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Research Article
Evaluating the Effects of Hypotensive Drug Valsartan on Angiogenesis and Associated Breast Ductal Carcinoma Cell Metastasis

[1]Ashok Munjal, [1]Rekha Khandia, [1]Saumi Paladhi, [2]Megha Pandey, [1]Arpana Parihar, [1]Chandrashekhar Pathe, [3]K. Rajukumar, [4]Talha Bin Emran, [5]Taha Alqahtani, [5]Ali M. Alqahtani, [6]Ali H. Alamri, [5]Kumarappan Chidambaram and [7]Kuldeep Dhama

[1]Department of Biochemistry and Genetics, Barkatullah University, Bhopal 462026 MP, India
[2]Translational Medicine Center, All India Institute of Medical Sciences, Bhopal 462020 MP, India
[3]ICAR-National Institute of High-Security Animal Diseases, Bhopal 462043, India
[4]Department of Pharmacy, BGC Trust University Bangladesh, Chittagong 4381, Bangladesh
[5]Department of Pharmacology, College of Pharmacy, King Khalid University, Abha 62529, Saudi Arabia
[6]Department of Pharmaceutics, College of Pharmacy, King Khalid University, Abha 62529, Saudi Arabia
[7]Division of Pathology, Indian Veterinary Research Institute, Izatnagar, Bareilly 243 122, UP, India

Abstract

Background and Objective: Valsartan is an anti-hypertensive drug routinely used to treat patients with hypertension. It is a Type 1 Angiotensin receptor (AT1) receptor blocker and AT1 receptor blocker possess anti-neoplastic activity, hence the anti-cancer activity of this drug can be speculated. However also reports are showing pro-angiogenic and hence the pro-tumorigenic activity of valsartan, therefore the drug was envisaged for potential pro-tumorigenic activities. **Materials and Methods:** The effects of valsartan as a pro-angiogenic/anti-angiogenic molecule was tested in chicken embryos using an MCF-7 ductal carcinoma cell line. Post-treatment number of branch points as an indicator of angiogenesis were counted and histopathology was done. Statistical analysis was done using Tukey's HSD procedure to determine the difference in angiogenesis between the groups. **Results:** The result suggested that valsartan is a proangiogenic drug that enhances new blood vessel arborization and intussusceptive angiogenesis. The breach in vascular wall integrity was also observed post valsartan treatment that may serve for cancer cell metastasis. **Conclusion:** Valsartan shows proangiogenic activity and alter the normal structure of blood vessels that can attribute to cancer cell metastasis at least in the chicken chorioallantoic membrane model.

Key words: Anti-hypertensive drug, valsartan, angiogenesis, leaky vessels, tumour metastasis, chorioallantoic membrane, AT1 receptor

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Corresponding Author: Rekha Khandia, Department of Biochemistry and Genetics, Barkatullah University, Bhopal 462026 MP, India Tel: +61-414-701-759

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

The unhealthy lifestyle has increased the problems associated with hypertension and the number of affected persons is estimated to increase to 1.5 billion by 2025[1]. Hypertension is the most widespread condition worldwide, causing 9.4 million deaths yearly and affecting more than 1 billion individuals. The detrimental consequences of hypertension including stroke, coronary artery disease and heart failure can be prevented by Antihypertensive Therapies (AHTs)[2,3]. Abraham *et al.*[4] reported valsartan as a non-peptide Angiotensin II Receptor Blocker (ARB) hypotensive drug and its mechanism of action is similar to losartan, lisinopril, enalapril, amlodipine and hydrochlorothiazide[5]. It is known to reduce vasoconstriction and inhibits aldosterone synthesis, leading to reduced blood pressure. In comparison to losartan, valsartan shows a higher affinity towards the AT1 receptor but the affinity is poorer than the affinity with candesartan, telmisartan and olmesartan. A single dose is prescribed per day for lowering blood pressure as valsartan has a half-life of ~7 hrs. It is partially metabolized in the liver whilst a major part is eliminated unchanged via biliary secretions. Approximately, 7-13% of the drug is excreted through the renal system and the remaining is excreted in feces[6]. It has been approved by the Food and Drug Administration (FDA) in 2007 for use in patients of age 6-16 years with hypertension[7]. There is considerable interest to find out the role of antihypertensive drugs in the initiation of various types of cancer and therefore many systematic reviews and meta-analyses have been conducted by researchers to elucidate this aspect[8-11]. The ARBs are preferred for patients exhibiting adverse reactions to Angiotensin Converting Enzyme (ACE) inhibitors[12]. However, the risks of hypotension, renal dysfunction and hyperkalemia are observed higher in ARB receiving patients than placebo[12]. Also the research data by Sipahi *et al.*[13] showed the association of cancer with the use of Angiotensin Receptor Blockers (ARBs). Whereases Zhao *et al.*[9] found contradictory results. A recent review encompassing the longitudinal studies showed an association between anti-hypertensive drugs and cancer risks, however, more rigorous and evidence-based clinical research is warranted for better clinical decision making[14]. The study carried out by Thorn *et al.*[15] revealed that sacubitril/valsartan therapy enhanced angiogenesis with an expansion in myocardial perfusion. Further, the increase in capillary density was observed with an attenuation of Cardiomyocyte (CM) growth.

