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Effectiveness of quality and quantity mononuclear cells for enhancing wound healing in diabetic ischemic limb animal model

Pongpol Sriphan[1] | **Nuttapol Chruewkamlow**[2] | **Chutipon Sathan-ard**[2] | **Phakawan Phutthakunphithak**[2] | **Somponnat Sampattavanich**[3] | **Tauangtham Anekpuritanang**[3,4] | **Romgase Sakamula**[3] | **Thanaphon Likhityungyuen**[3] | **Chumpol Wongwanit**[1] | **Chanean Ruangsetakit**[1] | **Nuttawut Sermsathanasawadi**[1]

[1]Division of Vascular Surgery, Department of Surgery, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand

[2]Research Department, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand

[3]Department of Pharmacology, Faculty of Medicine Siriraj Hospital, Siriraj Center of Research Excellence for Systems Pharmacology, Mahidol University, Bangkok, Thailand

[4]Department of Pathology, Faculty of Medicine, Siriraj Hospital, Mahidol University, Bangkok, Thailand

Correspondence
Nuttawut Sermsathanasawadi, Division of Vascular Surgery, Department of Surgery, Faculty of Medicine Siriraj Hospital, Mahidol University, 2 Wanglang Road, Bangkoknoi, Bangkok 10700, Thailand.
Email: nuttawut.ser@mahidol.ac.th

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Abstract

This study set forth to investigate the efficacy of Quality and Quantity mononuclear cells (QQMNCs) for promoting wound healing and limb salvage in a severe ischemic wound model using diabetic mice. Female BALB/c nude mice induced with diabetes were used to create ischemic limb models in a controlled experimental design. Intramuscular injections of human QQMNCs were compared to phosphate-buffered saline (PBS) and peripheral blood mononuclear cells (PBMNCs) relative to their effects on wound healing and limb salvage. In vitro analysis demonstrated that the QQMNC group had significantly higher median percentages of CD34+ cells, CD34+CD133+ cells, CD206+ cells, and FOXP3+ cells compared to the PBMNC group (all $p < 0.05$), which suggests an enhanced regenerative and immunomodulatory profile. Kaplan–Meier survival analysis showed a significantly higher number of completely healed wounds in the QQMNC group than in the PBMNC group ($p = 0.044$). The histological evaluation showed that the QQMNC group had a significantly thinner epithelial thickness than the PBMNC ($p = 0.032$) and PBS groups ($p = 0.002$), and a significantly greater T cell density than the PBS group ($p = 0.033$), which suggests more efficient tissue repair. Moreover, the QQMNC group exhibited the highest percentage of minor tissue loss (57% for forefoot and toe gangrene), and the lowest incidence of severe limb loss (0% for lower leg gangrene). The findings of this study highlight the effectiveness of QQMNCs for promoting wound healing and limb salvage in diabetic ischemic animal model; however, clinical trials are needed to further assess their efficacy in this clinical context.

KEYWORDS

diabetic ischemic limb animal model, effectiveness, quality and quantity mononuclear cells, wound healing

Key messages

- QQMNCs significantly enhance wound healing and reduce severe limb loss in diabetic ischemic models, outperforming PBMNCs.
- The study found that QQMNCs have a higher percentage of regenerative cells and better tissue repair capabilities, leading to thinner epithelial layers and more organized tissue structure.
- These findings suggest that QQMNCs could be a valuable therapeutic option for patients with chronic limb-threatening ischemia, particularly those who are not candidates for revascularization.

1 | INTRODUCTION

Chronic limb-threatening ischemia (CLTI), which is a severe clinical syndrome that is characterized by pain at rest, chronic non-healing ulcers, and/or gangrene, significantly increases the risk of impaired wound healing and amputation.[1] Diabetes mellitus (DM) is one of the most important risk factors for major amputation in CLTI patients.[2,3] Revascularization and appropriate wound care significantly reduce the overall amputation rate.[4–7] Approximately 20% of CLTI patients for whom effective revascularization is not feasible are classified as no-option CLTI (NOCLTI) patients.[8] In diabetic patients with NOCLTI, alternative treatments, including cell-based therapies and hyperbaric oxygen therapies, have shown potential for improving clinical outcomes.[9] Among these therapies, intramuscular transplantation of bone marrow or peripheral blood mononuclear cells (PBMNCs) has been proven safe and effective for wound healing, but has not consistently decreased amputation rates.[10–14] Quality and Quantity mononuclear cells (QQMNCs), which are mononuclear cells (MNCs) that are cultured in Quality and Quantity (QQ) media (which consists of various cytokines and growth factors) have shown convincing potential for enhancing angiogenesis and reducing inflammation compared to that of PBMNCs.[15–20] The aim of this study was to investigate the efficacy of QQMNCs for promoting wound healing and limb salvage in a severe ischemic wound model using diabetic mice.[21]

2 | MATERIALS AND METHODS

2.1 | Study design and ethical approval

This prospective experimental study was conducted at the Division of Vascular Surgery, Department of Surgery, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand during January 2023 to March 2024. Patients diagnosed with diabetes and chronic limb-threatening ischemia (CLTI) caused by atherosclerosis were recruited from our centre's CLTI clinic. Diagnosis of CLTI required established peripheral artery disease (PAD) and ischemic rest pain, gangrene at any part of the foot, or a non-healing ulcer present for at least 2 weeks. Additionally, at least one of the following hemodynamic criteria was required: ankle-brachial index (ABI) <0.6, absolute ankle pressure (AP) <70 millimetres of mercury (mmHg), absolute toe pressure (TP) <40 mmHg or transcutaneous pressure of oxygen ($TcPO_2$) <40 mmHg. The exclusion criteria included patients who had received a blood transfusion within the preceding 2 weeks, known cases of human immunodeficiency virus (HIV) and individuals with a diagnosis of malignancy.

