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Anti-Angiogenic Activity of L-Type Voltage Gated Calcium Channel Blocker, Nifedipine: An In-Vitro and In-Vivo Study

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ABSTRACT

Angiogenesis is the development of new blood vessels. A wealthy number of ion channels are found on the endothelial cells. These ion channels play a vital action in cell proliferation and in related angiogenesis. We aimed to investigate the effects of Nifedipine (L-Type voltage gated calcium channel blocker). The anti-angiogenic activities of Nifedipine was investigated by measuring its effects on number of branches formed, angiogenic score, number of sprouts formed, weight of sponge implanted, Hemoglobin content and histopathological studies by *in-vitro* (aortic ring assay) and *in-vivo* (sponge implantation method) methods. The test and standard drug (Bevacizumab) groups were compared with the control group using One-way ANOVA, followed by post hoc test, the Dennett's test to compare mean of all the groups with the control mean. The results revealed that Nifedipine treatment led to significant inhibition of proliferation and related angiogenesis in the dose dependent manner and were quite comparable with the standard antiangiogenic drug Bevacizumab. These scientific findings indicate the clinical benefits of Nifedipine in pathological situations involving excessive angiogenesis. Negative regulation of cell cycle progression at various checkpoints and cell migration may be the underlying molecular mechanisms for antiangiogenic action.

Keywords: Angiogenesis, aortic ring assay, sponge implantation method.

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INTRODUCTION

Angiogenesis is the generation of new blood vessels from pre-existing vasculature[1]. Dis regulation of angiogenesis results in many pathological conditions. Historically the best known are cancer, psoriasis, arthritis, blindness, additional disorders such as obesity, asthma, arthrosclerosis and infectious diseases are included and the list is still growing[2,12,13]. Angiogenesis plays a vital role in the pathological progress, growth and metamorphosis of tumor[3]. The newly developed blood capillaries can support cancer growth by supplying nutrients and oxygen and by taking away waste products. Metastasis also depends on angiogenesis, as tumor cells are shed from a primary tumor and grow at their target organs through the blood capillaries[4,16]. So inhibition of angiogenesis and reducing the formation of new blood vessels may offer therapeutic opportunities in this way[5, 6].

Endothelium is a multifunctional surface for signal transduction. It regulates several basic processes in vascular system like control of blood flow, blood pressure, coagulation, platelet aggregation, vessel permeability, wound healing and angiogenesis. Very surprisingly endothelial cells (EC) express abundant variety of ion channels. Only a few ion channels have been identified at the molecular level although some of them are distinctly emerging. One of the important functional targets of ion channels on endothelium is Calcium signaling. It is important for the EC to activate Calcium entry upon various stimuli. Electrogenesis in EC is potentiated by K^+ channels, Ca^{2+} activated K^+ channels and volume regulated anion channels (VRAC)[7, 8]. Recently the abundance of ion channels in the plasma membrane of non-excitable cells has raised questions about their functional role in angiogenesis.

Ion-channels are pore forming transmembrane proteins which plays an important role in many physiological process, Like Cell volume regulation, muscle contraction, neural depolarization, hormonal release etc. Ion channels are one of the most important players in EC function. So they may be a strong cause behind the EC dysfunction linked to many pathological conditions. Based on the above observations we have selected L-type voltage gated calcium channel blocker, Nefidipine to evaluate the anti-angiogenic effect at three different doses against two different models i.e. Rat aortic ring assay (*in vitro*), Sponge implantation method (*in vivo*).

MATERIALS AND METHOD

Chemical and lab wares:

Pure drug, Nifedipine was purchased from Sigma Aldrich Pvt. Ltd., Ketamine, Xylazin, Tramodol and Gentamycin injections were purchased from local medical stores, Matrigel was purchased from Becton Dickinson India Pvt. Ltd. (Gurgaon, India). Dulbecco's modified Eagle's medium

(DMEM) was supplied by Life Technologies (India) Pvt. Ltd. 24 well plates were purchased from Hi Media Laboratories Pvt. Ltd, India. All the chemicals used in the research are of AR grade.

Equipments:

High resolution digital camera, trinoccular microscope, surgical catguts (5/0) suture, curved needles, micropipettes, oven etc.

