Section: Pharmacology

Original Research Article

# A STUDY ON THE EFFECTS OF ASCORBIC ACID ON ANGIOGENESIS USING ZEBRAFISH EMBRYO MODEL

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## ABSTRACT

**Background:** Angiogenesis is a normal process, required for normal tissue repair and growth. Zebrafish is used as an alternative vertebrate model to study angiogenesis as it possess a complex circulatory system comparable to mammals with reasonable counterparts and evaluation of blood flow is extremely easy to score in zebrafish embryos, making it an ideal model for the study of angiogenesis. This study elucidates angiogenic potential of Ascorbic acid by the way of stimulating the formation of major blood vessels such as dorsal aorta, cardinal vein and intersegmental vessels and normal embryonic development. **Materials and Methods:** This Experimental study was conducted in the zebrafish embryos model for a period of year one duration. Vitamin C dissolved in hanks solution and added to the culture plate and the zebrafish embryos were added. The median lethal concentration LC 50, RBC staining and alkaline phosphatase staining were carried out. Quantitative analysis like Whole embryo Endogeneous Alkaline phosphate (EAP) assay was done. **Result:** Vitamin C exposed zebrafish embryos were showing significant ISV formation from time of exposure in the staining methods.The development of blood vessels were confirmed with the endogeneous ALP staining which is a quantitative method .The vessel growth of 50µl and 100µl compared showed that p value was 0.054 which shows mild significance and pearson correlation was 0.475 then 100µl and 200µl was compared which shows p value <0.001 which showed more significance with correlation of 0.762. then vessel growth of 50µl and 200µl was compared showed p value of <0.001 and correlation of 0.758 and showed much more significance. Hence the vessel growth was confirmed with Endogenous ALP staining assay. **Conclusion:** We conclude that vitamin C enhances angiogenesis in zebrafish embryo model at concentration of 200µg/ml. Hence Vitamin C is an angiogenesis inducer in zebrafish embryo model.

## INTRODUCTION

Angiogenesis is a normal process, required for normal tissue repair and growth. Pathological angiogenesis is characterized by the persistent proliferation of endothelial cells and blood vessel formation. This complex process plays an important role in tumor growth, invasion, and metastasis. Recent studies have linked the involvement of circulating endothelial precursor cells (EPCs) to pathologic angiogenesis. Tumor cells signaling vascular proliferation induce endothelial phenotypic expression of the bone marrow progenitor cells.[1] Angiogenesis is essential to support the nutritional demands of solid tumors undergoing rapid growth.[2] Most angiogenesis inhibitors currently being tested work either by neutralizing endothelial cell growth factors, inhibition endothelial cell proliferation, preventing turnover of basement membrane, or blocking capillary formation. Unfortunately, most of these agents are toxic and have high risk of adverse effects. In hopes of finding less toxic angiogenesis inhibitors, substances derived from natural sources, such as flavonoids, sulphated carbohydrates, or triterpenoids are being examined, as are natural health products such as herbs, phytochemicals, and antioxidants.[3]

In regard to antioxidant angiogenesis inhibitors, vitamin C is of particular interest for a variety of reasons. First, it is has already been shown to have anti- tumor effects in certain experimental and


