ASSESSMENT OF THE EFFECT OF LOW-LEVEL LASER THERAPY (808nm) ON THE PROLIFERATION AND MIGRATION OF FIBROBLASTS FROM CHRONIC WOUND TISSUE

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SUMMARY

Objectives: *1/To evaluate the morphology and proliferation of cultured dermal fibroblasts derived from patients with chronic wounds. 2/Evaluating the effect of low-level laser therapy (LLLT) 808nm on the proliferation and migration of fibroblasts from chronic wound tissue.*

Subjects and Methods: *A prospective study was conducted on 36 dermal samples from 12 patients with pressure ulcers and diabetic ulcers. Dermal samples were taken in the operating room and fibroblasts were isolated according to the procedure of Freshney RI (2003). Fibroblasts obtained in P3 generation will be cultured on 6 plates and divided into groups of laser irradiation with different energy levels and a control group (without laser). Conduct LLLT projection with energy levels of 5J; 4J; 3.5J; 3J; 2.5J and exposure times were: 60, 48, 42, 36, the 30s, respectively, for 3 consecutive days to evaluate proliferation and migration between the Laser group and the control group. Cells were counted at 24 h after the last laser exposure using the trypan blue experiment.*

Results: *The wound base fibroblasts (position 1) proliferated slowly, showed signs of aging, did not retain their phenotype, died floating on the surface of the culture plate, and could not trypsin to the P4 generation. Fibroblasts at positions 2, and 3 (wound margins and healing skin adjacent to the wound) could be isolated to the P3, P4, and P5 generation and did not change morphology. After LLLT irradiation, the number of cells in the laser group with energy levels: 3.5J; 3J, and 2.5J increased higher than the control group; with the highest increase at the energy level of 3J. LLLT dose of 3J with a corresponding exposure time of 36s increased the migration rate of fibroblasts when compared with the control group, completely covering the culture plate on 3rd day.*

Conclusion: *Fibroblasts derived from patients with pressure ulcers and diabetic ulcers can be isolated from the wound edge and healed skin adjacent to the wound without changing morphology when cultured. After LLLT irradiation (808 nm) on isolated fibroblasts, the effect was dose-dependent. The 3J dose did not change fibroblast morphology; or induce biostimulation, proliferation, and migration of cultured fibroblast samples derived from chronic wound patients.*

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***Keywords**: Low-level laser therapy, fibroblasts, chronic wound.*

I. INTRODUCTION

Chronic wounds tend to increase with the increase in life expectancy and chronic medical diseases. In 2017, chronic wounds affect 5.7 million Americans with an annual cost of $20 billion. This is a burden on society because it impacts not only economically but also significantly affects the quality of life of patients and their families, causing pain, psychological disorders, social isolation, hospitalization, and even death [1].

Currently, there are many methods of wound treatment, both traditional and modern. However, the actual process of treating many wounds, including chronic wounds, is still a challenge. Research to find new treatments to support traditional methods is also a director in the future. And low-level laser therapy is a new approach to the treatment of wounds, which has been proven by many documents to be a promising method. This study evaluates the effectiveness of laser at the cellular level, studying two basic processes of fibroblasts in wound healing: Proliferation and migration to the wound. This is a modern research model that helps to elucidate the mechanism of action of a wide range of drugs and topical wound therapies [2].

The study aims to: 1/ Evaluate the morphology and proliferation of cultured dermal fibroblasts derived from chronic wound patients. 2/ Evaluation of the effect of LLLT (808nm) on the proliferation and migration of fibroblasts from chronic wound tissue.

II. SUBJECTS AND METHODS

2.1. Subject

**** Criteria for selecting study patients:***

- 06 patients with pressure ulcers and 06 patients with diabetic ulcers were treated as inpatients at the Wound Healing Center - Le Huu Trac National Burn Hospital.

- Age: Aged 18 years and older.

