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Role of Vascular Endothelial Growth Factor-C during Stem Cell Therapy Using Autologous Bone Marrow Mononuclear Cells in Patients with Lower Limb Lymphedema

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Abstract

Introduction: Vascular endothelial growth factor-C (VEGF-C) is the primary lymphangiogenic factor that stimulates lymphangiogenesis by signaling via specific receptor, vascular endothelial growth factor receptor 3 (VEGFR3). This study was conducted to evaluate the change in the level of VEGF-C before and after autologous bone marrow mononuclear cell transplantation for treatment of Lower limb lymphedema. **Patient and methods:** Forty patients with lower limb lymphedema were divided into two groups. **Group I** included 20 patients with chronic lower limb lymphedema who underwent autologous bone marrow mononuclear cell transplantation. **Group II** included 20 patients with chronic lower limb lymphedema who were exposed only to compression therapy as a control group. VEGF-C level in the diseased limbs was measured in both groups at the beginning of the study then 3 and 6 months respectively. **Results:** Group I included 20 patients, 8 patients were male (40%) and 12 patients were females (60%) with mean age 29.5 ± 12.15 while group II included 20, 10 patients were male (50%) and 10 patients were females (50%) with mean age 39.5 ± 11.5. In group I, the specimens were taken at 3 and 6 months after transplantation showed a marked decrease in the VEGF-C level with statistically significant p value, 0.02 and 0.001 respectively. In group II the level of VEGF-C after compression therapy alone at 3 and 6 months interval showed fluctuation with statistically non-significant p value, 0.64 and 0.55 respectively. **Conclusion:** VEGF-C is essential for regulation of lymphangiogenesis. The level of VEGF-C was found elevated in pa-

tients with lymphedema and decrease after autologous mononuclear bone marrow cells, however these results were statically non-significant.

Keywords

Lymphangiogenesis, VEGF-C, Bone Marrow Mononuclear Cells

1. Introduction

The main functions of the lymphatic system are fluid homeostasis, lipid uptake in the intestines, as well as in immune cell trafficking and adipose metabolism. Vascular endothelial growth factor C (VEGF-C) is the primary lymphangiogenic factor which regulates lymphangiogenesis during development through signaling its receptor, vascular endothelial growth factor receptor 3 (VEGFR3). During adult life, lymphatic vessels are mostly quiescent except during wound healing that follows injury which is often accompanied by lymphangiogenesis. [1]

Lymph stasis can accompany anatomical or functional lymphatic disorders as a result of both congenital and postnatal abnormalities. Because the lymphatic circulation provides the normal conduit for the return of interstitial fluid and protein to the blood circulation, abnormal lymph stasis creates an accumulation of protein and cellular metabolites in the extracellular space, resulting in an increase in tissue colloid osmotic pressure, water accumulation and elevation of the interstitial hydraulic pressure. [2] Formation of new lymphatic vessels in the adult importantly occurs in adult tissues during some pathological conditions as inflammatory process, steps of wound healing and tumor metastasis. [3] The formation of new adult lymphatic vessels can occur either through lymphangiogenesis (*i.e.*, sprouting from preexisting vessels), or through lymphvasculogenesis, (*i.e.*, de novo formation of lymphatic vasculature from lymphatic endothelial progenitor cells (LECP) that originate from the bone marrow) [3], or another non-vascular source. [4] Inflammation and tumors induce new lymphatic vessel formation and adult BM-derived myeloid progenitors promote lymphangiogenesis, lymphvasculogenesis or both processes. Inflammatory lymphangiogenesis was attributed to VEGF-C derived primarily from macrophages [4], extensive lymphangiogenesis triggered by macrophage derived inflammatory mediators suggesting that VEGF-C and other products of activated macrophages are major contributors to the postnatal formation of new lymphatic vessels. [5]

Bone marrow derived cells (BMDCs), including endothelial progenitor cells (EPCs), have been suggested to contribute to lymphangiogenesis through two distinct mechanisms namely through their incorporation into growing lymphatic vessels in association with vasculogenesis and via the secretion of lymphangiogenic growth factors. [6] The role of hematopoietic stem cells (HSCs) and endothelial progenitor cells (EPCs), derived from BM for blood vessel formation is largely accepted. This has raised a question as to whether LEPCs can also be de-

rived from BM as well. [7] First potential evidence of LEPCs in hematopoietic organs was reported by Salven *et al.*, that a subpopulation of human fetal liver co-expresses CD34, VEGFR3 and CD133. [8]

This study introduces a new therapeutic approach to treat patients with lower limb lymphedema by using bone marrow derived mononuclar cells (BMMNC). Autologous bone marrow was harvested from the lymphedema patient and BMMNC were separated then injected to the same patient. Tru-cut needle biopsies were taken before and after the procedure and histopathological examination for new lymphatic vessel formation was done using immunohistochemistry. Level of VEGF-C was measured in the biopsy samples to delineate its important role in regulation of lymphangiogenesis process. Clinical parameters were monitored as limb circumference and life style improvement with correlation to the histopathological change and alteration in VEGF-C level. A control group was included in this study and only compression therapy was done for this group.

