EVALUATING THE CHANGEMENT OF HYDROXYPROLINE (HYP) IN CHRONIC WOUND TISSUE AFTER TREATMENT WITH PLATELET - RICH PLASMA

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ABSTRACT

***Background and aims**: Proline hydroxylation is essential for collagen synthesis in wound healing. Therefore, hydroxyproline (Hyp quantification may be a suitable marker of wound healing in chronic wound tissue.*

***Material and method**: This is a descriptive longitudinal study including 30 referral patients from Wound Healing Center, National Burn Hospital, from May 2019 to May 2020. Thirty patients with 33 chronic wounds were was injected subcutaneously inside and around the periphery of the chronic wound. 500mg of skin tissue from the chronic wound before and after PRP treatment were taken for the measurement of Hyp levels at the first and second of follow-up.*

***Results**: Mean Hyp concentration in wound granulation tissue after 2 weeks of PRP treatment (3.75 ± 1.15pg/mg) was significantly higher than mean concentration in the skin before PRP treatment (2.43 ± 1.01pg/mg).*

***Conclusion**: Our results showed that PRP therapy had a beneficial effect on wound healing of the chronic wound through the stimulation to increase Hyp.*

***Keywords**: Chronic wound, wound healing, hydroxyproline (Hyp).*

1. INTRODUCTION

In recent decades, the number of people suffering from impaired wound healing has increased. Approximately 1 - 2% of the population of the United States and Europe is affected by chronic wounds, requiring a financial commitment of 2 - 4% of total health budgets from governments and an average of 6000 - 10,000 EUR per patient per annum [1-3].

Chronic wounds are often characterized by increased levels of inflammatory cells that are associated with elevated levels of proteases; these appear to degrade the ECM components, growth factors and receptors that are essential for healing. The extracellular matrix (ECM) is the largest component of the dermal skin layer and the synthesis of ECM is a key feature of wound healing. Proline and its structural derivative hydroxyproline (HYP)

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constitute the triple collagen chain structure that forms the main collagen.

Collagen plays an important role in the tissue repairing steps acting as the main extracellular scaffolding in normal individuals. Reduction in HYP levels in tissue may therefore alter the structure of collagen, compromising its structural integrity and consequently, delaying the repair process. Therefore, biochemical quantification of tissue HYP content may be a viable approach in predicting the outcome of the wound healing process in chronic wounds. In regards to the importance of HYP in repairing steps, in some studies, suppression of high HYP concentrations as collagen deposition led to control of stricture in caustic esophagus models [4]. On the contrary, positive effects of collagen synthesis enhancement on healing reflected by HYP content, through inducing substances such as epidermal growth factor [5], nitric oxide [6], L-arginine [7], hyperbaric oxygen, extracellular matrix products [8] and global heat [9] have been shown. The aim of this study is the measurement and comparison of hydroxyproline quantities in chronic wounds before and after autologous PRP treatment.

2. METHODS

2.1. Patients and inclusive criteria

Thirsty patients of age 18 - 65 years ailing from chronic wounds of > 4 weeks (such as pressure ulcers, venous ulcers, arterial ulcers, or diabetic foot ulcers) were enrolled into the study, who were admitted to Wound Healing Center, National Burn Hospital and were treated with autologous PRP from May 2019 to May 2020.

Inclusion criteria: After examined inclusion (non-infected wound and full-thickness without exposure of bone, ligaments, or tendons.

Exclusion criteria: Pregnant women, patients with severe cardiovascular disorder and patients with a bleeding disorder were excluded. Individuals with systemic disease or history of anticoagulant, immunosuppressive were excluded. We too excluded patients with leprosy patients, liver cirrhosis, HIV positive status.

2.2. Preparation of Platelet Rich Plasma (PRP)

PRP was prepared using an advanced rapid point-of-care technology, the New-PRP^{pro} Kit (Genne World Corp., Vietnam) at the patient's bedside. 28ml Blood and anticoagulant were thoroughly mixed before transferring to the processing device, to prevent the formation of blood clots. The aspirated whole blood was then processed using the New-PRP^{pro} Kit processing device at the patient's bedside. The device works by separating peripheral blood into three distinct layers; erythrocytes settle at the substratum, above that the plasma layer containing a rich concentrate of platelets (PRP) and platelet-poor plasma (PPP) as the top layer. After centrifugation, 7 - 8ml of PRP was harvested from the processing device using an aseptic technique.

