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ORIGINAL ARTICLE

Expression levels of NPPB, ITGB6, CPNE4, EML5, and ITSN1 in fresh exudates swabbed from critically colonised and infected full-thickness wounds in rats

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Abstract

Pressure injury management requires reliable identification of critical colonisation due to lack of infection signs. Our research group previously proposed the mRNAs *natriuretic peptide B* (*Nppb*), *integrin subunit beta 6* (*Itgb6*), *copine 4* (*Cpne4*), *echinoderm microtubule-associated protein like 5*, and *intersectin 1* as candidate markers in pooled exudates of critically colonised wounds. However, it is unclear whether mRNAs or proteins of the candidate genes would be suitable as biomarkers in fresh exudate. Therefore, this study aimed to evaluate the validity of the mRNAs and proteins as fresh exudate markers for critical colonisation. Three wound models of normal healing, critical colonisation, and infection were created in rats. Fresh swab-collected exudates were collected, and mRNA and protein expression levels were measured. In the fresh wound exudates, the detection frequency of *Itgb6* tended to decrease in the critically colonised and infected wounds ($P = .067$), and those of *Cpne4* and *Nppb* tended to be lower in the infected wounds than in the normal healing and critically colonised wounds ($P = .006$ and .067, respectively). In contrast, there was no difference in protein expression in the exudates. This study suggests that *Itgb6* mRNA in fresh exudates is a promising biomarker for critical colonisation.

KEYWORDS

critical colonisation, infection, *integrin subunit beta 6*, pressure injury, wound exudate

Key Messages

- pressure injury management requires reliable identification of critical colonisation due to the lack of infection signs

List of Abbreviations: CPNE4, copine 4; EML5, echinoderm microtubule-associated protein like 5; ITGB6, integrin subunit beta 6; ITSN1, intersectin 1; NPPB, natriuretic peptide B; PI, pressure injury; PWD, post-wounding day.

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- this study aimed to evaluate the validity of the candidate mRNAs and proteins as fresh exudate markers for critical colonisation
- mRNA and protein expression levels were measured in wound tissues and fresh exudates
- in the fresh wound exudates, the detection frequency of *Itgb6* tended to decrease in the critically colonised and infected wounds, and those of *Cpne4* and *Nppb* tended to be lower in the infected wounds than in the normal healing and critically colonised wounds
- this study suggests that *Itgb6* mRNA may have distinguished critically colonised and infected wounds from normal healing wounds

1 | INTRODUCTION

Pressure injury (PI) is a localised injury to the skin and/or underlying tissue, usually over a bony prominence, resulting from sustained pressure, including pressure associated with shear.[1] PIs have physical, psychological, and social impacts as well as financial burdens for patients, their families, and society.[2,3] A global systematic review reported that the prevalence of PIs in hospitalised adult patients was 11.8% to 13.9% in Europe, North America, Asia, the Middle East, South America, and Australia.[4] Although the PI prevalence in Japan has dramatically declined to 1.47% to 2.48% in the last two decades owing to the dedicated efforts of researchers and clinicians, PIs remain a serious problem. Ageing is a risk factor for PIs and just over 60% of PI patients were older than 65. Older adults also often have underlying diseases, such as diabetes and stroke, which further elevate the risk of PI.[5] Not only in Japan, but worldwide, the elderly population will increase from 9% in 2019 to 16% in 2050.[6] Thus, PI is a critical problem in all regions with an ageing population.

The most severe complication of PI is wound infection, which can cause septicaemia and death.[7,8] PI infection inhibits wound healing and can lead to poor quality of life because of extended hospital stays, pain for patients, and difficult patient handling for caregivers.[2] Therefore, the identification of infection is a high priority in PI management.

Wound infection occurs when the balance between host immunity and bacterial virulence is lost.[9] The progression of a tissue from a healthy state to an infected state is often conceptualised in terms of four phases: contamination, colonisation, critical colonisation, and infection. Infection, the state in which bacterial virulence predominates over host immunity, can be identified by clinical signs, such as redness, swelling, warmth, and pain. Critical colonisation, which is a state of balance between host immunity and bacterial virulence, impedes the progress of wound healing. Antiseptic ointments are required for critical colonisation, but not for colonisation. Therefore, critically colonised PIs should be distinguished from colonised PIs. However, their identification is difficult because the clinical signs associated with infection are not clearly evident.[10] Indeed, critical colonisation in non-healing PIs is generally only diagnosed retrospectively when antimicrobial treatments prove their effectiveness in enhancing the progress of wound healing.[9] However, PIs must be monitored for more than 2 weeks in order to identify delayed healing. Therefore, the identification of critical colonisation without monitoring is essential for PI management. NERDS, an assessment tool that considers five clinical signs, was developed to assess critical colonisation.[11] However, its sensitivity and specificity are 32% to 70% and 47% to 86%, respectively, suggesting a need for more reliable methods to detect critical colonisation in PIs.

