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Title

Hypoxic culture enhances the antimicrobial activity of amnion-derived mesenchymal stem cells, thereby reducing bacterial load and promoting wound healing in diabetic mice

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Ethics approval statement

The protocols used in our study were approved by the Institutional Animal Care and Use Committee, Hokkaido University School of Medicine, and were conducted in accordance with the Guidelines for the Care and Use of Laboratory Animals, Hokkaido University

- Amnion-derived mesenchymal stem cells (AMSCs) secrete the antimicrobial peptide LL-37
- LL-37 secretion is increased when AMSCs are cultured under hypoxic conditions
- AMSCs inhibit *Staphylococcus aureus* growth *in vitro*
- AMSCs inhibit the growth of *Staphylococcus aureus* in infected wounds of diabetic mice

Abstract

Background: Conditioned medium from amnion-derived mesenchymal stem cells (AMSCs) enhances wound healing, a process that is further improved under hypoxic culture conditions. Diabetic foot ulcers are difficult to treat and are frequently complicated by a high rate of bacterial infections, mainly *Staphylococcus aureus*, which can lead to limb amputation and death. Here, we topically applied conditioned medium from AMSCs cultured under hypoxic conditions to *S. aureus*-infected wounds in diabetic mice to investigate its effect on bacterial counts and wound healing.

Methods: We prepared conditioned medium by culturing AMSCs under 21% or 1% O_2 and investigated its effects on *S. aureus*. We infected skin wounds of diabetic mice with *S. aureus* and treated these with hydrogels containing the conditioned medium to examine its effect on bacterial inhibition and wound healing.

Results: Conditioned medium from AMSCs cultured under 1% O_2 contained higher levels of the antimicrobial peptide LL-37. It significantly inhibited *S. aureus* growth *in vitro*, reduced bacterial counts in infected wounds, and facilitated wound closure in diabetic mice.

Conclusions: Hydrogels containing conditioned medium from hypoxically cultured AMSCs inhibited the growth of *S. aureus* and promoted wound healing in a mouse model of diabetic wounds.

Keywords

Mesenchymal Stem Cells; Diabetic Foot; Bacterial Infections; Cathelicidins; LL-37; Wound Healing

1. Introduction

Diabetic foot ulcers are deep tissue-destructive lesions that occur primarily in the lower extremities of diabetic patients and are refractory and have a poor prognosis. In 2021, approximately 537 million adults worldwide were living with diabetes, a number expected to increase to 783 million by 2045. Diabetic foot ulcers affect around 15% of these patients, and 14%–24% of these ulcers result in lower extremity amputations [1-3]. Infections in diabetic foot ulcers increase the risk of lower extremity amputation. The 5-year mortality rate for patients who undergo leg or thigh amputation is extremely high, ranging from 52%–80% [4]. Infections caused by methicillin-resistant *S. aureus* (MRSA) are associated with poor prognosis, leading to higher rates of reamputation and prolonged hospital stays [5]. Infection control of diabetic ulcers is very important, and the development of more effective treatments is desired.

Mesenchymal stem cells are found in a range of tissues, such as fat and bone marrow, and can differentiate into mesenchymal cells such as adipocytes, chondrocytes, and osteocytes [6]. Mesenchymal stem cells promote wound healing and inhibit bacterial growth by releasing various cytokines [7-11]. Hypoxia enhances the paracrine action of mesenchymal stem cells [7,8,12-16]. Stem cells derived from the bone marrow and adipose tissue inhibit the growth of *Escherichia coli*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus*, thereby improving osteomyelitis and pneumonia [17-19]. These effects are mediated via the secretion of the antimicrobial peptide LL-37 [17,18], which can kill bacteria, fungi, and viruses. The effects of LL-37 extend to antibiotic-resistant bacteria, including methicillin-resistant *S. aureus* [20,21]. No studies have examined the antimicrobial activity of amnion-derived mesenchymal stem cells (AMSCs) or the potential strategies to enhance it.

AMSCs can be obtained in large quantities from placental amniotic membranes after birth, which is less invasive to the donor and avoids ethical issues [22,23]. We previously showed that conditioned medium from AMSCs inhibits the proliferation and activation of keloid fibroblasts *in vitro* [24] and promotes wound healing in a diabetic mouse model [7]. In the present

study, we hypothesized that the topical application of conditioned medium from AMSCs cultured under hypoxic conditions would inhibit bacterial growth and promote wound healing in diabetic wounds infected with *S. aureus*. These findings could have a substantial impact on the field of diabetic wound healing by providing a novel, non-invasive therapeutic option targeting both infection control and tissue regeneration.

2. Material & Methods

2.1. Bacterial Strains and Growth Conditions

S. aureus (ATCC 29213) was used for all experiments. The bacteria were first grown on tryptic soy agar (#236950; BD, Franklin Lakes, NJ) under conditions of 37°C, 21% O_2, and 5% CO_2 for 24 h. A single colony from this initial growth was inoculated into 8 mL tryptic soy broth (#211825; BD) and incubated under the same conditions with shaking at 50 rpm for further expansion.

2.2. Propagation of Amnion-Derived Mesenchymal Stem Cells

AMSCs were kindly provided by Kaneka Corp. (Osaka, Japan) [25]. Cells from the third to fourth passages were used and incubated at 37°C in an atmosphere containing 21% O_2 and 5% CO_2 until they reached sub-confluency.

2.3. Preparation of Conditioned Medium

Upon reaching sub-confluency, the AMSCs were washed twice with phosphate-buffered saline (PBS) to remove residual serum. The culture medium was then replaced with antibiotic-free α-MEM (Life Technologies, Carlsbad, CA), and the cells were cultured under either normoxic (21% O_2) or hypoxic (1% O_2) conditions for 48 h to create normoxic conditioned medium (NCM) and hypoxic conditioned medium (HCM), respectively. To remove any cellular debris, the

conditioned medium was collected, filtered through a 0.2-μm filter, and immediately stored at −80°C until further use [7]. Additionally, standard medium (SM) was prepared by passing antibiotic-free α-MEM through a 0.2-μm filter, and immediately stored at −80°C until further use. SM served as a control to evaluate any potential antibacterial activity of the medium itself, independent of factors from AMSCs [7].

2.4. Evaluation of LL-37 Concentrations in Conditioned Medium using an Enzyme-Linked Immunosorbent Assay

The presence of LL-37, an antimicrobial peptide, in HCM, NCM, and SM was evaluated using an enzyme-linked immunosorbent assay kit (Hycult Biotech, Uden, The Netherlands).

2.5. Bacterial Inhibition by Conditioned Medium in vitro

A 12-well plate was prepared with HCM, NCM, or SM, each combined with an equal amount of tryptic soy broth (#211825; BD). Six wells were prepared for each medium. Using a spectrophotometer at a wavelength of 600 nm, an *S. aureus* suspension was prepared and added to each well to achieve a concentration of 5,000 colony-forming units (CFU)/mL. This was then cultured at 37°C under 21% O_2 and 5% CO_2 for 2 h. Aliquots of 50 μL were taken from each well and plated onto tryptic soy agar (#236950; BD), and the number of colonies was counted after 16–18 h.

