



## Study of Association of Hypoxia Inducible Factor 1 Alpha In Wound fluid with Clinical Outcome in Diabetic patients

**First and Corresponding Author:** Dr Sanjith Kamath N, Post graduate Trainee, Department of General Surgery, Justice K S Hegde Medical Academy, Deralakatte Mangalore, INDIA.

**Email:** [sanjithkamath34.sk@gmail.com](mailto:sanjithkamath34.sk@gmail.com)

**Second Author:** Dr Praveen M Pawar, Assistant Professor, Department of General Surgery, Justice K S Hegde Medical Academy, Deralakatte Mangalore, INDIA.

**Email:** [praveenmpawar2@gmail.com](mailto:praveenmpawar2@gmail.com)

**Third Author:** Dr Lancelot Lobo, Professor, Department of General Surgery, Justice K S Hegde Medical Academy, Deralakatte Mangalore, INDIA.

**Email:** [drl\\_lobo@yahoo.com](mailto:drl_lobo@yahoo.com)

**Fourth Author:** Dr Prakash Patil, Senior Scientist - G1, Central Research Laboratory, Justice K S Hegde Medical Academy, Deralakatte Mangalore, INDIA.

**Email:** [prakashpatil@nitte.edu.in](mailto:prakashpatil@nitte.edu.in)

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### ABSTRACT

**Background:** Vacuum assisted Closure (VAC) dressing produces a localized hypoxia and thereby it helps in wound healing, but the molecules (HIF 1 alpha and VEGF) involved in healing process have not been studied yet in humans however studied in-vitro and in-vivo. Present study was aimed to study association of hypoxia inducible factor 1 alpha in wound fluid with clinical outcome in diabetic patients. **Material and Methods:** Present study was single-center, prospective, observational study, conducted in patients of age 18-80 years, either gender, with diabetic foot ulcers, undergoing Vacuum Assisted Closure therapy. Wound fluid sample of 5-15cc was collected after application of VAC on day 1, day 3 and towards the end of VAC removal to study effects of HIF 1 alpha and VEGF. **Results:** In present study, among 35 patients satisfying study criteria, majority were male (85.7 %) & mean age was  $58.46 \pm 10.93$  years. Before VAC treatment, majority patients had Bates-Jensen wound category of extreme (74.3 %) followed by moderate (25.7 %). While after VAC treatment, majority patients had BJ wound category of moderate (45.7 %) followed by extreme (42.9 %), mild (11.4 %). In our study we found that correlation between the parameters HIF 1 alpha (before VAC removal) (ng/ml) & VEGF before VAC removal shows a Poor Positive correlation(  $p = 0.718$ ), with BJ Wound Score after VAC showing a poor negative correlation with both HIF 1 alpha and VEGF ( $p=0.987$ ), ( $p= 0.316$ ), with VEGF being higher in BJ wound category before VAC-extreme group (  $p =0.488$ ), though not statistically significant. In our study, BJ score before VAC & HIF 1 alpha day 3 showed a Moderate Negative correlation, being statistically significant in long standing diabetic foot ulcer patients, resulting in stabilization of HIF 1 alpha. **Conclusion:** Effects of HIF 1 alpha relating with VEGF, an angiogenic factor. Although not very large, the BJ wound result appraisal scale was also associated with the wound liquid proteins.

**Keywords:** diabetic patients, HIF 1 alpha, VEGF, VAC treatment, Bates Jensen

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## **INTRODUCTION**

Diabetes mellitus, characterized by elevated blood glucose levels, is a chronic metabolic disorder that now affects over 400 million people worldwide and is expected to more than double in prevalence by the year 2030. Endothelial breakdown leads to impaired damage healing, which is a major cause of complications in diabetes including both micro- and macro-vascular diseases.<sup>1</sup>

VEGF, a major angiogenic factor involved with collagen synthesis and epithelialization, is only delivered when hypoxia regulates production of HIF1 alpha, therefore hypoxia plays a crucial role in wound mending. On day 2, macrophages take over as the principal source for production of wound fluid cytokines and protein, such as angiogenic factors like VEGF; in these conditions, restricted hypoxia is crucial for the activation of macrophages, and therefore the production of HIF 1 alpha is crucial for macrophage recruitment.<sup>2</sup>

