EXPERIMENTAL STUDY

The contribution of probiotics to combined cellular therapy in skin wound healing in diabetic rats

Emine Müge Karakayalı,[1] İbrahim Tuğlu[2]

[1]Department of Medical Microbiology, Izmir Democracy University Faculty of Medicine, İzmir-*Türkiye*
[2]Department of Histology and Embryology, Manisa Celal Bayar University Faculty of Medicine, Manisa-*Türkiye*

ABSTRACT

BACKGROUND: Diabetes-related wound care is still a major issue due to chronic and hon-healing ulcers that are prone to infection and ultimately amputation. In recent years, cellular therapy (CT) products such as mesenchymal stem cells (MSC), platelet-rich plasma (PRP), and stromal vascular fraction (SVF) have been widely used. A combined cellular therapy (CCT) has not yet been tested as a triple combination, although its use alone and in dual combinations has been investigated. Probiotics (PB) accelerate healing by altering the intestinal microbiota. This study aims to examine the role of PB in enhancing the effects of CCT on diabetic wound healing.

METHODS: A 1×1 cm2 full-thickness cutaneous wound was created after administering 40 mg/kg streptozotocin intraperitoneally (STZ i.p.) to induce a diabetic (DB) animal model. Animals were divided into four groups: DB, DB+PB, DB+CCT, and DB+CCT+PB, each with six adult Albino rats. The wound edges were treated with a total of 300 µL of solution, consisting of 30 µL each of 100 µL 1×106 MSC, 100 µL SVF, and 100 µL PRP as CCT. PB was administered orally at a dose of approximately 200 mg daily. Histochemical analyses were performed using hematoxylin and eosin (HE) and Masson's trichrome (MT). Immunohistochemical analyses were conducted for endothelial nitric oxide synthase (eNOS), Caspase-3, interleukin-10 (IL-10), vascular endothelial growth factor (VEGF), and Collagen I. The intestinal microbiome was examined through metagenomic analyses of taxonomic structure.

RESULTS: Combined cellular therapy provided more effective and faster healing in DB animals. It was discovered that PB further accelerated this process, leading to greater improvement. CCT was observed to reverse high eNOS, Caspase-3, and IL-10 expression, as well as low VEGF and Collagen I levels. Moreover, PB therapy significantly enhanced the positive effects of CCT. CCT in combination with PB significantly improved wound healing by preventing oxidative stress, apoptosis, and inflammation, while promoting vascularization and collagen organization.

CONCLUSION: Probiotic support was considered important for diabetic wound healing and was suggested to improve patients' quality of life.

Keywords: Cell therapy; diabetic skin; rat; probiotics; wound healing.

INTRODUCTION

Diabetes-related wound ulcers are slow-healing and costly complications characterized by impaired vascularization, inflammation, oxidative stress, apoptosis, and reduced signaling that are important for healing. Mesenchymal stem cells (MSC), platelet-rich plasma (PRP), and stromal vascular fraction (SVF) are frequently used to treat these disorders, providing an effective therapeutic strategy.

Mesenchymal stem cells are vital for treatment due to the cellular support they provide, the growth factors they release, and the regenerative environment they create.[1] PRP has gained popularity in recent years, both for its growth factors and for the support it offers to MSC. SVF is widely used in

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Address for correspondence: Emine Müge Karakayalı
Department of Medical Microbiology, Izmir Democracy University Faculty of Medicine, İzmir, Türkiye
E-mail: mituglu@yahoo.com

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plastic surgery, where it has shown highly successful results in cellular regeneration and wound healing when administered alone or in combination with MSC.[2,3] However, the optimal treatment has not yet been fully defined. The role of probiotics (PB) in intestinal health has long been recognized.[4] Nevertheless, the literature provides no information on combined cellular therapy (CCT) with probiotic supplementation.[5,6] For diabetes-related (DB-related) skin wound healing, maintaining a healthy microbiota, tissue integrity, and absence of infection are crucial. The use of PB has been shown to be effective in addressing these atypical structural alterations, including epithelization, matrix formation, vascularization, leukocyte infiltration, and inflammation.

Immunomodulation, pathogen reduction, and tissue homeostasis are also involved.[7,8,9] MSC are important in regenerative medicine because they accelerate wound healing by increasing cell numbers, promoting vascularization, and reducing inflammation.[10,11] PRP has long been used in dermatology and plastic surgery.[5] It contributes significantly to wound healing by stimulating cell migration, proliferation, matrix formation, and vascularization, owing to its abundance of growth factors.[3,12] SVF is also considered highly useful for DB skin wound healing by providing support with diverse cell populations, including medium-sized fat cells.[13,14] Although there is evidence that their dual use as CCT enhances wound healing, the effects of their triple combination have not been thoroughly investigated.[15]

Combined cellular therapy improves wound healing in diabetic chronic wounds by reducing oxidative stress and apoptosis through the stimulation of vascularization and signaling pathways. Similar mechanisms underlie the effects of PB, but their contribution to CCT has not been fully recognized. The aim of this study is to determine the impact of combining CCT and PB to achieve more effective treatment and improved patient quality of life.

MATERIALS AND METHODS

Experimental Procedures

Animals

The Ethics Committee for Experimental Animal Research at Manisa Celal Bayar University (18/02/2020/77.637.435) approved the experiments. All experimental procedures were performed in accordance with the Institutional Animal Care and Use Committee of X Medical University (IACUC; No. 77.637.435), ensuring compliance with ethical guidelines for animal research. This study employed 24 healthy male Wistar albino rats, aged 8 weeks and weighing 250±50 g, obtained from the Manisa Celal Bayar University Experimental Animal Center. Sample size was determined using G-Power V.3.1 (Heinrich Heine University of Düsseldorf). The rats were housed in ventilated cages (21°C, 12-hour dark/light cycle) within a pathogen-free animal facility, with free access to food and water. The animals were randomly divided into four groups: Group 1, DB (n=6); Group 2, DB+PB (n=6); Group 3, DB+CCT (n=6); and Group 4, DB+CCT+PB (n=6). No anesthesia or analgesia was administered during the procedures, in accordance with the approved protocol and bioethical guidelines. Rats were to be euthanized before the planned end of the experiment if they showed severe signs of distress; however, no such incidents occurred.

Induction of Diabetes

A single dose of 55 mg/kg freshly dissolved streptozotocin (STZ, Sigma, St. Louis, MO) was administered intraperitoneally (i.p.) to all diabetes groups. Development of diabetes was confirmed in the experimental groups three days after STZ administration by measuring glucose levels in tail vein blood samples. According to Cil et al.,[1] rats with blood glucose levels 250 mg/dL were classified as diabetic.

