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RESEARCH ARTICLE

ACTIVITY OF EPIGALLOCATECHIN-3-GALLATE ON CHICK EMBRYO-AN IN OVA ANTI-ANGIOGENESIS STUDY

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ABSTRACT

Neovascularization is the growth of the vascular system which plays a major role in both health and diseases. Angiogenesis is a complex process involving extensive interplay between cell soluble factors and extra cellular matrix components. This assay is the most widely used assay for screening of angiogenic activity. In this study we report the development and validation of a modified quantitative and objective *in vivo* chick embryo assay with Digital image analysis to screen compounds for anti-angiogenic activity by studying the vessel thickness. The thickness of the blood vessel of group 2 was statistically significant ($p<0.001$) when compared to group 1. A significant reduction ($p<0.001$) in the vessel thickness was found in group 3, 4 and 5. The known anti-cancer compound cyclophosphamide (CPA) was used as a standard to study the anti-angiogenic activity of EGCG on chorioallontoic membrane (CAM) of chick embryo.

INTRODUCTION

Vasculogenesis is a process of blood vessel formation occurring by a *de novo* synthesis of endothelial cells. Angiogenesis is the sprouting, splitting and remodeling of existing blood vessels (Han and Liu, 1999) while vasculogenesis is confined to early embryonic development and is responsible for the formation of the primary vasculature, including the main blood vessels of heart and lungs (Patan, 2000). The blood vessels are composed of endothelial cells which control the passage of nutrients, white blood cells and other materials between the blood stream and the tissues (Alberts *et al*., 1994). Cell-cell contacts and cell basement contacts are mediated by adhesion molecules such as cadherins and integrins respectively. These are extremely important and loss of either or both can lead to local destabilization of the endothelium and endothelial cell apoptosis (Lobov *et al*., 2002). On receiving a net angiogenic stimulus, endothelial cells in capillaries near the tumor gets activated and loosen the tight contacts with adjacent cells (Papetti and Herman, 2002). These cells secrete protein kinase C, a proteolytic enzyme which limits beta-catinin that solicits angiogenesis (Kwon *et al*., 2010). The angiogenic factors such as vasculoendothelial growth factor (VEGF), angiopoietin-1and 2 have emerged as important regulators of angiogenesis (Leung *et al*., 1989; Davis *et al*., 1996; Maisonpierre *et al*., 1997).

CPA is commonly used to treat various types of cancer and some autoimmune disorders. Phosphoramide mustard a metabolite of CPA forms inter-strand and intra-strand cross linkages with DNA strands at guanine N-7 position, this DNA is unable to replicate and ultimately leads to cell death (Andrew and Stephen 2011). CPA induces bone marrow suppression, darkening of the skin; alopecia, hemorrhagic cystitis etc. are the side effects of cancer patients are treated with this drug.

Epigallocatechin-3-gallate (EGCG) is a most abundant catechins in green tea and have many therapeutic properties such as anti-oxidant (Morre *et al*., 2003), anti-cancer (Pyrko *et al*., 2007), anti-angiogenic (Fassina *et al*., 2004), cyclooxygenase-2 inhibitor (Subbaramaiah *et al*., 2003) and cell cycle arrest through G_2/M phase in sarcoma cells (Shishodia *et al*., 2005).

The chick embryo chorioallantoic membrane (CAM) is an extra embryonic membrane is widely used to study tumor angiogenesis and demonstrates a functional assay to screen agents for anti-angiogenic activity (Quigley and Armstrong, 1998). The highly vascularised extra embryonic membranes of the chick embryo possess experimental advantages, including ease of preparation and low costs as well as the absence of a mature immune system (Leene *et al*., 1973), but nevertheless involve the complexity of the whole angiogenic process (Ribatti *et al*., 1996). An angiogenic response occurs in 72-96

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hours after stimulation in the form of increased vessel density around the implant, with the vessels rapidly converging toward the center like spokes in a wheel (Ribatti *et al*., 1995). When tumor grafts of increasing size (1-4mm) are implanted on the 9-day CAM, grafts larger than 1 mm undergo necrosis and autolysis during the 72 hour prevascular phase then they shrink rapidly until the onset of vascularization, when rapid growth resumes (Knighton *et al*., 1977). Conversely, the vessels become less dense around the implant and eventually disappear when anti-angiogenic compound is treated (Annabelle and Evangeline 2011).

