Cancer Research Journal
2014; 2(3): 47-62
Published online June 30, 2014 (http://www.sciencepublishinggroup.com/j/crj)
doi: 10.11648/j.crj.20140203.12

Assessment methods of angiogenesis and present approaches for its quantification

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To cite this article:
Ghulam Jilany Khan, Lubna Shakir, Sara Khan, H. Sadaf Naeem, Muhammad Ovais Omer. Assessment Methods of Angiogenesis and Present Approaches for Its Quantification. *Cancer Research Journal.* Vol. 2, No. 3, 2014, pp. 47-62. doi: 10.11648/j.crj.20140203.12

Abstract: Angiogenesis is the extension of blood vessels from the prevailing vessels. It is an essential process in the formation and development of blood vessels, so it is supportive in healing of wound while its inhibition may help to restrict the size of tumor. To quantify the effect of an agent on angiogenesis, a standardized preclinical screening assay is necessary. The objective of this review is to document all the methods of angiogenesis assessment and specific techniques to quantify the results, majorly focusing on the modes of calculation and concerns that researchers need to make before reaching to any conclusion. Scientific research on angiogenesis relies on different models for assessment, including *in vitro, in vivo* and *in ovo* models. Many of these assays are practiced to test the effect of both pro and anti angiogenic agents. The findings articulated in this study will help to provide better visions into the pathophysiology of diseases associated with angiogenesis and to establish new as well as appropriate angiogenic or anti angiogenic treatment strategies for the future. Nevertheless, angiogenesis is a vigorous process and the techniques to evaluate this process with thorough assessment of physiological characteristics of new blood vessels will be advantageous over the older systems of angiogenesis assessment methods.

Keywords: Angiogenesis, Angiogenesis Assays, Anti-Angiogenic, Quantification Techniques, *In vitro, In vivo, Inovo*, Treatment Strategies, Blood Vessels, Healing, Non-Invasive

1. Introduction

Angiogenesis is a combination of two Greek words, “angeion” and “genesis” means vase and birth respectively. It is a common and most important process in the formation and development of vessels, so it is supportive in healing of wound and granulation tissue called physiological angiogenesis[1]. Pathological Angiogenesis is associated with many diseases including retinopathies, arthritis, and psoriasis [2]. To maintain natural balance between formulation and inhibitory factors, body controls angiogenesis. Disturbance in this balance results in either excessive acceleration in growth or too much inhibition of formation of new blood vessels. The general concept is that development of tumor is dependent on angiogenesis and needs vascular growth. In the absence of vascular growth the tumor will not be malignant and remain inactive [3]. That’s why the anti angiogenic drug development is of great interest for therapeutic purposes against cancer.

Basically the process of angiogenesis is divided into 2 main therapies which are anti-angiogenic and pro-angiogenic. Anti angiogenic means reduction in formation of blood vessels which is main step in reduction of tumor growth [4] and this process requires lots of oxygen and nutrient to proliferate. It was first time studied by Folkman in 1971 that before the initiation of angiogenesis process, the growth of solid tumors remain restricted to 2-3

millimeter in diameter [5]. Tumor derives blood supply from neighboring tissues which is an important step in tumor growth [6]. Pro-angiogenic therapy is explored for the treatment of various diseases like cardiovascular disease and coronary artery disease. Further studies on angiogenesis proved its worth in wound healing disorders and atherosclerotic diseases [7].

The tumor vasculature comprises of vessels which are formed from the preexisting nearby vessel network of the body. This tumor vasculature is a result of angiogenesis stimulation response of the nearby host vessels to cancer and stroma cells. The observations that a neoplastic mass cannot grow beyond a few millimeters in diameter without recruiting new vessels and that blood-borne metastasis cannot be initiated unless the neoplastic cells have access to blood vessels have led to significant efforts in angiogenesis research [8].

Angiogenesis is also a vital step in the evolution of cancer from inactive to malignant state, suggesting the use of angiogenesis inhibitors. A renowned surgeon, John Hunter, in 1794, mentioned the growth of blood vessel growth in reindeer antlers and used the term angiogenesis for this phenomenon [9] . Later on Arthur Hertig who was a pathologist used this term for medicines in 1935. After a brief description of relationship between angiogenesis and tumor growth by Surgeon Judah Folkman angiogenesis became a known word by the community of biomedical sciences. Dr. Judah Folkman was the pioneer of research in tumor angiogenesis [10] which was started in 1960's while the angiogenic diffusible factors were initially identified by Greenblatt and Shubick[11]. Dr. Henry Brem and Dr. Judah Folkman discovered the first anti-angiogenic agent in 1975. In 1980's, vascular growth factors were identified which proved the importance of concept of angiogenesis in animal models. In 1990's, Clinical Trials of angiogenic inhibitors were conducted which were not very successful. In 2004, FDA approved the role of Bevacizumb an angiogenic inhibitor for metastatic colorectal CA. In 2007, Bevacizumb and irinotecan proved efficacious for glioblastoma[12].

Sprouting angiogenesis and intussusceptive angiogenesis are the two major types of angiogenesis. Sprouting angiogenesis occurs when angiogenic factor acts on receptors of endothelial cells which release protease enzyme that degrade the basement membrane to let endothelial cells flourish into surrounding matrix to form compact buds connecting adjacent vessels. These little buds (sprouts) form loops to become lumen as cells migrates to the site of angiogenesis. Intussusception is also known as splitting angiogenesis. In intussuseeption, the capillary walls extend and divide the vessel into two branches[13].

There are two major angiogenic factors, Fibroblast growth factor (FGF) is the firstly discovered angiogenic factor with its prototype members FGF1 and FGF 2. Second angiogenic factor is vascular endothelial growth factor (VEGF). It increases the number of capillaries in a given network. Almost all tissues develop vascular network which provide them nutrients, oxygen and enable them to eliminate metabolic wastes. Once formed the vascular network is firm enough that it can regenerate slowly.

Procedure of maturation of vessels is a step wise alteration from actively growing vessel bed to the quiescent fully formed and functional network [14]. The balance between inhibiting and activating factors controls the tissue activity of angiogenesis. Signaling system of angiogenesis for regulation of creation and migration of endothelial cells, providing the basis for the formation of vessel, are vascular endothelium growth factors (VEGF) and their receptors. The formation of the embryonic vascular system is mainly dependent on this VEGF signaling system. During the formation of tumor, the neoangiogenesisis also related with this signaling system activation. The different factors and receptors of VEGF family were the key factors of this system. Various other signaling systems also participate actively in regulation of the important steps of development of vessels still VEGF remains a major factor for endothelial cells in *in vivo* and *in vitro* for the formation of blood vessels [15]. Specific signaling system Dll4/Notch controls the selection of endothelial cells to start angiogenesis. Vascular wall formation is another important step in the vessel formation for which the involvement of mural cells (smooth muscle cells and pericytes) is required. Vascular wall formation involves a couple of particular signaling system of PDGFB/PDGFRβ (Platelet derived growth factor subunit B/ PDGF receptor β) as well as angiopoietins system (Ang1, Ang2, and their receptor Tie2). Thorough investigation on these key molecules may lead to the development of new techniques to study angiogenesis (Figure 1).

