE 16.1 (StataCorp, College Station, TX).

3 | RESULTS

3.1 | Establishment of Category I PI rat models by pressure with and without shear loading (experiment 1)

The macroscopic findings are shown in Figure 2. Category I PIs were observed in all animals in the S2 and P3 groups and in two animals in the P2 group. One animal in the P2 group demonstrated a dot-like redness. All Category I PIs disappeared 7 days after loading in the S2 and P3 groups.

Thrombosis, infiltration of inflammatory cells, and bleeding in the dermis were examined by HE and MT staining (Figure 3). Sections from all five groups showed thrombosis. Infiltration of inflammatory cells was frequently observed in the S2 and P3 groups, whereas mild infiltration was observed in the other groups. Bleeding was frequently observed in all samples from the S2 and P3 groups and occasionally observed in the P2 group, whereas it was not found in the P1 and S1 groups, a finding which was in agreement with the macroscopic observation of persistent redness. These findings indicated that we successfully created Category I PI rat models in the S2 and P3 groups.

3.2 | Candidate miRs responsive to pressure with and without shear loading (experiment 2)

Figure 4 shows the relationship of miR expression between the control, shear (Figure 4A), and pressure groups (Figure 4B). The candidate miRs that were selected from the microarray profile are shown in Table 1. miR-328b and miR-503-5p were detected in the shear group only, and miR-652-5p was detected in the pressure group only. miR-25-5p, miR-92b-5p, and miR-877 were detected in all groups. The expression levels of miR-25-5p and miR-877 in the shear group, and those of miR-92b-5p in the pressure group were more than double those of the control group.

3.3 | Identification of candidate miRs by time-course expression (experiment 3)

The expression levels of four mature miRs (miR-25-5p, miR-92b-5p, miR-503-5p, and miR-652-5p) and six pre-miRs (miR-25, miR-92b, miR-328b, miR-503, miR-652, and miR-877) were examined by real-time RT-PCR at 0, 1, 6, 12, and 24 hours after loading. Because the *Ct* values of U6 were higher than 27, four samples were excluded from the control group at 6 hours, from the pressure group at 6 and 12 hours, and from the shear group at 6 hours.

Since amplification of miR-328b, miR-503, and miR-652 was detected in only some of the samples, the frequency of their detection was analysed (Table 2), and we found that no statistical difference was present in their detection frequency at any time point. In contrast, miR-25, miR-25-5p, miR-92b, miR-92b-5p, miR-503-5p, miR-652-5p, and miR-877 were detected in all samples; therefore, their expression levels were analysed quantitatively (Figure 5). The relative expression levels of miR-92b in the pressure and shear groups were 2.3 ± 1.3 and 2.9 ± 1.0, respectively, at 6 hours after loading, and 1.8 ± 0.1 and 2.0 ± 0.9, respectively, at 24 hours after loading; levels which were significantly higher than the control group (P = .014, .004, .001, and <.001, respectively). The relative expression level of miR-877 at 6 hours after loading was significantly increased only in the shear group (2.8 ± 0.9) compared with the control group (P = .016).

4 | DISCUSSION

To the best of our knowledge, this is the first study to investigate miR expression profiles in PI development. The results revealed that the expressions of miR-92b and miR-877 were enhanced prior to PI development in response to pressure with shear loading. These miRs are promising biomarkers to determine which is the critical external force and help healthcare professionals to intervene earlier to prevent PI development.

In experiment 1, two different Category I PI rat models were successfully created due to pressure with and without shear loading. Under the same loading magnitude (1 kg), the loading duration required for Category I PI development in the pressure with shear force (2 hours) group was shorter than that required in the pressure without shear force (3 hours) group. These results are consistent with previous studies,[24,25] indicating that a combination of pressure and shear force is more destructive than pressure alone as it generates greater stress in the skin and the underlying tissue and decreases the internal blood flow more effectively.

Based on the results of experiment 1, we determined the loading conditions of experiments 2 and 3. In experiment 2, the maximum loading condition of the device (10 kg, 6 hours) was applied to animals in order to maximise the differences in miR expression among the groups. This condition exceeded the loading required for PI development and resulted in severe persistent redness at 1 hour after loading. In experiment 3, a shorter duration than that used in experiment 1 was utilised to represent the process of PI development. The redness of all animals disappeared within 1 hour after loading. These results indicate the internal validity of the experimental animals used in experiments 2 and 3.

The role of miR-92b in lung cancer, glioma, and oesophageal cancer has been extensively studied.[26-28] Previous studies revealed that miR-92b is responsive to hypoxia in pulmonary artery smooth muscle cells,[29] and that overexpression of miR-92b results in carcinogenesis via activation of nuclear factor-κB signalling,[30] which promotes cell proliferation and inhibits apoptosis.[31] Because external forces induce PI development via tissue hypoxia, increased expression of miR-92b is probably a protective response of the skin tissue to pressure and shear loading. Therefore, our findings suggest that miR-92b is a promising biomarker to evaluate mechanical loading and a possible target to inhibit PI development.

Another of the candidate miRs, miR-877, is a known tumour suppressor that inhibits cell proliferation by targeting *cyclin-dependent kinase 14* and *forkhead box M1*.[32,33] In the current study, a significant increase in miR-877 expression was found only in the shear group. However, the studies on miR-877 are insufficient to explain these different responses to pressure with and without shear loading. Further studies are required to explore the role of miR-877 in the response to pressure with shear loading in the skin.

Notably, expression analysis revealed temporal increases in miR-92b and miR-877 at 6 hours after loading. This finding is probably due to miR biogenesis. When cells respond to external stimuli, it takes time to trigger the pathway. In general, primary miRs are transcribed from genomic DNA and are subsequently cleaved into pre-miR. Then, the pre-miR is exported into the cytoplasm, followed by cleavage into a mature miR duplex. Either the 5′ or 3′ strand of the mature miRNA is loaded into an RNA-induced silencing complex and then binds to the target mRNA where the miR induces target degradation or translational inhibition of the mRNA.[20]

Combined measurements of miR-92b and miR-877 are thought to be applicable to the risk assessment of PIs. A high level of miR-92b can indicate an increased risk of PI due to mechanical loading, and the miR-877 level can indicate which force, pressure or shear, requires immediate care. This is a possible assessment to choose the appropriate care to redistribute the mechanical loading to the skin and to reduce the risk of PI. Therefore, the temporal alteration of miRs should be considered for clinical application, possibly in combination with load sensors. A load sensor can indicate the appropriate timing to examine miR expressions. Our research group previously developed skin blotting, which is an innovative skin assessment tool to extract proteins non-invasively from the skin.[34] Future studies are required to establish a similar methodology for miR extraction from the skin as well as to reveal the mechanisms of PI development involving miR-92b and miR-877.

5 | CONCLUSIONS

In the present study, we established Category I PI rat models due to pressure with and without shear loading. Then, we identified miRs whose expressions were enhanced in the process of PI development by microarray and real-time RT-PCR. Consequently, miR-92b was identified as a marker responsive to pressure both with and without shear loading, and miR-877 was identified as a marker responsive to pressure with shear loading. These results indicate that miR-92b and miR-877 are promising biomarkers to determine for which external force healthcare professionals should intervene.

ACKNOWLEDGEMENT

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CONFLICT OF INTEREST

One of the authors (Takeo Minematsu) belongs to the department sponsored by Saraya Cooperation, Osaka, Japan. The company had no role on the study concept, design, data collection and analysis, and manuscript drafting.

DATA AVAILABILITY STATEMENT

Data openly available in a public repository that issues datasets with DOIs.

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