2.2 | Preparation and characterization of mononuclear cells (MNCs)

2.2.1 | PBMNC and QQMNC preparation

Following established protocols, PBMNCs were isolated from 15 millilitres (mL) of peripheral venous blood from study patients. Half of the MNCs were cultured QQ media for 7 days in a 5% carbon dioxide (CO_2) 37 degrees Celsius (°C) environment to generate the QQMNCs used in this study.[19]

2.2.2 | Characterization

The PBMNCs and QQMNCs were compared in quantities of progenitor cells (CD34+CD133+), M2 macrophages (CD206+) and regulatory T cells (FoxP3) by flow cytometry according to established protocols.[16,20] A colony-

forming assay (CFA) was also performed as previously described.[19]

2.3 | Animal model

2.3.1 | Induction of diabetes

A total of 17 female BALB/c nude mice (Nomura Siam International, Bangkok, Thailand) aged 8 to 10 weeks were used for this experiment. Diabetes mellitus (DM) was induced via a single intraperitoneal injection of 200 mg/kg of streptozotocin (STZ) (Sigma-Aldrich Corporation, St. Louis, MO, USA).[22] At 10 days after injection, the presence of DM was confirmed by fasting blood glucose levels exceeding 150 mg/dL.[23] All mice maintained a fasting blood glucose level above 150 mg/dL for at least 4 weeks.[18] Mice were housed under controlled conditions (24°C ± 1°C, 55% ± 5% humidity, and a 12-h light/dark cycle). These protocols, which are approved by the Mahidol University Animal Center (COA no. 001/2023), ensured reproducibility, accuracy and ethical compliance.

2.3.2 | Ischemic limb model and wound creation

Study mice were anaesthetised via intraperitoneal injection of 80–100 mg/kg ketamine and 10 mg/kg xylazine.[24] A small incision was made over the common femoral artery, which was then isolated and ligated using a 10–0 non-absorbable monofilament suture. Successful ligation was confirmed by cessation of pulse distal to the ligation site and groin-to-toe blood flow measurement by laser Doppler perfusion imaging (LDPI) (moorLDI research software; Moor Instruments, Devon, United Kingdom).[19] The skin incision was closed with sutures, and antiseptic or antibiotic ointment was applied. After ligating the femoral artery, a 6 mm punch biopsy created a full-thickness excision extending from the skin to the premuscular fascia on the ischemic left hindlimb. Postoperative care included placing the mice in a warm and clean recovery area, monitoring for distress or complications and providing appropriate pain relief and care to ensure their comfort and well-being.

2.3.3 | MNC injection

One day after the operation, the wound inflicted to each study mouse received an intramuscular injection of 10 μL of phosphate-buffered saline (PBS), 1×10^4 cells/10 μL of PBMNCs, or 1×10^4 cells/10 μL of QQMNCs that was injected beneath the wound. Additionally, intramuscular injections of 50 μL of PBS, 5×10^4 cells/50 μL of PBMNCs, or 5×10^4 cells/50 μL of QQMNCs were administered into four muscle groups (i.e., gastrocnemius, tibialis anterior, semimembranosus and rectus femoris) that were identified by the bony landmarks of the hip, knee and calcaneus.[16,18]

2.4 | Wound healing assessment

2.4.1 | Wound healing progression

Following surgery, wounds and affected limbs were evaluated on days 0, 3, 6, 13, 20 and 30. The wounds were photographed and digitally measured using ImageJ software (National Institutes of Health [NIH], Bethesda, Maryland, USA, and the Laboratory for Optical and Computational Instrumentation [LOCI], University of Wisconsin, Madison, WI, USA). The percentage of wound closure was measured on days 0, 3, 6, 13, 20 and 30 by photogrammetric analysis using the following formula: Wound closure percentage = [1−(area of the wound on examination day/area of the wound on day 0)] × 100. Complete wound healing was defined as complete scab off and full epithelialization with no ulceration. Levels of limb loss were classified as no gangrene, minor tissue loss (toes and forefoot gangrene), major tissue loss (midfoot to total foot gangrene) and severe limb loss (total leg gangrene). The results were compared among and between the PBS, PBMNC and QQMNC groups.

2.4.2 | Histologic analysis of the wound

On day 30, the mice were euthanized, and a full-thickness excision extending 3 mm beyond the origin of wound edge was performed.[18] Harvested samples were dissected and paraffin embedded. Four μm thick sections were prepared and processed for haematoxylin and eosin (H&E) staining to measure epithelial thickness and granulation tissue area. Wound maturity was assessed via H&E staining and analysis of digitalized images using SlideViewer version 2.7 (3DHistech Kft., Budapest, Hungary). Immunologic response in the wound was assessed by immunofluorescence staining of sections with CD3+, CD68+ and CD45+ markers, which indicated T cell presence, macrophage density and overall immune cell infiltration, respectively. These 3 analyses used the PhenoImager Fusion (Akoya Biosciences, Marlborough, Massachusetts, USA) for alignment, segmentation and

classification with the QuPath program (version 0.5.1; The University of Edinburgh, United Kingdom).

2.5 | Vascular regeneration assessment

2.5.1 | Laser Doppler perfusion imaging (LDPI)

LDPI was used to sequentially assess blood flow in diabetic mice with ischemic limbs. Perfusion measurements were evaluated with LDPI from the heel to the toes before and immediately after arterial ligation, and on days 0, 3, 6, 13, 20 and 30 postoperation. The foot perfusion ratio was calculated by comparing perfusion in the ischemic limb to perfusion in the normal contralateral limb. The foot perfusion ratio results were compared between and among the PBS, PBMNC and QQMNC groups.

2.5.2 | Histologic analysis of mouse muscle

Vascular regeneration was detected via immunofluorescence staining of muscular paraffin sections with CD31+ antibody. This analysis used the PhenoImager Fusion (Akoya Biosciences, Marlborough, Massachusetts, USA) for alignment, segmentation, and classification with the QuPath program (version 0.5.1; The University of Edinburgh, United Kingdom).

2.6 | Sample size calculation and statistical analysis

The primary outcome of this study was the percentage of wound closure. To calculate the sample size, we used data specific to the vasculogenic and wound healing effects of QQMNC therapy in diabetic mice from the study by Tanaka, et al. They reported the percentage of wound closure at 14 days to be approximately 80% in the QQMNC group and 50% in the PBMNC group, which resulted in a margin difference of 30% between the 2 groups. Using a standard deviation of 15%, an alpha error of 0.05, and a beta error of 0.20, we determined that 5 mice per group were required for the present study. The sample size was increased to 6–7 mice per group to ensure adequate statistical power, despite any losses to preserve the validity of the comparison between the QQMNC and PBMNC groups.[18] All data analyses were performed using PASW Statistics software (version 26.0; SPSS, Inc., Chicago, IL, USA). Normally and non-normally distributed data are reported as mean ± standard deviation (SD) and median and range (maximum [max], minimum [min]), respectively. The Wilcoxon signed-rank test (paired nonparametric test) was used to compare the results of in vitro studies between the PBMNC and QQMNC groups. Kaplan–Meier survival analysis and repeated measures analysis of variance (ANOVA) were used to assess wound healing and tissue perfusion. Histopathological data were analysed using one-way ANOVA followed by Tukey's HSD post hoc test or Pearson's correlation analysis. A p-value of <0.05 was considered statistically significant for all tests.

