Comparative Experimental Studies of Few L-Type and T-Type Ca\(^{2+}\) Channel Blockers Against *In-Ovo* and *In-Vitro* Models of Angiogenesis

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Abstract

Angiogenesis is the development of new blood vessels from pre-existing one. The ion-channels on endothelium plays vital role in cell proliferation and related angiogenesis. We aimed to investigate the effects of L-type (Verapamil and Diltiazem) and T-type (Ethosuximide) Calcium Channel Blockers (CCBs) on neovascularization. The effects on neovascularization were investigated by *in-ovo* (CAM, Chick Chorioallantoic membrane) and *in-vitro* (aortic ring assay) methods. Each test drug was tested for at least 3 doses and the anti-angiogenic effect was compared with Suramin as standard and normal control groups. Various vital parameters were recorded during the experiment like the number of blood capillaries, sprouts formation, angiogenic score etc. The L-type Ca\(^{2+}\) channel blockers Verapamil at the dose of 50µM, 110 and 220 µM/disk has shown significant \((p < 0.001)\) reduction in the number of branching points in CAM assay. For the further confirmation, angiogenic activity was evaluated *in vitro* by rat aortic ring assay method; the area of sprouts was reduced by the medium and high dose of verapamil. Diltiazem has demonstrated modest anti-angiogenic activity by both the models, whereas T-type calcium channel blocker ethosuximide has not shown any effect on the neovascularization. Among all the tested drugs verapamil has shown the promising anti-angiogenic property. Thus verapamil and diltiazem may have anti-angiogenic activity defines novel beneficial effects in angiogenic mediated pathological conditions in addition to their main indications in cardiovascular complications.

Keywords: Angiogenesis, CAM Assay, L-Type Ca\(^{2+}\) Channel Blockers, Rat Aortic Ring Assay, T-type Calcium Channel Blocker

1. Introduction

Blood capillaries play a vital role in governing normal physiological process such as wound healing, during embryonic development and corpus luteum formation endometrium formation etc\(^1\). In addition, it involves in many pathological processes like diabetic retinopathy, arthritis, inflammation, and many other conditions\(^2,3\). Angiogenesis is responsible for the pathological progress and metamorphosis of tumor\(^4\). The newly developed blood capillaries supplement cancer growth by supplying nutrients, oxygen and by taking away waste products. Angiogenesis also supports Metastasis\(^5\).

Angiogenesis is sequential and highly co-ordinated system involves several steps including basement membrane proteolysis, migration of individual endothelial cells, and endothelial cell proliferation\(^6\).

Inhibition of angiogenesis and arresting development of blood capillaries has emerged as a promising strategy for the treatment of cancer\(^2\). Many researchers are
2.2 Chick Chorioallantoic Membrane Assay

Fertilized chicken eggs were collected at day ‘0’. They were grouped randomly into various groups as per the treatment schedule. The eggs were incubated at 37°C. On the 2nd day, 2-3 ml of albumin was withdrawn from the narrow end of the egg by creating a small hole. Then after window was sealed with cellophane tape and again incubated. On the 7th day of incubation a square window was created in the shell and filter paper disk (3mm×3mm×1mm) was implanted on top of the CAM tissue (yellowish forge wheel like structure) membrane. The test drug was initially applied on the filter paper disk and dried in the oven after drying these disks were placed on the CAM tissue. The group I (normal control) was impregnated with distilled water; the tests drugs were loaded on the filter paper at three doses as shown in the Table 1 and suramin was used as a standard. The eggs were property sealed again by using cellophane tape and kept it back to the incubator and they were incubated undisturbed till day 14. On the 14th day of incubation, the eggs were taken out from the incubator and the CAM tissues directly beneath each filter paper was resected with the help of forceps.

The anti-angiogenic effect of the test drugs was quantified by comparing the quantity of blood capillaries mainly under the area of the disk (where the drug was loaded) by capturing the images before and after treatment. The scale of 0-2 was used for the rating. In this assay the mortality rate is around 30-40% so to get at least 8-10 viable eggs in each group, 18 eggs kept for each sample.

The average score for each test dose was calculated and the interpretation of anti-angiogenic effect was done as follows:

- Average score ≤ 0.5 = no anti-angiogenic effect (inactive).
- 0.5 ≤ average score < 1 = weak anti-angiogenic effect.
- 1 < average score < 1.5 = good anti-angiogenic effect.
- Average score ≥ 1.5 = strong anti-angiogenic effect.

For every test drug, 10-15 eggs were used in parallel. For the evaluation of the effects a scoring system was used.

### 2.1 Materials

**Chemical and lab wares:** Diltiazem, Verapamil, Nifedipine, Flunarizine dihydrochloride and Ethosuximide was purchased from Santa Cruz, USA. Corning® Matrigel® Growth Factor Reduced (GFR) Basement Membrane Matrix was purchased from Al Genome International scientific and laboratory products Dubai, UAE. All the chemicals used in the research are of AR grade.

