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Establishment of Impaired Angiogenesis Using Human Placental Mesenchymal Stem Cells under Micronutrient Deficiency

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Abstract

Micronutrient deficiency is well studied under many diseases. Angiogenesis, formation of new blood vessels is a break through interest of many researchers in diseases like cancer, diabetes. In this pilot study we aimed to comprehensively investigate the relationships of micronutrient deficiency and angiogenesis. We examined the results with the help of markers like VE-cadherin, VEGF, VWF, CD-31. Out of this study we conclude that micronutrient deficiency delays angiogenesis and weakens the existence of blood vessel.

Keywords: Diabetes; Angiogenesis; Micronutrients

Introduction

Diabetes is one of the most costly and burdensome chronic diseases of our time and is a condition that is increasing in epidemic proportion throughout the world [1]. It is well known that diabetic patients suffer premature vascular disease along with a host of other disorders related to blood glucose imbalances. Although the compliance for medications is very good, compliance is not consistent for diet and lifestyle modifications. Medical nutrition therapy for people with diabetes should be individualized, with consideration given to the individual's usual food and eating habits, metabolic profile, treatment goals, and desired outcomes [1,2]. The short-term consequences of micronutrient malnutrition during pregnancy on the mother and infant are fairly well understood, but the long-term costs of these deficiencies have yet to be fully elucidated. Although there is evidence from animal experiments and observational studies in humans to suggest a link between intrauterine micronutrient status and the potential risk of chronic diseases, the understanding of molecular mechanisms of these effects for many micronutrients is weak. High concentrations of plasma total homocysteine are also known to be associated with the risk of developing diabetes-related cardiovascular disease, nephropathy and proliferative retinopathy. Co-supplementation of pyridoxine (vitamin B6), folic acid (vitamin B9), and cobalamin (vitamin B12) has been used to decrease levels of plasma homocysteine and the risk of cardiovascular disease [2]. One reason is that excess blood glucose creates more glycation reactions in the body whereas glycation end products are linked to a range of diabetes-related conditions, particularly kidney disease (nephropathy), visual loss (retinopathy), and nerve damage (neuropathy). While the benefits of conventional vitamin B6 (pyridoxine) is well documented, the unique form of vitamin B6, that specifically interferes with toxic glycation reactions, offers hope in combating the ravages of protein degradation involved in normal aging and the accelerated glycation reactions suffered by diabetics [3]. A further factor underlying the association of high-dose vitamin B6, B9, B12 supplements and metabolic dysfunction in diabetic nephropathy could be the effect of high-dose folic acid on metabolite transport via the folate transporter 1 (RFC-1) [4].

As mentioned above, blood vessel formation is a common diabetes complication. Although micro- vascular complications increase morbidity and lead to premature mortality, the major cause of death in individuals with diabetes is CVD, accounting for approximately 65% of all diabetes-related deaths [5]. Studies show that increase in the level of homocysteine, which is highly associated with CVD in turn delays Angiogenesis [6]. A key mechanism that appears to contribute to blood vessel damage in people with diabetes has been identified by researchers recently and shows that the damage appears to involve two enzymes, fatty acid synthase (FAS) and nitric oxide synthase (NOS), which interact in the cells that line blood vessel walls. As we already know that in diabetes there's a defect in the endothelial cells that line the blood vessels and diabetic patients shows depressed levels of fatty acid synthase, for the first time it's been able to link those observations together [7]. Drugs that modulate vessel formation can be used to prevent the disease from occurring; blood vessel production in mice reaches its lowest point just after the halfway point in a mouse's life. Coincidentally, this is also when the adipose cells in mice secrete the least amount of Vascular Endothelial Growth Factor (vegf), a protein that regulates vessel formation. It's known that VEGF controls vessel formation in adipose tissue and that fewer vessels are consistent with a higher risk of type 2 diabetes [8]. In order to manage blood glucose levels, patients with type 2 diabetes are often treated with oral medication - such as metformin - insulin injections, or a combination of both. Kieffer et al. note, however, that such treatments can cause gastrointestinal problems, weight gain and low blood glucose levels, and some patients may not even respond to them. With these factors in mind, they developed fully functioning insulin secreting beta cells in vitro from human mesenchymal stem cells and encapsulated to transplant into the mice, causing the mice to experience better glucose metabolism and an improvement in responsiveness to insulin. Mesenchymal stem cells offer new opportunities in the treatment of diabetes, but they also raise many scientific questions that need to be addressed, particularly those related to safety and efficacy.