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clinical settings, provided that ascorbate concentrations reach the millimolar range.[4]
Secondly, its role in supporting the later stages of wound healing, particularly collagen formation and strengthening of extracellular matrix, may counter tumor-induced neovascularization. Moreover, its antioxidant activity may regulate some aspects of endothelial cell migration, since some aspects of this are promoted by nitric oxide and other oxidants.[5]
Zebrafish is used as an alternative vertebrate model to study angiogenesis as it possess a complex circulatory system comparable to mammals with reasonable counterparts and evaluation of blood flow is extremely easy to score in zebrafish embryos, making it an ideal model for the study of angiogenesis.[5] The significant similarity in protein sequences, conservation of developmental processes leading to organogenesis, and common appearance of pathophysiologic mechanisms all contribute to pull out zebrafish in biomedical research.[6] The cardiovascular system is the first functional organ to develop in the vertebrate embryo. It forms through several distinct, but parallel, processes.
The primordial vascular system is defined by vasculogenesis (Risau and Flamme, 1995). During this process, endothelial progenitor cells (angioblasts) differentiate in the embryonic and extra-embryonic mesoderm shortly after gastrulation and merge to form a primitive vascular network. This vascular plexus is extended and modified through a series of morphogenic processes that include the formation of new capillaries, by both sprouting and nonsprouting angiogenesis (intussusception), the remodelling of pre-existing vascular plexi into small and large vessels, and the pruning of blood vessels (Risau, 1997). The concomitant maturation of the developing vasculature involves the recruitment of perivascular cells, such as pericytes and smooth muscle cells, to the vasculature and the deposition of a specific basement membrane, leading to the stabilization of the vasculature. While the embryonic vascular system develops in anticipation of the demands of the growing embryo for oxygen and nutrients, angiogenesis in the adult organism occurs in response to the metabolical requirements of tissues and is efficiently triggered by hypoxia. A variety of diseases are associated with either insufficient or excess blood vessel growth. For example, the insufficient compensatory formation of blood vessels in ischaemic tissues is a major problem in coronary heart disease, or stroke. On the other hand, deregulated blood vessel growth is involved in the pathogenesis of diabetic proliferative retinopathy, or solid tumour growth (Folkman, 1995).
This article focuses on the role of the principal endothelium-specific receptors and their ligands which regulate the morphogenesis and growth of the vascular system: the vascular endothelial growth factor (VEGF)/VEGF receptor (VEGFR) system.
The zebrafish (Danio rerio) has become a popular model for studying genetics and developmental biology. The transparency of these animals at early developmental stages permits the direct visualization of tissue morphogenesis at the cellular level. Furthermore, zebrafish are amenable to genetic manipulation, allowing researchers to determine the effect of gene expression on the development of a vertebrate with a high degree of genetic similarity to humans.
As the human genome sequence project is complete and efforts to sequence the zebrafish genome are ongoing, this animal model is also well positioned to contribute to understanding function of human genes.
The effect of Ascorbic acid on blood vessel formation were investigated. Studies showed that high concentrations of ascorbic acid affect the initial phase of cell migration and tube vessel formation and thereby inhibits angiogenesis. However detailed studies on the pharmacological activity are sparse. Evidence seems to support the view that anti-angiogenic agents would destroy both cancer cells and endothelial cells. Thus this study elucidates angiogenic potential of Ascorbic acid by the way of stimulating the formation of major blood vessels such as dorsal aorta, cardinal vein and intersegmental vessels and normal embryonic development.

## MATERIALS AND METHODS

**Study design:** Experimental study
**Study sample:** Model organism: zebrafish embryos
**Study duration:** 1 year
**Zebrafish Animal Husbandry:**
Aquarium of Zebrafish was set up at first and wild type adult zebrafish (3-5 months old) both males and females was obtained from the local supplier at India. The adult male and female fish were maintained in separate tanks in large 50–60 liter aquaria with a maximum density of 1gm fish/liter chlorine free water. The pH of the water was maintained between 6.2 - 7.5 at 28±1ºC with a constant light–dark (14–10 hrs) cycle.
The water was continuously aerated and renewal of the water was done in a semi-static manner. Fish tanks was cleaned and water was changed regularly to maintain cleanliness of the fish and the tank. Also the fish was provided with feed twice a day and varied diet consisting of commercial dry flake food, artemia, bloodworms and live brine shrimp. The health condition of the fish was checked every day.
**Breeding of zebrafish**
Prior to 3-5 days of collection of embryos, the fish will be fed twice a day with blood worms. Prior to a day of collection of embryos, during the time of dusk, 8 male fish will be allowed in female tank containing 4 fish. The embryos collection tray with a nylon mesh on top will be kept underneath water in order to collect embryos and to avoid prey by zebrafish.
**Collection of embryos**
The next day during dawn the embryo tray will be removed and embryos will be collected to a 100 mm glass petridish. The eggs will be washed 3-4 times with embryo medium (Hank's medium) to remove


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debris prior to starting of experiment to avoid contamination by debris. Embryo medium will be prepared as per standard protocol for culturing embryos. The embryos will be collected at weekly intervals and various developmental stages will be identified. The stages of development will be fixed as hours of post fertilization (hpf). After collecting the embryos, they are checked for viability and different stages of development of embryos were photographed in microscope. In these, the zebrafish and embryos were well maintained with aquaria water which was replaced daily with fresh treatment embryo medium so as to maintain a purity in the experiment.

**Figure 1: Maintenance of zebrafish**

**Figure 2a: Breeding tank**

**Figure 2 b: Breeding tank**

**Embryo medium preparation**

Working solution of Embryo medium for 500 ml was prepared as per standard protocol

**Figure 3: Collected embryos in culture plate**

**Constituents of embryo medium**

Hank's solution 1:
8.0g Nacl & 0.4g Kcl – in 100ml double dilution water
Hank's stock solution 2:
0.358g Na2HPo4 anhydrous & 0.6g KH2Po4 – in 100ml double dilution water
Hank's stock solution 4:
0,72g CaCl2 in 50ml double dilution water56
Hank's solution 5:
1.23g MgSo4 X 7H2O in 50ml double dilution water
Hank's stock solution 6:
0.35g NaHCO3 in 10ml double dilution water

**Embryo Medium preparation**

To prepare 500ml of Embryo medium:
Take 5.0ml of Stock 1
0.5ml of Stock 2
5.0ml of Stock 4
479.5ml double dilution water
5.0ml of Stock 5
5.0ml of Stock 6
Check pH of the solution with pH meter and adjust to 7.2

**Vitamin C:** purchased from Sigma and dissolved in Hanks solution.

**Preperation of stock solution (Test solution)**

0.01g of ascorbic acid in 10 ml of embryo medium such that 1ml of embryo medium contains 1mg of ascorbic acid and stored in room temperature.