2. Patients and Methods

The present study was conducted in Vascular Surgery Department, Faculty of Medicine, Tanta University. A number of 40 patients with chronic lower limb lymphedema were recruited in the period from March 2019 till March 2022. They were divided into two groups. **Group I** included 20 patients with chronic lower limb lymphedema underwent autologous bone marrow mononuclear cell transplantation for purpose of regeneration of new lymphatic vessels for achieving drainage of the accumulated lymphatic fluid. **Group II** included 20 patients with chronic lower limb lymphedema were exposed only to compression therapy as a control group. VEGF-C level in the diseased limbs was measured in both groups at the beginning of the study then 3 and 6 months respectively.

Inclusion criteria: patients with chronic lower limb lymphedema Grade I, II & III. The age ranged between 18 years and 60 years.

Exclusion criteria: We excluded patients with chronic lower limb lymphedema grade IV from this study. Patients with hypercoagulable conditions as thrombocytosis or history of chronic lower limb ischemia, congestive heart failure (ejection fraction less than 30%), were also excluded. Patients with active local or systemic infection or organ dysfunction were considered unsuitable to be included in this study.

Ethical Consideration: Informed consent was obtained from all participating patients. Approval of the Ethical committee of Faculty of Medicine Tanta University was obtained with approval number (2603/06/14). All the procedures followed during the research were in accordance with the ethical standards and Helsinki Declaration of 1975, as revised in 2008.

2.1. Patient Evaluation

2.1.1. Evaluation for Suitability of Transplantation

All the recruited patients were exposed to thorough clinical examination to check the feasibility of transplantation. Abdominal examination was done to ex-

clude any enlargement in the liver or spleen. Neck and axilla were examined for any palpable enlarged lymph nodes. Hematological parameters were evaluated such as presence of anemia, leucopenia or thrombocytopenia. Viral markers were done for HIV, hepatitis B and C. Renal functions were evaluated by testing the levels of blood urea and serum creatinine. Liver functions tests also were evaluated including serum albumin, AST and ALT.

2.1.2. Evaluation of the Affected Extremity

Local examination of the affected lower extremity was performed to determine the exact diagnosis of lymphedema and to exclude the presence of lipidema or any clinical manifestations of chronic venous insufficiency as hyperpigmentation, venous ulceration or secondary varicose veins in the lower limb.

The degree of affection of the physical activity was observed before the intervention with presence or absence of any pain or sense of heaviness with determination if it is mild, moderate or severe affection on the life style. Presence of infection was excluded by assessment of the affected limb which was examined for redness, ulceration, bullae, hotness or presence of any discharge.

2.2. Group I (Mononuclear Cell Transplantation Group)

2.2.1. Patient Preparation before Bone Marrow Harvest

Patients were injected with recombinant human Granulocyte Colony Stimulating Factor (rhGCSF) (GeneLeukim Injection from Shandong Geneleuk Biopharmaceutical Co., Ltd., Jinan, Shandong, China). Each 1 ml vial contained 300 microgram Filgrastim administered subcutaneously in a dose of 5 μg/kg per day for 5 days to mobilize stem/progenitor cells. All patients were given a prophylactic dose of anticoagulant (Enoxaparin 40 mg subcutaneous) to decrease the expected risk of venous thrombo-embolism. Complete blood picture (CBC) was done before administration or rh GCSF and repeated daily to ensure its response. This effect appears in the form of increased white blood cell count (WBCs).

2.2.2. Bone Marrow Aspiration

Under complete antiseptic precautions, a prophylactic dose of antibiotic Ceftriaxone (1 g via intravenous injection) was given to all patients prior to bone marrow (BM) harvest. Two approaches were used to obtain a BM aspirate: the first one was from the anterior superior iliac spine; and the second one from the posterior superior iliac spine (which gives a higher amount of bone marrow aspirate). A volume of 100 - 150 cc of BM was aspirated from the iliac crest through anterior superior or posterior iliac spine of the patient then sent to the laboratory to separate the mononuclear cell fraction. All steps were performed under sterile conditions in a laminar flow hood in the Clinical Pathology Department, Tanta University Hospital.