- Grade IV

*** Exclusion criteria:** Patients with infectious diseases, hepatitis, and inflammation at the biopsy site.

2.2. Research material

**** Tools and equipment:***

- Device B-Cure Laser Pro (Good Energies®, Israel) maximum power 250mW, pulse frequency 13 kHz

- CO_2 incubator, inverted microscope, optical microscope, erythrocyte counting chamber, centrifuge, sterile air filter hood, refrigerator -80, -20, 4^0C, biopsy equipment.

**** Chemicals and consumables:***

- Dulbecco's Modified Eagle's Medium (DMEM), Fetal Bovine Serum (FBS), Trypsin/EDTA 0.25%, Phosphate Buffered Saline (PBS), Pettri, culture plate, plastic pipette, Eppendorf tube and consumable chemicals other consumption.

2.3. Research Methods: Prospective research

2.3.1. Clean the skin before surgery to remove tissue and preserve tissue samples

Shower the whole body from the day before surgery and clean the skin area right before surgery.

- Wash the skin area with medical soap (Microshiedl).

- Rinse with sterile filtered water.

- Disinfect with Betadine 10%.

- Disinfect with alcohol 70% by volume.

- Wash with 0.9% saline.

- Blot dry

2.3.2. Tissue biopsy to isolate fibroblasts

Perform biopsies with a sterile scalpel No. 15 at 3 locations: position 1 (wound base), position 2 (wound edge), and position 3 (healed skin adjacent to the wound) with sample size: 0.5 × 0.5cm [3]. Preserve tissue samples in 5% AB 1X DMEM solution and store them in the refrigerator until fibroblasts are isolated and purified.

2.3.3. Tissue culture, isolation, and preservation of fibroblasts

Isolation of NBS according to the procedure of Freshney RI (2003) developed based on the tissue culture method of Harrison (1907) and Carrel (1912) [4].

Wash the skin sample 3 times with PBS solution, and cut off the fat.

- Cut the skin into small pieces, each piece is 2mm in size.

- Inoculate 20 skin samples into bottles with an area of $25cm^2$ with 1ml of culture medium.

- Place the bottle in an incubator at 37^0C with 5% CO_2 in the air.

- After 2 days, add to the bottle 1ml of the medium.

- After 3 - 5 days, add medium to reach 5ml of culture at the time of inoculation.

- Then change the medium every 3 - 4 days, follow up when the cells reach at least 50% of the surface area of the bottle, then separate NBS from the skin sample and multiply the number of NBS by using the transfer procedure. Trypsin/EDTA in the amount of $0.1mL/cm^2$. Select NBS from the 3rd subculture to store in deeply cold conditions, cell preservation medium is DMEM and 10% DMSO.

2.3.4. Determination of the number, survival rate and quality of cells

**** Count the number of cells in the Neubauer counting chamber***

To determine the number of cells to inoculate at the correct rate and evaluate the cell's ability to multiply. Count the number of cells under the microscope per unit area equal to $1mm^2$.

The number of cells was calculated by the formula: C = n/v (n: number of cells counted in the counting chamber; v: counting volume (ml); C: cell concentration (cells/ml).

In the Neubauer counting chamber there is a volume of $0.1mm^3 = 1.10^{-4}$ ml so the formula is: **$C = n \times 10^4/ml$**

**** Determination of survival rate of cultured fibroblasts***

Using the trypan blue staining method of the author Kaltenbach et al [5] to evaluate the number of cells in the culture medium after applying LLLLT. This test provides a direct assessment of the total number of viable cells in the samples because the trypan blue dye can only penetrate the porous, permeable membrane of fatally damaged cells, which is detectable. under an optical microscope.

- Collect cell suspensions in culture bottles using trypsin and centrifugation.

- Mix 1 drop of cell suspension with 1 drop of 0.4% trypan blue.

- The solution was incubated for 5 min so that the trypan blue dye could pass through the cytoplasmic membrane of nonviable cells, changing their color to blue. The solution was then taken to a hemocytometer and examined with an inverted microscope to determine the total number of cells and non-viable cells were captured in blue.