3 | RESULTS

3.1 | CLTI patient characteristics

This study enrolled 9 human CLTI patients with a mean age of 69.22 ± 11.37 years, and 7 (78%) were male. All patients had DM and hypertension (HT), and all met the criteria for CLTI diagnosis. Clinically, gangrene was observed in 6 (67%) patients, and non-healing ulcers were observed in 3 (33%) patients.

3.2 | In vitro analysis

Flow cytometry analysis revealed that the QQMNC group had significantly higher percentages of CD34+ cells, CD34+CD133+ cells and CD206+ cells when compared to the PBMNC group, which indicates enhanced angiogenic and anti-inflammatory capabilities. The percentage of FOXP3+ regulatory T cells was also significantly higher in the QQMNC group than in the PBMNC group, which indicates better immune regulation. The QQMNC group also demonstrated a higher colony-forming unit (CFU) count than the PBMNC group, which suggests more colony-forming potential. These results are summarized in Table 1.

3.3 | In vivo wound healing

3.3.1 | Wound healing progression

The mean wound closure percentage on day 13 in the PBS, PBMNC, and QQMNC groups was 47.31% ± 31.11%, 38.86% ± 16.77%, and 59.52% ± 17.46%, respectively ($p > 0.05$ between and among groups). Kaplan–Meier survival analysis showed a significantly higher number of healed wounds in the QQMNC group than in the PBMNC group ($p = 0.044$) (Figure 1). The epithelial thickness was 112.88 ± 50.78 μm, 90.54 ± 18.18 μm and

TABLE 1 Outcomes of in vitro study compared between the PBMNC and QQMNC groups.

Evaluated parameters	PBMNC group [median (min–max)]	QQMNC group [median (min–max)]	*p-value*
CD34+ cells (%)	0.2 (0–0.40)	12.9 (7.23–39.00)	0.018
CD34 + CD133+ cells (%)	0.0 (0–0.09)	1.9 (0.18–9.00)	0.018
CD206+ cells (%)	0.9 (0.2–7.0)	42.0 (3.9–56.8)	0.018
CFU count (2×10^5 cells/dish)	1 (0–1)	3 (2–9)	0.102
FOXP3+ cells (%)	0.6 (0–1.8)	1.6 (1.1–3.6)	0.018

Note: A *p*-value <0.05 indicates statistical significance.
Abbreviations: CD, cluster of differentiation; CFA, colony-forming assay; CFU, colony-forming unit; FOXP3, forkhead box P3; PBMNC, peripheral blood mononuclear cell; QQMNC, quality and quantity mononuclear cell; %, percent.

34.68 ± 9.01 μm in the PBS group, the PBMNC and the QQMNC group, respectively (Figure 2A). The QQMNC group had significantly less epithelial thickness compared to both the PBMNC group ($p = 0.032$) and the PBS group ($p = 0.002$) (Figure 2). The QQMNC group also demonstrated a more well-organized tissue structure, including thinner epithelium and less granulation tissue than that observed in the PBMNC and PBS groups, which suggests enhanced tissue organization and improved wound healing[25–34] (Figure 2A). These findings suggest that QQMNC treatment reduces epithelial thickness and promotes better tissue maturation, which effectuated improved wound healing outcomes in diabetic ischemic mice.

3.3.2 | Immunofluorescence analysis

T cell density was 159.20 ± 54.87 cells/mm^2, 307.00 ± 40.84 cells/mm^2 and 414.00 ± 218.85 cells/mm^2 in the PBS group, the PBMNC group and the QQMNC group (Figure 3A, Table 2). The QQMNC group had a significantly higher T cell density than the PBS group ($p = 0.033$) (Figure 3G). Although the QQMNC group had lower macrophage densities than the PBMNC and PBS groups, the difference in both of those comparisons was not statistically significant (Figure 3H).

3.4 | Vascular regeneration

3.4.1 | LDPI and tissue salvage

The QQMNC group showed a trend towards improved blood flow and the highest rate of minor tissue loss. Over half (57%) of QQMNC mice had forefoot and toe gangrene; however, the QQMNC group had the lowest rate of severe limb loss with 0% of cases progressing to total leg gangrene compared to the PBMNC and PBS groups (both comparisons $p > 0.05$) (Figure 4).

3.4.2 | Intramuscular vessel density in the ischemic limb

Histologic assessment of vascular density in the intramuscular area that was injected, as indicated by CD31+ staining, demonstrated a trend towards increased blood vessel density in the QQMNC group (Figure 5); however, this increase did not reach statistical significance.

4 | DISCUSSION

Our data demonstrate significantly higher percentages of CD34+, CD34+CD133+ and CD206+ cells in the QQMNC group, which indicates a more robust presence of progenitor cells and anti-inflammatory M2 macrophages compared to the PBMNC group. The QQMNC group also showed significantly more FOXP3+ regulatory T cells, which is crucial for immune regulation.[35] These findings are consistent with the work of Masuda et al. and Tanaka et al., who demonstrated the capacity of QQMNCs to enhance angiogenesis and reduce inflammation via in vitro conditioning.[16,18]

The QQMNC group exhibited more rapid wound closure than the PBMNC group, underscoring the superior ability of QQMNCs to promote wound healing in diabetic ischemic animal model. Histopathologic analyses revealed that wounds treated with QQMNCs had thinner epithelial layers and smaller areas of granulation compared to those treated with PBMNCs. As a result, QQMNCs led to more mature dermal tissue and better-organized tissue regeneration than that observed in PBMNC mice.[25–34] Our in vivo results showed that the PBMNC group experienced more pronounced inflammation than the QQMNC group. Consistent with that observation, previous studies reported elevated levels of inflammation to be associated with delayed wound healing, which may explain the delayed wound healing observed in our PBMNC group due to the inflammatory process.[36,37] Further investigation is warranted to