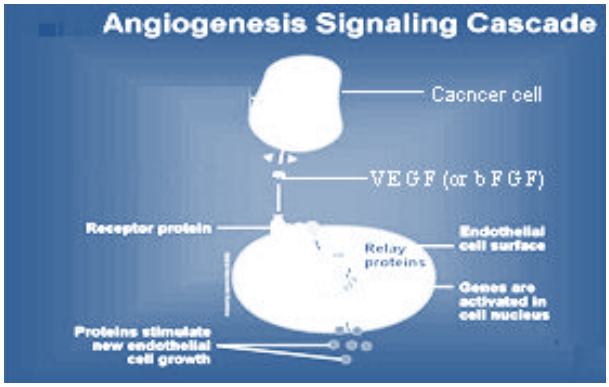


Figure 1. *Chemical signaling of angiogenesis (courtesy to National Cancer Institute)*

Angiogenesis is regulated by a complex cascade of cellular and molecular events. There is a balance of angiogenesis inducers and inhibitors in our body. In order to control the excessive angiogenesis in our body some angiogenesis inhibitors are usual part of the body's control mechanisms, some are recommended as drugs, and few are present in our daily diet. Initially these Angiogenesis inhibitors were called as a "silver bullet" due to their use in the treatment of various cancer types, but in practice this

term has not been used. The drugs used to inhibit these blood vessels are also used to treat cancer. Endogenous inhibitors of angiogenesis are VEGFR-1, Angiopoiten2, Angiostatin, SPARC (scalable processor architecture), Prolactin, Maspin and Vasostatin while exogenous inhibitors are Bevacizumb, Itraconazole, Thalidomide and Prolactin [16].

Various studies were conducted to study angiogenesis. Thousands of articles have been published in different scientific journals to prove the effects of angiogenesis in different life threatening diseases [17]. In 1971, 3 articles in Pubmed on Angiogenesis were published out of which 2 were from Folkman's lab. Till 1981, 34 research articles on angiogenesis were published. Till 2011, 50,967 researches had been conducted out of which 27,287 studies were related to cancer.

It is believed that angiogenesis, in which vascular supply from adjacent tissues is derived by cancer cells, is a vital step in growth and metastasis of tumor [6]. With the help of angiogenesis process, cancerous cells got the sufficient supply of nutrients and oxygen. When a tumor cell has multifaceted system of blood capillaries, the shedding of the cells from the primary tumor begins [18].

Angiogenesis proceeds side by side along the development of new tissue (granulation tissue) to heal wound [19]. Angiogenesis is stimulated by the certain factors which are released by the wounded tissue [20]. This whole process is regulated by cell-ECM (extracellular matrix molecule) interactions, cell-soluble factor interactions and cell-cell interactions [20]. A blood capillary consists of a hollow tube lined with endothelial cells. Collagen IV which is the main component of outer basement membrane of tube consists of fibronectin and proteoglycans. When degradation of these basement membranes starts, the process of angiogenesis also initiates which is further followed by the process migration of endothelial cells out of these vessels [19].

Inflammation and angiogenesis both can stimulate each other. Angiogenesis can help in the process of innervations of articular cartilage, a source in osteoarthritis pain. Pain can also be stimulated by the sensitization of sensory nerves by inflammatory mediators and these sensitized nerves can be a source of neurogenic inflammation and start angiogenesis [21].

For the management of cardiovascular disease, Angiogenesis could be an excellent therapeutic target. It is a potent, physiological process that helps to counter by means of production of new collateral vessels in certain conditions when blood [22].

As angiogenesis plays a vital role in the pathophysiology of many diseases, scientists are trying to utilize this knowledge for the development of new treatment strategies. For example, the prohibition of angiogenesis has been used to arrest the growth of tumor and metastasis, similarly in case of rheumatoid arthritis where the focus is to minimize the infiltration of inflammatory cells and soluble mediators, treatment strategy based on angiogenesis could be a successful therapy [23]. Impaired development of new blood vessels has been observed in many diseases. For example, restricted growth of blood vessels is associated with peptic ulcers and bowel atresia [24]. Increased vascular growth has been verified in non-malignant diseases such as, systemic lupus erythematosus, rheumatoid arthritis, psoriasis, atherosclerosis and proliferative retinopathy [25]. In1994, The Angiogenesis Foundation used a term 'common denominator' for angiogenesis while describing most prevailing diseases of society. Further studies showed that in particular diseases states, the body loses control of angiogenesis which exhibits the necessity and importance of studies on angiogenesis. This clearly shows the further need of research in this field which may be fruitful to develop good therapies. Several Diseases associated with angiogenesis are given in table 1.

Table 1. *Diseases associated with angiogenesis*

Excessive	Insufficient
• Cancer	• Stroke
• Rheumatoid arthritis	• Infertility
• Aids complication	• Scleroderma
• Psoriasis	• Ulcers
• Blindness (Diabetic retinopathy)	• Heart disease

In comparison of conventional chemotherapeutic drugs, Anti-angiogenic agents may be less toxic. Treating with anti-angiogenic agents patients may not experience certain side effects like uncontrollable vomiting, or diarrhea and hair loss. However treatment with anti-angiogenic drugs would be very dangerous if given to pregnant women and also have a potential to stop the normal reproductive cycle in women. Still other side effects of treatment with anti-angiogenic agents in people are being determined. Different researchers at the FDA are monitoring and establishing the database of these side effects to assess the risks associated with anti-angiogenic therapies [26].