Among the *in vivo* systems, CAM model is widely used in the study of angiogenesis and demonstrates a functional assay to screen agents for antiangiogenic activity (Auerbach *et al*., 2003). Chick embryo models have been the object of diverse research for 100 years and are commonly referred to as "hen's egg tests (HET)" (Lüpke, 1986; Schöffl *et al*., 1992). Concerning their application in the angiogenesis field, it has been shown that the data evaluation is statistically valid (Spielmann *et al*., 1997).

A novel quantitative evaluation of the early CAM assay was developed based on determination of lengths of veins and arteries (Hazel, 2003). The CAM assay is relatively simple and inexpensive and thus suitable for large scale screening. The chick embryo assay can be defined as ease of performance, high robustness, fast, objective and quantitative evaluation and good reproducibility of results. Also this is the best model system to study the induction of angiogenesis and anti-angiogenic drugs by tumor cells (Brooks *et al*., 1994; Presta *et al*., 1999). In this study, we report the development of a modified early *in-ovo* chick embryo assay to screen agents for anti-angiogenic activity on primary blood vessels. The changes in the distribution and density of CAM vessels next to the implant which are evaluated *in-vivo* by means of a stereomicroscope at regular intervals following the graft procedure. To investigate the extra-embryonic vessel systems, fertilized hens' eggs are maintained at 37.5°C in humidified air in order to allow the growth of the embryos under physiological conditions. Research is performed in or *ex-ovo* with about 10-day-old CAMs (Ribatti *et al*., 2001; Richardson and Singh, 2003).

MATERIALS AND METHODS

Experimental Design

Embryonated eggs at the age of 8 days were obtained from the Poultry Research Centre, Chennai and it was maintained at 37.0±0.5°C and 62% relative humidity in an incubation chamber till the entire period of experiment. The present study consisted of five groups. Group 1-chick embryo without any drug treatment (control), group 2-chick embryo induced with angiogenin, group 3-chick embryo treated with EGCG, group 4-chick embryo treated with CPA and group 5-chick embryo treated with EGCG+CPA. Treatment was given for three days with respective drugs in groups 2 to 5.

Preparation of Angiogenin

Polymorphonuclear leucocytes were isolated from human blood cells by differential centrifugation techniques using Ficoll hypaque from Sigma Co., USA (density of 1.077-1.080). Cells were in RPMI-1640 medium for 48 hours and provided 5% CO_2 in a CO_2 incubator. (Patrizia *et al*, 2002).

Preparation of EGCG

58.4g of EGCG was present in 100g of total polyphenol content of green tea, obtained after solvent extraction and purification was done with high performance liquid chromatography (Chitra and Krithika, 2013). EGCG was filter sterilized through membrane filter of 0.2µm in size and the final concentration was 3650µg/mL and stored at 4°C until analysis.

Preparation of CPA

22mg/kg body weight/day of commercially available CPA (Sigma Co., USA) was used for three days by standard procedure of Nayak and Abhilash (2008).

CAM Assay

Eight days old embryonated eggs were purchased and experiment was started on 9th day. CAM area was located using light source and marked, without disturbing the embryo, a small hole was made using dentist drill. Drug of the interest was introduced in to the respective groups using the fine needle without rupturing the membrane. Waited till all the drugs were absorbed by the embryo and the hole was closed with warm wax and these eggs were incubated at 37.0±0.5°C and 62% relative humidity in an incubation chamber. This procedure was repeated for three days. On the fourth day the eggs were cut open and captured the Images usinf Stereomicroscope. And Tissues were removed for the biochemical assays.

Capturing of Images and Measurement of vessel thickness

After completion of the treatment period the egg shell of the embryo was carefully opened and placed on the sterile petri dish. The vessel thickness was captured using Stereomicroscope and these pictures were transferred to the Microsoft Excel sheet for the measurement of vessel thickness using Image Proplus Software version 6.0.

Preparation of Tissue Homogenate

The embryo was washed three times with phosphate buffered saline, pH 7.4, the heart, liver and eye lens were carefully removed by dissection. 1% homogenate was prepared using the same buffer and the homogenate was used for the following assays.