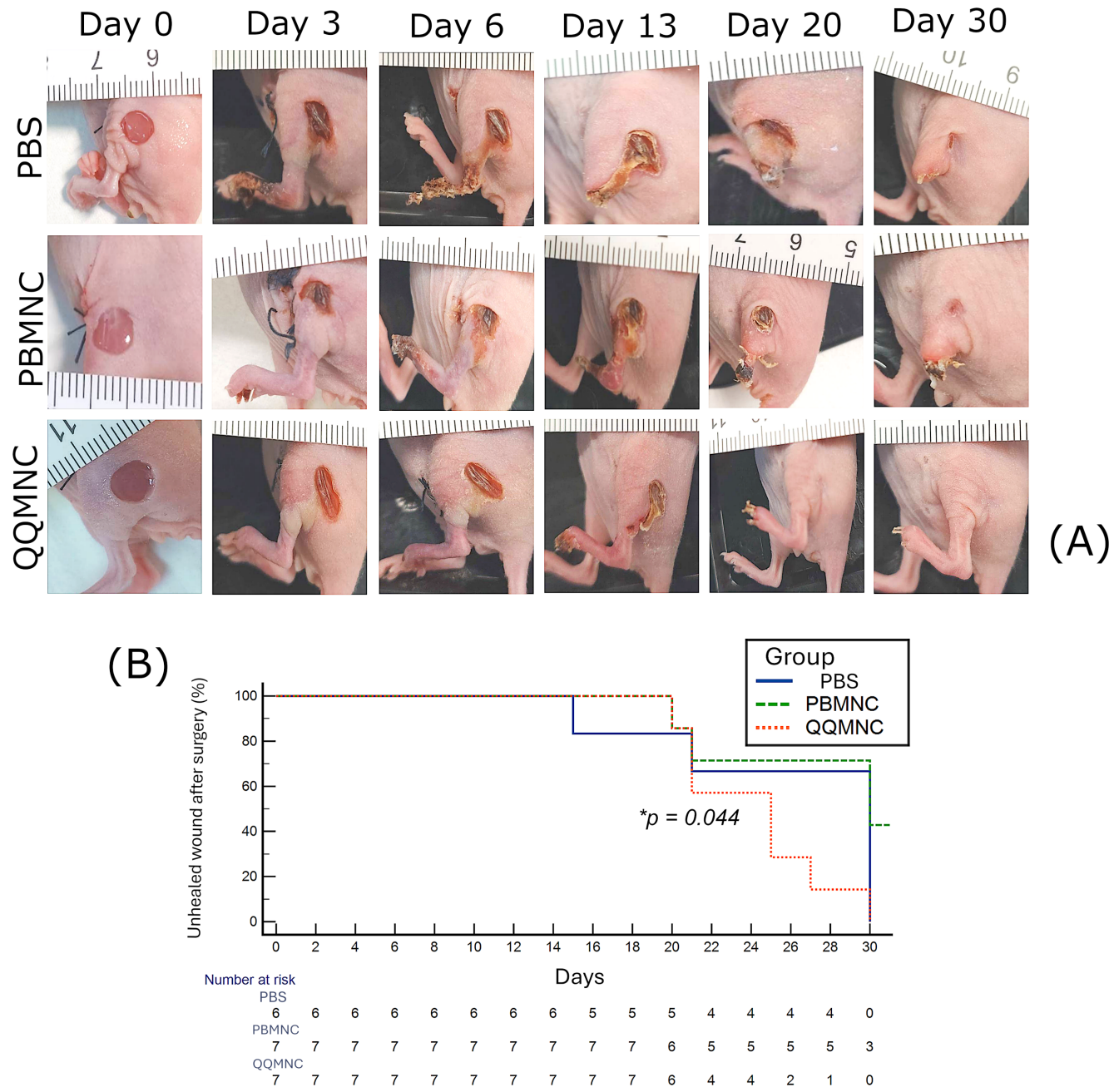

FIGURE 1 (A) Representative images of wound healing in diabetic ischemic mice in the PBS, PBMNC and QQMNC groups captured on day 0, day 3, day 6, day 13, day 20 and day 30. The images illustrate the progression of wound healing with the QQMNC ($n = 7$) group showing accelerated healing compared to the PBS ($n = 6$) and PBMNC ($n = 7$) groups. (B) Kaplan–Meier survival analysis showing the probability of complete wound healing over time among the three groups. The QQMNC group demonstrated a significantly higher complete healing rate ($p = 0.044$) compared to the PBMNC group. The number-at-risk table below the curve shows the remaining subjects in each group at each time point. *<0.05. PBMNC, peripheral blood mononuclear cell; PBS, phosphate-buffered saline; QQMNC, quality and quantity mononuclear cell.

fully elucidate the mechanisms underlying these findings and to determine the potential therapeutic implications of QQMNCs for modulating inflammation and promoting wound healing. Moreover, immunofluorescence staining revealed a higher density of CD3+ T cells in the QQMNC group, which suggests that CD3+ T cells may play a role in wound healing in athymic mice, which is a hypothesis that should be further explored.

Our analysis of tissue loss levels demonstrated that the QQMNC group had less tissue necrosis than the PBMNC and PBS groups. QQMNCs and PBMNCs enhanced blood flow to the ischemic limb, as evidenced