2.6. Induction of Diabetes in Mice

The experimental protocol was approved by the Animal Care and Use Committee of Hokkaido University (approval no. 2022-23-1). Five-week-old male ICR mice (Japan SLC, Hamamatsu, Japan) were kept in a temperature-controlled room maintained at 24°C on a 12-h light/dark cycle. Food and water were provided *ad libitum*.

After a 1-week acclimatization period, diabetes was induced in the mice by

intraperitoneal injection of 180 mg/kg streptozotocin (Fujifilm Wako Pure Chemical Corporation, Osaka, Japan). Blood glucose levels were monitored from the tail vein using a glucometer (One Touch Ultra Vue; LifeScan, Malvern, PA) [26]. Polyuria was noted, requiring daily cage changes. Blood was collected from the tail vein 4 weeks later, and mice with a blood glucose level exceeding 300 mg/dL were included in the experiment.

2.7. Creation of Wounds and Bacterial Infection

The mice were anesthetized with 2.5% isoflurane, and the dorsal and ventral hair was removed completely using a surgical clipper and depilatory cream. The skin of the mice was disinfected with a 0.05% chlorhexidine gluconate solution, and two 8-mm diameter round full-thickness wounds were created symmetrically to the spinal line on the panniculus carnosus using a scalpel [7]. *S. aureus* cultured in tryptic soy broth was centrifuged at 1,500 rpm for 5 min. The supernatant was removed, and the bacterial pellet was washed twice with 2 mL PBS. Using a spectrophotometer at a wavelength of 600 nm, a bacterial suspension of 20,000 CFU/mL was prepared. A total of 0.05 mL of this bacterial suspension was dripped onto the full-thickness skin defects of the mice. After bacterial inoculation, the wounds were covered with a sterile film (IV3000; Smith and Nephew, Watford, UK), and infection was confirmed 48 h later by the appearance of pus and erythema.

2.8. Evaluation of Bacterial Inhibition and Wound Closure Ratio in vivo

Conditioned medium gels were prepared by adding 7% carboxymethylcellulose to SM, NCM, or HCM [7]. The day of infection establishment was set as day 1; photographs of the wounds were taken on days 1, 2, 3, 5, 9, and 10, using a single-lens reflex camera (EOS X10i; Canon, Tokyo, Japan). Measurement of the wound area was performed using Fiji software (National Institutes of Health, Bethesda, MD), and the wound closure ratio was calculated according to the following formula:

Wound closure ratio (%) = (1 – [remaining wound area / initial wound area]) × 100.

After each photography session, 0.05 mL hydrogel was applied to the wounds and covered with IV3000 and Tegaderm. Six mice were assigned to SM, NCM, or HCM in each experiment to measure bacterial counts and histological evaluations of the wounds.

On day 10, the mice were euthanized. The wounds were sampled with a 3-mm skin punch. The samples were homogenized using a BioMasher II homogenizer (Nippi, Tokyo, Japan) with 100 µL PBS; after serial dilution with 100 µL PBS, 10 µL homogenate was plated on Baird-Parker agar medium (BD). This was incubated at 37°C for 16–18 h under 21% O_2 and 5% CO_2. The number of colonies was then counted.

2.9. Histology and Immunohistochemistry

Specimens were fixed in 4% paraformaldehyde (Fujifilm Wako Pure Chemical Corporation) and embedded in paraffin prior to hematoxylin-eosin and Masson's trichrome staining, Gram staining, and immunohistochemical analysis. Sections (4-µm thick) were incubated with the following antibodies: rabbit anti-myeloperoxidase (MPO; 1:2,000; #A0398; Agilent Technologies, Santa Clara, CA), rabbit anti-CD31 (1:2,000; #ab182981; Abcam, Cambridge, UK), and rabbit anti-Ki-67 (1:150; #ab16667; Abcam). A goat anti-rabbit secondary antibody conjugated with horseradish peroxidase (Histofine® Simple Stain™ Mouse MAX PO; Nichirei Biosciences, Tokyo, Japan) was used as a secondary antibody for MPO, CD31, and Ki-67.

Digital images of the slides were created using a whole-slide scanner (NanoZoomer Digital Pathology; Hamamatsu Photonics, Hamamatsu, Japan) and visualized using NDP.view2 software (Hamamatsu Photonics). Quantification of the Gram-, MPO-, CD31-, and Ki-67-positive areas was performed using Fiji software (National Institutes of Health) by observing three randomly selected fields of view (×40 magnification) for each specimen.

Histological wound healing was assessed from hematoxylin-eosin and Masson's trichrome staining using a histology scoring system. Briefly, total scores were calculated with the

sum of the following four items rated on a scale of 0, 1, or 2: percentage of epithelial elongation relative to the wound (Re-epithelialization), thickness of granulation that grew on the wound (Granulation tissue), degree of regeneration of collagen and skin appendages (Remodeling), and thickness of the regenerated dermis (Scar elevation index) [27]. The higher the total score, the more advanced the wound healing.

2.10. Statistical Analysis

Data are shown as the mean ± standard error. Comparisons were performed using the Tukey–Kramer test. $P < 0.05$ was considered statistically significant. Statistical analysis was performed using JMP Pro (ver. 17.0.0; JMP Statistical Discovery LLC, Cary, NC).

3. Result

3.1. High LL-37 Concentrations in HCM

The concentrations of LL-37 were 1.26 ± 0.13 ng/mL in HCM, 0.60 ± 0.26 ng/mL in NCM, and below the detection limit in SM. HCM had significantly higher LL-37 concentrations than NCM (Fig. 1).

3.2. Conditioned Medium Under Hypoxic Conditions Inhibits Bacteria in vitro

SM exhibited *S. aureus* counts of 760 ± 40 CFU/mL, NCM exhibited 543 ± 140 CFU/mL, and HCM exhibited 360 ± 53 CFU/mL *in vitro*. HCM demonstrated a statistically significant inhibition of *S. aureus* growth by 52.6% compared with SM. Additionally, NCM exhibited a 28.5% inhibition of bacterial growth compared with SM, although this difference was not statistically significant (Fig. 2).

3.3. Conditioned Medium Under Hypoxic Conditions Inhibits Bacteria in a Mouse

Model

In *S. aureus*-infected wounds of the diabetic mouse model, SM exhibited *S. aureus* counts of 1,750 ± 480 CFU/mL and NCM was 1,800 ± 800 CFU/mL, while HCM was 70 ± 30 CFU/mL. NCM did not significantly reduce *S. aureus* levels compared with SM. However, HCM significantly inhibited the growth of *S. aureus* by 96.0% compared with SM and by 96.1% compared with NCM (Fig. 3).

3.4. Conditioned Medium Under Hypoxic Conditions Accelerates Wound Healing

Regarding wound healing promotion, SM showed a 40.1% ± 3.8% reduction in wound area and NCM showed a 42.0% ± 5.2% reduction in wound area. However, for HCM, the reduction in wound area was 76.0% ± 4.1%, which was significantly higher than for SM and NCM (Fig. 4). These findings indicated that HCM significantly accelerated wound healing.