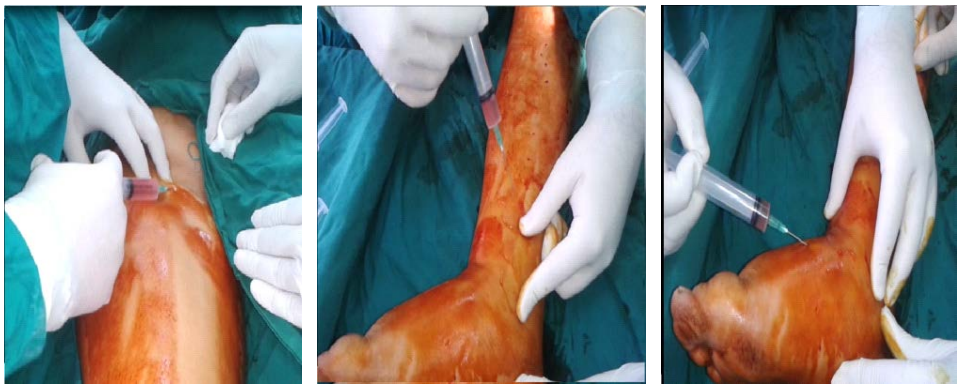
2.2.3. Steps of Separation of Bone Marrow Mononuclear Cells (BM MNCs)

The bone marrow aspirate harvested above was diluted at a ratio of 4:1 with

clinical buffer (Clini MACS PBS/EDTA buffer 1000 ml, CE approved for clinical use catalogue number 25 - 700, from Miltenyi Biotec Company, Bergisch Gladbach, Germany). The diluted cell suspension was then carefully layered over 15 ml of Ficoll-Paque (GE Electric, Pharmacia, Piscataway, NJ, USA) in a 50 ml conical tube, and then centrifuged at 2000 rpm for 20 minutes at 20˚C in a swinging out bucket rotor without brake. The upper layer was aspirated leaving the mono-nuclear cell layer undisturbed at the interphase containing lymphocytes, monocytes, and thrombocytes. The middle layer was carefully transferred to a new 50 ml conical tube. The cells were then washed twice with clinical buffer, mixed gently and centrifuged at 1200 rpm for 15 min at 20˚C. Then the supernatant was carefully and completely removed. The cell pellet was re-suspended in the appropriate amount of clinical buffer with the final volume of 300 µl of clinical buffer for up to 10^8.

2.2.4. Protocol of Mononuclear Cell Transplantation (Figure 1)

Bone marrow mononuclear cells transplantation was done in patients in group I under general anesthesia with complete antiseptic precautions after sterilization using Povidone Iodine 10% solution from the groin to the toes. A sterile bag containing the freshly separated bone marrow mononuclear cells was transferred from the laboratory to the operating room to be injected within 2 hours at room temperature. The targeted transplantation sites were planned to be at the expected lymphangiogenesis locations around the inguinal lymph nodes, along lymphatic vessels that accompany the superficial venous system of the lower limb namely, great saphenous vein (from the start at the foot till the end at the saphenofemoral junction), short saphenous vein till the popliteal fossa. Additional transplantation sites were distributed into the lymphedematous tissue in the web spaces of the foot, around the ankle and circumferentially in the leg with spacing 3 - 4 cm in between and depth of 1.5 - 2 cm. Multiple punches of true-cut needle biopsies were taken from different sites in the foot, leg and thigh to measure the level of VEGF-C before intervention.



(a) (b) (c)

Figure 1. Sites of transplantation of bone marrow derived mononuclear cells in Groin (a) Leg (b) and foot (c) for treatment of lower limb lymphedema.

2.2.5. Post Transplantation Care

Compression therapy using multilayer crepe bandage was applied immediately after stem cell transplantation for two weeks. The compression therapy was applied for two weeks and released for two weeks alternatively. CBC was repeated daily to be sure that the leucocytic count became back to normal (below 11,000/cc).

Group II (control group):

Compression therapy using multilayer crepe bandage was applied and continued for two weeks and stop compression therapy for the following two weeks. There was no active intervention in Group II this means no bone marrow aspiration or stem cell transplantation was done for Group II.