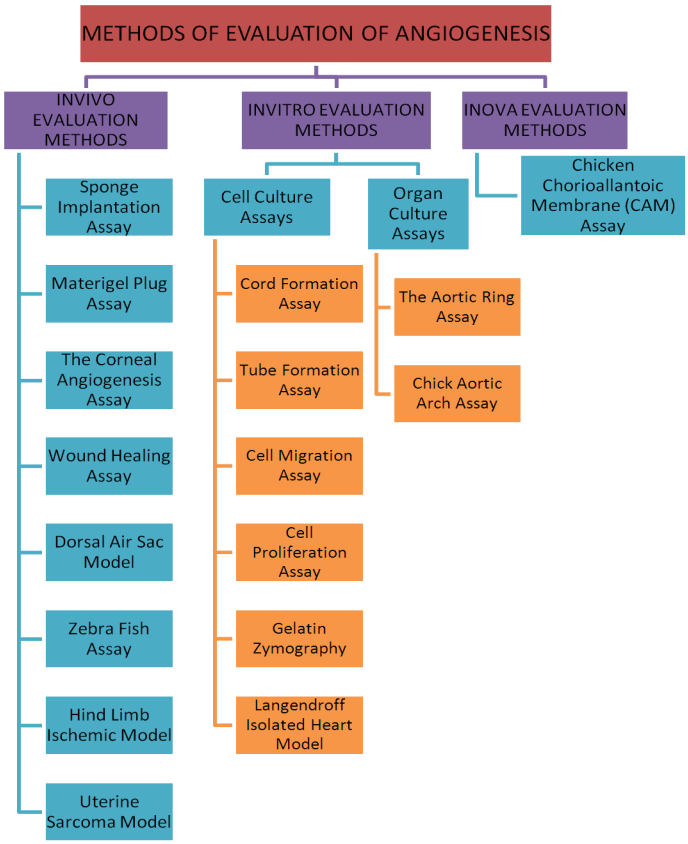
The major problem in the field of angiogenesis studies is the accurate interpretation of the highly fluctuating and inconsistent results acquired from different tests which are currently in practice. This assessment by Auerbach and his collogues in 1996 showed a clear gap for the development of new strategies and to establish step wise guidelines of each assay which are in practice to analyses the angiogenesis studies[27].

The potential benefits include the ability to evaluate the new natural and therapeutic remedies for patients suffering from different diseases and to whom medical therapy is ineffective. Furthermore, by gaining an understanding of how these growth factors work in the human we shall be able to further intensify our understanding of a process whose mysteries carry the potential to eradicate a great number of diseases that afflict mankind, especially cancer and ischemia [28].

2. Methods of Evaluation of Angiogenesis

One of the most technical challenges in the studies of angiogenesis is selection of the appropriate assay. There are many *in vitro*, *in vivo* and *in ovo* angiogenesis assays. It has been proved from previous researches that it is necessary to use a combination of assays for accurate identification of the cellular and molecular events in angiogenesis and to find complete range of effects of a given test compound (Table 2).

Table 2. *Methods of evaluation of angiogenesis (Curtsey to Yousaf M.Q.)*



The following methods have been used to quantify image obtained from light microscope observations: (a) blood vessels enclosed in a silicone ring laid randomly in a treated zone are manually counted [29] ; (b) binary images of vessels are manually drawn or automatically thresholded and then global parameters like vessel areal density [30], vessel length density [31], fractal dimension [32] or complexity measurements [33] are determined; (c) individual parameters like the end-points, number of drawn vessels [31] and the length density of radially arranged capillaries during vasculogenesis [34] are determined. This last method, which is completely automatic, is appropriated to characterize large changes in vascularity. From scanning electronic microscopy, mercox cast images are used to determine automatically vessel areal density and pillar mean area. However, the number and the mean diameter of vessels were still manually determined [35] (d) Enzyme Linked Immuno Sorbent Assay (ELISA) of quantification can be used for the estimation of angiogenic growth factors. This assay is performed by using specific angiogenic growth factor ELISA kit according to the manufacture's protocol. The mostly 96-well micro liter plates are used for this study. (e) Hemoglobin Determination in Matrigel Plugs is particularly for the quantification of matrigel plug assay. In this method the Matrigels are dissected from the mice and weighed. Then they are homogenized for 5-10 min in ice at 10,000 rpm on micro centrifuge for 6 min and supernatant is collected for hemoglobin measurements. The supernatants are mixedwith Drabkin's reagent and hemoglobin in the samples is quantified calorimetrically at 540 nm in spectrophotometer [36], this method was easy and not as much of cost. (f) Capillary Density Estimation.A previously developed Krogh-type theoretical model was used to estimate capillary density in human skeletal muscle based on published measurements of oxygen utilization, arterial partial pressure of oxygen, and blood flow during tough workout. The model assumes that oxygen intake in tough workout is limited by the ability of capillaries to deliver oxygen to tissue so it is strongly reliant on capillary compactness, defined as the number of capillaries per unit cross-sectional area of muscle.

3. *In Vivo* Evaluation Methods

In vivo tests are not easy to perform and take more time than *in vitro* assays. The process of quantification is also more complicated. However, these *in vivo* assays are vital because of the complex nature of vascular responses to test reagents [37], that no *in vitro* model can fully achieve.

The earliest of these *in vivo* assays involved the preparation of diffusion chambers made with Millipore filters[38], as well as various other chamber techniques designed to monitor visually the progress of neovascularization of implanted tumors. Histologic observations even provide more complete information regarding *in vivo* angiogenesis studies [39]. Improved techniques for monitoring blood flow by Doppler or radiologic approaches augment the information available from standard histologic observations [40]. Different types of *in vivo* angiogenesis assays include Sponge Implantation Assay, Matrigel Plug Assay, The Corneal Angiogenesis Assay, Wound Healing Assay and Dorsal Air Sac Model.

3.1. Sponge Implantation Assay

Sponge implantation model has been optimized and adapted to characterize essential components and their roles in blood vessel formation in a variety of physiological and pathological conditions. As a direct consequence of advances in genetic manipulation, mouse models (i.e., knockouts, severe combined immunodeficient [SCID], nude) have provided resources to delineate the mechanisms regulating the healing associated with implants. However, differences in shape, size and composition of these sponges make it difficult to compare. Furthermore, implantation if these sponges can cause nonspecific immune responses that may themselves lead to an angiogenic response[41].

Procedure

In this method the sponge is prepared by using sterile absorbable gel foam. The gel foam is cut and treated with sterile agarose along with the test substance which is used for angiogenesis study. The animals are anaesthetized and an incision is given at midline and gel piece is inserted at subcutaneous layer. Animals are endorsed to improve and at 14th day the animals are sacrificed with an excessive dose of sodium phenobarbitoneand gel foams are harvested from the mice carefully without any remaining of the peritoneum [42](Figure 2). Then these sponges are weighed by placing them into pre weighed 1 ml tube of double distilled water and for homogenization kept on ice for 5 to 10 minutes. The supernatant of the sample is collected by centrifugation with a speed of 10,000 revolutions per minute (rpm) on a micro centrifuge for 6 minutes. The resultant supernatant is used to measure the hemoglobin level by filtering it through a 0.22 µm filter. Fifty micro liters of supernatant are mixed with Drabkin's reagent, and kept at room temperature for 15–30 minutes. Hemoglobin (Hb) in the samples is then quantified[43].

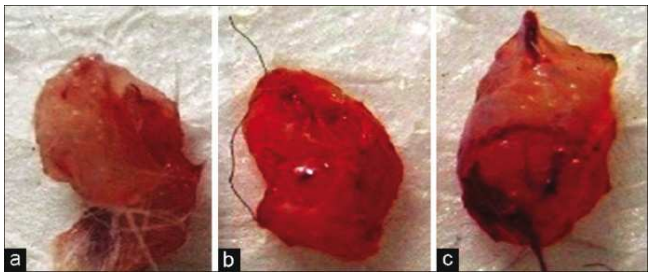


Figure 2. *Sponge implantation method*[90]

Quantification

Neovascularization can be assessed by a variety of methods including immune histological staining (e.g. the CD31/34 or integrin status of vessels in the sponge), the levels of a radioactive tracer in blood and the blood/ hemoglobin content of the sponge [44]. Hemoglobin (Hb) from the samples is quantified calorimetrically at 540 nm in a spectrophotometer. The resultant level of hemoglobin in the sample is then compared with a known amount of hemoglobin assayed in parallel and the results are expressed as Hbµg/mg [43].

