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ANGIOSTATIC PROPERTY OF CHITOSAN ACETATE IN THE CHICK CHORIOALLANTOIC MEMBRANE (CAM), *IN OVO*

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ABSTRACT
Chitin is the most ubiquitous natural polysaccharide after cellulose on earth. A wide variety of medicinal applications for chitin and chitosan (deacylated chitin) have been reported. In the present investigation the salt of chitosan-chitosan acetate was used to study its effect on angiogenesis using Chorioallantoic Membrane (CAM) assay *in ovo*. The solution of chitosan acetate was tested by administration at 48, 72 and 96 hours of incubation in chick eggs. Morphometric and microscopic examination of CAM indicated inhibition of neovasculature. The inhibition was reported in number and growth of secondary and tertiary blood vessels. There is 16.36%, 20.29% and 30.52% inhibition in number of secondary and 9.42%, 10.99% and 20.20% inhibition in number of tertiary blood vessels after 48hrs, 72hrs, and 96hrs of chitosan acetate treatment. It indicates that chitosan acetate affects endothelial growth factors having angiostatic property and may be used to control tumor growth.

INTRODUCTION

Angiogenesis or formation of new blood vessels from pre-existing vasculature is the most regulated process. It starts early in embryogenesis and continues throughout life. It plays vital role in physiology and in pathology. It is necessary for wound healing, growth and action of female reproductive organs. Moreover disturbance in regulation leads to pathogenesis. Excessive angiogenesis is reported in malignancy, psoriasis, arthritis, retinopathy, obesity and asthma. Insufficient angiogenesis leads to heart and brain ischemia, hypertension and osteoporosis.

Angiogenesis process begins with degradation of capillary basement membrane, endothelial cell (EC) proliferation, directed migration of ECs, tubulogenesis, vessel fusion, vessel pruning and pericytes stabilization. This process is under the control of some factors- angiogenic growth factors like fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF), angiopoietin and epidermal growth factor (EGF). The angiostatic growth factors are angiostatin, endostatin and thrombo spondin. The modulation of the angiogenesis is considered as therapeutic strategies of great importance for human health. In 1970, Folkman hypothesized that tumor growth is angiogenesis- dependent and an antiangiogenic strategy might constitute a new therapeutic approach for treatment of solid tumors (Folkman, 1974).

The chitosan, a cationic polysaccharide generated commercially is useful in variety of applications including biomedicines (Berger *et al*., 2005). N-acetyl chitooligosaccharides, particularly hexamer and heptamer, display notable antitumor activity against- sarcoma 180 solid tumors in BALLB/C mice as well as in MM-46 solid tumor implanted in C3H/ HC mice (Suzuki *et al*., 1986). According to Harish Prashanth and Tharanathan (2005), depolymerized products of chitosan are inhibitors of angiogenesis. Chitosan-glycerol phosphate-hydroxyethyl cellulose (chitosan-GP-HEC) acts as angiostatic agent (Ahmadi *et al*., 2010). The main objective of the present study was to elucidate the effect of chitosan salt on angiogenesis by using chick CAM assay.

Chitin is the most abundant natural polymer next to cellulose. It is found in exoskeleton of most invertebrates like crustaceans, insects as well as in fungi. Chitin can be isolated from marine crustaceans, mainly because a large amount of waste is available as a by-product. Crustacean shell consists of 30-40% protein, 30-50% calcium carbonate and 20-30% chitin. Chitosan (CS) is derived from chitin by the process of deacetylation. Chitin, CS and their derivatives having more than 200 applications in biomedicine, food, biotechnology, agriculture and cosmetics. The key problem is about solubility of chitin and chitosan. In many applications salts of CS are used. Mostly used salts are CS acetate and CS chloride. The biological and chemical characteristic of CS acetate is same as that of chitin (Natthan *et al*., 2012). It is biocompatible and nontoxic natural polymer (Dutta *et al*., 2004). These are used in cosmetics, like in shampoo, as hair tonic and in lipstick (www.geocites.com). The biomedical applications include tissue engineering (Wang *et al*., 2003). One of the CS derivative chitooligoschhares (COS), having ability to trigger cancer cell apoptosis (Prashanth and Tharanathan, 2005). CS acetate bandage can act as an effective topical antimicrobial dressing (Stevenson *et al*., 2003).