Methods:

Experimental animals and maintenance:

A total of 30 Wistar albino rats weighing in between 150-200 g were purchased from Teena labs Pvt. Ltd, Hyderabad, India. The animals were maintained at a controlled temperature (22–25^{o} C, 45% humidity) on a 12:12-h dark–light cycle. CPCSEA guidelines were strictly followed and the studies were approved by the Institutional animal ethical committee (IAEC), (Ref: CPCSEA /1657/ IAEC /CMRCP/PhD-14/30) CMR College of Pharmacy, Hyderabad, India. All the surgical procedures like preparation of rat Aortic rings and implantation of sponges were done by same investigator to increase the reproducibility of the process. The aortic rings and sponge implantation were randomized to eliminate potential bias in the degree of injury within the different groups.

Rat aortic ring assay method:

Rat Aortic Ring Assay method is widely used *in vitro* method for the evaluation of angiogenic and antiangiogenic drugs [19, 29]. A healthy male wistar rat weighing in between 180 to 200 gm sacrificed by cervical dislocation, thoracic cavity was opened and the visceral organs were separated. Thoracic aorta was identified and isolated by cutting both the ends. Immediately it was transferred to cold PBS supplied with aeration. Fibroadipose tissue was removed. Aorta was cut into 1mm ring sections and washed with DMEM (Dulbecco's modified Eagle's medium). These rings were put into the 24 well plates with 150µl of Matrigel. Rings were overloaded with matrigel and were allowed to polymerize for 1-2 hours at 37^{o} C and then exposed to hypoxic conditions for 2 hours. This hypoxic condition stimulates the formation of sprouts from the rings. This was reoxygenated for 7 days and the abundance of blood vessels was quantified. (Figure 1). Based on the scientific literature survey the doses were expressed as µM/ml and the three concentrations were selected based on the previous study. Group I was treated as control, group II standard, III, IV and V were provided with 10, 20 and 30 of Nifedipine respectively. [10, 11] Length of the branches formed was measured under microscope at 400 X magnification using stage micrometer and area of neovascularization was analyzed. (For results refer Figure 2).

Sponge implantation method:

Experimental Procedure:

Rats were anaesthetized by a cocktail of Ketamine (80 mg/kg) and Xylazine (5 mg/kg) and sponges were implanted *s.c.* Sponges of 2 cm diameter and 8 mm thickness were sterilized in 70% ethanol for 3 hours and then boiled at 70^{o} C for 30 min. All the surgical instruments were sterilized by autoclaving at 121^{0}C for 25min. Then the skin was cut open by surgical blade and sterilized sponge was inserted subcutaneously by forming an air pocket and sutured back by 5/0 silk sutures. Two such sponges were implanted on the mid-dorsal line of the body. After the animal recovered from anesthesia, they were kept separately one in a cage and were given regular diet and water. To reduce the surgical pain, Tramadol at a dose of 0.9 mg/kg was injected *i.m* twice a day. Gentamicin at a dose of 2 mg/kg was injected *i.m* for three days after surgery. In order to avoid further pain tramadol at the dose of 50 mg/kg *p.o.* was administered for next 1 week[9, 14].

Based on the literature survey the three concentrations were expressed as mg/kg [15]. The LD 50 values were obtained from acute toxicity studies, 1/10th of the LD 50 was used to select three concentrations, then using the dose conversion formulae of Jang-Woo Shin, et al., 2010, and animal doses were calculated. [20] Animals were divided into 5 groups by keeping 6 animals in each. Group I was served as sham and group II received 0.25 mg of Bevasizumab , III, IV and V were treated with the Nifedipine 0.75, 1.5 and 3.0 mg/kg respectively for successive 13 days. On 14th day the animals were sacrificed by cervical dislocation and the sponges were dissected out carefully without giving much traction on the sponges. All the dissections were done by the same person to avoid individual variations. Sponges were stained with hematoxylin and eosin, then observed under trinocular hi-definition microscope and photographs were captured. Wet weights of sponges were calculated on digital balance of 0.01 mg sensitivity (Figure 3 & 4). Immediately after weighing, hemoglobin content was calculated in all the sponges as per the method prescribed by Tahergorabi and Khazaei, 2012.