3.5. Conditioned Medium Under Hypoxic Conditions Improves the Histological Findings of Wounds

Gram staining revealed that HCM had 90.5% and 89.3% lower *S. aureus* counts at the wound site than SM and NCM, respectively, which was consistent with the results of the direct assessment of tissue bacterial content. HCM had an 86.8% smaller MPO-positive area than SM and an 84.7% smaller area than NCM, indicating that HCM reduced neutrophil infiltration. HCM had 624% and 481% larger CD31-positive areas than SM and NCM, respectively. HCM had 1,552% and 454% larger Ki-67-positive areas than SM and NCM, respectively. HCM also promoted angiogenesis and cell division under *S. aureus* infection (Fig. 5).

In Masson's trichrome staining, HCM showed a higher re-epithelialization rate, more granulation tissue, greater regeneration of collagen and skin appendages, and more dermal regeneration than NCM and SM. Therefore, the histology score was significantly higher for HCM (3.67 ± 0.38) than for SM (0.66 ± 0.38) and NCM (1.17 ± 0.44) (Fig. 6).

Histological analysis revealed that HCM-treated wounds exhibited enhanced angiogenesis, re-epithelialization, and collagen regeneration compared with SM and NCM.

4. Discussion

We investigated the inhibitory effect of hypoxically cultured AMSCs on the proliferation of *S. aureus* in diabetic conditions. The results demonstrated that (1) conditioned medium from AMSCs inhibited the proliferation of *S. aureus*, (2) AMSCs secreted the antimicrobial peptide LL-37, (3) in a diabetic mouse model with *S. aureus*-infected wounds, conditioned medium inhibited the proliferation of *S. aureus* and promoted wound healing, and (4) the aforementioned effects were enhanced when AMSCs were cultured under hypoxic conditions. To our knowledge, this is the first study to demonstrate that AMSCs secrete the antimicrobial peptide LL-37, exhibit antimicrobial activity *in vivo*, and have enhanced antimicrobial activity under hypoxic culture conditions.

It is well established that mesenchymal stem cells derived from adipose tissue, bone marrow, and umbilical cord blood possess antimicrobial activity both *in vitro* and *in vivo* [7,8,12-16]. Bone marrow-derived mesenchymal stem cells and their conditioned medium have inhibitory effects on the proliferation of *E. coli*, *S. aureus*, and *P. aeruginosa* [17]. Moreover, in a mouse model of *E. coli* pneumonia, conditioned medium from human mesenchymal stem cells inhibits bacterial growth and neutrophil infiltration [18]. These effects are due to bioactive substances in the conditioned medium [17,18]. However, it has been reported that there is no difference in antimicrobial activity between mesenchymal stem cells derived from adipose tissue and those derived from bone marrow [17].

Previous studies have indicated that the antimicrobial activity of mesenchymal stem cells is due to the secretion of LL-37 by the cells [17,18,28]. It is well established that humans and other mammals as well as birds and fish possess antimicrobial peptides called cathelicidins [29,30], which are expressed in neutrophils, macrophages, natural killer cells, and epithelial cells.

Cathelicidins are induced by inflammatory pathways, the vitamin D pathway, and endoplasmic reticulum stress [20,31-34]. In humans, LL-37 is the only identified cathelicidin and is formed by the cleavage of its precursor, the 18-kDa human cationic antibacterial polypeptide [31]. LL-37 exerts antimicrobial activity against both Gram-positive and -negative bacteria by binding to bacterial lipopolysaccharides and lipid bilayers and permeabilizing microbial membranes, thereby inducing cell death [35-37]. Moreover, LL-37 exhibits antifungal and antiviral properties [20,38]. In addition to its direct effects against pathogens, LL-37 also exerts antimicrobial activity through indirect mechanisms such as recruiting monocytes, neutrophils, and CD4+ T cells to the site of pathogen entry and activating dendritic cells [39-41]. The combination of LL-37 with antibiotics can achieve greater efficacy than antibiotics alone. In addition to its effects against drug-resistant bacteria, LL-37 is also effective against a wide range of other pathogens, including fungi and viruses, making LL-37 a promising treatment option for infections [42,43].

We previously demonstrated that culturing mesenchymal stem cells under hypoxic conditions enhances their wound-healing effect through a paracrine effect [7]. Not only are AMSCs readily available from the amniotic membrane of postpartum placental tissue, a source typically considered medical waste, but their collection poses no risk to the donor, minimizing ethical concerns [22,23]. In addition, AMSCs exhibit superior proliferative capacity compared with other mesenchymal stem cells, suggesting their potential high efficacy when used clinically [44]. However, no studies to date have reported that AMSCs have antimicrobial activity. Based on the above findings, we hypothesized and demonstrated that AMSCs secreted LL-37 and that hypoxic culture increased its secretion. However, the relationship between hypoxic conditions and the antimicrobial activity of various cells has not been fully elucidated. The antimicrobial activity of menstrual blood-derived mesenchymal stem cells against *S. aureus* is attenuated under hypoxic conditions [45]. Moreover, macrophages enhance their antimicrobial activity through increased expression of β-defensin 2 in a hypoxic environment [46]. The activation of hypoxia-inducible factor-1 (HIF-1) by hypoxic conditions and the administration of a HIF-1 activator

increases the secretion of LL-37 by human urothelial cells and inhibits *E. coli* growth in urinary tract infection in diabetic mice [47]. There is a paucity of literature on the relationship between hypoxic conditions and antimicrobial activity, and a consensus on this matter has yet to be reached [48]. However, in light of several studies showing that HIF-1α is activated in MSCs under hypoxic conditions [49,50], and that activation of HIF-1α activates transcription of the *CAMP* gene encoding LL-37 [51], it is conceivable that HIF-1α is activated by hypoxia in AMSCs, which promotes transcription of the *CAMP* gene and increases expression of LL-37, thereby enhancing antimicrobial activity.

It is known that AMSCs exhibit anti-inflammatory effects by promoting the secretion of anti-inflammatory cytokines [52,53]. The administration of AMSCs or their conditioned medium suppresses the infiltration of neutrophils and macrophages in mouse colitis and in pigs following endoscopic submucosal dissection of the esophagus and rectum [54-56]. We previously demonstrated that in a mouse model of non-infectious diabetic wounds, HCM prepared under the same conditions as in the present study suppresses neutrophil and macrophage infiltration and the expression of inflammatory cytokines such as interleukin (IL)-1β, IL-6, chemokine ligand (CXCL)-1, and CXCL-2 [7]. However, bone marrow-derived mesenchymal stem cell conditioned medium activates neutrophil bacterial phagocytosis and the formation of neutrophil extracellular traps, thereby reducing methicillin-resistant *S. aureus* counts in a mouse model of infected skin wounds [28]. The present study demonstrated the significant suppression of neutrophil infiltration. This is believed to be because bacterial counts were sufficiently reduced by the neutrophils activated by the mesenchymal stem cell conditioned medium and the LL-37 present in the conditioned medium, which resulted in decreased neutrophil activity by day 10.

