ANGIOGENIC AND ANTIANGIOGENIC INFLUENCE OF STREPTOZOTOCIN AND INSULIN ON CHORIOALLANTOIC MEMBRANE OF CHICK EMBRYO


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Abstract: Angiogenesis, the development of new blood vessel from pre-existing vessels, is a key process in the formation of the granulation tissue during wound healing. The appropriate development of new blood vessels, along with their subsequent maturation and differentiation establishes the foundation of functional neo-vasculature. Angiogenesis has been evaluated by in vivo and in vitro systems using different types of endothelial cells isolated either from capillaries or large vessels. Therefore, in present study, the induction of angiogenesis by insulin and streptozotocin has been evaluated in chorioallantoic membrane (CAM) of the avian embryo. Streptozotocin and insulin were administered to two respective groups of 8 days old chick embryo by making window on the chorioallantoic membrane. Parallel control was maintained, where embryos received phosphate buffered saline (PBS) of pH 7.4. On 12th day of incubation window was opened to reveal the progressive blood vessels pattern formation in CAM in insulin treated embryos with the help of stereomicroscopic study. In controls, no such changes were observed. Biochemical study by spectrometric analysis and histological study was also carried out to understand the difference between insulin treated, streptozotocin treated and control embryos.

Key words: Chorioallantoic membrane, Streptozotocin, Insulin, Angiogenesis

INTRODUCTION

Angiogenesis, the process of new blood vessel formation, is a natural event that occurs under both normal and pathological conditions [1-4]. The activated endothelial cells migrate and proliferate to form new vessels. The endothelial cells are surrounded by layers of peri-endothelial cells, small blood vessels, which are surrounded by pericytes and large blood vessels, surrounded by smooth muscle cells [5]. The formation of blood vessels is thought to depend on a precise balance of positive and negative regulation, involving both stimulating and inhibiting factors [6,7].

In normal state, two distinct processes can be seen. One process utilizes endothelial progenitor cells, usually derived from bone marrow and initiate endothelial growth and vascular tube formation. The second process utilizes existing vasculature to generate new vessels, and is highly dependent on endothelial cell activation and protease secretion [8]. Under pathological conditions, many of the steps involved in normal vessel formation are repeated. However, the structures formed are often functionally abnormal, possibly due to an imbalance in the angiogenic process [9,10]. Multiple factors contribute to angiogenesis, including soluble growth and differentiating factors, extracellular matrix.
components, membrane-bound receptors, and intracellular signaling molecules [11]. Angiogenesis dependent diseases are controlled by using chemotherapy, immunotherapy and radiation therapy to inhibit the stimulating or stimulate the inhibiting factors [12-14].

One of the most frequently used angiogenesis assays is the chick chorioallantoic membrane assay (CAM assay) [15,16]. The extra-embryonic chick embryo chorioallantoic membrane is formed by fusion of the chorion and the allantois. It is in direct contact with the shell and contains a very thick capillary network [17]. The membrane can be used in vivo, which utilizes a sealed window cut into the shell [18] or in vitro [19].

Streptozotocin is an antitumour antibiotic consisting of a nitrosourea moiety interposed between a methyl group and a glucosamine. It is naturally occurring chemical that is particularly toxic to the insulin-producing β-cells of the pancreas in mammals [20]. It is used in medicine for treating certain cancers of the Islets of Langerhans and also used in medicinal research to produce Type I diabetes in animal model [21]. It is toxic to cells by causing damage to the DNA causing inhabitation of DNA synthesis. It is known to interfere with biochemical reactions of NAD and NADH, and inhibit enzymes involved in gluconeogenesis [22].

In present study the attempt has been made to understand the effect of streptozotocin and insulin on angiogenesis and antiangiogenesis on chorioallantoic membrane (CAM) of chick embryo. It was investigated whether streptozotocin acts as angiogenic inhibitor or stimulator. It is also possible to understand, whether it has prominent effect on development of major blood vessel and capillary plexus on CAM of chick embryo. It may able to add some knowledge on toxic effects of these compounds.

**MATERIALS AND METHODS**

Present study deals with the quantitative analysis of angiogenesis in chick embryo, by using Chorioallantoic membrane (CAM) base as the direct use of surfactants on the top of the growing CAM as a suitable method [23].

Sufficient numbers of fertilized, white, leghorn chicken eggs were wiped with a soft cloth to remove dirt-dust and unwanted materials. The eggs were then kept in egg incubator (Innovative India LTD. DTC-96) for incubation under constant humidity at 37°C. Constant humidity was maintained by keeping petri dish filled with water inside incubator. Eggs candling was carried out as per the standard procedure [18,24]. Fertility of fresh eggs has been identified by observing cell division prior to ovipositor, which results in blastoderm. The eggs were marked further in the position of blastoderm, which is facing towards sky with the help of nontoxic marker pen. These eggs were further kept in incubator for two days. On the 3rd day, eggs were again candled for confirmation of cell division which shows development of blasto-derm. These eggs were used for further experimental study.

