(Turkish Title: OVO, IN VITRO VE VIVO GÜNLÜKLERİNDEN FLUNARİZİNİN ANTİ-MİYOGRAFİK AKTİVİTESİ**)**

Özet

Giriş ve Amaç: Hücre proliferasyonunda T-tipi kalsiyum kanallarının tutulumu ve hücre göçü içindeki sodyum kanallarının rolü anjiyogenezde kapsamlı olarak incelenmiştir. Bu çalışmada, ikili bir sodyum / kalsiyum kanal blokeri olan Flunarizin; anti-anjiyojenik potansiyelini değerlendirmek için seçildi. Bu, patolojik olarak aşırı anjiyogenezin neden olduğu hastalıklarda terapötik olarak yararlı olabilir.

Metot: İyon kanalı blokörünün anti-anjiyojenik aktivitesi, civciv korioallantoik membran deneyi (ovoda), sıçan aortik halka deneyi, endotelyal hücre proliferasyon analizi, transwell migrasyon deneyi, matrigel kord benzeri morfojenez deneyi (in vitro) ve sünger implantasyonu ile tarandı. yöntem (in vivo). Test ilacının anti-anjiyogenik aktivitesi standart anti-anjiyojenik ilaç olan Bevacizumab ile karşılaştırıldı ve buna ek olarak, test yanıtları, 500mM'lik bir maksimum konsantrasyonda anjiyojenik faktör VEGF ile karşılaştırıldı.

Bulgular: Tüm gruplar kontrol grubu ile tek yönlü ANOVA kullanılarak ve post hoc testi ile karşılaştırıldı, Dunnett testi ile tüm grupların ortalamaları kontrol ortalaması ile karşılaştırıldı. Chick chorioallantoic membran testinde dallanma noktaları ve anjiyojenik skorlar değerlendirildi ve 10-5M ve 10-4M'de anlamlı sonuçlar gözlendi. Aort halkası analizinde, filiz alanında 5-10µM azalma gözlendi ve sünger implantasyon yönteminde test edilen üç Flunarizin konsantrasyonunda sünger ağırlığında anlamlı bir azalma, kan damarlarının sayısı ve hemoglobin içeriği gözlendi. İnsan umbilikal ven endotelyal hücreleri üzerindeki çalışmalarda, test ilacı (1-100 nM), proliferasyonun önemli ölçüde engellenmesini, doza ve kordon benzeri tüplerin ağ uzunluğunun doza bağımlı bir şekilde azaldığını gösterdi.

Sonuçlar: Flunarizin, T-tipi kalsiyum ve sodyum kanallarını bloke ederek hücre proliferasyonu, migrasyon ve kord benzeri tüp oluşumunu inhibe ederek önemli anti-anjiyogenik etkiye sahiptir. Flunarizin'in bu iyon kanal modülatörünü yeniden üretmek için yapısal modifikasyonları ile ilgili daha ileri çalışmalar, kök nedeninden aşırı anjiyogenezden kaynaklanan hastalıkları tedavi edebilecektir.

Anahtar Kelimeler: Anti-anjiyogenez, civciv korioallantoik membran deneyi, sıçan aortik halka deneyi, sünger implantasyon yöntemi, insan umbilikal ven endotel hücreleri, flunarizin.

Abstract

Background and Objective: The involvement of T-type calcium channels in cell proliferation and the role of sodium channels in cell migration have been extensively studied in angiogenesis. In the present study Flunarizine, a dual sodium /calcium channel blocker; was selected to evaluate its anti-angiogenic potential. This can be therapeutically beneficial in the diseases caused due to pathologically excess angiogenesis.

Methods: The anti-angiogenic activity of ion channel blocker was screened by chick chorioallantoic membrane assay (*in ovo*), rat aortic ring assay,endothelial cell proliferation assay,transwell migration assay, matrigel cord-like morphogenesis assay (*in vitro*) and sponge implantation method (*in vivo*). The anti-angiogenic activity of the test drug was compared with the standard anti-angiogenic drug Bevacizumab and in addition, the test responses were compared with the angiogenic factor VEGF, at a maximal concentration of 500pM.