2.3. Measurement of VEGF-C Level

2.3.1. Timing of Biopsy

In Group I, the level of VEGFC was measured in all patients with chronic lower limb lymphedema, 3 biopsies were taken one before and two biopsies were taken at 3 months, 6 months after autologous stem cell transplantation**. In Group II**, the level of VEGF-C was measured in all patients with chronic lower limb lymphedema, 3 biopsies were taken one before and two biopsies were taken at 3 months, 6 months after starting compression therapy.

2.3.2. Method of Biopsy Taking

Multiple samples using true cut needle biopsy were taken from groin, thigh, leg and foot, two punches from each site. **In Group I,** the biopsy specimens were taken before autologous stem cell transplantation and then at 3 months, 6 months after the transplantation. **In Group II,** initial biopsy was taken at the start of the recruitment then after 3 and 6 months from the start of the compression therapy.

2.3.3. Biopsy Specimen Preservation

Lymphedematous tissues were obtained in Wassermann tubes and labeled and preserved at −20˚C in deep freezer and repeated freeze-thaw cycles were avoided.

2.3.4. Measurement of VEGF-C Level

VEGF-C level was measured by quantitative Enzyme Linked Immune sorbent Assay (ELISA) kit catalog number EK0588 manufactured by Boster Biological Technology Co, Ltd. Manufactured in Fermont.

2.3.5. Principal of the Assay

VEGF-C is under the control of special human gene encoding it is VEGFC. The protein encoded by the gene is a member of the platelet-derived growth factor/vascular endothelial growth factor (PDGF/VEGF) family is active in angiogenesis, lymphangiogenesis and endothelial cell growth and survival, and can also affect the permeability of blood vessels. This secreted protein undergoes a complex proteolytic maturation, generating multiple processed forms which

bind and activate VEGFR-3 receptors. Only the fully processed form can bind and activate VEGFR-2 receptors. This protein is structurally and functionally similar to vascular endothelial growth factor D (VEGF-D). The C terminus of VEGFC has cysteine-rich repeat units characteristic of the Balbiani Ring 3 Protein (BR3P) of the midge Chironomus tentans. The standard product used in this kit is recombinant human VEGF-C consisting of 125 amino acids with the molecular mass of 23 Kda after glycosylation.

2.3.6. Sample Preparation

After weight measurement of the sample using the sensitive balance, melting was done by adding equal volume of phosphate buffer saline (PH = 7.4) then thoroughly homogenated and collected in Epindorf's tubes. Then the samples were centrifuged at 3000 rpm for 10 min. The supernatant was taken and assayed immediately or aliquoted and stored samples at −20˚C.

2.3.7. Reagent Preparation and Storage

VEGF-C standard solution was prepared no more than 2 hours prior to the experiment. Standard solution 10,000 pg/ml of human VEGF-C was prepared by adding 1 ml sample diluent buffer into one tube; the tube was kept at room temperature for 10 min and was mixed thoroughly. Then standard solution 4000 pg/ml of human VEGF-C was prepared by adding 0.4 ml of the above 10,000 pg/ml VEGF-C standard solution into 0.6 ml sample diluent buffer and mixed thoroughly.

2.3.8. Assay Procedure

The Avidin-Biotin-Peroxidase Complex (ABC) working solution and Tetramethylebenzene (TMB) color developing agent was kept warm at 37˚C for 30 min before use. When reagents were mixed completely and evenly with addition of 0.1 ml per well of the 4000 pg/ml, 2000 pg/ml, 1000 pg/ml, 500 pg/ml, 250 pg/ml, 125 pg/ml, 62.5 pg/ml human VEGF-C standard solutions were aliquoted into the precoated well plate. 0.1 ml of the sample diluent buffer was added into the control well (Zero well). 0.1 ml of each properly diluted sample was added) to each empty well. Each human VEGF-C standard solution and each sample was measured in duplicate.

2.4. Follow up Parameters

Patients in both groups were evaluated in regular intervals for the degree of lymphedema improvement. Limb circumference was measured at fixed levels as foot, above ankle, mid leg, below knee, above knee & mid-thigh. Patients also were evaluated for any improvement in walking ability and sense of heaviness in the extremity during standing or ambulation. The level of VEGF-C was measured initially and after 3 and 6 months. Histopathological examination of the biopsy specimen with immunohistochemistry uses CD34 stain to detect lymphatic vessels.