From review of published literature, there is no report about CS acetate and angiogenesis. To study angiogenesis chick CAM is the novel material. Hence we propose to study the

effect of CS acetate on angiogenesis by using chick CAM assay. The waste material from food processing unit may be channelized into best for medicinal purpose.

MATERIALS AND METHODS

Extra pure CS acetate was gifted by India Sea Foods, Kochi (Kerala) with Batch No. CA-21/3AN-5/0512. Dextrose with Normal Saline (DNS) was purchased from March Bioscience Ltd, Kheda.

Chorioallantoic membrane (CAM) assay (*in ovo*)

CAM assay for screening the effect of CS acetate on angiogenesis was performed by window method. Fertilized eggs of *Gallus gallus* were purchased from Assistant Commissioner of Animal Husbandry, Central Hatchery, Kolhapur. These were properly sterilized and incubated in aseptic incubator adjusted at 37.5°C temperature with 70-75 relative humidity. The eggs were grouped into four groups- normal, sham controlled, DNS controlled and CS acetate treated. The treatment was given at 48 hrs, 72 hrs and 96 hrs of incubation. The development is continued up to 144 hrs of incubation. After day 6 the CAM was evaluated.

The dose was selected on the basis of mortality, abnormality and toxicity study (Patil and Gonjari, 2010). After completion of scheduled time, the eggs were treated according to Table 1.

The window method is used as described by Korn and Cramer (2009) with some modifications.

The windows were by removing shell at broad end, in aseptic condition 1mg/ml CS acetate was spread on CAM in DNS. The window was sealed with medicated tape in experimental group. One group of eggs was incubated as normal. The embryos of operated control group were sham operated and other group was with administration of 1 ml DNS as a control. All eggs were incubated for 144 hrs.

Evaluation of CAM angiogenesis

The CAM evaluation was made by measuring CAM area with some modifications, which was described by Melkonian *et al*. (2002). The CAM area was calculated-

Area = (1/2 A) x (1/2 B) x ð, where A-longest length, b- longest width and ð = 3.14.

The CAM was studied morphometrically as well as histologically. For histological preparation the CAM was fixed in CAF fixative. After paraffin embedding sections were cut at 5μm thickness.

Statistical analysis

The data was expressed in Mean ± SE. The statistical significance between groups were analyzed by using one-way ANOVA. The values of $p < 0.05$, $p < 0.1$ and $p < 0.001$