Estimation of Total Protein

Total protein was estimated by the method of Lowry *et al*. (1951). Briefly, to the tissue homogenate added alkaline copper reagent and Folin's Ciocalteu Phenol reagent. Tubes were incubated for 20minutes at room temperature and color developed was read at 620nm in a UV-Visible Spectrophotometer. Bovine serum albumin was used as a standard.

Estimation of Malondialdehyde Product (MDA)

Malondialdehyde levels were estimated by the method of Ohkawa *et al*., 1999. Briefly, to the tissue homogenate added 10% trichloacetic acid, centrifuged the contents at 3000 rpm for 20 minutes in a high speed refrigerated centrifuge. To the supernatant added 0.002M thiobarbituric acid was added. Heated the contents in a boiling water bath for 30 minutes and cooled to room temperature. Absorbance was measured at 532 nm in a UV-Visible Spectrophotometer. 10nmoles/ml of

1,1',3,3'-tetra ethoxy propane in 20mM Tris-HCl buffer was used as a standard in the place of tissue homogenate.

Estimation of Reduced Glutathione (GSH)

GSH was estimated by the method of Ellman, 1959. Briefly, the tissue homogenate was precipitated with 20% trichloro acetic acid and centrifuged the contents for 10 minutes at 3000rpm. To the supernatant added Ellman's reagent prepared in phosphate buffer of pH 8.0. Color developed was read at 412nm in a UV-Visible Spectrophotometer. Absorbance values were compared with a standard curve generated from known GSH. The GSH level in tissue was calculated as micromol/mg protein.

Statistical Analysis

Statistical analysis of the data was performed using SPSS software. One-way analysis of variance (ANOVA) was used and level of significance was calculated. $p<0.05$ was considered as significant.

RESULTS

The group 2 embryo showed a very thick blood vessel and the thickness was statistically significant ($p<0.001$) when compared to group 1. A significant reduction ($p<0.001$) in the vessel thickness was found in group 3, 4 and 5.

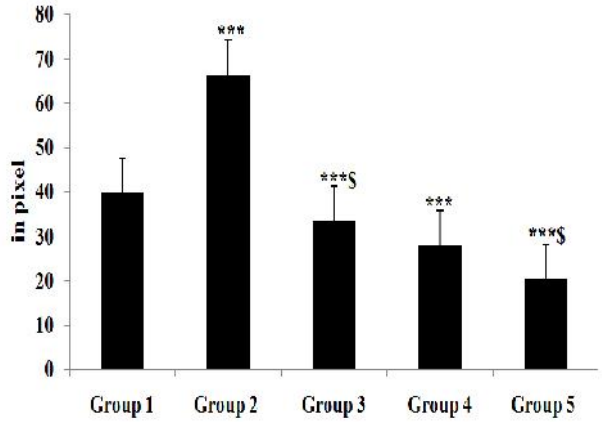

Fig.1 Blood vessel thickness (Major vessels)

Groups 2 to 5 were compared with group 1. Group 3 & 5 were compared with group 4; ***-$p<0.001$; $-$p<0.0001$. Group 1-control, group 2-chick embryo induced with angiogenin, group 3-chick embryo treated with EGCG, group 4-chick embryo treated with cyclophosphamide and group 5-chick embryo treated with EGCG+cyclophosphamide.

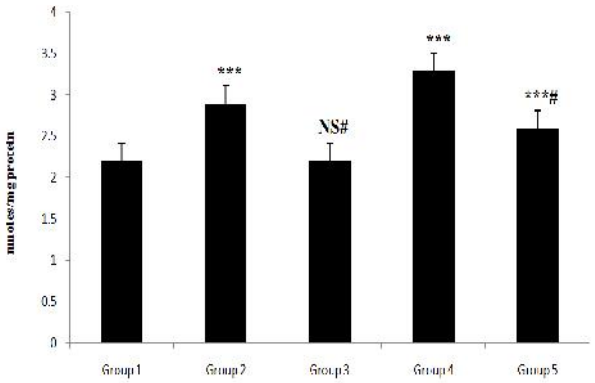

Fig. 2 Levels of Malondialdehyde in Heart of Chick Embryo

Groups 2 to 5 were compared with group 1. Group 3 & 5 were compared with group 4; ***-$p<0.001$; $-$p<0.0001$. Group 1-control, group 2-chick embryo induced with angiogenin, group 3-chick embryo treated with EGCG, group 4-chick embryo treated with cyclophosphamide and group 5-chick embryo treated with EGCG+cyclophosphamide.