3.2. Matrigel Plug Assay

The matrigel plug angiogenesis assay is a simple *in vivo* technique to detect the formation of new blood vessels in the transplanted gel plugs in nude mice. The matrigel matrix is mainly derived from the engelbroth-holm-swarm of mouse sarcoma, and its composition is similar to the basement membrane proteins. The matrigel can induce differentiation of a variety of cell types such as mammary epithelial cells, hepatocytes, and endothelial cells. Matrigel plug assay has become the method of choice for many studies involving *in vivo* testing for angiogenesis [45]. This assay permits a more precise visualization of the angiogenic process. It provides accurate information, no histological analysis is required for this and protocols of image analysis lends itself to photographic documentation (Figure 3).

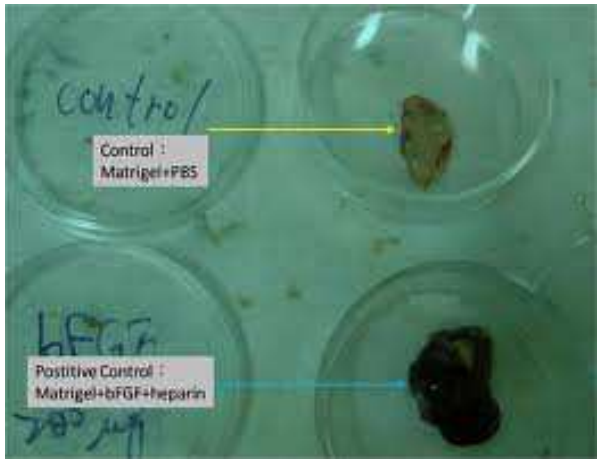


Figure 3. *Matrigel plug method*[91]

Procedure

Apparatus required for this assay includes *Nude* female mouse, 6 to 8 week-old, Matrigel matrix, Formalin solution-neutral nude buffered 10%, Anti-rat CD34, Biotin goat Anti-Rat IgG, Streptavidin-peroxidase conjugate, DAB (diaminobenzidine), Tumor cells, Paraffin, Hematoxylin and eosin, AperioScanscope CS-S microscopic slide scanning system, Tumor cell culture set up, Hemocytometer, Centrifuge machine, 24G syringe.

In this assay, angiogenesis-inducing compounds such as bFGF or tumor cells are introduced into cold liquid Matrigel which after subcutaneous injection, solidifies and permits penetration by host cells and the formation of new blood vessels., while in parallel pro-angiogenic or anti-angiogenic agents (test material) is added to the matrigel (forming the single solid gel plug) and is injected into the subcutaneous space of the animals of other group. The stimulation of formation of the new blood vessels invading the Matrigelis observed. The Matrigelis then harvested and the new vessel formation in the plug is assessed[46].

Quantification

Different methods have been used to quantify tubule formation, yet no method has been considered as the best quantification method for assessing the value of angiogenic stimulants or inhibitors in this Matrigel plug assay. Quantification of angiogenesis in this assay is achieved either by measuring hemoglobin or by scoring selected regions of histological sections for vascular density. However, the hemoglobin assay may be misleading because blood content is much affected by the size of vessels and by the extent of stagnant pools of blood [45].

3.3. Corneal Angiogenesis Assay

During the process of formation of new capillaries, the role of various cells, growth factors, tissues and some other factors can be determined with the use of the corneal angiogenesis assay. The original method was developed for rabbit eyes[47], but has been adapted to mice [48]. The basis of cornea assay is the induction of an angiogenic inducer (tumor tissue, cell suspension, growth factor) into a corneal pouch to produce vascular outgrowth from the limbal vasculature of surroundings. The cornea assay has an advantage of evaluating the new formed blood vessels as the cornea is avascular in the beginning. It is a distinctive feature of this assay. Improper use of the assay coupled with lack of thorough evaluation has generated unrelated, confusing, and even wrong results (Figure 4).

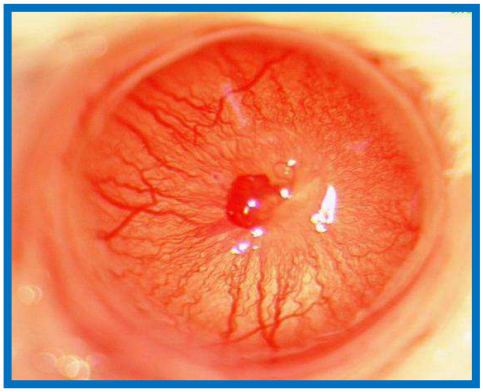


Figure 4. *Corneal angiogenesis assay*[92]

Procedure

A pouch is created in the cornea and the tumor or the tissue to be tested (test substance, when introduced in this pouch, causes the ingrowth of new blood vessels from the peripheral limbal vasculature. Slow release substances like ethylene vinyl copolymer or Hydronis used for this introduction [49].

Quantification

The quantification procedure comprises the measurement of the area of vessel penetration, advancement rate of vessels under the influence of angiogenic stimulus, in case of fluorescence, histogram analysis or pixel beyond the threshold (background) is recorded. The vascular response can be monitored by direct observation throughout the course of the experiment [50].

3.4. Wound Healing Assay

It is simple, less expensive, and an earliest proposed method. It is based on the opinion of cell migration into a wound which is formed on cell monolayer [51]

Procedure

Two circular holes of approximately 5 mm in diameter are punched with a tissue puncher through the dorsal skin of an anesthetized mouse. Wound size, scar formation and re-epithelization of the wounds should be recorded daily by photography and by measuring the wound area with calipers. Treatment can consist of pro-or anti-angiogenic compounds and their effects on angiogenesis is determined post mortem after the regenerated tissue has been excised, fixed and stained. Transgenic or knock-out mice can be used for study of the specific effects of specific genes (Figure 5).

Figure 5. *Inflammation, tissueformation and remodeling*[93]

Quantification

The impact of different culture conditions can be assessed by spotting the change in the size of the wound area. For further examination, more detailed measurements of the cellular behavior are mandatory. An application of spontaneous cell tracking in phase contrast microscopy images to wound healing assay has also been recently introduced. In which the performance of cell under three different culture conditions is investigated. This cell tracking system can chase individual cells during the process of healing and provides elaborative spatio-temporal measurements of cell behaviors including the density of cell its mitosis event's statistics and even its migration rate and direction too.