**Depigmentation of Zebrafish**

Depigmenting of zebrafish is done by adding 1-phenyl 2- thio urea (PTU) was added in the following concentration.

Stock concentration: 7.5%, from the stock concentration 0.002% which is 5.333µl was added to the 20ml of embryo medium and this is added to the 6 well tissue culture plate during 50% epiboly stage of development of embryos. PTU is added along with the treatment and the embryos are added as 20 for each well in 6 well tissue culture plate and incubated at 280C upto 72 hours.


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**Figure 4: Depigmentation of embryos in 6 well plate**

**The Test**

Group I (Control): Consisted of 20 embryos each in 6 wells of a tissue culture plate at 50% epiboly stage with embryo medium and were incubated.

Group II (Vitamin C ): Consisted of 20 embryos each in 6 wells of tissue culture plate at 50% epiboly stage and were treated with 50, 100, 200, 400,500, 800µg of Vitamin C respectively. At the end period, the developmental changes such as embryo survival rate, hatching rate, heart rate, yolksac edema, tail, head and eye deformities was assessed in allexperimental embryos. After assessing the toxicity dose which was repeated for three times in three separate breeding and then the angiogenesis study was performed for those concentrations below the toxicity level.

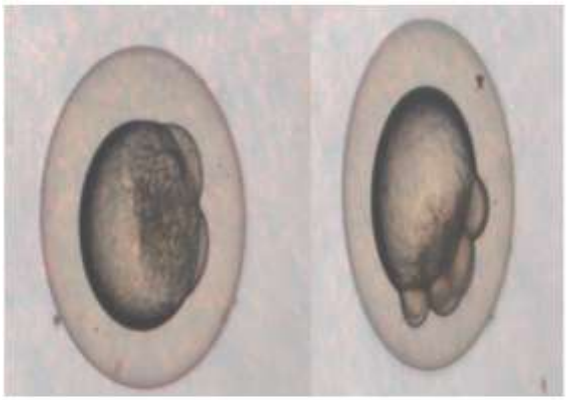

**Figure 5: Early stages of development**

**Determination of median lethal concentration LC 50**

The eggs were exposed to different concentrations of Vitamin C. Controls were also run simultaneously. Behavioural manifestations, condition of embryo and mortalities were noted every 24h upto 72h. The number of dead larvae was recorded for each of the concentrations and data were used to determine the median lethal concentration (LC50). For this study, 6 well tissue culture plate was used in which each well had 20 embryos and totally 120 embryos were studied. This determination of lethal dose was repeated hence totally 360 embryos were studied

**RBC staining:**

RBC staining is the technique which uses o-dianisidine used to study the expression of globin. RBC staining was performed to visualise the formation of blood vessels in zebrafish embryos. O-Dianisidine was used to stain embryo red blood cells in blood vessels.

- Embryos were dechlorinated with protease
- Incubated for half and hour at 28ºC
- Washed with embryo medium for 3-4 times
- Fixed with 4% paraformaldehyde for half and hour
- Embryos washed with 1X PBS for 3-4 times
- Embryos stained in dark using O-Dianisidine and incubated for half and hour
- Then examined under microscope and photographed

**Alkaline phosphatase Staining**

**A) Materials**

- Fixation Buffer: 10ml 4% paraformaldehyde+1ml 10%Triton-X 100; make upto 11ml
- Rinse Buffer: 10ml 10XPBS +5ml 10% Triton-X100 + 84ml distilled water + 1ml normal horse serum; makes 100ml
- Staining Buffer: 1ml 5M NaCl+2.5ml 1M MgCl2 + 5ml 1M Tris pH 9-9.5 +500µl 10% Tween-20 + 41ml distilled water; makes 50ml
- Staining solution: 45µl NBT + 35µl BCIP + 10ml staining buffer 5960
- NBT- 4-Nitro Blue Tetrazolium -100mg/ml in 70% dimethylformamide
- BCIP- 5-Bromo-4-Chloro-3-indolyl-phosphate, 50mg/ml in dimethylformamide

