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Study of angiogenesis in cervical intraepithelial lesion and carcinoma using chick chorioallantoic membrane (CAM) assay

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Abstract
Background: Carcinoma cervix is the most common cancer among women in developing countries and most common cause of female mortality. Agiogenesis plays an essential role in tumor growth and invasion. CAM assay has been widely explored to study tumour angiogenesis and effect of anti-angiogenic drug. Itraconazole, an anti-fungal drug has high anti-angiogenic activity.
Aim of the study: To study of effect of angiogenesis on cervical intra epithelial lesion and carcinoma using chick chorioallantoic membrane (CAM) assay and the effect of itraconazole on angiogenesis.
Materials and Methods: This is a prospective cross sectional study conducted at Department of Obstetrics and Gynaecology, at D. Y. Patil Hospital and Research centre, Kolhapur over a period of 2 years. 100 patients between ages of 21-65 years with abnormal cervix on visual inspection were included for study.
Results: High grades of angiogenesis were found in this order: Carcinoma, CIN III, CIN II, and normal tissue. After giving Itraconazole drug average angiogenic score was reduced in CIN and Carcinoma.
Conclusion: From the study, this was concluded that although the result proves itraconazole to be a prominent anti-angiogenic and anti-cancer drug, there are limitations in the study due to less number of samples. Further in-depth research is more randomised samples are needed to validate these findings.

Keywords: angiogenesis, carcinoma cervix, chorioallantoic membrane, itraconazole

Introduction
Cervical cancer arises from cervix and invades and metastasized. Many women get infected with Human Papilloma virus throughout life but not in every woman carcinoma occur [1]. Virus stays for years and in some women with other risk factor it cause infection and cellular changes which turn into first cervical intraepithelial lesion and then cancer [2]. Usually patient does not have any complaint in early phase but in advance phase pelvic pain, bleeding vagina and bleeding after sexual intercourse occurs. The risk factors are: Sexual and reproductive factors, socio-economic factors, viruses like herpes simplex virus (HSV), human papilloma virus (HPV), human immunodeficiency virus (HIV) in cervical carcinoma and other factors who also play their role are smoking, poor diet, use of oral contraceptives, other hormones [3, 4]. Cervical cancer can be preventing by screening and early detection. Angiogenesis as a physiological process involves the growth of new blood vessels from pre-existing vessels and plays a central role in embryonic and normal developments and wound healing. It has also important roles in the aetiology of many diseases such as chronic inflammatory disorders, cancer, and some pregnancy related diseases such as intrauterine growth restriction and preeclampsia [4], Some approved anti-angiogenic agents such as bevacizumab, sorafenib, sunitinib, and thalidomide are used clinically as effective drugs for several types of cancer, and many new agents are in phase II trials. The chick embryo chorioallantoic membrane (CAM) is an extraembryonic membrane that is commonly used *in vivo* to study both new vessel formation and its inhibition in response to tissues, cells, or soluble factors. Quantitative or semi-quantitative methods may be used to evaluate the amount of angiogenesis and anti-angiogenesis. Thanks to the CAM system, angiogenesis could be investigated in association with normal inflammatory and tumor tissues, and soluble factors inducing angiogenic or anti-angiogenic effects could be identified [5]. Itraconazole, an anti-fungal drug has high anti-angiogenic activity. Itraconazole is a triazole antifungal agent prescribed to patients with fungal infections. It has also recently been explored as an anticancer agent for patients with basal cell carcinoma, non-small cell lung cancer, and

prostate cancer and other cancers. Functionally, the anti-angiogenic activity of itraconazole has been shown to be linked to inhibition of glycosylation, VEGFR2 phosphorylation, and trafficking and cholesterol biosynthesis pathways and through inhibition of headgehog signaling [6]. Despite all this existing evidence, Itraconazole, so far is not explored as an anti-angiogenic drug in chick embryo [7]. Hence, the present study was done in our tertiary care center to study of effect of angiogenesis on cervical intra epithelial lesion and carcinoma using chick chorioallantoic membrane (CAM) assay and the effect of itraconazole on angiogenesis.

Materials and Methods

This is a prospective cross-sectional study conducted at Department of Obstetrics and Gynaecology, at D. Y. Patil Hospital and Research Centre, Kolhapur. The study period was 2 years (August 2016 to August 2018). The sample size for the study was colposcopy of 100 unhealthy cervix on visual examination.

Inclusion Criteria

1. Unhealthy cervix (clinically suspicious cervix, cervical erosion, hypertrophied cervix, growth on cervix)
2. Sexually active age group age between 21 to 65 years
3. Patients willing to take part in the study

Exclusion Criteria

1. Unmarried women
2. Pregnant women
3. Patients with age less than 21 years and more than 65 year
4. Patients not willing to take part in the study

The sample was collected during colposcopy with punch biopsy forceps in sterile technique. The suspected cervical tissue biopsy collected during colposcopy was viewed under light photomicroscope after staining. The cervical cells were isolated from tissue biopsy.