3.5. Dorsal Air Sac Model

The dorsal air sac model is used to examine the *in vivo* effects of substances against the angiogenic response triggered by cancer cells [52]. This idea was initially presented by Algire[53]and the procedure was developed by Selye by means of monitoring the vascularization of tumor grafts [54]. This assay is relatively easy to test, still the care should be practiced to not to exasperate the external layer to which the chamber is placed, because it may produce false results. It provides continuous non-invasive monitoring of vascular networks *in vivo* for long duration and also helps to explain the physiological properties of new blood vessels [41].

Procedure

Both sides of a Millipore ring are covered with filters (0.45mm pore size) and the resultant chamber is carefully filled with a tumor cell suspension; this is then implanted into the preformed dorsal air sac of an anaesthetized mouse. Following treatment with the compound of interest, the chamber is carefully removed and rings of the same diameter placed directly upon the sites that were exposed to a direct contact with the chamber. Following treatment with the multifarious of interest, the compartment is carefully detached and rings of the same diameter placed directly at the position where the compartment is attached.

Quantification

The results of angiogenesis can be evaluated by using a dissecting microscope and calculating the blood vessels which are newly formed within the observational area highlighted by means of ring. Still, it is difficult to differentiate between the pre-existing and new blood vessels. In recent practices, Evans blue is injected into the mice, which leaks out of the angiogenic vessels and accumulates in interstitial spaces whereas this dye is retained within the pre-existing vessels. The accumulation of this dye in the interstitial spaces is then considered as a semi-quantitative measure of angiogenesis. This quantification technique is not very much accurate it only gives a relative measurement [55].

3.6. Zebrafish Assay

Zebrafish (Daniorerio) is a tropical freshwater fish. It is used as a model system for studies of developmental biology and new drug discovery [56]. This fish is progressively in use for the studies on angiogenesis because of some unique characters of development of blood vessels, such as the dorsal aorta, posterior cardinal vein, sub intestinal veins and inter segmental vessels in fish embryo. Zebra fish produce hundreds of offspring per week which increases the availability of experimental objects at large scale for research purposes at a very low cost [56].

Procedure

In this assay, early embryos of zebra fish are used which range from a size of 1-2mm. in order to test lipophilic substances, 15-25 embryos are put into a well and test substance is inserted along with water. Proteins/peptides or compounds of high molecular weight may not be tested this way. To test prteins/peptides or the compounds of high molecular weight must be injected into the yolk sac of embryo after 20 hours of fertilization. The embryos are allowed to grow and after particular period, the development of blood vessels is studied which on comparing with control group clearly indicates the effects of test substances on angiogenesis [57]. Dorsal aorta and inter segmental vessels can be easily monitored[58].

Quantification

The embryos of zebrafish are transparent which make the direct observation of development of blood vessels more easy and exact with a simple low-power binocular microscope. Furthermore, some of the simple techniques like microangiography or endogenous alkaline phosphate staining of blood vessels are also very much helpful for the quantification of angiogenesis. Scientists have also developed new transgenic zebrafish lines with fluorescent (GFP-labeled) blood vessels, which have significantly streamlined the imaging of vessels which is quite helping in quantification [59].

3.7. Hind Limb Ischemia Model

It is observed that blood vessels can amend and alter in unfavorable conditions or stimuli to compensate the blood supply to the tissues. Keeping this theory in mind different scientists developed a model to study angiogenesis and arteriogenesis. For this purpose, the researchers use the ischemic hind-limb model of rabbit. It is an animal model in which ischemia is produced by femoral artery ligation [60].

Procedure

The rabbit is anaesthetized and an incision is made on the skin overlying the middle portion of the hind limb of the animal. After the incision proximal end of the femoral artery and distal portion of saphenous artery are identified and ligated. The small capillaries and side branches of the arteries are separated to make them more prominent and observable and at the end overlying skin is then closed [61].

Quantification

Test substance can be either injected into the hind limb artery or can be given orally. The comparison of development of blood vessels can be made between treated and controlled group of animals. In order to quantify the progress in blood vessel formation, an intra-arterial bolus injection of the vasodilator sodium nitroprusside (300µg in 1 ml saline), iodinated contrast media (Isovue-370) is infused intra arterially at a constant rate of 60 ml / min for 5s. The hind limb of the rabbit is then positioned 20 cm below the output beam of a fluoroscope (General Electric, Steno scope). Perfusion of the hind limb arteries is observed on a monitor in real time. Images are taken exactly 4 seconds after the start of contrast media infusion. These images are then analyzed to determine the development and growth progress of collateral vessels [62].

3.8. Uterine Sarcoma Model

On the basis of histology, Uterine sarcomas can be divided into four subtypes:endometrial stromal sarcomas,carcinosarcomas,adenosarcomas anduterine leiomyosarcomas (ULMS)[63]. Uterine carcinomas are found in the main body of the uterus only [64]. Yevgeniya and his colleagues developed a genetically engineered mouse tumor model in which the female mouse developed uterine tumors. This tumorpossesses the same histology as that of of human ULMS. This mouse is designed with specific genetic modifications that alter expression of oncogenes or tumor suppressor genes [65].

Procedure

In Uterine Sarcoma model Yevgeniya and his colleagues used female mouse and developed uterine leiomyosarcomas. Tumor suppressor gene p53 and/or BRCA1 were conditionally inactivated with the use of tissue-specific Cre-Lox system where Cre is driven by the female reproductive system-specific Amhr2 promoter [65]. The conditional deletion of p53 in the female reproductive tract results in ULMS development and concurrent inactivation of p53 and BRCA1 further augments tumor progression by means of angiogenesis [66]. In this way a tumor is developed in the mouse and then the effect of the test material on tumor angiogenesis is evaluated by means of color Doppler ultrasound imaging technique [67].

Quantification

The effect of low-intensity ultrasound irradiation can be observed over a short time period. It was firstly experimented and investigated by the Makoto with IC50 of low-intensity ultrasound against sarcoma cells**.** Based on the IC50, only the tumor site received radiation (using Sonitoron 2000: Richmar, USA) for 3 minutes, 3 times a week, under the condition of 1MHZ, 50%-DF and 2.0 w/cm2 *in vivo*.The results of development of blood vessels around the tumor were then obtained by color Doppler ultrasound imaging and quantified on the basis of prefixed controlled scale [68].

4. *In Vitro* Evaluation Methods

In vitro method of evaluation is of great importance which can be practiced in short period of time. If quantified thoroughly and properly it may produce accurate and reliable outcome. *In vitro* kinds of tests are observable at its best as they provide valuable initial information. Multiple tests should be conducted to obtain maximally reliable outcome [69].