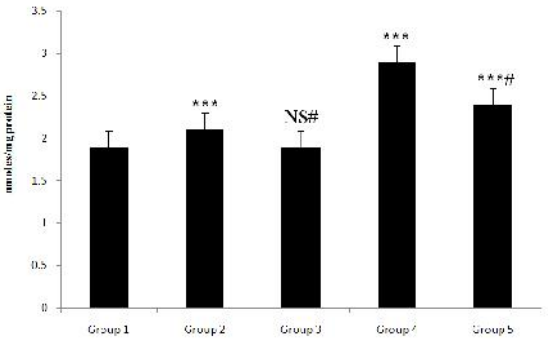

Fig. 3 Levels of Malondialdehyde in Liver of Chick Embryo

Groups 2 to 5 were compared with group 1. Group 3 & 5 were compared with group 4; ***-$p<0.001$; $-$p<0.0001$. Group 1-control, group 2-chick embryo induced with angiogenin, group 3-chick embryo treated with EGCG, group 4-chick embryo treated with cyclophosphamide and group 5-chick embryo treated with EGCG+cyclophosphamide.

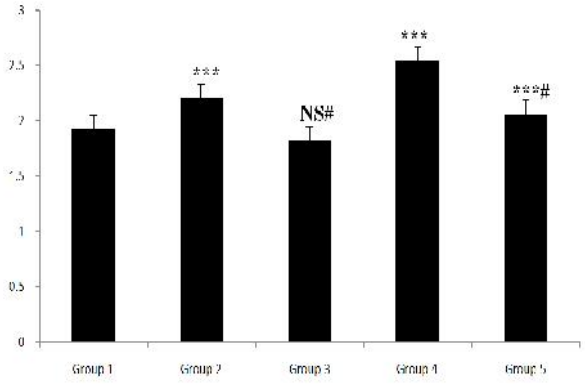

Fig. 4 Levels of Malondialdehyde in Eye Lens of Chick Embryo

Groups 2 to 5 were compared with group 1. Group 3 & 5 were compared with group 4; ***-$p<0.001$; $-$p<0.0001$. Group 1-control, group 2-chick embryo induced with angiogenin, group 3-chick embryo treated with EGCG, group 4-chick embryo treated with cyclophosphamide and group 5-chick embryo treated with EGCG+cyclophosphamide.

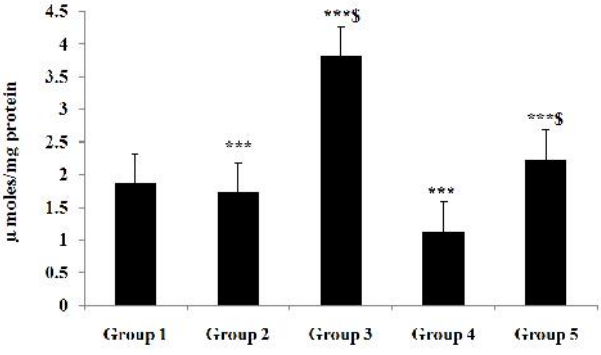

Fig.5 Levels of Reduced Glutathione in Heart of Chick Embryo

Groups 2 to 5 were compared with group 1. Group 3 & 5 were compared with group 4; ***-$p<0.001$; $-$p<0.0001$. Group 1-control, group 2-chick embryo induced with angiogenin, group 3-chick embryo treated with EGCG, group 4-chick embryo

treated with cyclophosphamide and group 5-chick embryo treated with EGCG+cyclophosphamide.