There have been contradictory reports regarding the angiogenetic potential of valsartan. Hatzopoulos *et al.*[16] reported a reduction in angiogenesis in a rat model of retinopathy whereas Zhang *et al.*[17] reported an enhancement in the angiogenesis in the experimental domestic pig model. Since angiogenesis has been associated with tumour growth and development, valsartan may likely have an anti-tumorigenic effect. Several studies have been carried out to get a deeper insight into the role of valsartan in the progression of neoplastic diseases. However, there is a discrepancy in reports available regarding the role of valsartan for the development/prevention of cancer. The relationship between the use of valsartan and relative increase in the risk of cancer or cancer-related death has been denied after analyses of 324168 participants from randomized trials[18]. Contrarily a non-significant 22% increase in cancer-related mortality has been observed in the valsartan group in comparison to the captopril group by Dézsi[19]. Besides this, valsartan induced incidence of melanoma in a patient has been recently reported[20]. Therefore, there is an urge to investigate the effects of valsartan on angiogenesis and risk assessment for cancer progression belonging to various organs and types like sarcoma, carcinoma etc. following treatment with this anti-hypertensive drug valsartan.

The Chorioallantoic Membrane (CAM) is a highly vascularized 2 dimensional tissue, which is a popular model to study invasive tumours due to its immature immune system[21]. The main advantage of this model includes clear visibility, more accessibility and fast developmental growth of the CAM along with options of manipulation of vascular functions[22,23]. Various studies have been conducted to test several pro-and anti-angiogenic agents using the CAM model. In this context, compounds such as growth factors[24,25], hormones[26-29], pro-angiogenic molecules, antibiotics[30], natural molecules[31], organometallic compounds[32,33], gases[34], antibodies and synthetic small molecules[33,35,36] have been tested. Due to an immature immune system, it could be used as an analogue to the athymic nude mice model. The experiments are easy to handle and inexpensive with high reproducibility of results. As per the report prepared by the staff of the Division of Animal Welfare, Office for Protection from Research Risks, National Institute of Health, Bethesda, Maryland, in the chicken embryo of gestation age less than 14 days, the nervous system is not well developed and it does not feel pain. Therefore, prior approval from the animal ethical committee is not required, which further simplifies the experimentation.

In the present study, we investigated the angiogenic potential of valsartan, as well as assessed of risk of MCF-7 ductal carcinoma growth and metastasis in the chicken embryo chorioallantoic membrane model.

MATERIALS AND METHODS

Study area: The study was conducted at the Department of Biochemistry and Genetics, Barkatullah University, Bhopal, India between January-July, 2019.

Cell cultures and treatments: Embryonated Cobb430Y chicken eggs at the age of 0 days were procured from Rajdhani Agro products, Shop No. 3, Villa residency, Lal Ghati, Indore Road, MP, India. The eggs were cleaned with 70% ethanol and kept in a humid incubator at 36±1°C. At the age of 9 days, the eggs were candled for viability and dead egg were separated and only viable and healthy eggs with active and motile embryos were taken for study.