In the process of wound healing, HCM demonstrated significantly superior outcomes compared with NCM and SM. AMSCs secrete angiogenic factors, including vascular endothelial growth factor (VEGF)-A, basic fibroblast growth factor (b-FGF), epidermal growth factor, insulin-like growth factor 1, and IL-8. It is well documented that conditioned medium enhances

283 angiogenesis in skin wound models [52,57-59]. We demonstrated that HCM has high levels of
284 VEGF-A and b-FGF and that applying this conditioned medium significantly promotes
285 angiogenesis and wound healing of non-infected wounds in diabetic mice [7]. In the present study,
286 as in our previous research, HCM promoted active angiogenesis and cell proliferation. As a result,
287 it was found that HCM aids wound healing by promoting epithelialization and angiogenesis
288 through paracrine action under *S. aureus* infection. In particular, HCM promoted re-
289 epithelialization, granulation, and regeneration of collagen and skin appendages due to its high
290 growth factor content and scored highly in a histology scoring system (Fig. 6).

291 Furthermore, LL-37 is known to activate the proliferation and migration of
292 keratinocytes and fibroblasts, which are essential for wound healing. In addition, by reducing
293 bacterial load and biofilm formation, LL-37 promotes wound healing by creating a more favorable
294 environment for tissue regeneration and repair [60,61]. In our previous study, we concluded that
295 VEGF-A and b-FGF [7], which are abundant in HCM, are important for wound healing, and that
296 LL-37, which is increased by hypoxic culture, also seemed to play an important role. It is possible
297 that there are pathways beyond the direct actions of these bioactive substances. Epidermal stem
298 cells are known to promote diabetic wound healing via the Notch signaling pathway [62].
299 However, future research needs to investigate whether the AMSC conditioned medium has a
300 similar pathway.

301 As mentioned above, AMSCs, like other MSCs, promote wound healing by increasing
302 the release of growth factors such as VEGF-A and b-FGF through hypoxic culture [7].
303 Furthermore, the present study has revealed that LL-37 exerts an antibacterial effect similar to
304 that of other MSCs. However, there are no reports that the amount of LL-37 produced by other
305 MSCs increases through hypoxic culture, and there is no evidence to suggest that LL-37, which
306 increases through hypoxic culture, promotes wound healing [63]. The present study is the first to
307 demonstrate that AMSCs have antibacterial properties and that the antibacterial properties of
308 MSCs are enhanced by hypoxic culture.

Hypoxically cultured AMSCs are also expected to be effective under other conditions. For example, biofilms present a problem in device infections; however, LL-37 can destroy biofilms and hypoxically cultured AMSCs may also be effective [61]. In addition, because human umbilical cord MSCs are reported to promote wound healing in mice with severe burns, it is expected that hypoxically cultured AMSCs will not only promote wound healing with severe burns but also have an effect on bacterial infections [64].

This study is limited in that it did not investigate antimicrobial substances other than LL-37. In general, mesenchymal stem cells secrete other antimicrobial substances, including β-defensin 2, hepcidin, and lipocalin [28]. However, the effects of hypoxic culture on these substances have not been examined. Further investigation of other antimicrobial substances would provide a more comprehensive understanding of the antimicrobial activity of AMSCs. Other limitations of this study include the focus on a single bacterial strain, *S. aureus*. Although *S. aureus* is a major cause of diabetic wound infections, future studies should also investigate the effects of other clinically important pathogens such as *P. aeruginosa* and MRSA. In addition, because the duration of infection was relatively short, it is possible that the long-term effects of AMSCs therapy on chronic wound healing could not be fully assessed. Further studies using longer-term models are needed to fully evaluate the sustained effects.

5. Conclusion

Conditioned medium obtained from hypoxically cultured AMSCs contained LL-37 and exhibited antibacterial activity. Furthermore, this conditioned medium inhibited bacterial growth and promoted wound healing when applied to skin wounds of diabetic mice infected with *S. aureus*. These findings suggest that conditioned medium obtained from hypoxic cultured AMSCs may be a potential treatment for infected diabetic foot ulcers.

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Data availability

The datasets generated and analyzed during the present study are available from the corresponding author on reasonable request.

CRediT authorship contribution statement

Riku Ishii: Conceptualization, Methodology, Investigation, Writing – original draft, Data curation. **Shunsuke Ohnishi:** Methodology, Writing – review & editing, Supervision. **Masahiro Hojo:** Conceptualization. **Kosuke Ishikawa:** Methodology, Writing – review & editing, Supervision. **Emi Funayama:** Writing – review & editing. **Takahiro Miura**: Writing – review & editing., Supervision. **Naoto Okubo:** Writing – review & editing. **Kazufumi Okada:** Data curation. **Yuhei Yamamoto:** Conceptualization,Writing – review & editing, Supervision. **Taku Maeda:** Methodology, Writing – review & editing, Supervision.

Declaration of competing interest

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Declaration of competing interest

The authors declare neither competing interests nor conflict of financial interest.

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Figure captions

Fig. 1. LL-37 concentrations in conditioned medium from amnion-derived mesenchymal stem cells. LL-37 concentrations were significantly higher in HCM compared to NCM ($p < 0.05$), and no LL-37 was detected in SM. Culturing AMSCs under hypoxic conditions increased the secretion of LL-37. Data are shown as the mean ± standard error. *$p < 0.05$. *SM*, standard medium; *NCM*, normoxic conditioned medium; *HCM*, hypoxic conditioned medium.

Fig. 2. Antimicrobial activity of conditioned medium from amnion-derived mesenchymal stem cells against *Staphylococcus aureus*. HCM significantly inhibited the growth of *S.aureus* compared to SM. Data are shown as the mean ± standard error from six independent replicates ($n = 6$). *$p < 0.05$. *SM*, standard medium; *NCM*, normoxic conditioned medium; *HCM*, hypoxic conditioned medium.