Results: All the groups were compared with the control group using one-way ANOVA, followed by post hoc test, the Dunnett's test to compare the mean of all the groups with the control mean.In the chick chorioallantoic membrane assay, number of branching points and angiogenic score were evaluated and significant results were observed at 10^{-5}M and 10^{-4}M. In the aortic ring assay reduction in the area of sprouts were observed with 5-10µM and a significant reduction in weight of sponges, number of blood vessels formed and hemoglobin content were observed at all the three tested concentrations of Flunarizine in the sponge implantation method. In the studies on human umbilical vein endothelial cellsthe test drug (1-100nM) showed significant inhibition of proliferation, migration and decrease in network length of cord like tubes in a dose dependent manner.

Conclusions: Flunarizine has significant anti-angiogenic action byinhibiting the cell proliferation, migration and cord like tube formation, which have resulted by blocking the T-type calcium and sodium channels. Further studies on the structural modifications of Flunarizinefor repurposing this ion channel modulator will be able to treat the diseases due to excess angiogenesis from the root cause.

Key Words: Anti-angiogenesis, chick chorioallantoic membrane assay, rat aortic ring assay, sponge implantation method, human umbilical vein endothelial cells, flunarizine.

1.Introduction

The term angiogenesis or neovascularization means the formation of new blood vessels from existing vasculature. Blood capillaries supply oxygen: more capillaries can increase tissue oxygen conduction and hence improve energy production; fewer capillaries results in ischemia, hypoxia and even anoxia in the tissues[1]**.**Thus, angiogenesis is important for both normal physiology and in pathological conditions [2-4].

Endothelial cell (EC) structure and functional integrity is important in the maintenance of the vessel wall and circulatory functions, and most of these endothelial functions are regulated by ion channels[5,6].Role of ion channels in pathophysiology of diseases has been extensively discussed[7-9**].** Despite their prime role in several diseases, there are hardly few drugs targeting specifically the ion channels as therapeutics Inhibitors for the treatment of diseases caused due to excessive angiogenesis. Such clinically approved ion channel modulators with well known safety profiles may be reframed in the treatment of many disease saving significant time and money.

In the present study,Flunarizine (FLN), a dual Na^+/ Ca^{2+} channel blocker was selected to screen its anti-angiogenic potential**.** FLN, diphenylpiperazine analog acts on both Na^+ and Ca^{2+} channels [20]. The test drug is a T-type calcium channel blocker which has been studied as extensively unregulated in most of the tumor types. The anti-angiogenicpotential of the test drug FLN was tested at three different doses in different methods by an *in ovo* method, chorioallantoic membrane(CAM) assay; an *in vitro* method, rat aortic ring assay,endothelial cell proliferation assay,transwell migration assay, matrigel cord-like morphogenesis assay; and*in vivo* method, sponge implantation assay.

2.Material and Methods

2.1.Chemicals

Flunarizine, ketamine, xylazine and tramadol werepurchased from N.R. CHEM, India. Matrigel was purchased from Becton Dickinson India Pvt.Ltd, Gurgaon India. Gel foam and Dulbecco's modified Eagle's medium were supplied by life technologies (India) Pvt.Ltd. Well plates were purchased from Hi media laboratories Pvt. Ltd, India. Bevacizumab, VEGF, penicillin, streptomycin, amphotericin,

gentamycin, heparin, bovine serum albumin, gelatinand M199 were obtained from Sigma-Aldrich (India). All the chemical and reagents used in the study are of AR grade.

2.2.Equipment

All the equipment of CMR College of pharmacy was used. BOD incubator, Dona analytical balance, Digital P^H meter , Ever Shine 697 homogenizer, laminar airflow unit, labomed trinocular microscope were purchased from MH enterprises, Hyderabad, India.

2.3.Experimental Animals

Fourty two healthy male Wistar Albino rats weighing in between 150-200 g were selected for *in vivo* methods and for *in vitro* assay. Animals were obtained from Teena Labs Pvt Ltd, Hyderabad, Telangana state, India. Fertilized leghorn chicken eggs were selected for *in ovo* assay. All the procedures were performed according to the CPCSEA under a protocol approved by the Institutional Animal Ethics Committee (IAEC) with project license numbers **(CPCSEA /1657/ IAEC/CMRCP/PhD-15/42)**

