COMPARATIVE EVALUATION OF THE ANTI-ANGIOGENIC PROPERTIES OF VITAMIN B COMPLEX AND ASCORBIC ACID USING DUCK EMBRYO CHORIOALLANTOIC MEMBRANE (CAM) ASSAY

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ABSTRACT

With tens of millions of people diagnosed with cancer each year, and with the increasing attention on the use of vitamins as an important part of human health, investigating the effect of essential vitamins on angiogenesis which is a critical step in cancer development plays a significant role in fighting the disease. The study compares the effects of vitamin B complex (Vitamin B1 + B6 + B12) and ascorbic acid on angiogenesis using the Chorioallantoic Membrane (CAM) assay. The CAM of ten-day-old duck embryos were treated with various concentrations (1mg/ml, 3mg/ml, and 5mg/ml) of commercially available vitamin B complex and ascorbic acid, and the vascularization of the CAM was determined after two days of incubation. Results revealed that vitamin B complex and ascorbic acid have anti-angiogenic properties at peaks in dosages of 5mg/ml and 3mg/ml respectively. However, all concentrations of both vitamins have a significant effect on the growth of secondary blood vessels. The result of the post hoc test showed that vitamin B complex at a dosage of 5mg/ml exhibited the highest anti-angiogenic activity.

Keywords: angiogenesis, anti-angiogenic property, vitamin B complex, CAM assay

INTRODUCTION

Cancer is known to be one of the leading causes of death worldwide. It is also considered to be responsible for an estimated death of 9.6 million in 2018. Based on statistics, 1 out of 6 deaths is because of cancer. The most common cancers affect the lung, breast, colorectal, prostate, skin, and stomach. Moreover, about 70% of deaths from cancer affect low- and middle-income countries (WHO, 2018). A lot of synthetic agents are utilized to cure cancer, but these are known to be highly toxic (Bisht et. al, 2011). Cancer medicines are also valued very high, therefore a lot of people in developing countries cannot afford these medicines causing an increase in the mortality rate. Efforts to find a cure for cancer never stopped and are still being intensified in the present day. Government, and even private institutions focus on developing drugs that primarily aims to lower the mortality rate, with the hopes of preventing the disease altogether. Currently, cancer remains to be a national health priority in the country. It is the third leading cause of death in the country, after heart diseases and vascular diseases. According to a study done by the University of the Philippines' Institute of Human Genetics, National Institutes of Health, about four Filipinos die of cancer every hour, or approximately 96% of patients daily.

Vitamins have been recognized for their role in keeping the body's normal functioning due to several studies that proved their relationship with human health and diseases. At present, it is known that vitamins can be used in the prevention and treatment of cancer, but more studies still have to be conducted to obtain conclusive results. With the widespread usage of vitamins, investigations of their effects on angiogenesis will be of high importance.

Angiogenesis is the growth of new capillaries from blood vessels which are already existing. This process is mediated by a cellular event which eventually leads to neovascularization. According to Yoo and Kwon (2013), angiogenesis is associated with tumor growth because of its relation to the formation of new blood vessels. The standard of care for different types of cancer is by blocking the formation of new blood vessels in tumors, known as anti-angiogenesis therapy. In this type of therapy, it is expected that the tumor will be “dormant” (Yang, Xu, Mu, and Xie, 2017). Chorioallantoic membrane (CAM) assay, has been utilized to study angiogenesis in various types of cancers.

CAM assay is less costly and time-consuming as compared to animal models which is why it is an attractive alternative for the *in vivo* assessment of potential cancer (Lokman, Elder, Ricciardelli, and Oehler, 2012). It is ideal to use CAM assay because it is simple, low-cost, quick, and it also allows to screen many samples given a restricted time. Moreover, it does not include procedures that require approval from the ethics committee for animal experimentation (Ribatti, 2016).

The Vitamin B-complex includes all the known essential water-soluble vitamins except for vitamin C. These include thiamine (Vitamin B1), riboflavin (Vitamin B2), niacin (Vitamin B3), pantothenic acid (Vitamin B5), pyridoxine (Vitamin B6), biotin, folic acid, and the cobalamins, Vitamin B12. Vitamins B1, B2, B3, and biotin participate in different aspects of energy production, Vitamin B6 is essential for amino acid metabolism, and Vitamin B12 and folic acid facilitate steps required for cell division (Peace Health, 2005). Previous studies have reported the effects of these individual vitamins on angiogenesis. Still, no study has been available reporting the effects of the use of the combinations of these vitamins on angiogenesis. Saghiri et al. (2017) report that the anti-angiogenesis effect of most of the vitamins acts by altering the cell cycle, or the cell receptors involved in cell migration and proliferation. These vitamins affect cell pathways such as PKB/Akt, NAS (+)-dependent, and Sirtuin (SIRT) mediated responses, decreasing DNA synthesis or induction of cell cycle arrest and apoptosis to exercise their effects.