Skin Defect

Two full-thickness skin defects measuring 1×1 cm^2 were created in each rat. CCT and CCT+PB were administered to the wound sites.[9]

Cellular Therapy Applications

Rat inguinal fat tissue was mechanically fragmented and transferred into the culture medium, where attached cells were proliferated and differentiated into MSC. Characterization for MSC surface markers was performed by immunohistochemistry (IHC), showing CD90 positivity (Ab44898, Abcam) and CD45 negativity (Sc2590, Santa Cruz), which were also used for SVF characterization. Rat inguinal fat tissues were collected and treated with collagenase enzyme before centrifugation at 1500 g for 5 minutes, and the stromal vascular fraction in the pellet was aspirated using a syringe and prepared for application.[2] Blood from a rat heart was drawn into a heparinized tube with a 1 cc syringe and centrifuged at 300 g for 5 minutes. After thrombin activation, the supernatant was centrifuged at 3000 rpm for 10 minutes and prepared for application. The final solution contained 100 µL 1×10^6 MSC, 100 µL SVF, and 100 µL PRP, applied to the wound edges as 30 µL per site.[16]

Probiotics

Until the completion of the experiment, probiotic preparation was administered daily via gavage. The formulation contained a mixture of non-infectious bacteria, including 2.5×10^9 Enterococcus faecium, 2.5×10^9 Lactobacillus acidophilus, 2.5×10^9 Lactobacillus rhamnosus, 2.5×10^9 Bifidobacterium longum, and 2.5×10^9 Bifidobacterium bifidum (NBL Probiotic Gold, Nobel-Farma NCFM®). This formulation was administered orally from the beginning of the trial until euthanasia, at a daily dose of 200 mg, corresponding to approximately 200,000-210,000 CFU (colony-forming units), given at the same time each morning.[9,17]

Histopathology

ImageJ was used to assess wound closure on images taken

for macroscopic evaluation on day 7 of treatment. Samples were collected and fixed in 4% formaldehyde. Coronal sections were then prepared from paraffin-embedded blocks and stained with hematoxylin-eosin (HE) and Masson's trichrome (MT). Images were examined digitally and analyzed using morphometric scoring. Skin epithelialization and dermal healing during wound repair were graded on a scale from 1 to 5.[18]

Immunohistochemistry

Immunohistochemical staining was performed as previously described.[2,10] Primary antibodies were used as follows: endothelial nitric oxide synthase (eNOS) for oxidative stress (Sc-654, Santa Cruz), Caspase-3 for apoptosis (sc-56053, Santa Cruz), interleukin-10 (IL-10) for inflammation (Sc-52560, Santa Cruz), vascular endothelial growth factor (VEGF) for vascularization (anti-VEGF; Ab1316, Abcam), and Collagen I for connective tissue (mouse monoclonal type IC, 2456-Sigma, USA). Samples were incubated overnight. Immunoreactivity was detected using a 3,3-diaminobenzidine (DAB) kit (Santa Cruz Biotechnology).

Formalin-fixed, paraffin-embedded tissues were cut into 4-µm sections. To inhibit endogenous peroxidase activity, deparaffinized and dehydrated slides were treated with 3% hydrogen peroxide (H_2O_2). Slides were then rinsed in phosphate-buffered saline with Tween-20 (PBS-T) and blocked with 1% bovine serum albumin. The avidin–biotin peroxidase system (Santa Cruz Staining System, ImmunoCruz sc-2051, Santa Cruz, CA) was employed for immunohistochemical analysis.

Staining intensity was evaluated in five separate microscopic fields and graded as weak (+), moderate (++), or strong (+++). For each intensity, cells were counted, and the corresponding score was calculated using the formula: H-Score=Pi (intensity of staining + 1), where Pi represents the percentage of stained cells at each intensity level (0-100%). H-scores were independently assessed by at least two blinded observers.[2,19]

TUNEL Assay

To detect apoptotic cell death, the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining technique was used. Following deparaffinization, TUNEL staining was performed according to the manufacturer's instructions using the ApopTag® Peroxidase In Situ Apoptosis Detection Kit (Millipore, Massachusetts, USA). After labeling, TUNEL-positive cells were identified and the data were statistically analyzed. The apoptotic index was used to determine apoptosis. Apoptotic cells were counted in five selected areas, and the apoptotic index was calculated using the following formula: (number of apoptotic cells / total number of cells)×100.[19]

Statistical Analysis

Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by post hoc multiple comparisons with GraphPad Prism 8 (San Diego, CA). Values of $p \leq 0.05$ were considered statistically significant.[19]

Polymerase Chain Reaction (PCR)

The goal of this experiment was to carry out metagenomic bacterial profiling on samples from four different groups. For this purpose, the 16S rDNA V3-V4 gene region was targeted for next-generation sequencing. PCR was performed as described in the referenced protocol [file:///Users/nemo/Downloads/RP21-086/RP21-086.html - fn1].

DNA Isolation

Stool samples were collected at the end of the experiment and frozen at -80°C within four hours of collection. Total DNA was extracted from fecal samples using the Stool Total DNA Purification Kit (Norgen Biotek Inc., Thorold, ON, Canada). As suggested by Klindworth et al.[20] and applied in this experiment, the primers selected were those with the highest efficiency. For library preparation in the current project, a two-step PCR procedure was adopted, following the referenced documentation https://support.illumina.com/documents/documentation/chemistry_documentation/16s/16s-metagenomic-library-prep-guide-15044223-b.pdf. KAPA HiFi HotStart ReadyMix (Roche) was employed, and 25 PCR cycles were performed independently for each sample. Distillation and purification were carried out at the end of both PCR stages using the Agencourt AMPure XP kit (Beckman Coulter).[20] Following isolation, DNA quantification was performed using Qubit (Thermo Fisher Scientific, Waltham, MA, USA). Library preparation and sequencing were conducted according to the manufacturer's protocols.