2.4.Chick Chorioallantoic Membrane Assay

This is an *in ovo* angiogenesis assay for identification and quantification of antiangiogenic agents. Eggs were collected from the hatchery at day 0' and checked for any damage. They were randomly grouped into control, VEGF, Bevacizumab and three test concentrations,each containing six eggs. Eggs were disinfected using ethanol then incubated under condition of constant humidity and at a constant temperature of 37^0C. On the 3rd day, a hole was drilled at the narrow end and 2-3 ml of albumin was withdrawn with 18 gauge hypodermic needle. The hole was sealed with sterile tape and put back for incubation. On the 7th day, a window was opened on the shell, sterile gel foam or sponge (3mm×3mm×1mm) piece was placed on top of the membrane. The control group was given saline; the test and standard groups were impregnated with their respective doses. Eggs were incubated till day 14. On the 14th day,CAM tissues directly beneath sponge were removed from control and treated CAM samples. Tissues were placed in 10% formalin, stained with hematoxylin-eosin, and then examined under trinocular microscope. The vessel branching points in the square area were counted and analyzed for each treatment group. The resulting angiogenesis index is the mean ± SEM of new branch point in each set of samples. 'Angiogenesis score 1-4 was given to each egg based on number of branching points. If number

of branching points is ≥35, the angiogenesis score is 4. If branches are between 25-34, score is 3 and for 15-24, the score is 2. If the points are <15, the score is 1'.The concentrations (10^{-6}M, 10^{-5}M, 10^{-4}M) were selected based on the results of the previous studies. In the previous study the concentration of 10^{-5}M has shown sub maximal efficacy of the drug.The classical molarity formula M=m/V was used to find out the required drug amount to provide 10^{-4}M concentration.First the concentration of 10^{-4}M was prepared, and then the other concentrations were prepared from the earlier by serial dilutions[10-13**]**.

2.5.Rat Aortic Ring Assay

This method is a widely used *in vitro* assay for the evaluation of both angiogenic and antiangiogenic compounds. One healthy male Wistar Albino rat for each group was selected. It was sacrificed by cervical dislocation, thoracic cavity was cut opened and the visceral organs were separated. Thoracic aorta was identified and isolated by cutting both the ends. Immediately it was transferred to cold phosphate buffer solution (PBS) supplied with aeration. Fibro-adipose tissue was isolated, the proximal and distal 2mm segments of aorta were cut away. Aorta were cut into 1mm ring sections and washed with PBS. These rings were placed in the 24 well plates with 150µl of matrigel. Rings were overloaded with matrigel and were left to polymerize for 1-2 hours at 37º C. Then they were exposed to hypoxia for 2 hours. This hypoxic condition stimulates formation of sprouts. The rings were reoxygenated and then incubated for seven days. Area of sprouts was quantified by the measurement of length and abundance of microvessel-like extension from the explants**[**14-16]**.**

2.6.Sponge Implantation Method

In the sponge implantation method, the surgical procedure was done by a single investigator to increase the reproducibility of the process. Sponges were implanted subcutaneously(*s.c).* All the surgical instruments used in study were sterilized by autoclaving at 121^0C for 25min. The sponges of 2cm diameter and 8mm thickness were prepared and sterilized by soaking in 70% ethanol for three hours and then boiling at 70ºC for half-an-hour. This *in vivo* method was carried out by anaesthetizing the rats by a cocktail of Ketamine (80 mg/kg) and Xylazine (5 mg/kg). Then the skin was cut open by surgical blade. Sterile sponge was implanted subcutaneously by creating an air pocket which was sutured back by 5/0 silk sutures. Two such sterile sponges were implanted on the mid-dorsal line of the body. When the

animal recovered from anesthesia, they were allowed to have normal diet and water. The animals after the surgery were caged individually. Tramadol at a dose of 0.9 mg/kg was injected intramuscularly (*i.m)*twice a day in the morning and evening, Gentamycin at a dose of 2mg/kg was injected *i.m* in the morning. The analgesic and antibiotic drugs were given for the three days post-operatively. Standard and test drugs were given to the sponges of their respective groups for 13 days after the implantation. On the 14^{th} day animals were sacrificed and the sponges were dissected out. Sponges were weighed, amount of hemoglobin and number of vessels per sponge were quantified**.** The drug concentrations were expressed as mg/kg. The therapeutic human range of each drug in the subcutaneous route was obtained from the literature and three animal doses were calculated by the formula

$$\text{Dose of the animal} = \frac{\text{Surface area of animal}}{\text{Surface area of Human}} \times \text{Human dose}$$

Where,

Rat surface area =0.025sq.m

Human surface area=1.6sq.m

First the highest concentration of each drug was prepared, and then the other concentrations were prepared from the earlier by serial dilutions[17-19].

