Can aerobic exercises normalize endothelial growth factor (VEGF) in patients of peripheral arterial disease?

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Abstract:

Purpose:
The purpose of the current study was to find out the effect of eight weeks of aerobic training on vascular endothelial growth factor (VEGF) protein and capillarization in peripheral arterial disease (PAD) patients. Therefore, we aimed to compare VEGF and capillarization of PAD subjects (n=10), who trained aerobic exercise for eight weeks against that of normal healthy subjects (n=9).

Methods:
The muscles biopsies were taken from the middle part of vastus lateralis muscle at baseline and post -training. Vascular endothelial growth factor and capillarization was determined, using the immuno- histochemistry and RT-PCR protocol used previously for assessment of skeletal muscles and angiogenesis.

Results:
The peripheral arterial disease subjects had lower vascular endothelial growth factor (30%) as compared to control group at baseline ($P<0.01$). Capillary to fiber ratio was almost similar in both groups before the training intervention. However, after eight weeks of aerobic exercises, there was about 65% increment in VEGF ($P<0.05$), 20% increment in capillary to fiber ratio ($P<0.01$) and 10% increment in capillary density in peripheral arterial disease group compared with control group. There was also some increment in fiber size too in PAD group but no changes were seen in myofiber distribution.

Conclusion:
In summary, aerobic training significantly increase the vascular endothelial growth factor (VEGF) level in muscle tissue of peripheral arterial disease group. There was also improvement in capillary to fiber ratio, capillary density and fiber area, which shows that aerobic training, is the effective strategy to combat and prevent peripheral arterial disease.

Key words: *Aerobic exercises, VEGF, Capillarization, peripheral arterial disease.*

Introduction:

Angiogenesis is the natural process characterized by the formation of new blood vessels. It is the important process which plays key part in restoration of blood flow to the tissues after injury or insult[1]. Angiogenesis may be physiological or pathological and normally it is very effective mechanism of regeneration after tissue injury or insult. However sometimes angiogenesis also occurs in pathological conditions like in growth of tumor[2].

Angiogenesis is controlled by the existence of physiological balance between the angiogenetic stimulator and endogenous inhibitors[1].There is many pro-angiogenic factors identified but VEGF is recognized as best pro-angiogenic growth factor because it is very specific for vascular endothelium[3]. Vascular endothelial growth factor (VEGF) is one of the potent glycoprotein which is very specific mitogen for the cells of endothelium. Its size ranges about 34-45 KD[4].

Inactivity or sedentary life style leads to different chronic diseases in the form of obesity and in the form of arterial insufficiency. There are about 5 million people only in USA suffered from obstructive vascular disease i.e. peripheral arterial disease (PAD) and it is one of the main reason of morbidity and impairment[5]. Exercises are the effective tool which can be helpful for improving good quality of life in such type of diseases by promoting angiogenesis[6]. In response of aerobic exercises in peripheral arterial disease, there are adaptive changes in skeletal muscles in the form of angiogenesis or arterio-genesis. There is increased capillary density and mitochondrial activity in exercises skeletal muscles[7]. Exercise training is one of the few physiological processes that activate organized angiogenic response in adults and results in formation of new blood vessels[5].

Exercises induced hypoxia is potent stimulus for angiogenesis by the up regulation of vascular endothelial growth factor[7]. Exercises to improve endurance or aerobic exercises may cause the formation of new blood vessels but it does depend on intensity and type of exercise[8]. In response to endurance exercises, there are number of adaptive changes occurs, mostly in musculoskeletal and cardiovascular system. Aerobic exercises induce skeletal muscle angiogenesis which helps in aerobic capacity of skeletal muscle and increase microcirculation[9].