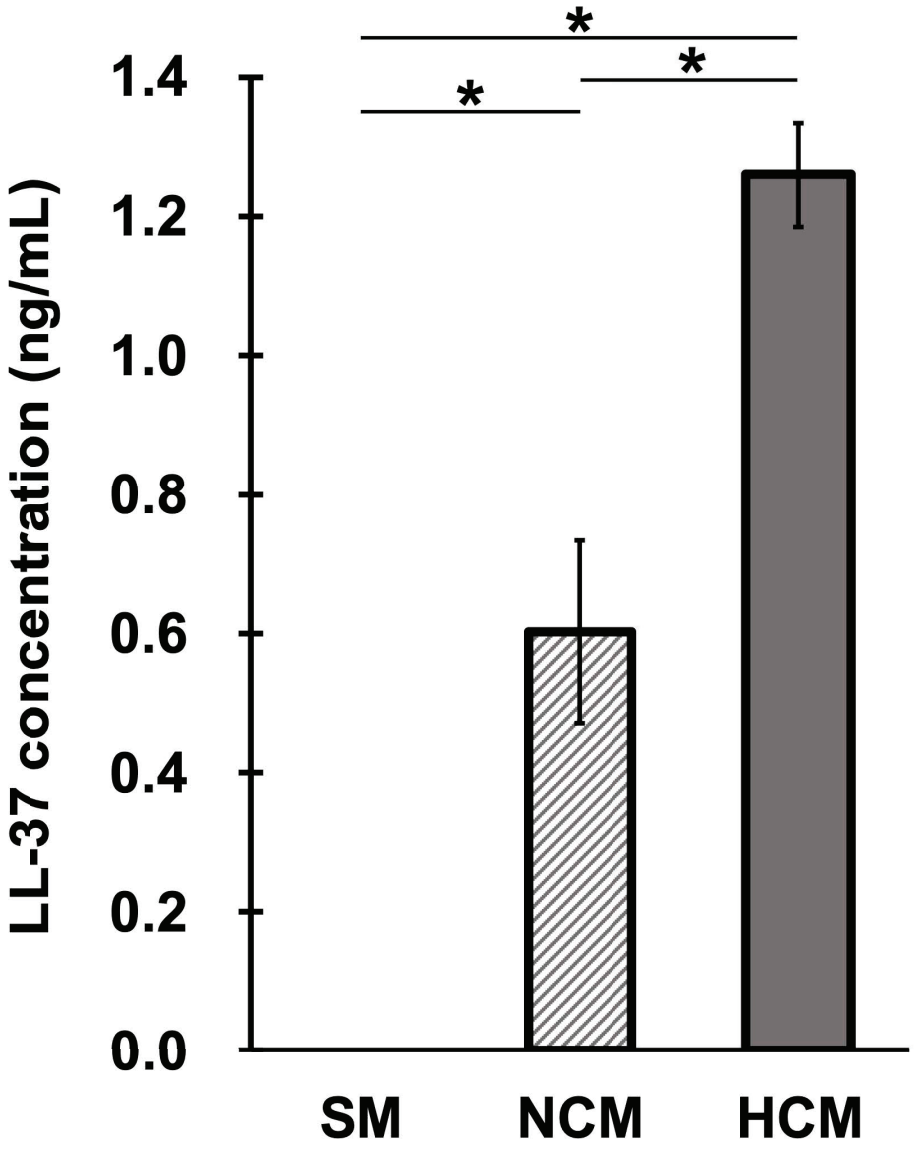
Fig. 3. Bacterial inhibition by conditioned medium from amnion-derived mesenchymal stem cells in *Staphylococcus aureus*-infected wounds in diabetic mice. (*Left*) Colonies sampled from *S. aureus*-infected wounds of diabetic mice, homogenized, and incubated on tryptic soy agar for 2 h. (*Right*) Colony-forming units counted on day 10. HCM significantly reduced the bacterial load compared to SM and NCM, indicating its potential to enhance infection control in diabetic wounds. Data are shown as the mean ± standard error from six independent replicates ($n = 6$). *$p < 0.05$. *SM*, standard medium; *NCM*, normoxic conditioned medium; *HCM*, hypoxic conditioned medium.

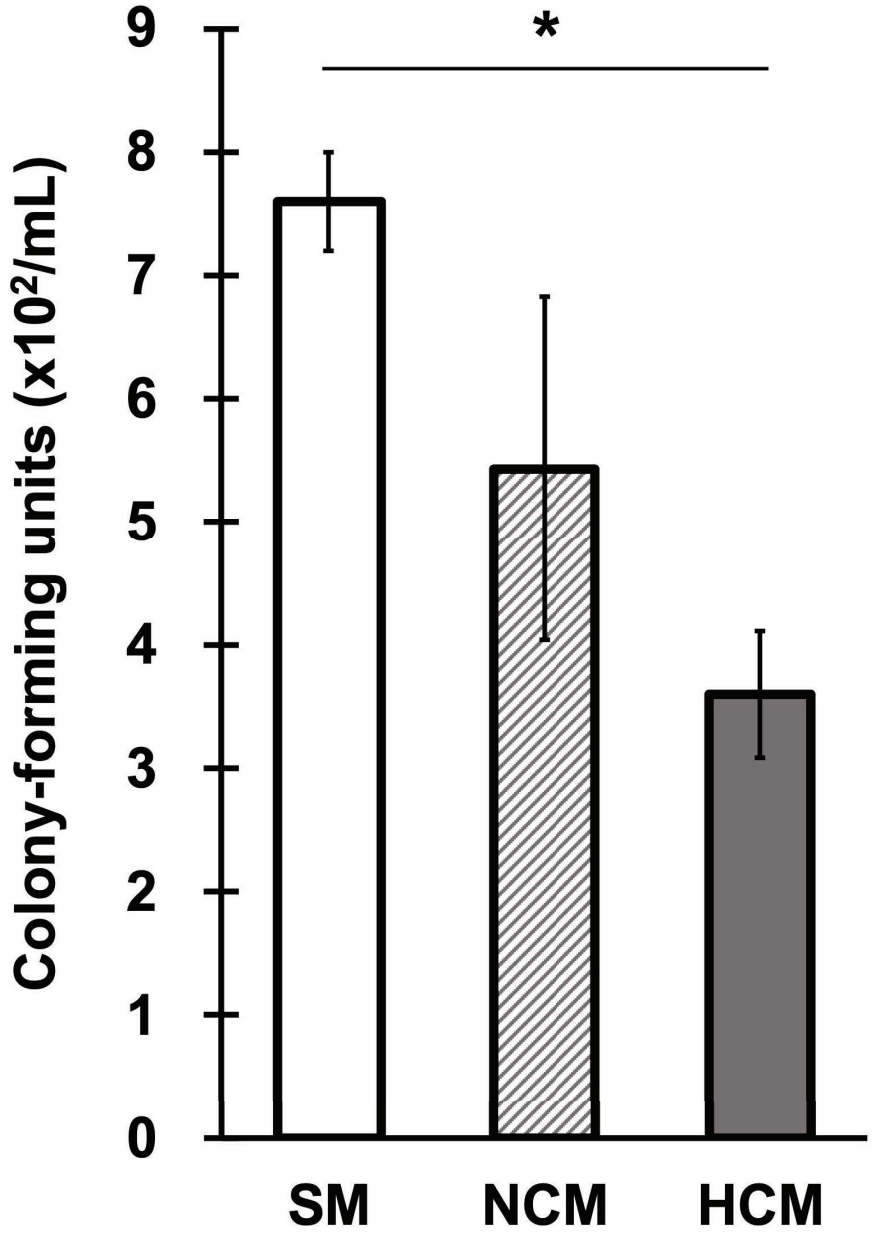
Fig. 4. Effect of conditioned medium from amnion-derived mesenchymal stem cells on the wound closure ratio in diabetic mice. (*Left*) Macroscopic images of wounds on days 0 and 10. (*Right*) Wound closure ratio on day 10. The group treated with HCM showed a significantly greater reduction in wound area compared to SM and NCM ($p < 0.05$). Data are shown as the mean ±

