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ASSESSMENT OF HYDROXYPROLINE LEVELS IN NON-ISCHEMIC DIABETIC FOOT ULCERS DURING RECOVERY. A PROSPECTIVE CASE-CONTROL STUDY

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Abstract

Background and aims: *Proline hydroxylation is essential for collagen synthesis in wound healing. Therefore, hydroxyproline quantification may be a suitable marker of wound healing in diabetic tissue.* ***Material and method:*** *This is a prospective–case-control clinical study including 90 referral patients from our clinics in Golestan hospital affiliated to Jundishapour Medical University, Ahwaz-Iran, during a period of 18 months. Three groups were recruited: intervening diabetics with non-ischemic foot ulcers, diabetics without foot ulcers (normal diabetics) and non-diabetics without foot ulcers (normal non-diabetics) as control groups (n=30 per group). 500 mg of granulation tissue from ulcers after treatment and 500 mg of normal skin from both control groups were taken for the measurement of hydroxyproline levels.* ***Results:*** *13 (43.3%) males and 17 (56.7%) females in trial group were analysed. There was no significant differences in age, gender, and BMI between groups. Mean hydroxyproline concentration in wound granulation tissue (140.44 µg/g) was statistically different from the mean concentration in the skin content of normal diabetics (173.22 µ g/g,) and the skin content of non-diabetics (178.83 µg/g) (p=0.001). There were no statistically significant differences between the mean values of normal diabetics and non-diabetics (p=0.63).* ***Conclusion:*** *Our results showed the presence of a lower quantity of hydroxyproline in diabetic patients with foot ulcers compared to control groups. This raises the issue of its effectiveness in delaying the repair process in diabetics. Therefore, compensating for tissue hydroxyproline deficit can be a clue in improving diabetic tissue repair.*

key words: *diabetic foot ulcer, wound healing, hydroxyproline.*

Background and aims

Diabetes mellitus is the most common endocrine disease. Fifty percent of patients, in spite of good control, suffer from diabetic foot - a condition which results in over 50% amputation rate and 50% mortality rate within three years post amputation [1]. The risk of amputation associated with diabetes is forty folds higher compared to non-diabetics, and the possibility of counterpart foot amputation during the latter three years is 30 percent [2]. Apart from microangiopathy and accompanied circulatory deficits, failure of healing process

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due to collagen deposition imparement is supposed to be applicable. Proline and its structural derivative hydroxyproline (OHP) 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 OHP 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 OHP content may be a viable approach in predicting the outcome of wound healing process in diabetic foot ulcers. In regards to the importance of OHP in repairing steps, in some studies, suppression of high OHP concentrations as collagen deposition led to control of stricture in caustic oesophagus models [3]. On the contrary, positive effects of collagen synthesis enhancement on healing reflected by OHP content, through inducing substances such as amino acid mixture [4], zhuhuang frost [5], epidermal growth factor [6,7], nitric oxide [8], L arginine [9], hyperbaric oxygen [10-13], extracellular matrix products [10] and global heat [14] have been shown.

Although many deficiencies such as the lack of cellular responses to inflammatory mediators, growth factors, cytokines, angiogenesis and matrix turnover contribute to failure to heal [15], it seems that collagen products are the main factors involved. Therefore, collagen content deficiency points out the necessity of healing acceleration for foot ulcer improvement and better management, especially in diabetics in whom the promotion of healing is impaired [16].

The aim of this study is the measurement and comparison of hydroxyproline quantities in non-ischemic diabetic foot ulcers during repair with normal skin of other diabetics and normal skin of non-diabetics.

Materials and methods

We designed a prospective case-control study, which included 30 diabetic patients with severe and infected foot ulcers that were referred to our surgical clinics in Ahwaz Jundishapour University of Medical Sciences, Golestan Hospital in Iran, for a period of 18 months. Additionally, for control groups, we included 30 normal diabetic patients without ulcer and 30 normal non-diabetic patients who both underwent aesthetic operations like abdominoplasty with tissue resection. We analysed samples from these resections, considering ethics. All patients gave their written informed consent prior to commencement of the study.