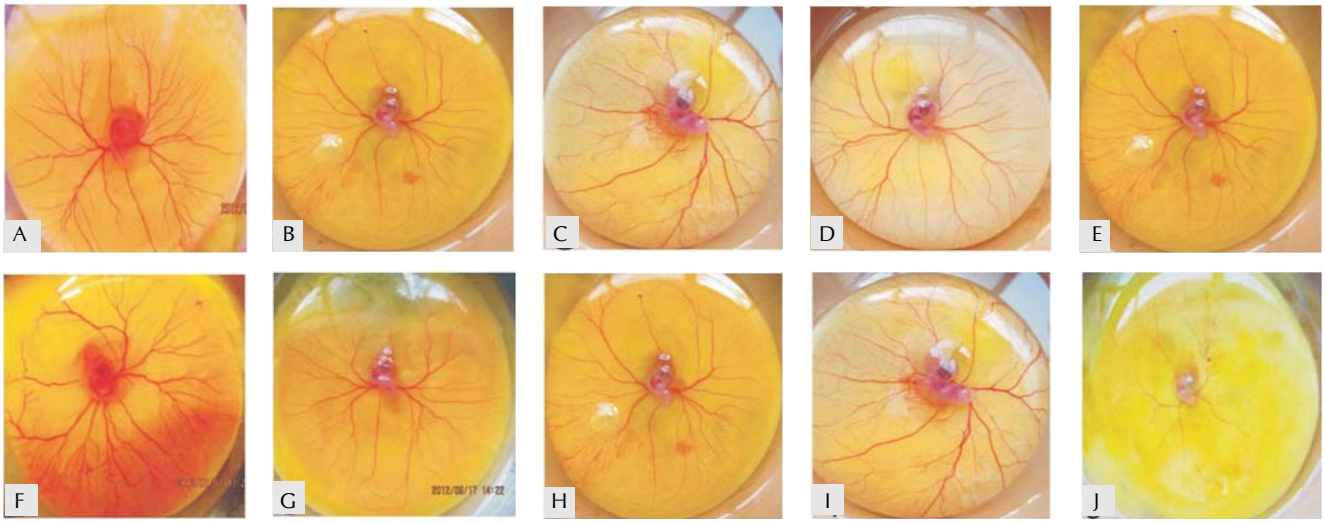

Plate 1: Antiangiogenic effect of chitosan acetate on chick CAM (A - Normal, B to D - Sham controlled, E to G - DNS controlled, H to J - Chitosan acetate treated

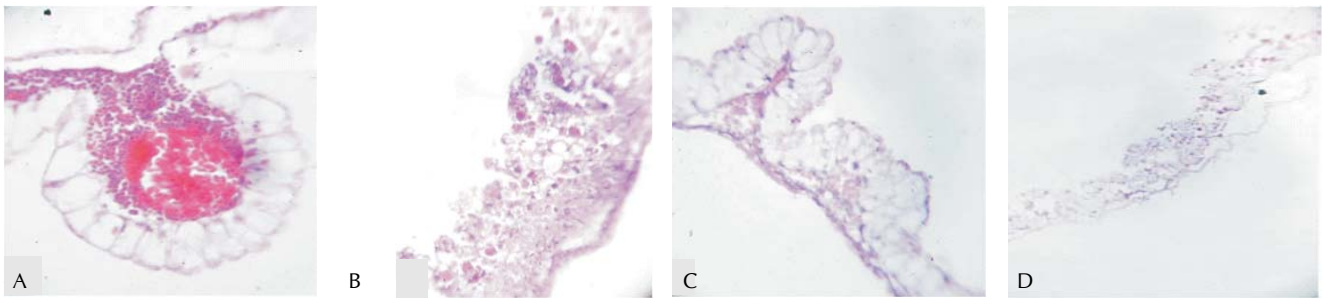

Plate 2: T. S. of CAM (stained with H-E) (A - Normal, B - Sham Controlled, C - DNS controlled, D - Chitosan acetate treated

were considered as significant.

RESULTS AND DISCUSSION

Morphometric study

For study of effect of CS acetate on CAM, the normal CAM was studied after 144 hrs, which provide the model for vasculogenesis and angiogenesis.

During the present investigation the CAM was studied morphometrically. At 48 hrs of treatment sham controlled embryos showed no significant but very marginal decrease in number of secondary blood vessels. DNS controlled embryos after 72 hrs treatment showed marginal increase in number of secondary and tertiary blood vessels. Moderate decrease in the number of secondary and tertiary blood vessels was observed at 48 hrs of CS acetate treatment. At 72 hrs of CS acetate treatment showed significant change in the number of secondary and tertiary blood vessels. Highly significant decrease in number of these blood vessels was observed after 96 hrs of CS acetate treatment as shown in plate1 and in Table 2.

16.36%, 20.29% and 30.52% inhibition was observed in secondary blood vessels at 48 hrs, 72 hrs and 96 hrs of treatment respectively. Inhibition of tertiary blood vessels at 48 hrs, 72 hrs and 96 hrs of CS acetate treatment was 9.42%, 10.99% and 20.20%.

