CHAPTER – IV
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Angiogenesis is a normal process in growth and development. It involves the growth of new blood vessels from preexisting vessels. In sprouting angiogenesis biological signals known as angiogenic growth factors activate receptors present on endothelial cells present in pre-existing veins. The activated endothelial cells begin to release enzymes called proteases that degrade the basement membrane in order to allow endothelial cells to escape from the original vessel walls. The endothelial cells then proliferate into the surrounding matrix and form solid sprouts connecting neighbouring vessels. As sprouts extend towards the source of the angiogenic stimulus, endothelial cells migrate in tandem, using adhesion molecules called integrins. Sprouting occurs at a rate of several millimeters per day (Burri, 2004).

Another method of angiogenesis is intussusceptive angiogenesis, also known as splitting angiogenesis. In this type of vessel formation, the capillary wall extends into the lumen to split a single vessel in two. Intussusception is important because it is a reorganization of existing cells. It allows vast increase in the number of capillaries without a corresponding increase in the number of endothelial cells and important in embryonic development.

Besides the differentiation between sprouting angiogenesis and intussusceptive angiogenesis there exists more common differentiation between the following types of angiogenesis.

Vasculogenesis : Formation of vascular structures from circulating or tissue resident endothelial stem cells (angioblasts), which proliferate into de novos endothelial cells. This form particularly relates to the embryonic development of the vascular system.

Angiogenesis : Formation of thin walled endothelium lined structures with or without smooth and pericytes (fibrocytes). This form plays an important role during the adult life span, also as 'repair mechanism' of damaged tissues.

Anteriogenesis : Formation of medium sized blood vessels possessing tunica media plus adventitia.

At present as above differentiation is not a sharp one, quite often the term "Angiogenesis" is used summarizing all different types and modifications of arterial vessels growth (Rubanyi, 2000, Kornowski *et. al.,* 2000; Laham & Baim, 2005).

The modern clinical application of the angiogenesis can be divided into two main areas – Antiangiogenic therapies, and proangiogenic therapies. Antiangiogenic therapies are being employed to fight cancer and malignancies (Folkman, 1996), which require an abundance of oxygen and nutrients to proliferate. Proangiogenic therapies are being explored as options to treat cardiovascular diseases which is a major cause of death. One of the first applications of proangiogenic methods in humans was a German trial using fibroblast growth factor 1 (FGF-1) for the treatment of coronary artery disease (Schumacher *et. al.*, 1998). Clinical research in therapeutic angiogenesis is ongoing for a variety of atherosclerotic diseases like

coronary heart disease, peripheral arterial disease, wound holding disorders etc.

Recently shark cartilage has generated intense interest in both public and medical circles because of theoretical justification for its clinical use in disease, including cancer, psoriasis, age related macular degeneration and arthritis, which involve angiogenesis. Clinical trials and recent patents on shark cartilage have demonstrated its antitumor activity and its ability to relieve pain and inflammation associated with tumor activity and diseases involving angiogenesis (Lane, 1993).

The antitumor efficacy of shark cartilage has been investigated for a long time. More than 7 antitumor factors have been isolated from shark cartilage (Lee and Langer, 1983). Experimental results have suggested that its antitumor mechanisms include inhibiting the proliferation of tumor cells, restricting and damage induced by hydrogen peroxide, inhibiting the production of a PG like substance and stimulating the immune system (Gomes *et. al.,* 1996). A potent angiogenesis inhibitor, U-995 has been purified from the cartilage of the blue shark *Prionace glauca* and it is composed of two single peptides (Sheu *et. al.,* 1998). Antiangiogenic effects of U-995 may be due to interference with the proliferation and migration of umbilical vein endothelial cells as well as inhibition of collagenolysis, thereby leading to inhibition of both angiogenesis and tumor cell growth (Sheu *et. al.,* 1998).

Antiproliferative activity and apoptotic effect of tick salivary gland extracts on human HeLa cell have been studied by Kazimirova *et. al.*, (2006).

In the present investigation salivary and digestive gland of *A. pulmonata* was studied for its effect on angiogenesis using the chorio-allantric membrane of chick embryo. The salivary and digestive gland extracts were prepared in acetone and alcohol and the developing embryos were treated with the extracts by window method.

The observations on the experiments have already been given in chapter III. In the present investigation as in case of tick salivary glands (Kazimirova *et. al.*, 2006) inhibitory effects on angiogenesis have been observed.

Effects of HBSS and salivary as well as digestive gland of *A. pulmonata* seen on mortality of chick embryos. Normal and HBSS treated embryos showed 5% mortality which is very common and salivary gland acetone extract and alcohol extracts of *A. pulmonata* showed the mortality 30% and 25% respectively among the embryos treated at 48 hrs of incubation. The embryos treated in later stages showed more survival and less mortality (Table III.1). This is probably due to more effect of extract on the early angiogenesis.

Chick chorioallantoic membrane (CAM) angiogenesis model is used for the study. Salivary gland and digestive gland extracts were prepared in both alcohol and acetone (1 mg/ml). For control HBSS medium is used. One set is developed as normal up to 144 hrs. Control set was developed in HBSS for 144 hrs. Other 4 groups were treated by extracts of salivary glands in acetone and

alcohol and digestive gland in acetone and alcohol. All experimental are treated with extract at 48, 55, 66, 72, 88 and 96 hrs. of incubation. The eggs are opened at 144 hrs of incubation for observations.