Sequencing

Bidirectional reading was performed using the iSeq 100 i1 Reagent Kit on the Illumina iSeq 100 next-generation sequencing platform (Illumina, CA, USA) by Gen-Era (Gen-Era Diagnostik, TR). This process was carried out in accordance with the manufacturer's instructions.[20]

Bioinformatic Analysis

Quality control was performed using FastQC [https://www.bioinformatics.babraham.ac.uk/projects/fastqc/] Trimmomatic tool [http://www.usadellab.org/cms/?page=trimmomatic (CTtp://www.usadellab.org/cms/?page=trimmomatic)] was used to remove low-quality base reads, adaptor contaminants, and chimeric sequences from the Genomes OnLine Database (GOLD). Reads were aligned to target organisms using the Greengenes database for taxonomic profiling with the Ribosomal Database Project (RDP) Classifier. Following alignment, operational taxonomic unit (OTU) groups were determined for each sample. Data reporting, statistical analyses, and data visualization were performed using R [https://www.r-project.org/] scripts (file:///Users/nemo/Downloads/RP21-086/RP21-086.html - fnref5).[21]

RESULTS

Diabetic Data

Body weights in the DB group (24.68±4.72 mg) decreased significantly ($p<0.05$) in the DB+PB (21.88±3.46 mg) and DB+CCT (18.25±3.34 mg) groups, with a very significant decrease ($p<0.01$) observed in the DB+CCT+PB group (16.44±4.76 mg) when baseline and endpoint measurements were compared.

Fasting blood glucose (FBG) levels in the DB group (351.68±25.48 mg/dL) were significantly lower in the DB+PB (325±25 mg/dL) and DB+CCT (325±25 mg/dL) groups ($p<0.05$), and very significantly lower in the DB+CCT+PB group (300±25 mg/dL) ($p<0.01$).

MSC Characterization

In MSC culture, MSC characterization markers showed positivity for CD90 (H-score: 254.22+23.92) and negativity for CD45 (H-score: 44.36±18.68) (Fig. 1).

Samples of Combined Cellular Therapy

High numbers of MSC were visualized in smear preparations of the SVF, MSC, and PRP combination used as CCT (Fig. 2).

Metagenomic Bacterial Profiling

Prevotella spp., Escherichia coli, and Barnesiella were found to be increased in DB samples. While Prevotella was reduced in DB+CCT, E. coli decreased, and Barnesiella was found to be high in this group. In the DB+CCT+PB group, Prevotella increased, while Barnesiella decreased and E. coli was completely eliminated. Bacteroides were reduced in DB but increased with the use of CCT and PB. Clostridium IV was detected in DB, but not in the CCT or PB groups. Additionally, Lactobacillus was absent in DB but present in both DB+CCT and DB+CCT+PB groups. Other notable groupings that were determined to have higher numbers, upon examination of their relative proportions, were Faecaliatea in DB, Ruminococcus in DB+CCT+PB, and Halothermothrix, Falsiporphmonas, and Alloprevotella in DB+CCT (Fig. 3a-3b).

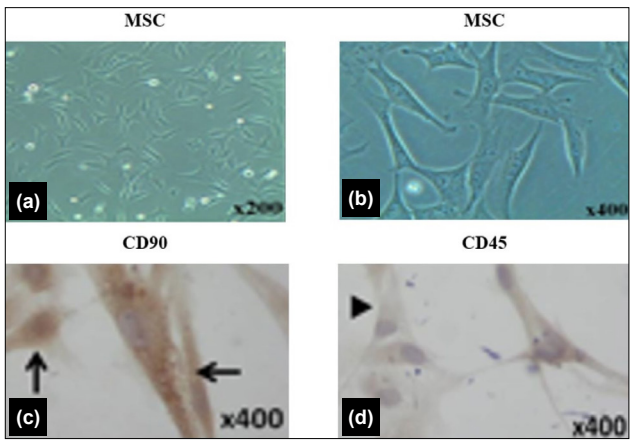


Figure 1. Phase-contrast images showing mesenchymal stem cells (MSC) attached, spread, and proliferated in culture **(a,b)**. Characterization was evaluated by CD90-positive (**c**) and CD45-negative **(d)** labeling using immunocytochemistry.

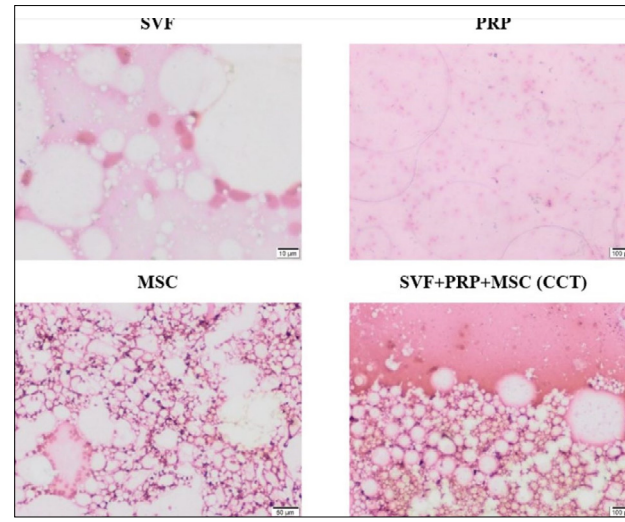


Figure 2. Light microscopy images of smear preparations from combined cellular therapy (CCT) samples.

Macroscopic Histopathology

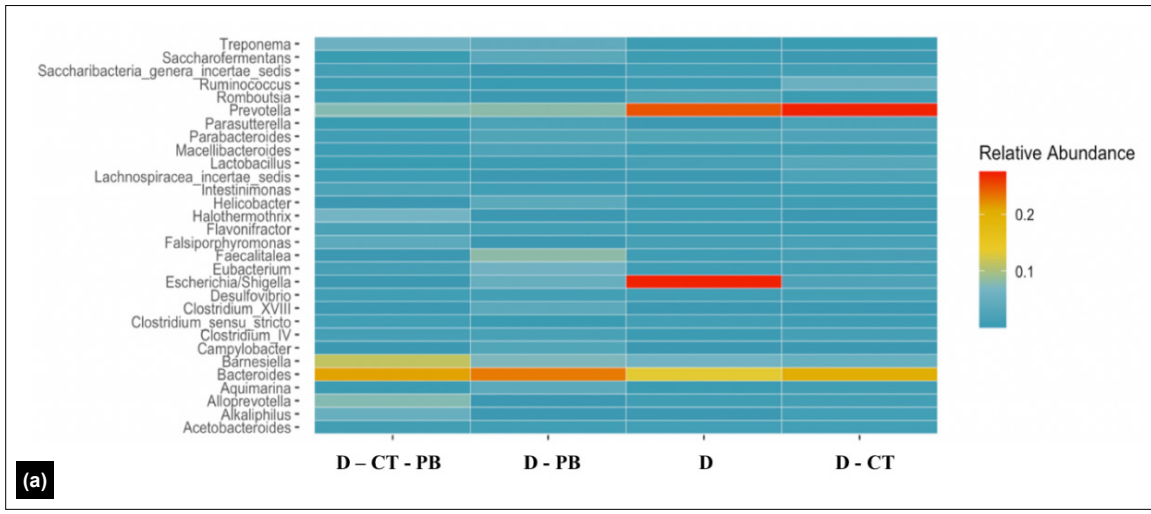
In the macroscopic assessment carried out with ImageJ, all groups showed differences at day 7 (Fig. 4). CCT and PB applications each had a significant effect ($p<0.01$), while their combination produced an even greater effect ($p<0.001$). In wound healing analysis, DB+CCT+PB was found to close wounds significantly ($p<0.05$) compared to DB+CCT.

