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ANJİYOJENİK VE ANTİANJİYOJENİK ÖZELLİĞİ BELİRLEMEDE KULLANILAN *in vitro* ve *in vivo* YÖNTEMLER

METHODS FOR ASSAYING ANGIOGENIC-ANTIANGIOGENIC FEATURE *in vitro* and *in vivo*

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Özet

Anjiyogenez damar ağlarından yeni kan damarlarının gelişimidir ve yara iyileşmesi, dişi üreme sistemi, fetal gelişim gibi fizyolojik durumlar; sedef hastalığı, diabetik retinopati, tümör gelişimi ve metestaz gibi patolojik durumlar için gerekli bir süreçtir. Anjiyojenez analizleri ajanların anjiyojenik veya anti-anjiyojenik özellikleri belirlemek için kullanılmaktadır. Anjiyogenik aktivite *in vitro* ve *in vivo* yöntemler kullanılarak değerlendirilir. Bu yöntemlerin herbirinin avantaj ve dezavantajları bulunmaktadır. İdeal bir metot kolayca ölçülebilir, hassas ve güvenilir olmalıdır. Bu çalışmanın temel amacı, bu alanda çalışan araştırmacılara anjiyojenik karakteri belirlemek için mevcut olan yöntemleri tanıtmaktır.

Anahtar Kelimeler: Angiogenesis assays, *in vivo, in ovo, in vitro*

Abstract

Angiogenesis is the growing of new blood vessels from vasculature network and essential in many conditions like wound healing, female reproductive system, fetal development as well as psoriasis, diabetic retinopathy, tumour growth and metastasis. Angiogenesis assays are maintained to determine the angiogenic or anti-angiogenic properties of the agents. Angiogenic activity can be determined by using *in vitro* and *in vivo* systems. These methods have advantages and disadvantages yet. Nevertheless an ideal method should be easily measurable, precise and reliable. The main objective of the review is introduce the methods available to determine the angiogenic character to the researchers working in this field.

Key Words: Angiogenesis assays, *in vivo, in ovo, in vitro*

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1. INTRODUCTION

Angiogenesis is the process of the growing of new vessels from preexisting vessels (Ulus et al., 2018, pp. 1537-1550). (Figure 1). Angiogenesis is a vital process both in physiological – wound healing, ovulation, embryogenesis, fetal development- and pathological conditions – tumour development, progression, spread and metastasis, diabetic retinopathy, proriasis, rheumatoid arthritis. Angiogenesis which is a complex mechanism is a response to the increasement in requirements of oxygen and nutrient in tissue mass (Tong et al.,2004, pp. 101-109). The complex process of angiogenesis contains extracellular matrix degration, migration, proliferation of endothelial cells, tube formation, and sprouting of new capillary branches (Koparal et al., 2010, pp. 754–758; Ulus et al., 2018, pp. 1537-1550) (Figure 2).

Figure 1: Angiogenesis prosess (Rajabi and Mousa, 2017, pp. 34).

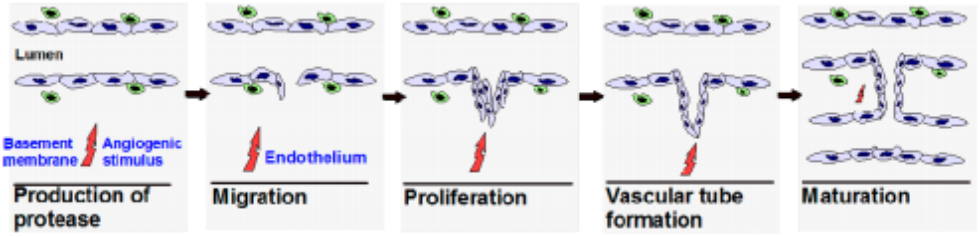

Figure 2: Steps of angiogenesis (Rajabi and Mousa, 2017, pp. 34).

Angiogenesis regulation by pharmacological agents is a promising therapy. Therefore, in vivo and in vitro angiogenic studies are ongoing (Ulus et al., 2018, pp. 1537-1550).

The requirements below should be met by an ideal angiogenesis study model:

- The quantitive measurement of new vessel development should be done.
- A measure for the functional characteristics of new blood vessels should be provided by the model
- Newly grown blood vessels should be distinguishable.
- Any in vitro response should be demonstrated in the in vivo model (Özgürtaş, 2009, pp. 67-69).