**B) Procedure**

1. Fix the embryos in room temperature for 1 hour in fixation buffer
2. Rinse 1 time in rinse buffer
3. Wash 5 times for 10 mins at room temperature in rinse buffer
4. Wash 2 times for 5mins in staining buffer
5. Stain in 1ml of staining solution. Colour development takes about 5- 30mins
6. To stop reaction, wash 3 times in rinse buffer without horse serum, then fix with 4% paraformaldehyde for 30mins and store in fixative at 4ºC

**Quantitative Analysis**

**Whole embryo Endogeneous Alkaline phosphate (EAP) assay:**

Using this quantitative assay format, large compounds can be rapidly screened for many agents that affect angiogenesis in zebrafish. The quantitative measurement is more sensitive than visual inspection. In order to prosper a high-throughput assay for assessing angiogenesis, vessel growth induction or inhibition, we subsequently used a 96 well microplate format to quantitative endogenous alkaline phosphatase activity in individual embryo using soluble EAP substrate. The quantitative differences were then measured using the optical microplate reader for EAP activity. The measured optical density is then tabulated and % vessel growth was checked


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for each concentration after entering in excel sheet. This assay was repeated for three times in three different breeding times and the average was taken.

**A) Materials**

- 70% and 100% ethanol
- Diethanolamine buffer
- p-nitrophenyl phosphate disodium salt
- 2M NaoH

**B) Procedure**

1. Embryos were first treated with 100µl of ice-cold 70% ethanol for 10mins.
2. Then dehydrated and permiabilized in 100% ethanol for 30mins
3. Washed three times with diethanolamine buffer, 10 mins each
4. Incubated in 100µl of 0.5mg/ml p-nitrophenyl phosphate disodium salt for 30 mins at room temperature
5. 50µl of 2M NaoH was added to stop reaction
6. Then the optical density of soluble end product was measured at 405nm(OD405) using a ELISA microplate reader.

$$\% \text{ vessel growth} = \frac{(OD_{\text{treated day 3}} - OD_{\text{control day 1}})}{(OD_{\text{control day 3}} - OD_{\text{control day 1}})}$$

Each assay was repeated at least three times. No additional maintenance was required as the embryo yolk sac supplies nutrients during the experiment.


**Figure 6: ELISA microplate reader**

**Statistical Analysis:** The data were represented as mean ± standard deviation (S.D) of at least three separate and consequetive experiments in triplicate. Comparisons between different groups were analysed using the one way analysis of variance(ANOVA). P-values <0.05 were considered significant. The vessel growth of zebrafish was calculated mean ± SD and the correlation was found with pearson correlation.

## RESULTS

Angiogenesis is an important mechanism in development. The sprouting of new blood vessels is very important in developing embryo.

The following parameters were determined.

I)Determination of lethal dose concentration LC50

II) Morphological assays

III) Phenotypic parameters

IV) Angiogenesis

### I) DETERMINATION OF MEDIAN LETHAL CONCENTRATION LC 50

- The LC50 was determined as 400 µg/ml as at 24 hpf only 50% of embryos were live.
- The result showed none of the zebrafish survived beyond 48hrs at higher concentration above 400 µg/ml.
- At 400 µg/ml, of 360 embryos only 189 embryos survived whereas, there was no significant difference in the mortality at low concentrations 50, 100, 200 µg/ml.

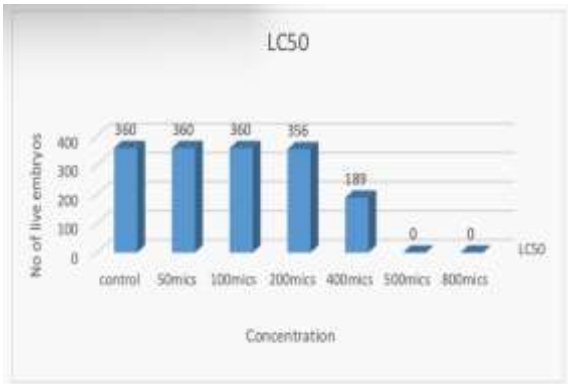


**Figure 7: Determination of LC 50 at various concentration**

### II) MORPHOLOGICAL ASSAYS

Embryos of control group and tested groups were maintained in wells of culture plates at 28±2ºC until 72 hpf and were inspected for mainly the parameters like survival rate, hatching rate and heart rate

**1) Survival Rate**

The test determined the mortality in zebrafish embryos exposed to various concentration of ascorbic acid of 50, 100, 200µl for 72hpf. The observed percentages of zebrafish survival of ascorbic acid treated comparing with control are shown in figure 19 at 72 hours post fertilisation.

- The percentage of survival rate was defined as (the number of larvae survived/ the initial number of embryos) x100.

In control the survival rate was 98.65% , 50µl was 98.40%, 100µl was 98.25% and 200µl was 98%.