Determination of Hemoglobin content:

The sponges were soaked in double distilled water and homogenized 5min in a cooling centrifuge and the liquid was separated, and then centrifuged at 10,000 rpm for 5 minutes [26] .The supernatant liquid was placed in the cell count machine and the hemoglobin content was estimated as g/dl. (For results refer figure. 5)

Determination of Number of blood vessels formed per sponge:

The sponges were cut into two halves, soaked in normal saline at 4°C for 1 h and then put in 75% ethanol for 30 min and finally fixed in 90% ethanol. Paraffin sections (10 pm) were prepared and stained with Hematoxylin and Eosin (Figure.6). The slides were observed under trinocular

microscope and the circular spaces amidst the fibroblast growth regions represent the blood vessels formed in the sponges. (For results refer figure. 7)

Statistical analysis:

The statistical analysis was carried out by using graph pad Prism 5. Results were presented as mean ± SEM. The differences between the groups were compared by one way ANOVA followed by post hoc Dunnett's test. Results were considered statistically significant at p value<0.05.

RESULTS AND DISCUSSION

Aortic Ring Assay:

The quantification parameter used in this is area of neovascularization. A significant decrease in the area of sprouts was observed by the treatment with 10^{-5}M and 10^{-4}M Nifedinipe in aortic rings. Our results indicated that Nifedinipe has inhibitory effects on angiogenesis similar to Bevacizumab.

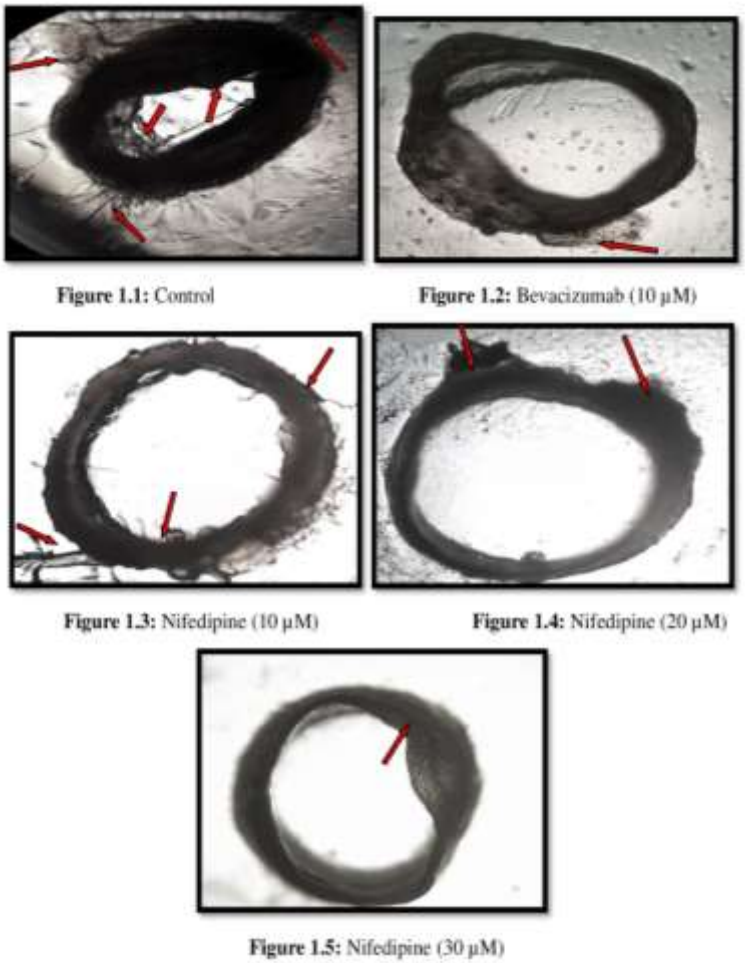

Figure 1.1: Control

Figure 1.2: Bevacizumab (10 µM)

Figure 1.3: Nifedipine (10 µM)

Figure 1.4: Nifedipine (20 µM)

Figure 1.5: Nifedipine (30 µM)

Figure 1: Effect of test drug on sprouting of Aortic rings in Aortic Ring Assay of angiogenesis

Effect of Nifedipine on Aortic rings was analyzed. Rats aortic rings were maintained in culture and the different concentrations of test substances were added to the rings. The area of sprouts was quantified in mm^2. Data is expressed as mean ± SEM. The ANOVA test followed by Dunnett's test was used to compare the data. Findings with p values ＜0.05 were considered statistically significant.