**Equipment:** Incubator, high resolution digital camera (Cyber-Shot 6.0, Sony, Tokyo, Japan), micropipettes, oven etc.

**Experimental animal and maintenance:** A total of 03 Wistar albino rats weighing in between 150-200 g were purchased from central animal house of Northern Border University, Saudi Arabia. The animals were maintained at a controlled temperature (22–25°C, 45% humidity) on a 12:12-h dark–light cycle. The animals were provided with adequate standard diet and ecofriendly conditions throughout the experiment. All the experiments were carried out between 9:00-16:00 hours. National Committees of BioEthics (NCEB) guidelines projected by Saudi Arabia were strictly followed and all the studies were approved by the Local Committee of BioEthics (LCEB), (Ref: 8/38/A).

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For every test drug, 10-15 eggs were used in parallel. For the evaluation of the effects a scoring system was used.
Briefly, the score obtained from above equation was assigned as follows:

\[
\text{Average score} = \frac{\text{Number of (score 2)} \times 2}{\text{Total number of eggs (score 0, 1, 2)}} + \frac{\text{Number of (score 1)} \times 1}{\text{Total number of eggs (score 0, 1, 2)}}
\]

**Score system of anti angiogenic activity:**

Score < 0.5 % = no anti-angiogenic effect  
Score 0.5 to 0.75 % weak anti-angiogenic effect  
Score > 0.75 to 1 = good anti-angiogenic effect  
Score > 1 % very good anti-angiogenic effect.

### 2.3 Rat Aortic Ring Assay

Aortic Ring Assay (in vitro) method is one of the popular methods used for the screening of pro-angiogenic and anti-angiogenic activity. A healthy male Wistar rat weighing in between 200-210 gm sacrificed by CO$_2$ over dose thoracic cage was opened and the surrounding organs were detached. Thoracic aorta was isolated and immediately it was transferred to cold PBS supplied with aeration. While isolating much traction was avoided to maintain the integrity of the aorta. Aorta was cut into 1mm ring size and washed with DMEM (Dulbecco’s modified Eagle’s medium)\(^1\). These rings were placed in the 24 well plates with 150µl of corning Matrigel without growth factor. Initially, matrigel was stored at -20°C to liquefy and the micropipettes tips also were kept at -20°C to avoid any gelling effect of tips on the matrigel. Rings were completely immersed in matrigel and were allowed to polymerize for 1-2 hours at 37°C and then challenged 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. Doses were selected based on the IC$_{50}$ values and from earlier literature. Three doses were selected for each test drug; Verapamil (55, 110, 220 µM)\(^1\), Diltiazem 1, 10 100 µM\(^2\), ethosuximide (0.3, 0.6 and 1.2 mM)\(^3\). Dosing schedule is mentioned Table 1. Development of microcapillary and length of the capillaries formed was measured under the microscope at 400 X magnification using stage micrometer.

### 2.4 Statistical Analysis

Data were expressed as means ± standard deviation. Statistical analysis was performed using one-way ANOVA followed by Dunnett test. \(p = 0.05\) level of significance. All the statistical analysis was done by using graph-pad Prism 7.

**Table 1.** Treatment schedule L-type and T-type calcium channel blockers for anti-angiogenic activity

| Group No | Treatment                          | Dose per Ring |
|----------|-----------------------------------|---------------|
| 1.       | Control                           | Distilled water |
| 2.       | Standard Drug (Suramin)           | 38.54 µM      |
| 3.       | Verapamil (Low dose)              | 55 µM         |
| 4.       | Verapamil (Medium dose)           | 110 µM        |
| 5.       | Verapamil (High dose)             | 220 µM        |
| 6.       | Diltiazem (Low dose)              | 1 µM          |
| 7.       | Diltiazem (Medium dose)           | 10 µM         |
| 8.       | Diltiazem (High dose)             | 100 µM        |
| 9.       | Ethosuximide (Low dose)           | 0.3 mM        |
| 10.      | Ethosuximide (Medium dose)        | 0.6 mM        |
| 11.      | Ethosuximide (High dose)          | 1.2 mM        |

