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COMPARISON OF 3 DIFFERENT TECHNIQUES TO ASSESS ANGIOGENESIS USING THE CHICK EMBRYO CHORIOALLANTOIC MEMBRANE MODEL

Renuka Munshi*[1], Rima Mittra[1] and Tanvi Patil[1]

[1]Department of Clinical Pharmacology, TN Medical College & BYL Nair Ch. Hospital, Mumbai, India.

***Corresponding Author: Dr. Renuka Munshi**

Department of Clinical Pharmacology, TN Medical College & BYL Nair Ch. Hospital, Mumbai, India.

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ABSTRACT

Angiogenesis is the growth of blood vessels from the existing vasculature. A variety of methods have been used to assess angiogenesis. One widely accepted is the Chick Chorioallantoic membrane (CAM) model. Quantification of blood vessels in the CAM model using Stereomicroscope and the Image Analysis software, is one of the easy, reproducible and validated technique, but it is not economical. Hence, there is a need for an alternative technique which has the consistency of the Image analysis method but is cost effective. Thus, the present study was aimed to compare 3 different techniques of CAM model, 2 known and one novel (Drabkin's method), in determining the angiogenic potential of drugs. 6-day old embryonated White Leghorn eggs were used for the CAM model. The angiogenic potential of 3 standard drugs *viz.*, VEGF, Erythropoietin and Heparin was assessed using 3 techniques, *viz.*, Drabkin's method, Overall quantification of blood vessels (using Stereomicroscope) and Localized quantification of blood vessels (using discs).6-day old embryonated eggs were inoculated with the known drugs respectively, and incubated. Untreated eggs served as the normal control. On day 12, CAM was excised from the eggs and the effect of the drugs on angiogenesis was evaluated by the 3 techniques and compared. All techniques evaluated in the study demonstrated comparable results with significant Pearson's correlation ($r = 0.9877$, $p<0.0001$). Significant correlation between the Drabkin's method results and both the image analysis methods demonstrated that Drabkin's method is a relatively cheaper, reproducible and a reliable technique to screen drugs for their angiogenic potential.

KEYWORDS: Angiogenesis; CAM; Drabkin's method; Image Analysis methods.

INTRODUCTION

Angiogenesis is the growth of blood vessels from the existing vasculature.[1] It is physiologically tuned by a fine balance between the stimulatory and inhibitory growth factors. An angiogenic switch is a critical progression point in a range of pathologies.[3] A persistent unregulated angiogenesis leads to the cause of many diseases[4] *viz.* cancers, cardiovascular disease, blindness, arthritis, complications of AIDS, diabetes, Alzheimer's disease and more than 70 other major health conditions affecting children and adults in developed and developing nations. In many serious disease states the body loses control over angiogenesis. This results in angiogenesis-dependent diseases where new blood vessels either grow excessively or insufficiently.[2,5] It follows that control of angiogenesis offers hope in the treatment of many illnesses and an effective pro / anti-angiogenic therapy may have wide spectrum applicability.

The classical *in-vivo* models include the rabbit ear chamber, hamster cheek pouch, dorsal skin chamber, dorsal skin and air sac model, avascular corneal pocket assay, murine matrigel plug assay and the chick chorioallantoic membrane (CAM) assay. Owing to the simplicity of the CAM model, it is the most widely used *in vivo* model to assess the extent of angiogenesis.

CAM is an extra-embryonic membrane and a vascular system, thus providing a simple and easy alternative to the rodent models.[6] It is used to study both new vessel formation and its inhibition in response to tissues, cells, or soluble factors.[7] Also it is more physiological than other *in vitro* assays.

Numerous methods of quantifying the CAM angiogenesis response have been published in previous years. Semiautomatic image analysis methods are one of the validated and excepted methods for quantifying angiogenesis. Semi-quantitative scoring methods dependon a subjective grading scale for response, or numerical grading scale for calculating the coefficient of angiogenesis. These methods are useful for screening many novel substances but can give inconsistent and false results in inexperienced hands, with Potentially significant results requiring further exploration and confirmation. The choice of method to use depends on available equipment and technical backup.

The various biochemical methods provide objective parameters of angiogenesis andthe methods are generally quicker than image analysis.[8] Drabkin reagent is well known for colorimetric determination of hemoglobin concentration in whole blood in *in-vivo* models.[9,10] It is based on the oxidation of hemoglobin and its derivatives (except sulfhemoglobin) to methemoglobin in the presence of alkaline potassium ferricyanide. Methemoglobin reacts with potassium cyanide to form cyanmethemoglobin, which has maximum absorption at 540 nm. The color intensity measured at 540 nm is proportional to the total hemoglobin concentration.[11,12] In this study, the application of this redox reaction was subjected in the CAM model.