The efficacy of angiogenic and anti-angiogenic agents can be tested with the help of angiogenesis assays. In order to establish angiogenic and antiangiogenic effects, there are many in vitro and in vivo methods (Khan et al 2014, pp. 47-62).

2. ANGIOGENIC-ANTI-ANGIOGENIC METHODS

2.1. in vitro Angiogenesis Methods

Many *in vitro* angiogenesis assays have been developed. As *in vitro* angiogenesis evaluation methods can be implemented in a short while and they supply precise outcome if quantified corrrectly, they have a great significance (AlMalki et al., 2014, pp. 251–256). Usage of different types of endothelial cells isolated from either large blood vessels or capillaries is the way to execute *in vitro* assays. Human umbilical vein endothelial cells (HUVECs), Human micro vascular endothelial cells (HMVECs), Chicken endothelial cells (CECs), Bovine aortic endothelial cells (BAECs) are the mostly used cell lines (Staton, 2004, pp. 233–248; Veeramaniand and Veni, 2010 pp. 2379-2387). Every single step of the angiogenic process can be studied with these modals. It is the most efficient modal in the aspects of cost and effort in comparison with *in vivo* systems (Veeramaniand and Veni; 2010 pp. 2379-2387).

2.1.1. Endothelial cell proliferation assays

Endothelial cells which are established in culture are able to cell division (Staton et al., 2004, pp. 233–248). Cell proliferation, major targets in anti-cancer therapy (Huyck et al., 2012 pp. 382-392). As cell proliferation assays are easily executable and highly reproducible, these properties make the assays precise in quantification (Staton et al., 2004, pp. 233–248). A couple of assays for determining cell proliferation exist and they vary on these measured aspects: DNA synthesis, metabolic activity, antigens associated with cell proliferation and ATP concentration (Madhavan, 2007, pp. 12-14; Menyhárt et al., 2016, pp. 300-319; Schlfiter et al., 1993, pp. 513-522; Seminario-Vidal L et al., 2009, pp. 25-36).

2.1.1.1. DNA synthesis cell proliferation assay

In a cell, segmentation occurs after DNA replication as it is a necessity. Biochemical pathway corraletes with DNA synthesis which is specific for segmentation. That means the number of the new DNA synthesis is equal to the number of the segmentation. (Madhavan, 2007, pp. 12-14). A direct measurement is mostly done with the help of a marked nucleoside into genomic DNA. The tritiated thymidine ([3H]dT) and BrdU (bromodeoxyuridine) methods are two of the examples (Waldman et al., 1991, pp. 718-722; Grattner, 1982, pp. 474-475).

Burns et al, (2006, pp. 1121-1127) gave proof BrDU marked dead cells after granting them into the mice brains. The time of cell splitting is marked by using BrdU in experiments. The usage of BrDU lets the researchers examine where those cells went and what type of cells they turned into (Madhavan, 2007, pp. 12-14).

2.1.1.2. Metabolic cell proliferation assay

Metabolic activity of cells allows for another method of measuring cell proliferation. In order to detect viable cells, diverse tetrazolium compounds have been used. MTT,XTT, MTS and WST1 are widely used tetrazolium salts（Terzioğlu et al., 2013 pp. 74-89; Riss, 2013, pp. 305-335）. These tetrazolium compounds are basically in two categories: 1) MTT is positively charged and with ease penetrates into viable eukaryotic cells. 2) Compounds such as MTS, XTT, WST-1 are negatively charged and do not penetrate cells. The second category compunds are generally used with an intermediate electron acceptor transforming electrons from the

cytoplasm or plasm membrane enable the reduction of the tetrazolium into the colored formazan product (Riss et al., 2013, pp. 305-335).

The cleavage of a tetrazolium salt by succinate dehydrogenase -a mitochondrial enzym- leads to the generation of a colored formazan product which underlie these methods. The colored formazan product can be quantified by spectrophotometry formazan (Ginouves et al., 2014 pp. 2131–2138; Mosmann, 1983, pp. 55–63).