Recently, two methods for identifying critical colonisation have been developed. An increased temperature of the wound bed compared with the periwound skin as measured by thermography was reported to predict delayed healing of PIs.[12] A high temperature of the wound bed indicates tissue inflammation[13]; however, wound inflammation is not only caused by critical colonisation, but also by tissue damage due to external force. Biofilm is another factor contributing to critical colonisation. It has been reported that detecting biofilm is effective for predicting critical colonisation or infection.[14] However, the detection of biofilm is not sufficient to identify critical colonisation because biofilm formation begins with bacterial attachment to the wound surface.[15] Thus, an appropriate method for identifying critical colonisation has not yet been established.

Our study group has been focusing on wound exudates for wound assessment because these can be obtained noninvasively and contain inflammatory cells, cytokines, and growth factors that reflect the PI condition.[16-18] Asada et al found increased mRNA levels of *natriuretic peptide B* (*Nppb*), *integrin subunit beta 6* (*Itgb6*), *copine 4* (*Cpne4*), *echinoderm microtubule-associated protein like 5* (*Eml5*), and *intersectin 1* (*Itsn1*) in critically colonised and infected wounds compared with non-infected wounds using high-

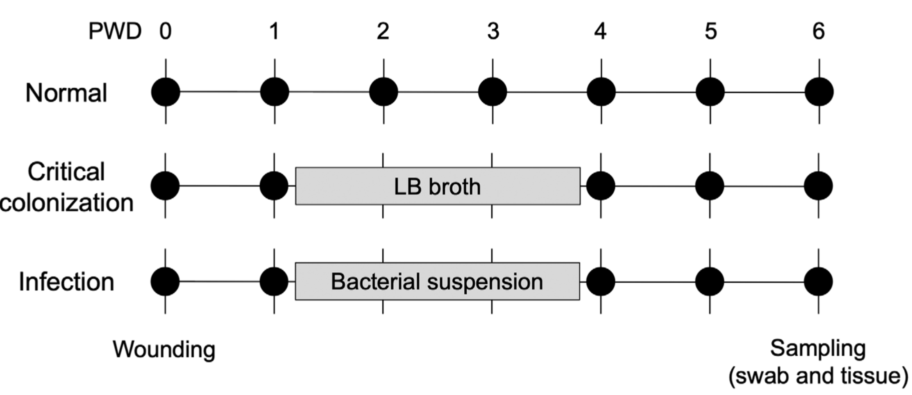

FIGURE 1 Experimental schedule. N: normal group, CC: critical colonisation group, I: infection group. Black circle: wound cleansing, photograph, and dressing change; grey box: treatment of wounds with LB broth or bacterial suspension; PWD: post-wounding day

throughput mRNA expression analysis of rat pooled exudates.[19] In addition, they conducted a cross-sectional analysis in which the mRNA levels of these genes were examined in 11 PIs, and the sensitivity and specificity in distinguishing critically colonised and infected PIs from normal PIs were both 100%.[19] These mRNAs in the pooled exudates may be candidate biomarkers for the detection of critical colonisation. However, pooling of wound exudates has low clinical feasibility compared with collecting fresh exudates using a swab because occlusion of wounds increases risk of infection. Therefore, fresh exudates should be targeted for clinical application.

In this study, we aimed to evaluate the validity of NPPB, ITGB6, CPNE4, EML5, and ITSN1 as mRNA and protein markers in fresh wound exudates for the detection of critical colonisation. We hypothesised that some mRNAs or proteins would be highly expressed in critically colonised and infected wounds compared with normal healing wounds.

2 | METHODS

2.1 | Animals

In total, 15 six-month-old male Sprague–Dawley rats were purchased from Japan SLC (Shizuoka, Japan), and randomly assigned to three groups: normal, critical colonisation, and infection groups. The rats were maintained under controlled conditions of $23 \pm 2^{\circ}$C, $45 \pm 10\%$ humidity, and 12-h/12-h light/dark cycles, and had ad libitum access to feed and drinking water.