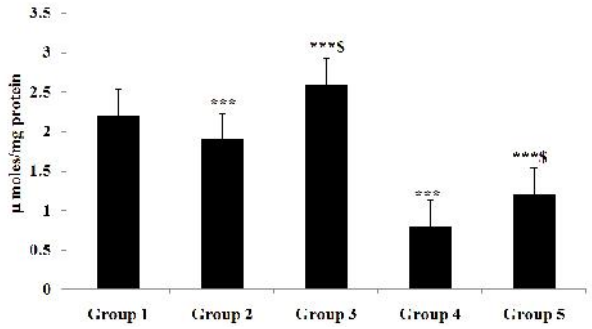

Fig.6 Levels of Reduced Glutathione in Liver of Chick Embryo

Groups 2 to 5 were compared with group 1. Group 3 & 5 were compared with group 4; ***-p<0.001; $-p<0.0001. Group 1-control, group 2-chick embryo induced with angiogenin, group 3-chick embryo treated with EGCG, group 4-chick embryo treated with cyclophosphamide and group 5-chick embryo treated with EGCG+ cyclophosphamide.

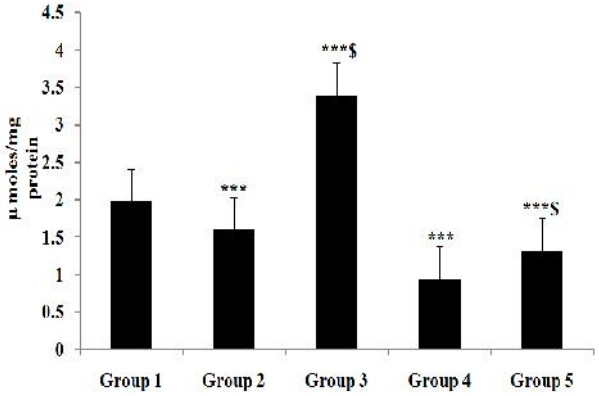

Fig.7 Levels of Reduced Glutathione in Eye Lens of Chick Embryo

Groups 2 to 5 were compared with group 1. Group 3 & 5 were compared with group 4; ***-p<0.001; $-p<0.0001. Group 1-control, group 2-chick embryo induced with angiogenin, group 3-chick embryo treated with EGCG, group 4-chick embryo treated with cyclophosphamide and group 5-chick embryo treated with EGCG+cyclophosphamide.

Cell cytotoxicity was assessed in terms of lactate dehydrogenase release. Pro-oxidant and anti-oxidant were assessed in terms of MDA and GSH respectively. Activity of LDH in liver, heart and eye lens were assessed and it was significantly increased in group 2 ($p<0.05$) and group 4 ($p<0.001$) when compared to group 1. No significant change was noticed in group 3 compared to group1.

The levels of MDA was significantly increased ($p<0.001$) in group 2 and 5 when compared to group 1. Group 3 showed no significant change in the levels of MDA in heart tissue. The levels of MDA in liver was significantly increased in group 2 ($p<0.05$) group 4 ($p<0.001$) and group 5 ($p<0.01$) when compared to group 1. The inter comparison of group 3 & 5 showed a significant reduction ($p<0.0001$) when compared to group 4. There was no significant change in group 3 was noticed. The levels of MDA in eye lens of group 2 & 4 ($p<0.001$) showed a significant variation when compared to control. The inter comparison of group 3 & 5 showed a significant variation ($p<0.0001$) when compared to group 4. No significant change in group 3 & 5 were noticed.

The cellular antioxidant, GSH protects the cells from free radical damage. The levels of GSH in liver was significantly decreased in group 2 ($p<0.05$) and 4 ($p<0.001$) when compared to group 1. In group3 showed a significant increase ($p<0.001$) in the levels of reduced glutathione when compared to group 1.

DISCUSSION

In-ovo angiogenesis model ie. CAM assay is the most widely used method for the most of the angiogenesis evaluation studies (Theodore *et al*., 2004). Angiogenesis involve the sprouting of new capillary like structures from existing vasculature and is regulated by pro and anti-angiogenic factors (Carmeliet, 2000). In this assay agents are applied to preexisting vessels. Since it is possible that the anti-angiogenic activity of test substances only targets small immature capillaries or neovascularisation and that the more stable vessels are unaffected, the differentiation of these effects will be an advantage. Thus, determination of total vessel area alone may not identify significant activity of agents targeting mainly small capillaries. To attain this goal, image analysis was programmed for the determination of major vessel areas.