2.**1.1.**3. **MTT Assay**

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (van Meerloo et al., 2011, pp. 237-245) tetrazolium reduction assay, the first homoheneous cell viability essay, was developed for a 96-well format which was suitable for high throughput screening (HTS) (Mosmann, 1983, pp. 55–63). As the total mitochondrial activity is related to the number of viable cells for most cell populations, the assay is mainly used for measuring the *in vitro* cytotoxic effects of drugs on cell lines or primary patient cells (van Meerloo et al., 2011, pp. pp. 237-245). MTT is converted into purple colored formazan product by viable cells with active metabolism and the maximum absorbance is near to 570 nm. The cells cannot convert MTT into formazan when they die and therefore color formation is useful and proper marker for only viable cells. Although the exact cellular mechanism of MTT reduction into formazan is not fully understood, it probably involves a reaction with NADH or similar reducing molecules tronsforming electrons to MTT (Kumaravel and Begum, 2015, pp. 2023-2030).

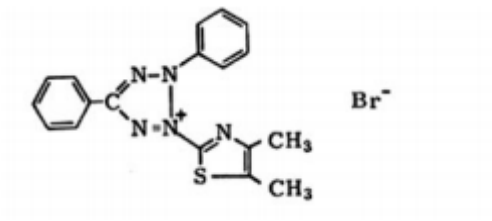

Figure 1. Structures of MTT (Sa´nchez and Königsberg 2006, pp. 209-212)

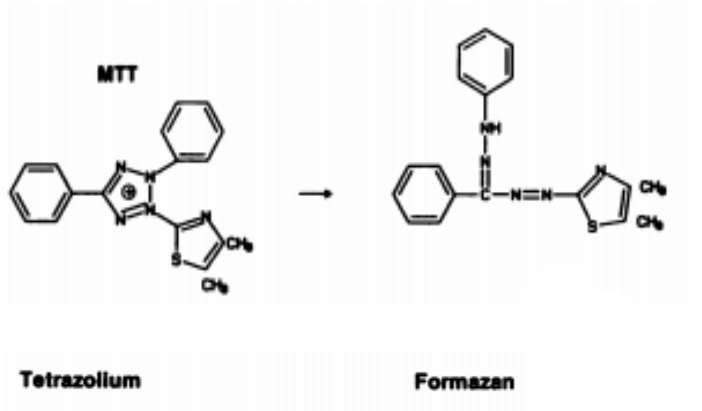

Figure 2. MTT tetrazolium and formazan (Scudiero et al., 1988, pp. 4827-4833).

2.1.1.4. XTT Assay

The XTT assay has been used under diverse conditions in order assess the viability of different organism such as mammalian cells, bactaria and fungi (Scudiero et al.,1988 pp. 4827-4833; McCluskey, 2005, pp. 379-387).

XTT (2,3-bis-(2- methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide) (Huyck et al, 2012, pp.382-392; Silva et al., 2008, pp. 364-369). which is a new tetrazolium salt, has been synthesized by Paull and colleagues (1988, p.911). XTT assay is a simple, single-step process for measuring cell proliferation (Huyck et al, 2012, pp.382-392). Bioreduction of XTT produces a highly colored formazan product and this is in contrast to other tetrazolium salts like water-soluble Mq" F Scudiero et al. (1988, pp. 4827-4833), studied on the use of bioreduction of XTT with human tumor cells and showed that electron coupling agents were able to potentiate bioreduction of XTT (Roehm et al., 1991, pp. 257-265).

XTT is reduced into a colored soluble formazan by cells with active metabolism (Huyck et al., 2012, pp. 382-392). The formazan product is easily measured in cellular supernatants as it is a water-soluble product. XTT methods are expected to be used much in order to study fungal growth and drug susceptibility. As a result, it is important to comprehend better the uses and limitations of them (Kuhn et al, 2003, pp. 506–508). Three components named XTT, menadione and phosphate buffer saline (PBS) are involved by XTT reduction assay formulation (Silva et al., 2008, pp. 364-369). As the bioreduction of XTT is inefficient, addition of an electron-coupling agent such as phenazine methosulfate, menadione or coenzyme Q0 (CoQ) can potentiate it (Xie et al, 2011, p. 93).

In the existence of metabolic activity, XTT is converted to a colored formazan. The mitochondrial succinoxidase, cytochrome 9450 systems and flavoprotein oxidases are the primary mechanism of XTT-to-formazan conversation (Altman, 1976, pp. 1–56; Kuhn et al., 2003, pp. 506–508). Formazan product is easily measured in cellular supernatants as it is water-soluble. XTT methods are expected to be used much in order to study fungal growth and drug susceptibility (Kuhn, 2003, pp. 506–508).