Mesenchymal stem cells (MSCs) are considered one of the most promising cell types for therapeutic applications and are, hence, most intensively evaluated in pre-clinical and clinical settings. The inherent propensity of fetal derived tissues restraining an endogenously high expression of VEGF motivated us to choose PDMSCs as the source depicting that it is sufficient for engendering the angiogenic signal [8,9]. The characteristics that demonstrate MSC's high therapeutic potentials

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include the potential for multiple lineage differentiation, the ability of secreting growth factors that can promote cell growth and tissue repair; the function of immunomodulation and immunosuppression and the limited expression of major histocompatibility complex (MHC) II and co-stimulating molecules which allow MSCs to be used across MHC barriers. Human placenta is an attractive source of mesenchymal stem cells (MSC) for regenerative medicine. The cell surface markers expressed on MSC's have been proposed as useful tools for the isolation of MSC from other cell populations [10]. However, the correlation between the expression of MSC markers and the ability to support tissue regeneration in vivo has not been well examined.

In the absence of Vit B6 & Vit B9 micro nutrients the level of homocysteine increases in blood leading to hyperhomocysteinemia which has been implicated in endothelial dysfunctioning and cardiovascular diseases [11]. The functions of Vitamin B9 and B6 are closely associated including the intimate relation between metabolism of two vitamins in making red blood cells and to help iron work better in the body. It work together to produce S-adenosylmethionine (SAMe) a compound involved in immune function and mood [12-14]. Apart from micronutrient deficiency related diseases folic acid and vitamin B6 may have roles in the prevention of disorders of CNS development, mood disorders, and dementias, including Alzheimer's disease and vascular dementia in elderly people [15]. This necessitates the development of suitable model systems to understand the impact of micronutrient deficiency versus angiogenesis not only to delineate the mechanisms but also for the pharmacological complications including obesity, type-2 diabetes, insulin resistance etc. In the present study, we use human placental mesenchymal stem cells as an *in-vitro* model system under micronutrient deficiency to study angiogenesis.

Materials and Methods

Human Placental derived Mesenchymal stem cells cultured in Basal DMEM High Glucose containing 10% FBS, Penstrep and Glutamax were collected. A definite protocol is followed to culture from the original source which is done aseptically in a biological safety cabinet. Cells are washed thoroughly with Dulbecco's phosphate-buffered saline (DPBS) containing antibiotics and then fresh media is added as soon as the arrival. Processed well in 60 mm dishes (BD Biosciences, USA) in High glucose Dulbecco's modified Eagle's medium (DMEM) (Himedia), supplemented with 10% FBS (Himedia) with glutamax (500 µl)(Invitrogen) and penstrep (500 µl)(Invitrogen).

Tube formation assay in normal and micronutrient deficient condition

For Matrigel assay, the *in-vitro* endothelial ring formation assay, the wells are coated with Matrigel under cool room temperatures, as the gel solidifies above 25°C. The 12 well plates were coated with 350 µl Matrigel and incubated at 37°C for 1 hour. PDMSC's cultured in normal growth media containing 10%FBS and in restricted media with 10%FBS were seeded at a density of 50,000cells/well and incubated with 50 ng of vascular endothelial growth factor. Ring formation was observed and qualitatively analyzed using a bright field phase contrast microscope (Nikon eclipse TE 2000-s) and photographs were taken at every 2 hours.