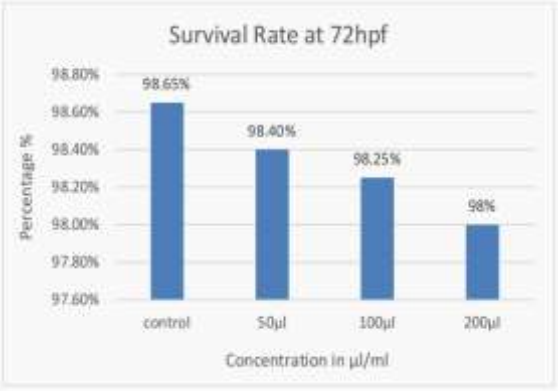


**Figure 8: Survival rate at different concentrations at72hpf**


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**2) Hatching rate of zebrafish:**

- The percentage of the Hatching rate was defined as (the number of larvae survived/ the initial number of embryos) x 100.
- In both the groups 70% and 90% of the embryos hatched out at 48 and 72 hpf respectively.

The 99% of the embryos were hatched out in control group by 72 hpf whereas in treated group 99% for 50µl, 100% for 100µl and 98% for 200µl.

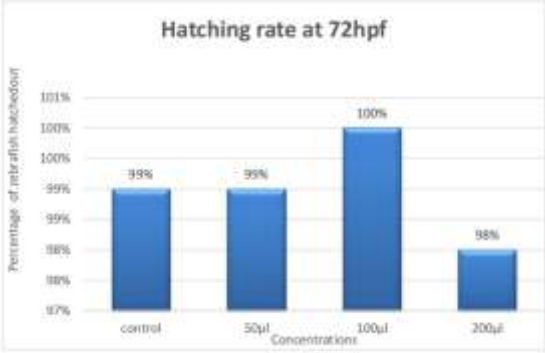


**Figure 9: Hatching rate at different concentrations of embryo at 72 hpf**

**3) Heart rate measurement**

The embryos were placed into a well slide with a small amount of embryo medium. Heart rates were visually determined using compound light microscope and counting ventricular contraction.

- Figure 10 shows heart rate of embryos at different concentrations at 72 hpf
- The treated embryos show 98bpm, 105bpm and 99bpm at increasing concentration whereas 100bpm for control group.

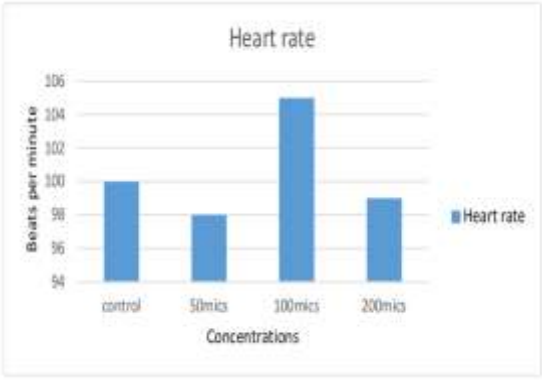


**Figure 10: Heart rate of different concentrations of embryo at 72 hp**

## III) PHENOTYPIC PARAMETERS

The phenotypical abnormalities like skeletal malformations, pericardial edema and yolk sac width were inspected at 24, 48 ,72 hpf was repeated for consequentively for three times for different set of zebrafish and the average was taken. The other phenotypic parameters like Body length, yolk sac length, head circumference, eye circumference, tail width are being noted and compared with control and different concentrations of the drug and they are tabulated and shown in the table 1.

- For comparing the phenotypic parameters 120 embryos were taken in both control group as well as in treated group.

**Control 50µl 100µl 200µl**

**Figure 11: Embryos treated with control, 50µl,100µl and 200µl at 24hpf, 48hpf and 72hpf**

- Figure 11 shows embryos of different concentrations and control at 24, 48 and 72 hpf which shows no major phenotypic changes
- Morphometric deformities were also inspected for and were been photographed if specific when compared to control at various specific time intervals.

## IV) ANGIOGENESIS STUDY

**1)** The angiogenesis study was carried out with ideal concentration of 200µg/ml of vitamin C **RBC staining**

This technique was performed for higher concentration of vitamin 200µl and was compared along with control. The figure shows red blood cells which is stained in tail region of 72 hours post fertilisation of zebrafish.

- In this study mainly the intersegmental vessels were concentrated which is compared for control embryos and 200µl of vitamin C at 72 hpf.
- The result shows that vitamin C treated zebrafish shows similar RBC staining as compared to that of the control.