During the present investigation the CAM was studied morphometrically. At 48 hrs of treatment sham controlled embryos showed no significant but very marginal decrease in number of secondary blood vessels (Fig.1). DNS controlled embryos after 72 hrs treatment showed marginal increase in number of secondary and tertiary blood vessels. Moderate decrease in the number of secondary and tertiary blood vessels was observed at 48 hrs of CS acetate treatment. At 72 hrs of CS acetate treatment showed significant change in the number of

Table 1: CS acetate treatment schedule at different developmental stages of chick embryo

Groups	Exposure to treatment (hrs.)			Treatment (hrs.)
	48	72	96	
I	-	-	√	48
II	-	√	-	72
III	√	-	-	96

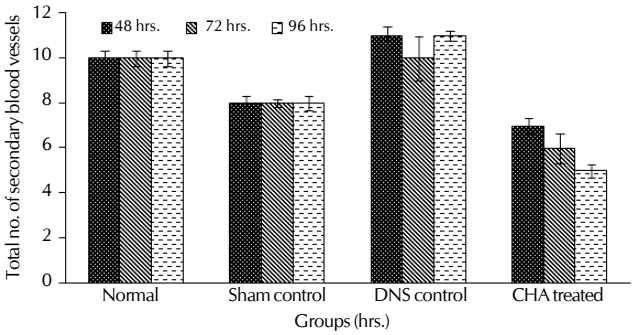

Figure 1: Chitosan acetate influnced alteration in secondary blood vessels (144 hrs of development)

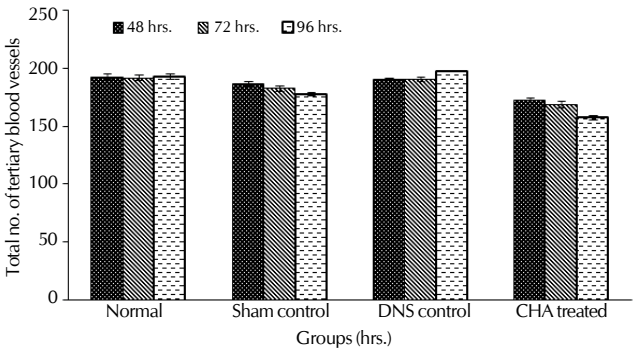

Figure 2: Chitosan acetate influnced alteration in tertiary blood vessels (144 hrs of development)

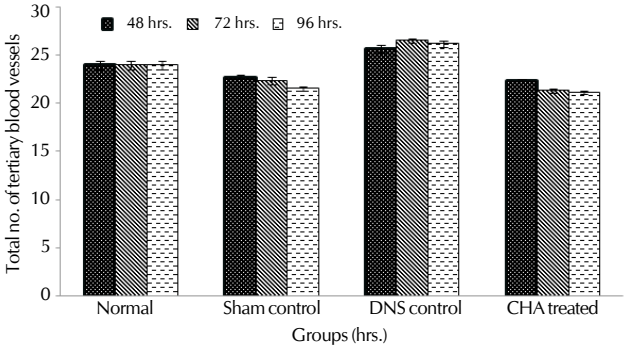

Figure 3: Chitosan acetate influnced alteration in CAM area (144 hrs of development)

Table 2: Effect of chitosan acetate on number of blood vessels and CAM area of chick CAM