Wound scratch assay in normal and micronutrient deficient condition

For the wound scratch assay a 100% confluent plate of PDMSC's were wounded by a scratch on the monolayer by using a 1000 µl pipette tip. To determine the endothelialization potential of PDMSC's secrete pro-angiogenic fators, wound scratch assay was performed by PDMSC-condition media of cells cultured in growth media with 10% FBS for 72 hours. Conditioned media were prepared in each cell type by growing the cells in 70% confluent plates in growth media with 10% FBS for 48 h. The respective media were collected and centrifuged at 1800 rpm for 10 min to remove cellular debris. The supernatant was stored at 4°C till use. After the wound is made, debris was removed by washing with PBS and the area of wound is recorded at every 6 hours for migration by using a bright field phase contrast microscope (Nikon eclipse TE 2000-s) and photographs were taken. The effect of wound healing was calculated as the percentage of remaining cell-free area (at 14 h) compared with the initial wound area.

The defined area was counted for number of cells before the wound is made and the percentage is taken as 100%. Spotting the same defined area, a scratch is introduced where the cells in the injury area was visualized and the number of cells which had migrated into the initially cell-free scratch area was counted and percentage of wound healing was calculated against the 100% of the initial unwounded cell count in the same area.

Semi quantitative PCR

Total RNA from PDMSCs differentiated in normal and restricted conditions was introduced to dispase to isolate the cells from Matrigel and separated the cell pellet by centrifuging at 1800 prm for 15 min. Further the cells are taken out and added 0.5 ml of TRI reagent according to the manufactures instructions (Sigma Aldrich, St. Louis, MO, USA). Reverse transcription reactions were performed in a 20 ml volume with 1-5 mg of total RNA using the AMV-Reverse transcriptase (Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer's protocol. Using the cDNA, expression of mRNA related to angiogenesis was investigated using GAPDH as a positive control. Specific primers from the cDNA were amplified by using RT-PCR master mix (Platinum PCR Super Mix, Invitrogen, US), DEPC water and the respective primers (IDT). VEGF, VE-cadherin, CD-31, VWF were checked for confirming the angiogenic property.

Results

Culturing of placental derived mesenchymal stem cells (PDMSC)

Placental derived Mesenchymal stem cells was used for the study due to their low level expression of immune stimulatory molecule HLA class 1 and higher levels of the immune suppressive molecules PDL-1 and CD1a, compared to bone marrow derived mesenchymal stem cells. Apart from their tri- lineage differentiation potentiality into adipo, osteo and chondro in vitro, it also differentiates into endothelial cells. Current clinical trials focus mainly on the availability of qualitative Mesenchymal stem cells for the study of Angiogenesis. To understand the series of angiogenic development in micro nutrient deficiency, initially cells were cultured in growth media lacking Folic acid and Vitamin B9 and later taken for angiogenic assays and Molecular analysis.

Characterization of human placental MSC's

Mesenchymal stem cells were isolated from human placenta (hPDMSc), confluent cultures were established and were cultured through a number of passages. Cells were characterized for percentage expression of MSC- specific positive markers CD 90, CD 105 and MSC negative marker negative marker CD 34, CD 45, HLA-DR by flow cytometry. Further to confirm angiogenic potential, we investigated presence of vegf, ve-cadherin, cd31, vwf. Thus, isolated and expanded

cells were confirmed to be a homogenous population of MSCs before subjection to differentiation.

*In **vitro*** **differention studies:** At the fourth passage hPDMSC s were induced to differentiation into osteogenic, adipogenic and chondrogenic lineages using previously described protocol. Angiogenic differentiation was assayed using Matrigel and wound scratch assays as described below.

Osteogenic differentiation: Cells were induced to differentiate into the osteogenic lineage by using induction medium containing ascorbic acid (500 mg/ml), b-glycerophosphate (10 mM) and dexamethasone (1 mM) (Sigma Aldrich, St. Louis, MO, USA) and cultured for 18 days. Osteogenic differentiation was determined by staining with 2% Alizarin Red for 15 min. Observation of a bright red to brown color indicated the formation of calcium deposits.

Adipogenic differentiation: Transformation into adipogenic lineage was accomplished using induction medium containing dexamethasone (1 mM), isobutyl methyl xanthin (IBMX 0.5 mM), indomethacin (200 mM) and insulin (1 mg/ml) in growth medium. Cells were cultured and maintained for 10-15 days in this medium till the appearance of lipid droplets were observed. Lipid deposition was visualized by staining with oil red 'O' stain.