**a) Control b) Vitamin C(200µg/ml)**

**Figure 12: RBC staining of zebrafish embryos at 72hpf (Control) and 200µl of Vitamin C**


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**2) Alkaline phosphatase staining**

Alkaline phosphatase staining which was employed to highlight the presence of key planner of angiogenesis namely intersegmental vessels (ISV) in vitamin C treated embryos. The figure 13 shows the images of zebrafish with alkaline phosphatase staining where the blood vessels of various regions are shown which says that staining of vitamin C treated zebrafish was similar to that of control group.

- Vitamin C exposed zebrafish embryos were showing significant ISV formation from time of exposure.
- The intact embryos of the Vitamin C exposed zebrafish embryos displayed no signs of necrosis with the increasing concentrations.

**a) Control b) Vitamin C**

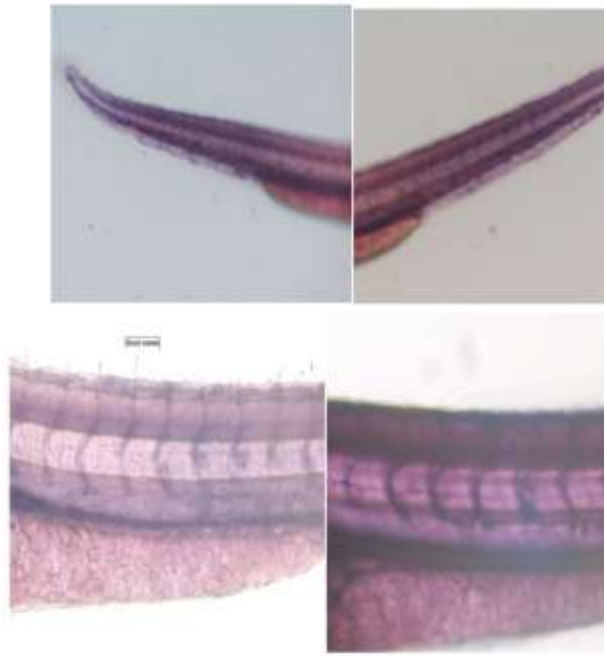

**Figure 13: Effect of a) control and b) vitamin C (200µl) at 72 hrs post fertilisation in alkaline phosphatase staining**

**3) Quantitative assay**

**Endogenous alkaline phosphatase (EAP)**

For the determination of endogenous alkaline phosphatase assay, the optical density of control day 1 and day 3 and the optical density of treated group with different concentrations of 50,100, 200 µg/ml at day 3 were taken. The percentage of vessel growth was then determined from the optical density from the following formula

$$\% \text{ vessel growth} = (OD_{\text{treated day 3}} - OD_{\text{control day 1}}) / (OD_{\text{control day 3}} - OD_{\text{control day 1}})$$

**Table 1: Development of zebrafish embros (72hpf) treated with Ascorbic acid**

| Parameters | Control | Ascorbic acid-50µl | Ascorbic acid-100µl | Ascorbic acid- 200µl | p-value |
|---|---|---|---|---|---|
| Body length | 1384.3±54.2 | 1388±32.9 | 1400±33 | 1407.5±93.4 | >0.05 |
| Yolk sac length | 521.2±29.6 | 490.0±30.1 | 500.0±28.0 | 498.0±32.1 | >0.05 |
| Head circumference | 105.5±20.9 | 103.2±20.1 | 105±18.4 | 102.5±12.0 | >0.05 |
| Eye circumference | 54.4±3.4 | 58.0±5.1 | 53.6±4.0 | 58.8±9.0 | >0.05 |
| Tail width | 100.7±6.4 | 98.8±7.0 | 99.0±2.0 | 99.7±6.3 | >0.05 |

The table 2 shows the percentage of vessel growth of the three different concentrations of 50,100,200 µg/m


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**Table 2: Vessel growth in percentage – EAP Assay**

| % Vessel growth 50µl | % Vessel growth 100µl | % Vessel growth 200µl |
|---|---|---|
| 0.79075 | 0.0539 | 0.59755 |
| 0.67655 | 0.6136 | 0.83645 |
| 0.1041 | 0.71185 | 0.51315 |
| 3.27575 | 0.2267 | 0.48 |
| 0.22085 | 0.3934 | 1.97625 |
| 0.68815 | 1.0479 | 0.92785 |
| 0.054185 | 0.20525 | 0.45535 |
| 1.4934 | 2.6881 | 3.87675 |
| 0.50435 | 0.19865 | 0.4072 |
| 1.90885 | 1.48725 | 1.7219 |
| 1.36055 | 0.3111 | 0.7068 |
| 5.37345 | 7.6442 | 6.131 |
| 0.49115 | 0.3446 | 0.6976 |
| 0.2254 | 7.9526 | 4.6043 |
| 0.0606 | 0.17355 | 0.9923 |
| 0.0196 | 0.69755 | 0.44 |
| 1.077980313 | 1.5468875 | 1.585278125 |

The table 3 shows 1.08% vessel growth in 50µl, 1.54% in 100µl and 1.58% vessel growth in 200µl of vitamin C treated embryos.