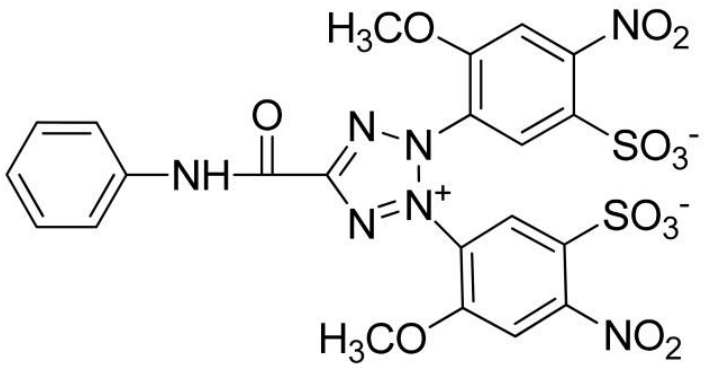

Figure 3. Structure of tetrazolium salt XTT (31). (Shimamura and Ukeda, 2012, pp. 148-158).

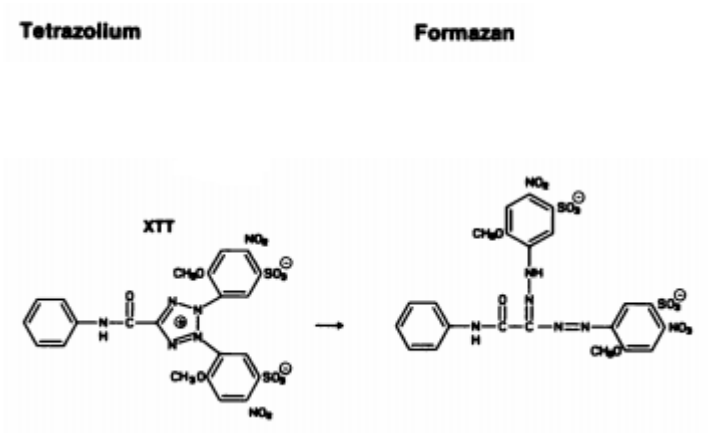

Figure 4. XTT tetrazolium and formazan (Scudiero et al., 1988, pp. 4827-4833).

2.1.1.5. MTS Assay

The MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (O'Toole et al., 2003, pp. 47-54; Yin et al., 2013, pp. 68-72), is an alternative to MTT. MTS is water-soluble and less toxic compared to MTT (Wang et al., 2010, pp. 1-10).

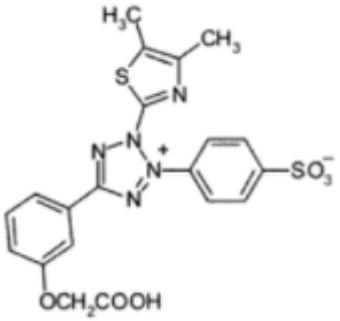

Figure 5. Structures of MTS tetrazolium salt (Berridge, 2005, pp. 127 -152).

Being able to measure the cell number without having to disturb cells is the major advantage of MTS assays (Huang et al., 2004, pp. 406-412).

It is introduced by some studies that MTS in-vitro cytotoxicity assay is suitable method for assing cell viability. It has been found that the assay is easy for using, accurate and quickly indicates the toxicity (Malich et al., 1997, pp. 179-192).

2.1.1.6. WST1 Assay

The WST-1 (disodium mono{4-[3- (4-iodophenyl)-2-(4-nitrophenyl)-2H-tetrazol]-3-ium-5-yl] benzene-1,3-disulfonate}) (Szumilak et al., 2017, pp. 307-313) assay, is an effective test to measure cell viability (Yin et al., 2013).

Cellular dehydrogenases with the existence of intermediate electron acceptor such as mPMS (1-methoxy-5-methyl-phenazinium methyl sulfate) can reduce the WST-1 reagent to highly water-soluble formazan (Yin et al., 2013, pp. 68-72; Berridge et al., 2005, pp. 127 -152). This mechanism is shown in the Figure 6. The formazan produced by WST-1 higher sensivity than XTT and MTS. Although the standart incubation period of WST-1 is 2 hours (Yin et al., 2013, pp. 68-72; Ishiyama et al., 1993, pp. 1118 -1122).