4.1. Cell Culture Assays

Endothelial Cells are the most important tool for *in vitro* studies of angiogenesis. Whereas its use in *Invivo* models for rapid and objective testing of anti or pro-angiogenic compounds is infrequent because grafting human endothelial cells that establish a human vasculature in mice is unique and highly skilled technique. ProQinase has combined these features in its spheroid-based *in vivo* angiogenesis assay to allow fast *in vivo*testing and screening of antibodies and compounds. Human umbilical vein endothelial cells (HUVEC) are the commonly used human endothelial cell for *in vitro* angiogenesis assays. These are easily isolated by perfusion of the umbilical vein by the enzyme collagenase or trypsin and have been successfully cultured since 1973 [70]. However, as the process of angiogenesis involves the microvasculature instead of macrovasculature, these are far from ideal circumstances because of the basic difference in lineage and may lead to inadequate or erroneous responses.

Sources of endothelial cells

Large / Macro-Vascular Sources	Small / Micro-Vascular (HMVEC) Sources
Aortic (e.g., HAEC), Vein (e.g. HUVEC), Umbilical pulmonary artery	Brain Lung Dermis (e.g., HDMEC) Myocardium

One problem with endothelial cell assays is the phenotypic differences in endothelial cells which are not of same kind always. This difference can be clearly observed between the endothelial cells of large vessels and endothelial cells of microvascular origin for example Human umbilical vein endothelial cells and human dermal microvascular endothelial cells respectively[71]. In addition, another limitation is that *in vitro* endothelial cells may exhibit altered characteristics when compared the resting condition with flowing culture conditions as well as when put in comparison by means of attachment to different matrices. Therefore even if *in vitro* analysis methods are quick, easily assessable and dependably reproducible, it bounds the study of the complex physiological communications which take place *in vivo*. Furthermore, the valuations of secondary effects of a compound (which are employed on non-endothelial cells in turn producing chemicals which act on endothelial cells) are difficult to imitate. So *in vitro* assays should be carried out using endothelial cells from more than one source, or more importantly, be followed up with one or more *in vivo* assay of angiogenesis.

4.1.1. Cord Formation Assay

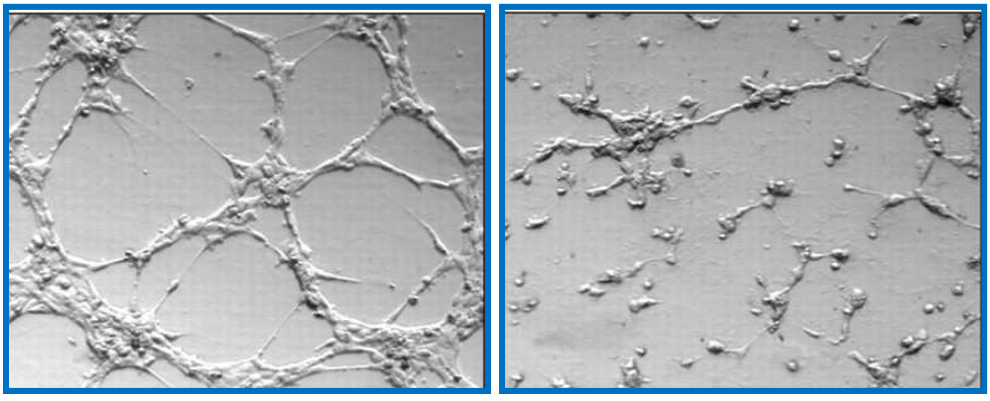
It is a quite simple and easily executable method.

Procedure

In this assay the growth factor reduced Matrigel is used. The endothelial cells are incubated in 1% FBS-containing growth medium for 12 hrs. Then these are trypsinized and resuspended in the same medium and dispersed onto the Matrigel (after treating with the test substance). After this the Matrigel it is then pipette out into a well of a 48-well plate and polymerized for 30 min at 37^0C [72].

Quantification

After 18 hrs Cord formation in each well is monitored and photographed using an inverted microscope. The tubular lengths of the cells are measured using electrophoresis and the results are interpreted with the size, shape and growth of cords [72](Figure 6).



Control +ve inhibitor

Figure 6. *Cord formation assay*[100]

4.1.2. Tube Formation Assay

One quick assessment of angiogenesis is the measurement of the ability of endothelial cells to form three-dimensional structures which give rise to the formation of tube [20]. The assay measures the ability of endothelial cells, plated at sub confluent densities with the appropriate extracellular matrix support, to form capillary-like structures (tubes). Researchers adopt this assay to find the capability of compounds to indorse or obstruct the formation of tube. Compounds that can prevent tube formation could be useful in numerous diseases, such as cancer, in which tumors trigger and excite the formation of new vessels to get nutrients for its growth.

Short culture period, easy to set up, amenable to high throughput analysis and quantifiable, are the key advantages of this this assay. While large variation of tube-forming capability among different groups of endothelial cells which is surely not acceptable to obtain consistent and reliable data is its major drawbacks (Figure 7).[20]

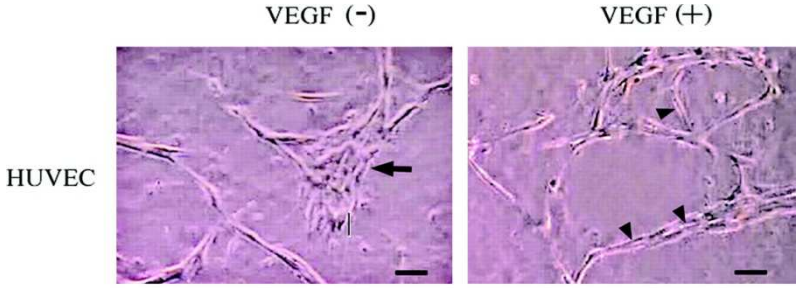


Figure 7. *Tube formation assay*[101]

Procedure

The endothelial cells are isolated and cultured in medium of gelatin coated flasks. After gelation at 37°C for 30 min, the gels are overlaid with basal medium supplemented with test substances at desired concentrations. Endothelial tube formation on extracellular matrix gel closely mimics the *in vivo* environment and may be used to test angiogenesis inhibitors before *in vivo* testing [73].

Quantification

The quantification of this assay is quite easy. Gels are examined by microscope and the tube length is determined for each well by electron microscopy and then compared with the results of control groups [74]. Many of the commercially available preparations of Matrigel do not promote tube formation *in vitro* that is why the concentration of proteins must be monitored and maintained as per standards to avoid the false results. Another limitation of this assay is that cultured cells of non-endothelial origin, for example fibroblasts may also respond to Matrigel and may lead to false observations [75].

4.1.3. Cell Migration Assay

There are several tests that can be used to determine the migratory response of endothelial cells to angiogenesis inducing or inhibiting factors [76].The assay is equally useful for testing inhibitory factors like endostatin or TNP 470, and motility increasing factors, for example fibroblast growth factor-2 (FGF-2) or vascular endothelial growth factor (VEGF), and due to its ease, allows the utilization of many different endothelial and non-endothelial cell types to control for specificity of the observed response.[76].