- The number of viable cells was calculated by subtracting the number of non-viable cells from the total number of cells. Number of cells/ml = n × 10^4

- The percentage of viable cells in the sample is determined as follows:

Percentage of living cells (%) = (Number of living cells/Total number of cells) x 100%.

**** Evaluation of cell quality***

Monitor cell shape, cytoplasmic status and cell nucleus by examining cells on an inverted microscope, optical microscope at 40 - 400X magnification. The senescence state of the cells was determined by each transfection, with the characteristic: Elongated cells, and loss of characteristic shape. Sparse cell populations do not grow vortex despite changing the medium many times. The number of cells decreases gradually, there are many cell fragments, and the cytoplasm contains vacuoles.

2.3.3. Procedure to evaluate the effect of LLLT on fibroblast proliferation

P3 generation dermal fibroblasts were inoculated in plates at a density of 5 × 104 TB/well and maintained in DMEM growth medium supplemented with 10% FBS and 1% AB 1X, maintained in a 37°C incubator with 5% CO_2. Cells were cultured on a 6-well plate, divided into a control group (no laser) and an LLLT group (808 nm) with energy levels of 5; 4; 3.5; 3, 2.5J, and the corresponding projection time is 60, 48, 42, 36, the 30s; irradiated daily for 3 consecutive days. 24 h after the last irradiation, count the cells using the Trypan blue test [6].

Evaluation criteria include the number of cells obtained, from which the optimal energy level is found. Cell structure morphology was assessed by inverted microscopy.

2.3.4. Procedure to evaluate the effect of LLLT on fibroblast migration

Evaluation of the effect of laser on the migration process of fibroblasts is done through the experiment of scraping in an in vitro wound model through the following steps: P3 generation fibroblasts are implanted in a plate at high density. 5×10^4 TB/well and maintained in a 37^0C incubator with 5% CO_2 until 90% coverage was achieved. A sterile 5 ml pipette tip was used to scratch the surface of in vitro wound cultures in two groups: the control group and the laser irradiation group with similar widths to evaluate the effect of the laser on migration. the population of fibroblasts. After every 24 h, change the medium, irradiate the laser, observe the migration of cells at the edge of the incision to the center to fill the gap under the microscope, and take pictures [6].

Evaluation criteria are cell morphology, complete healing time of scraping and the number of cells obtained.

III. RESEARCH RESULTS

3.1. Some patient characteristics and ulcers

Table 3.1. Distribution of patients according to age and duration of ulcers

Characteristics	Pressure ulcer group (n = 6)	Diabetic ulcer group (n = 6)
Patient age (years)	55.66 ± 21 (22 - 84)	63.5 ± 13.5 (44 - 77)
Ulcer duration (weeks)	6.83 ± 3.3 (4 -12)	6.5 ± 3.67 (2 - 12)

Comment: The average age of diabetic ulcer patients was higher than the pressure ulcer group, and the mean ulcer duration in both groups was over 6 weeks.

Table 3.2. Location of the ulcer

Position of the ulcer	Pressure ulcer group (n = 6)	p (%)	Diabetic ulcer group (n = 6)	p (%)
Sacrum	6	100%		
Lower extremities			5	83.3%
Body area			1	16.67%

Comment: Pressure ulcers are more common at the sacral site, and diabetic ulcers are common in the lower extremities.