Chondrogenic differentiation: The hMSC chondrogenic differentiation medium is offered by lonza as a BulletkitTM Medium (catalog no: PT-3003) which includes both the basal media and the necessary supplements (with the addition of TGF-β3 [sold separately, catalog no: PT-4124]) for chondrogenic differentiation of human bone marrow derived mesenchymal stem cells. The basal medium and the necessary supplements were purchased separately for the 21 day differentiation protocol. To quantify we have adapted previously published methodology to assess the success of the chondrogenic differentiation by staining with Alcian blue staining.

Analysis of angiogenic markers: After observing a delay in angigogenic lineage commitment in restricted micro nutrient condition, to ascertain the expression of angiogenic and vascularization markers in normal and restricted PDMSCs, mRNA transcripts were profiled using RT-PCR. A panel of markers VEGF, VE-Catherin, VWF and CD-31 is being used to compare the endothelialization at 10^{th} hour in both conditions. This result precisely signifies the endothelial lineage commitment.

Discussion

We investigated the effect of micro nutrient deficiency in angiogenesis where a detained developmental process is observed. The sequential behavior of delayed angiogenic development can cause delayed replication, abnormal cell cycling and increased apoptosis [16,17]. This probably explains the relevance of micro nutrients for an apt angiogenic functioning specifically vitamin B6 and B9. *In-vitro* analysis of tube formation assay is further studied at its cellular level by checking the mRNA transcripts for endothelial expression taken at a prticular time. Our data also support the earlier published study that the placental MSC has an inherent property to secrete migratory factors which is an essential component of angiogenesis. Wound healing is a dynamic process involving the following phases: rapid hemostasis, appropriate inflammation, Mesenchymal cell differentiation, proliferation and migration to the wound site, suitable angiogenesis, prompt re-epithelialization and tissue remodeling including proper synthesis, cross-linking and alignment of collagen to provide strength to the healing tissue[18]. Delayed wound healing due to production of angiogenic inhibitors causes endothelial cell dysfunctioning and death, platelet activation leading to the release of tumor [19] and can cause impaired wound healing by affecting one or more phases of the process. Another major challenge in future is to study the mechanism of Vit B6 and Folic acid on angiogenesis which also involves its association with the activation of intracellular pathways that converge on the reestablishment of endothelial tube functioning.

Angiogenic differentiation and pro-angiogenic potential-migration property

The process Angiogenesis is regulated by a tight balance between pro- and anti-angiogenic agents involving a cascade of events of which migration of capillary endothelial cells is an important component. The developmental stages of angiogenesis include Initiation, Proliferation/ Invasion, Maturation/ Differentiation [20]. Once angiogenesis is initiated, it activates different types of cell population like endothelial cells, fibroblast, macrophages and mast cells which in turn releases various types of cytokines, chemokines and vegf. Angiogenic factors like VEGF, PDGF, FGF, TNF-α and adenosine are released. During this stage, changes occur in the blood vessel wall which allows the migration and proliferation of EC's towards the target tissue. Endothelial cell migration is a process which requires the integration of signals elicited by chemotactic, haptotatic, and mechanotactic stimuli which in turn is associated with the activation of intracellular pathways that converge on cytoskeleton remodeling [21]. Proteolytic digestion of basement membrane causes EC junction to dissociate and further during maturation, EC's forms a tube like structure and it restores the tight EC junction complexes. During this maturation stage, there are several Angiostatic factors which get released like Endostatin, Angiostatin, Angiopoietin-2, Interferon gamma (IFN-γ), Nitric oxide. Predominantly, when the existing EC's are exposed to stimuli,

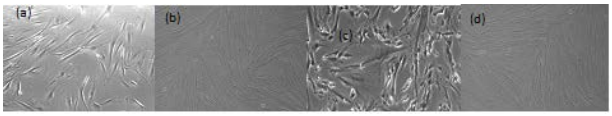

Figure 1a: Cultured PDMSC's in normal and restricted growth media: a) Cells maintained in Basal Media containing 10%FBS and antibiotics (Normal condition) at passage 4 in day 2. b) Confluent cells in normal condition at day 4. c) Cells grown under restricted conditions in Basal media without vitamin B12 and Folic acid with 10%FBS and antibiotics at passage4 in day 2. d) Cells attained confluence at day 4.