On the 3rd day of incubation, windows were prepared and 2-3 ml of egg albumin was removed for the detachment of the developing Chorioallantoic membrane from the shell, windows were sealed quickly with cellophane tape and eggs were returned to the incubator for further incubation [18]. Three sets of eggs were prepared and on 8th day the windows were re-opened and 200 IU of streptozotocin solution was loaded on the CAM of set one. 200 IU of insulin was loaded on the CAM of set two and phosphate buffer saline (PBS) was loaded on set three as sham operated. These eggs again sealed quickly and returned to the incubator for further incubation. These Eggs were examined daily under egg’s candler to find out developmental changes in the chorioallantoic membrane (CAM). During the examination under egg’s candler, eggs showing dark patches on the shell were discarded and rest were continued to incubate. On the 10th day half of the eggs were used for CAM assay and other were continued to incubate till day 12th. On 12th day windows were opened again and CAM was observed for light and stereomicroscopic study. Streptozotocin, insulin and PBS treated CAM were fixed in Bouin’s fixative and processed further for histological studies. CAM from freshly open eggs was observed on stereomicroscope and photographs were taken [19,25].

**CAM assay**: CAM assays were carried out as per the standard protocol [26]. On 10th day eggs were divided in to three groups as streptozotocin, insulin and PBS treated. Each group was further divided into three sub-groups scontaining six CAM, likewise nine sub-groups were prepared. Three different
concentration of Trypan blue was prepared as 0.01%; 0.1%; and 1% respectively for the treatment on CAM. Windows were reopened and drops of sterile water were placed onto the shell membrane to avoid the capillary bleeding. 200 IU of insulin was loaded with the help of tuberculin syringe on each CAM. After 20 seconds each CAM was washed with sterile water. Immediately after 20 second each CAM were treated with Trypan blue. The group one was treated with 0.01% trypan blue (1mM). The treated sub-group-1, 2 and 3 were kept for 0.5 minutes, 1 minute and 2 minutes respectively. Group two and three were also treated similarly. The dyed CAM were excised carefully, separated, homogenized and extracted with formamide. The absorbent of the extracts were measured with the help of spectrophotometer (Thermo specectronic -20D) at 595 nm. All samples were run in duplicate.

RESULTS

Histological and stereomicroscopic image study: Histological sections of phosphates buffer saline (PBS) control CAM when observed on the 10th day of incubation showed the capillary plexus beneath the ectoderm, showing normal development in the large mesodermal vessels (Figs. 1,2). Stereoscopic microscope images of CAM of PBS control also showed normal development in the blood vessels pattern formation. It also showed dendritic branching pattern of major blood vessels in 10th day of control CAM and well developed capillary plexus with normal branching (Fig. 3). Histological sections of treated CAM show the remarkable changes in the capillary plexus after treatment of streptozotocin solution on capillary plexus, when observed on 10th day of development. The CAM showed some changes in formation of angiogenic cells. The large and small vessels in the mesoderm showed an immediate migration towards the ectoderm with definitive capillary formation. Overall structure indicated antiangiogenic effects, when compared with the control (Figs. 4,5). Stereoscopic microscope images of streptozotocin solution treated CAM when observed on 10th day of incubation showed negative effects of blood vessels formation. The dendritic branching pattern characteristic of major blood vessels in the 10th day on incubation of streptozotocin treated was affected as compared to control. Capillary plexus showed disruption of normal branching after the treatment. It appeared thick and coarse. Areas where plexus should form showed abnormal branching. It also indicated major disruption of branching after the treatment (Fig. 6).

CAM assay: The treatment of streptozotocin registered significant increase in all nine sub-groups of CAM absorbed Trypan blue. As the treatment period of trypan blue increased, absorption also increased. In group two, trypan blue absorption showed significant increase as compared to groups PBS control and streptozotocin treated CAM (Table 1). Histological sections of insulin treated CAM when observed on 10th days showed the effects on the capillary plexus. The treatment of insulin showed almost complete capillary plexus formation with appearance of numerous mesodermal vessels (Figs. 7,8). Stereoscopic microscope image of insulin solution treated CAM when observed showed progressive effects on blood vessel formation. The dendritic branching pattern formation was prominent as characteristic of major blood vessels. It also showed the development of capillary plexus with prominent branching formation after the insulin treatment (Fig. 9).