The animal models of the infection group were prepared according to the method described by Kitamura et al[20] (Figure 1). A wild-type strain of *Pseudomonas aeruginosa* (PAO1, NBRC106052, National Institute of Technology and Evaluation, Tokyo, Japan) was cultured in Luria–Bertani (LB) broth at 37°C and the bacterial density was adjusted to $OD_{600} = 1.0$, which is equivalent to 2.36×10^9 colony-forming units/mL. A 1.2 cm × 1.2 cm gauze was incubated in 2 mL bacterial suspension at 37°C for 1.5 hours to allow for bacterial colonisation on the gauze. A full-thickness round wound with a diameter of 2 cm was created on the dorsal skin, the centre of which was located on the posterior median line and at the 8:7 point between the acromion and greater trochanter. The wounds were first covered with a perforated film dressing (Mepitel One, Mölnlycke Health Care AB, Göteborg, Sweden) and then overlaid with an absorbent pad dressing (Derma Aid, ALCARE Co., Ltd., Tokyo, Japan). A water-repellent coating agent (3M™ Cavilon™ No String Barrier Film, 3 M, St. Paul, Minnesota) was applied to the periwound skin to prevent contact dermatitis caused by wound exudates. On post-wounding day (PWD) 1, the gauze incubated with bacterial suspension was fixed to the surface of wounds with perforated film dressing, overlaid with the absorbent pad dressing, and left for 3 days. The gauze with bacteria was removed on PWD 4, and the wound was cleansed daily with sterilised saline and covered with new dressings until PWD 6.

We established a new critical colonisation model by modifying the method described by Kitamura in the preliminary experiments. In this model, the gauze was incubated in 2 mL of LB broth without bacteria at 37°C for 1.5 hours and applied to the wound using the same method utilised in the infection group. For the normal group, the wound was washed with sterilised saline and the dressing was changed daily.

2.2 | Sample collection

Wound tissues and fresh wound exudates were collected on PWD 6. Fresh wound exudates were obtained via swabbing using the Levine technique after wound cleansing.[21] Total RNA and proteins were isolated using the AllPrep DNA/RNA/Protein Mini Kit (Qiagen, Venlo, Netherlands). Immediately after euthanasia, the wound and periwound skin tissues were harvested and cut into three parts as follows: the left half of the tissue was used

for histological analysis, the cranial right quarter for RNA extraction, and the caudal right quarter for protein extraction. The tissue for histology was fixed with 10% neutral buffered formalin and embedded in paraffin. Following tissue fixation with RNAlater (Merck, Burlington, MA), total RNA was extracted in an automatic nucleic acid extraction device (Maelstrom™ 8 Autostage, Taiwan Advanced Nanotech Inc., Taoyuan, Taiwan) using the TANBead Nucleic Acids Extraction Kit (Taiwan Advanced Nanotech Inc.). For protein extraction, the tissue was homogenised with 5-mm- and 3-mm-diameter zirconia beads in RIPA buffer (Nacalai Tesque, Inc., Kyoto, Japan) using a shaking homogeniser (Shakeman 6, Bio-Medical Science Co., Ltd., Tokyo, Japan). The homogenates were centrifuged at 10 000 × g, 4°C for 10 minutes and the supernatants were collected as protein.

2.3 | Macroscopic observation

During the experimental period, the macroscopic appearance of the wound and surrounding skin was recorded at the time of the dressing change using a digital camera (Sony Corp., Tokyo, Japan). Erythema on the periwound skin and necrotic tissue on the wound bed were observed. The wound areas were measured using the ImageJ software (National Institutes of Health, Bethesda, MD) and the wound areas relative to that on PWD 1 were calculated.

2.4 | Histological analysis

Three-micrometre-thick tissue sections were stained with haematoxylin and eosin. Histological features were recorded using an inverted microscope (BZ-X710, Keyence Corp., Osaka, Japan).

2.5 | Reverse transcription quantitative polymerase chain reaction (RT-qPCR)

Ten microliters of RNA was used for complementary DNA (cDNA) synthesis on a thermal cycler (Bio-Rad Laboratories, Inc., Hercules, California) using the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, Waltham, Massachusetts). qPCRs were performed in triplicate in a Mx3000P qPCR System (Agilent Technologies, Santa Clara, California) using the following TaqMan Gene Expression Assay Kits: *Nppb* (Rn00580641_m1), *Itgb6* (Rn01747277_m1), *Cpne4* (Rn01445085_m1), *Eml5* (Rn01518272_m1), and *Itsn1* (Rn00570225_m1). The thermal cycling conditions were as follows: 95°C for 10 minutes, followed by 50 cycles of 95°C for 15 seconds and 60°C for 1 minutes. The 18S ribosomal RNA gene was used as an internal control. Each reaction was performed with 2.5 μL of cDNA diluted 50-fold. Presence of expression was examined in each wound. When the gene expressions were confirmed in each wound, gene expression levels were calculated using the ΔΔCt method. The relative expression levels (Log_2 folds) were used for statistical analysis.