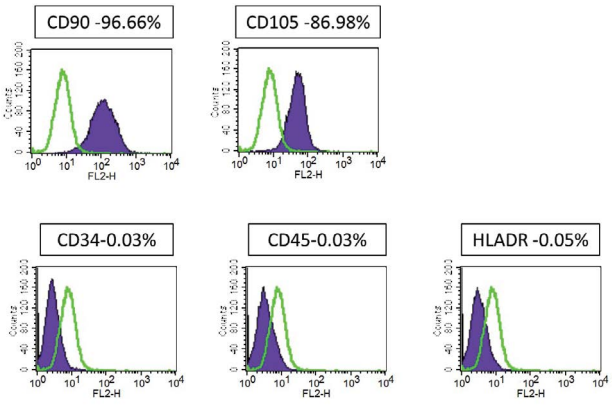

Figure 1b: Characterization of PDMSC's by Flow cytometry showing positive population for CD90, CD105 and negative for CD34, CD45, HLA-DR.

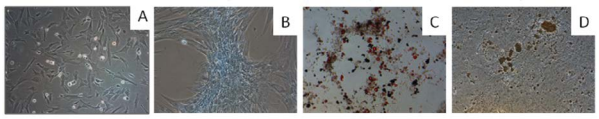

Figure 1c: Trilineage Differentiation in PDMSC's: (A) represents undifferentiated and unstained cells. PDMSCs could successfully differentiate into chondrocytes (B), adipocytes(C) and osteocytes (D).

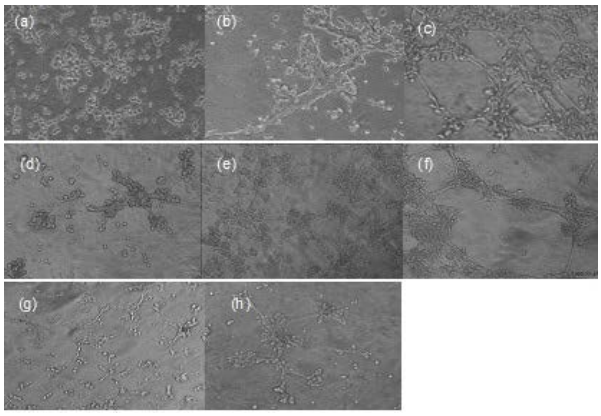

Figure 2a: PDMSC's grown in Normal media condition (DMEM+10%FBS+Antibiotics) is seeded in a 12 well plate coated with matrigel showing Endothelial Tube Formation within 12hrs.VEGF, vascular endothelial growth factor is added at a rate of 50ng/ml. a) Cell seeded at a density of 50000cells/well and morphological changes are observed after 2hrs. b) Tube like structures is protruding from the cell clusters after 4hrs. c) A tube like structure connecting different clusters is formed at 12hrs and Ring formation is clearly visible. *In-vitro* endothelial tube formation assay in restricted condition. PDMSC'S grown n restricted media condition (DMEM without folic acid and vitamin B12+10%FBS+Antibiotics) is seeded in a 12well plate coated with matrigel showing Endothelial Tube Formation within 15hrs. As treated in control, 50ng/ml VEGF is added exogenously. d) Cell seeded at a density of 50,000 cell/well. e) Though morphological changes are observed, compared to control, initiation of cell clustering is slow at 2hrs, even after 4 hrs there is no much change observed. f) After 6hrs branching is initiated and tube formation is noticed at 8hrs after seeding. Stable rings are formed by the end of15hrs. g) Formed tube gets destabilized within 24hrs under restricted condition whereas h) After 24 hrs the tubes formed started destabilizing gradually.