DISCUSSION

The chick chorioallantoic membrane (CAM) assay is a well-established model of angiogenesis that can be used to test pro- and anti-angiogenic conditions and molecules [18,27,28]. The allantois is an extraembryonic membrane, composed of endoderm and mesoderm, in which primitive blood vessels begin to take shape on 3rd day of incubation. Primitive vessels continue to proliferate into an arterio-venous system until 8th day, and the vascular system attains its final arrangement on 18th day, just before hatching. The main function of CAM is to mediate gas exchanges with the outer environment [29].

In the fasting state the plasma glucose level is maintained by glycogenolysis and gluconeogenesis. The main regulatory of these processes and ultimately of the plasma glucose level are under the influence of four hormones insulin, glucagon, adrenalin and cortisol [30-32]. Insulin increases malonyl-CoA (thus increasing fatty acid synthesis) and increases the glycogen stores; both actions reduce carnitine acyl transferase (CAT) activity and inhibit ketogenesis. Insulin is a hormone of the fed state and it is released in response to rising blood

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Table 1: Effect of Streptozotocin and insulin on Chorioallantoic membrane of chick embryo. Dependency of the amount of trypan blue absorbed (nmol) onto the CAM on the concentration of surfactant used. Mean ± SE of six eggs. Figure in parenthesis denotes number of estimations. Data analysed by ANOVAs analysis test. P > 0.05 i.e. P value is statistically significant. P < 0.05 i.e. P value is statistically non-significant. a = significant

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In present study histology of insulin treated CAM showed almost complete capillary plexus formation with appearance of numerous mesodermal vessels. Stereomicroscopic study of insulin treated CAM showed thick and coarse appearances of the blood vessel. Insulin can cause hypoglycemia in cell and can causes glycogenolysis. The treatment of insulin can stimulate uptake of glucose into the cell and can stimulate several anabolic processes by anabolic pathway [38]. The present observation indicated the effect of insulin on capillary plexus is mediated through insulin-like growth factor binding protein.

Explantation of figers:

Fig. 1: Histological sections of control CAM showing capillary plexus (arrow head) beneath the ectoderm. A large mesodermal vessel (arrow) representing its normal structure. (10 X). Fig. 2: Magnified portion of figure 1 of control CAM showing well-formed large mesodermal vessel with blood cells. (40 X). Fig. 3: Stereomicroscopic image of CAM of phosphate buffer saline (PBS) control showing normal development of blood vessels pattern formation. The dendritic branching pattern characteristic of major blood vessels in day of 10 control CAM’s. Arrow head denotes area where capillary plexus was developing, shows the normal branching (arrow heads). Fig. 4: Histological section showing effect of Streptozotocin solution on capillary plexus formation of day 10th of development. The CAM has clusters of angiogenic cells (small arrowhead). Note large and small vessels in the mesoderm (arrows) with little definitive capillary plexus immediately beneath the ectoderm. Overall structure indicates anti-angiogenic effect of Streptozotocin (STZ) treatment. (10 X). Fig. 5: Magnified photograph of the figure 4 showing anti-angiogenic effect on capillary plexus formation. Also note some mesodermal vessels (small arrow) migration that made contact with basal lamina beneath the ectoderm. Mesodermal blood vessels are present but are few and angiogenic clusters are rare. (40 X). Fig. 6: Stereomicroscopic image of CAM treated with Streptozotocin solution, showing progressive effects of Streptozotocin blood vessel formation. The dendritic branching pattern characteristic of major vessels is visible. Arrow heads denote areas where the capillary plexus should form but unlike the control, the vessel appeared thick and coarse, shows abnormal branching and normal dendritic branching is lost. Figs. 7, 8: Histological sections showing the effect of insulin solution on capillary plexus in CAM treated with insulin solution on day of 8 and examined on day 10. The capillary plexus is completely formed and many mesodermal vessels could migrate to ectoderm to form plexus. (X10 and X40). Fig. 9: Stereomicroscopic image of insulin treated CAM showing progressive effect of insulin solution on blood vessel pattern formation. The prominent dendritic branching pattern shown with characteristic of major blood vessel on day 8th of insulin treatment and the same were observed on day 12th. Arrow heads denote developing capillary plexus. Prominent branching formation shows after the insulin treatment. Arrow head denotes areas where the capillary plexus are formed which are more prominent than that of control.