Microscopic Histopathology

After microscopic examination, skin samples were stained with HE and evaluated for re-epithelization (RE), capillary count (CC), inflammatory cells (IC), fibroblast count (FC), collagen fibers (CF), and sebaceous glands (SG). CCT and its combination with PB significantly improved RE, SG, CC, and neovascularization. As a result of the inflammatory reaction, significant differences were observed in edema, congestion, and polymorphonuclear cell infiltration. Fibrosis was considerably lower in the DB+PB group compared to the DB group. Neovascularization and monocyte counts were found to be similar. Positive differences in edema, obstruction, polymorphonuclear activation, fibrosis, neovascularization, and monocytes were observed between the DB and DB+PB groups. The combination of CCT and PB was also found to improve RE, as shown in Figure 5.

MT staining revealed increased regenerative capacity of the extracellular matrix (ECM), demonstrated by properly structured, thicker, undulating collagen fibers and more extensive collagen deposition (Fig. 6). Additionally, the combination of CCT and PB significantly accelerated wound healing compared to either treatment alone ($p<0.001$).

Oxidative stress was evaluated by eNOS IHC (Fig. 7). In the DB group, CCT and its combination with PB produced highly significant effects ($p<0.001$). These applications reduced free



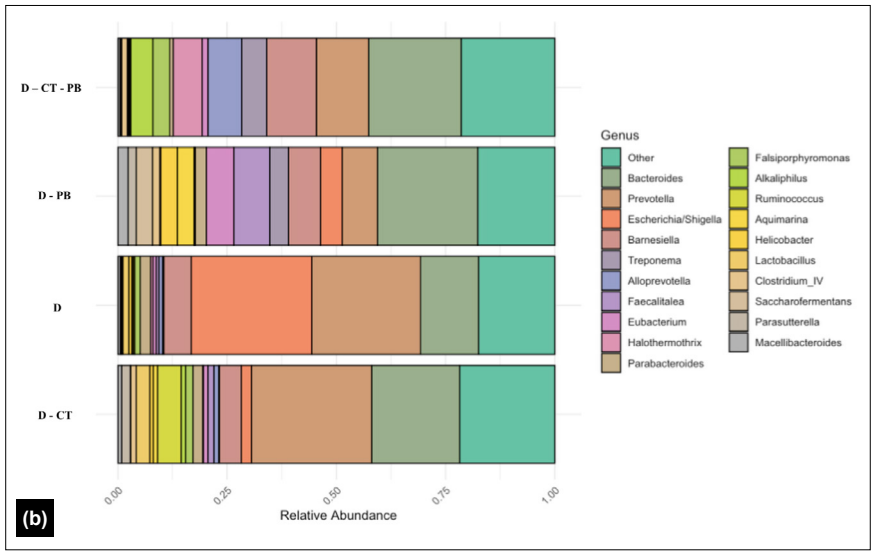
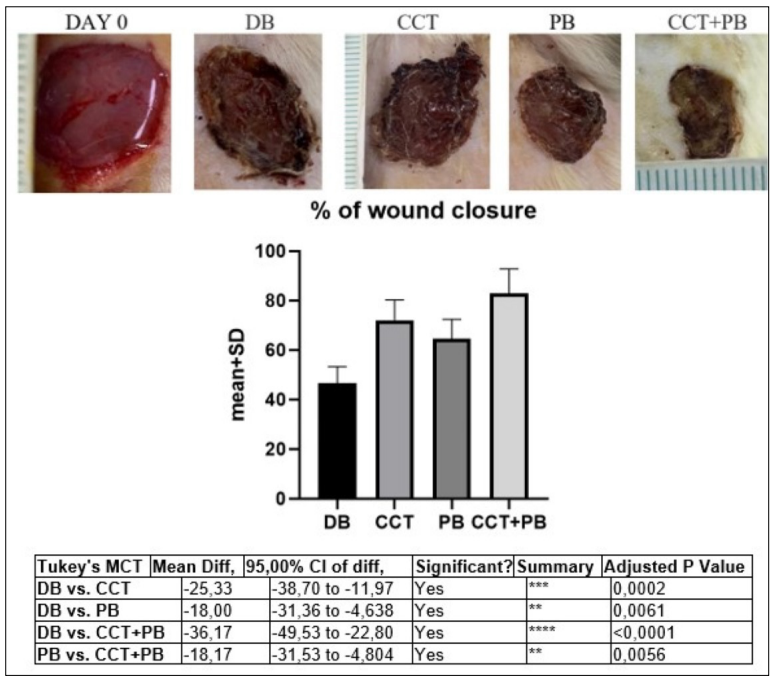
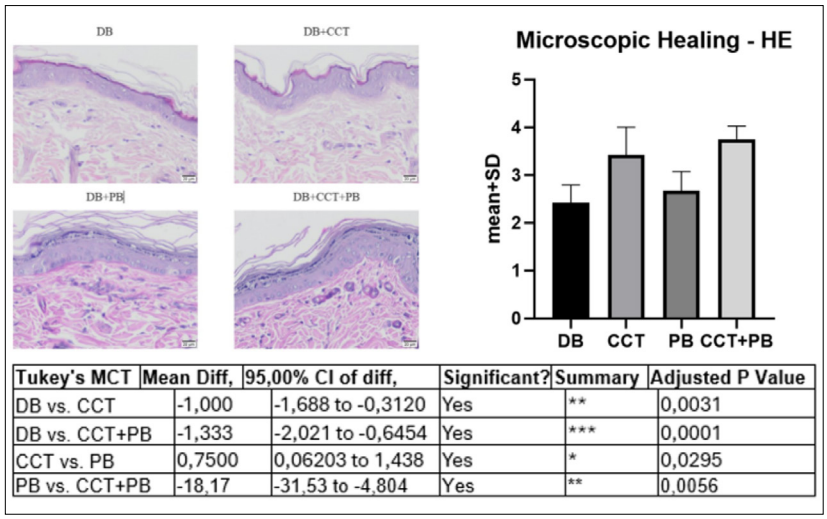


Figure 3. (a) Results of metagenomic analysis. **(b)** The top 30 genera with the highest prevalence (top) and the top 20 genera with the highest prevalence (bottom).