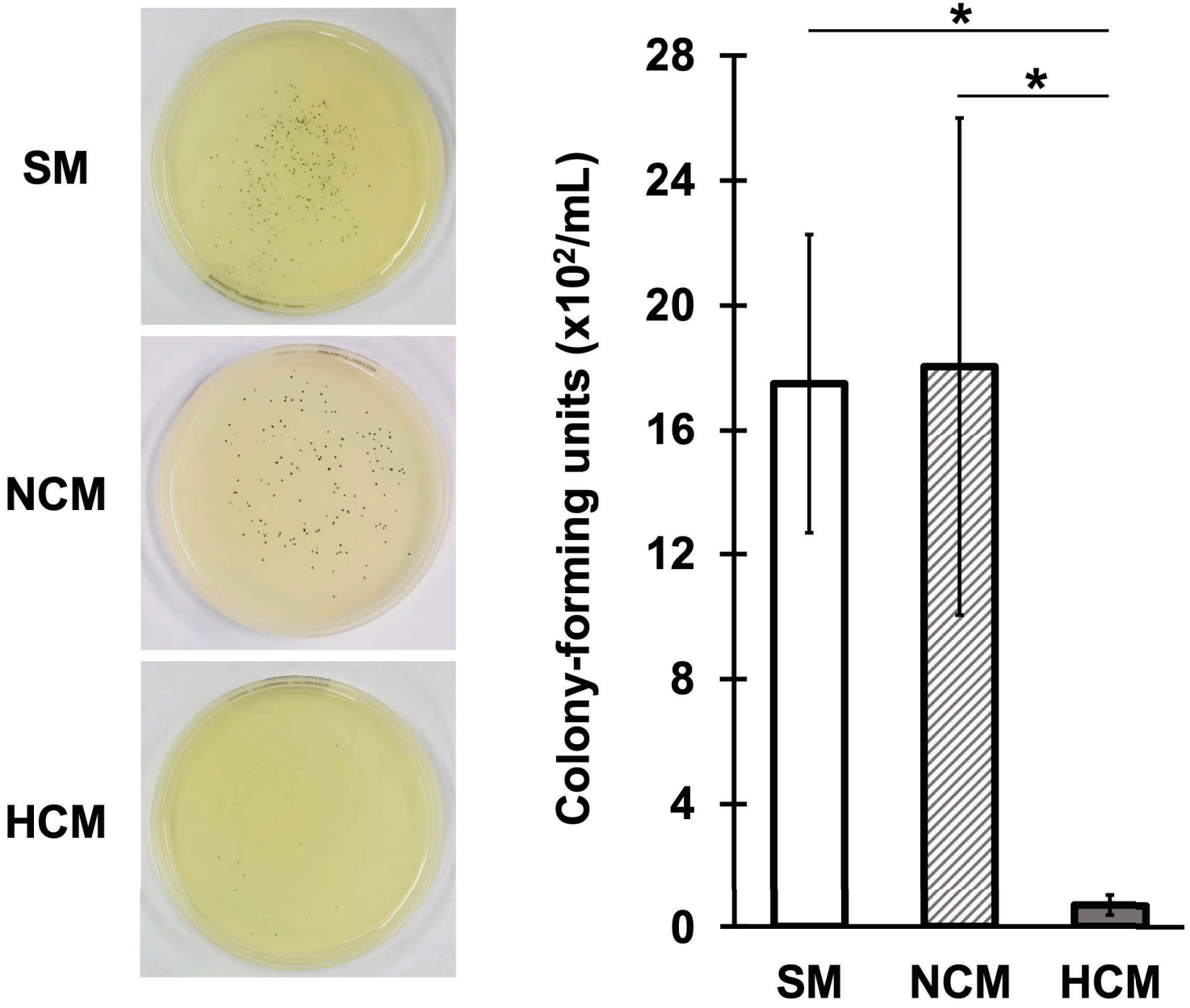
standard error. $n = 6$, $*p < 0.05$. Scale bar = 500 μm. *SM*, standard medium; *NCM*, normoxic conditioned medium; *HCM*, hypoxic conditioned medium.

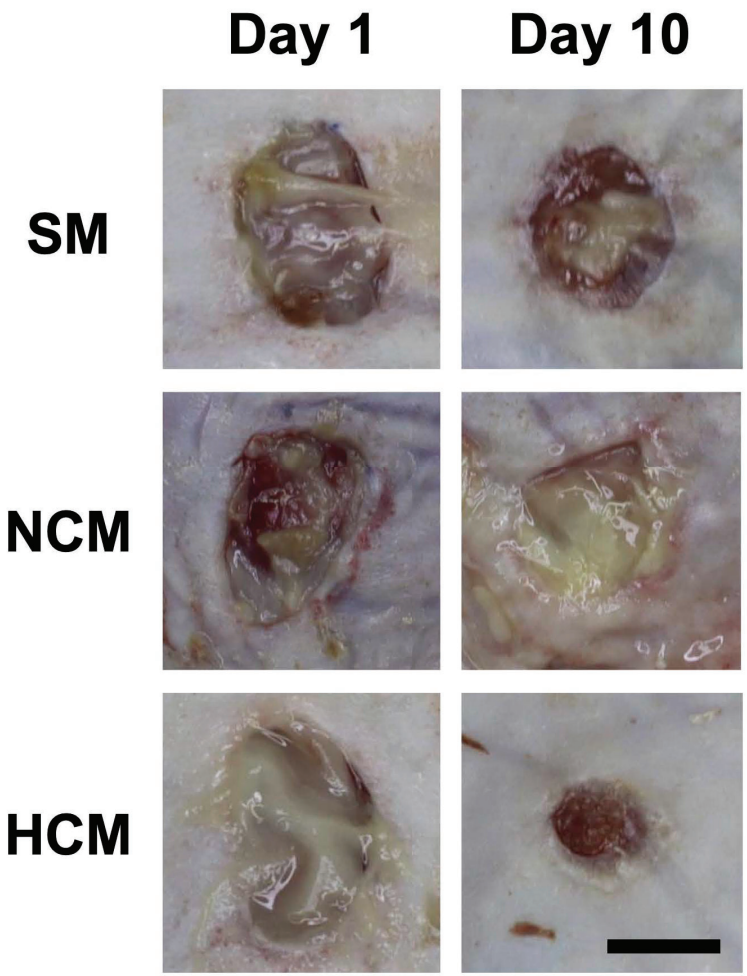
Fig. 5. Effect of conditioned medium from amnion-derived mesenchymal stem cells on the histological findings of wounds in diabetic mice. (*Above*) Representative histological images and (*Below*) percentages of the Gram-, myeloperoxidase (MPO)-, CD31-, and Ki-67-positive areas. There was less bacterial growth and fewer neutrophils in the group treated with HCM. The group treated with HCM exhibited increased angiogenesis and cell proliferation. Data are shown as the mean ± standard error. $n = 6$, $*p < 0.05$. Scale bar = 25 μm. *SM*, standard medium; *NCM*, normoxic conditioned medium; *HCM*, hypoxic conditioned medium.