2.5. Statistical Analysis

The objective of this study is to determine the degree of change in the level of VEGFC after autologous transplantation of bone marrow mononuclear cells in comparison to control group in which just compression therapy was applied. The primary hypothesis is that therapeutic lymphangiogenesis can be produced by stem cell therapy with subsequent improvement of lymphedema symptoms and decrease of the elevated level of VEGFC. The data of patients were entered into a data base and analyzed using statistical software (Statistical Package for Social Science (SPSS) Version 26.0 (2020) for Windows, SPSS, Chicago, IL, USA). Paired samples t test was used to prove the differences between before and after intervention. Chi square test was performed for determination of the p value to compare the rates and the probability ratio between the studied groups. Statistical significance was assumed at a value of $p < 0.05$. So p value above 0.05 is considered statistically non-significant.

3. Results

Group I included 20 patients with age ranged from 13 to 55 years with mean value (29.5 ± 12.15), 8 patients were male (40%) and 12 Patients were females (60%). Group II included 20 patients with age ranged from 18 to 59 years with mean value (39.5 ± 11.5), 10 patients were male (50%) and 10 patients were females (50%). The duration of lymphedema in group I ranged from 2 to 18 years with a mean value (9 ± 7.33) years while in group II ranged from 1 to 12 years with a mean value (7 ± 4.66) years as shown in Table 1.

3.1. Clinical Outcome

3.1.1. Heaviness, Pain and Life-Style Affection

Sense of heaviness and discomfort from lymphedema was assessed by Visual Analogue Scale (VAS) from 1 to 10 degrees. In group I, VAS was 7.55 ± 1.45 before stem cell therapy then it became 4.66 ± 0.75, 2.75 ± 0.86 and 1.30 ± 0.77 after one month, 3 months and 6 months respectively. This was associated with improvement in walking ability and overall patient satisfaction in 16 patients (80%) The p-value started to be statistically significant after the first month. In group II, VAS did not show any statistically significant change after compression therapy ($p > 0.05$). Before compression therapy, the mean value of visual analogue scale was 7.56 ± 2.38 then it became 7.33 ± 1.37, 6.50 ± 2.16 and 6.33 ± 2.50 after one month, 3 and 6 months respectively as shown in Table 2.

3.1.2. Difference in Circumferential Measurements

In group I, there was a significant reduction in the mean value of the measurement of the limb circumference at the selected levels namely, ankle, mid-leg, below and above the knee and mid-thigh. The p-value was 0.362 after one month, 0.028 after 3 months and 0.014 after 6 months. In group II, there was fluctuation in circumferential measurement results. The p-value was 0.253 after

Table 1. Comparison between the two studied groups as regards gender, age and duration of lymphedema.

Parameter		Group I		Group II	
		N.	%	N.	%
Gender	Male	8	40%	10	50%
	Female	12	60%	10	50%
	Total	20	100%	20	100%
	X2	0.218			
	p-value	0.677			
Age(in years)	Range	13 - 55		18 - 59	
	Mean ± SD	29.5 ± 12.15		39.5 ± 11.5	
	t-test	0.963			
	p-value	0.528			
Duration of lymphedema (in years)	Range	2 - 18		1 - 12	
	Mean ± SD	9 ± 7.33		7 ± 4.66	
	t-test	0.045			
	p-value	0.821			

p-value ≤ 0.050 significant*. p-value ≤ 0.001 highly significant**. No statistical significant differences between two groups (p > 0.050) as regards the age, gender and duration of lymphedema.

Table 2. Follow up of pain and sense of heaviness using visual analogue scale of studied groups.

Visual analogue scale (VAS)	Group I			Group II		
	Mean	SD	p-value	Mean	SD	p-value
Before	7.55	1.45	-	7.56	2.38	-
After 1 month	4.66	0.75	**0.001****	7.33	1.37	**0.241**
After 3 month	2.75	0.86	**0.001****	6.50	2.16	**0.460**
After 6 month	1.3	0.77	**0.001****	6.33	2.50	**0.550**

p-value > 0.050 non significant. p-value ≤ 0.050 significant*. p-value ≤ 0.001 highly significant**.

one month, 0.412 after 3 months and 0.349 after 6 months which are all statistically non-significant as shown in Table 3 and Figure 2.

3.1.3. VEGFC Level

The level of detection of VEGFC in the specimen taken by true-cut biopsy needle was found to be 62.5 pg/ml. In group I, VEGF-C level before mononuclear cell transplantation found high elevated. The mean value was 1589.25 with SD 1438.5. In group II, VEGF-C level was also high with mean value 1607.45 and SD 1371.5.

Table 3. Circumferential measurements in both groups at ankle, mid-leg, below knee, above knee and mid-thigh levels.