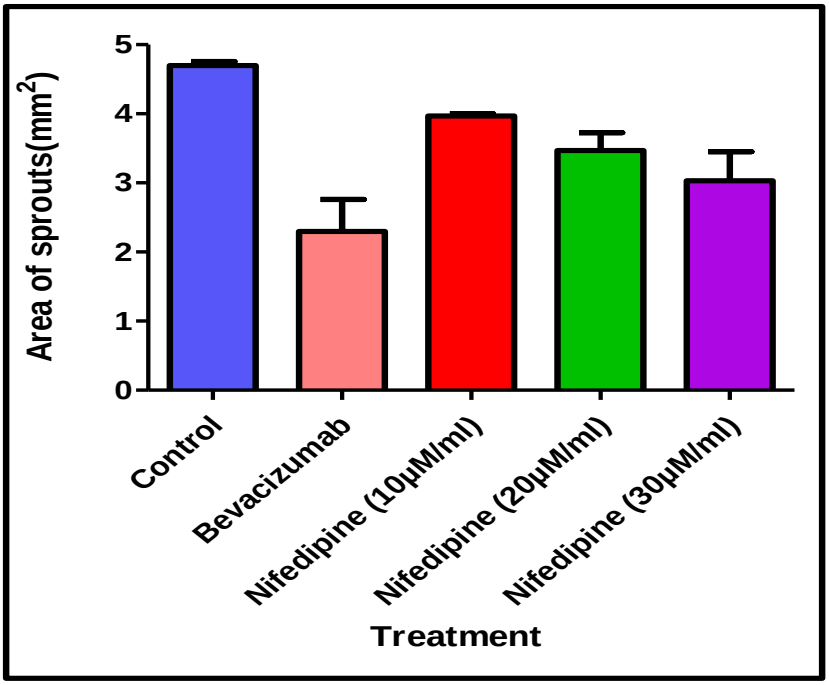

Figure 2: Graph showing the effect of Treatment groups on the area of sprouts formed in Aortic Ring Assay of angiogenesis

All the results were expressed as Mean ± SEM; n=6 The ANOVA test followed by Dunnett's test was used to compare the data. Findings with p values ＜0.05 were considered statistically significant and ns is insignificant.