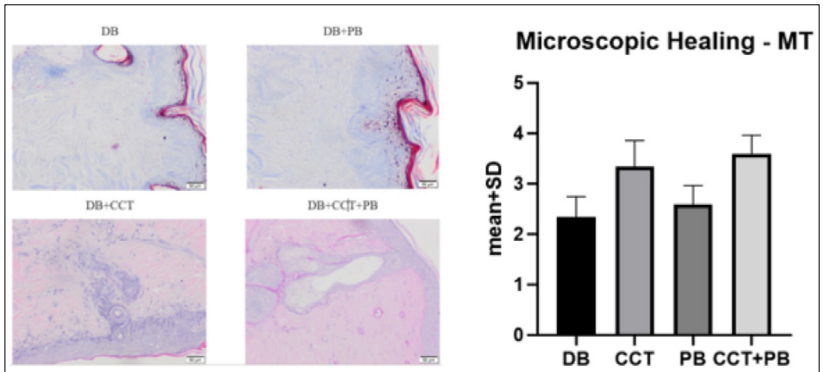
Tukey's MCT	Mean Diff,	95,00% CI of diff,	Significant?	Summary	Adjusted P Value
DB vs. CCT	-25,33	-38,70 to -11,97	Yes	***	0,0002
DB vs. PB	-18,00	-31,36 to -4,638	Yes	**	0,0061
DB vs. CCT+PB	-36,17	-49,53 to -22,80	Yes	****	<0,0001
PB vs. CCT+PB	-18,17	-31,53 to -4,804	Yes	**	0,0056

Figure 4. Macroscopic analysis of the contribution of probiotics (PB) to the effect of combined cellular therapy (CCT) on wound healing.



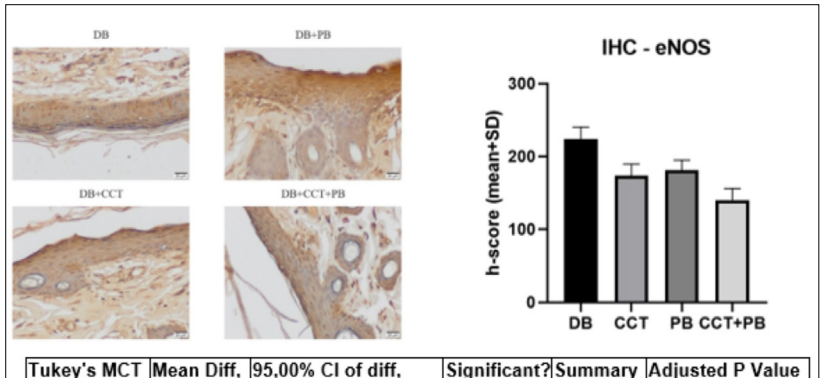
Tukey's MCT	Mean Diff,	95,00% CI of diff,	Significant?	Summary	Adjusted P Value
DB vs. CCT	-1,000	-1,688 to -0,3120	Yes	**	0,0031
DB vs. CCT+PB	-1,333	-2,021 to -0,6454	Yes	***	0,0001
CCT vs. PB	0,7500	0,06203 to 1,438	Yes	*	0,0295
PB vs. CCT+PB	-18,17	-31,53 to -4,804	Yes	**	0,0056

Figure 5. Microscopic analysis of the contribution of probiotics (PB) to the effect of combined cellular therapy (CCT) on wound healing by hematoxylin and eosin (HE) staining.



Tukey's MCT	Mean Diff,	95,00% CI of diff,	Significant?	Summary	Adjusted P Value
DB vs. CCT	-1,000	-1,684 to -0,3160	Yes	**	0,0029
DB vs. CCT+PB	-1,250	-1,934 to -0,5660	Yes	***	0,0003
CCT vs. PB	0,7500	0,06599 to 1,434	Yes	*	0,0285
PB vs. CCT+PB	-1,000	-1,684 to -0,3160	Yes	**	0,0029

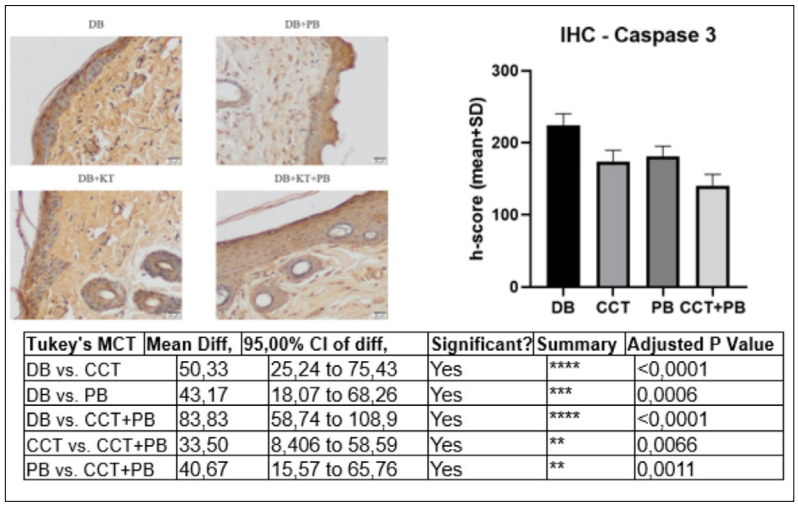
Figure 6. Microscopic analysis of the contribution of probiotics (PB) to the effect of combined cellular therapy (CCT) on wound healing by Masson's trichrome (MT) staining.



Tukey's MCT	Mean Diff,	95,00% CI of diff,	Significant?	Summary	Adjusted P Value
DB vs. CCT	50,33	25,24 to 75,43	Yes	****	<0,0001
DB vs. PB	43,17	18,07 to 68,26	Yes	***	0,0006
DB vs. CCT+PB	83,83	58,74 to 108,9	Yes	****	<0,0001
CCT vs. CCT+PB	33,50	8,406 to 58,59	Yes	**	0,0066
PB vs. CCT+PB	40,67	15,57 to 65,76	Yes	**	0,0011