- The percentage of vessel growth has been compared for two concentrations as shown in table 5 where 50 and100µg/ml was compared shows p value of 0.054, 100 and 200µg/ml was compared, the p value was <0.001 and 50 and 200µg/ml was compared, the p value was <0.001 and the pearson correlation has been shown in the table 3.

**Table 3: Vessel growth comparing each concentration**

| Vessel growth | Mean + SD | p value | Pearson correlation |
|---|---|---|---|
| vessel growth with 50 µg/ml<br>vessel growth with 100 µg/ml | 1.08 + 1.39<br>1.55 + 2.45 | 0.054 | 0.475 |
| vessel growth with 100 µg/ml<br>vessel growth with 200µg/ml | 1.55 + 2.45<br>1.91 + 2.98 | < 0.001 | 0.762 |
| vessel growth with 50 µg/ml<br>vessel growth with 200 µg/ml | 1.08 + 1.39<br>1.91 + 2.98 | < 0.001 | 0.758 |

The figure 14 a is the line diagram representing weak correlation between %vessel growth of 50µg/ml and 100µg/ml.

- The Figure 14 b is the line graph showing % vessel growth comparing 100µg/ml and 200µg/ml which shows strong linear correlation between the concentrations.
- The Figure 14 c is the line graph which represents stronger linear correlation between 50µl and 200µl of vitamin C concentrations.

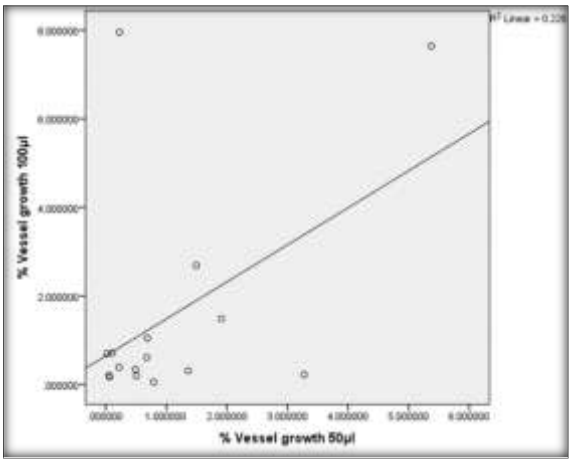


**Figure 14 a): %vessel growth comparing 50µg/ml and 100µg/ml**


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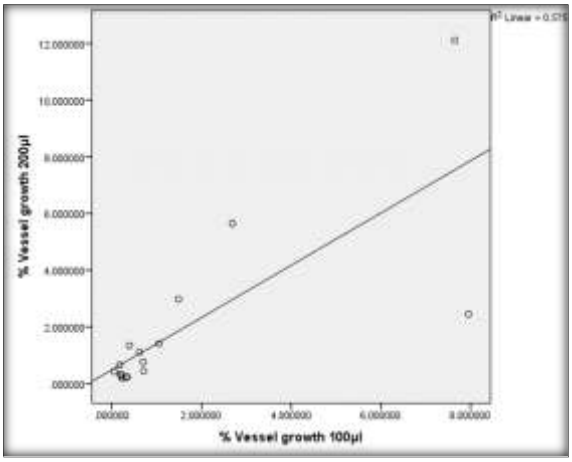