Four study groups each comprised of 8 eggs (group I: PBS control, group II: MCF-7 cells inoculation group, group III: Valsartan treatment group, group IV: MCF-7 cells and valsartan treatment group) were separated. The wider end of the egg was drilled and a hole of 2×2 mm was made. Through this hole, the study material was inserted and overlaid onto the CAM. The study material contained antibiotic (ampicillin 50 μg mL^{-1}, streptomycin 10 μg mL^{-1}) and antimycotic solution (amphotericin 10 μg mL^{-1}). The control group was considered as 1st group and treated with 100 μL PBS, the 2nd group, eggs were inoculated with 50,000 MCF-7 cells (50 μL), the 3rd group, was treated with valsartan (50 μL) equivalent to human daily dose (the human dose was taken as 80 mg per day considering the human weight as 60 kg, so per egg 80 μg drug was introduced) and 4th group eggs were treated with 50,000 MCF-7 cells (50 μL) and valsartan (50 μL) equivalent to the human daily dose. The eggs were sealed with cellophane tape and returned to the incubator for additional 72 hrs. Seventy-two hrs post-incubation the shells were opened by removing the cellophane tape and 5 mL of 10% formalin was flooded over the CAM to fix cells. After that randomly the CAM was excised and on average 40 fields per treatment, the group was photographed in 40X magnification using a Nikon microscope.

Branch point counting: The number of branch points is the measure of angiogenesis. The more the number of branch points, the more will be the angiogenesis. For each image photographed at 40X magnification, the absolute number of branch points, which is an indicator of new blood vessel arborization, was calculated using Digimizer image analysis software version 4.6.1 (MedCalc Software BVBA). Here the length of the branch points was not considered. For each observation, a minimum of 10 frames was processed and the mean of each group was calculated and compared statistically with other treatment groups.

Histopathological observation: The excised CAM was fixed into 10% formaldehyde solution and dehydrated gradually using an increasing percentage of alcohol. The CAM tissue was later embedded in paraffin and sections of 5 μM thickness was prepared. The sections were stained with standard Hematoxylin and Eosin stain (H and E stain) for light microscopy analysis. Images were captured using a digital camera attached to the Nikon microscope. Histopathology images were captured at 400X magnification.

Statistical analysis: The Tukey's HSD procedure within ANOVA was done to facilitate pairwise comparisons of data. The test was performed using IBM SPSS statistics version 18.0 software. Tukey's HSD test allowed us to determine the statistically significant difference, if there is between groups.

RESULTS

Branch point counting: After completion of 72 hrs, the eggs shell was opened to harvest CAM tissue. The collected CAM was subjected to microscopic analysis at 40X. The images showing branch points in CAM tissue is presented in Fig. 1a (control group), Fig. 1b (MCF-7 cells inoculated group), Fig. 1c (valsartan treated group) and Fig. 1d (Inoculated with MCF-7 cells and valsartan treated group). The analysis revealed an increase in the number of branch points in all treated groups when compared to the control group.

Tukey's HSD procedure in the present study demonstrated a significant difference in the number of branch points between control and valsartan treated CAMs. Cancer cells secrete several growth factors that promote angiogenesis i.e., the number of branch points. There was a significant difference in the number of branch points in MCF-7 treated ($p<0.00001$), valsartan treated ($p<0.00001$) and MCF-7 and valsartan treated group ($p<0.0001$) in comparison to control (Table 1). In all the treatment groups' number of branch, points were increased. However, there is no significant difference in branch points observed between the valsartan treatment and MCF-7 inoculation group whereas valsartan+MCF-7 treatment group showed a promising increase in branch points. These results indicated that