2.6 | Western blotting

The protein concentration in each sample was measured using a Pierce BCA Protein Assay Kit (Thermo Fisher Scientific). Ten microliters of tissue homogenates (adjusted to 1 μg/μL) or exudate samples (unadjusted concentration) were loaded onto 4% to 20% polyacrylamide gels. The samples were separated via electrophoresis (200 V, 30-60 minutes) and transferred to a polyvinylidene difluoride membrane using the iBlot Dry Blotting System (Thermo Fisher Scientific). The five candidate markers were identified by immunostaining using a vacuum-driven immunodetection system (SNAP i.d. 2.0, Merck). The membranes were blocked with Blocking One (Nacalai Tesque) for 10 minutes. Primary antibodies against the following proteins were used: NPPB (1:400 dilution, #PA5-96084, Thermo Fisher), ITGB6 (1:200 dilution, #STJ191874, St John's Laboratory Ltd, London, UK), CPNE4 (1:1000 dilution, #CSB-PA850253LA01HU, Cusabio Technology, Houston, Texas), EML5 (1:400 dilution, #LS-C477270, LifeSpan Biosciences, Seattle, Washington), and ITSN1 (1:500 dilution, #LS-C803928, LifeSpan Biosciences). The secondary antibody was peroxidase-conjugated anti-rabbit IgG (1:1000 dilution, Jackson ImmunoResearch, West Grove, Pennsylvania). Immunoreactivity was visualised using a chemiluminescent substrate for peroxidase (Immobilon Forte Western HRP Substrate, Merck) and captured by chemiluminescence image capture (Lumicube, Liponics, Tokyo, Japan). β-actin was used as a loading control, and the expression level of each marker relative to β-actin expression was used for statistical analysis. Qualitative evaluation was conducted when the proteins were detected in only some wounds, and quantitative evaluation was conducted when the proteins were detected in all wounds.

2.7 | Statistical analysis

Data are expressed as the mean ± standard deviation (SD). One-way analysis of variance (ANOVA) with

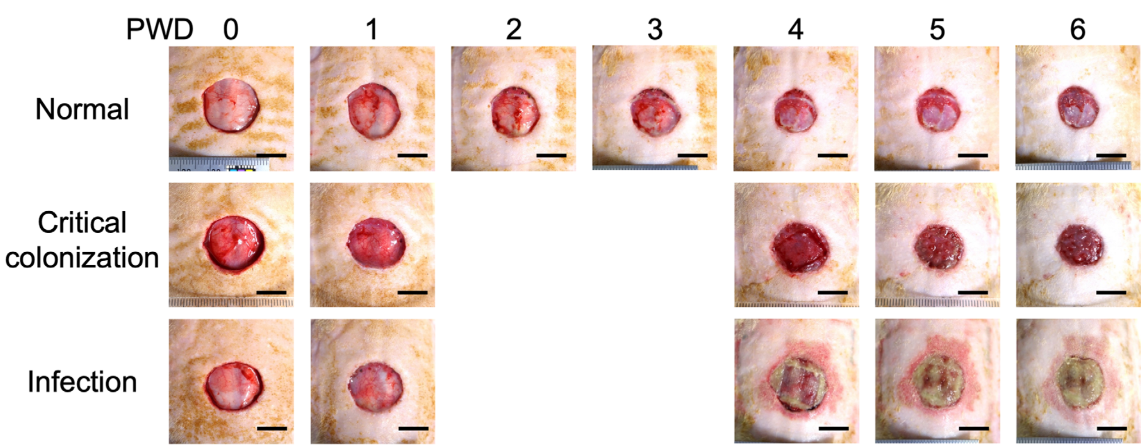

FIGURE 2 Macroscopic findings. N: normal group, CC: critical colonisation group, I: infection group. PWD: post-wounding day. Scale bar = 1 cm

Tukey's post-hoc tests was used to compare the continuous variables, and Fisher's exact test with Bonferroni correction was used for the categorical variables. Statistical significance was set at $P < .05$. All data were analysed with R version 4.0.0 (R Foundation for Statistical Computing, Vienna, Austria).

2.8 | Ethical consideration

The study protocol was approved by the Animal Experimental Committee of the School of Medicine, the University of Tokyo (H20-164), and the experiments were conducted in accordance with the guidelines published by the Japanese Association for Laboratory Animal Science (1987).