Confirmed diabetic patients admitted for management of non-ischemic foot ulcers in the form of infected-gangrenous ulcers, and clinically good toe capillary filling (≤2 seconds), with at least a palpable dorsalis pedis pulse were included in the study. Surgical debridement, antibiotic therapy, daily scrub and washing until control of ulcer toward healing and granulation formation along with blood glucose control by medical team have been scheduled and performed. The tissue sample collection was carried out by collecting a fixed 500 mg of granulation tissue which had filled the cavity of healing ulcer (5×5×10 mm), 500 mg of normal diabetic patient's full-thickness skin from aesthetic resected sites (3×5×10 mm), and 500 mg of normal full-thickness skin of non-diabetics during their elective aesthetic surgery from their tissue resections. The quantity of 500 mg of tissue was considered for convenience of calculations as µg/g of hydroxyproline and less tissue resections.

Samples were preserved in tube glasses containing 6-Normal hydrochloric acid solution. After the preparation, the hydroxyproline level was measured and calculated in the samples

using spectrophotometry. This method was previously described by Edwards and O'Brien [17].

Excluding criteria were diabetic patients with pulseless peripheral arteries, diabetics with ischemic wounds or pure gangrene who were candidates for amputation, diabetics were required non-surgical management and who were under corticosteroids or immune suppression therapy. Body mass index (BMI), blood glucose three times a day, quantities of daily insulin, duration of diabetes, duration of wound evolution before referral and admission, duration of wounds recovery, age, gender, and other demographic features were also considered as variables in intervening diabetics with foot ulcers.

Statistical analysis

Data was analysed using SPSS software version 13 (IBM, NY, USA). For analysing the difference in the mean of continuous variables between the groups we used ANOVA and t test. Post hoc analysis was performed through Tukey's HSD, Tamhane's post hoc test and Games-Howell test. We considered the level of significance α of 0.05.

Table 1. Demographic status and conditions of groups included in the trial.

Variables		Min	Max	Mean	SD
Total Diabetics -With foot ulcers (n=30) -Normal diabetics (n=30) (34 males, 26 females)	Age (years)	33	80	56.87	12.72
	Blood Glucose (mg/dl)	112	276	193.87	45.35
	BMI (kg/m^2)	19	33	25.43	3.26
Diabetics with foot ulcers 13 males, 17 females n=30	Duration of diabetes (years)	1	30	11.10	6.98
	Duration of wound recovery With treatment (days)	7	65	15.30	10.16
	Duration of wound involvement Before referral and admission (weeks)	1	30	6.13	5.80
Normal Non-diabetics n=30	Age (years)	30	80	52.50	11.34
	Blood Glucose (mg/dl)	67	105	89.63	9.39
	BMI (kg/m^2)	19	30	24.80	2.96

Min (minimum), Max (maximum), SD (standard deviations).

Table 2. Hydroxyproline concentrations (µg per gram of tissue) in the 3 study groups.

Groups	No.	Min	Max	Mean	SD	SEM
Diabetics with ulcers	30	81.23	198.15	140.44	30.18	5.51
Normal non-diabetics	30	98.31	292.47	178.83	51.80	9.45
Normal diabetics	30	96.89	285.00	173.22	36.80	6.72
Total	90	81.23	292.47	164.17	43.62	4.59

Min (minimum), Max (maximum), SD (standard deviations), SEM (standard error of mean).

Results

From 30 intervening diabetic patients, 13 cases were males (43.3%) and 17 females (56.7%). Eight cases had wounds in the right lower extremity and 22 patients in the left lower extremity. There were no cases of upper extremity involvement. Patient characteristics for each group are presented in Table 1.

Unpredictable, because of more prevalent higher BMI attributed to diabetics, in our study there was no significant difference in mean BMI between diabetics (25.43 kg/m^2) and control groups (24.8 kg/m^2) (p=0.672).

The differences in hydroxyproline concentration are shown in Table 2.

The mean concentration of skin OHP in the normal non-diabetic control group was 178.83

µg/g (**95% CI** 98.31-292.47). The mean concentration of OHP in granulation tissue of repairing ulcers in intervening diabetics was 140.44 µg/g (81.23 –198.15), and for skin of normal diabetics as the other control group, was 173.22 µg/g (96.89 –285) as shown in Table 3. Final result of hydroxyproline concentration comparisons by ANOVA test is also shown in Table 4. 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 = 0.001, there was a certain difference between the intervening and other control groups regarding OHP concentration.