2.3. Treatment procedure

The chronic wounds were firstly debrided to remove any necrotic and infected tissues and the wound bed was cleaned thoroughly with betadine solution. Based on the wound size and bed, autologous PRP solution was injected subcutaneously inside and around the periphery of the chronic wound. An antiseptic agent dressing was used to cover the wound area (Betablast Silver, Aquacel Ag...). The dressing was changed on day 3 post-treatment; the wound was irrigated with normal saline and assessed for the presence of any form of infection.

Following which the autologous PRP injection was frequently performed once a week and for 3 weeks.

2.4. Parameters studied

The wound biopsy was performed before PRP injection (T0) and at the first (T1) and second week (T2) of studied progress for Hydroxyoriline estimation.

Tissue preparation for Hydroxyproline estimation

We based on the modified H.Stegemann (1967) to quantify Hydroproline

- Principles: We completely hydrolyzed to convert collagen of skin sample into amino acid by HCl 6N. Then oxidizing Hydroxyproline (Hyp) (Hyp contributes to 57% of total amino acids in collagen) by Chloramine T (~ 7%) in citrate buffer to obtain Pyrol derivatives. Finally, the Pyrol derivatives are mixed with para dimethylamino benzaldehyde to form the color solution with 560nm maximum absorption wavelength.

- Hydrolyze 2-3mg tissue sample by HCl 6N at 115^0C in 24 hours

- Evaporate acid with water in a cabinet with a temperature higher than 200^0C.

- Prepare the standard solution of Hydroxyproline: Retrieved 5mg oxaprozin and entered in 100ml of distilled water to get 0.05mg/ml oxyproline solution (S1), then take out 5ml (S1) and add 20ml of distilled water to obtain 0.01mg/ml Hyp solution.

- Concentration of Hyp in tissue samples will be calculated by the formula:

Hyp = A x 20 / m (mg/g tissue)

Statistical Analysis

Results for each biopsy were pooled. Where appropriate, results are presented as the mean ± the standard error of the mean. The data were analyzed by ANOVA and Duncan test using SPSS software. The level of significance was assumed to be $P < 0.05$.

3. RESULTS

3.1. Patients and chronic wound characteristics

Thirty patients were treated with PRP injections around the wound periphery. Among the included patients, 21 (70%) were males and 9 (30%) were females with a mean age of 49.52 ± 12.38 years old. These patients had 33 wounds and their positions were extremities (10 (30.3%)), sacrum (21(63.63%)), head (1 (3.03%)) and back (1 (3.03%)). Wound size mean was 55.62 ± 29.81 cm^2 (min-max: 25-108) (Table 1). Additionally, among the ulcers treated, there were 3(10%) venous ulcers, 5 (16.67%) diabetic ulcers, 20 (66.67%) pressure ulcers and 2 (6.67) others. The duration of the chronic wounds presented by the patients' pre-treatment ranged from 4 to 12 weeks with a mean duration of 6 weeks (Table 1).

Table 1. Duration of the wound persisted before PRP treatment wound position and wound size

Duration (weeks)	No. of Patients (n)	Percentage (%)
4 - 6	10	33.33
7 - 9	17	56.67
9 - 12	3	10
Wound position		
Extremities	10	30.3
Sacrum	21	63.63
Back	1	3.03
Head	1	3.03
Wound size (n = 33)	X±SD	Min-max
	55.62 ± 29.81cm^2	25 - 108

3.2. Hydroxyproline concentration after PRP therapy

The mean concentration of Hyp before PRP treatment was 2.43 ± 1,01pg/mg (min-max: 0.27 - 5.42). The mean concentration of Hyp in the tissue of chronic wounds after 1 week and 2 weeks under PRP treatment was 2.87 ± 1.26 pg/mg (min-max: 0.56 - 5.48) and 3.75 ± 1.15pg/mg (min-max: 0.62-5,98) as shown in (Table 2). The final result of hydroxyproline concentration comparisons by the ANOVA test is also shown in (Table 3). Accordingly, as the achieved F ratio = 7.817 was greater than the critical level of F distribution in the conventional chart and extracted p-value = 0.05, there was a certain difference between before PRP treatment and after PRP treatment 2 weeks regarding Hyp concentration. Regarding statistic results, the effect size was 0.152. Post hoc comparisons to evaluate pairwise differences among group means were conducted with Tukey's HSD test. Tests revealed the significant pairwise difference was between before and after PRP treatment 2 weeks.