Procedure for determining hemoglobin content: The sponges after removal from rats were soaked in double distilled water and homogenized completely over ice platform for 5min. The homogenate was centrifuged at 10,000 rpm in cooling centrifuge for 5 min and the supernatant liquid obtained was used to estimate hemoglobin content (g/Dl).

Procedure for determining number of blood vessels formed per sponge: The sponges were bisected, fixed in saline at 4°C for 1 h. The sponges were immersed in 75% ethanol for thirty min, finally kept in 10% formalin and paraffin sections (10 pm) were prepared, stained with hematoxylin-eosin. The prepared slides were then observed under trinocular microscope. The circular spaces amidst the fibroblast regions present were counted as they represent vessels formed in the sponges.

2.7.Endothelial Cell Culture

Human umbilical vein endothelial cells (HUVECs) were grown on gelatinized dishes in M199 supplemented with 15% fetal calf serum, 50 U/ml penicillin, 50 mg/ml streptomycin, 50 mg/ml gentamycin, 2.5 mg/ml amphotericin B, 5 U/ml heparin, and 150–200 mg/ml endothelial cell growth supplement. Cells were used between passages 1 and 3. Each experiment shown derives from three independent repeats, each time using different pools (isolates) and/or passages of cells [24].

2.8.Endothelial Cell Proliferation Assay

The HUVECs were seeded in 24-well plates at a density of 6,000 cells/cm^2 and incubated overnight in Dulbecco's modified Eagle's medium. Cells were exposed to different concentrations of FLN, Bevacizumab, VEGF or vehicle and allowed to proliferate for 48 hours. At the end of this incubation time, the cells were trypsinized, and their number was determined using a Neubauer hemocytometer [25].

2.9.Transwell Migration Assay

The capacity of endothelial cells to migrate through a pore-bearing membrane was assessed using 6.5 mm diameter transwell chambers with polycarbonate membrane inserts (8 mm pore size). Control or endothelial cells were serum starved overnight. Cells were trypsinized, and $1*10^5$ cells were added to each transwell in 100 ml of serum-free medium containing 0.2% bovine serum albumin in the control and in the presenceof different concentrations of FLN (1nM,10nM,100nM), Bevacizumab and VEGF. Cells were allowed to migrate for 4 hours, after which the non-migrated cells at the top of the transwell filter were removed with a cotton swab. The migrated cells on the bottom side of the filter were fixed in Carson's solution for 30 minutes at room temperature and then were stained with toluidine blue. Migrated cells were scored and averaged from eight random fields per transwell as previously described elsewhere [26].

2.10.Matrigel Cord-Like Morphogenesis Assay

The formation of cord-like structures by endothelial cells (HUVECs) was assessed in growth factor-reduced matrigel. The cell groups were plated in 96-well plates pre-coated with 45 ml of matrigel per well. After 8 hours of incubation, cord-like structure formation was quantified. One image per well was analyzed and used for the statistical analysis [25,27].

2.11. 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. In the statistical analysis all the groups were compared with the control group. Results were considered statistically significant at p value<0.05.In all the groups of CAM assay, rat aortic ring assay and sponge implantation method, n=6. (Fig. 2 & 3)

3.Results

In the chick chorioallantoic membrane assay (*in ovo*), the dual ion channel blocker exhibited marked anti-angiogenic activity at all the tested concentrations, In rat aortic ring assay (*in vitro*), reduction in the area of sprouts were observed. A noticeable reduction in weight of sponges and inhibition in the growth of new blood vessels; and a very sharp reduction in hemoglobin content were observed, which was better than the standard drug response (in vivo).

3.1.Results of Chick Chorioallantoic Membrane (CAM) Assay

In the Assay,on the 14^{th} day the CAM tissues directly beneath the sponge were removed from control and treated CAM samples. The vessel branching points in the square area equal to the region of each sponge were counted(Fig .1). Angiogenesis score 1-4 was given to each egg based on number of branching points. Effect of the drug treatment on the two evaluation parameters that is, number of branching points and angiogenic score are represented in the Fig.2A & 2B. The results of three doses of FLN, the standard anti-angiogenic drug Bevacizumab and VEGF were statistically compared with the control results. Significant results were observed with all the three test dose selected;10^{-6} M, 10^{-5} M and10^{-4} M.