Vitamin C is known to be an antioxidant helping to protect cells from the damage caused by free radicals and as well helps the immune system work properly to protect the body from diseases (Carr and Maggini, 2017). Studies have reported that Vitamin C, most especially in high dosage, acts as an angiogenesis inhibitor by changing the metabolic activity of endothelial cells, decreasing their ATP levels and cell proliferation. Vitamin C has been also reported to inhibit the formation of nitric oxide (NO), as NO is an important regulator of angiogenesis (Mikirova et al., 2008)

With current inhibitors of angiogenesis exhibiting host toxicity and resistance after prolonged use, the search for natural alternatives is needed. This investigation supports the utilization of natural products and vitamins for potential angiogenesis regulatory activity. This study focused on comparing the effects of the commercially available Vitamin B complex (*Vitamin B1 + B6 + B12)* and ascorbic acid on angiogenesis through the Chorioallantoic Membrane (CAM) assay using ten-day-old duck embryos. The effect of different dosages (1mg/ml, 3mg/ml, and 5mg/ml) of vitamin B complex and ascorbic acid on the growth of secondary blood vessels was evaluated and compared to determine which among the treatment concentrations would exhibit the highest anti-angiogenic activity and could be used as a potential protective agent from cancer.

OBJECTIVES OF THE STUDY

This study aimed to evaluate the anti-angiogenic properties of Vitamin B complex and Ascorbic Acid using Duck Embryo Chorioallantoic

Membrane (CAM) Assay. Specifically, this study sought to:

1. determine and compare the antiangiogenic activity of the varying concentrations of vitamin B complex and Ascorbic Acid in terms of;
   1.1. primary blood vessel growth
   1.2. secondary blood vessel growth
2. determine the significant difference in the blood vessel growth between the treatment and control groups; and
3. determine the concentration of vitamin B complex and ascorbic acid that has the highest anti-angiogenic property.

METHODOLOGY

Preparation of Varying Treatment Concentrations

Vitamin B complex (Vitamin B1 + B6 + B12) and ascorbic acid (vitamin C) were acquired from a retail store in Batangas City. The Vitamin B complex tablets were pulverized using a mortar and pestle. The different concentrations of Vitamin B complex and ascorbic acid were prepared: T1=(vit B complex-1 mg/ml); T2 (Vit B complex-3mg/ml); T3 (Vit B complex-5mg/ml); T4 (Vit C -1mg/ml); T5 (vit C-3mg/ml); and T6 (Vit C-5mg/ml). The desired concentrations of each variable were prepared by diluting it with distilled water using the formula:

$$C1V1=C2V2$$

Where C1V1 is the initial concentration and volume of the vitamin and C2V2 is the treatment concentration and final volume of the vitamins.

Administration of the Treatment of the Duck Eggs

The duck eggs were incubated at 37°C and 70% humidity until the tenth day. On the tenth day of incubation, the eggs were removed from the incubator and swabbed with 70% ethanol for sterilization. Then, the air sac of each of the egg was determined using a flashlight. Without damaging the eggs, holes were punctured near the edge of the air sac using a sterile pithing needle. After which, 0.1ml of each treatment were induced to the eggs using a 1ml sterile syringe. The punctured holes were resealed using melted candle wax. Then, the eggs were returned to the incubator for another 48 hours.

Observation of CAM

After 48 hours of incubation, the duck eggs were removed from the incubator and the vascularization of the CAM was determined. To examine the CAM, the eggshells were removed then the embryos was extracted and placed in a sterile petri dish. The image of each embryo was taken using a camera phone. The vessels were manually counted and the data gathered was verified using the Image J Software. The branching of blood vessels (primary, secondary, or tertiary) was also identified and compared among the varying treatment concentrations.

Data Gathering and Statistical Analysis

One-way ANOVA was utilized to determine the significant difference between the treatments. For further analysis, the Scheffe Post Hoc test was also used in determining the significant difference of the blood vessels branching among treatment groups.