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which may act as a suppressor by inhibiting angiogenesis [39]. The effects of insulin on the CAM assay indicated that insulin acts as pro- and anti-angiogenic [18,27,28,40]. The amount of trypan blue absorbed at the treated site is an indicator of the damage in the CAM after the treatment of insulin onto the CAM [26,41]. The treatment of insulin showed significant increase in trypan blue absorption with most significant increased with treated CAM as compare to streptozotocin and PBS control. The present observations indicated angiogenesis effects of insulin on CAM, which may promote the cellular changes in the blood vessel or it may also promote the cellular uptake of glucose, which may cause further hypoglycemia leading to blood glucose concentration below the normal range. From the far going it is clear that the insulin may have angiogenic and antiangiogenic effect on the chick chorioallantoic membrane [42].

Streptozotocin induced diabetes but it was also shown to increase mRNA levels in skeletal muscle or cardiac muscle. However, the protein content decreases in skeletal but remains unchanged in cardiac muscle [43- 45]. Further it can cause uncontrolled hyperglycemia which may result insulin deficiency [46, 47]. Streptozotocin can induce new kidney tumor and has ability to induce renal carcinomas in mice [48- 51]. It can also induce diabetogenic condition and can act as a diabetogenic agent. It can also cause hyperglycemia. Effect of hyperglycemia on angiogenesis in chick CAM assay was as a model of active neoangiogenesis [40]. Hyperglycemia decrease angiogenesis, therefore, impair angiogenesis without altering the expression level of vascular growth factor through induction of apoptosis and decrease proliferation of endothelial cells. It also affects arterioles and their side branches [40,52]. In present study histology of CAM showed the changes in the capillary plexus after treatment of streptozotocin which was observed on the 10th day of incubation. CAM also showed notable changes as formation of angiogenic cells. The large and small vessels in the mesoderm showed immediate migration towards the ectoderm with definitive capillary formation.

Stereomicroscopic studies of streptozotocin treated CAM showed effect on blood vessel pattern formation as the disruption of capillary plexus and in normal branching. The vessels appeared thick and coarse, with loss of small capillaries. Histological study of streptozotocin treated chorioallantoic membrane show dapparence of thick blood vessels as direct effect on capillary plexus formation and capillary development. The treatment of streptozotocin may cause hyperglycemia in the CAM of chick embryo and may leads to affect angiogenesis in the CAM. It may also cause active neo-angiogenic effect of Streptozotocin on model animal. The hyperglycemic condition may results into decreased angiogenesis. Present observations correlate with earlier observations, which were able to show that hyperglycemia can induce defects in angiogenesis in the CAM membrane in model animal [40]. It showed immediate migration towards the ectoderm, indicating antiangiogenic effect. Stereomicroscopic study showed disruption of normal branching after the treatment with changes in branching pattern formation, which may relate to the effect of streptozotocin on CAM as hyperglycemic. It further suggested that hyperglycemia may induce defects in angiogenesis after the treatment of this drug. The present study also corelates with the earlier observations made by Engerman, [52] who showed that the treatment of streptozotocin induces the hyperglycemic condition.

In vitro test on chorioallantoic membrane of chick as a respiratory organ serving for sensitivity test in chick lung, and complete tissue with blood vessel can provide different evidence on toxicity of chemical [26,54]. Trypan blue staining is one of the methods in order to measure quantitatively the damage occurred in the CAM. Aqueous trypan blue can be absorbed by the surfactant of the CAM. The amount of absorption depends on the concentration and period of the treatment [26,41]. The trypan blue staining method is a promising test to predict the toxicity of wide variety of chemicals [26,40,53]. In present study the treatment of streptozotocin registered significant increase in all nine sub-group of CAM which was treated with trypan blue. With the increase of trypan blue treatment period, the absorption is also increases. The amount of trypan blue absorbed at the treated site as an indicator of damage to the CAM [26,41]. Trypan blue employed in this study have been widely used as reagent that distinguish between the live or dead cells, which showed that the trypan blue method as a promising test to predict the toxicity and also the effects of various chemicals on angiogenesis [26,41].
Significant increase in trypan blue absorption after streptozotocin treatment to the CAM, indicated the damage caused to the chick chorioallantoic membrane. The present results also correlate with the earlier observations made by Hagino et al. [26] on trypan blue absorption study, as an indicator to the damage caused by the treatment.

CONCLUSION

Streptozotocin causes hyperglycemia, which may induce defects in angiogenesis in the chick chorioallantoic membrane. It can act as anti-angiogenic agent that causes specific anti-angiogenic effects which may directly inhibit growth of capillaries. It may also cause non-specific anti-angiogenic effects that could suppress neurovascularization indirectly via the inhibition of angiogenesis. However, insulin may promote the cellular changes in the blood vessel or cellular uptake of glucose, which could lead to angiogenesis and antiangiogenesis in the chick chorioallantoic membrane.

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REFERENCES