3 | RESULTS

3.1 | Animal models of critical colonisation and infection wounds

The macroscopic findings are shown in Figure 2. Granulation tissue was observed in the normal and critical colonisation groups, and there were no inflammatory findings in the surrounding skin in these groups on PWDs 4-6. In the infection group, the wound bed was fully covered with necrotic tissue on PWD 6, and the amount of necrotic tissue increased gradually. Redness was observed in the surrounding tissue on PWD 6. Infection signs were found only in the infection wounds.

On PWD 1, the absolute wound area was 2.75, 2.70, and 2.69 cm^2 in the normal, critical colonisation, and infection groups, respectively, and there were no differences among the groups ($P = .761$). The relative wound areas are shown in Figure 3. The wound areas relative to PWD 1 in the infection group were larger than those in the normal group on PWDs 4 to 6 ($P < .001$, $P = .006$, and $P = .005$, respectively). The relative wound areas in the critical colonisation group were larger than those in the normal group on PWDs 4 and 5 ($P < .001$ and $P = .042$, respectively), but there was no significant difference on PWD 6. No significant differences in relative wound areas were observed between the critical colonisation and infection groups.

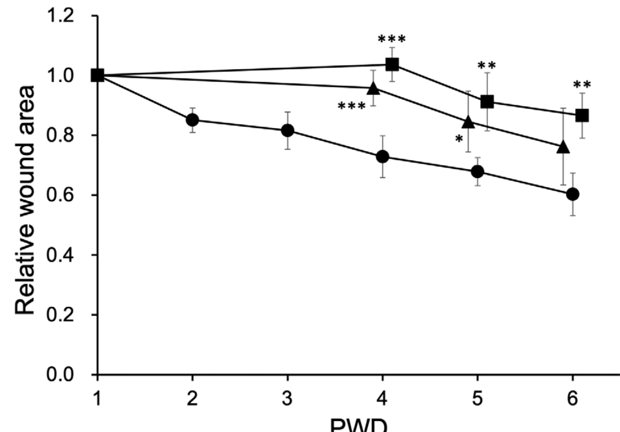

FIGURE 3 Time course changes in wound area. Wound areas relative to PWD 1 were calculated in the normal (circle), critical colonisation (triangle), and infection groups (square). Data are presented as the mean ± SD and were analysed using ANOVA followed by Tukey's post-hoc tests. ***$P < .001$, **$P < .01$, *$P < .05$ vs normal group. PWD: post-wounding day

In the histological analysis, re-epithelialisation was observed in the normal group. Less re-epithelialisation was found in the critical colonisation and infection groups than in the normal group. Necrotising granulation tissue, in which few fibroblasts were observed and collagen fibres were degraded, was found in the deeper granulation tissue layer at the centre of the wound in

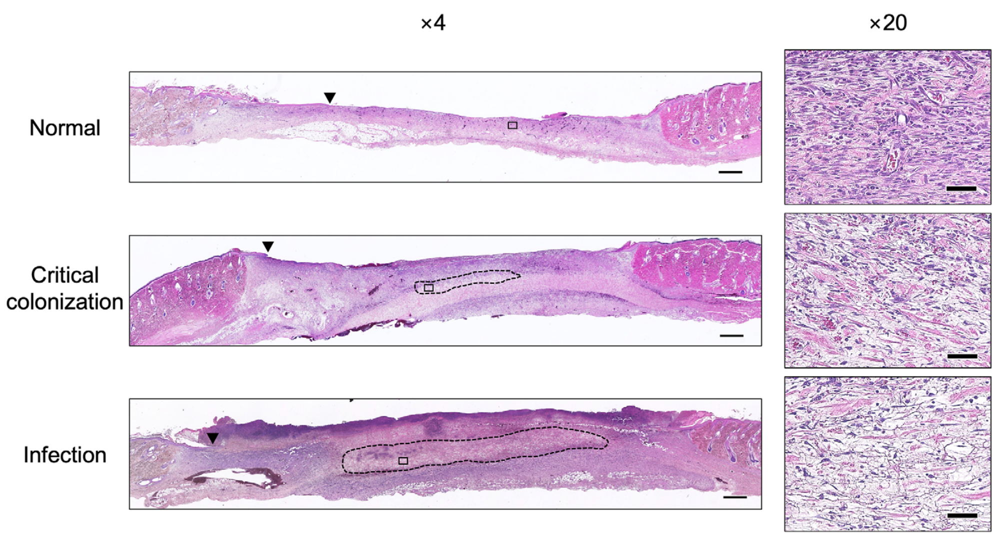

FIGURE 4 Histology of granulation tissue. N: normal group, CC: critical colonisation group, I: infection group. Low magnification images show whole wounds. Black arrowheads point to the tip of re-epithelialising tissue, and the areas encircled by black broken lines indicate the necrotising tissue. High magnification images, which show parts of granulation tissues, are consistent with the areas with the black boxes in the high magnifications. Scale bars for low magnification images = 1 cm and high magnification images = 200 μm