3.2. Isolation and proliferation of fibroblasts

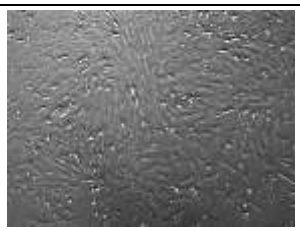
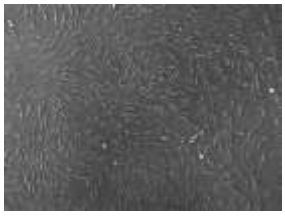
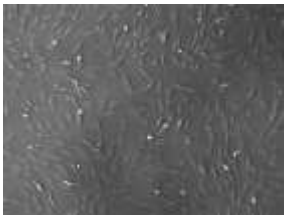
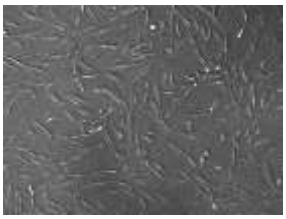
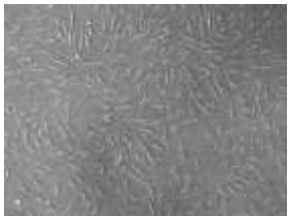
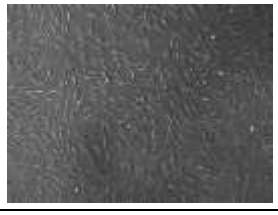
	Location 1	Location 2	Location 3
Fibroblasts grow from dermal samples			
Fibroblast proliferation			
Generation P1			
Generation P2			
Generation P3			

Photo 3.1. Tissue samples in a culture of fibroblast isolates of patient Nguyen Thi Bich H.44T. Medical record number: 4430

Comment:

- Location1: Growing mainly endothelial cells, with few fibroblasts that proliferate slowly, do not retain the shape of a rhombus, cells die a lot and have much debris floating on the surface of the culture plate. In the P3 generation, the cell does not proliferate and cannot trypsin to the P4 generation.

- Location 2, 3: Fibroblasts proliferate strongly, scattered with some dead cells at position 2, cell morphology is rhombic, belonging to the type of cells that adhere to the surface of the culture plate and just create a single layer, no overlap is seen. After 4 days to 6 days, reach 80% - 90% of coverage and trypsin can multiply to P4, P5 generation.

Table 3.3. The rate of fibroblast growth and isolation over generations at sites

	Location 1	Location 2	Location 3
Fibroblast growth rate	100%	100%	100%
Generation P1	83.3%	91.7%	100%
Generation P2	41.7%	91.7%	100%
Generation P3	0%	83.3%	91.7%

Comment: 100% of the samples at all 3 sites had fibroblasts. However, when trypsin passes through generations, there is a great change. Position 1 proliferates the least, by the P3 generation, there are not enough cells left to trypsin to P4. Cells at position 2,3 can trypsin to the P4 and P5 generation. Fibroblasts in position 3 proliferate the best, with little aging and death.

Table 3.4. Time of growth and isolation through generations (days)

Time (day)	Location 1 (n = 12)	Location 2 (n = 12)	Location 3 (n = 12)	p
Average days of fibroblast growth	6.5 ± 2.19	5.42 ± 1.73	4.33 ± 1.56	p1/2 = 0.249 p2/3 = 0.106 p1/3 = 0.022
Time of fibroblast isolation (Passage 1)	10.3 ± 3.4	11.55 ± 6.4	10.75 ± 5.22	p1/2 = 0.695 p2/3 = 0.286 p1/3 = 0.462
Time of fibroblast isolation (Passage 2)	9.4 ± 4.33	6.27 ± 1.95	7.33 ± 3.89	p1/2 = 0.246 p2/3 = 0.755 p1/3 = 0.392
Time of fibroblast isolation (Passage 3)		5.7 ± 1.33	5.36 ± 2.73	p2/3 = 0.219

Comment: Fibroblasts in 3 locations all grew, fastest at position 3 (healed skin next to the wound) with an average growth time of: 4.33 days. Position 1 fibroblasts (wound background) grow the slowest with an average growth time of: 6.5 days. The

difference between 1 and 3 is significant with p = 0.02.

- Up to the P1, P2 generation, there was no difference in the isolation time of the 3 sites

- By the P3 generation, only positions 2 and 3 could be isolated from fibroblasts and there was no difference between the two positions with p > 0.05.