Figure 3.1: Control group

Figure 3.2: 0.25 mg Bevacizumab

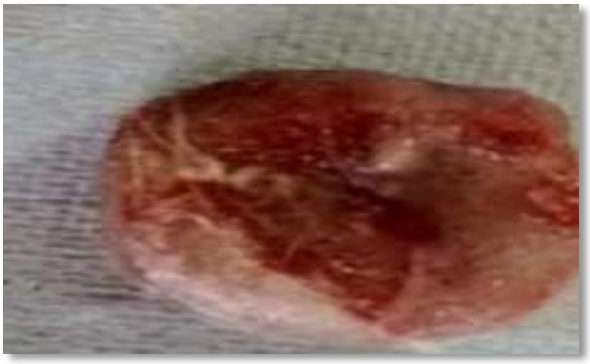

Figure 3.3: 0.75 mg/kg Nifedipine

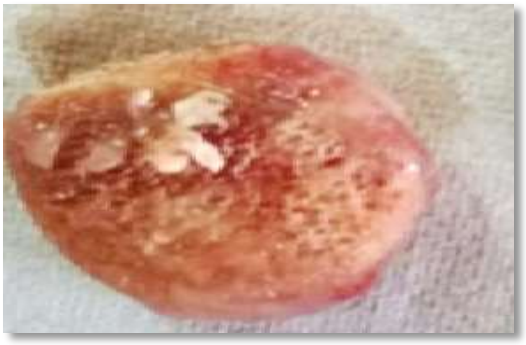

Figure 3.4: 1.5 mg/kg Nifedipine

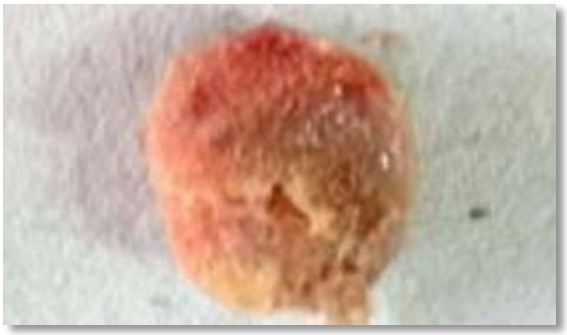

Figure 3.5: 3.0 mg/kg Nifedipine

Figure 3: Isolated sponge after 13 days of treatment

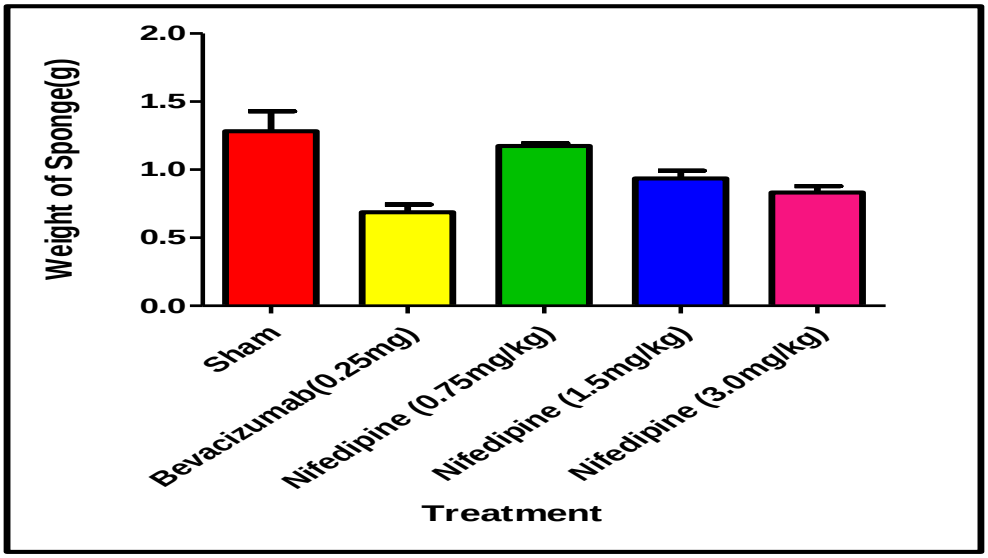

Figure 4: Graph showing the effect of Treatment groups on wet weight of Sponge

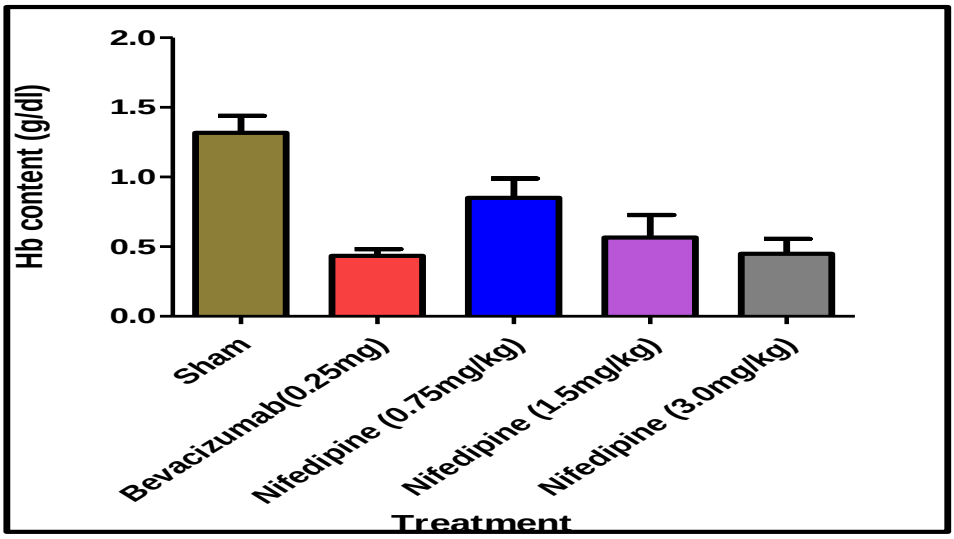

Figure 5: Graph showing the effect of Treatment groups on Haemoglobin content per sponge.

All of the data obtained from the experimental groups have been compared to the disease control group. The data was analyzed by one-way ANOVA followed by Dunnett's test using graph pad prism5.0 software.