TABLE 1 Detection frequencies of the five candidate markers in the rat wound tissues

		Normal	Critical colonisation	Infection	*P*
NPPB	mRNA	5/5	5/5	5/5	1.000
	protein	5/5	5/5	5/5	1.000
ITGB6	mRNA	4/5	5/5	3/5	.725
	protein	5/5	5/5	5/5	1.000
CPNE4	mRNA	5/5	5/5	5/5	1.000
	protein	5/5	5/5	5/5	1.000
EML5	mRNA	1/5	0/5	1/5	1.000
	protein	5/5	5/5	5/5	1.000
ITSN1	mRNA	5/5	5/5	5/5	1.000
	protein	0/5	4/5	4/5	.021

Note: Data were analysed using Fisher's exact test.

the infection group (Figure 4). Two out of five wounds had necrotising granulation tissue in the shallow granulation tissue layer in the critical colonisation group. In the critical colonisation and infection groups, all wounds showed a decreased density of collagen fibres at the wound edge.

3.2 | mRNA and protein levels of the five candidate markers in rat wound tissues

The mRNA and protein levels of the five candidate markers in rat wound tissues were examined on PWD 6. ITSN1 protein was not detected in the normal group, but it was detected in four out of five wounds in both the critical colonisation and infection groups (P = .021, Table 1). In contrast, *Nppb*, *Cpne4*, and *Itsn1* mRNAs and NPPB, ITGB6, CPNE4, and EML5 proteins were detected in all samples, and their expression levels of mRNAs and proteins were quantitatively analysed (Figures 5 and 6). The relative mRNA expression levels of *Cpne4* were significantly decreased in the critical colonisation and infection groups when compared with the normal group (P = .028 and P = .010, respectively). Similarly, *Itsn1* mRNA expression was significantly lower in the critical colonisation and infection groups than in the normal group (P = .008 and P = .004, respectively). There was no statistically significant difference in any of the protein levels.

3.3 | mRNA and protein levels of the five candidate markers in rat wound exudates

As both mRNAs and proteins of all candidate genes were detected only in some of the exudate samples on