Preparation of Stock Solution

- Itraconazole (1mg/ml): 5mg of Itraconazole was weighed and dissolved in 5 ml DMSO (Dimethyl sulfoxide)
- Cyclophosphamide (1mg/ml): 5mg of Cyclophosphamide was weighed and dissolved in 5 ml medical grade NS (0.9%).
- Both of the stock solutions were diluted serially from 1000µg/ml to 31.5µg/ml

The effect of Itraconazole and Cyclophosphamide on cell viability was assessed by MTT assay. Briefly, carcinoma cells were suspended in DMEM to $5x10^4$ cells/ml and aliquots were put into each well of 96 well plates. After 24 hours, the media was changed with different concentration of Itraconazole and Cyclophosphamide and incubated for 48 hours in CO2 incubator. After exposure the supernatant was removed and 100 µl MTT solutions were added in each well and incubated for 4 hours in CO2 incubator. After incubation, MTT solution was discarded and formed crystal was dissolved in DMSO (100 µl). The optical density of each well was determined at 540 nm.

Chick Chorioallantoic Membrane assay

Incubation of eggs was done at 37 ^{0}C and humidity 60-70% on day 0. Eggs were pricked on 5'o clock direction at narrow end on day 3/4.

I. Optimization of Drug concentration In CAM assay

a) The shells of fertilized eggs were disinfected and incubated at 37.50C in humidified (65-70%) incubator.
b) Before experiment, the air chamber of eggs was marked and live embryos were selected under an egg candler. Excessively misshapen eggs or eggs with cracked or thin shells were not used.
c) The shell above the air chamber was punched in a biosafety cabinet. The various concentrations of drugs (Itraconazole and Cyclophosphamide) i.e. 10µg, 20 µg, 30 µg and 40µg/ml in appropriate vehicle solution were injected onto the air chamber at developmental stage day 8 under aseptic conditions.
d) After dose administration (using sterile BD syringe), punctured area was sealed with sterilized adhesive tape and was returned to the incubator for further development.
e) 1 × HBSS injected eggs were used as control.
f) The experiment was done in duplicates with n=6 for each group.

II. Making of Pre-cancerous and cancerous (xenograft) model of Cervical Cancer

a) The shells of fertilized eggs were disinfected and incubated at 37.5 ^{0}C in humidified (65-70%) incubator.
b) Before experiment, the air chamber of eggs was marked and live embryos were selected under an egg candler. Excessively misshapen eggs or eggs with cracked or thin shells were not used. On Day 4, the eggs were made ready for injection of cervical cells (CIN II, CIN III and squamous cell carcinoma) derived from biopsies taken during Colposcopy.
c) On day 5, $1x10^6$cells/ml was injected on CAM area, and eggs were re incubated for 72 hrs. Other the cells derived from squamous cell carcinoma was incubated for 7 days.
d) On day 8, the eggs were assessed for pro-angiogenic effect of cervical cells and observations were recorded.

(The experiment was done in three biological replicates and three technical replicates.)

III. Administration of Itraconazole in Pre-cancerous model of Cervical Cancer

a) On day 8, optimized concentration of Itraconazole (40ug/ml) was administered focally by using Whatman filter paper (No.2) on CAM vasculature of pre-cancerous model.
b) The eggs were sealed and re incubated for 48 hrs.
c) On day 10, the eggs were assessed for anti-angiogenic effect.

Macroscopic analysis

The eggs were open and anti-angiogenic effect was assessed on all eggs after 48 hours of injection. CAM was surgically removed from eggs in a bowl. Photographs of the developing CAM and its vasculature of both control and Itraconazole and Cyclophosphamide treated eggs were obtained with stereomicroscope and exported to Image J software for image analysis. The CAM area and the number of blood vessels were assessed.

Histological preparation

The CAM was surgically removed and fixed in 10% buffered formaldehyde for 10 hours, dehydrated in graded alcohol, cleared in xylene and embedded in paraffin. 5µm thick sections

were cut in a plane parallel to the surface of the CAM and stained by haematoxylin-eosin (H & E) which was observed under a light photomicroscope.

Angiogenesis scoring

The inhibitory effects of the drugs on angiogenesis in chorioallantoic membrane were evaluated and assessed according to the scoring system used previously in several studies (65, 66 and 67).

Score anti-angiogenic effect

0	Absent
0.5	Weak
1	Moderate
2	Strong

According to this scoring system, a score of < 0.5 meant that there was no anti-angiogenic effect; a score of 0.5 to 1 indicated a weak or moderate anti-angiogenic effect, and a score of >1 implied a strong anti-angiogenic effect.

Statistical method

Statistical analysis was done using SPSS 20. Data was presented using frequency, percentage, mean and standard deviation. Association between two variables studied using chi-square and fisher exact test whenever necessary. Comparison between two continuous variables was done using unpaired t test. Test considered significant if p value is less than 0.05.