3.2. Results of Rat Aortic Ring Assay

Photographs showing the abundance of microvessel-like extensions from the explants are represented in the Fig.1. Significant reduction in the area of sprouts was observed with 5µM and 10µM of the drug. (Fig.2C)

3.3. Results of Sponge Implantation Method

In the sponge implantation method, the evaluation parameters are weight of the sponge, number of vessels per sponge, haemoglobin content and the histopathology of the sponge. A moderate reduction in weight of sponges and a prompt inhibition in the growth of new blood vessels and hemoglobin content were observed at 1.0 mg/kg and 10 mg/kg of the drug (Fig.2D,2E and 2F).The sections of the sponges were observed under trinocular microscope. The circular spaces amidst the fibroblast regions were counted as they represent new vessels formed in the sponges. In the VEGF group large numbers of vessels wereidentified, in the standard very less microvessels were formed due to strong anti-angiogenic action and the test drug showed a dose dependent decrease in the number of blood vessels per sponge (Fig.1).

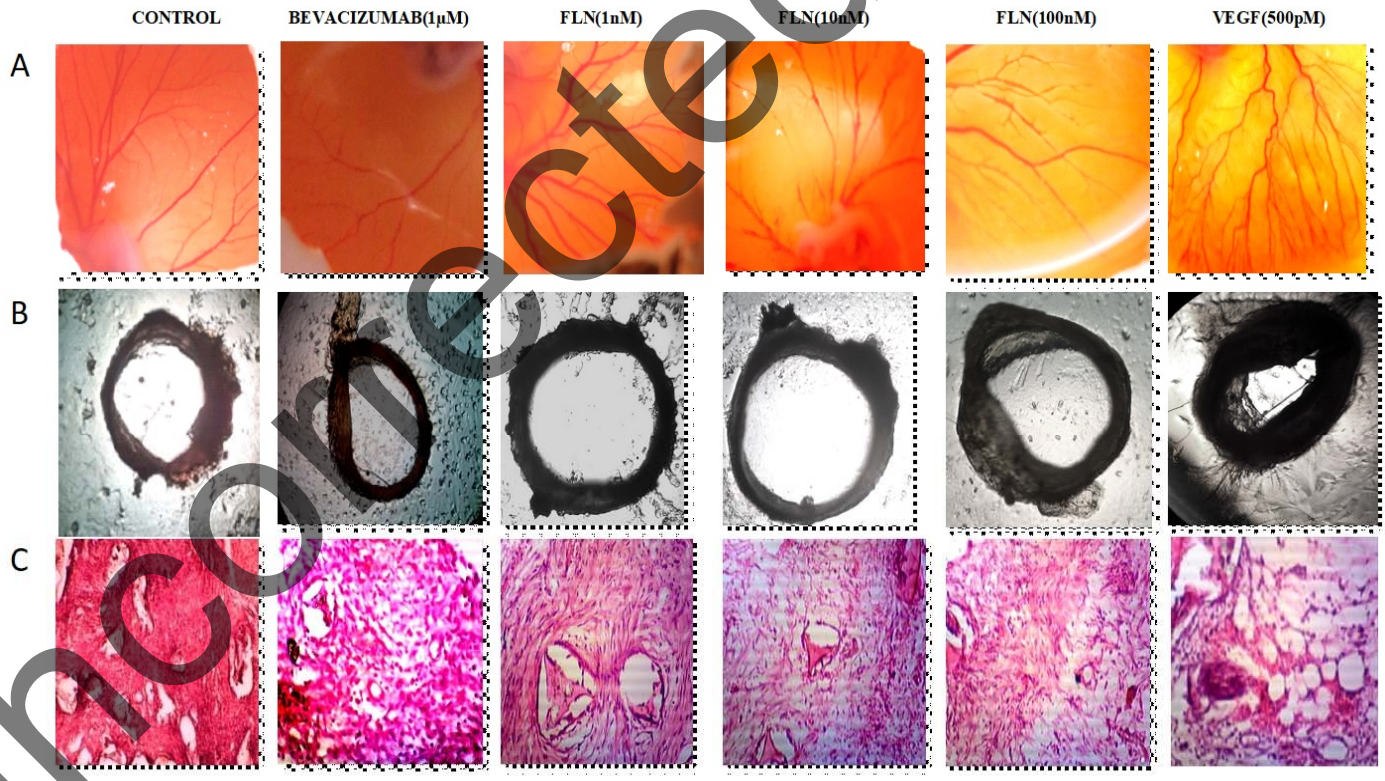


Fig. (1): FLN inhibited angiogenesis *in ovo*, *in vitro* and *in vivo*. (A)In the CAM assay number of branching points from each major vessel were counted. (B) Photographs of explants in the aortic ring assay shows the micro vessel like extensions. (C) The histological sections of the sponges show circular spaces amidst the fibroblast region represent the newly formed blood vessels.