Table 2. Comparison of the mean concentration of Hyproxyproline before and after PRP treatment

Time	n	Mean	Standard Deviation	Standard Error	95% Confidence interval for mean		Min	Max
					Lower Bound	Upper Bound		
T0	30	2.43	0.52	0.087	2.23	2.61	0.27	5.42
T1	30	2.87	0.61	0.104	2.66	3.08	0.56	5.48
T2	30	3.75	1.09	0.122	3.1	4.03	0.62	5.98
Model	Fixed effect		0.95	0.092	2.96	3.75		
	Random effects			0.208	2.12	5.24		

Table 3. The final result of ANOVA analysis of the mean Hyp concentration before and after PRP treatment

	Sum of Squares	df	Mean Square	F	Sig
T0 - T2	257.98	2	128.12	7.817	0.05
T0 - T1	1435.50	87	16.05		
Total	1693.76	89			

4. DISCUSSION

Hyp quantity, matrix metalloproteinases (MMP), tissue inhibitors of MP (TIMP) and also the ratio of MMP/TIMP [9,10] measured in the healing steps are some examples. We preferred to study Hyp concentration because the main collagen composition of the healing tissue consists of it. It has been demonstrated that Hyp deposition is impaired in chronic wound healing [11].

Platelets are produced in the bone marrow and have growth factors that are the main factor in healing and tissue return. Growth factors are stored in alpha granules and once released; induce cellular growth, proliferation and differentiation in different cells. Thereby this event leads to an increase in collagen, elastin, intracellular matrix, vascularity and tissue healing. Platelet activation and aggregation, in addition to accelerating coagulation, provide a bolus of secreted proteins and α-granule contents to the immediate area, all of which help initiate and accelerate the inflammatory response by the host.

Examples of such secreted proteins include arachidonic acid metabolites, heparin, serotonin, thrombin, coagulation factors (factor V), adhesive proteins (fibrinogen and von Willebrand factor), plasma proteins (immunoglobulin-γ and albumin), cell growth factors (platelet-derived growth factor (PDGF), platelet-derived angiogenesis factor, transforming growth factor-α(TGF-α), TGF-β and basic fibroblast growth factor (bFGF)), enzymes (heparinase and factor XIII) and protease inhibitors (plasminogen activator inhibitor-1, α2-macroglobulin and α2-antiplasmin).

Following platelet-induced hemostasis and release of TGF- β1 and PDGF, the formation of granulation tissue is facilitated by chemotaxis of neutrophils, monocytes, fibroblasts and myofibroblasts, as well as by synthesis of new extracellular matrix (ECM) and neoangiogenesis [12]. In response to wounding, the fibroblasts migrate into the wound bed and initially secrete collagen type III, which is later replaced by collagen type I. Synthesis and deposition of these collagens by fibroblasts is stimulated by factors including TGF- β1, -β2 and - β3, PDGF, IL-1α, -1β and -4, and mast cell tryptase. Once sufficient collagen has been generated, its synthesis is stopped; thus, during tissue repair, production, as well as the degradation of collagens, is under precise spatial and temporal control [12].

In this study, we found that there is a significant difference in collagen content, expressed as hydroxyproline level, between before Autologous PRP treatment and after 2 weeks of treatment ($p = 0.05$). There was no difference between Hyp concentration in the chronic wound before PRP treatment and after a week of PRP treatment ($p = 0.63$).

5. CONCLUSION

In conclusion, PRP therapy had a beneficial effect on wound healing of the chronic wound through the stimulation to increase Hyp.

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