Because of Vascular endothelial growth factor's importance in tissue repair and its production due to an adaptive response to hypoxia, angiogenesis has been named one of the eight most important biological processes in the human body.<sup>3,4,5</sup> Vacuum assisted Closure (VAC) dressing produces a localized hypoxia and thereby it helps in wound healing, but the molecules (HIF 1 alpha and VEGF) involved in healing process have not been studied yet in humans however studied in-vitro and in-vivo. Present study was aimed to study association of hypoxia inducible factor 1 alpha in wound fluid with clinical outcome in diabetic patients.

## **MATERIAL AND METHODS**

Present study was single-center, prospective, observational study, conducted in Department of General Surgery, K.S Hegde Charitable Hospital Deralakatte Mangalore and, Central research laboratory K.S. Hegde Medical Academy NITTE (Deemed to be University), MANGALORE, India. Study duration was of 18 months (April 2021 to September 2022). Study approval was obtained from institutional ethical committee.

### **Inclusion criteria**

- Patients of age 18-80 years, either gender, with diabetic foot ulcers, undergoing Vacuum Assisted Closure therapy, willing to participate in present study

### **Exclusion criteria**

- All immunocompromised patients except diabetes
- All patients with progressive Chronic Obstructive Pulmonary Disease
- Patient who undergo chemotherapy or radiation therapy
- Patient who is on steroid therapy

Study was explained to patients in local language & written consent was taken for participation & study. Diabetic patients who admitted in were assessed and enrolled in the study based on the above inclusion and exclusion criteria after taking informed consent. A case study was used for clinical details of patient which includes demographic data i.e.; characteristics of individual such as age, sex, height, weight, body mass index any chronic health conditions, drugs for any condition, diabetic diagnosed time, on any drugs (Oral hypoglycemic drugs or Injectable Human insulin), any kind of malignancy, any kind of steroid drugs, if ulcer detailed timeline (history with clinical examination) of it was included.

Wound fluid sample of 5-15cc was collected after application of VAC on day 1, day 3 and towards the end of VAC removal to study effects of HIF 1 alpha and VEGF which was estimated by ELISA according to manufacturer's instructions at serial interval of time in our case series study.

HIF 1 alpha levels measured by Human HIF 1 alpha GEN ELISA Test (KIT-KBH0422), Standard calibration range of HIF 1alpha:0.156-10ng/ml, lower limit of detection in kit was 0.061ng/ml. VEGF levels measured by Human VEGF GENELISA Test (KIT - KB1155), The kit had a lower limit of detection of 11.6 pg/ ml, which is much lower than the standard calibration range of 31.2-1000 pg/ml. The clinical wound outcome in diabetic patients was assessed using Bates- Jensen Wound assessment tool.

The IBM statistical package SPSS version 20 was used for data entry and analysis. Characterization of the individual characteristics was done by mean and standard deviation. Correlation of HIF 1 alpha levels with VEGF levels were done by Karl- Pearson correlation co-efficient. Significance of HIF 1 alpha levels and VEGF levels correlated with clinical outcome in VAC dressing in diabetic patients by Receiver operating curves. A p-value of <0.05 was considered significant.

## RESULTS

In present study, among 35 patients satisfying study criteria, majority were male (85.7 %) & mean age was  $58.46 \pm 10.93$  years.

**Table 1:** General characteristics

	No. of patients	Percentage
Mean age (mean $\pm$ SD)	$58.46 \pm 10.93$	
Gender		
Male	30	85.7
Female	5	14.3

Before VAC treatment, majority patients had Bates-Jensen wound category of extreme (74.3 %) followed by moderate (25.7 %). While after VAC treatment, majority patients had BJ wound category of moderate (45.7 %) followed by extreme (42.9 %), mild (11.4 %).