**Figure 14 b): %vessel growth comparing 100µg/ml and 200µg/ml**

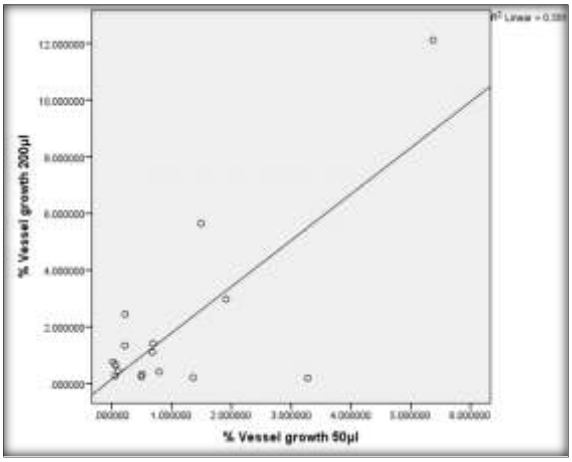


**Figure14c): % vessel growth comparing 50µl and 200µl**

## DISCUSSION

Vitamin C has a 5-hydrocarbon ring which is similar to that of glucose. L- Ascorbic acid becomes a weak sugar acid, with an attached hydrogen ion, which is similar to other alfa hydroxy acids. It forms a mineral ascorbate with the metal ion. There is always a marked interest in synthesis of physiologically active and chemically stable ascorbate molecules as ascorbic acid is unstable in nature, when exposed to light. Vitamin C has antioxidant properties, is essential for collagen synthesis and also has anti-inflammatory action and depigmentation effect. Vitamin C is a naturally occurring molecule with multiple advantageous effects. It has an excellent safety profile, it finds increasing use in hyperpigmentation, photoageing, tissue inflammation and also wound healing. There are many ongoing researches which has been directed towards improving drug delivery into the dermis so as to stimulate collagen production and also for scavenging free radicals. For toxicity testing and also for angiogenesis studies, zebrafish is one of the important and developing tools.

Though this vitamin C is devoid of side effects, there are also some rare adverse effects like renal stones reported in mankind. This study evaluates the effects of vitamin C in zebrafish embryos to find the toxicity level and also to study about its angiogenesis property. The embryos were treated with varying concentrations of Vitamin C like 50, 100,200,400,500, 800µg/ml which revealed that LC50 was 400µg/ml. This was repeated thrice to check the reproducibility of the results. The median lethal dose of Vitamin C in 72 hours post fertilisation zebrafish embryos was confirmed to be 400µg/ml and above this concentrations all embryos were dead. Subsequent test was conducted with the intermediate and below ranges of vitamin C like 250, 300 and 350µl which showed there was no significant death of the embryos. The growth, mortality, development and morphometric analysis was similar to the control groups. The embryos were daily observed under a dissection microscope for observing mortality, survival rate, hatching rate and morphological changes when compared to that of the control.

The concentrations like 50,100,200 µg/ml of vitamin C for survival rate, hatching rate showed no significant changes which was similar to the control group which was similar to raskin et al study,[7] but was different from akinola et al study,[8] which shows that as the concentration of curcuma longa extract increases the hatching out rate was decreased whereas heart rate was similar to the akinola et at study.

The morphological parameters and phenotypic parameters were also seemed to be similar and does not shows major changes when compared between control and different concentrations of vitamin C. No major phenotypic deformities were observed in our study whereas in a study conducted by uppuluri et at,[9] shows that there was phenotypic changes like abdominal elongation of yolk sac, tail bending, hemorrhages, stunted growth and pericardial edema has been observed after exposing to neem root extract.

The angiogenesis study was then done for the below levels of the toxicity for 50,100,200µl and during each study consecutive controls were also done to compare the effects. Angiogenesis study was carried on by RBC staining which was done for the higher concentration of vitamin C 200µl and was compared with control. In this RBC staining mainly intersegmental vessels were focussed and was repeated for thrice which showed the staining of RBC was similar in both the group of control and 200µl of vitamin C group which is shown in figure 21 at 72 hours of post fertilisation. This RBC staining result was not similar to rishipal et al study,[10] where cinnamon extract showed to exhibit inhibition of angiogenesis which was confirmed with impairment of intersegmental vessels(ISV) formation.

The angiogenesis study was with ALP staining which showed the vessel growth was similar to that of the control group and 200µl of vitamin c treated group.


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This was compared for mainly the yolk sac region, the cardiac vessels, body region and the tail which was similar in both group of zebrafish which showed that vitamin C enhances the angiogenesis in zebrafish which was similar to that of vivek sagaraj et al study,[11] which showed that there was increased vessel formation of CoCl2 which was shown by ALP staining and was compared with an angiogenesis inhibitor like SU 5416.

Angiogenic vessel activity was determined by endogenous alkaline phosphatase activity, which is present primarily in vessels during early development(0-72 hpf). This study was carried at 72hpf which showed peak activity of alkaline phosphatase and vessel growth is normally enhanced at 72 hpf.

The development of blood vessels were confirmed with the endogeneous ALP staining which is a quantitative method and the tables 4 and 5 shows the result. This showed that the vessel growth of 50µl and 100µl compared showed that p value was 0.054 which shows mild significance and pearson correlation was 0.475 then 100µl and 200µl was compared which shows p value <0.001 which showed more significance with correlation of 0.762. then vessel growth of 50µl and 200µl was compared showed p value of <0.001 and correlation of 0.758 and showed much more significance.

Hence the vessel growth was confirmed with Endogenous ALP staining assay.

**Limitations:** This vessel growth can be further confirmed by RT-PCR testing of VEFG-A expression. In future studies can be carried with the confirmation with RT-PCR technique.

## CONCLUSION

We conclude that vitamin C enhances angiogenesis in zebrafish embryo model at concentration of 200µg/ml. Hence Vitamin C is an angiogenesis inducer in zebrafish embryo model.

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