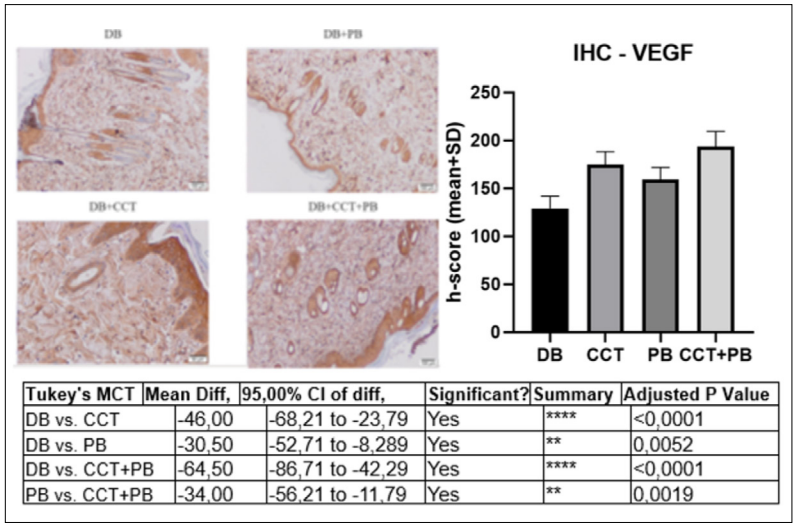
Figure 7. Microscopic analysis of the contribution of probiotics (PB) to the effect of combined cellular therapy (CCT) on wound healing by endothelial nitric oxide synthase (eNOS) immunolabeling.

radicals, which cause damage, thereby accelerating the wound healing process. Administration of CCT or PB alone significantly reduced oxidative stress ($p<0.05$), as determined by eNOS staining with H-score analysis. It was discovered that CCT or PB treatments alone significantly reduced oxidative stress ($p<0.05$). Additionally, we found that their combination



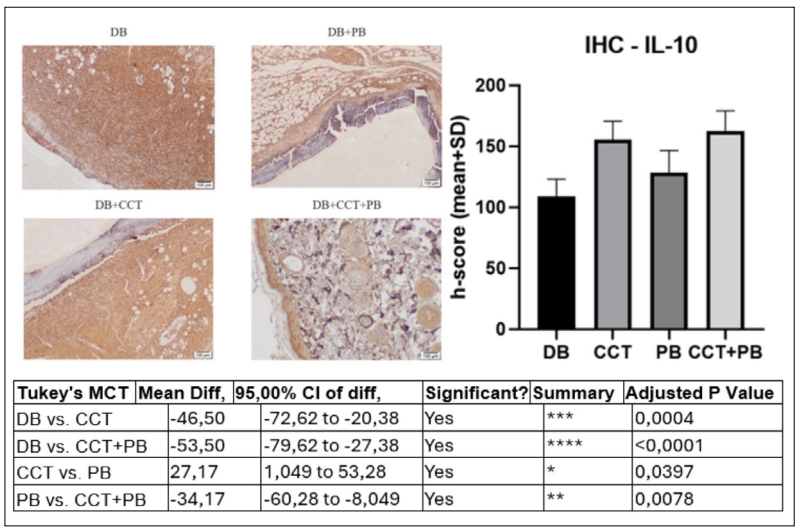
Tukey's MCT	Mean Diff,	95,00% CI of diff,	Significant?	Summary	Adjusted P Value
DB vs. CCT	50,33	25,24 to 75,43	Yes	****	<0,0001
DB vs. PB	43,17	18,07 to 68,26	Yes	***	0,0006
DB vs. CCT+PB	83,83	58,74 to 108,9	Yes	****	<0,0001
CCT vs. CCT+PB	33,50	8,406 to 58,59	Yes	**	0,0066
PB vs. CCT+PB	40,67	15,57 to 65,76	Yes	**	0,0011

Figure 8. Microscopic analysis of the contribution of probiotics (PB) to the effect of combined cellular therapy (CCT) on wound healing by Caspase-3 immunolabeling.



Tukey's MCT	Mean Diff,	95,00% CI of diff,	Significant?	Summary	Adjusted P Value
DB vs. CCT	-46,00	-68,21 to -23,79	Yes	****	<0,0001
DB vs. PB	-30,50	-52,71 to -8,289	Yes	**	0,0052
DB vs. CCT+PB	-64,50	-86,71 to -42,29	Yes	****	<0,0001
PB vs. CCT+PB	-34,00	-56,21 to -11,79	Yes	**	0,0019

Figure 9. Microscopic analysis of the contribution of probiotics (PB) to the effect of combined cellular therapy (CCT) on wound healing by vascular endothelial growth factor (VEGF) immunolabeling.

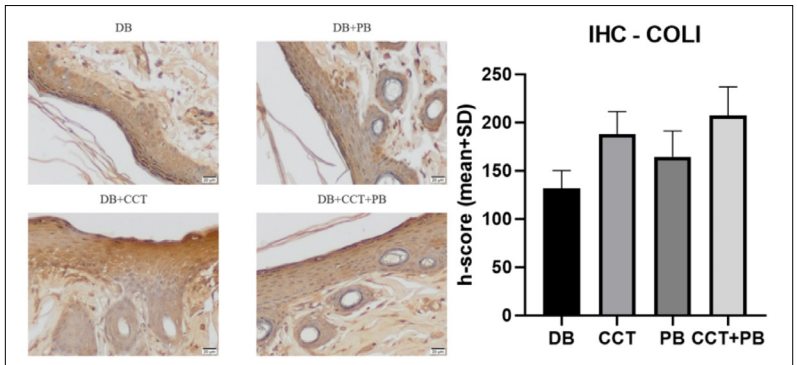


Tukey's MCT	Mean Diff,	95,00% CI of diff,	Significant?	Summary	Adjusted P Value
DB vs. CCT	-46,50	-72,62 to -20,38	Yes	***	0,0004
DB vs. CCT+PB	-53,50	-79,62 to -27,38	Yes	****	<0,0001
CCT vs. PB	27,17	1,049 to 53,28	Yes	*	0,0397
PB vs. CCT+PB	-34,17	-60,28 to -8,049	Yes	**	0,0078

Figure 10. Microscopic analysis of the contribution of probiotics (PB) to the effect of combined cellular therapy (CCT) on wound healing by interleukin-10 (IL-10) immunolabeling.

significantly decreased free radical levels ($p<0.05$).

Caspase-3 IHC was performed to assess apoptosis (Fig. 8). Both CCT and PB were shown to be significant in the DB group ($p<0.001$). H-score analysis of Caspase-3 staining showed that their combination produced an even greater reduction in apoptosis ($p<0.001$).



Tukey's MCT	Mean Diff.	95,00% CI of diff.	Significant?	Summary	Adjusted P Value
DB vs. CCT	-56,32	-96,44 to -16,20	Yes	**	0,0042
DB vs. CCT+PB	-75,34	-115,5 to -35,22	Yes	***	0,0002
PB vs. CCT+PB	-42,57	-82,68 to -2,450	Yes	**	0,0051

Figure 11. Microscopic analysis of the contribution of probiotics (PB) to the effect of combined cellular therapy (CCT) on wound healing by Collagen I immunolabeling.