Histopathological Report

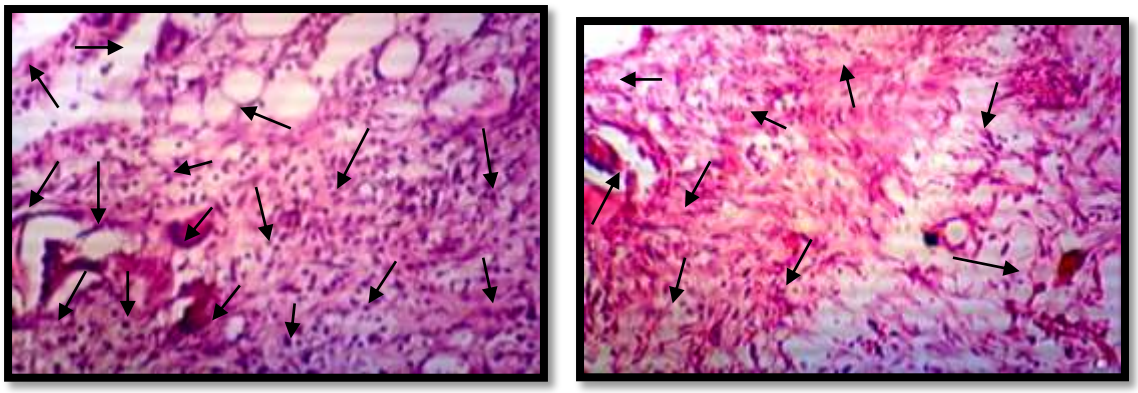

Figure 6.1: Control

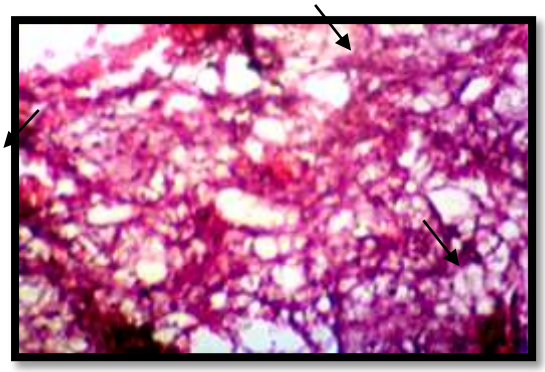

Figure 6.2: 0.25 mg Bevacizumab

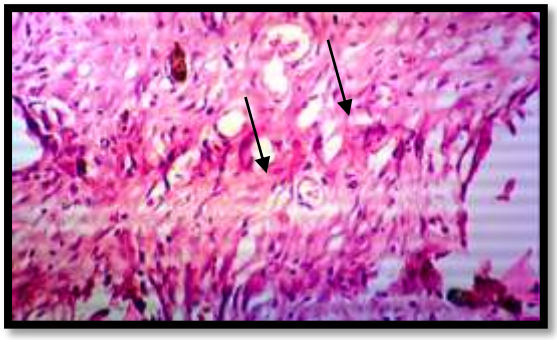

Figure 6.3: 0.75 mg/kg Nifedipine

Figure 6.4: 1.5 mg/kg Nifedipine

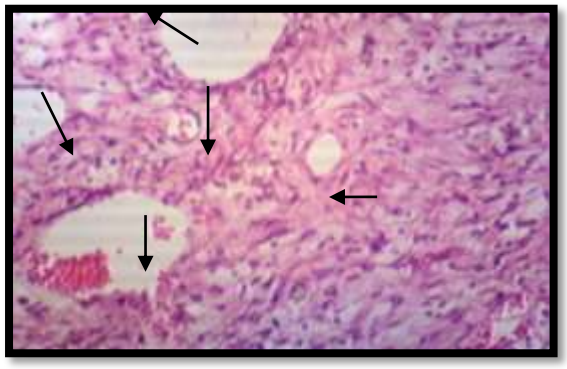

Figure 6.5: 3.0 mg/kg Nifedipine

Figure 6: Representative photographic results showing new capillaries formed in the control and test groups. Image from Trinocular Microscope at magnification of 400 X. All sections were stained with H&E. Scale bar, 50 mm. Arrows indicate the newly formed microvessels

Number of blood vessels:

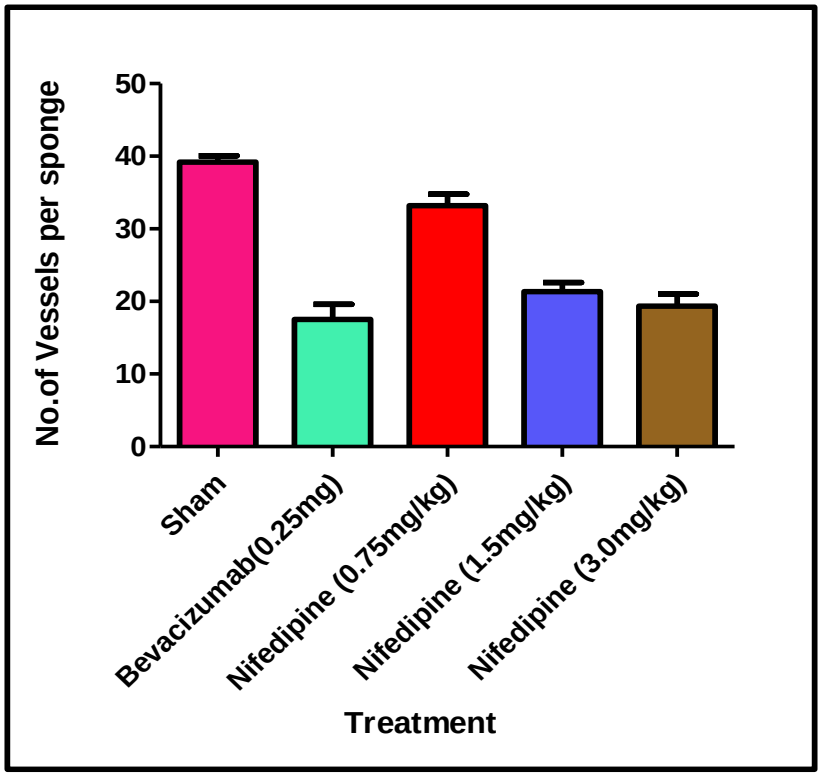

Figure 7: Graph showing the effect of Treatment groups on Number of blood vessels formed per sponge.

All of the data obtained from the experimental groups have been compared to the diseased control group. The data was analyzed by one-way ANOVA followed by Dunnett's test using graph pad prism5.0 software. Values are significant at * $p<0.05$. Comparison of the test groups were done with disease control group. Values are expressed as mean ± SEM;

Sponge Implantation Method:

In sponge implantation method, weight of the sponges, number of blood vessels formed and hemoglobin content were estimated. At 10^{-6}M and 10^{-5}M doses of Nifedinipe significantly less sponge weights, reduced hemoglobin (Hb) content and less number of blood vessels per sponge compared to the control group was reported. The number of new branches formed the preexisting vessels were quantified from the histology slides. The circular area with a central fibroblast represents the newly formed vessels. The test drug at 10^{-5}M produced similar results as the standard antiangiogenic drug, Bevacizumab.

Angiogenesis can happen only after the proliferation and migration of vascular endothelial cells. All these critical steps are controlled by various extracellular signals. VEGFs, FGFs are good examples of peptides binding to tyrosine kinase receptors which trigger the intracellular signaling cascades. The angiogenic factors increase the cytosolic calcium mainly through two pathways:

entry through the opening of calcium permeable channels in the endothelium that is from an extracellular medium. [30] Secondly, release from intracellular organelles like endoplasmic reticulum. In this research we have focused on blocking the increase in cytosolic calcium through decreasing the permeability of channels on endothelium.

Calcium signals are involved at various critical levels in the control of the multi stepped process of angiogenesis. [24] EC, the leading player, leaves the existing blood vessels to organize into a new tube. In this process they leave the vessel by piercing into the matrix, divide and finally stop their mitogenic activity to form a new tube. [18] Here migration and proliferation are the checkpoints, regulated by calcium dynamics. And these calcium dynamics are controlled by extracellular agents such as vascular endothelial growth factors (VEGFs), fibroblast growth factors (FBFs), platelet derived growth factors (PDGFs), epidermal growth factors (EGFs). A rise in free cytosolic calcium stimulates the cell cycle progression, the exit from dormant early G1 phase, the G1/S transition and other regulatory points during S and M phase.[25]

Nifedipine a L-type voltage gated calcium channel blocker, inhibits the involvement of calcium-calmodulin dependent kinase type II (CaMKII). In the absence of the blocker, the protein calmodulin interacts with the ion and stimulates the calcium dependent cell proliferation. A great amount of literature supports the direct involvement of CaMKII at several transition points during cell cycle progression. Calcium concentration extracellularly and in ER is much greater than free cytosolic calcium concentration. This enables calcium entry from extracellular source and release from intracellular stores in a passive way to elevate free cytosolic Calcium in all cell types.