Results

A total 103 patient of unhealthy cervix in visual examination were studied, and out of which 7 were found having cervical intraepithelial neoplasia and 6 were of carcinoma. Colposcopy was done in 97 women; 36 (37.11%) women were present for cervical intraepithelial lesion, however, 61 (62.89%) women were negative for cervical intraepithelial lesion. [Table 1 and Fig 1] Figure 2 shows the effect of Itraconazole on cancer cells in MTT assay. Here, Cyclophosphamide was taken as positive control which is FDA approved anti-cancer drug. The X-axis in the graph represents % viability of cancer cells in the culture while Y-axis represents drug dosage concentration in µg/ml. The viability of the cancer cells in the cell cultures decreased significantly with increase in drug concentrations of Itraconazole. The LC50 value i.e. value at which 50% of the cells are viable of both the drugs in all the cell line were determined and found to be 500µg/ml. On the basis of the results of MTT assay, a range of drug concentration was selected from 10-40µg/ml. The injection of respective drug concentration showed anti-angiogenic effect on CAM vasculature as compared to control. Highest drug concentration showed least nomber of tertiary blood vessels (40µg/ml). It was further validated by Image J software, which showed significant decrease in no. of junctions, nodes and branches. (Fig 3 and 4). Figure 5 shows the effect of CIN stages on angiogenesis. Table 2 shows the effect of CIN stages on tertiary blood vessels. On day 10 of incubation, the eggs injected with Cervical cells derived from CIN II and CIN III stage of cervical biopsy were opened carefully inside a bowl filled with water. The eggs showed pro-angiogenic effect on CAM vasculature. There was increase in number of tertiary blood vessels as well as capillary density as compared to control, which was assessed manually under stereomicroscope. The graph plotted based on no. of tertiary blood vessels clearly showed increase in tertiary blood vessels of CIN stages as compared to eggs injected with vehicle control & cervical cells of healthy cervix. There was no change in CAM vasculature of eggs injected with vehicle control i.e. 1x HBSS. From the tertiary blood vessel counting, it is evident that high grades of angiogenesis were found in this order: Carcinoma, CIN III, CIN II, and normal tissue. This proves the increasing angiogenesis as the grades of cervical malignancy increases. Although the angiogenesis is more prominent in carcinoma, due to the formation of tumor keeps increasing in xengraft mode, it cannot be subjected to quantification (Table 8). Thus, the quantification of carcinoma has been excluded from the graphical representation. The pre-cancerous model of cervical cancer was taken as control to assess the effect of Itraconazole. The optimized dose of Itraconazole i.e. 40ug/ml was loaded on Whatmann filter paper no.2 and was placed on CAM vasculature of CIN model (Xenograft model). On day 12 of incubation, the eggs treated with Itraconazole showed anti-angiogenic effect on CAM vasculature. This anti-angiogenic effect was assessed by using a scoring system. The score was recorded according to reference table and average score was calculated using formula. It was found that Itraconazole has imparted strong anti-angiogenic effect on CIN II samples as average score was >1, however, CIN III samples showed average score =1 and slight more than that i.e. 1.33, considered as moderate anti-angiogenic response. On day 11 of incubation, the eggs injected with Cervical cells (1x106 cells/ml) derived from squamous cell carcinoma of cervix were opened carefully inside a bowl filled with water. The eggs showed tumor growth on CAM vasculature. There was increase in vasculature for the nourishment of tumor. The same model were focally treated with Itraconazole 40 µg/ml aseptically, and incubated further for 48 hrs. On day 13, the eggs were opened carefully to assess the anti-angiogenic effect. Itraconazole had shown anti-angiogenic effect around the vicinity of whatmann filter paper, which was assessed on the basis of scoring system. [Fig 5, 6, 7, 8 and 9; Table 3]

Table 1: Result of colposcopy examination in total number of patient

Colposcopy finding	No. of Patients	Percentage
cervical intraepithelial lesion present	36	37.11%
cervical intraepithelial lesion absent	61	62.89%
Total	97	100%