3.3. Evaluation of the effect of LLLT on cultured fibroblast proliferation

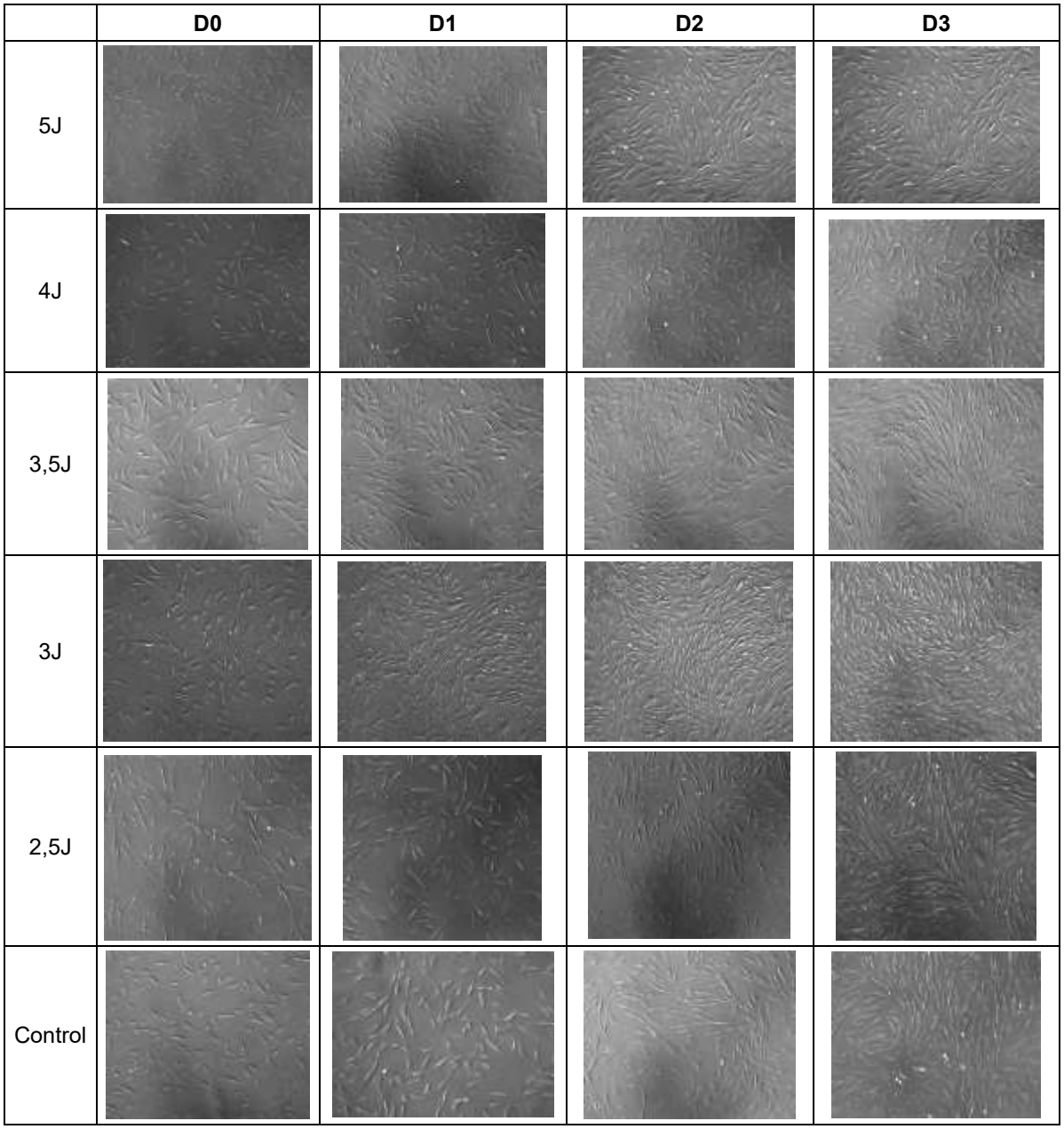


Photo 3.2. Proliferation images of fibroblasts after LLLT irradiation with different energy levels at different time points before irradiation (D0), 1 day after irradiation (D1), 2 days (D2), 3 days (D3)

Comment: The cell morphology after laser irradiation remained unchanged and the cell density at the energy level of 3J was the highest.