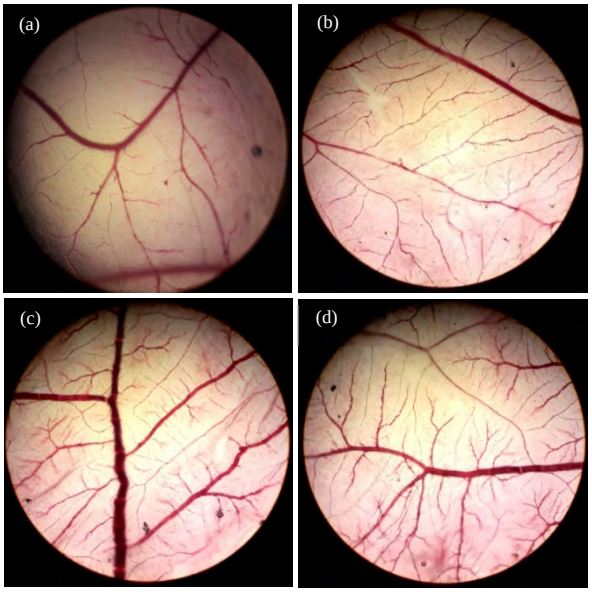

Fig. 1(a-d): Representative images of CAM from each group showing arborization of vessels, (a) Control group I, (b) MCF-7 cells group II, (c) Valsartan group III and (d) Valsartan+MCF-7 group IV
CAM: Chorioallantoic membrane

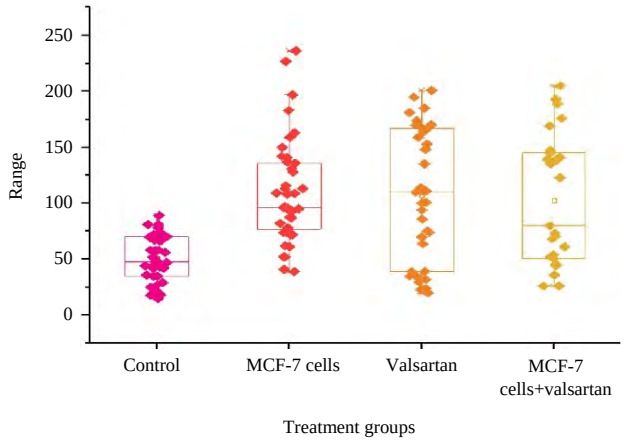

Fig. 2: Jitter and box plot for branch point analysis in CAM of an embryonated egg
X-axis represents various treatment groups and Y-axis represents the range for the number of branch points

valsartan treatment increases the number of branch points, which is a parameter of neovascularization. The arborization was exacerbated in the group treated with both valsartan and MCF-7 cells. The data in Fig. 2 represents the results of branch point analysis between the groups. Each branch point is the measurement of the degree of arborization and the more the number of branch points, the more the arborization is considered. The notches explain approximately 95% confidence interval for the median. The minimum and the largest value is indicated as a whisker. In MCF-7 treated CAM one out of whisker is an outlier point, having a value more than 1.5 than the box height.

Histological analysis: Histological analysis revealed a well-organized vascular system, with scanty mesodermal blood vessels and numerous epidermal blood vessels with the normal architecture of CAM in the control group. The blood vessels were with intact endothelium and connective tissue layer. Blood vessels were filled with nucleated RBCs. Multilayered allantoic epithelial and uni-layered chorionic epithelium was seen (Fig. 3a-b). In the valsartan treatment group also, intussusceptive blood vessels were observed. (Fig. 3c-d). These intussusceptive blood vessels were either of arterial or venous origin. Intussusceptive angiogenesis is an additional and alternative mechanism to endothelial sprouting, which generates blood vessels in a faster mode with less metabolic demand in comparison to sprouting angiogenesis, a putative strategy used by tumours. Parallel presence of 2 or more blood vessels with almost equal diameter was taken as intussusceptive blood vessels. Sparse coverage of mesenchymal and connective tissue layer around vessel was common that making the vessel leaky and tortuous. The intussusceptive blood vessels were mostly leaky with an improper formation of the mesenchymal and connective tissue layer. Apart from these medium-sized blood vessels in the mesodermal area, significant numbers of blood vessels in the peripheral epidermal area were also visible. In MCF-7 inoculated CAM, hyperchromasia with an aggregated cellular composition which is indicative of the establishment of cancer cells was observed (Fig. 3e). Also, aggressive

Table 1: Results of *post hoc* Tukey HSD (beta) test for pairwise comparison between groups

Pairwise comparison	HSD 0.05 = 29.4201, 0.01 = 35.8683	Q-score 0.05 = 3.6769, Q 0.01 = 4.4828	Level of significance (p-value)
Control: MCF-7 cells	59.52	Q = 7.44	p<0 .00001***
Control: Valsartan	55.99	Q = 7.00	p<0.00001***
Control: Valsartan+MCF-7 cells	51.86	Q = 6.48	P<0 .0001***
MCF-7 cells: Valsartan	3.53	Q = 0.44	p = 0.98945^{NS}
MCF-7 cells: Valsartan+MCF-7 cells	7.66	Q = 0.96	p = 0 .90581^{NS}
Valsartan: Valsartan+MCF-7 cells	4.13	Q = 0.52	p = 0.98335^{NS}