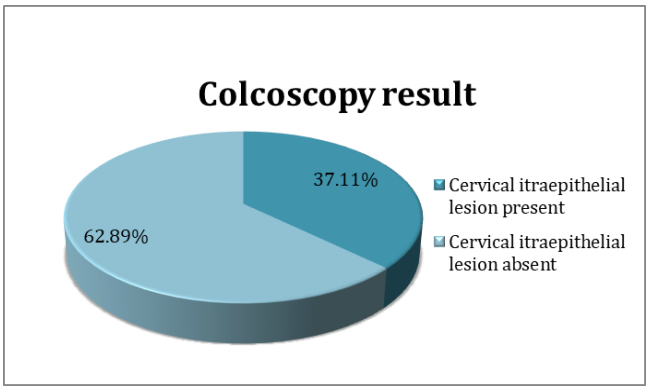
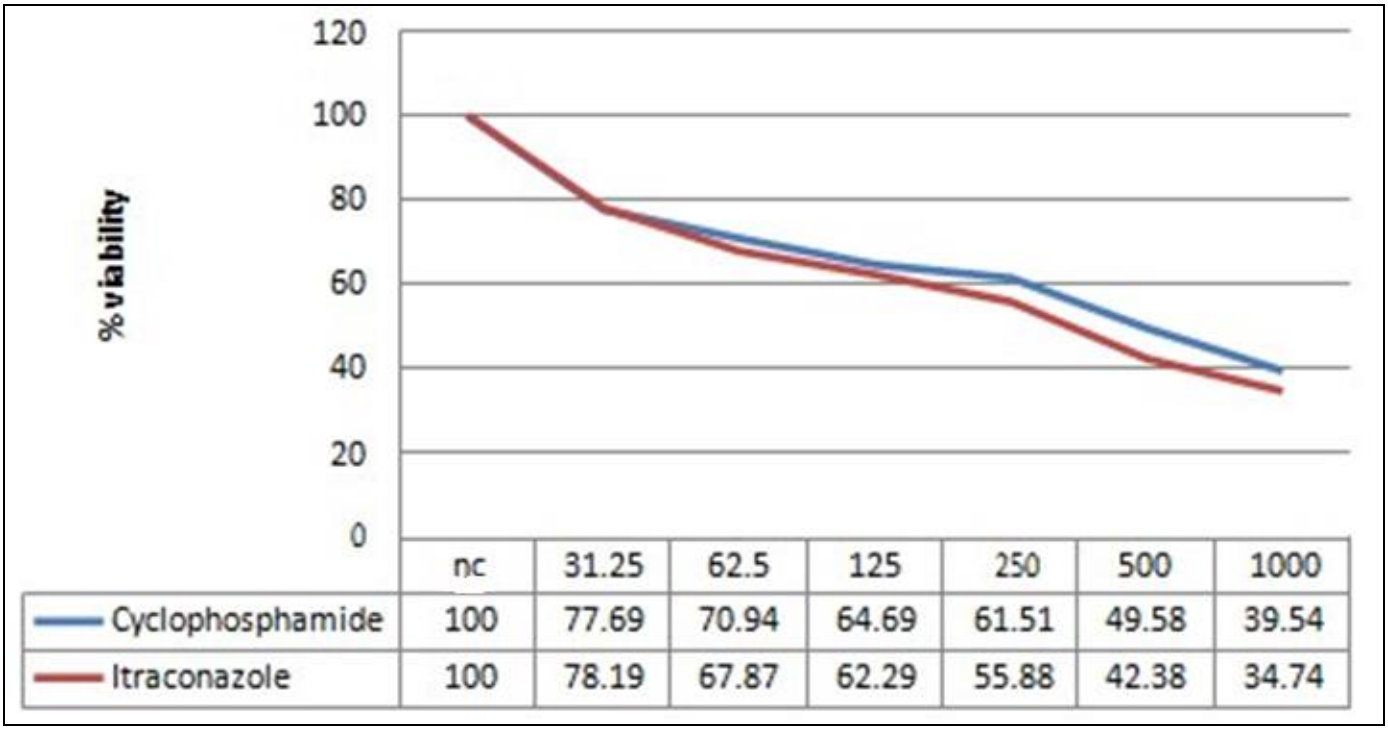


Fig 2: Effect of Itraconazole and Cyclophosphamide on cervical cancer cells



	nc	31.25	62.5	125	250	500	1000
Cyclophosphamide	100	77.69	70.94	64.69	61.51	49.58	39.54
Itraconazole	100	78.19	67.87	62.29	55.88	42.38	34.74