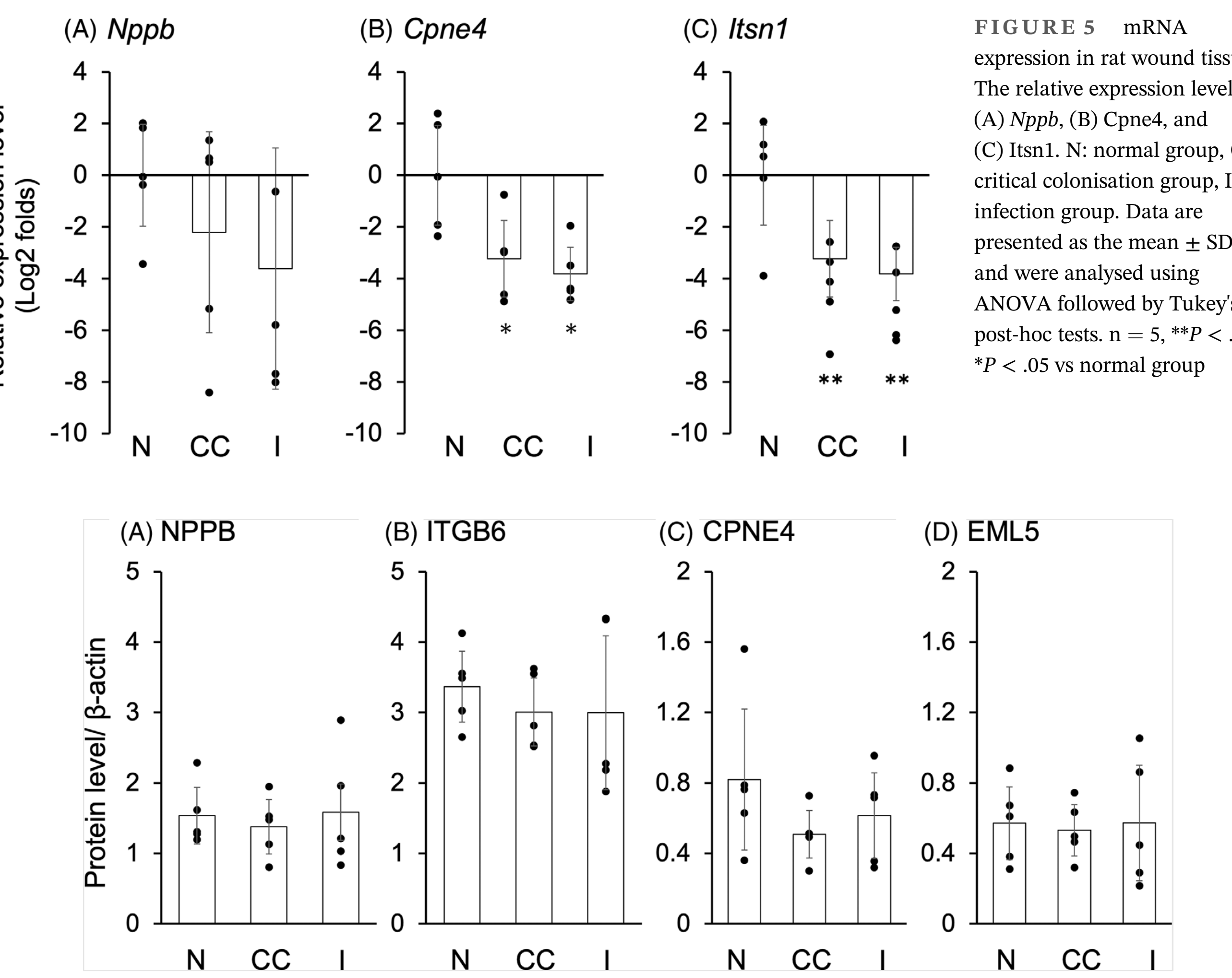

FIGURE 5 mRNA expression in rat wound tissues. The relative expression levels of (A) *Nppb*, (B) Cpne4, and (C) Itsn1. N: normal group, CC: critical colonisation group, I: infection group. Data are presented as the mean ± SD and were analysed using ANOVA followed by Tukey's post-hoc tests. n = 5, **$P < .01$, *$P < .05$ vs normal group

FIGURE 6 Protein detection in rat wound tissues. The protein levels of (A) NPPB, (B) ITGB6, (C) CPNE4, and (D) EML5. N: normal group, CC: critical colonisation group, I: infection group. Data are presented as the mean ± SD and were analysed using ANOVA followed by Tukey's post-hoc tests. n = 5

TABLE 2 Detection frequencies of the five candidate markers in the rat wound exudates

		Normal	Critical colonisation	Infection	*P*
NPPB	mRNA	5/5	5/5	2/5	.067
	protein	4/5	3/5	4/5	1.000
ITGB6	mRNA	4/5	2/5	0/5	.067
	protein	4/5	3/5	4/5	1.000
CPNE4	mRNA	5/5	4/5	0/5	.006
	protein	3/5	3/5	4/5	1.000
EML5	mRNA	0/5	0/5	0/5	1.000
	protein	3/5	2/5	2/5	1.000
ITSN1	mRNA	5/5	5/5	3/5	.286
	protein	4/5	5/5	3/5	.725

Note: Data were analysed using Fisher's exact test.

PWD 6, they were qualitatively analysed (Table 2). Exudates from the infected wounds were completely negative for *Cpne4*, whereas *Cpne4* was detected in four out of five critically colonised wound exudate samples and all the normal exudate samples ($P = .006$). The mRNA expression frequencies of *Nppb* and *Itgb6*

tended to differ among the groups (both $P = .067$). *Nppb* mRNA expression tended to decrease only in the infection group, and *Itgb6* mRNA expression tended to decrease in both the critical colonisation and infection groups.

4 | DISCUSSION

This study identified *Itgb6* mRNA as a potential biomarker in fresh exudates to distinguish critically colonised and infected wounds from normal healing wounds. The detection frequencies of *Cpne4* and *Nppb* were lower in exudates of infected wounds than in those of normal and critically colonised wounds.

The three wound models were normal healing, critical colonisation, and infection. Delayed healing was observed in critically colonised wounds without any signs of infection. Histological analysis revealed the presence of necrotising granulation tissue, which was a common feature of the infected wounds, but was also found in some critically colonised wounds, suggesting the internal validity of the critical colonisation model. Kitamura et al prepared control wounds using the same method as that used in the critically colonised group in this study; however, they did not find delayed healing in these wounds because they did not compare the wounds to complete control wounds without gauze application. Our novel findings indicate the possibility of creating a critically colonised wound model without bacterial application. Because the wounds were sealed with nutrients for the bacteria for 72 hours, commensal skin bacteria may have proliferated on the wound surface and their burden may have been outweighed by the host's immunity in the critical colonisation group. The distribution of bacteria and biofilms in the critically colonised wounds remains to be investigated.