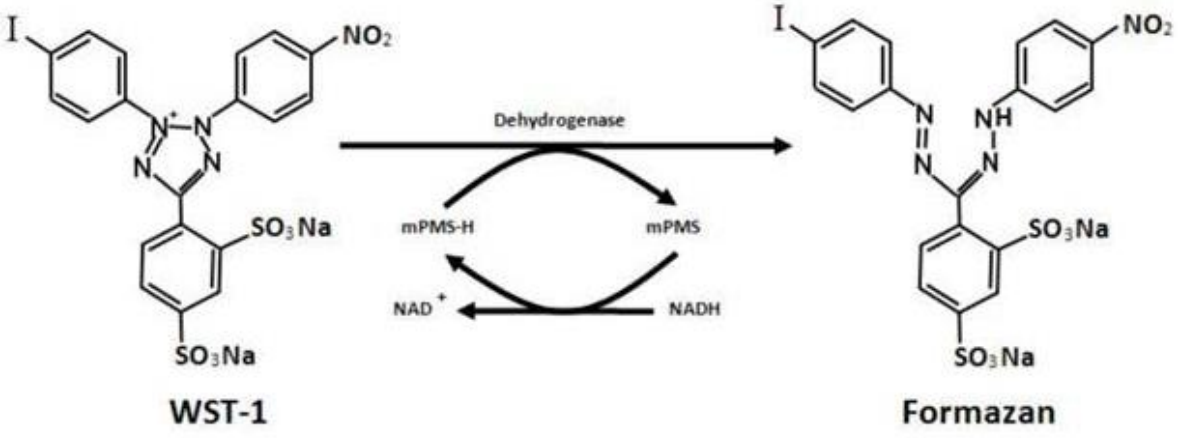

Figure 6. Schematic mechanism of the WST-1 reduction (Yin et al., 2013, pp. 68-72).

2.1.1.7. Measuring [ATP]

The quantity of ATP in cells correlates with cell viability. Cells become unable to synthesize ATP and the remaining ATP is devoured by endogenous ATPases. This results with precipitious and quick fall of the quantity of ATP (Riss et al., 2003, pp. 6-12).

2.1.1.8. Resazurin Reduction Cell Viability Assay

Resazurin can enter the viable cells in case of being reduced to the fluoresecnt resorufin product. The resazurin-to-resorufin coversation is in accordance with the quantity of metabolically active, living cells which exist in a popluation (Riss et al., 2003, pp. 6-12).

2.1.2. Cell migration assays

A process named chemotaxis lets endothelial cells move (Staton, 2004). There are some test which are applicable to detect the migratory response of endothelial cells to angiogenesis-including/inhbiting factors (Auerbach et al., 2003, pp. 32–40). The blind-well chemotaxis chamber which modified Boyden chambers is one of the most frequently used one (Auerbach et al., 2003, pp. 32–40).

With the help of colloidal gold-plated coverslips serving as substrate for the movement of cells, a track left the collodial gold enables the measurement of directional properties and the total area (Staton, 2004, pp. 233–248; Zetter 1987, pp. 135-144). As cells migrates on an “alien” substrate which is not present *in vivo*, this can be named as the main limitation of these assays (Staton, 2004, pp. 233–248).

2.1.3. Wound Healing Assay

This is an easy, cheaper and earliest-propesed method. The method puts forward the idea of cell migration into a wound formed on cell monolayer (Khan et al., 2014, pp. 47-62)..

This assay represents an aspect of wound healing through the process: The confluent endothelial monolayer is wounded by using a scrapping tool and endothelial cells migrates back to restore the monolayer (Auerbach et al. 1991, pp. 32–40; Staton et al., 2004, pp. 233–248).

2.1.4. Tube Formation Assay

Compounds which are able to inhibit tube formation could be beneficial in a large number of diseases like cancer. The compunds prevents the tumors to initiate new blood vessel growth which enables to have nutrients to grow (Al Malki et al., 2014, pp. 251–256).

Measuring the ability of endothelial cells to form capillary like structures giving rise to the formation of tube is a rapid assessment of angiogenesis. The ability of endothelial cells of forming capillary like structures is measured in this assay (Khan et al., 2014, pp. 47-62).

2.1.5. Gelatin zymography

Gelatin zymography assay can also be named as MatrixMetalloproteinase (MMP) assay (Veeramani and Veni, 2010, pp.2379-2387). Gelatin zymograpy is a helpful qualitative tool to determine and analyse the level and type of the gelatinases give in different cell types/tissues at any given time and/or after different treatments (Toth and Fridman, 2001, pp. 163-174).