Fig 2: Effect of Itraconazole and Cyclophosphamide on cervical cancer cells

Concentration of cyclophosphamide and itraconazole

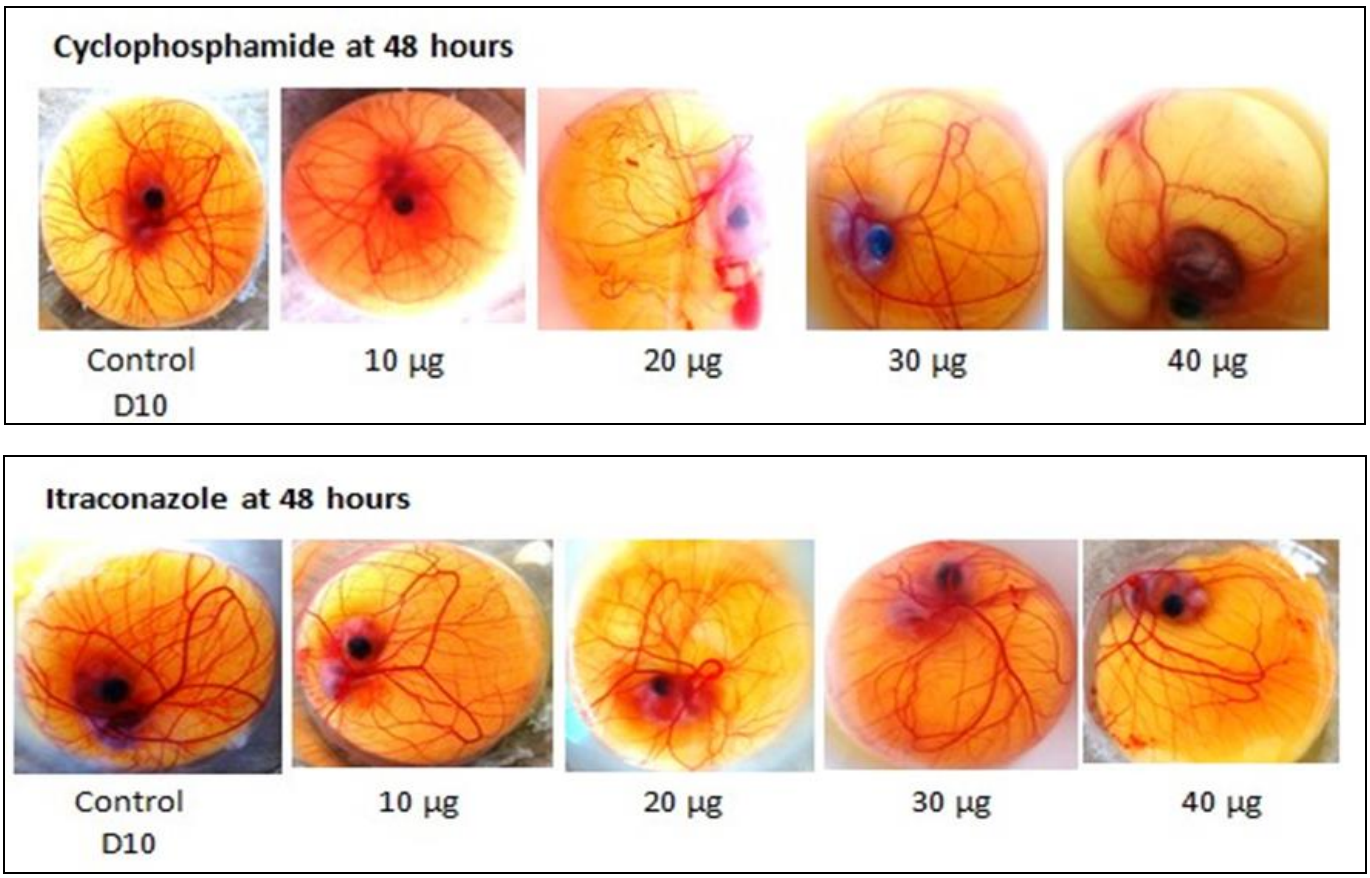


Fig 3: Chick chorioallantoic membrane assay and Optimization of Drug concentration in CAM assay

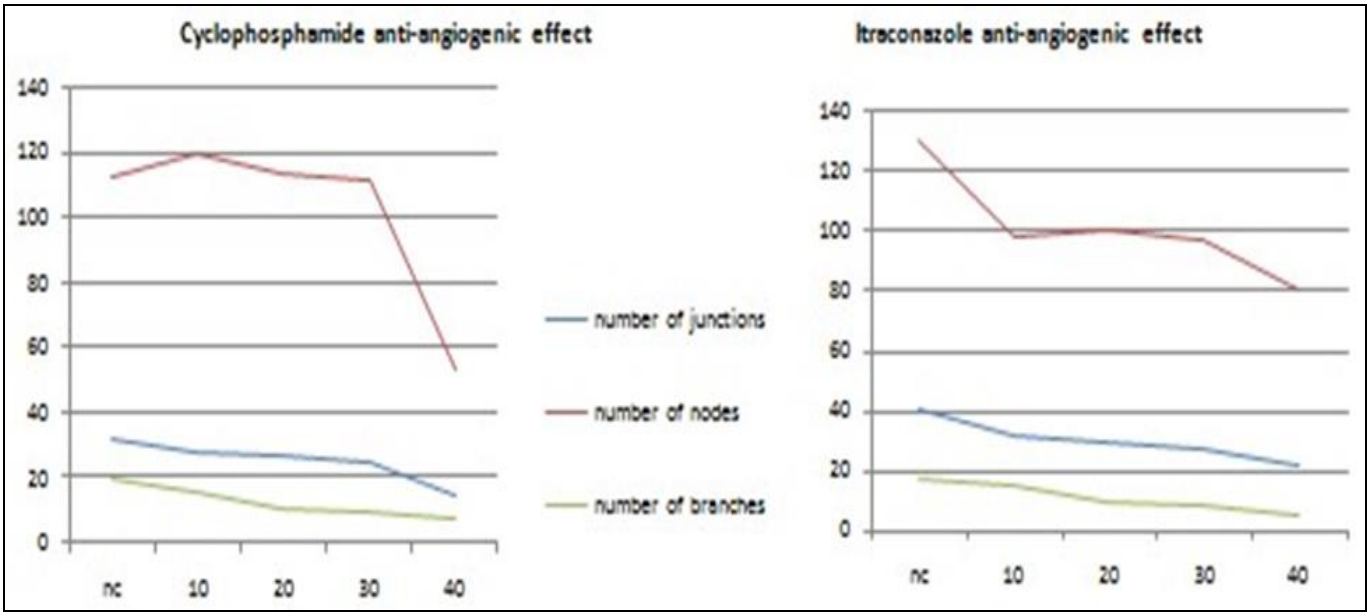
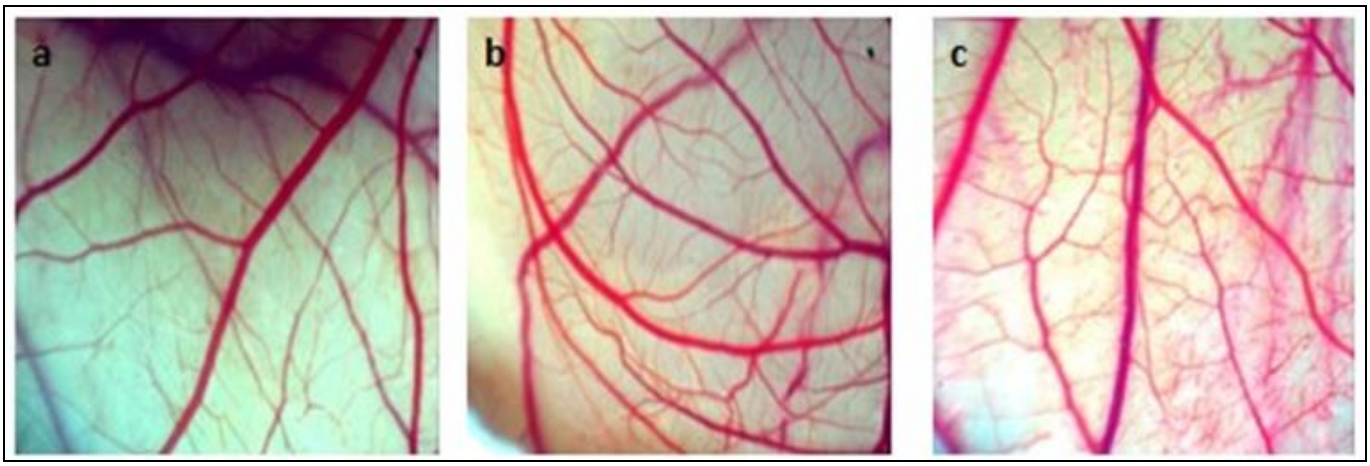