When VEGF staining was evaluated using the H-score method (Fig. 9), CCT ($p<0.01$), PB ($p<0.05$), and their combination ($p<0.001$) were each observed to significantly increase vascularization. Labeling was confirmed in both newly formed and mature vessels.

IL-10 staining (Fig. 10) demonstrated that CCT ($p<0.01$), PB ($p<0.05$), and their combination ($p<0.001$) considerably reduced inflammation.

Collagen I labeling (Fig. 11) indicated that CCT ($p<0.01$) and PB ($p<0.05$) significantly improved matrix formation and increased H-scores. Their combination further enhanced connective tissue formation ($p<0.001$).

DISCUSSION

Diabetes-related chronic skin ulcer wound healing remains a significant issue. Growing evidence indicates that MSC, PRP, and SVF are effective treatment options.[22] PRP is effective due its high concentration of growth factors, MSC provide cellular support and secretions, and SVF is useful because of the medium-sized fatty acids it contains. Dual combinations of these products have been shown to exert synergistic effects on diabetic wounds.[23] Another product, probiotics, which has gained popularity in recent years, has also been found effective in diabetic wound healing.[24,25] In our study, the effects of these products on full-thickness wound healing, and the role of PB, were investigated using their triple combination as CCT. To date, no research employing this strategy has been reported in the literature. We found that CCT and PB applications provided faster and more efficient treatment. Furthermore, metagenomic analysis revealed that PB made a significant contribution to this effect. This was achieved through reductions in oxidative stress (via eNOS), apoptosis (via Caspase-3), inflammation (via IL-10), and fibrosis (via Collagen I), along with increased vascularization (via VEGF).

The diabetic model with poor healing capacity was shown to be consistent with other studies in the literature.[26] Wound closure was significantly greater with CCT at day 7, and PB therapy was found to significantly enhance this effect. Similarly, earlier studies reported that MSC application accelerated healing at 7 days.[27] In comparison with individual treatments, the combined use of MSC and PRP has also been shown to improve wound closure within a comparable timeframe.[28]

Another study investigating PB use demonstrated considerable and rapid recovery in a 2×2 cm^2 wound defect within seven days.[9,29,30] The results of our study align with relevant data in the literature, indicating that the contribution of PB to the effect of CCT was significant. The skin-microbiota interaction is vital for preventing infections, accelerating wound healing, and preventing scar tissue formation. L. rhamnosus, L. acidophilus, L. plantarum, L. casei, L. delbrueckii subsp. bulgaricus, and B. genera were identified as the most frequently used strains in 14 culture studies, eight animal studies, and 18 clinical studies in a prior meta-analysis. In culture investigations, these strains were found to exert effective pressure on wound pathogens, reduce wound infections in animal studies, and prevent infection at surgical wound sites in clinical studies.

They were also observed to accelerate wound healing in several studies. These characteristics are particularly important for PB use during antibiotic therapy.[31] Several mechanisms explain the role of PB in wound healing, including eliminating pathogenic bacteria, replacing pathogenic bacteria, protecting epithelial barrier integrity, promoting epithelial cell and fibroblast migration, modulating intraepithelial lymphocytes, natural killer (NK) cells, and macrophages, and increasing cytokine production.[32] In addition, our study revealed that the high E. coli levels observed in the DB group decreased following PB treatment. To maintain good dental health, the balance of oral microbiota is very important. A shift in this balance toward dysbiosis may delay wound healing by impairing the function of MSC. Probiotics appear to be effective in preventing bacteria-induced oral inflammatory diseases. An increase in Prevotella species in mucosal regions has been linked to periodontitis, bacterial vaginosis, rheumatoid arthritis, met-

abolic disorders, and low-grade systemic inflammatory diseases in human studies.[33] In our study, CCT was also found to reduce the number of Prevotella species in DB. A study combining adipose-derived MSC and PRP revealed that healing progressed more quickly and maturely through epithelial regeneration, matrix organization, connective tissue development, vascularization, and modulation of inflammation in diabetic skin. Histomorphometric analysis indicated that the primary recovery variables included increased epithelial thickening, reduced prolonged and severe inflammation, reversal of infiltration, and increased immune cell activity.[34]

Probiotic supplementation has also been shown to affect these variables, particularly by reducing infiltration and pathogenic bacteria.[9,35] The fact that the parameter values obtained in our study are comparable to those reported in the literature indicates that the combination of CCT and PB operates through these mechanisms. Lactobacilli and B. bacteria are effective PB for wound healing because they reduce oxidative stress and apoptosis. Furthermore, L. plantarum has been shown to prevent oxidative stress and apoptosis in pregnant rats with kidney and liver damage caused by endosulfan.[36] Lactic acid bacteria have also been found to promote keratinocyte proliferation through topical application and to accelerate re-epithelization by reducing pro-inflammatory cytokine release from keratinocytes. In addition, L. salivarius SGL 19 and L. fermentum SGL 10 demonstrated anti-pathogenic effects against S. aureus and S. pyogenes, while L. brevis SGL 12 and L. paracasei SGL 04 inhibited S. aureus and S. pyogenes, respectively.[37] P. aeruginosa impairs wound healing in burn-induced skin infections in animal models by triggering inflammation and cell death. This infection was inhibited by increased IL-18 cytokine levels, particularly in wounds treated with L. plantarum. L. plantarum interacts with fibroblasts, epithelial cells, and inflammatory cells to regulate a wide range of cytokines and chemokines. These interactions with pathogenic bacteria produce an anti-inflammatory effect. Additionally, Lactobacillus enhances phagocytosis of P. aeruginosa and provides protection against pathogen-associated apoptosis. These properties support the application of L. plantarum as a new generation treatment for burn wounds.[38] In a previous study, lactic acid bacteria and intestinal microbiota added to drinking water reduced wound healing time by half compared to control animals. Moreover, L. reuteri was shown to accelerate wound healing by upregulating oxytocin and decreasing fibroblast mortality through Caspase-3 regulation.[39]

Caspase regulation plays an important role in the apoptotic mechanisms of wound healing. In a previous study, lactic acid and intestinal microbiota added to drinking water reduced wound healing time by half compared to control animals.[39] In a rat wound healing study, Caspase-3 mRNA was shown to increase by day 3, Caspase-8 by day 5, and Caspase-9 at both time points. These expressions were observed in polymorphonuclear leukocytes (PMNL) and inflammatory mononuclear cells.[40] In another study, no skin wound healing was observed in mice deficient in Caspase-3 and Caspase-7, which are initiators of apoptosis.[41] In the follow-up of skin flap wound healing, Caspase-3 activity was assessed for apoptosis using the TUNEL technique. Increased levels of reactive oxygen species were shown to trigger Caspase-3 and promote apoptosis, while reperfusion was associated with increased Caspase-3 activity.[42] The results of our study revealed that apoptosis was prevented by CCT, and this effect was more pronounced with the addition of PB, findings compatible with the relevant literature. Fibrosis is prevented by anti-inflammatory cytokines, with IL-10 being a key factor in scar tissue prevention. IL-10 induces M2 macrophage polarization while shifting from glycolysis to oxidative phosphorylation. Thus, effective wound healing is ensured by decreasing M1 macrophage-related inflammation.