Voltage operated calcium channels are pentameric in structure with 1α, 2β, γ and δ subunits. All important channel properties like, binding sites for agonists and antagonists, voltage – sensor, ion-conducting pore are exhibited by α subunit. Voltage gated calcium channels are classified as L, N, P/Q, R and T types based on electrophysiological and pharmacological properties. The kinetics and dynamics of the subtypes of the channel differ.

Several calcium channel blockers, with various chemical structures and different selectivity and specificity are available. All these blockers are broadly placed into 2 groups: the inorganic (ionic) blockers and the organic blockers. The organic blockers like carboxyamidotriazole inhibits intracellular pathways involving phospholipase-C, InsP3 and arachidonic acid release, which attributes to their anti-proliferative action, as well as blocks the calcium channels. Inorganic blockers having a divalent transition ions (Ni^{2+}, Cd^{2+}, and Co^{2+}) and lanthanides, both voltage dependent and independent channels are competitively blocked. They reduce calcium entry, proliferation and thus the related angiogenesis [23].

The test drug is a dihydropyridine derivative and so is an ionic blocker. Nifedipine the most widely used blocker of L-type voltage dependent calcium channels is effective even at µM concentration range. Most of the dihydropyrinine derivatives are specific calcium influx pathway blockers. But uniquely Nifedipine can block voltage insensitive calcium influx and can be a very effective suppressor of proliferation and migration. Based on the above observations among all the various calcium channel blockers available, Nifedipine was selected to evaluate its antiangiogenic potential.

Rat Aortic assay is a major model implemented in this research. The assay is based on the generation of neovascular capillary network from the endothelial cells when the aortic ring is incubated in matrigel under hypoxic conditions. The neovascular capillary network generated from the rings is very similar to those formed during *in-vivo* angiogenesis. In this assay the major steps of angiogenesis like endothelial cell invasion, cell migration, proliferation, differentiation, and new vessel formation can be reported. Hypoxia created during the assay induces the free radical formation provoking angiogenesis. Nifedipine has antioxidant and free radical scavenging property which rescues the endothelium from oxidative stress [22]. In the blood vessels, production of free radicals is majorly responsible for the oxidative damage of endothelium resulting in its dysfunction which is the basic root cause for diseased conditions. As superoxide anion scavenger and anti oxidant, Nifedipine would resume the normal function of the endothelium. There was a drastic change in the area of sprout formation by the Nifedipine treated group in dose dependent manner. Anti-proliferative action of Nifedipine may be due to block of serum activated calcium entry in fibroblasts, which is required for the mitogenic activation and further causes the block in G2/ M phase of the cell cycle [21].

The treatment with Nifedipine reduced the wet weight of the sponges significantly. Estimation of hemoglobin content of the sponges is one of the sensitive markers for angiogenesis[17]. There was dose dependent decrease in the sponge Hb content that was observed and compared with the negative control group. Angiogenesis after implantation of the sponge was estimated and augmented neovascularization in the test compound treated animals was analysed. Histopathology of the control and test compound treated implants demonstrated noticeable divergences. As depicted in figure 6, in the control sponges, there is high degree of neovascularization compared to test groups. Though not equal, Nifedipine showed quite comparable antiproliferative potency as Bevacizumab.

The rat aortic ring assay bridges the gap between *in ovo* and *in vivo* methods. Aortic rings cultured in matrigel give rise to microvascular network with branching endothelial channels. Use of intact

vascular tissue gives a more similar *in vivo* environment for angiogenesis to occur than those with the isolated endothelial cells.[27, 28]Area of neovascularization was quantified and the test drug Nifedipine gave significant antiangiogenic results.

Based on the *in vitro* and *in vivo* studies Nifedipine action can be correlated to inhibit cell adhesion, spreading, proteolysis, migration and angiogenesis. The scientific postulations in this research should offer in future, a critical task of identifying the gene encoding the calcium channels involved in mitigenic induced calcium entry. Furthermore, Structural modifications in Nifedipine can evolve a new molecular target which will allow a more specific and more potential antiangiogenic compound.

CONCLUSION

Based on the scientific finds in the research, we report here that L-type voltage gated calcium ion channel blocker; Nefidipine inhibits new vessel formation in both the models. These results suggest that Nefidipine may be useful in the therapy of angiogenic-dependent tumor growth and other angiogenic-dependent diseases on specific molecular modifications for endothelial cell targeting.

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