Fig 4: Image J analysis



a) Control (D10); b) Egg injected with CIN II cells; c) Egg injected with CIN III cells

Fig 5: Effect of CIN stages on Angiogenesis

Table 2: Effect of CIN stages on tertiary blood vessels

	Control (HBSS)	Normal Cervix tissue	CIN II	CIN III
Gr.1	170	184	262	315
	162	176	246	300
	165	181	243	311
Average	165.67	180.33	250.33	308.67
SD	4.04	4.04	10.21	7.77
Gr.2	178	190	256	290
	159	174	263	305
	164	176	251	320
Average	167	180	256.67	305
SD	9.85	8.72	6.03	15.00
Gr.3	172	192	243	322
	161	176	252	328
	175	190	260	308
Average	169.33	186.00	251.67	319.33
SD	7.37	8.72	8.50	10.26

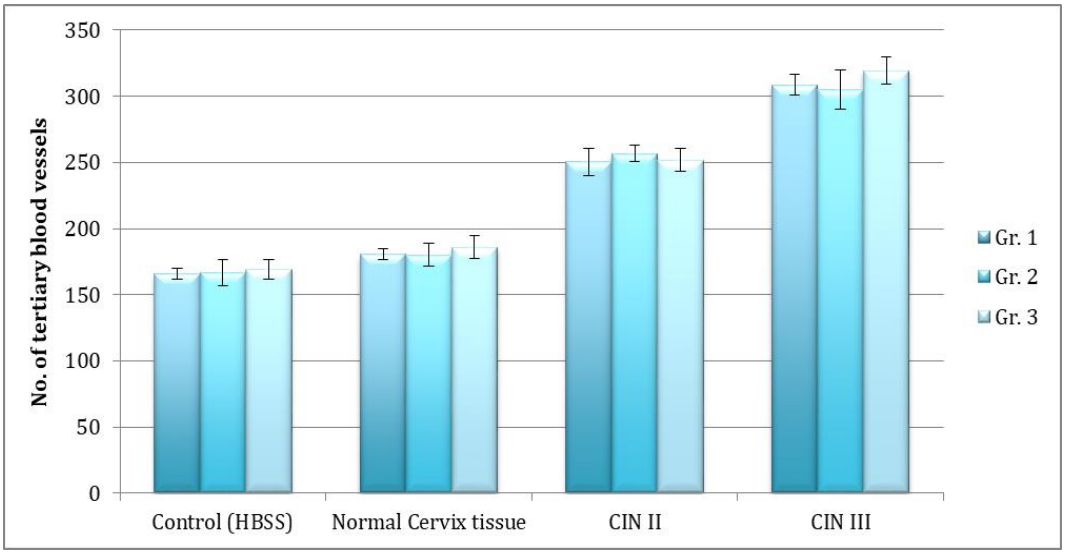
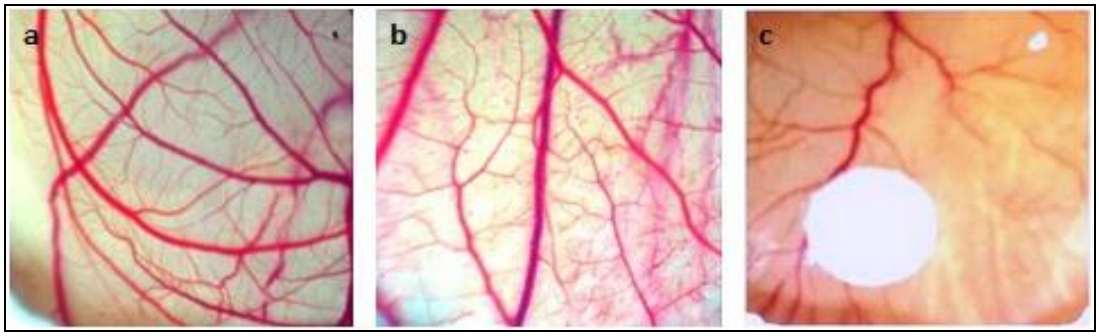