Procedure

Cell cultures are prepared and maintained according to standard cell culture procedures. The cell lines HT1080, HeLa and NIH3T3 are cultured in DMEM medium with 10% fetal calf serum. The night before the migration experiment, the cells are deprived in serum-free DMEM medium containing 0.2% bovine serum albumin. Then these cells are harvested, washed twice in PBS, and re-suspended in serum free DMEM medium with 0.2% BSA to obtain an appropriate final concentration (e.g. 106cells/ml). 24 well Thin Cert TM cell culture inserts with 8 µm pores and translucent PET membranes are placed in the wells of a CELLSTAR®, a 24 well cell culture plate. 600 µl of serum-free DMEM medium with 0.2% BSA and varying concentrations of FCS are added to each well of the cell culture plate (lower compartment). 200 µl of the cell suspension was added to each cell culture insert, and the plate with inserts was incubated for 20 hrs in an incubator at 37°C and 5% CO2. Subsequently, the cell culture medium was removed from each well of the cell culture plate and replaced with 450 µl DMEM medium containing 0.2 % BSA and 8 µM Calcein-AM. The plate with inserts was incubated for 45 min in a cell culture incubator at 37°C and 5 % CO_2. Thereafter, the culture medium was removed from the Thin Cert TM cell culture inserts, and the Thin Cert TM cell culture inserts were transferred in the wells of a freshly prepared 24 well plate containing 500 µl Trypsin-EDTA per well. This plate was incubated for 10 min in a cell culture incubator at 37°C and 5 % CO2 with sporadic agitation. The Thin Cert TM cell culture inserts were castoff and 200 µl of the Trypsin-EDTA solution (now having separated migratory cells) was transferred from each well of the 24 well plates into a new well of a flat-bottom black 96 well plate. At the end, migratory cells were reckoned in the black 96 well plate [77]. Endothelial cells (100 cells/well) are then placed in the well, along with test medium and incubated for 24 hrs.

Quantification

Finally, the migratory cells are quantified after 24hrs in the black 96 well plate with a fluorescence plate reader at an excitation wavelength of 485 nm and an emission wavelength of 520 nm.

4.1.4. Cell Proliferation Assay

The proliferation studies are based on cell counting, thymidine incorporation (or) Immuno-histochemical staining for proliferation (or) cell death.

Procedure

Endothelial cells are isolated and cultured in medium at 37°C in a humidified atmosphere containing 5 % CO_2. Cell proliferation is determined using a 5-bromo-2'-deoxyuridine colorimetric assay kit. Then the endothelial cells are seeded onto gelatin coated well plates in the presence and absence of test material and incubated at 37°C for 48 hrs. Then 10 ml of 5-bromo-2'-deoxyuridine is added to each well and the cells are further incubated at 37°C for 6 hrs.

Quantification

The obtained cells are fixed and incubated with anti-BrdU and then detected by the substrate reaction. The reaction is stopped by the addition of 1 M H_2SO_4 and the absorbance is measured by using micro plate reader at 450 nm with 690 nm correction [78].

4.1.5. Gelatin Zymography / Matrix Metalloproteinase (MMP) Assay

Gelatin Zymography is the most sensitive method to detect the activity of both gelatinases but quantification problem compromises its use. Matrix metalloproteinase activities of the myocardial tissue are measured with the use of sodium dodecyl sulphate-poly acryl amide gels.

Procedure

Gelatin is used as a substrate and is incorporated into poly acryl amide gels. Test material is diluted to a final protein concentration by adding distilled water and mixed with Sodium dodecyl sulphate sample buffer onto the gel and electrophoreses is done [79].

Quantification

After electrophoreses, the obtained gel is incubated in activity buffer and analyzed by densitography[79]. During electrophoresis the inhibitors dissociate from the MMP and do not interfere with detection of the enzymatic activity of gelatinase and metalloproteinase. On the other hand, sandwich ELISA can discriminate between MMP/TIMP complexes and free MMPs, resulting in determination of a potential active fraction [80].

4.1.6. Langendorff Isolated heart Model

This method is an example of *in vitro* coronary artery ligation model. This procedure can be executed by using the "isolated buffer perfused heart model."

Procedure

By tracheotomy heart is isolated and the main branch of left circumflex coronary artery is sutured. Heart is placed in iced buffer after excision. Heart is then hanged by using the aortic root on a Langendorff apparatus for retrograde non-recirculating buffer perfusion (modified Krebs-hens let solution) at a constant pressure of 85 mm of Hg with a regular supply of 95% O_2 and 5% CO_2 and a maintained temperature of 37^{o}C [81].

Quantification

There are number of physiological parameters that can be measured in the perfused heart preparation. (ECG) is readily obtained using surface electrodes of monopolar or bipolar construction and is of interest in studies involving arrhythmias. Microelectrodes implanted in the surface myocytescan also be used for electrical measurements. Oxygen consumption can be determined with dual oxygen electrodes, one placed in the perfusate stream entering the heart, the other monitoring the effluent leaving the coronary sinus. This effluent can be removed through the use of a peristaltic pump and then transferred to the second oxygen electrode. Similarly, ion selective electrodes can be placed in the effluent or perfusate stream or oxygenation chamber of the Radnoti perfused heart apparatus, permitting measurement of pH and other cations and anions. Radio labelled compounds can be used for metabolic studies, the release or uptake of various ions or substrates. Optical studies have been performed on the fluorescence of endogenous or exogenous fluorescent compounds [81].

4.2. Organ Culture Assays

In angiogenesis not only endothelial cells are involved but also their surrounding cells/organs participate and this fact lead to development of more recent methods of angiogenesis assessment which are called organ culture methods [82]

4.2.1. The Aortic Ring Assay

The angiogenesis can also be evaluated by culturing rings of mouse aorta in three dimensional collagen gels with some modification of the method originally reported for the rat aorta [82].

Procedure

In it, the isolated rat aorta is cut into segments that are positioned in culture, generally in a matrix-containing environment such as Matrigel for the next 7–14 days, the explants are checked for the development of endothelial (and other) cells as this is affected by the addition of test substances.The aorta ring assay bridges the gap between *in vivo* and *in vitro* models. By using intact vascular explants, it reproduces more accurately the environment in which angiogenesis takes place than those with isolated endothelial cells. To study the role of enzymatic systems such as serine-protease and matrix metalloprotease highly related to angiogenesis, the rat aortic ring assay defined by Nicosia (1990) to the mouse was developed [82]

Quantification

Quantification is completed by measurement of the length and profusion of vessel-like additions from the explant. Use of endothelium-selective components such as fluorescein labeled BSL-I [83] allow quantification by pixel counts.The advantage of the rat aortic system is that cultures can be maintained in the absence of serum, in a more chemically defined environment allowing the evaluation of pro- or anti-angiogenic compounds. The main interest of the mouse system is to exploit the recent generation of transgenic mice and to study the consequence of deficiencies, mutations and conditional expression of gene products.[83]

4.2.2. The Chick Aortic Arch Assay

The chick aortic arch assay represents a major modification of the rat aortic ring assay. Initially developed for the unambiguous purpose of testing thalidomide (which had previously been shown to have limited effects in rodents but strong effects in chick embryos).