**Table 2:** BJ wound category before VAC and after VAC removal

Bates-Jensen wound category	Before VAC	after VAC removal
Mild		4 (11.4 %)
Moderate	9 (25.7 %)	16 (45.7 %)
Extreme	26 (74.3 %)	15 (42.9 %)

On comparison of the mean values of BJ score before VAC ( $46.8 \pm 6.99$ ) and BJ score after VAC ( $37.14 \pm 6.56$ ), the mean values of BJ score before VAC is higher with a difference of 9.657, is statistically significant ( $p < 0.001$ ). Comparing HIF 1 alpha day 1, HIF 1 alpha day 3 and HIF 1 alpha (before VAC expulsion), the difference between the means is not statistically significant ( $p > 0.05$ ), but the HIF 1 alpha day 1 means are higher. Similarly, Comparing VEGF day 1, VEGF day 3 and VEGF (before VAC expulsion), the difference between the means is not statistically significant ( $p > 0.05$ ), but the VEGF day 1 means are higher

**Table 3:** Showing descriptive statistics of the cases:

	Mean $\pm$ SD	Median (IQR)	Range	P value
BJ score before VAC	$46.8 \pm 6.99$	47(40,51)	34 - 60	<0.001
BJ score after VAC	$37.14 \pm 6.56$	35(34,41)	26 - 61	
HIF 1 alpha day 1	$0.13 \pm 0.09$	0.1(0.06,0.19)	0.03 - 0.37	> 0.05
HIF 1 alpha day 3	$0.12 \pm 0.08$	0.09(0.06,0.14)	0.02 - 0.31	
HIF 1 alpha (Before VAC removal) (ng/ml)	$0.1 \pm 0.07$	0.07(0.05,0.13)	0.04 - 0.29	
VEGF day 1 pg/ml	$97.02 \pm 81.96$	63.37(47.38,129.59)	13.17 - 307.41	> 0.05

VEGF day 3 pg/ml	93.05 ± 105.18	49.61(44.49,83.83)	9.21 - 426.43
VEGF (Before VAC removal)	92.3 ± 104.08	49.53(46.56,91.63)	13.22 - 473.67

The correlation between the parameters BJ score before VAC & HIF 1 alpha day 1 shows a Poor Negative correlation and is not statistically significant (p=0.542). The correlation between the parameters BJ score before VAC & HIF 1 alpha day 3 shows a Moderate Negative correlation and is statistically significant (p = 0.047). The correlation between the parameters BJ score before VAC & HIF 1 alpha (before VAC removal) (ng/ml) shows a Moderate Negative correlation (p =0.137), not statistically significant.

**Table 4:** Showing correlation between HIF 1 alpha and BJ score before VAC

Parameters being correlated	Correlation(r)	p value
BJ score before VAC & HIF 1 alpha day 1	-0.107	0.542
BJ score before VAC & HIF 1 alpha day 3	-0.338	0.047
BJ score before VAC & HIF 1 alpha (before VAC removal) (ng/ml)	-0.256	0.137

The correlation between the parameters BJ score before VAC & VEGF day 1 shows a Poor Positive correlation and is not statistically significant (p = 0.694), correlation between the parameters BJ score before VAC & VEGF day 3(pg/ml) shows a Poor Negative correlation (p = 0.28), not statistically significant & correlation between the parameters BJ score before VAC & VEGF before VAC removal shows a Poor Negative correlation, (p = 0.759), not statistically significant

**Table 5:** Showing correlation between VEGF and BJ score before VAC

Parameters being correlated	Correlation(r)	p value
BJ score before VAC & VEGF day 1	0.069	0.694
BJ score before VAC & VEGF day 3 (pg/ml)	-0.188	0.28
BJ SCORE before VAC & VEGF before VAC removal	-0.054	0.759

The correlation between the parameters BJ score after VAC & HIF 1 alpha day 1 shows a Poor Positive correlation and is not statistically significant (p value=0.269), correlation between the parameters BJ score after VAC & HIF 1 alpha day 3 shows a Poor Negative correlation (p= 0.858), not statistically significant & correlation between the parameters BJ score after VAC & HIF 1 alpha (before VAC removal) (ng/ml) shows a Poor Negative correlation (p = 0.987), not statistically significant.