Measurement level In centimeter (Mean ± SD)		Group I (stem cell therapy)				Group II (control group)			
		Before	After 1 month	After 3 month	After 6 month	Before	After 1 month	After 3 month	After 6 month
Ankle		28.6 ± 3.1	27.3 ± 3.4	26.45 ± 3.1	25.8 ± 3.1	31.2 ± 4.3	29.4 ± 4.5	30.7 ± 4.5	30.2 ± 2.8
Mid-leg		37.8 ± 4.9	35.1 ± 4.3	34.05 ± 3.6	33.1 ± 3.1	39 ± 4.69	37 ± 5.8	38.5 ± 4.5	39.3 ± 4.8
Below knee		39.9 ± 5.24	38.4 ± 3.4	37.2 ± 3.6	36.8 ± 3.2	43.1 ± 4.8	42.4 ± 5	43.4 ± 4.8	42.9 ± 3.5
Above knee		45.9 ± 5.2	43.83 ± 5.3	42 ± 3.5	41.5 ± 3.3	45.9 ± 6.4	**45.3 ± 6.8**	47.2 ± 6.8	**45.6 ± 6.9**
Mid-thigh		54.2 ± 3.9	53.6 ± 3.4	52.5 ± 3.1	50.2 ± 3.9	54.6 ± 4.7	54.3 ± 3.9	53.6 ± 3.6	53.8 ± 4.1
Paired t-test	p-value	-	**0.033**	**0.35**	0.012	-	**0.282**	**0.395**	**0.351**
	T	-	**1.288**	**1.18**	**3.11**	-	**0.711**	**0.311**	**1.330**

p-value > 0.050 non significant. p-value ≤ 0.050 significant*. p-value ≤ 0.001 highly significant**. SD: Standard Deviation.

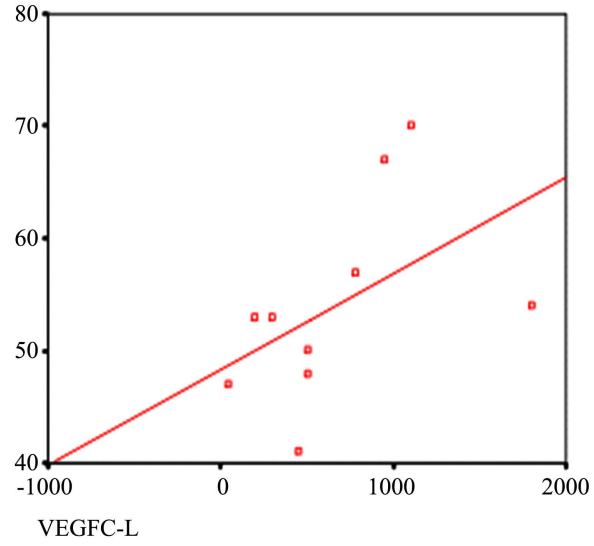
(a) (b)

Figure 2. (a) Circumferential measurement at the ankle (30.5 centimeter) before bone marrow derived mononuclear cells. (b) Circumferential measurement at the ankle (26.5 centimeter) after bone marrow derived mononuclear cells.

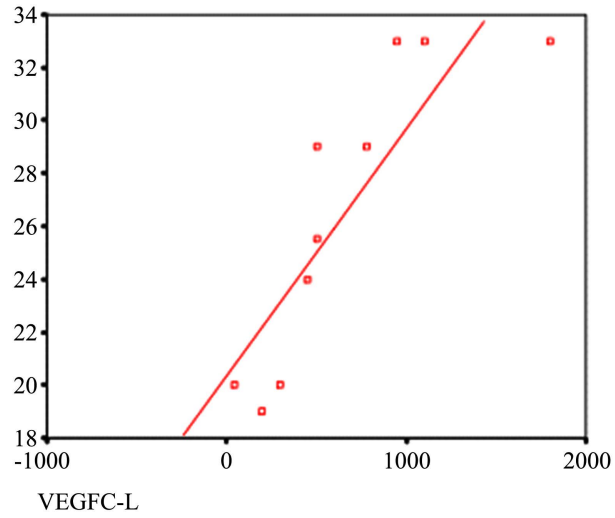
In group I, the specimens taken at 3 and 6 months after transplantation showed marked decrease in the VEGFC level with statistically significant p value, 0.02 and 0.001 respectively. In group II the level of VEGFC in the subsequent specimens taken after compression therapy alone at 3 and 6 months interval showed fluctuation with statistically non-significant p value, 0.64 and 0.55 respectively. All these data were explained in Table 4.