Table 3.5. The number of cells measured after LLLT irradiation with different energy levels by trypan blue test

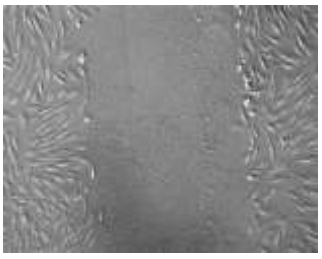
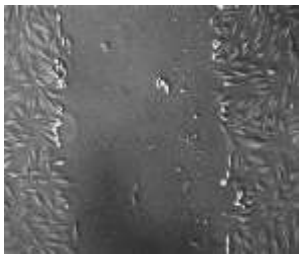
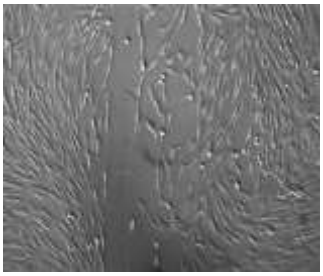
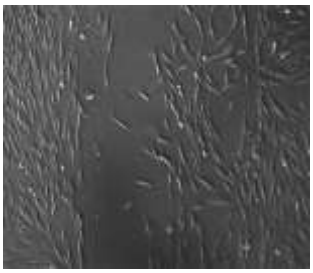
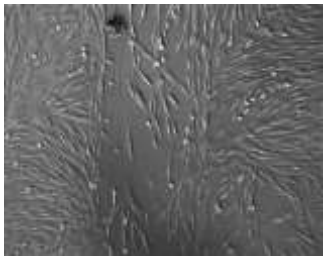
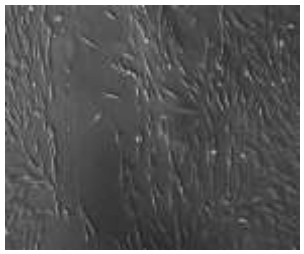
Average cell count	Energy levels					Control
	5J	4J	3,5J	3J	2,5J	
	$4.25x10^5$±0.44	$4.38x10^5$±0.44	$5.04x10^5$±0.39	5.03 $x10^5$±0.32	4.88 $x10^5$±0.38	4.35 $x10^5$±0.46
p			p = 0.076	p = 0.047	P = 0.076	

Comment:

- With the energy level of 4.5J: no difference was observed between the irradiated group and the control group, even at the dose of 5J, the average number of cells had a slight decrease, the 5J energy level may cause inhibition cells.

- With a dose of 2.5; 3; 3.5J: the number of cells increased more than the control group, the highest increase was at 3J with $p < 0.05$.

3.3. Evaluation of the effect of laser on cultured fibroblast migration

	LLLT (3J)	Control
D0		
D1		
D2		

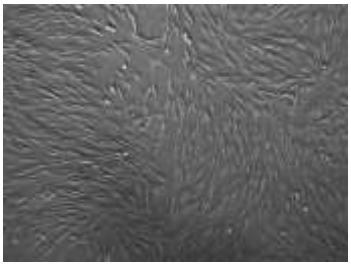
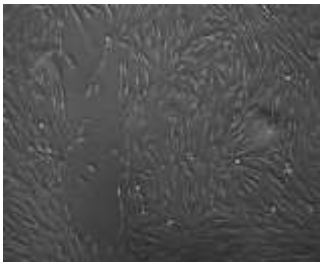
D3		
Average cell count	$4.93x10^5$±0.49	$3.9x10^5$±0.65

Photo 3.3. Image of migrating fibroblasts between the LLLT 3J projection group and the control group

Comment:

D1: Fibroblasts in the two groups started to migrate into the experimental wound.

D2: The migration of fibroblasts into the experimental wound was thicker in the laser group than in the control group.