Fig 6: Graphical representation of effect of CIN stages on tertiary blood vessels

Administration of Itraconazole in Pre-cancerous model of Cervical Cancer



a) Egg injected with CIN II cells; b) Egg injected with CIN III cells; c) Egg treated with Itraconazole (40ug/ml)

Fig 7: Effect of Itraconazole on CIN

Table 3: Effect of Itraconazole in CIN and carcinoma

Average angiogenic score	Itraconazole drug study Result positive
Absent	**0**
Weak	**0**
Moderate	**5**
Strong	**4**
Total	**9**

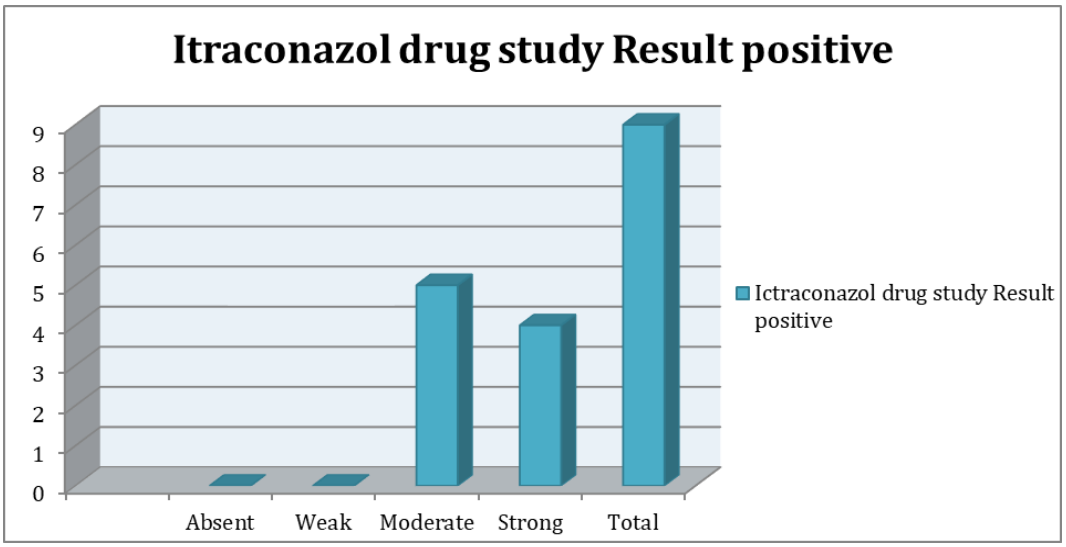
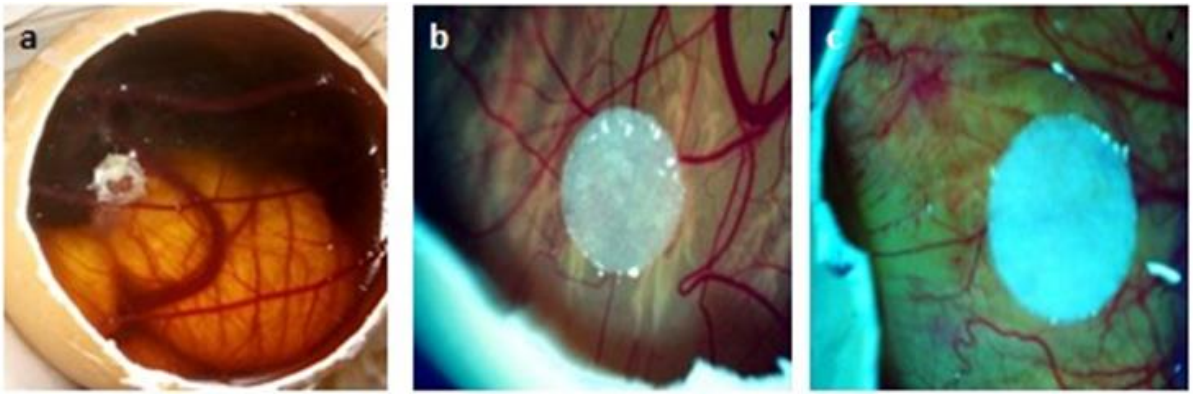