As connective tissue degrading enzymes like gelatinase quickly cleave gelatin and it is easily included in poly acryl amide gels, gelatin is used as a substrate (Veeramani and Veni, 2010, pp. 2379-2387)

For instance, it can be detected which gelatinases are sent in tumor cells with various degrees of invasive potential and whether they sprout from existent cancer cell lines or from tumor biopsies (Toth and Fridman, 2001, pp. Fridman, 2001, pp. 163-174).

2.1.6. The Aortic Ring Assay

Angiogenic and anti-angiogenic factors can be evaluated with aortic ring assay. The blood vessels sprouting out from aortic rings employ smooth muscle cells and pericytes to associate with the endothelial cell tube which means they are anatomically analogous with neovessels *in vivo*. There are some disadvantages of the aortic ring assay. First, blood vessels outgrowth *in vivo* exist on microvessels, not from major vessels like aorta. Second, the blood vessel growth can be affected by the inconsistancy in the managing of the rings and the quantity of the surrounding tissue which remains on the blood vessel (Bellacen and Lewis, 2009, pp. 1-2)

2.1.7. The Chick Aortic Arch Assay

This method is the modification of the rat aortic ring assay (Padhani and Newman, 2001, pp. 886–890; Tahergorabi and Khazaei, 2012, pp. 1110-1126). The method performed quickly and takes 1-3 days. By incubating chick aortic arch ring in culture medium containing test substances, the assay can be executed. The aortic arches are isolated from 12-14 chick embryos and cut into 1mm rings. Then cultured in matrigel containing plate (Seunghyun et al., 2008, pp. 5-14; Veeramani and Veni, 2010, pp. 2379-2387).

2.1.8. Langendorff Isolated heart Model

The method is an instance of *in vitro* coronary artery ligation model. By using the “isolated buffer perfused hearth model”, the procedure can be performed (Veeramani and Veni, 2010, pp. 2379-2387).

Since Langendorff the isolated heart models have been used successfully for the study of hearts in mammals. Mechanical features of the heart and coronary flow and metabolical study

of the isolated organ can be examined with the help of these sorts of models. In a model like this, a support animal is used to keep the isolated organ perfused. Though these hearth models need more complicated preparations than the preparation with solutions crystalloids, they enable the model to be much closer to the physiological situation (Silveira et al., 2008, pp. 14-22).

2.2. *in ovo* Evaluation Methods

2.2.1. Chicken Chorio-Allantoic Membrane (CAM) Assay

Both the angiogenesis and anti-angiogenesis substances can be examined with this method (Veeramani and Veni, 2010, pp. 2379-2387). The fusion of the chorion with the allantoic membrane forms the chick chorioallantoic membrane (CAM). In this double mesodermic layer, a highly rich vascular network grows and serves as the respiratory organ of the embryo till the hatching time. CAM vascularization has a dense artery and vein network and the network forms a delicate and elaborate capillary plexus (Blacher et al., 2005, pp. 169-180).

The CAM assay is quick, simple and cheap (Padhani, 2001, pp. 886–890). CAM assay model is very useful on observing the development of embryo because proceeding a research on other systems such as mammalian systems is impossible. CAM model enables the manupilation of the development of loating chick embryo and the changes occuring in nutritious egg yolk (Khan et al., 2014, pp. 47-62).

The test matter is carried out in the following two ways: in slow-release polymer pellets which are absorbed by gelatin sponges; air-dired onto plastic discs. Then through a cut in the eggshell, these are inserted into the CAM. As 7-8-day old chick embryos do not have a strong immune system, tumor induced angiogenesis study can be performed. By counting the number of the blood vessels in the area decided with a stereomicroscope, the angiogenic effects of test matter can be learned (Staton et al, 2004, pp. 233–248).

There are some limitation on this assay. CAM masks distinguished new capillaries from existing ones as it has a quick morphological change. Also 7-9-day old CAM has an inflammatory reaction to a variety of test matter which can prevent the recognition of new vasculature (Padhani, 2001, pp. 886–890).

2.3. *in vivo* Angiogenesis Evaluation Methods

As in-vivo tests of angiogenesis are difficult to execute and more time-consuming than in-vitro assays, very few tests are executed at one time. Complication of the process of quantification is another handicap. Nevertheless the *in vivo* tests have a great importance due to the complexed character of vascular responses to tests substance is not able to fully succeed (Khan et al., 2014, pp. 47-62).

2.3.1. Sponge Implantation Assay

This assay has been developed to characterize the core elements and their roles in angiogenesis under a diverse physicological and pathological conditions (Couffinha et al., 1997, pp. 1673–1685). Sponge implantation model has been developed to characterize the necessary components and their roles in blood vessel formation physiologic and pathologic conditions (Andrade and Ferreira, 2016, pp. 333-343).