Observations in diameter and area of CAM after the administration of salivary and digestive gland extracts showed reduction in the mean diameter and area of CAM. At 48 hrs. alcohol extracts showed inhibition i.e. 36.22% CAM area. The embryo treated in the earlier period of incubation i.e. 48, 55, 66, 72 showed more inhibition of angiogenesis. Maximum depletion in the CAM area due to salivary and digestive gland extracts in alcohol was observed in 66 hrs. treated embryos i.e. 52.73% and 50.32% respectively. Maximum depletion in the CAM area due to digestive gland extracts in acetone was observed in 55 hrs. treated embryos i.e. 30.24% and 43.47% respectively. The alcohol extract of both salivary and digestive gland has more inhibitory effect on mean diameter and area of CAM.

When all the embryos treated with HBSS as control and if compared with normal embryos showed stimulatory effect on mean diameter and area of CAM.

Primary vitelline veins (PVV) :

Alterations in the primary vitelline veins after the administration of both salivary and digestive gland extracts of *A. pulmonata* were observed in relation with number and area occupied by them. Both right and left sides are considered separately.

Very minute effect of extracts was observed on the number of primary vitelline veins as these veins are already formed before 48 hrs. of treatment. New PVV's are not developed and after 66

hrs. of treatment 50% of reduction in the number of PVV on left side in both salivary and digestive gland alcohol and acetone extracts of *A. pulmonata*. No significant change is observed in the number of PVV in embryos under study.

Maximum inhibition of area of PVV was observed in the eggs treated with salivary gland extracts both in acetone and alcohol at 48 hrs. especially on the left side i.e. 50% and 60% respectively.

Maximum inhibition of area of PVV was seen in the embryos treated at 96 hrs. of incubation with digestive gland extracts in acetone and alcohol on the right side i.e. 52.37% and 64.29% respectively.

In most of the experimental conditions it is observed that alcohol extract showed more effective inhibitory effect than acetone extract in both salivary glands and digestive glands.

Secondary vitelline veins (SVV) :

Observations on SVV of CAM after the treatment of salivary and digestive gland extracts of *A. pulmonata* revealed followed features.

Maximum depletion in the number and area i.e. 67.67% and 63.50% was observed respectively in the 48 hrs. salivary gland acetone extract treated embryo studied at 144 hrs. in acetone and alcohol respectively. Where as salivary gland alcohol extract treated at 48 hrs. salivary gland alcohol extract treated at embryos studied at 144 hrs. showed depletion in the number and area as 67.67% and 60.38%.

Salivary gland extract in acetone inhibition in area of SVV is found to hanging widely with maximum 65.29% at 55 hrs and

59.63% at 72 hrs. The same figures for the alcohol extracts are 60% at 66 hrs of treatment as maximum.

Digestive gland acetone extract showed comparatively less inhibition of area of SVV whereas alcohol extract has comparatively more enhanced inhibition.

Tertiary vitelline veins (TVV) :

In the entire experiments it is observed that tertiary vitelline veins are most affected and show maximum inhibition.

Maximum number and area of TVV was found to be inhibited when the embryos are treated at 48 hrs. with salivary gland extract in acetone and alcohol.

The embryos treated with salivary and digestive gland extract in acetone showed 68.68% and 80.32% reduction in number and area of TVV. Whereas alcohol extract showed 70.70% and 8032% depletion in the number and area of TVV respectively. Similar results with less intensity were observed in the digestive gland extract.

Both salivary and digestive gland extracts had profound effect of inhibition on the number and area of SVV of chick embryo.

Salivary glands and digestive glands had inhibitory effect on angiogenesis. The inhibitory effect of sulphated and non sulphated glycosamino glycans on the normal outgrowth of capillaries was tested in the chick embryo chorioallantoic membrane (CAM). The results show that sulphation of glycosaminoglycans and polysaccharides increases or induces an antiangiogenic effect (Hahnenberger, and Jakobson 1991). A potent angiogenesis inhibitor u-995 has been purified from the cartilage of the blue shark *Prionace*

gluca (Sheu *et. al.,* 1998). Above research indicate that sulphated and non sulphated mucopolysaccharides had an antigiogenesis effect. In the present investigation salivary gland and even digestive gland contain mucins and glycogen contents respectively in greater proportion.

Same of these complex carbohydrate might be playing a role in antiangiogenesis. More elaborate studies will be helpful to isolate and identify antiangiogenic factor from animal origin.

Conclusion :

In conclusion angiogenesis is influenced by salivary and digestive gland extracts of *A. pulmonata*. Decrease in angiogenesis is highly significant evidently 48, 55 and 72 hrs as compared to remaining hrs. HBSS independently stimulates angiogenesis. Comparatively salivary gland had more significant inhibition through alcohol extract than acetone extract. A complex carbohydrate may be in the form of sulphated or non sulphated micropolysaccharides and glycogen like molecule from digestive gland might be playing important role in antiangiogenesis.