NS: Non significant and ***p<0.001

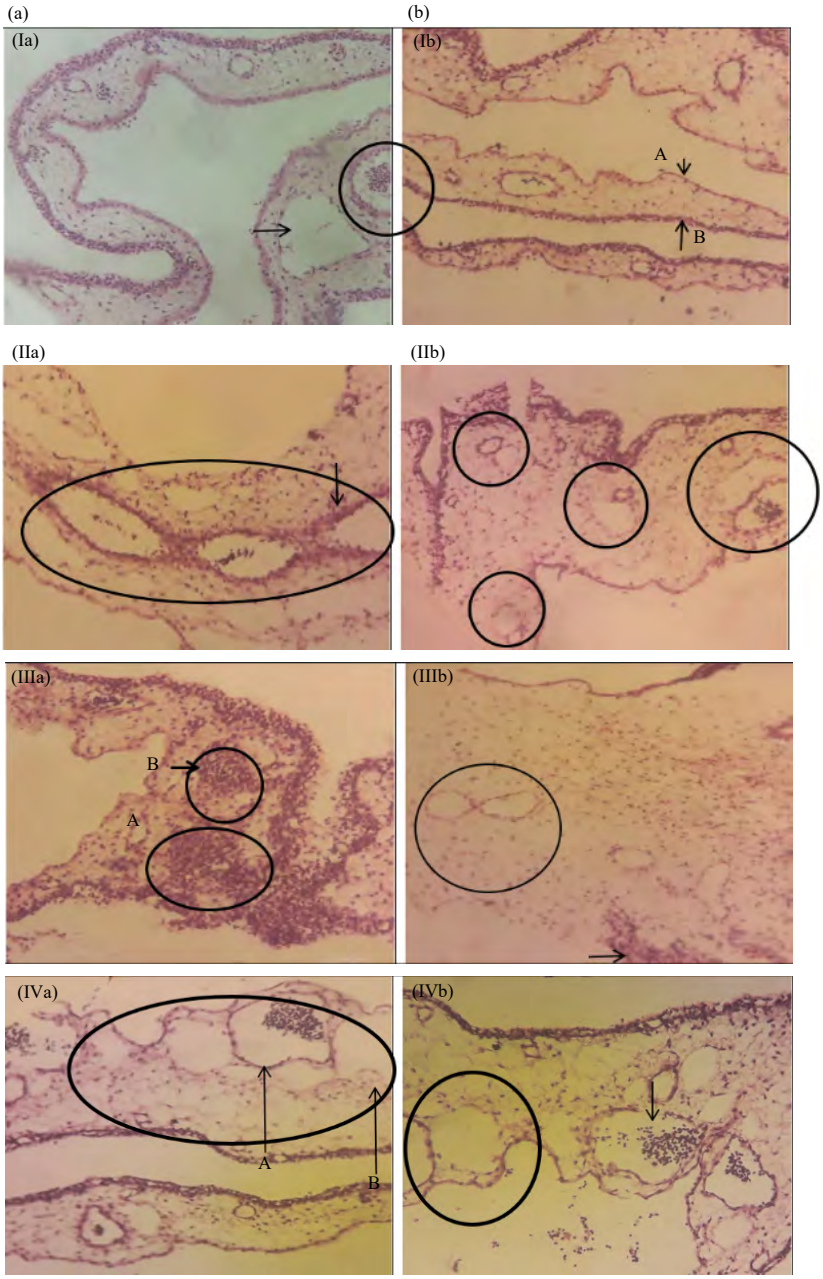

Fig. 3(a-b): Representative images of histological analysis, (Ia) Control CAM the circle A is indicating the arteriole present in the CAM. The arrow is showing venule along with well architectured endothelial cells along with mesenchymal and connective tissue layer, (Ib) Arrow A is showing the endoderm whilst arrow B is showing the ectoderm of CAM, (IIa) Valsartan treated CAM, the proliferation of endothelial cells was observed (arrow). Apart from that numerous proliferating blood vessels with sparsely arranged mesenchymal and connective tissue layers can be seen (elliptical), (IIb) Circles are showing intussusceptive angiogenesis, (IIIa) MCF-7 cells treated CAM exhibited proliferative thickening of allantoic epithelium with a focal accumulation of hyperchromatic cells in the mesodermal area. Also, the arrow is indicating developing blood vessel, (IIIb) Inside the circle, intussusceptive angiogenesis and arrows are indicating hyperchromatic regions in the CAM indicating the establishment of cancer cells, (IVa) Elliptical showed intussusceptive angiogenesis. Arrow A indicated blood vessel lined with endothelium cells and mesenchymal and connective tissue whilst arrow B is indicating blood vessel lined with endothelium cells only and (IVb) Circle is showing a blood vessel with not well-defined margin, a leaky blood vessel

Arrow is showing breached blood vessels from where the cancer cells may metastasize CAM: Chorioallantoic membrane (Magnification x400)

angiogenesis was observed which was evident with the presence of intussusceptive angiogenesis with numerous mesodermal blood vessels (Fig. 3f). Similarly, a large number of epidermal blood vessels along with intussusceptive leaky blood vessels was observed in MCF-7 inoculated CAM (Fig. 3g-h).

DISCUSSION

Valsartan has been currently indicated for treating hypertension in more than 80 countries and is effective against black and white, men and women. Apart from the treatment of blood pressure-related problems, it is also prescribed in case of heart failure and myocardial infarction. Huang *et al.