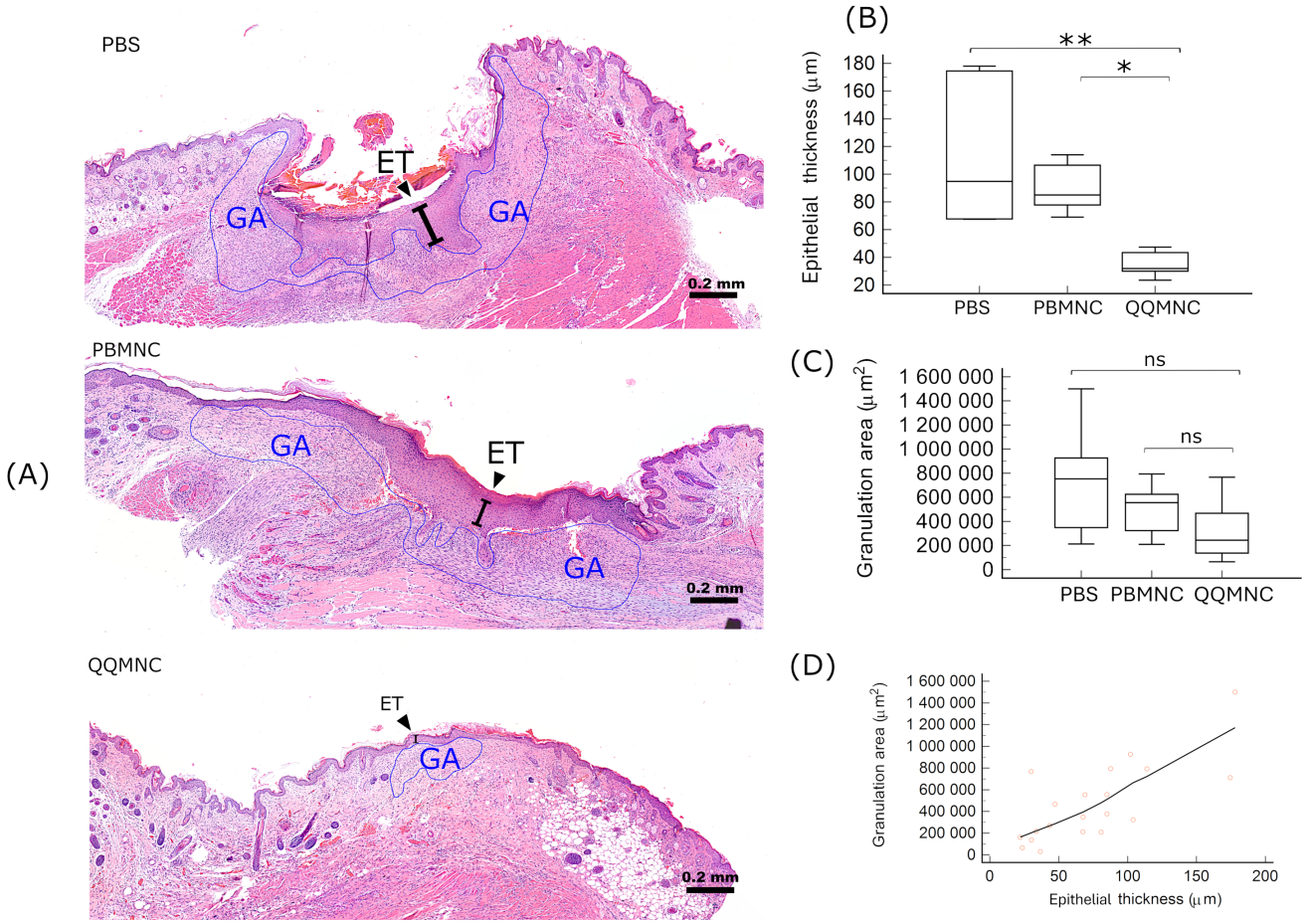

FIGURE 2 (A) Representative histological images of wound tissue sections from the PBS, PBMNC and QQMNC groups stained with H&E, which highlight the epithelial thickness (ET) and granulation area (GA). The QQMNC group shows a more well-organized tissue structure, including thinner epithelium and less granulation tissue compared to the PBMNC and PBS groups. (B) Box plot showing the epithelial thickness compared among the PBS ($n = 6$), PBMNC ($n = 5$), and QQMNC ($n = 6$) groups. The QQMNC group had a significantly thinner epithelium than the PBS and PBMNC groups. Results shown as mean plus/minus standard deviation. (C) Box plot demonstrating the granulation area compared among the PBS ($n = 6$), PBMNC ($n = 6$), and QQMNC ($n = 6$) groups, which showed no significant difference for any comparison. Results shown as mean plus/minus standard deviation. (D) Scatter plot illustrating the positive correlation between epithelial thickness and granulation area ($r = 0.737$, $p = 0.0002$). 'ns' denotes no significant difference. *$p < 0.05$; **$p < 0.01$. PBMNC, peripheral blood mononuclear cell; PBS, phosphate-buffered saline; QQMNC, quality and quantity mononuclear cell.

by laser Doppler imaging. Notably, the QQMNC group exhibited a trend towards greater improvement in blood flow compared to the PBMNC group, which is further supported by the increased intramuscular vessel density in the QQMNC group. These findings align with previous research, which serves to strengthen the evolving belief that QQMNCs improve blood flow more effectively than PBMNCs.[16,19]

Previous studies by Masuda et al. and Tanaka et al. identified key mechanisms through which QQMNCs exert their therapeutic effects. QQMNCs promote angiogenesis by secreting pro-angiogenic factors, such as vascular endothelial growth factor B and angiopoietin-1, which stimulate endothelial cell proliferation and the organization of new blood vessels, which improves tissue perfusion in ischemic conditions.[16] Additionally, QQMNCs modulate the immune system by decreasing proinflammatory M1 phenotype macrophages and increasing anti-inflammatory M2 phenotype macrophages, which reduces inflammation and promotes tissue repair.[18] This immune modulation enhances the regenerative environment by controlling chronic inflammation. Moreover, QQMNCs facilitate the expansion of endothelial progenitor cells (EPCs), which play an essential role in vascular regeneration and tissue repair. The Phase 1/2a clinical trial showed that QQMNCs significantly improve wound healing in diabetic patients, which is likely due to their combined favourable effects on angiogenesis and immune modulation. Together, these mechanisms highlight the potential of QQMNCs' to treat CLTI