IL-10 also reduces pro-inflammatory cytokine IL-6 and contributes to healing by inhibiting macrophage inflammatory signals such as tumor necrosis factor-alpha (TNF-α). Furthermore, it prevents the formation of fibrosis and scar tissue by reducing collagen production through extracellular matrix regulation.[43,44] Our findings were found to be consistent with the literature.

Vascular endothelial growth factor is a growth factor family that promotes vascular and lymphatic growth and remodeling, increases vascular permeability, and recruits inflammatory cells to the wound site. Its topical application has been shown to be highly effective in diabetic wound healing and to positively stimulate stem cells. In addition to its local effects on wound healing, systemic effects have also been demonstrated in diabetic mice. VEGF-stem cell (VEGF-SC) interactions promote rapid healing by increasing the number of circulating stem cells and enhancing their mobilization from bone marrow to the wound site.[25,45,46] Alongside other growth factors, the use of PB in wound healing resulted in increased VEGF expression, which directs angiogenesis. VEGF, elevated from the onset of wound healing, aids in the acceleration and maturation of healing, particularly in diabetic wounds.[47-49]

Collagen produced by fibroblasts is another crucial factor in wound healing. Collagen fibers and their products are reduced in poorly healing wounds such as those in diabetes mellitus (DM).[50] In one experiment, PB administration was reported to increase collagen production along with angiogenesis, thereby accelerating and improving wound healing.[46] Additionally, full-thickness skin wound healing induced by PB supplementation in DM rats resulted in increased Collagen I levels that persisted from day 3 to day 10. This was accompanied by an increase in Collagen III. During the recovery phase, Collagen I was replaced by Collagen III, allowing for the development of more mature collagen.[9,39,46]

CONCLUSION

Diabetic wound care is still a major issue due to chronic and non-healing ulcers that are prone to infection and may ultimately lead to amputation. MSC, PRP, and SVF are cellular

therapy (CT) products that have recently gained popularity.[51-53] Because of the alterations they cause in the intestine, PB hasten wound healing. The aim of this study was to investigate the role of PB in diabetic wound healing when used together with CCT. PB supplementation to CCT, which was highly effective, was shown to be advantageous based on morphological, histological, immunohistochemical, and metagenomic analyses.

This contribution was achieved by reducing apoptosis and oxidative stress, supporting the development of a thicker and better-organized matrix, and promoting vascularization. It is suggested that routine clinical practice may be an appropriate setting for the use of PB as a treatment for slow-healing wounds. Furthermore, it was emphasized that this contribution might be crucial for hastening wound healing and improving patients' quality of life.

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DENEYSEL ÇALIŞMA - *ÖZ*

Diyabetik sıçanlarda deri yarasında kombine hücresel tedaviye probiyotiklerin katkısı

AMAÇ: Diyabetik (DB) yara bakımı, kronik ve iyileşmeyen, enfeksiyona yatkın ve ampütasyona yol açan ülserler nedeniyle hala çok önemli bir sorundur. Mezenkimal Kök Hücreler (MKH), Trombositten Zengin Plazma (TZP) ve Stromal Vasküler Fraksiyon (SVF), son yıllarda yaygın olarak kullanılan Hücresel Terapi (HT) ürünleridir. Tek başlarına ve ikili kombinasyonlarda kullanımları üzerine çalışmalar olmasına rağmen, üçlü kombinasyon olarak Kombine Hücresel Terapi (KHT) çalışılmamıştır. Probiyotikler (PB), bağırsakta yaptıkları mikrobiyata değişiklikler ile iyileşmeyi hızlandırır. Bu çalışmada, KHT ile oluşan DB yara iyileşmesinde PB katkısının incelenmesi amaçlandı.

GEREÇ VE YÖNTEM: DB model, erişkin Albino sıçanlarda 40 mg/kg STZ i.p. uygulanmasıyla oluşturuldu. 1x1 cm^2 tam kat kutanöz yara yapıldı. Denekler, DB, DB+PB, DB+CCT ve DB+ CCT+PB olmak üzere her biri 6 yetişkin içeren gruplara bölündü. CCT olarak, 100 µL $1x10^6$ MSC, 100 µL SVF ve 100 µL PRP içeren toplam 300 µL solüsyonun her biri 30 µL olacak şekilde yara kenarlarına uygulanması ile yapıldı. PB, yaklaşık 200 mg/günlük dozlarda oral yoldan uygulandı. Histokimyasal analizler HE ve MT ile gerçekleştirildi. İmmünohistokimyasal analizleri için eNOS, Kaspaz 3, IL10, VEGF ve Kolajen I kullanıldı. PB için taksonomik yapı fekal metagenomik analizi ile gösterildi.

BULGULAR: DB yaranın CCT ile daha etkin ve hızlı kapandığı görüldü. PB ile bu sürecin hızlandırıldığı ve daha etkin iyileşme sağlandığı saptandı. Ayrıca KHT ile yüksek eNOS, Kaspaz 3 ve IL-10 ekspresyonlarının azaltıldığı ayrıca düşük VEGF ve Kollajen I tersine çevrildiği gözlendi. PB uygulamasının KHT ile oluşan bu olumlu etkilerine anlamlı bir katkı sağladığı gösterildi. PB katkısı ile KHT oksidatif stresi, apoptozu ve inflamasyonu önleyip vaskülarizasyon ve kollajen organizasyonunu artırarak yara iyileşmesini önemli ölçüde geliştirdi.

SONUÇ: DB yara tedavisinde KHT ile PB katkısının önemli olduğu ve hastanın kötü yaşam kalitesini düzeltebileceği düşünüldü.

Anahtar sözcükler: Diyabetik cilt; kombine hücre tedavisi; probiyotikler; yara iyileşmesi.

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