**Table 6:** Showing correlation between HIF 1 alpha and BJ score after VAC

Parameters being correlated	Correlation(r)	p value
BJ score after VAC & HIF 1 alpha day 1	0.192	0.269
BJ score after VAC & HIF 1 alpha day 3	-0.031	0.858
BJ score after VAC & HIF 1 alpha (before VAC removal) (ng/ml)	-0.003	0.987

The correlation between the parameters BJ score after VAC & VEGF day 1 shows a Poor Negative correlation and is not statistically significant (p =0.604)

The correlation between the parameters BJ score after VAC & VEGF day 3(pg/ml) shows a Poor Negative correlation (p =0.316), not statistically significant

The correlation between the parameters BJ score after VAC & VEGF before VAC removal shows a Poor Negative correlation and is not statistically significant with a p =0.679

**Table 7:** Showing correlation between VEGF and BJ score after VAC

Parameters being correlated	Correlation(r)	p value
BJ score after VAC & VEGF day 1	-0.091	0.604
BJ score after VAC & VEGF day 3 (pg/ml)	-0.174	0.316
BJ score after VAC & VEGF before VAC removal	-0.072	0.679

However also in our study we found that correlation between the parameters HIF 1 alpha (before VAC removal) (ng/ml) & VEGF before VAC removal shows a Poor Positive correlation( p = 0.718), with BJ Wound Score after VAC showing a poor negative correlation with both HIF 1 alpha and VEGF (p=0.987), (p= 0.316), with VEGF being higher in BJ wound category before VAC-extreme group ( p =0.488), though not statistically significant.

In our study, BJ score before VAC & HIF 1 alpha day 3 showed a Moderate Negative correlation, being statistically significant in long standing diabetic foot ulcer patients, resulting in stabilization of HIF 1 alpha, in patients undergoing NPWT even in chronic hypoxic conditions, thus helping in inflammatory process and remodeling.

**Table 8:** Showing Pearsons's correlation between HIF 1 alpha and VEGF values

Parameters being correlated	Correlation(r)	p value
HIF 1 alpha day 1 & VEGF day1	-0.082	0.64
HIF 1 alpha day 1 & VEGF day 3(pg/ml)	-0.021	0.903
HIF 1 alpha day 1 & VEGF before VAC removal	0.009	0.957
HIF 1 alpha day 3 & VEGF day 1	-0.216	0.213
HIF 1 alpha day 3 & VEGF day 3(pg/ml)	0.076	0.665
HIF 1 alpha day 3 & VEGF before VAC removal	0.089	0.61
HIF 1 alpha (before VAC removal) (ng/ml) & VEGF day 1	-0.175	0.316
HIF 1 alpha (before VAC removal) (ng/ml) & VEGF day 3 (pg/ml)	-0.022	0.899
HIF 1 alpha (before removal) & VEGF before VAC removal	0.063	0.718

## DISCUSSION

Type 2 Diabetes mellitus a major socioeconomical medical problem that results in loss of lower limb every 30 seconds. Hence it becomes a priority for devising new strategies for treatment to prevent this complication.<sup>6</sup> Angiogenesis and granulation tissue production are two aspects of wound healing that rely on regional differences in oxygen levels and the transcription factor HIF 1 alpha.<sup>7</sup>

Chronic hypoxia, brought on by diabetes and aging, prevents the yield of HIF 1 alpha post-translational qualities such as vascular endothelial growth factor (VEGF) and stromal cell-determined factor (SDF-1), reduces collagenous grid development, brings about flawed granulation tissue development and dermal recovery, and slows the healing of foot ulcers in diabetic patients.<sup>8</sup> For this reason, many angiogenetic growth factors, cell motility, and the recruitment of endothelial progenitor cells all rely on HIF 1 alpha.<sup>9</sup>