The test reagent is either directly injected or incorporated to the sponge (Hu et al., 1995, pp. 601–610; Fajardo et al., 1992, pp. 539–544). These are placed into the center of the sponge. Immunohistological staining, the blood or hemoglobin ingredient of the sponge or the levels of a radioactive tracer in blood (Mahadevan et al., 1989, pp. 415–419; Plunkett and Hailey, 1990, pp. 510–517) consist of the methods which enable the assessment of neovascularization. However the factors of sponge like shape, size, composition make comparisons between different studies challenging. In addition to challenging factors, implementation can lead to nonspecific immune responses which may cause an angiogenic response (Staton et al., 2004, pp. 233–248; Dellian et al., 1996, pp. 59–72).

2.3.2. Matrigel Plug Assay

The assay enables to detect the formation of new blood vessels in the transplanted gel plugs in nude mice. The matrigel matrix is generally based on the engelbroth-holm-swarm of mouse sarcoma and the composition of the matrix is like the basement membrane proteins. Extracellular matrix components and growth factors compose it (Tahergorabi and Khazaei, 2012, pp. 1110-1126). The matrigel can cause differentiation of several cell types like mammary epithelial cells, hepatocytes and endothial cells (Tahergorabi and Khazaei, 2012, pp. 1110-1126).

The Matrigel plug assay has been recently the favorite method for for numerous studies including in-vivo testing for angiogenesis (Madhavan, 2007, pp. 12-14; Menyhárt et al., 2016, pp. 300-319). Angiogenesis-inducing coumpunds like bFGF (FGF-2) or tumor cells put into cold liquid Matrigel in the assay. The step is followed by subcutaneous injection and it solidifies and allows subsequent penetration by hot cells causing vascularization. Assassment of angiogenic reactions in the Matrial plug can be done either by detection of hemoglobin content or by analysis of histological preparations, stained to increase the visibility of blood vessels and to allow the detection of vascular density in chosen areas (Akhtar, 2002, pp. 75-80).

2.3.3. Corneal Angiogenesis Assay

The cornea is an avascular site, so new vessels saturating from limbus to the corneal stroma can be names as newly formed (Staton et al., 2004, pp. 233–248; Gimbrone et al., 1974, pp. 673-684; Muthukkaruppan and Auerbach, 1979, pp. 1416–1418).

In the method, new blood vessels formation is stimulated by placing test substance into micropockets which are made on the cornea (Andrade and Ferreira 2016, pp. 333-343).

In the assay, corneal stroma of an experimental animal is used to made a “pocket” which rabbits were used originally. Then the method was adapted for rats and mice. The slow-release

pellet or polymer implantation which contains angiogenic material cause an angiogenic respond (Staton et al., 2004, pp. 233–248; Presta et al., 1999, pp. 2417–2424; Gimbrone(2) et al., 1974, pp.413–427)

Corneal angiogenesis assay can determine some factors like the role of various cells, growth factors and tissues during the process of new cappillaries formation. Altough the method has been adopted for mice, the original one was developed for rabbits (Gimbrone (2) et al., 1974, pp. 413–427; Muthukkaruppanet al., 2000, p.65) Induction of an angiogenic inducer into a corneal pouch underlies the cornea assay. This enables the vascular outgrowth from limbal vasculature of surroundings. Though the cornea is avascular in the beginning, the assay enables the evaluation of newly formed blood vessels. This is a remarkable qualification of the assay. Unrelated, confusing and even wrong results have been obtained because of inappropriate use of the assay and undetailed evaluation process (Khan et al., 2014, pp. 47-62).

2.3.4. Dorsal Air Sac Model

The dorsal air sac model is used to determine the *in vivo* angiogenic effects of test material against stimulated by cancer cells (Khan et al., 2014, pp. 47-62; Semba et al., 2004, pp. 1430-1438).

The dorsal air sac assay (DASA) is a simple and convenient *in vivo* assay to measure angiogenesis and angiogenesis inhibition by substances of interest. In the assay, tumor cells that release angiogenic factors are placed in a diffusion chamber that consists of plastic or rubber ring covered with cellulose membrane filters on both sides. The chamber is implanted into a dorsal air sac under the skin of mice. Angiogenic factors released from the tumor cells induce angiogenesis in the mouse air sac fascia attached to the chamber. The newly formed bloood vessels are readily recognizable and these blood vessels may be quantified. The chamber-bearing mice may be treated with antiangiogenic agents by systemic administration and the degree of the angiogenesis inhibition can be measured and quantified after the mice are sacrified (Semba et al., 2004, pp. 1430-1438).