D3: Fibroblasts completely covered the experimental wound in the laser group but did not completely cover the control group.

After trypsin counting cells: The number of cells obtained in the laser group was more than in the control group

IV. DISCUSS

Studies evaluating chronic wound therapies on fibroblasts are currently still mainly performed on healthy fibroblasts because many studies have shown that fibroblasts from chronic wounds typically reduced proliferation and premature aging [7].

Models to study wound fibroblasts (such as diabetic fibroblasts) are often generated in vitro rather than using fibroblasts derived from diabetic ulcer patients themselves. Therefore, the successful isolation of chronic wound-derived fibroblasts is the basis for experimental studies on cultured cells that can help evaluate potential therapy on target wound cells. Authors around the world have also successfully isolated fibroblasts from venous ulcers, diabetic ulcers and pressure ulcers [3, 8, 9]. We successfully cultured and isolated 36 skin samples from 12 patients with pressure ulcers and diabetes, none of which were contaminated with wound preparation before tissue collection.

Among 36 fibroblast dermal samples, at position 1, the phenotype could not be maintained, in the process of trypsin through generations, there was aging and floating death on the surface of the culture plate until the P3 generation did not have enough cell density for trypsin to reach P4. Fibroblasts in the healing skin adjacent to the wound (position 3) and the wound edge (position 2) have no change in morphology, have a rhombus shape, are cells that adhere to the surface of the culture plate, and create single class only. Cells at positions 2 and 3 have the good proliferative ability and can be isolated to the P3, P4, P5 generation.

In the study of Harold Brem et al. [10], when isolated fibroblasts from 4 tissue samples of patients with venous ulcers were also found, fibroblasts still retained their phenotype and fibroblasts from healed skin adjacent to the wound showed the best response, cells from the wound

base showed moderate response and cells from the wound edge showed a minimal response.

Research on fibroblasts from chronic wounds is the basis for conducting research and testing new therapies, including LLLT. In this study, we conducted laser irradiation on fibroblasts with different energy levels to find the optimal dose with the potential biostimulation effect of LLLT on fibroblasts, which is directly related to the wound healing process. The results showed a significant increase in the number of cells after LLLT irradiation with doses of 2.5; 3 and 3.5J/cm^2, in which the 3J dose is the most optimal ($p < 0.05$). This result is also consistent with the study of the author Basso et al [6, 11] evaluating the impact of LLLT on the proliferation and migration of human gingival fibroblasts also found an energy dose of 3 J/cm² is optimal. A cell culture review by AlGhamdi, K.M., A. Kumar, et al. [12] showed that LLLT doses of 0.5 to 4.0 J/cm^2 enhanced the proliferation rate of cell lines. cell.

The migration and proliferation of fibroblasts are events necessary for tissue healing. In this study, the effect of LLLT on the migration capacity of fibroblasts, using an optimal 3J energy level, was evaluated by an in vitro wound healing assay. The results showed that the experimental wound in the laser group was completely covered by day 3, while the fibroblasts in the control group had migrated into the experimental wound but did not completely cover it. The number of cells obtained in the laser group was higher than in the control group. The results demonstrate that the LLLT can increase the migratory capacity of fibroblasts.

Kreisler et al. [13] also reported an increase in cultured fibroblasts in vitro after direct and sequential LLLT irradiation. The study of Ma et al. [14] also showed that 830 nm laser irradiation on normal fibroblasts increased collagen proliferation and synthesis. These results are consistent with the results of several studies that demonstrated that LLLT can induce a positive effect on cells by increasing growth factor expression [15, 16].

V. CONCLUSION

Chronic wounds caused by pressure ulcers and diabetic ulcers can be isolated from fibroblasts at the wound edges and in healing skin adjacent to the wound without changing morphology when cultured. Low-level laser therapy (808 nm) with 3J energy level did not change cell morphology; induce biostimulation, proliferation and migration of cultured fibroblast samples derived from chronic wound patients.

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