Treatment (hrs) and dose	Groups	No. of blood vessels		CAM area(sq.cm)
		Secondary	Tertiary	
48(1mg/ml)	Normal	10 ± 0.341	193 ± 2.44	24.0 ± 0.44
	Sham control	8 ± 0.341	187 ± 2.109	22.66 ± 0.33
	DNS control	11 ± 0.40	191 ± 0.988	25.66 ± 0.42
	CHA treated	7 ± 0.331^{bpy}	173 ± 2.011^{crx}	22.33 ± 0.21^{az}
72(1mg/ml)	Normal	10 ± 0.341	192 ± 2.44	24.0 ± 0.44
	Sham control	8 ± 0.166	183 ± 2.755	22.33 ± 0.42
	DNS control	10 ± 0.988	191 ± 1.536	26.5 ± 0.22
	CHA treated	6 ± 0.653^{cqy}	169 ± 2.77^{brz}	21.33 ± 0.21^{cz}
96(1mg/ml)	Normal	10 ± 0.341	193 ± 2.46	24.0 ± 0.44
	Sham control	8 ± 0.341	178 ± 1.587	21.5 ± 0.22
	DNS control	11 ± 0.223	198 ± 0.881	26.16 ± 0.31
	CHA treated	5 ± 0.302^{arz}	158 ± 1.796^{crz}	21.16 ± 0.17^{cpx}

(Results expressed as mean ± S.E. of 6 embryos. P-values- a < 0.05, b < 0.01, c < 0.001 vs. Normal embryos. p < 0.05, q < 0.01, r < 0.001 vs. Sham control embryos. x < 0.05, y < 0.01, z < 0.001 vs. DNS control embryos)

secondary and tertiary blood vessels. There is significant decrease in CAM area after 96 hrs CS acetate treatment (Fig. 2 and 3). Highly significant decrease in number of these blood vessels was observed after 96 hrs of CS acetate treatment (Plate I and Table 2).

16.36%, 20.29% and 30.52% inhibition was observed in secondary blood vessels at 48 hrs, 72 hrs and 96 hrs of treatment respectively. Inhibition of tertiary blood vessels at 48 hrs, 72 hrs and 96 hrs of CS acetate treatment was 9.42%, 10.99% and 20.20%.

Histological study

Hematoxylene-eosine stained sections of CAM were observed under light microscope (plate II). Thickness of CAM area was decreased due to CS acetate treatment. The marginal increase in thickness was observed after 72 hrs of DNS treatment CAM (Plate II). In histological sections the ectoderm and endoderm of control and treated CAMs were similar in thickness. In contrast, the mesoderm of treated CAMs was thinner and appeared having less extracellular matrix. In chitosan acetate treated CAMs capillary plexus were few in number.

The nutrition and oxygen is the prerequisite for developing neoplastic mass. Tumors that outgrow 1-2 mm^3 cannot form masses without developing blood vessels from surrounding stroma by sprouting angiogenesis (Fidler, 2001). For some localized solid malignant tumors, surgery have curative potential, but the use of radiations cytotoxic chemotherapy is a more appropriate route of treatment for surgical unrespectable tumors (Long, 2003 and Evans, 2005). The discovery of angiogenic inhibitors provides hope for reducing the mortality and morbidity from carcinomas. There has been some positive outcome with the use of angiostatic drugs based on some clinical trials (Cobleigh *et al*., 2003).

Antitumor activity of chitosanoligosaccharides (COS) have been demonstrated by Prashanth and Tharanathan (2005). Chick CAM is mostly used angiogenic assay. The results of CAM assay showed that COS had the potential anti-angiogenic function. The COS affect tube formation during the tumor angiogenesis. These researchers also found that water soluble low molecular weight chitosan showed inhibition of formation of sprouts in CAM assay. The similar results have been observed in the present investigation by using chitosan acetate.

Since angiogenesis is indispensable for tumor growth and metastasis, early in1971, Folkman proposed that the growth and metastasis of tumor could be reduced by inhibiting angiogenesis.

In conclusion, CS acetate has a potential angiostatic polysaccharide probably inhibiting the migration and tube formation of endothelial cells. This study supports the development and use of CS acetate as a natural, non-toxic, safe and low cost angiostatic agent. However, further studies are required to elucidate the exact mechanism underlying the angiostatic property of chitosan acetate.

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