Occlusive dressing control achieved by means of NPWT therapy (VAC dressing) causes relative hypoxia acting as a stimulus for wound healing, resulting in accumulation of HIF 1 alpha and related angiogenic factors like VEGF, with removal of matrix metalloproteinases thus controlling new vessel formation.<sup>10</sup>

In the study done by Alizadeh et al.,<sup>11</sup> it was found that chronic prolonged hypoxia in diabetic wound results in destabilization of HIF 1 alpha reducing it levels even in NPWT therapy thus delaying wound healing. The average positive outcomes for HIF 1 alpha on day 1 and day 3 differed by 0.0122857 ng/ml, for HIF 1 alpha before VAC removal and 3.0 ng/ml after VAC removal, according to our review. In patients who were previously assigned to the

VAC group (N=26), where wound healing is expected to be sluggish, the mean benefits of HIF 1 alpha day 3 are higher by a difference of 0.0171429. Hence, between the parameters BJ score before VAC & HIF 1 alpha day 3 shows a Moderate Negative correlation, and is statistically significant (p value = 0.047) whereas correlation between the parameters BJ score after VAC & HIF 1 alpha (before VAC removal) (ng/ml) shows a Poor Negative correlation (p = 0.987)

In the study conducted by Grimm et al.,<sup>12</sup> there was significant reduction in HIF 1 alpha with decreased VEGF production after VAC treatment,<sup>42</sup> But in our study we found that correlation between the parameters HIF 1 alpha (before VAC removal) (ng/ml) & VEGF before VAC removal shows a Poor Positive correlation (p = 0.718) with BJ Wound Score after VAC showing a poor negative correlation with both HIF 1 alpha and VEGF (p=0.987), (p= 0.316), with VEGF being higher in BJ wound category before VAC-extreme group (p = 0.488), even though not statistically significant.

According to preliminary research conducted over the course of five days by Quinn TP et al.,<sup>13</sup> vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) expression were both increased after VAC treatment. Wounded tissue exposed to VAC at 75 and 150 mmHg showed significantly increased expression of vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF). However, in our study we found that, on comparison of the mean values of VEGF-day 1 and VEGF before VAC removal, the mean values of VEGF day 1 is higher with a difference of 4.7174286 with (p = 0.772), which was more than VEGF day 3, showing a negative correlation with BJ score before VAC and BJ score after VAC removal (p = 0.679), (p= 0.316), respectively, being higher in BJ wound category before VAC - extreme group.

Our review confirms the findings of a study by Paola et al.<sup>44</sup> that found that VAC treatment for diabetic foot ulcers resulted in faster wound bed planning (p=0.03) and faster conclusion (p=0.005) compared to standard injury care, where in our study the clinical wound outcome assessment done in diabetic patients using Bates Jensen wound outcome assessment scale showed the mean values of BJ score before VAC and BJ score after VAC, the mean values of BJ score before VAC is higher with a difference of 9.657 is statistically significant with a p value of <0.001.

This study is one of its kinds where in NPWT therapy, both the wound fluid parameters (HIF 1 alpha and VEGF) are studied together and correlated with wound outcome assessment done by Bates Jensen Wound assessment Scale in diabetic patients to assess the wound healing process, which is never being done till date in human subjects. Limitations of present study were shorter duration of study, being a non-randomized blinded study, technical hindrance reducing the compliance & different range of age group who may respond to NPWT therapy differently than what is being proposed.

Nonetheless, further research is required, and that may include recruiting more people like them for similar tests. Ulcers may continue to heal with the help of NPWT and liquid wound proteins because they eliminate infection and speed up the formation of granulation tissue and neovascularization, thus HIF 1 alpha acting as therapeutic tool in wound repair process.

## **CONCLUSION**

Since evaluating wound mending in diabetic patients, where HIF 1 alpha and VEGF were created in VAC treatment, has not yet been completed in human subjects, our review aimed to focus on the effects of HIF 1 alpha relating with VEGF, an angiogenic factor. Although not very large, the BJ wound result appraisal scale was also associated with the wound liquid proteins. The findings of this research are, in all honesty, preliminary and are dependent on the small sample size of patients analyzed.

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