3.1.4. Correlation between the Change in Both VEGFC Level and Limb Circumference

There was positive correlation between VEGFC level and circumference with statistically highly significant ($p \leq 0.001$) at the level of foot mid leg and below knee. There was positive correlation between VEGFC level and circumference at the above knee and mid-thigh with statistically significant ($p > 0.050$) as shown in Figure 3.



(a)



(b)

Figure 3. (a) Positive correlation between VEGFC level and circumference at the level of foot mid leg and below knee with statistically highly significant ($p \leq 0.001$). (b) Positive correlation between VEGFC level and circumference at the above knee and mid-thigh with statistically significant ($p > 0.050$).

Table 4. Level of vascular endothelial growth factor c in both groups though out the study.

VEGFC Level (in pg/ml)	Group I			Group II		
	Mean	SD	p-value	Mean	SD	p-value
Before	1589.25	1438.5	-	1607.45	1371.5	-
After 3 month	976.8	825	**0.032***	1516.5	1334.5	**0.64**
After 6 month	462.4	517	**0.001****	1621.25	1441.5	**0.550**

p-value > 0.050 non significant. p-value ≤ 0.050 significant*. p-value ≤ 0.001 highly significant**.

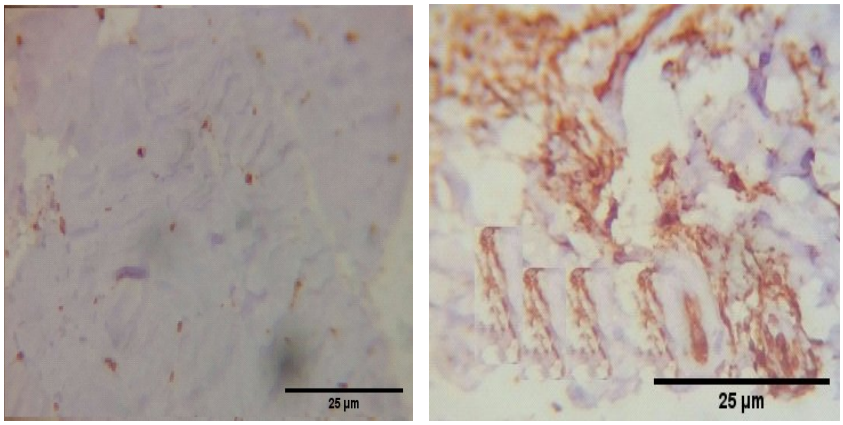
3.1.5. Histopathological Examination of Biopsy Specimens

Tru-cut Biopsies were taken from all patients before, 3 and 6 months after autologous transplantation of bone marrow derived mononuclear cells. The specimens were examined by immunohistochemistry for new lymphatic capillary regeneration. In both groups, all specimens taken before stem cell therapy showed lymphatic capillaries ranged from 0 - 4/High power field (HPF). In group I, all biopsy sample taken 3 months post-intervention showed new lymphatic capillaries ranged from 13 - 18/HPF while after 6 months showed 21 - 25/HPF. Biopsy samples from patients in group II showed no change (**Figure 4**).

4. Discussion

Lymphedema is characterized by an imbalance of lymphatic flow leading to accumulation of protein rich fluid in subcutaneous tissues. The consequent swelling may cause cosmetic and functional impairment, with significant physical and psychological morbidity. There is progressive damage to the lymphatic vessels with inflammation, fibrosis and more swelling, eventually leading to elephantiasis. [9] There is no cure for lymphedema but it is easily managed with early recognition and therapy. Those who do not have treatment tend to worsen rapidly and advanced disease tends to be more difficult to treat than early disease. [10]

Understanding of the molecular mechanisms of lymphangiogenesis has increased considerably in recent years. [11] Fundamental discoveries in lymphatic development have yielded relevant animal models for vexing clinical diseases that suffer from nonexistent of minimally effective treatments. Inherited and acquired lymphedema represent the current crux of research efforts to identify potential molecular therapies born from these early discoveries. [12] Lymphangiogenesis is the process of formation of new lymphatic vessels during both embryonic development and in adulthood. [13] Vascular endothelial growth factors



(a) (b)

Figure 4. (a) True cut biopsy before mononuclear cell transplantation stained by $CD34^+$ stain by immunohistochemistry showing scanty lymphatic vessels. (b): True cut biopsy 6 months after mononuclear cell transplantation stained by $CD34^+$ stain by immunohistochemistry showing new lymphatic vessel formation.