Procedure

The assay eludes the utilization of laboratory animals, is swift with a time of 1–3 days, and can be conducted in serum-free medium [48].

Aortic arches are separated from day 12–14 chick embryos and cut into rings analogous to those of the rat aorta. When the rings are placed on Matrigel, considerable development of cells occurs within 48 h, with the formation of vessel-like structures readily ostensible. If the aortic arch is everted before explanting, time of the procedure can be minimized up to 24 h. Both growth stimulating and inhibiting factors such as FGF-2 and endostatin respectively can be added to the medium and their measurement of effect can be done easily [84][48].

Quantification

Quantification of endothelial cell outgrowth of the aortic arch culture can be done with the help of fluorescein-labeled lectins such as BSL-I and BSL-B4 or by the use of process of staining of the cultures with labeled antibodies to CD31.Standard imaging techniques are effective for both the details of endothelial cells and for delineating the total

outgrowth area.

5. *Inovo* Evaluation Methods

5.1. Chicken Chorio-Alntoic Membrane (cam) Assay

The CAM assay is rapid, technically simple and inexpensive [85], [86], [87]. To observe the development of embryo, Chick chorioallantoic membrane assay model is very helpful to precede research as it is impossible in other systems like mammalian system [88].The development of floating chick embryo and the changes which occur in nutritious egg yolk are easy to manipulate with the help of CAM model(Figure 8). So, the use of this model is fruitful for research purposes [89] due to the creation of heart [90] and the neurohumoral cardiac control growth [91]. The basic mechanisms of fetal cardiovascular control are analogous in both the chicken and mammalian species [92].

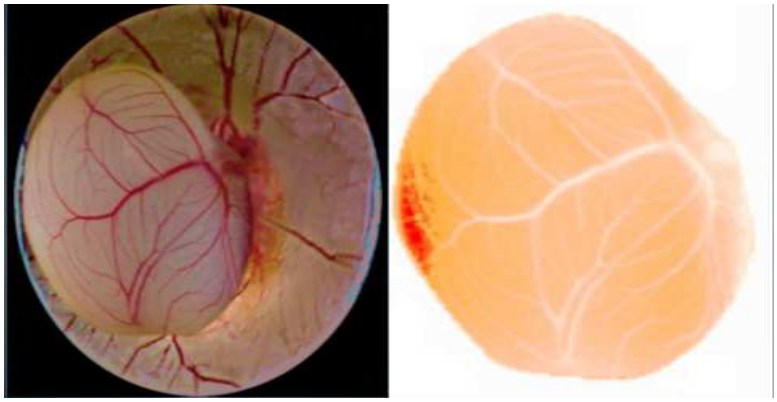


Figure 8. *CAM assay [85]*

Procedure

Fresh fertilized eggs are obtained and sprayed with 70% ethanol and subject to air dry. Eggs are incubated at 37^{o}C and 60-70% humidity. At day 4 of incubation, tiny window is made by removing shell membrane of egg; 4-5 ml of albumin is removed with the help of syringe and sealed with sterile parafilm. At day 5 of incubation, window is opened and 200 µl of each sample/test solution is applied on developing CAM. Windows are then sealed again with adhesive parafilm tape and eggs are kept in incubator for 24 hrs. After 24 hrs, CAM is separated and processed for further evaluation [87].

Quantification

In CAM assay, images of the developing CAM are taken which are then quantified. Previously various methods were used for quantification which were point counting of photographed images fractal analysis of digital images [32] and even subjective observations of variation in vasculature but now a days it is done by a different system which is Scanning Probe Image Processing (SPIP) system. This imaging of whole process explains the changes in blood vessels completely which gives better understanding of whole process of angiogenesis [84].

The SPIP technique was developed by Jorgensen and his colleagues [93]. A very fine quality camera is used to obtain the high definition images. Serial images in all 3 dimensions x, y and z were captured for an ideal measurement of 3D roughness parameters of whole angiogenesis processes on CAMs. After taking the image, quantification of all images is done with the help of software SPIP, its principle is based on particular system of surface analysis [94]. For the determination of different parameters, the particular x, y and z dimensions of each image are captured which are then quantified and evaluated to figure out the detailed effects of test sample. With the help of calibration and measurement, the diameters of different blood vessels are determined. Surface roughness (3D) [95] , the major parameters in 3D image analysis and several other parameters for example: surface area of the vessel (Sa), minimum height of the peak on the surface (Smin), maximum height of the peak on the surface (Smax), mean height of the peaks on the surface (Smean), developed surface area ratio (Sdr), ratio of void volume of the unit sampling at core zone, core roughness depth of the surface (Sk), texture index of the blood vessel (Stdi) and many others are measured with SPIP software for proper quantification of angiogenesis. With the help of calibration and measurement control command, the length and diameter of different blood vessels and area of CAM is measured and then compared with the control group results.

6. Conclusion and Prospective

During the last decade, it has become evident that angiogenesis plays a central role in the pathogenesis of various disease. Many angiogenic growth factors, particularly VEGF, could be detected. However, despite present efforts in this field of angiogenesis, there is still an inadequate amount of knowledge about the mechanisms regulating the complex dynamic process of blood vessel development. This can be overcome by the introduction of sophisticated *in vivo* and *in vitro* models. Thus, it may be possible in the future to develop effective anti-angiogenic treatment strategies for various therapies [85] [87].

Regardless of the discovery and progress of several agents in dealing the angiogenic diseases (mainly cancer), promising inhibitory action in preclinical evaluation *in vitro* and *in vivo*, and in some cases even tumor regression, failed in phase II/III of clinical trials. This discrepancy of results between preclinical and clinical trials demonstrates the necessity for arduous preclinical testing both *in vitro* and *in vivo* using appropriate models and the limitations of each assay should be dealt carefully so that the over interpretation of results can be minimized.

In vitro tests are beneficial, can be practiced expeditiously, and provide enough room for quantification, but must be deduced with extreme attention. *In vitro* tests deliver critical data and are essential first steps for validation. Various tests should be performed to get maximum benefit from *in vitro* tests. *In ovo*angiogenesis assay model (CAM assay) is mostly used as a pilot method for most of the angiogenesis evaluation studies.

In summarizing form , for understanding and interpreting the effects of a particular test materials on the process of angiogenesis, it is mandatory to use more than single type of *in vitro* assay to figure out the different steps in the angiogenic pathway, the utilization of different sources of endothelial cells and then following these with more than one *in vivo* assay to make sure that the outcomes seen *in vitro* are in accordance with that *in vivo* where other cells and extracellular matrix proteins are also having a definite share in the process of angiogenesis.

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