*[5], reported common side effects like headache, dizziness and fatigue in valsartan treatment groups, which were very similar to placebo-controlled studies. The AT1 receptors have been shown to express on various neoplastic tissues including squamous cell carcinoma, mammary adenocarcinomas, squamous cell carcinoma, lung adenocarcinoma and pancreatic cancer cells. Treatment with valsartan reduced AT1 receptor and p38 MAPK protein expression in aortic tissue of rats, those underwent aortic endothelial denudation using self-made balloon catheters. In vitro studies have suggested a higher selectivity of valsartan for AT1 receptor and the affinity is 30,000 times higher than with AT2 receptor. It has no substantial affinity for other unrelated receptors[37,38]. There are now growing incidences that are indicative of anti-angiogenic, anti-invasive and anti-neoplastic activities of Losartan and other similar AT1 blockers[39]. There is no noteworthy association between AHT use and breast cancer risk but the study of Ni *et al.*[40] have shown long-term ACEi/ARB beneficial effect. To prove this fact large, randomized controlled trials along with long-term follow-up are warranted to test the association of medications on breast cancer risk[40]. Lin *et al.*[41] have shown ARBs improve 5 years survival among patients with nasopharyngeal carcinoma and also exert antiproliferative and antiangiogenesis effects in nasopharyngeal carcinoma via PI3K/AKT signalling. Valsartan has been shown to inhibit physiological as well as pathological angiogenesis in the rat model of Retinopathy of Prematurity (ROP) by reducing neovascular tuft formation[16]. The process of neovascularization in experimental retinopathy of prematurity, which is also named Oxygen-Induced Retinopathy (OIR), valsartan reduce preretinal neovascularization and neovascular tufts by ~76 and 71%, respectively in comparison to control[42]. Acellular capillaries (devoid of pericytes and endothelial cells), were seen to be reduced by the use of valsartan with the dose 4-40 mg in 8 weeks-old hypertensive Ren-2 rats[26]. Contradictory reports have been observed regarding the effect of valsartan on angiogenesis. In the report of Li *et al.*[41], protection against ischemic brain damage in the C57BL/6 mice model has been reported through an increase in vessel density. Valsartan significantly improved the endothelial progenitor cells mobilization in circulation and induce neovascularization in experimental uremia induced in 8 weeks old-male C57BL/6J mice by subtotal nephrectomy[43]. Bangalore and Bhatt[18] and Dézsi[19] are other workers who also reported contradictory results. Considering the contradictory reports, here in the present study, we tried to evaluate whether the risk of mammary ductal carcinoma progression in presence of valsartan, really increases.