RESULTS AND DISCUSSION

1. Mean blood vessel growth of control and treatment

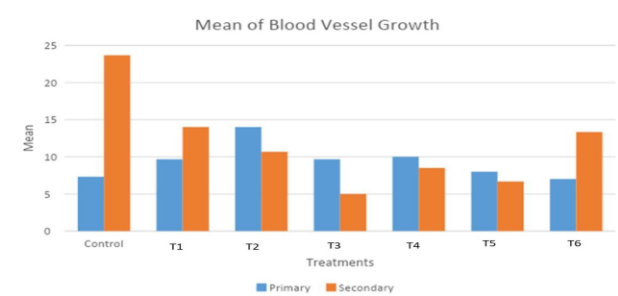

Figure 1. Mean blood vessel growth of control and treatment groups (Control: no treatment; T1: vit B-complex-1mg/ml; T2: vit B complex - 3mg/ml; T3-5mg/ml; T4: vit C- 1mg/ml, T5: vit C- 3mg/ml; T6: vit C-5mg.ml)

The results of the experiment on the use of different vitamins on blood vessel growth in duck embryos showed an increase in the primary blood vessels (Figure 1). However, the results also revealed that there was a decrease in the growth of secondary blood vessels (Figure 1). According to Jadhav & Kengar (2016), primary blood vessel growth is at its peak during the 10-11th day, this may account for the increase in the primary blood vessels of the treatment groups, since the vitamins are yet to be absorbed by the embryo, and thus the effect is exhibited on the secondary growth at which is optimum during the 12-14th day of incubation (Deryugina & Quigley, 2008).

2. Comparison of blood vessel formation (Primary & Secondary) among treatment groups

Table 1

Comparison of blood vessel formation (Primary & Secondary) among treatment groups (T1: vit B complex-1mg/ml; T2: vit B complex -3mg/ml; T3: vit B complex- 5mg/ml; T4: vit C-1mg/ml; T5: vit C- 3mg/ml, T6: vit C- 5mg.ml)

Treatments	Blood Vessels	
	Primary	Secondary
T1	-2.3333	9.6667*
T2	-6.6667	13.0000*
T3	-2.3333	18.6667*
T4	-2.6667	15.1667*
T5	-0.6667	17.0000*
T6	0.3333	10.3333*

with significant difference

Table 1 shows that using Scheffe Post Hoc test, the increase in primary blood vessel growth is not significant while there is a significant decrease in the secondary blood vessel growth, as compared to the controlled setup. Further, the result of the post hoc test also revealed that out of the six treatments, T3 (vit B complex-5mg/ml) showed the most significant effect in decreasing the number of blood vessel growth (Table 1). Overall, the decrease in secondary blood vessel formation could be accounted for by the anti-angiogenic property of Vitamin B and C (Saghiri et al., 2017).