An angiogenic or anti-angiogenic response observed in 72-96 hours after introduction of drug in the form of increased vessel density around the implant and this was reported by Ribatti *et al*. (1995). Therefore, measuring the vessel thickness in chick embryo after introduction of drugs is the best way to evaluate the potency of drugs of interest. In the CAM system anti-angiogenic agent was found to be active against microvessel growth, probably by inhibition of O_3 integrin associated microvessel endothelial cells (Sharma *et al*., 2001).

A variety of growth factors have been reported to induce CAM angiogenesis and one of the factor is that protein kinase C (PKC) activation. Tsopanoglou *et al*. (1993) reported to induce CAM angiogenesis in chick embryo at the age of 9 to 11 days. Angiogenin might enhance the activation of PKC by decreasing the intracellular cyclic Anenosine monophasphate (cAMP). The activation of PKC by decreasing the intracellular cAMP by angiogenin might enhance the vessel thickness in group 2. In group 3 the vessel thickness was reduced significantly this might be due to EGCG down regulates PKC by enhancing the cAMP level in chick embryo. Various cell signaling pathways involving mitotic signals, cell cycle regulation by EGCG and as mentioned above by Tsopanoglou *et al*. (1994). Matrix metalloproteinases (MMPs) play an important role in angiogenesis. EGCG may suppress angiogenesis through inhibition of MMP activation that leads suppression og angiogenic endothelial cell migration. EGCG suppressed the activation of MMP_2 in a dose dependant manner and also suggested that EGCG actually suppressed membrane type MMP on the endothelial cells in rats (Oku *et al*., 2003). Activation of STAT3/1 directly involves in the angiogenesis process, hence EGCG decreasing the activation of STAT3/1 which in turn inhibits angiogenesis (Manegazzi *et al*., 2001; Zhu *et al*., 2009). Vascular endothelial growth factor (VEGF) plays an essential role in angiogenesis hence the inhibition of VEGF prevents angiogenesis. Diana *et al*. (2010) reported that EGCG down regulates VEGF which in turn inhibits angiogenesis. This was supported by Jung *et al*. (2001) and Masuda *et al*. (2002). Anti- angiogenic activity and intra cellular distribution of EGCG was reported

(Piyaviriyakul *et al*., 2011). EGCG changes the early transcriptional pattern of angiogenesis in cervical tumor cells (Tudoran *et al*., 2012). In CPA and combination with EGCG treated group showed a decreased vessel thickness. This may be due to the anti-angiogenic activity of CPA. Low doses of CPA and with the combination of the natural flavonoids have improved anti-angiogenic and anti- tumor activity in lung carcinoma (Zhang *et al*., 2006; Touil *et al*., 2011)

Present results indicate that the CPA and angiogenin treated group showed an elevated levels of MDA, this might be due to the high amount of free radicals are generated in this group and which could not overcome by the body's first defence mechanism hence the chick embryo does not develop immunocompetence system and also no immunological rejection at this age. No significant level of MDA was found in EGCG treated group when compared to control and this might be due to its anti-oxidant potential (Leonilla *et al*., 2010).

Decreased levels of GSH in group 2, 3 and 5 which might be due to the development of cytotoxicity induced by angiogenin and a standard drug CPA (Suzuki *et al*., 1999). In EGCG treated group showed a significant increase in the GSH content might be due to its antioxidant nature. EGCG reduces the cytotoxicity evoked by H_2O_2 and increases the levels of the enzymes related to the oxidative stress, resulting in an enhanced cellular GSH content in $HepG_2$ cell line (Murakami *et al*., 2009).

CONCLUSION

In this study we demonstrated the quantification of the anti-angiogenic effects of EGCG in the modified early chick embryo model. The advantages of this assay are that it is time- and cost-effective and permits objective evaluation of the angiogenic activity of test compounds *via* Digital image analysis. The procedure provides differentiated values of major vessel areas. However, the approach with Digital image analysis in this early chick embryo model can, in general, also be applied to later CAM assays. This test system will be complementary to existing chick embryo assays and may be used to screen series of compounds for anti-angiogenic activity.

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