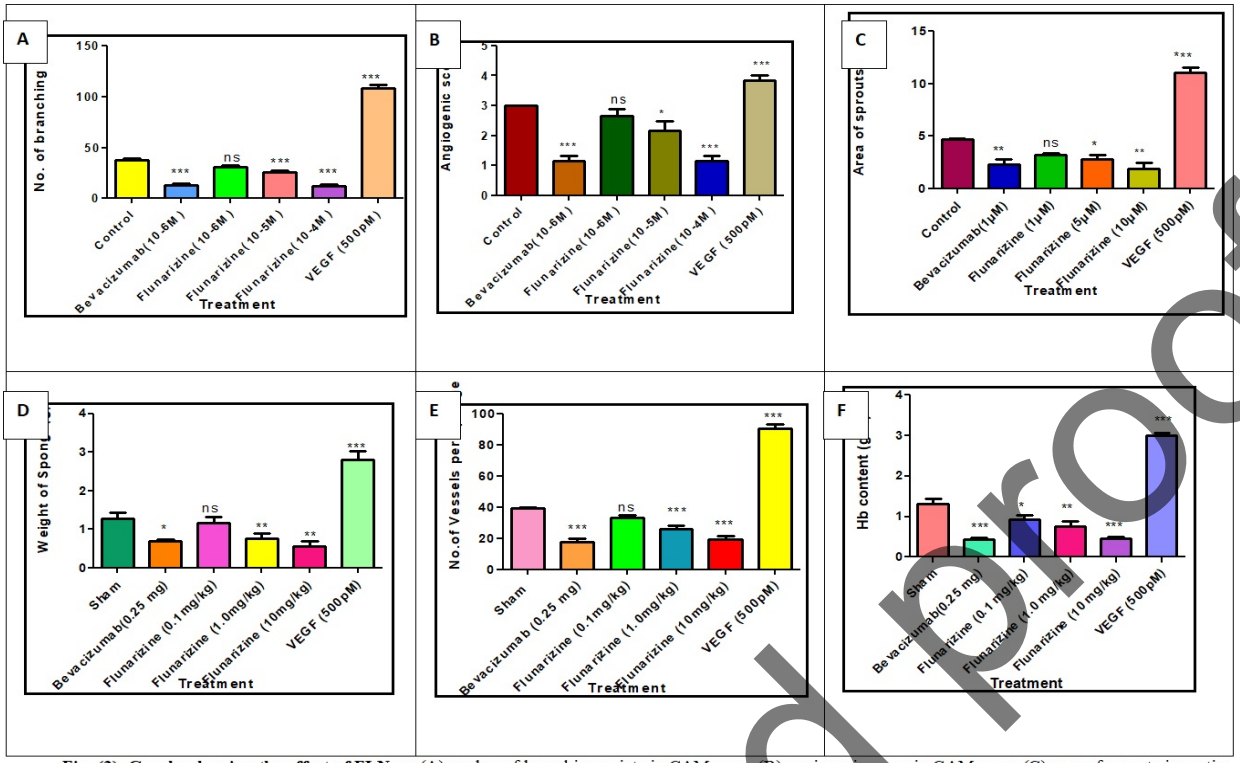
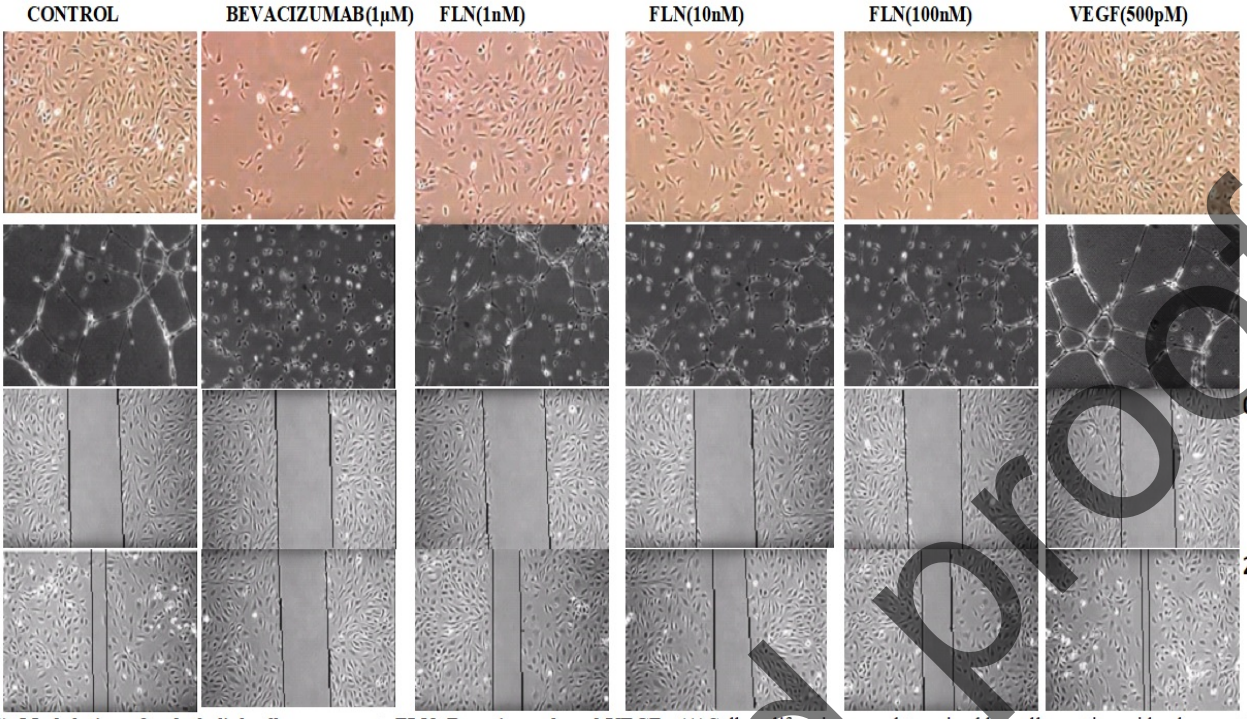