### 3. Results

#### 3.1 Chick Chorioallantoic Membrane (CAM) Assay

Not all the test drugs caused the inhibition of vessel development in the CAM Figure 1 depicts maximum inhibitory effects of verapamil and standard drug Suramin on the number of branching points. No changes in the growth patterns of the micro capillaries were observed by the ethosuximide treated groups at all the selected doses. Verapamil at medium and high dose, significantly (\(p<0.001\)) inhibited number of branching points by 17.3 and 7 at 110µM and 220µM concentration compared to control group. Diltiazem treated groups have shown slight but significant (\(p<0.001\)) reduction in the number of branching points by 30.66 and 24.16 compared to normal control group. (Figure 1)  
Test drug Verapamil also have shown very good angiogenic score in the range of 1 to 1.5. In fact, Verapamil has shown significant (\(p<0.001\)) dose-dependent increase in the angiogenic score at all the three doses i.e., 0.5, 0.75 and 1.3 at the concentration of 55, 110 and 220 µM/disk respectively. (Figure 2)  
Ethosuximide, a T-type calcium channel blocker has not demonstrated any inhibitory effects on vessel growth in the CAM.
It's essential to take note of that the evaluation strategy utilized does not recognize recently developed micro vessels (after utilization of the medication containing plates on the CAM) and those already present on the seventh day. Hence, 100% diminution of vascular thickness isn’t conceivable by this assay and the number of branching points 17.3 and 7 by the medium and high of verapamil can be considered as the maximum achievable limit.

Interestingly, no micro vessels were detected on the CAM under the disc containing 220 µM of verapamil, suggesting that this compound not only hinder the formation of new micro-vessels but also destroy or regression of existing capillaries.

### 3.2 Aortic Ring Assay

The CAM experiment demonstrated that verapamil could inhibit angiogenesis. To further determine whether verapamil inhibited angiogenesis *ex vivo*, the effect of verapamil and other test drugs on the sprouting of microvessels from the aortic rings was carried out. As shown in Figure 3, compared to the control, sprouts around the ring

![Figure 1.](image1.png)

**Figure 1.** Comparative effect of L-type and T-type calcium channel blockers on the number of branching points of the blood vessels by CAM assay of angiogenesis.

![Figure 2.](image2.png)

**Figure 2.** Comparative Anti-angiogenic effect (CAM-assay) of L-type, T-type Calcium Channel blockers and positive control.
treated with verapamil (55, 110 and 220 µM) and standard Suramin (38.54 µM) the sprouts around the rings were shorter. These data suggested that verapamil could inhibit the sprout length and density in the dose-dependent manner. Diltiazem also showed significant (p<0.01) dose-dependent decrease in the number and the length of microvessel sprouting compared to the normal group but the potency is less as compared to verapamil-treated groups.

![Figure 3](image.png)

**Figure 3.** Comparative effect of L-type and T-type calcium channel blockers on the area of sprouts formed in aortic ring assay of angiogenesis.

### 4. Discussion

Angiogenesis is one of the critical steps in tumor progression and metastasis. Pro- and anti-angiogenic factors regulate the formation of new blood vessels. Excessive angiogenic happens when the effects of angiogenic factors become preponderant on the anti-angiogenic factors leads to formation of new blood vessels supplying tumor tissue.

Most mammalian cells represent numerous types of calcium channels involved in the regulation of the membrane potential the transport of osmolytes, and cell-volume regulation but the exact role of these channels in cell angiogenesis is not clearly defined. Earlier, voltage-dependent calcium channels blockers were used and have shown potential antiproliferative effects in several tissues but none of these drugs are clearly specific to one type of channel but they all highlight the role of calcium channels in the control of cell proliferation but not defined their exact role in angiogenesis. Based upon these considerations in our study we have screened and compared the anti-angiogenic effect of voltage-dependent L-type (verapamil and diltiazem) and T-type (Ethosuximide) against CAM assay and aortic ring assay of angiogenesis. We report here that verapamil has shown the potent anti-angiogenic activity in both the models of angiogenesis in the dose-dependent passion. Diltiazem has also shown the activity but not as good as verapamil while ethosuximide has not shown any effect on the micro vessels formation. These results suggest that L-type calcium channels may play a role in the angiogenic cascade.

The CAM assay in the chick embryo is the most widely used vessel development assay in vivo. Initially, it was developed as a qualitative assay; ways were later reported for reliably quantifying the effects of promoters and inhibitors of angiogenesis on capillary development. It is particularly suitable for the primary screening of potential inhibitors of angiogenesis in a living organism because it is simple and quick and vessels grow spontaneously with no need for the addition of external growth factors.

In the chick embryo, CAM shows the densest vascular networks so it can be considered that pro-angiogenic and angiogenic factors are present in the vascular growth.

The results our study represents that verapamil had shown significant (p<0.001) decrease in the number of capillaries formation, branching points and significant (p<0.001) increase in angiogenic score in all three doses in the dose-dependent manner (Figure 1, Figure 2 and Figure 4) compared to the control group. Moreover, the tested drugs with has shown anti-angiogenic activity the actions are mediated through the receptors because only drug-treated groups have shown the decline in blood vessel growth when compared to normal control group. Hypoxia and hence the free radical formation is one of the provoking factors for angiogenesis. In response to hypoxia, there is release of HIF (Hypoxia Inducible Factors) i.e. angiogenic and Vascular Endothelial Growth Factors (VEGF), which lead to the formation and growth of new blood vessels are considered as the basic and major inducers of angiogenesis. Based on these findings, we assumed two possible mechanisms.