In the present study, to evaluate the risk of mammary ductal carcinoma progression in presence of valsartan, CAM tissue of chicken embryo was used. CAM is an extraembryonic membrane, providing a robust experimental platform and is known to support various types of tumours including myeloma, carcinoma, carcinosarcoma, glioblastoma, neuroblastoma, adenocarcinoma etc. by providing an extensive vascular network[22].

The number of branch points, which is used as an indicator of growing capillary plexus is determined in different groups and valsartan treatment was found to increase the angiogenesis in comparison to control that supports the finding of Li *et al.*[44], who demonstrated an increase in vessel density following the treatment with valsartan. At the histopathological level, intussusceptive angiogenesis was observed, into which pairs of blood vessels were observed. In the pair 1 vessel was with pericyte covering while the other 1 was without a pericyte sheath. Such vessel architecture is possibly arising due to the formation of new blood vessels from the older 1 through the intussusception, since this method of neovascularization is energetically less expensive and meets the demand of rapidly growing tumour cells. In MCF-7 treated CAMs, numerous epidermal blood vessels along with mesodermal blood vessels with/without a well-defined mesenchymal and connective tissue layer was observed. The blood vessels that are associated with the tumour, exhibit abnormal structural and functional aspects. These vessels are generally leaky and tortuous. The tumour vasculature is often associated with vessels lacking pericyte and sparse pericyte coverage has been found in both humans as wells as experimental solid tumours that lead to increased vessel permeability and poor vessel integrity. The studies have been demonstrated that vessels with ablated pericytes, instead of hampering tumour growth, favour blood vessel invasion and

metastasis of tumour cells[45]. With limited data, it is evident that in human tumours vessels lacking pericytes may induce early metastasis[46]. In our study, it is clear that individually both the valsartan treatment and MCF-7 inoculation resulted in intussusceptive angiogenesis however the phenomenon of pericyte ablation could not be demonstrated to the age of the embryo used in our experimental study. Pericytes are known to develop at about 14-15 days of the age of the embryo. Since the effects of valsartan is similar to the effects of MCF-7 cells on CAM concerning angiogenesis, similar results in the group where MCF-7 and valsartan both have been added are expected. As per expectation in the 4th group, intussusceptive angiogenesis was observed and the vessels were partially covered with a well-defined mesenchymal and connective tissue layer. Our results support the observation of Sui *et al*.[47] who demonstrated that valsartan treatment can improve myocardial infarction in rat model owing to the improvement of the microenvironment for migration of endothelial cells and thereby promoting angiogenesis.

In summary, it can be inferred that valsartan treatment may increase the risk of growth as well as the spread of mammary ductal carcinoma as depicted by the CAM model of chicken embryo. A report by Tchernev and Temelkova[20] is in support of our observation which states that valsartan induced incidence of melanoma in a patient has been reported and the drug is not from the manufacturers that withdrew their valsartan products from the market due to contamination of N-nitrosodimethylamine (NDMA) or N-nitrosodiethylamine (NDEA) which are potent carcinogens, so the drug may be the cause of melanoma. However, more data needs to be accumulated to ascertain the tumour promoting effects of valsartan.

CONCLUSION

Valsartan is a drug that is taken regularly for the treatment of hypertensive disorders. Our study shows valsartan have the proangiogenic role as demonstrated by enhancement of branch points, intussusceptive angiogenesis and presence of vessels with ablated pericyte and endothelial cell hyperplasia in the CAM model, therefore it may enhance the risk of tumour growth and spread through leaky, pericyte ablated vessels. At the same time, it could be having ameliorative effects while myocardial infarction. However, further studies are warranted to assess the effects of valsartan and till the time it is declared completely safe for cancer patients, it should be prescribed cautiously.

SIGNIFICANCE STATEMENT

Various contradictory reports have been published related to the effect of valsartan on angiogenesis, those report both the anti-angiogenic role as inhibition of both the physiological and pathological angiogenesis in the retinopathy proangiogenic role in the brain ischemia model. How valsartan may affect cancer progression in hypertensive patients, we tried to evaluate in the chicken CAM model. The study discovered that valsartan showed both the proangiogenic properties and altered the structure of blood vessels to support the cancer cell metastasis, at least in the case of ductal carcinoma. Hence the study warns the judicial use of valsartan as an anti-hypertensive drug in cancer patients and patients prone to the proangiogenic disorder.

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