3. Various concentrations of Vitamin B complex and Vitamin C

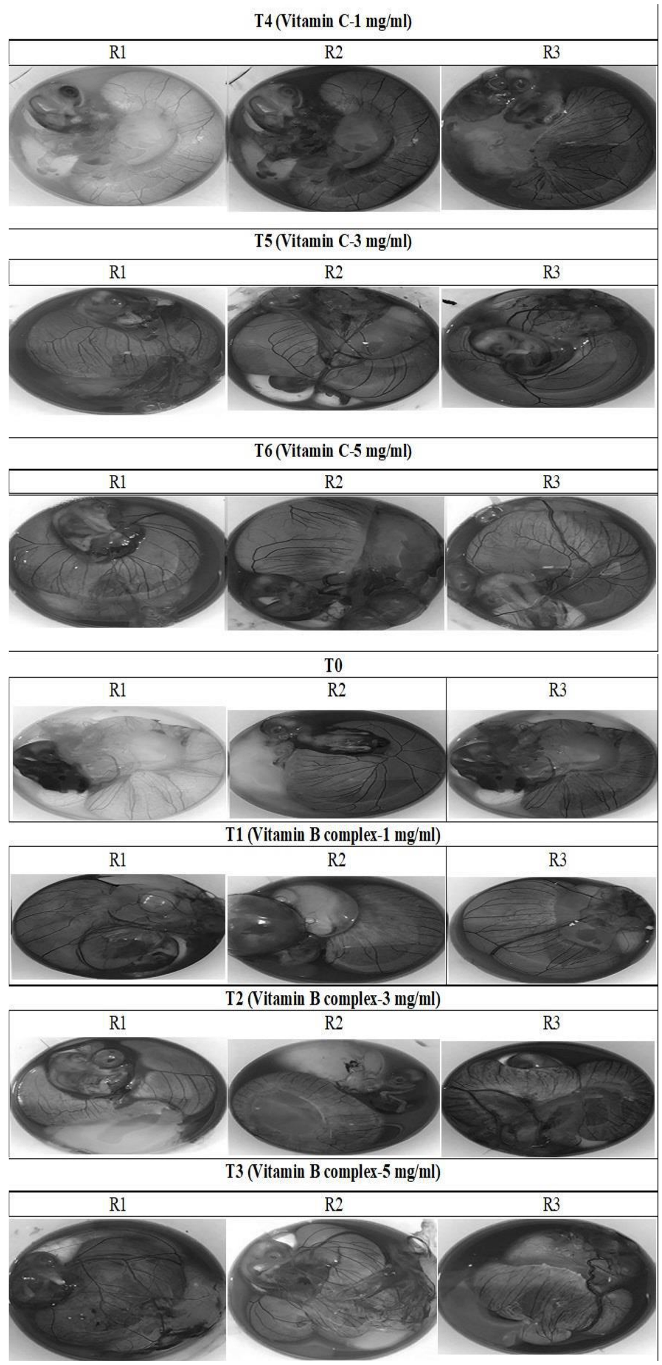

Figure 2. Duck embryos treated with various concentrations of Vitamin B complex and Vitamin C

Vitamin B-complex has the following sub-components: Vitamin B1, B6, and B12. According to Gadau et al. (2006), Vitamin B1 or thiamine simulates angiogenesis by inducing the proliferation of human endothelial progenitor cells and inhibiting apoptosis via PKB/Akt-mediated potentiation. On the other hand, Vitamin B6 and B12 are anti-angiogenic in nature. Vitamin B6 inhibits angiogenesis by inhibiting microvessel outgrowth and suppressing the proliferation of endothelial cells while Vitamin B12, Cobalamin, acts as a pro-angiogenic substance by inducing the production of nitric oxide (NO), prostaglandin E1, and prostacyclin leading to angiogenesis. Furthermore, Vitamin B12 reduces homocysteine levels in plasma, which is a significant anti-angiogenesis agent (Saghiri et al., 2017). The anti-angiogenic property of B6 and B12 could have masked the pro-angiogenic property of B1 and thus exhibited anti-angiogenesis. Among the treatment concentrations, 5mg/ml exhibited the lowest formation of the secondary blood vessel (Figure 2). The dosage at which the vitamins are of maximum potency in their angiogenic property is yet to be determined in order to determine its maximum effects.

According to Yeom et al. (2009), vitamin C or ascorbic acid inhibits NO in endothelial cells, maintains iron in the ferrous state, and regulates hypoxia-inducible transcription factor (HIF). This leads to carcinogenic effects induced by high concentrations of ascorbic acid through inhibition of angiogenesis and limiting angiogenesis and blood vessel formation. The data also shows that the treatment of 3mg/ml exhibited the lowest formation of the secondary blood vessel.

CONCLUSION

Results unveil that both vitamin B-complex and ascorbic acid have anti-angiogenic property at peaks in dosages of 5mg/ml and 3mg/ml, respectively. This is exhibited by the highest significant difference in the secondary blood vessel formation among the treatment concentrations and untreated group. Vitamin B6 inhibits angiogenesis by inhibiting microvessel outgrowth and suppressing the proliferation of endothelial cells while Vitamin B12, Cobalamin, acts as a pro-angiogenic substance by inducing the production of NO, prostaglandin E1, and prostacyclin leading to angiogenesis. Meanwhile, vitamin C carcinogenic effects are induced by high concentrations of ascorbic acid through inhibition of angiogenesis and limiting angiogenesis and blood vessel formation.

RECOMMENDATION

Since the Chorioallantoic membrane (CAM) assay is a preliminary assay used to determine the antiangiogenic property of certain substances, further evaluation can be done on the antiangiogenic property of vitamin B complex and ascorbic acid by utilizing different assays. Moreover, the concentrations of the treatment groups can be expanded to a higher and lower limit to determine the minimum and maximum concentration optimum for its antiangiogenic property. The researchers also recommend looking into Vitamin B complex and Ascorbic Acid's anti-carcinogenic and/or anti-mutagenic properties.

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