Fig. (2). Graphs showing the effect of FLN on (A)number of branching points in CAM assay (B) angiogenic score in CAM assay (C) area of sprouts in aortic ring assay (D) weight of sponge in sponge implantation method (E) number of vessels per sponge (F) Hemoglobin content per sponge in sponge implantation method. All the results were expressed as Mean ± SEM; n=6. . ***p<0.001, **p<0.01, *p<0.05 Vs Control, ns-Non significance.

3.4. Results of Endothelial Cell Proliferation Assay, Transwell Matrigel and Cord-Like Morphogenesis Assay

Na^+ and Ca^{2+} channels are important for cell proliferation, migration and cord like network formation. To further test the link between channel inhibition and anti-angiogenesis, endothelial cell based assays triggering proliferation and mobilization were performed.In the cell proliferation assay VEGF resulted in elevated proliferation (increase of 49%),whereas bevacizumab and the three doses of FLN showed significant inhibition of proliferation respectively (inhibition by 50%, 79.3%, 69.7%, 58.3%). In addition, test doses of FLN inhibited cell motility through transwell compartments comparable to vehicle control, respectively. To further assess the anti-angiogenic property of the test drug, cord like tube formation assay was performed. Significant inhibition was observed with the test doses (69.3%, 59.7%, 48.3% respectively).(Fig.3& 4)



3). Modulation of endothelial cell responses to FLN, Bevacizumab and VEGF . (A) Cell proliferation was determined by cell counting with a hemocytometer Representative images of tube formation after being treated with FLN for 24h following VEGF stimulation (**C**). Quantitative data of scratch wound-healing ition in HUVECs treated with FLN for 24h under VEGF stimulation. Cord-like network morphogenesis in vitro is affected by K_{ATP} modulation