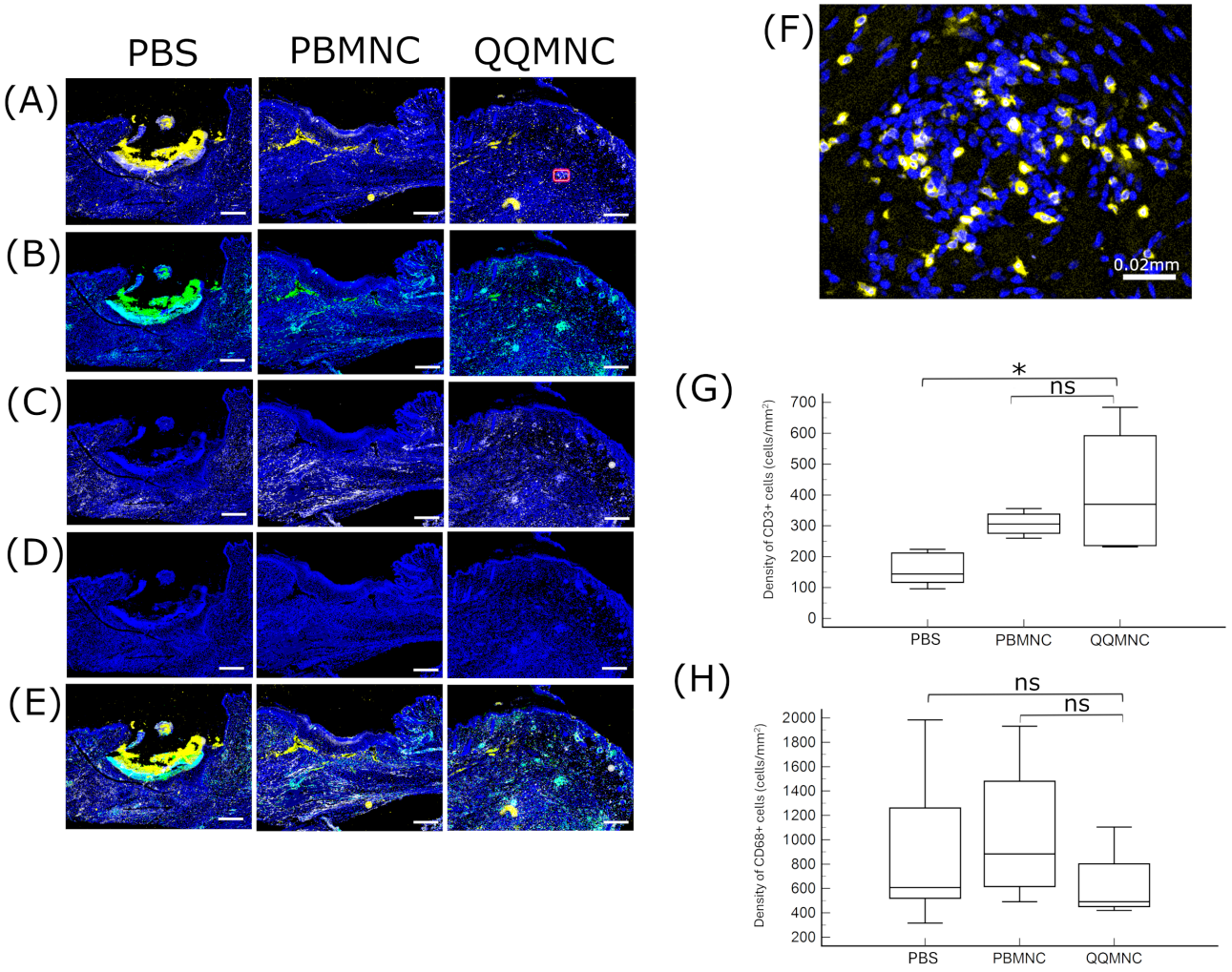

FIGURE 3 (A) Immunofluorescence staining of CD3+ cells with DAPI. (B) Immunofluorescence staining of CD45+ cells with DAPI. (C) Immunofluorescence staining of CD68+ cells with DAPI. (D) DAPI staining of cell nuclei. (E) Merged image of all markers (A-D). (Scale bar: 250 μm) (F) Magnified view of CD3+ cells in the red rectangle shown in the QQMNC image in row (A) (scale bar: 20 μm). (G) Box plot showing the density of CD3+ T cells (cells/mm^2) in the PBS (POI = 5), PBMNC (POI = 4), and QQMNC (POI = 4) groups. The QQMNC group showed non-significantly higher T cell density than the PBMNC group, but significantly higher than the PBS group. Results shown as mean plus/minus standard deviation. (H) Box plot showing the density of CD68+ macrophages (cells/mm^2) among the three groups. The PBS and PBMNC groups both showed non-significantly higher macrophage densities compared to the QQMNC group. Results shown as mean plus/minus standard deviation. 'ns' denotes no significant difference, determined through one-way ANOVA followed by Tukey's HSD post hoc test. *$p < 0.05$. CD, cluster of differentiation; PBMNC, peripheral blood mononuclear cell; PBS, phosphate-buffered saline; POI, point of interest; QQMNC, quality and quantity mononuclear cell.

TABLE 2 Outcomes of histologic and immunofluorescent analysis of diabetic ischemic limb mouse model at day 30.

Evaluated parameters	PBS group (mean ± SD)	PBMNC group (mean ± SD)	QQMNC group (mean ± SD)	*p*
Wound				
Epithelium thickness (μm)	112.88 ± 50.78	90.54 ± 18.18	34.68 ± 9.01	0.003
Granulation area (μm^2)	749 223.00 ± 457 361.03	487 930.80 ± 227 322.66	320 986.67 ± 258 027.01	0.108
Density CD3+ cells (cells/mm^2)	159.20 ± 54.87	307.00 ± 40.84	414.00 ± 218.85	0.039
Density CD68+ cells (cells/mm^2)	903.20 ± 654.42	1048.00 ± 628.72	627.00 ± 319 0.87	0.581
Intramuscular				
Density CD31+ cells (cells/mm^2)	447.02 ± 254.74	559.87 ± 442.85	626.27 ± 114.37	0.708

Abbreviations: CD, cluster of differentiation; mm^2, millimetre squared; PBMNC, peripheral blood mononuclear cell; PBS, phosphate-buffered saline; QQMNC, quality and quantity mononuclear cell; μm, micrometre; μm^2, micrometre squared.