Although quantification of blood vessels in the CAM model using Stereomicroscope and the Image Analysis software is an easy, reproducible and validated technique, but it is not economical. Hence, there is a need for an alternative technique which has the consistency of the Image analysis method however is also cost effective. With this background the present study was carried out to compare and correlate the 3 different techniques of CAM model *viz.*, Drabkin's method, Overall quantification of blood vessels (using Stereomicrocope) and localized quantification of blood vessels (using discs) to assess the angiogenic potential of drugs.

METHODS

Eggs: 3 sets of 6 day-old embryonated White Leghorn eggs (procured from Goregaon poultry farm, Mumbai), were used for all the 3 techniques used in the study. The eggs were surface sterilized using ethanol. In aseptic conditions, a window was made at the broader end of the egg (containing the air sac) and the respective drugs were inoculated and the eggs were sealed with the parafilm strips. The eggs were incubated at 37°C with 80% humidity. On day 12 of the embryo, CAM was excised from the eggs and the effect of the respective drugs on angiogenesis in the CAM model was evaluated.

Drugs

All the 3 techniques of CAM model were compared by evaluating the effect of known angiogenic drugs *viz.*, Vascular Endothelial Growth Factor (VEGF-10ng/ml) (Sigma-Aldrich Co.)[13,14], Erythropoietin (Ery-100U/ml) (ESPOGEN, Vacsera), Heparin (Hep-5U/ml).

Estimation of hemoglobin content (Drabkin's method)

1 set of embryonated eggs, incubated till the day 12, at the specified conditions, was used for the estimating the hemoglobin content by the Drabkin's method. The CAM was separated from egg shell and the blood of CAM along with the CAM were dispensed in 15ml of Drabkin's reagent (Hemacor D) which was homogenized at a desired speed for 5 mins and further centrifuged for 20mins at 2500rpm. Optical Density (O.D.) of the supernatant was measured against the Drabkin's reagent at 540nm on Spectrophotometer (SPECORD 200). The O.D is directly proportional to the hemoglobin content of the CAM.[11]

Image Analysis Methods: Quantification of blood vessels using the stereomicroscope

A] Overall Quantification

On day 12, from the 2nd set of inoculated eggs, CAM was separated and spread out on the petri-plate for stereomicroscopic examination. The images of four non-overlapping regions of CAM were taken and average count of the blood vessels was obtained[14] using '3.1 Analysis Software' which was further compared with that of the untreated eggs.

B] Localized Quantification by Disc Method

In this method, on day 6 of the embryo, instead of inoculating the drugs directly, a sterile Whattman filter paper no.1discs[15] containing the desired volume of drugs absorbed in them were placed near the main vein, on the developing CAM through the inoculation window which was then sealed using parafilm strips and incubated further till day 12 of embryo at 37°C. On day 12, the developed CAM separated with the disc was observed under the stereomicroscope for the neo-vascularization around the disc and images of the neo-vascularization were clicked and further rated on an analogue scale of 0 to 5.5.[15-17]

Statistical Analysis

The results obtained using all the techniques were analyzed using ANOVA followed by Tukey's post hoc tests on "Graphpad Instat 3 Software". The data was compared with untreated control. As the 3 techniques of CAM model performed, demonstrated results in different units the results were converted into Relative %angiogenesis by considering the highest values of angiogenesis obtained by VEGF to give 100% angiogenesis; as it has a highly specific mitogen for endothelial cells making it a high inducer of angiogenesis.[18] Relative % angiogenesis obtained using the 3 techniques were analyzed using Pearson's Correlation.

RESULTS

In the Drabkin's method, pro-angiogenesis was observed in the CAM subjected to VEGF (79.48±11.62%) and Erythropoietin (56.30±14.78%), whereas anti-angiogenesis were observed in the CAM subjected to Heparin (18.55±5.54%) when compared to the untreated CAM (29.30±6.13%).

Overall quantification of blood vessels demonstrated that both VEGF (92.84±3.76%) and Erythropoietin (83.31±19.60%) exhibited a pro-angiogenic response, whereas Heparin (32.07±9.72%) gave an anti-angiogenic response in comparison to the untreated CAM (61±12.35%).