Fig 8: Graphical representation of effect of Itraconazole in CIN and carcinoma



a) Xenograft model of squamous cell carcinoma of cervix (Day11); b) Administration of Itraconazole (Day11); c) Effect of Itraconazole (Day13)

Fig 9: Effect of Itraconazole on squamous cell carcinoma of cervix

Discussion

Angiogenic Score and Staging

In the present study mean angiogenesis Score of all patients was 167.33±6.67. Trend shows that angiogenesis score increases in order of cervical intraepithelial neoplasia CIN ¬III to CIN I, Normal Cervix tissue, Control. In a study by Landt S. *et al*. on "The Utility of an *In Vitro* Angiogenesis Score for Prognosis Assessment in Patients with Cervical Cancer", they found that median score of the entire sample was 53.3% and median score of the entire sample was significantly dependent on tumor stages, as the tumor stage increased angiogenesis score increased in order of CIN III to CIN I [8].

Itraconazole and Angiogenesis

After giving Itraconazole drug, Average angiogenic score was reduced. Out of 9 cases of dysplasia and cervical carcinoma, density of the capillaries around the disc was reduced moderately in 5 patients, and strongly reduced in another 4 patients. Similarly in a study by Ueda T *et al*. on "Itraconazole Modulates Hedgehog, WNT/β-catenin, as well as Akt Signaling, and Inhibits Proliferation of Cervical Cancer Cells" microarray analysis showed an 8-fold down-regulation in the expression of GLI1, WNT4 and WNT10A among Itraconazole-treated cells in patients of cervical cancer. Itraconazole is a multi-targeting anticancer agent and a promising therapeutic agent for cervical cancer [9]. In a study by Blake T. Aftab *et al*. on "Itraconazole inhibits angiogenesis and tumor growth in non-small cell lung cancer" mentioned that Itraconazole has potent and selective inhibitory activity against multiple key aspects of tumor-associated angiogenesis [10]. Angiogenesis is the major phenomena in development of all cancers. In tumor angiogenesis, the basement membrane in tissues is injured locally, where there is immediate destruction and hypoxia. The endothelial cells activated by angiogenic factors, then migrate, proliferate and stabilize. Thus, several anti-angiogenic drugs are being explored as effective anti-cancer drugs. For example, Balke *et al*. investigated eight osteosarcoma cell lines for their ability to form vascularized tumors on the CAM with or without imatinib [11]. They concluded that treatment with imatinib inhibited tumor angiogenesis and growth in their model. Rocha *et al*. demonstrated the anti-angiogenic effects of imatinib related to smooth muscle cells but not endothelial cells, and it was probably preventing vessel stabilization [12]. The anti-cancer drug, Itraconazole, is seemingly effective against few types of cancer, as reported above. It is shown to inhibit both the hedgehog signalling pathway and angiogenesis. Functionally, the anti-angiogenic activity of Itraconazole has been shown to be linked to inhibition of glycosylation, VEGFR2 phosphorylation and trafficking and cholesterol biosynthesis pathways. However, Itraconazole, so far is not explored as an anti-angiogenic drug in chick embryo, despite its activity being well established in other models. This forms the basis of the study.

For the first time, anti-angiogenic effect of Itraconazole on CAM vasculature was proven effectively with this pilot study. After the application of various concentrations of 10, 20, 30 and 40µg/ml, it caused clear changes in CAM vasculature as compared to control group. There was not much difference in anti-angiogenic effect of Itraconazole when compared with Cyclophosphamide on angiogenic scoring system. Both the drugs showed strong anti-angiogenic effect with average score >1. Cyclophosphamide of same concentrations, cyclophosphamide has higher anti-angiogenic effect than that of Itraconazole but the magnitude of difference in anti-angiogenic scores is not very high and Itraconazole has proven to have good anti-angiogenic response from the above study. MTT assay further proves the point of effectivity of Itraconazole on Cervical cancer cells. MTT assay of Itraconazole signifies that it has anti-cancer effect comparable with Cyclophosphamide. In comparison between average scoring results of CIN II and CIN III, Itraconazole imparted strong and weak anti-angiogenic effect on CIN II and CIN III respectively with dose of 40µg/ml; however increase in Itraconazole dose concentration more than 40µg/ml might impart strong anti-angiogenic effect on CIN III. Cyclophosphamide has known to show side effects like Blood in the urine, dizziness, confusion, or agitation, fast heartbeat, joint pain, shortness of breath, swelling of the feet or lower legs, unusual tiredness or weakness. Also, Cyclophosphamide is a teratogen [13]. Also, in our study Cyclophosphamide had teratogenic effect on embryo while Itraconazole has none. Thus Itraconazole is a safer drug that can be used for anti-cancer therapies.

Conclusion

From the study, this was concluded that although the result proves itraconazole to be a prominent anti-angiogenic and anti-cancer drug, there are limitations in the study due to less number of samples. Further in-depth research is more randomised samples are needed to validate these findings.

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