(VEGFs) are important regulators of angiogenesis and lymphangiogenesis. [11] VEGFs stimulate cellular response by binding to tyrosine kinase receptors (VEGFRs) that are specifically expressed in blood and lymphatic endothelial cells that line the luminal surface of vessels. VEGF-C and VEGF-D signaling induces lymphangiogenesis via VEGFR-3. [14]

The present study was designed to measure level of VEGF-C in patients with chronic lower limb lymphedema before and after mononuclear cell transplantation in group I which included 20 patients with age ranged from 13 to 55 years with mean value (29.5 ± 12.15), 8 patients were male (40%) and 12 patients were females (60%). Meanwhile only compression therapy was done in group II which included 20 patients with age ranged from 18 to 59 years with mean value (39.5 ± 11.5), 10 patients were male (50%) and 10 patients were females (50%).

In the present study we found gradual decrease in the level of VEGF-C after mononuclear cell transplantation. In group I, VEGF-C level before mononuclear cell transplantation found elevated. The mean value was 1589.25 with SD 1438.5. In group II, VEGF-C level was also high with mean value 1607.45 and SD 1371.5. In group I, the specimens taken at 3 and 6 months after transplantation showed marked decrease in the VEGFC level with statistically significant p value, 0.02 and 0.001 respectively. In group II the level of VEGFC in the subsequent specimens taken after compression therapy alone at 3 and 6 months interval showed fluctuation with statistically non-significant p value, 0.64 and 0.55 respectively.

Goicoechea-Diaz *et al.*, (2010) performed study on a 58 year old man were referred to them with severe swelling of both lower extremities that was unresponsive to conservative therapy. Bone marrow stem cells were implanted in the most affected extremity (right limb) by multiple circumferential injections into the leg and distal half of the thigh. In addition, cells were also injected into the dorsum of the foot. One week after cell implantation a mild improvement of foot swelling was appreciated at the treated extremity. Subsequently, progressive improvement of lymphedema was observed and striking improvement of bilateral lymphedema was obtained. [15]

Hou *et al.*, (2008) had a study on fifteen women with lymphedema, who had undergone breast cancer surgery and no radiotherapy 5 years before, served as the study group and received bone marrow stem cells (BMSCs) transplantation; 35 patients were measured as the control group treated with compression decongestive therapy. They were kept on follow-up for 1 year. At the end of the study they had a conclusion that autologous BMSCs transplantation for the treatment of breast cancer related lymphedema is effective and feasible than compression decongestive therapy. Result of this study showed that the role of VEGF-C is very important as lymphogenic factor during autologous bone marrow transplantation in patients suffering from chronic upper limb lymphedema. [16]

Tervala *et al.*, (2015) investigated the effect of VEGF therapy on lymphedema. VEGF-C improved lymphatic vessel function compared with that of controls. VEGF-C156S induced moderate lymphangiogenesis, but the effect remained sta-

tistically non-significant. Prolymphangiogenic growth factors (VEGF-C, -D, and -C156S) also improved lymph node survival as compared with those of the VEGF-A and control group. [17]

Maldonado *et al.*, (2011) performed a study including 20 women with lymphedema secondary to breast cancer surgery with axillary lymphadenectomy was conducted. Women were assigned at random to one of two groups. Study group of 10 women was injected with autologous stem cells (ASC) in the affected arm, whereas the other 10 women comprised the control group and received traditional compression sleeve therapy (CST). There was improvement in the volume of lymphedema in both groups. In the study group there was an overall volume reduction during the follow-up, whereas in the control group lymphedema recurred after compression sleeve was removed. [18] However, further studies and larger number of patients and longer duration of follow up are needed in order to obtain an accurate evaluation of VEGF-C and it is combination as therapeutic growth factor with autologous mononuclear cell transplantation in treatment of lymphedema.

5. Conclusion

VEGFC is a specific regulator and important factor in lymphangiogenesis. Autologous transplantation of bone marrow mononuclear cells showed a promising new therapeutic option for the patients with lymphedema. VEGFC level showed high levels in the extremities of lymphedema patients in both study and control groups. The group treated with mononuclear cell transplantation showed clinical improvement with marked decrease in the level of VEGFC but with statistically non-significant p value. Further studies with higher number of patients are required for more accurate interpretation of such outcome.

Conflicts of Interest

No interests of either financial or personal aspects.

Authors' Contributions

Authors from vascular surgery department selected the patients and performed transplantation of stem cells. Authors from clinical pathology department separated Bone Marrow Mononuclear cells. Author from faculty of science measured the level of VEGFC. Authors from pathology department examined the biopsy specimens by immunohistochemistry.

Availability of Data

All the data sheets were tabulated within the manuscript tables.

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