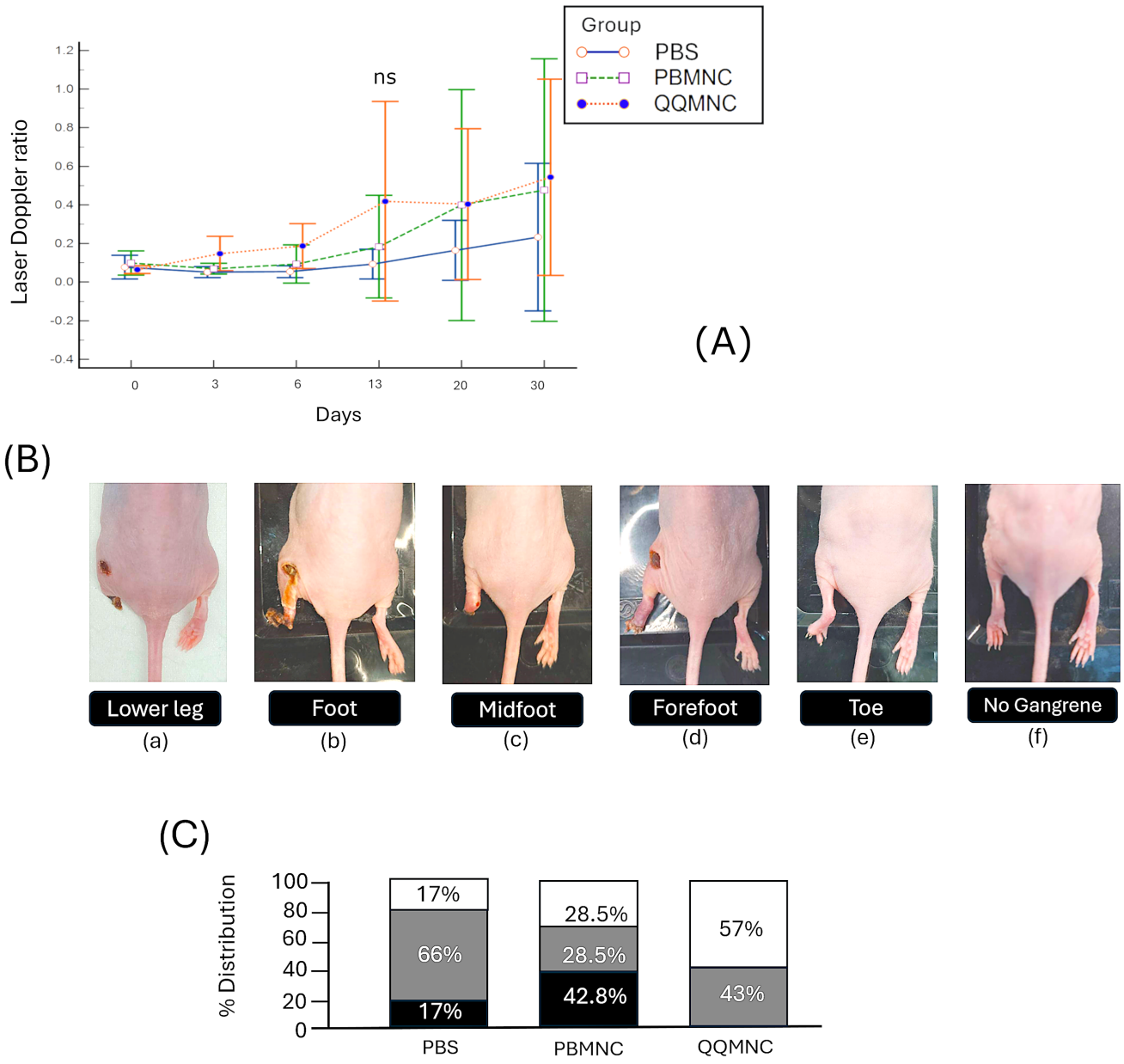

FIGURE 4 (A) Laser Doppler imaging of ischemic (left) feet versus normal (right) feet in diabetic ischemic mice, and the results are demonstrated as the mean of the laser Doppler ratio over time among the PBS ($n = 6$), PBMNC ($n = 7$), and QQMNC ($n = 7$) groups. Measurements were taken on day 0, day 3, day 6, day 13, day 20 and day 30. The QQMNC group showed an increase in perfusion over time compared to the PBMNC and PBS groups. (B) Assessment of limb loss in diabetic ischemic mice at day 30 visually stratified according to severity and distribution of gangrene across different parts of the lower limb. Limb outcomes included total leg gangrene (a), foot gangrene (b), midfoot gangrene (c), forefoot gangrene (d), toe gangrene (e) and no gangrene (f). (C) Bar graph showing the percentage distribution of limb loss levels among the PBS, PBMNC and QQMNC groups. The categories include minor tissue loss (defined as toes and forefoot gangrene) (white bar); major tissue loss (defined as midfoot to total foot gangrene) (grey bar) and severe limb loss (defined as total leg gangrene) (black bar). The QQMNC group demonstrated the highest percentage of minor tissue loss (57% for forefoot and toe gangrene), and the lowest percentage of severe limb loss (0% for lower leg gangrene). 'ns' denotes no significant difference. PBMNC, peripheral blood mononuclear cell; PBS, phosphate-buffered saline; QQMNC, Quality and Quantity mononuclear cell.

and other ischemic conditions by promoting vascular regeneration, modulating immune responses and enhancing tissue repair. Future studies should evaluate cytokine, growth factor and immune response after QQMNC treatment to evaluate their direct contribution to new vessel formation and tissue regeneration. Masuda et al. demonstrated the benefits of QQMNCs in an ischemic mouse model, and Tanaka et al. demonstrated the