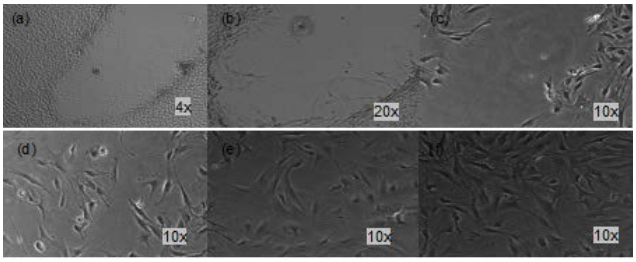

Figure 2b: Placental Derived Mesencynal stem cells grown in normal grwoth media is observed for its migration property. a) A scratch is made in a 100%confluent plate. b) Scratch shown in 20x magnification. c) After 12 hrs the cells are migrating towards the wounded area. (d, e, f), The migration is shown at different timings after 15, 20, 24 hrs.

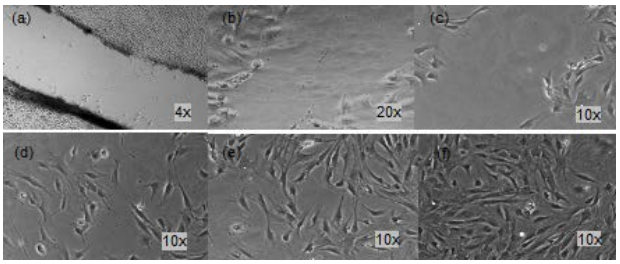

Figure 2c: Placental Derived Mesencynal stem cells grown in restricted media condition (DMEM without folic acid and vitamin B12+10%FBS+Antibiotics) are observed for its migration property. a) A scratch is made in a 100% confluent plate. b) Scratch shown in 20x magnification. c) After 12 hrs the wound seems to be clear unlike the normal case. d) A delayed migration is observed after 20hrs. e) 90% confluency is attained by 28hrs. f) Cells migration shown after 48hrs.

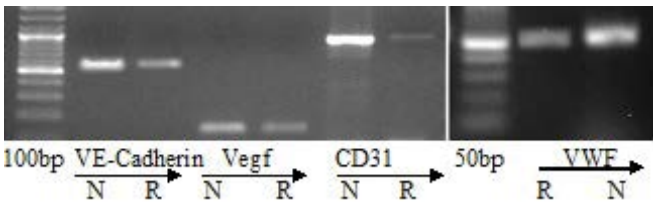

Figure 2d: Expression of Angiogenic markers in Normal and micro nutrient deficient conditions. By RT-PCR it was observed that there is a prominent difference in the endothelial expression in normal and restricted taken at a particular time point.

they detach from each other due to alterations through adherens in tight EC junction complexes. Further metalloproteinase are released which degrades the underlying basement membrane and surrounding structural elements. Hence angiogenesis occurs and is often associated with the increased capillary permeability which serves to enrich to adjacent interstitial compartments of plasma components. When the EC monolayer is destabilized, the cells then migrate towards the angiogenic stimulus through the extra vascular space via integrin (avβ3, avβ5) - mediated adhesion to matrix proteins. Along with this, concomitant proliferation of EC's lining the vessel wall to replace the previously migrated cells. These proliferated EC's forms cord-like structures which later canalize to form functional vessels, which are further stabilized by surrounding pericytes. In the absence of angiogenic stimulus, the ECM contributes to maintain the endothelial cells in a quiescent state [22].

Among the angiogenic factors, vegf and its receptors have been studied most extensively as the essential factor in regulating the functions of endothelial cells and maintaining the vascular system. It is a potent angiogenic agent that regulating all key steps involved in angiogenic process. Another major role of vegf is in the transition of an anti-angiogenic state to a pro-angiogenic phenotype [23]. Vascular endothelial (VE)-cadherin is a strictly endothelial specific adhesion molecule located at junctions between endothelial cells. In symmetry of the role of E-cadherin as a major determinant for epithelial cell contact integrity, VE-cadherin is of vital importance for the maintenance and control of endothelial cell contacts [15,24]. CD-31 is expressed at high levels on early and mature endothelial cells, platelets and leukocyte. The expression on endothelial cells is concentrated at junctions between adjacent cells [25]. Tie-1 and Tie-2 are expressed exclusively on endothelial cells and is a potent angiogenic regulators involved in