Localized quantification of angiogenesis demonstrated similar results with the pro-angiogenic activity seen in CAM treated with VEGF (99.47±2.21%) and Erythropoeitin (72.47±9.98%), while Heparin demonstrating an anti-angiogenic activity (27.79±12.66%), compared to the untreated CAM (48.57±7.93%).

The results obtained in all the 3 techniques were similar. The results obtained are tabulated in the Table 1 and Figure 1.

Table 1: Relative Percent Angiogenesis obtained using 3 techniques

Groups	Drabkin'sMethod	Overall Quantification	Localized Quantification
Control (%)	29.49±6.13	61±12.35	48.57±7.93
Erythropoietin (%)	56.30±14.78	83.31±19.60	72.47±9.98
VEGF (%)	79.48±11.62	92.84±3.76	99.47±2.21
Heparin (%)	18.55±5.54	32.07±9.72	27.79±12.66

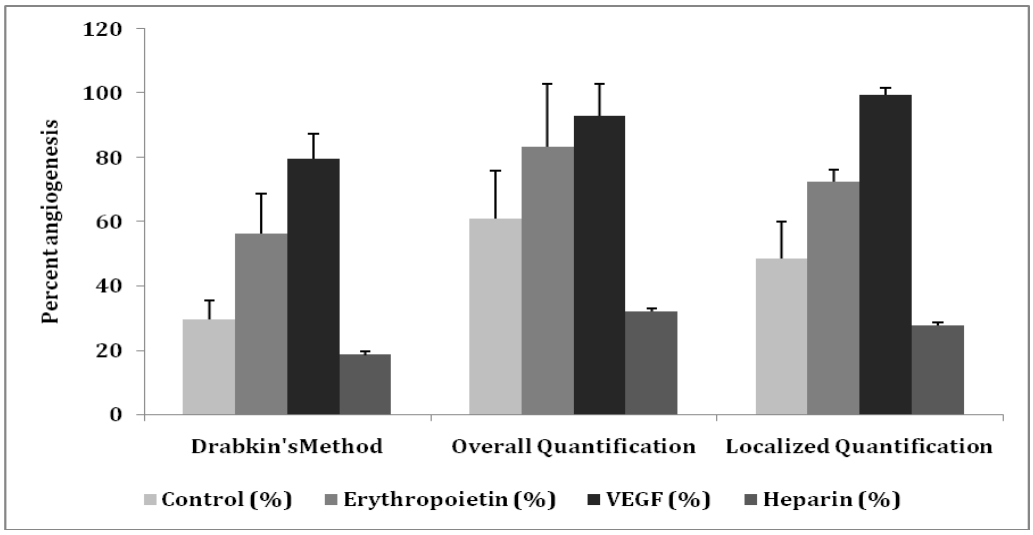

Fig 1: Relative Percent Angiogenesis obtained using 3 techniques

Correlation among the 3 techniques

The percent angiogenesis data obtained using all the 3 techniques of CAM model were analyzed using Pearson's Correlation to confirm that the correlation of Drabkin's method to the 2 Image analysis techniques of CAM model. The statistical analysis demonstrated that the results obtained using the Drabkin's method were in agreement to both the Image analysis techniques, *viz.*, Overall and Localized methods used in the study. The correlation results of the Drabkin's method with the Stereomicroscopic techniques using Pearson's Correlation was (r = 0.9877) which was extremely significant (p<0.0001). The correlation data obtained from the study is depicted in Figure 2.

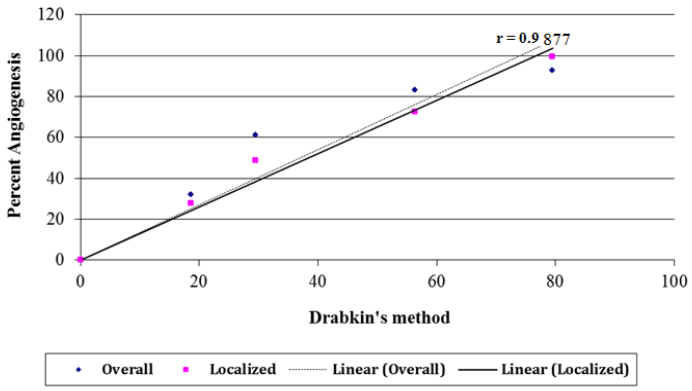

Fig 2: Correlation among the 3 techniques of the CAM model

DISCUSSION

In a normal healthy individual a perfect balance is maintained between the various intrinsic angiogenic modulators. Many diseases are driven by persistent unregulated angiogenesis.[4] Thus, the Angiogenic Switch is proven to be a critical progression point in a

range of pathologies.[3] Hence, the process of angiogenesis is being targeted for the treatment of various pathological diseases.