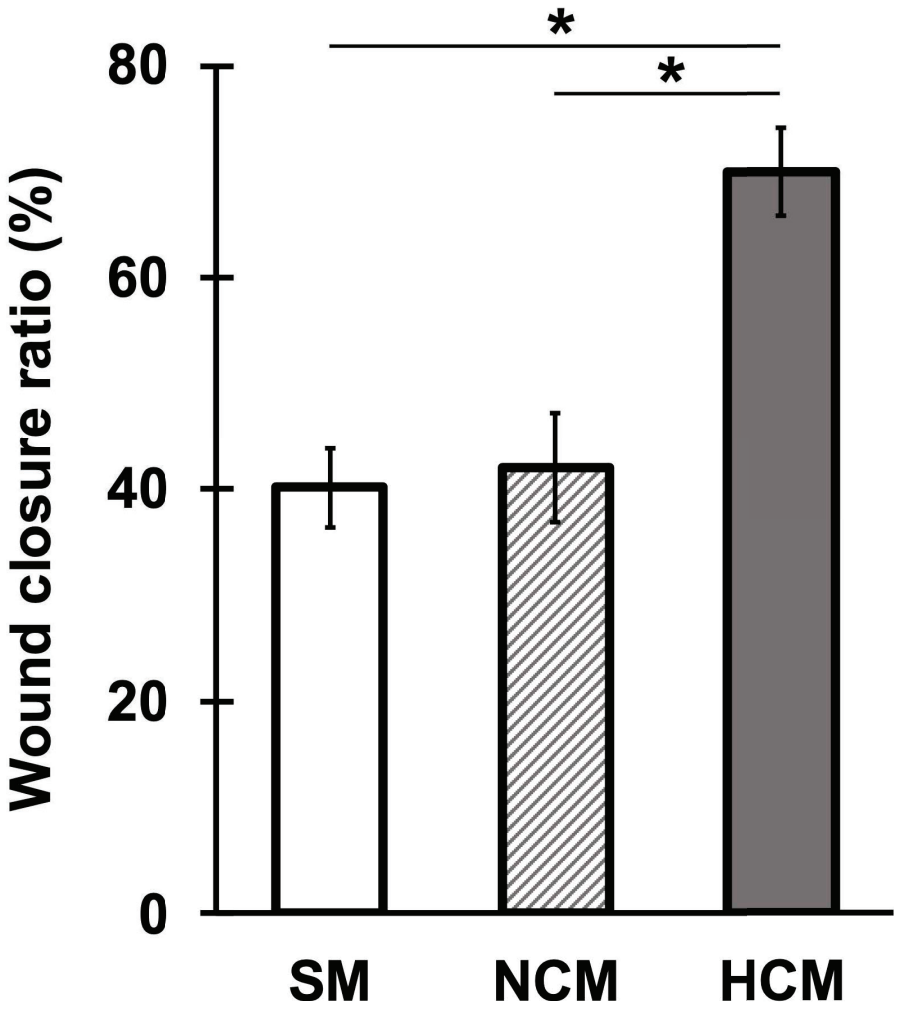
Fig. 6. Histology score of wound healing in diabetic mice. HCM scored significantly higher than SM and NCM ($p < 0.05$). **Re-epithelialization**: the black dotted lines show the original wounds and the red dotted lines show the elongated epithelium. Scale bar = 1 mm. **Granulation tissue**: the yellow dotted lines show the granulation and the black dotted lines show its thickness. Scale bar = 500 μm. **Remodeling**: the yellow dotted lines show the collagen and circular black dotted lines show regenerated skin appendages in the granulation tissue. Scale bar = 500 μm. **Scar elevation index**: the black dotted lines indicate the thickness of the epithelium, and in NCM and HCM, the left sides show normal epithelium and right side show regenerated epithelium. Scale bar = 500 μm. Data are shown as the mean ± standard error. $n = 6$, $*p < 0.05$. *SM*, standard medium; *NCM*, normoxic conditioned medium; *HCM*, hypoxic conditioned medium.

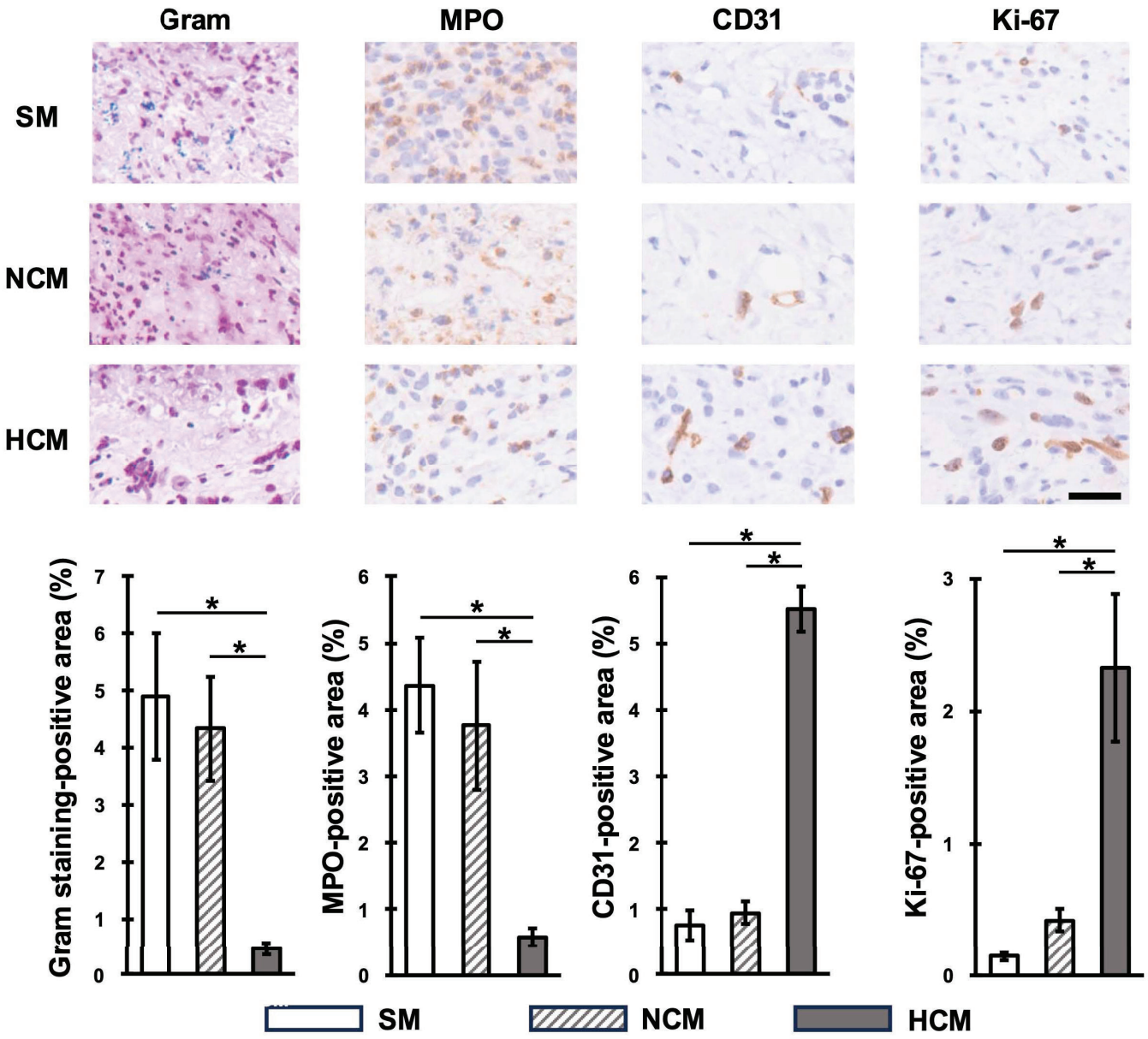

*
*
*
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
LL-37 concentration (ng/mL)
SM
NCM
HCM