Primer	sequence	Tm(˚c)	product size (bp)
Vegf	F- 5'ctacctccaccatgccaagt-3' R- 5'gcagtagctgcgctgataga-3'	52	109
VWF	F- 5'cggcttgcaccattcagcta-3' R- 5'tgcagaagtgagtatcacagccatc-3'	53	90
CD 31	F- 5'attgcagtggttatcggagtg-3' R- 5'ctcgttgttggagttcagaagtgg-3'	52	965
VE-cadherin	F- 5'acgggatgaccaagtacagc-3' R- 5'acacactttgggctggtagg-3'	52	596
Tie-2	F- 5'agaccagcacgttgatgtga-3' R- 5'tgggttgcttgaccctatgt-3'	60	282
Ang-1	F- 5'agcagcctgatcttacac-3' R- 5'atgatgatggtcgacggc-3'	50	115

Table 1: Angiogenic markers.

	0-2 hrs	4-6 hrs	8-10 hrs	12-15 hrs	After 15 hrs
Cells grown in Normal growth media seeded in Matrigel. (SD=5×10^6)	Cell morphology changes and initiated cell clustering	Tube formation is initiated within 4hrs.	Partial ring formation is observed	Within 12hrs complete ring formation happens.	Formed rings are stable
Cells grown in Restricted growth media seeded in Matrigel. (SD=5×10^6)	Cells remains the same	By late 4hrs cell morphology changes and cell started clustering.	Tube formation is initiated after 8 hrs.	Partial ring formation is seen.	Complete ring formation occurred.

Table 2: Comparison of tube formation in normal and nutrient deficient conditions.

vessel remodeling, vessel maturation and endothelial cell survival. Ang-1 is the ligand for Tie-2 and both components are required for vascular formation. Ang-1 binding to tie-2m causes phosphorylation and protects endothelial cells from apoptosis and this effect is antagonized by Ang2/Ang3. Ang-1 is believed to stabilize and prevent leakiness of vessels whereas Ang-2 destabilizes the existing vasculature causing vessel sprouting and new vessel growth in the presence of VEGF, but resulting in regression of vessels without the presence of VEGF. Nevertheless, Ang-1 promotes *in-vivo* angiogenesis in a Matrigel plug assay and Ang2 increases endothelial cell migration and sprouting [26].

MSC co-ordinate with endothelial cells to replenish the blood supply and participate in vascularization, which is a fundamental process in tissue repairment. MSC are also known to have paracrine effects and release growth factors, chemo attractants and immunomodulatory factors that aid in angiogenesis [27]. Our data supports our observation of a rapid ability of PDMSCs to cover the wounded region and endothelial tube formation in Matrigel. Though many works have been carried out in various aspects of endothelial cells, our interest was to study the mannerism of angiogenic development in micro nutrient deficiency condition. Spontaneous organization of endothelial ring formation under micro nutrient deficiency was studied for time dependant manner 2-15 hrs in Matrigel in comparison to normal with the addition of Vegf in both growth media. We were likely to observe that there is a delay in angiogenic initiation where the cell aggregation occurred very slowly. A complete ring formation is viewed after 15 hours in restricted condition and the tubes formed were not stable enough for long time as seen in normal condition. This observation apparently indicates the crucial role of Vitamin B12 and B9 in proper angiogenic functioning. Our studies also suggest that migratory factors are secreted by PDMSC as the condition media collected from PDMSC cultures are rich in factors promoting migration. A recent study indicated that the placental MSCs secrete factors promoting migration, immune modulation and angiogenesis [28].

The above discussion underscores the complexity of micro-nutrient deficient responses to angiogenic stimuli along with the diversity in the responses of the microvasculature to this condition. We examined the tube formation potentiality of Placental derived Mesenchymal stem cells (PDMSCs) under micro nutrient deficient(without Vitamin B6 and Folic acid) condition to study the nature of angiogenesis in anemic like diseases. The study is compared to angiogenic differentiation conducted in normal growth media in which both the conditions are supplemented with an addition of VEGF. It is well known that Vitamin B9/Folic acid is crucial for proper brain function, plays an important role in mental and emotional health and is essential for replication, synthesis and repair of nucleotides for RNA and DNA.

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