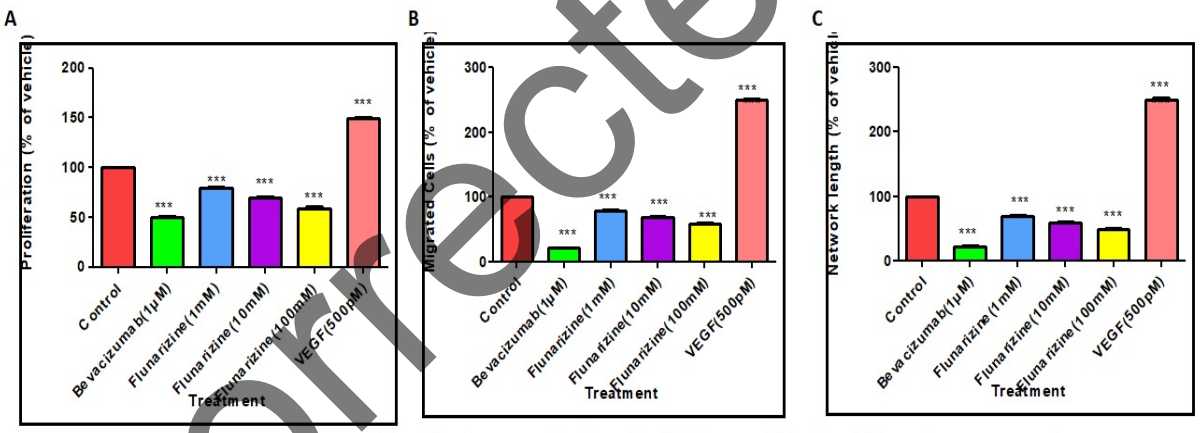


Fig. (4). Graphs showing the effect of FLN on (A)Proliferation assay (B) Migrated cells (C) Network length. All the results were expressed as Mean ± SEM; n=6. . ***p<0.001, **p<0.01, *p<0.05 Vs Control, ns-Non significance.

4. Discussion

FLN is a dual sodium /calcium blocker [20].It acts on sodium and Ca^{2+}channels, blocks influx of Ca^{2+} ions. Ca^{2+} ions have long been known as secondary messengers in various cellular signaling resulting in angiogenesis. The fact that deprivation of extracellular Ca^{2+} leads to cell growth arrest in G1/S indicates that Ca^{2+} is required for cell cycle progression [21, 22, 23].

One of Ca^{2+} regulation mechanisms is binding of calcium to calmodulin protein. Intracellular Ca^{2+} binds with calmodulin (CaM) II, in turn activates calcium-calmodulin-dependent protein kinases and regulates pro-survival transcriptional proteins.

In the chick chorioallantoic membrane assay, the ion channel blocker exhibited potent antiangiogenic activity at the all the three test concentrations of 10^{-6}M, 10^{-5}M and 10^{-4}M. Reduction in the area of sprouts in rat aortic ring assay was observed with 5µM and 10µM of FLN. A significant reduction in weight of sponges, number of blood vessels formed and hemoglobin content were observed at 1mg/kg and 10 mg/kg. The results revealed that FLN has significant inhibition of sprout formation and branching in the dose dependent manner. Modulation of endothelial cell response to FLN was significant at all the test doses of 1nM, 10nM and 100nM on the endothelial cell proliferation, migration and tube formation assays. FLN being a strong blocker of Ca^{2+} ions influx gave significant anti angiogenic results. This drug serves as good chemical templet, which can be structurally modified for more site specific actions for the anti angiogenic therapy.

5. Conclusion

The anti-angiogenic property of ion channel modulator, FLN was thoroughly evaluated by *in ovo*, *in vitro* and by *in vivo*studies. The test drug showed very potent anti-angiogenic activity, even better than the standard drug bevacizumab at a concentration range of 5-10µM.The very strong anti- angiogenic potential is due to effective blockage of Ca^{2+}influx. Na^{+}/Ca^{2+}dual blocker inhibits the Ca^{2+}influx with double the strength. Calcium dynamics play a crucial role in the critical steps of angiogenesis like cell migration, proliferation and even in cell death. Molecular modifications of the ion channel modulator used in the present study will evolve endothelial cell targeted chemical moieties. Furthermore, such endothelial targeted chemical moieties can be formulated suitably to achieve a site specific action which minimizes the side effects.

6. Acknowledgements

We thank the management of CMR College of pharmacy to support us to perform the research in the institution.

7. Conflicts of Interest

No conflict of interest was declared by the authors.

8. Funding Agency

The research was partially supported by University grants commission, minor research project grant.(Proposal Number: 3228; ROMRP-SERO-PHAR-2015-16-68663)

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