The aim of this study was to find out the affect of eight weeks of aerobic exercise training on the level of vascular endothelial growth factor (VEGF) and on capillarization in patient suffering from peripheral arterial disease. For this purpose we had taken subjects suffering from peripheral vascular disease and these subjects had taken part in eight weeks for aerobic exercises on treadmill under the supervision of physiotherapist. Muscle biopsies were taken for analysis of VEGF and capillarization at baseline and post training in both groups.

Material and methods:

Participants:

10 (6 male and 4 female) individual suffering from mild to moderate peripheral vascular disease diagnosed by their physician, selected randomly by clinical database and 9 (5 male, 4 female) normal healthy individuals as control group was participated in this study. All participants were recruited from Orebro city. They were not taking any medication and had not been engaged with any endurance or strength training programme. They were ranked sedentary according to their level

of activity (>30 minute moderate physical activity 3days/wk).They were participated voluntarily in this study after oral and verbal explanation of the methods and purpose of the study according to the guidelines of the research committee of Orebro University. They had been informed about related risks and benefits of the study and they could withdraw from study at anytime. The research was approved by the research department of the Orebro University. The participants were instructed to participate regularly in performing aerobic exercises. The physical characteristic of the subjects with peripheral arterial disease is shown in the table below.

	Male (n= 6)	Female (n= 4)
Age (years)	46±5.5	41±3
Weight (Kg)	97±3	85±3
Height (cm)	171±4.5	166±3
BMI (Kg/m^2)	32.2±2	32.5±1.8

Table-1 Physical characteristic of PAD group, data presented as mean and SD.

Study design:

All subjects were participated in eight weeks of aerobic exercise training under the supervision of Physiotherapist. The instructions and guidelines about aerobic exercise training were provided to each individual in written and oral form. The training programme consisted of total 40 minutes workout 4days/ week [10] for eight weeks [16] including aerobic training, warm up and cool down period. Started with 5-7 minutes of warm up exercises which consisted of light physical activities and stretching of large muscles groups of body was performed followed by paddling on cycle ergometer for 30 minutes with 50 rpm and cool down period of 5 minutes [11,17].

Visits:

Muscles biopsies were taken at pre and post training period. The subjects had visited in gym on regular basis for aerobic training 4 days per week for eight weeks. For sample collection of muscle biopsies, they had come in lab two day before and two day post intervention.

Muscle biopsy:

Muscle biopsies were taken in two separate occasions, pre-training and other one on post- training period from the middle part of vastus lateralis. First of all anesthetize the local area of vastus lateralis near to the muscle belly by using local anesthesia in the form of 1% xylocaine, make a cut of 1-2cm long and 0.3cm in diameter in vastus lateralis and then was taken biopsy with the help of weil-blakesely forceps[10]. The post training muscle biopsy was taken from same muscle and horizontally about 2cm apart from previous incision. Then muscle tissue was blotted, freeze in liquid nitrogen and store in -70 Celsius. The sample was divided into parts for further analysis. This is valid method and used previously by Charifi et al[10].

Immuno-histochemistry:

Frozen biopsies were dissected by using cryostat into 6 µm thick transverse section and then microscopic slides were prepared. Care was taken not to stretch or damage the specimen with no air bubbles. The section was fixed by immersion in acetone about -20 Celsius for 1 min followed by incubation for 2 minute in formaldehyde in room temperature. These sections were washed in 10 mmol/liter PBS containing 1 % of bovine serum albumin and retain for one hour. The identification of capillaries was performed by using monoclonal antibody CD31 as a endothelial cell marker in 1:10 dilution (Daku, Glostrup, Denmark; M0823). Capillaries and muscle fibers type composition were analyzed randomly selected cross-sectional areas and visualized at total magnification of ×400. Mean fiber area was quantified by using computer software TEMA (Version 95, Denmark). For each muscle biopsy, capillary density; capillary to fiber area, fiber cross sectional area was measured by using microscopy and TEMA software. CD31 marker is reliable and was used previously by Hansen et al[11].