In the blood capillaries, the formation of superoxide anions (O$_2^-$) and free radicals considered as a vital factor responsible for endothelial dysfunction. Verapamil
### Table 1: Effect of L-type and T-type Calcium Channel blockers on neovascularization by using CAM assay of angiogenesis.

| Treatment Conditions | Images | Description |
|----------------------|--------|-------------|
| Normal Control group | ![Image](image1) | Filter disk loaded with Distilled water | Effect on the blood capillaries |
| Before Suramin (38.54 µM) Treatment | ![Image](image2) | Filter disk loaded with Suramin (38.54 µM) | Effect on the capillaries after Suramin (38.54 µM) Treatment |
| Before Verapamil (55µM) Treatment | ![Image](image3) | Filter disk loaded with Verapamil (55µM) Treatment | Effect on the capillaries after Verapamil (55µM) Treatment |
| Before Verapamil (110 µM) Treatment | ![Image](image4) | Filter disk loaded with Verapamil (110 µM) | Effect on the capillaries after Verapamil (110µM) Treatment |
| Before Verapamil (220 µM) Treatment | ![Image](image5) | Filter disk loaded with Verapamil (220 µM) | Effect on the capillaries after Verapamil (220µM) Treatment |

**Figure 4.** Effect of L-type and T-type Calcium Channel blockers on neovascularization by using CAM assay of angiogenesis.
possesses antioxidant and free radical scavenging effects.

This action of verapamil might have protected the endothelium from oxidative damage\(^\text{28}\). Second, as mentioned earlier in response to hypoxia there is a release of VEGF which induces angiogenesis via Receptor Tyrosine Kinases (RTK). Earlier literature shows that the verapamil reduces the VEGF formation and hence angiogenesis. The potency of antioxidant activity and VEGF inhibition activity is verapamil > diltiazem, this could be the possible reason why verapamil has shown better anti-angiogenic effect than diltiazem\(^\text{28–30}\). Ethosuximide has not shown any anti-angiogenic effect in both the models of angiogenesis. The possible explanation is that T-type calcium channels are predominantly present in the neuronal cells and those might be absent on the extra-neuronal tissue. Moreover, ethosuximide does not possess any effect on VEGF and free radicals, unlike verapamil and diltiazem.

Rat Aortic assay is another major model which was implemented in this research. In rat aortic ring assay, endothelial cells originate from the inner surface to from the capillary structure of rings of rat aorta which is immersed in the corneal matrigel. This assay also has many advantages like no need for repeated passages in culture to generate vascular outgrowth, (which resemble in-vivo angiogenesis), development of microvessels composed of endothelial cells and pericytes, self-controlling nature of the angiogenic response, external growth factor is not needed as they are naturally present in the developing embryo. The aortic ring assay provides a very good model which covers all major steps of angiogenesis like cell invasion, migration, proliferation, differentiation, and new vessel formation\(^\text{31,32}\). By the treatment with the verapamil at all three doses i.e., 55, 110 and 220 µM produced a drastic change in the area of sprouts formation in dose-dependent manner. (Figure 3 and Figure 5)

![Figure 5](image-url)
Ca$^{2+}$ is one of the important signaling molecule involves in many physiological processes including muscle contraction, pacemaker activity, cell volume regulation, and is also an important component of regulation of cell cycle. So slight depletion or over loading then the normal limits of the intracellular calcium concentration may affect many physiological processes adversely. Therefore, overloading of calcium or disturbances in calcium signaling cause cell death$^{33-35}$.

Verapamil is an L-type calcium channel blocker. We found that verapamil in 55, 110 and 220 µM concentrations had a very good anti-angiogenic effect. However, diltiazem 1 and 10 µM concentration was a poor inhibitor of angiogenesis but at 100 µM shown good anti-angiogenic activity. The findings show that diltiazem has also an anti-angiogenic activity which is active and significant at high doses.

5. Conclusion

Based on our in-vitro studies we have shown that L-type calcium channel blockers are inhibitors of angiogenesis while T-type calcium channel blockers may not involve in cell proliferation and angiogenesis. Among the tested drugs verapamil has shown a very potent antiangiogenic activity as good as suramin. Our results reveal a probable physiological role for L-type calcium channel blockers in angiogenesis and suggest new insight to antiangiogenesis research by suggesting that L-calcium channel blockers play a key role in the angiogenic process and angiogenesis-dependent tumor growth. Verapamil and diltiazem are already in the clinical use for cardiovascular complications and considered as safe drugs. This additional new finding of these drugs will help prescriber to prescribe such drugs for the co-comorbid patients.

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7. Conflict of Interest

Authors don’t have any conflict of interest.

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