Table 3. Comparison of the mean concentration of hydroxyproline in all groups using ANOVA.

Groups		n	Mean	Standard Deviation	Standard Error	95% Confidence interval for mean		Min	Max	Between-Component Variance
						Lower Bound	Upper Bound			
Diabetics with wounds		30	140.44	30.18	5.51	129.17	151.72	81.23	198.15	
Normal non-diabetics		30	178.83	51.80	9.45	159.49	198.18	98.31	292.47	
Normal diabetics		30	173.22	36.80	6.72	159.48	186.97	96.89	280.00	
Total		90	164.17	43.62	4.59	155.03	173.30	81.23	292.47	
Model	**Fixed effects**			40.62	4.28	155.66	172.68			
	Random effects				11.97	112.66	215.68			374.96

Table 4. Final result of ANOVA analysis of the mean hydroxyproline concentrations between and within groups.

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	25798.25	2	12899.12	7.817	0.001
Within Groups	143554.50	87	1650.05		
Total	169352.76	89			

df (degree of freedom), F (F Ratio), sig (significance).

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 diabetics with ulcer, the intervening group and the other control groups of normal diabetics and normal non-diabetics.

Discussion

One of the most unpleasant complications for a diabetic patient is the chronic, resistant, slow progressing and delayed healing ulcers. This can be associated with life threatening infections, usually accompanied by bone deformity and silent fractures, known as diabetic Charcot foot. Since the characteristic findings of these feet are successive ulcers, many factors are supposed to be effective in its control. Social functioning and mental health are the two popular and important dependent factors have shown to affect the healing process of ulcers [18] and vice versa. Nevertheless, other documented and practically predictive factors in healing of foot ulcers also have been introduced and deserve to be considered.

OHP quantity, matrix metalloproteinases (MMP), tissue inhibitors of MP (TIMP) and also the ratio of MMP/TIMP [19,20] measured in the healing steps are some examples.

We preferred to study OHP concentration, because the main collagen composition of the

healing tissue consists of it. It has been demonstrated that OHP deposition is impaired in wounds healing in type 1 diabetes [21], and healing differs between diabetics with and without concomitant peripheral arterial disease [22]. Our patients had type 2 diabetes and had no prior peripheral arterial disease or any other ischemic conditions expected to affect their ordinary healing process. We found that there is a significant difference in collagen content, expressed as hydroxyproline level, between granulation covered diabetic healed ulcer, and the healthy skin samples of the other control groups formed by normal non-wounded diabetic patients and normal non-diabetics (p=0.001). There were no difference between OHP concentration in the healthy skin of diabetics and normal non-diabetic samples (p=0.63).

Low collagen concentrations (decreased OHP), prompt one of the probable causes of delayed ulcer repair in diabetics, independent of gender and BMI. Consequently, we advocate the usage of approved available drugs and applications shown to be effective on increasing tissue OHP concentrations. These, are postulated to promote and induce healing acceleration by some studies; for instance, local Tetracycline 3% [23] and growth factor. Hyperbaric oxygen tents also have been effective. Besides, increasing patient's optimism and quality of life have further been generative. Mental depression and low quality of life are associated with poor ulcer prognosis [18,24].

The advantages of our study can be defined as finding a defference that guides us toward recognizing a compensatory deficit which may be useful for better management of simple, non-complex wounds of diabetic patients and prevent their progression to amputation. On the other hand, limitations of our study are represented by the unability to generalize it to all diabetic wound conditions, it requires complementary approval for finding the real equity of HOP amongst the other deficiencies for diabetic wounds repair and multi factorial causes of ulcer formation.

Conclusion

Our study identified different hydroxy proline tissue contents between deffective healing diabetic ulcers compared to non-diabetics and other diabetics without foot ulcers. Therefore, we would conclude that OHP tissue compensation can be a novel therapy in acceleration of wound repair and prevention of progressive diabetic wounds.

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