RNA Isolation and Real time PCR analysis:

Approximately 20 mg of muscle tissues collected previously was frozen in liquid nitrogen and was homogenized in Dounce homogenizer in RIPA. RNA was isolated by using RNEasy fibrous tissue mini kit (Qiagen, Valencia, California, and USA) in accordance with the guidelines provided by the company. Quantification of total protein was done by using bicinchoninic acid (Bio-Rad Laboratories, Hercules, CA). After quantification, the isolated RNA was examined for integrity with the help of Agilent 2100 bioanalyzer and RNA 6000 Nano assay kit (Agilent technology, Santa Clara, CA, USA) and stored at -70 Celsius for analysis. Method validated previously by Ryan et al[12].

The mRNA content of VEGF was detected by real time polymerase chain reaction. Total mRNA obtained previously was reverse transcribed to cDNA with the help of SuperScript II first-strand synthesis kit (Invitrogen, Carlsbad, California, USA). The manufacturer instruction was followed. The cDNA was kept at -20ºC. For the gene expression of VEGF, quantitative real time Polymerase Chain Reaction (PCR) was executed on ABI Prism Sequence Detection System 7900HT (PE Applied Biosystems, Foster City, California, USA). VEGF was targeted gene in expression analysis, designed and provided by Applied biosystem (Foster City, California, USA). The probe was labeled by FAM and TAMRA as reporter and quencher dye. PCR cycles was consists of 50º C for 2 min, 95ºC for 10 min, 15 sec at 95ºC and one minute for 60℃. PCR amplification was correlated against standard curve. Recently, some researcher validated this method [11, 15].

Statistical analysis:

All data was presented as mean and standard deviation. Paired sampled T-test was used to obtain statistical significance values before and after training. Pearson Correlation coefficient was used to measure the degree of relationship between the variables.
P value less than 0.05 was considered significant.

Results:

Before aerobic training morphology analysis:

The participants with peripheral arterial disease had 30% lower capillary density (CD) than control group ($P<0.01$). Whereas, the fiber cross sectional area (FCSA) was slightly higher in subjects with peripheral arterial disease than control group. There was little difference in capillary to fiber ratio(C: F) between the two groups. The skeletal muscle fiber size before training was 5,251 ± 861 μm^2 and fiber type distribution in the peripheral arterial disease patients before training was 54.1% type I and 45.9 % Type II.

After the training period morphology:

The capillary to fiber ratio (C: F) in subjects with peripheral arterial disease was 20% higher ($P<0.01$) than before training (Fig. 1A). There is about 10% increment in capillary density but insignificant improvement in fiber cross sectional area in peripheral arterial disease group after training (Fig. 1B). There was improvement in fiber size area. The skeletal muscle fiber size after training was 6110 ± 1008 μm^2.

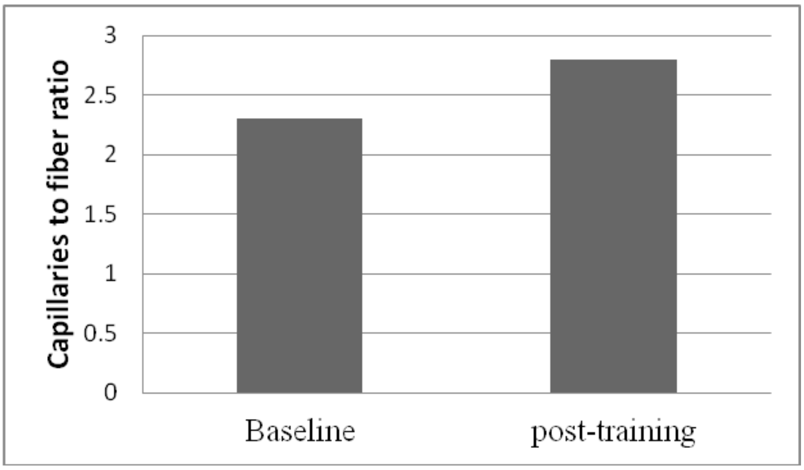

Fig. 1A- capillary to fiber ration in PAD subjects before and after aerobic training