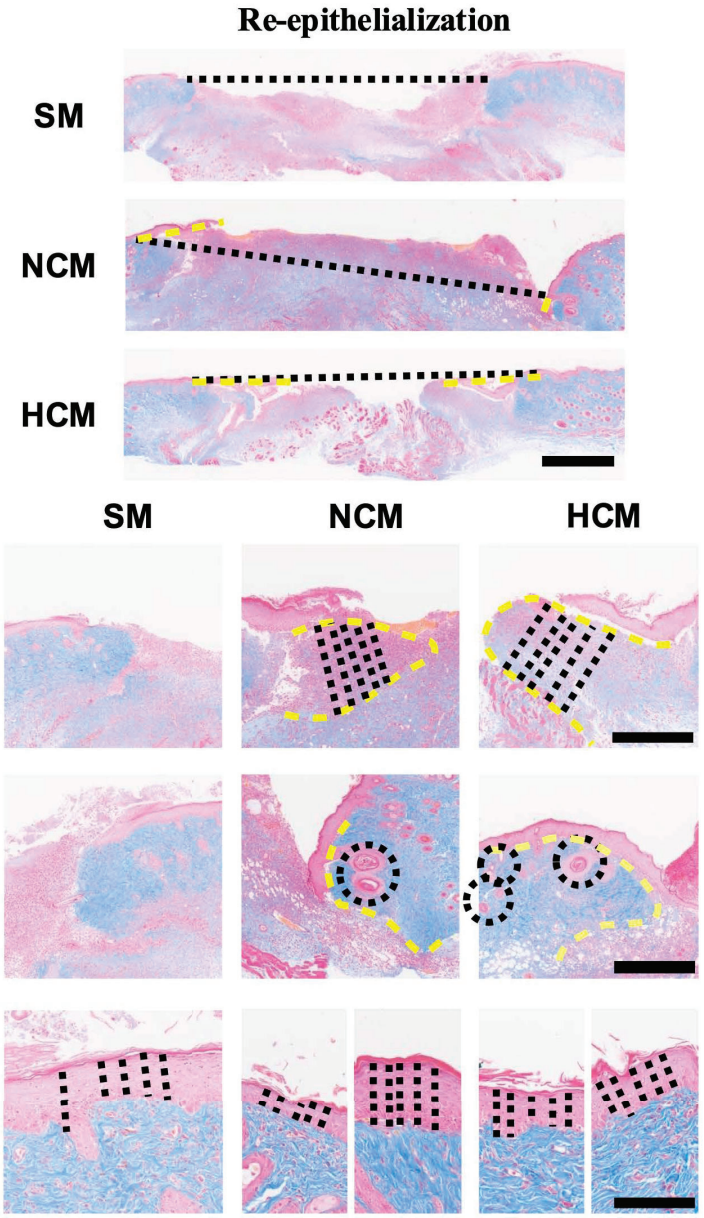

*
9
8
7
6
5
4
3
2
1
0
Colony-forming units (x10²/mL)
SM
NCM
HCM

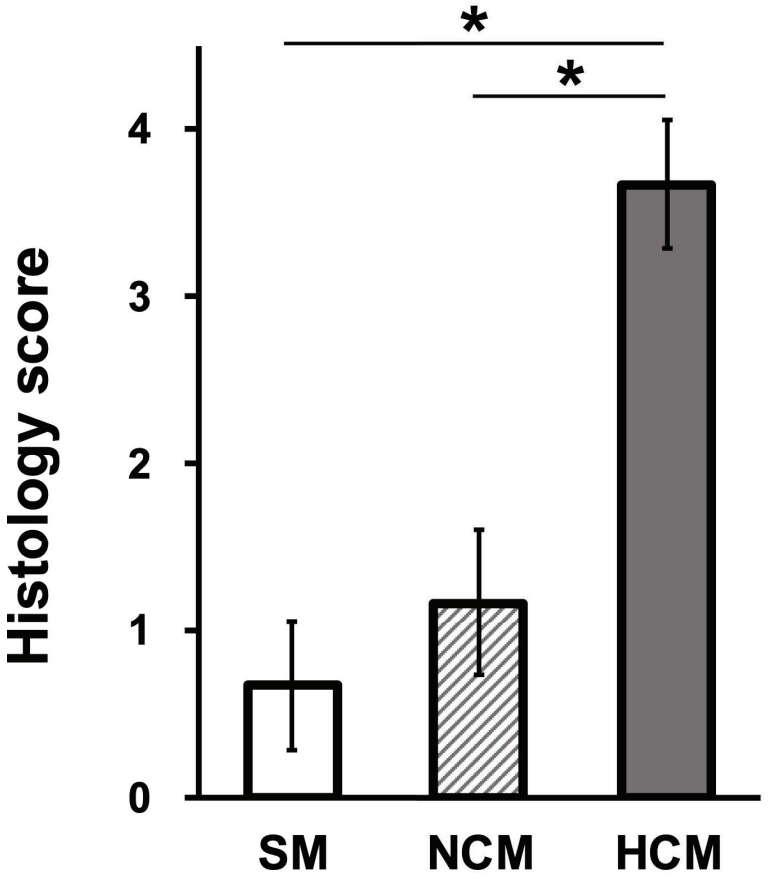

SM
NCM
HCM
Colony-forming units (x10²/mL)
28
24
20
16
12
8
4
0
*
*
SM
NCM
HCM


Day 1
Day 10
SM
NCM
HCM


Wound closure ratio (%)
80
60
40
20
0
*
*
SM
NCM
HCM


Gram
MPO
CD31
Ki-67
SM
NCM
HCM
Gram staining-positive area (%)
MPO-positive area (%)
CD31-positive area (%)
Ki-67-positive area (%)
*
SM
NCM
HCM





	SM	NCM	HCM
Re-epithelization	0.33	0.67	1.00
Granulation tissue	0.33	0.33	1.00
Remodeling	0.00	0.17	1.00
Scar elevation index	0.00	0.00	0.67
Total	**0.66**	**1.17**	**3.67**