Itgb6 mRNA expression was inhibited in the critical colonisation and infection groups. ITGB6 is an adhesion receptor for keratinocyte adhesion to the epidermal basement membrane during re-epithelialisation.[22] In the critically colonised and infected wounds, re-epithelialisation was not advanced compared with the normal healing wounds. Bacterial endotoxins can stimulate the expression and activation of matrix metalloproteinases,[23] which play a pathogenic role by interfering with basement membrane regeneration.[24,25] Under such conditions, excess ITGB6 that cannot bind to the basement membrane may inhibit *Itgb6* mRNA expression in keratinocytes. Therefore, the suppression of *Itgb6* mRNA expression in fresh wound exudates could reflect the epithelialisation suppression induced by critical colonisation and infection.

Nppb mRNA expression was detected in exudates of all wounds, except infected wounds. NPPB is cleaved into brain natriuretic peptide (BNP), which acts as a cardiac hormone. There are few reports on BNP and NPPB protein expression in wounds.[26,27] Enhanced *Nppb* mRNA expression has been observed in the epidermis at the wound edge of surgical incisions.[27] BNP regulates angiogenesis via guanylyl cyclase-A in ischemic muscles and the heart.[28] Therefore, *Nppb* mRNA expression may be associated with disrupted re-epithelialisation and angiogenesis in wound infection.

Cpne4, which encodes a calcium-dependent phospholipid-binding protein (CPNE4), has not been investigated in the field of wound healing. To the best of our knowledge, this is the first study to show the suppression of *Cpne4* mRNA expression in infected wounds. Our results suggest that *Cpne4* mRNA expression in wound tissues is associated with critical colonisation and infection, and that *Cpne4* mRNA expression in exudates is only associated with infection. Further studies are required to clarify the role of *Cpne4* in critical colonisation and infection of wounds.

Asada et al reported increased mRNA expression of the five candidate markers evaluated in this study in critically colonised and infected wounds[19]; however, these results are in contrast with the results of this study. There are at least two possible reasons for these discrepant results. The first is a difference in the inoculation method. In the study by Asada et al, a bacterial solution was injected into the granulation tissue of the wound in critical colonisation and infection model rats, whereas in this study, according to the method of Kitamura et al, bacteria were applied on the wound surface, which is recognised to mimic the natural route of bacterial invasion.[20] Critical colonisation is widely recognised as a superficial bacterial wound infection. The second reason may be a difference in the sampling method. In their animal study, Asada et al collected pooled exudates from below the film dressing for 24 hours, and in their clinical study, exudates were absorbed by a gauze dressing that covered the wound for 24 hours. In other words, they incubated the exudates outside the body for up to 24 hours, whereas in this study, fresh exudates were collected by swabbing. It is known that gene expression is altered significantly during primary culture of mammalian cells.[29] The results of Asada et al suggest that exudates of critically colonised and infected wounds include factors that enhance the expression levels of the five target genes during incubation outside the body, which may also be potential markers for identifying critical colonisation.

DESIGN-R® is the global standard tool for PI assessment, and it measures seven items: depth, exudates, size, inflammation/infection, granulation, necrosis, and undermining.[30] In

the recent revision, DESIGN-R®2020, critical colonisation was added to the category of inflammation/infection.[31] The identification of critical colonisation is important for expert nurses and general nurses. *Itgb6* is considered an applicable biomarker for critical colonisation. By monitoring *Itgb6* mRNA expression in exudates, critical colonisation may be identified based on decreased *Itgb6* mRNA expression. Furthermore, high *Itgb6* mRNA expression in hard-to-heal wounds may indicate the presence of wound healing-inhibiting factors other than bacteria. Technological breakthroughs are expected to develop a simple and rapid examination for mRNAs that can enable point-of-care testing of critical colonisation to improve selection of appropriate treatments for critically colonised wounds and shorten the healing time of PIs.

This study had some limitations. Histological analysis showed heterogeneity in wound status, especially the distribution of necrotising tissue, in the critical colonisation group. The large variation in wound status may have concealed actual differences among the groups. It is not clear whether the results of this animal study can be applied to clinical settings. Further clinical studies are required for generalisation of the present findings.

5 | CONCLUSION

In this study, three wound models of normal healing, critical colonisation, and infection were created in rats. In the critical colonisation and infection groups, *Itgb6* mRNA expression tended to decrease in fresh swab-collected exudates. This result suggests that *Itgb6* in fresh exudates is a promising biomarker for critical colonisation.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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