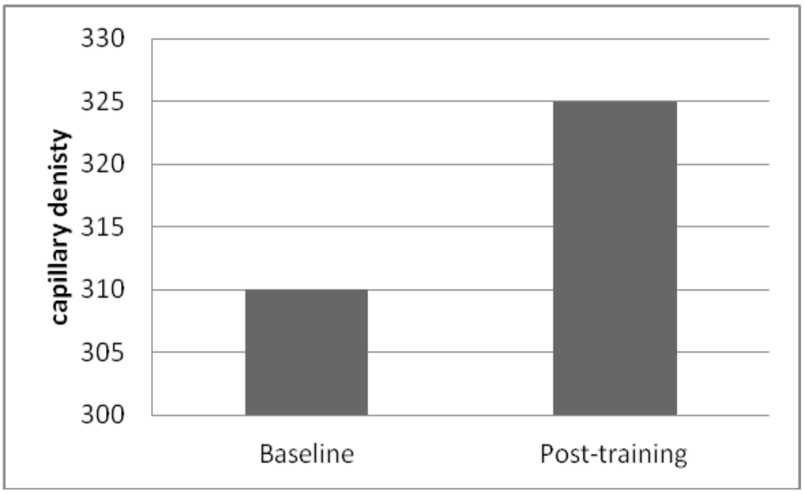

Fig. 1B- capillary density in PAD subjects before and after aerobic training.

VEGF protein level and mRNA in muscle biopsies:

Before training:

In peripheral arterial disease patients, there was not marked difference in the level of VEGF mRNA in comparison with control group. While the VEGF protein level was 30% lower in peripheral arterial disease participants than control group.

After training:

The level of mRNA did not alter significantly after training in subjects with peripheral arterial disease. However there was marked increase in VEGF protein level, about 65% (P<0.05) in peripheral arterial disease subjects. After training the VEGF protein level was approximately similar to the control group.

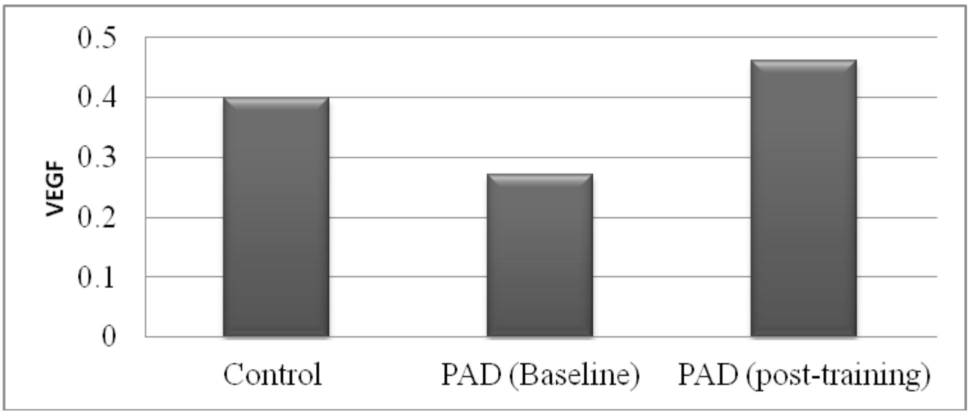

Fig. 2- VEGF Level in PAD subjects before and after aerobic training

Physical characteristics:

There was no marked difference regarding the sex and age in response to training for capillarization or VEGF protein (P>0.05) in subjects with peripheral arterial disease in comparison with control group. However, subjects with peripheral arterial disease differ in term of body mass index (BMI) and their physical activity level from control group. Subjects with PAD had more BMI (>32) but less physical activity than normal subjects.