The assay is easy to test, but care should be taken not to exasoerate the external later which the chamber is placed to as false results may be obtained. The assay ensure lasting noninvasive monitoring of vascular networks *in vivo* for a long period and it enables to explain physiological properties of new blood vessels (Khan et al., 2014, 47-62).

2.3.5. Hind Limb Ischemia Model

This method is generally used for the determine of angiogenic materials. Hind limb ischemia animal model is the model of peripheral arterial disease. The model has been extensively applied to the studies of vascular modelling. Heamodynamic changes undelie the mechanism of the model and the mechanism leads to new blood vessel formation (Veeramani and Veni; 2010, pp. 2379-2387).

The arteriogenesis mechanism is remarkable at the tied location whereas angiogenesis predominates in the ischemic location. Increasing endothelial cell proliferation and capillary

density enable to determine neovascularization. Histological measurement of angiogenesis is mostly done by capillary density model. (Tahergorabi and Khazaei , 2012, pp. 1110-1126).

The animal model of ischemia by ligating the vessels has been applied on a cat, canine, rabbit and rat. As rabbits have a less complete formation of collaterals than dogs and they are adequate on cost, manageable and easy to maintain, they are generally used as model animal for the study (Veeramani and Veni , 2010, pp. 2379-2387).

2.3.6. Zebrafish assay

The zebrafish (Danio rerio), which is a small tropical freshwater fish, can be used as a model system in drug discovery, development biology and angiogenesis studies. (Rubinstein, 2003, pp. 218–223). The similarties on the development of vascular anatomy of zebrafish with other vertabrates have been asserted (Staton et al., 2004, pp. 233–248).

Some properties of the fish such as rapid development, optical transparency, high number of offspring and straightforward strategies for forward and reverse genetic manupilation provide advantages for the model study (Chávez et al., 2016, pp. 1-15).

A number of embryos are usually put together in wells and test substance, in case of being small and lipophilic, added to water because in most experiments the embryos used are approximately 1-2mm. However test substances have to be injected into the yolk sac of embryos at 20 hours post fertilization (Staton et al., 2004, pp. 233–248).

2.3.7. Tumour models

In order to determine potential anti-cancer substance, many varied in-vivo tumor models have been developed. In immunodeficient rodents, tumors can be developed syngeneically (e.g subcutaneous), orthotopically (in the tissue of origin) or as xenografts. The effects of test substance on tumor size (diameter, area or volume) and animal survival can be determined at regular intervals (Staton (2) et al., 2004, pp. 601-606). Also in the investigation of anti-angiogenic drugs in tumor models have been applied specifically. Tumors need the development of blood vessels, which enable them to have oxygen and nutrients and remove the waste products, to grow beyond a certain size. Several histological analyses provide information to study on the effects on these blood vessels like vascular density, blood flow and/or thrombosis and tumor cell apoptosis/necrosis. Also a new drug's potential antiangiogenic effects can be tested against well-vascularized tumors. The test can be carried out on the vascular origin tumors like chemically induced haemangiosarcomas and Kaposi's sarcoma (Staton et al, 2004, pp. 233–248).

Compare of *in vitro* and *in vivo* assays:

- *in vitro* assays recognize direct effects on endothelial cell function while *in vivo* assays involve multiple cell types.

- *in vitro* assays analyse isolated processes of angiogenesis consisting of proliferation, migration and differentiation, or tubule formation of endothelial cells. Whereas *in vivo* assays analyse angiogenesis as a whole.
- Technical skill of handling animal *in vitro* assays is not needed to be compared with that of in- vivo assays.
- *in vitro* assays are less expensive than *in vivo* assays.

in vitro angiogenesis assays often can be quantified more easily in comparison with *in vivo* assay (Tahergorabi and Khazaei, 2012, pp. 1110-1126).

3. DISCUSSION

Angiogenesis, the formation of new blood vessels from pre-exiting vascular, has been identified and recognized by various investigations as a therapeutic approach to slow or treat neoplastic and non-neoplastic degenerative diseases; these include cancer, arthritis, diabetic retinopathy and others. It is important that the researchers know the methods to be used in finding new agents with angiogenic effect.

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