The chick embryo chorioallantoic membrane (CAM) was adapted as an angiogenesis assay by Folkman and coworkers, initially to examine the angiogenic activity of tumor tissues. CAM is an extra-embryonic membrane that is commonly used *in vivo* to study both new vessel formation and its inhibition in response to tissues, cells, or soluble factors.[7] The vascular system of CAM is directly accessible to observation and experimentation, and there are no metabolic or hormonal influences from the mother that directly affect angiogenesis. In addition, it is a more physiological model than *in-vitro* models because vascularization of CAM is subjected to regulations through fluxes, pressure, shear stress and growth factors.[18] The major features of both yolk sac and chorioallantoic membranes is the vascular system, which appears to be sufficiently similar to the mammalian counterpart to make this a useful *in-vivo* model and a possible alternative to rodent models.[19]

Quantification of Blood vessels by Stereomicroscopic methods are been established for many years. However the technique proves to be expensive due to the need of the stereomicroscope and the image analysis software, also the quantification is subjected to the bias interpretation of the experimenter. The alternative is the determination of hemoglobin, a validated indicator of angiogenesis. Classic techniques for determining blood hemoglobin were based on estimation of oxygen, carbon monoxide capacity, or iron content. These assays proved unreliable because of the heterogeneous nature of hemoglobin. Hence the study was planned to standardize a new and simple Drabkin's method which can be performed in laboratories with limited funds & infrastructure[11], for the estimation of hemoglobin content of the CAM model, to evaluate the extend of angiogenesis.

The Drabkin's method is an indirect method that measures the hemoglobin content in the CAM proportional to the extent of angiogenesis. The concentration of hemoglobin is directly proportional to its optical density and the content of hemoglobin is proportional to the extent of angiogenesis thereby obtaining the advancement of angiogenesis under the influence of the drug inoculated. It is new and relatively simple as well as cost-effective technique to screen drugs using the CAM model for their ability to modulate angiogenesis. The results (Optical Density) obtained by the method are based on spectro-photometric readings that can be used to analyze pro- as well as anti-angiogenic effect of drugs.

Image analysis methods, however, are direct method that depends on the visual assessment of the blood vessel growth, which are mainly useful to assess pro-angiogenic effect of drugs. The cost of the method is more as the method demands for a Stereomicroscope along with special software for the quantification of the blood vessels.

In the study, Drabkin's method was developed and compared to the known image analysis methods. The method was modified from the known Drabkin's method[12] and used to evaluate the extent of angiogenesis obtained by using the known standard angiogenesis modulating drugs. VEGF[9] is a known pro-angiogenic drug used as a standard in most of the angiogenesis related studies. Erythropoeitin[15] is also a well-established pro-angiogenic drug that is used in angiogenesis studies. Heparin[9] is a known anti-angiogenic drug, which was used in the study. The drugs used were studied for their effect on angiogenesis using Drabkin's method and compared with the known Image analysis methods.

The results for the extent of the angiogenesis obtained using Drabkin's method was compared with the well-known image analysis methods, *i.e.*, Overall quantification and Localized quantification of blood vessels. The results of the Drabkin's method demonstrated a perfect correlation with the known techniques of Image analysis methods; however the method was less sensitive as compared to the known methods used in the study. Hence the Drabkin's method can be used to screen drugs with potential pro or anti-angiogenic activity in laboratories with limited funds & infrastructure, followed by the confirmation studies using more sensitive techniques, which will help reduce the cost of screening angiogenesis modulating drugs.

CONCLUSION

In the present work, a new method to assess the hemoglobin content in the CAM by "Drabkin's method" was standardized and validated against the known image analysis methods to screen angiogenesis using chick embryo chorioallantoic membrane model. The advantages include reproducibility, reliable measurement of the angiogenic response, simple handling and direct calculation of the functional blood volume.

In conclusion, our data indicates that Drabkin's method can be used to demonstrate the angiogenic response in CAM model, similar to the results obtained by the known image analysis methods *i.e.* the techniques demonstrated correlated results (r = 0.9877) which was extremely significant (p<0.0001). Therefore, our model system may be a significant contribution to the characterization of pro or anti-angiogenic compounds in the CAM model.

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