Discussion:

Our current study demonstrated that, the aerobic exercise training in subjects suffered from peripheral arterial disease have a very positive effect on the level of VEGF protein level in the muscles. Furthermore, aerobic training augmented the angiogenic response and capillarization in peripheral arterial disease subjects. This is obvious in our results as there is marked increase in level of VEGF protein and capillarization in term of capillary to fiber ratio and in capillary density. Subjects with peripheral arterial disease had lower VEGF level and capillary density than normal subjects. There was about 65% increase in VEGF protein level, 20 % in capillary to fiber ratio, and 10% in capillary density, after the eight weeks of supervised aerobic training programme in subjects with mild to moderate peripheral arterial disease. The increase in capillary to fiber ratio and capillary density indicates that there was more capillary growth and oxygenation capacity in peripheral arterial disease subjects after aerobic training. These changes help in more uptake of oxygen and availability of more blood supply to the muscles. There were no significant changes in fiber type composition but improvement in muscle fiber size.

The present finding of enhancement of VEGF level with aerobic training in PAD subjects is not reported before but Hansen et al[11] have demonstrated the role of combined exercise training (strength + endurance exercises) in hypertensive patient. They had found significant response of exercise training on hypertensive patient in the form of improvement in the VEGF level and capillarization in term of capillary to fiber ration and increment in capillary density. Gavin et al[13] showed the acute systemic exercises have positive effect on pro -angiogenic factors but some researchers[13], demonstrated that, there is less angiogenic response in human after exercise training with increasing age. Jansen et al[8], discussed the intense intermittent enduring training can bring changes in VEGF level after 4 week of training. So it seems that exercises are the good intervention for angiogenesis but response depends mainly on the type, duration, frequency and intensity of exercise. More intense and long duration exercises seem to have more positive effects on angiogenesis. In the current study, the level of mRNA was almost similar before and after training in subjects with peripheral arterial disease. From these facts, it seems that increase in VEGF protein level in muscles have no direct association with VEGF mRNA level in response of aerobic training programme. The same finding was reported before[11]; increase in VEGF level is not dependent on VEGF mRNA level in muscles. In contrast to this, some studies by Lloyd et al[14], demonstrated that increase in VEGF level is directly related with mRNA level in skeletal muscles. In response of increase in mRNA the VEGF level is increases, in a manner that they are directly correlated with each other. This effect may be due to intensity and frequency of the exercise[8] or other factors like aging[12]. These factors suggested that, greater the stimulus in the form of intense exercise, there will more increment in mRNA and in return VEGF level. It might be the mRNA is the controlling machinery for VEGF level which regulates the VEGF level and angiogenesis in response of intensity and frequency of the stimulus. The stimulus for angiogenesis may be in the form of hypoxia or hypoglycemia[4].

It is worth of noticing in our results that subjects with peripheral arterial disease have more BMI and less physical activity in comparison with normal subjects. So, it seems overweight and sedentary lifestyle may be the causative factors for arterial disorders. There is no significant difference regarding the sex of the participants suffered from peripheral arterial disease. The reduction in BMI after aerobic training has positive effect in relieving the symptom of intermittent claudication. So, aerobic exercises can be used as therapeutic intervention for preventing and managing arterial diseases or other cardiovascular problems. It is obvious from the different studies that aerobic exercises play a vital role to combat different conditions. As, in our studies aerobic training bring positive changes on angiogenesis. Some studies demonstrated that the angiogenic capacity

decrease with age in response of exercises, so acerbic exercises can be used as preventive strategy to combat different conditions including peripheral arterial disease because mostly people suffered in their middle or later part of life. In early part of the life, proper acerbic exercises will be more helpful in preventing and managing these types of cardiovascular disorders. In short, current study suggests that aerobic exercise training is very useful for promotion angiogenesis, increase in VEGF level and more capillarization in skeletal muscles. So, aerobic exercise therapy can be used to get benefit in patient with peripheral arterial disease.

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