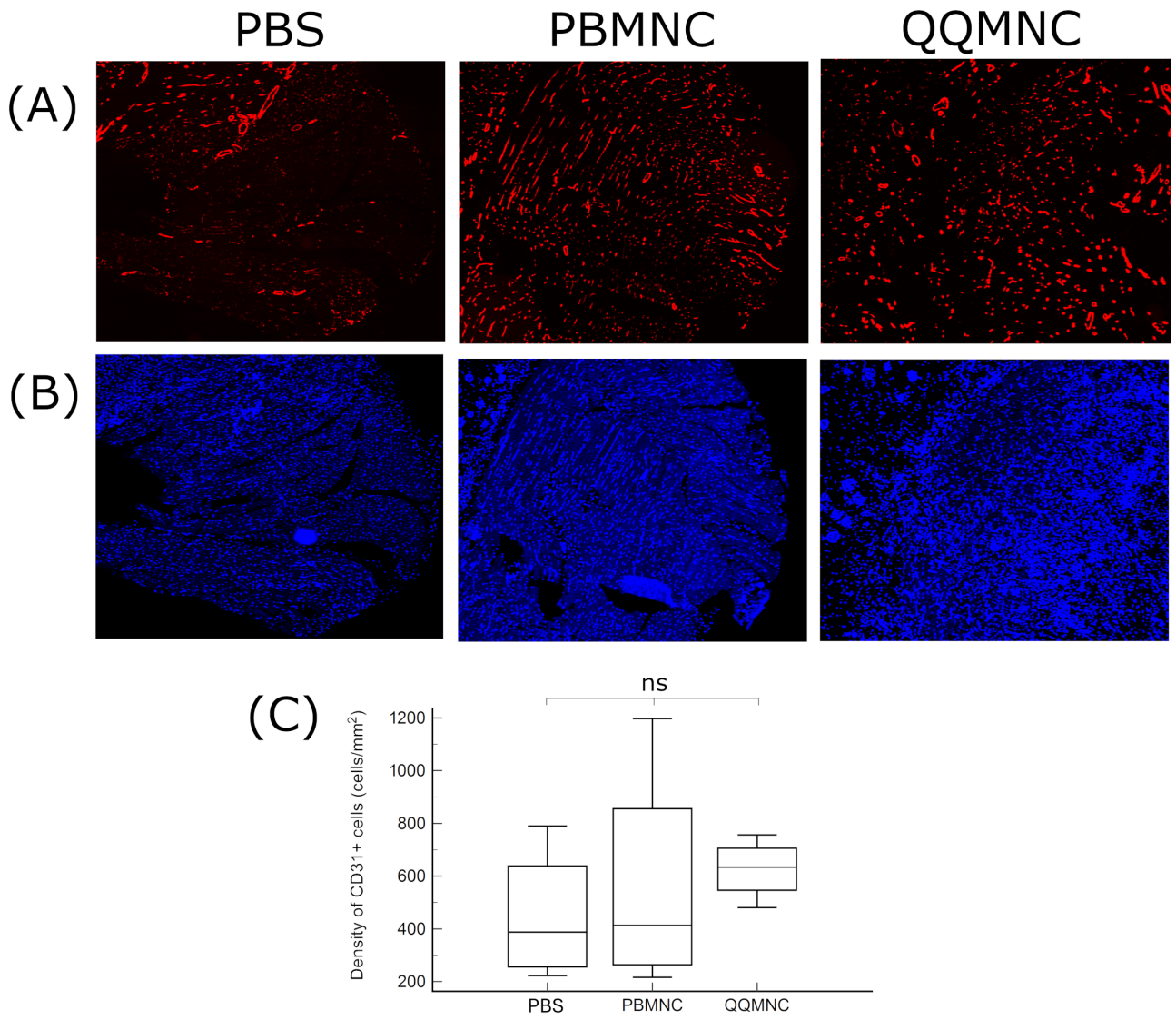

FIGURE 5 Representative immunofluorescence images of histological specimens of the injection area of muscle sections from the PBS (POI = 4), PBMNC (POI = 4), and QQMNC (POI = 4) groups. The immunofluorescence images show staining for (A) CD31 + cells (red colour) and (B) DAPI stain (blue colour). (C) Box plot showing the density of intramuscular vessels (CD31+ cells, cells/mm^2) in the PBS, PBMNC and QQMNC groups. The QQMNC group shows a more consistent vessel density than the PBS and PBMNC groups, which indicates better vascularization. The data are labelled as mean ± standard deviation (SD). 'ns' denotes no significant difference. CD, cluster of differentiation; PBMNC, peripheral blood mononuclear cell; PBS, phosphate-buffered saline; POI, point of interest; QQMNC, quality and quantity mononuclear cell.

benefits of QQMNCs in a diabetic mouse model. Our research expands on these findings by showing the benefits of QQMNCs in treating ischemic wounds in a diabetic mouse model.

Despite these promising findings, further research is needed to establish the optimal dosage of QQMNCs that is required to translate these results into human clinical practice. Tanaka et al. demonstrated the potential of QQMNCs in treating CLTI patients; however, a volume-based comparison between human and murine models suggests that the cell dosage used in preclinical studies may not be directly generalizable to humans. Therefore, future studies will focus on refining the dosing regimen to ensure the development of a cost-effective and biologically effective therapeutic strategy for human clinical trials.[17]

4.1 | Limitations

This study also has some mentionable limitations. First, we used BALB/c nude athymic mice in this study, so the

T cells observed likely resulted from either the transplanted cells or extrathymically developed immature T cells. Due to the immaturity of these T cells in athymic mice, they may be unable to fully differentiate into CD4+ or CD8+ cells, which may explain our inability to detect these subtypes in the current study. In future studies, we plan to include CD4 and CD8 T cell staining to better differentiate between CD4+ helper T cells (Tregs and Th2 cells) and CD8+ cytotoxic T cells. As such, it must be noted that the use of athymic mice (BALB/c nude) limits the generalizability of our findings to models with a fully functional immune system since these mice do not fully replicate complex immune interactions – particularly inflammatory response following QQMNC or PBMNC injection. This limitation adversely affects the direct translation of our results to humans since human patients have more complex immune and inflammatory responses that could impact the treatment's effectiveness. Therefore, the efficacy of QQMNCs should be further investigated in clinical trials. Despite this limitation, BALB/c nude mice are widely recommended for studying human erythropoietic cells in animal models. Second – despite the fact that we enrolled the minimum number of mice (20–21 total for an estimated 6–7 mice per group), we were only able to include a total of 17 mice [PBS group ($n = 6$), PBMNC group ($n = 5$) and QQMNC group ($n = 6$)] in our final analysis. The reason for this decrease in our mouse study population is that some mice died after receiving the injection of 200 mg/kg of streptozotocin to induce diabetes. The result is a potential decrease in the statistical power of our study, and a commensurate potential decrease in the robustness of our findings. This suggests the need for larger study to remedy this limitation. Increasing the sample size will help to reduce variability in key factors, such as blood sugar levels and residual collateral supply between groups. In a smaller study sample, these baseline differences can obscure the true effects of QQMNCs on angiogenesis and anti-inflammatory responses. A larger sample size would minimize this variability and allow for clearer and more accurate detection of QQMNC effects, such as wound closure percentage, LDPI and immunological assay. Therefore, future studies should enrol a larger study sample to improve the precision and reliability of these findings, which would help to confirm the true therapeutic impact of QQMNCs without the noise of baseline variability. The results of study in a larger study sample would strengthen the conclusions and provide a better window into the potential clinical applications of QQMNCs. Third, variability in the baseline characteristics of study mice, such as age and health status, could have influenced the outcomes, which highlights the importance of standardized variables in future research. Fourth, the 30-day follow-up period in our study enabled us to capture the remodelling phase and the initial effects of QQMNCs on wound healing. However, further research is needed to assess the long-term efficacy and safety of QQMNC therapy for promoting sustained tissue repair and vascular regeneration, especially in humans. Fifth and last, this study did not delve deeply into the mechanisms underlying the observed benefits of QQMNC injection. Future research should include detailed mechanistic investigations to elucidate the cellular and molecular processes involved in QQMNC-mediated wound healing. For example, we focused on CD31+ cells as markers for smaller vessels and capillaries; however, we did not perform a detailed analysis of αSMA+ large blood vessels. Further study should evaluate both CD31+ and αSMA+.

5 | CONCLUSIONS

The outcomes of this study demonstrate the effectiveness of QQMNCs for enhancing wound healing and limb salvage in diabetic ischemic animal models. The significant improvements in angiogenesis and tissue perfusion observed in the QQMNC group strongly suggest QQMNCs as a potential therapeutic option for enhancing wound healing and limb salvage in diabetic ischemic conditions, especially in patients with NOCLTI.

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CONFLICT OF INTEREST STATEMENT

All authors declare no personal or professional conflicts of interest and no financial support from the companies that produce or distribute the drugs, devices, or materials described in this report.

DATA AVAILABILITY STATEMENT

Data will be made available to the Editor-in-Chief upon reasonable request to the corresponding author.

ETHICS STATEMENT

The Siriraj Institutional Review Board (SIRB) of the Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand, reviewed and approved the study protocol (approval no. Si752/2022). The animal studies were performed after approval from the Siriraj Laboratory Animal Research and Care Center of the Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand approval No. 001/2566 (SI-ACUP 013/25).

INFORMED CONSENT

Written informed consent to participate in this study was obtained from all enrolled healthy human volunteers.

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