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Investigations on Angiogenic Potential of Ranitidine Using Chorioallantoic Membrane (CAM) Assay

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Abstract: The uncontrolled growth of cells in body often results in metastasis of overgrown tissue into other parts of the body. This medical condition is known as cancer. Angiogenesis, formation of new blood vessels from the preexisting ones, plays an immense role in supporting development and progression of cancer by providing oxygen and nutrients to these abnormally dividing cells. Chorioallantoic membrane (CAM) of chick embryos is a well-known model system to study angiogenesis and for identification of angiogenic and anti-angiogenic molecules. Recently, Ranitidine, a commonly used antacid, has been banned in many countries due to its potential carcinogenic activity. Nitrosodimethylamine is thought to be the main contaminant and reason for this activity of Ranitidine. To our knowledge, this is the first report that evaluated the angiogenic potential of Ranitidine using chick embryo CAM model. Our preliminary observations demonstrate significant angiogenic activity of Ranitidine which may impart carcinogenic potential to it.

Keywords: Angiogenesis, Angiogenic molecules, Chorioallantoic membrane, Nitrosodimethylamine, Ranitidine, Cancer

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Introduction

Cancer, one of the serious medical complications, is characterized by uncontrolled growth of body cells which can migrate from one position to other within the body. Naturally, the newly formed or migrated tissue requires supply of oxygen,

nutrients and also timely removal of the cellular waste. To suffice this, body forms new network of blood vessels and the process by which these new blood vessels arise from the pre-existing vasculature is known as angiogenesis. A battery of

molecules acts as either activators (angiogenic molecules) or inhibitors (anti-angiogenic) of angiogenesis. The expression levels of these angiogenic and anti-angiogenic molecules determine the aggressiveness of cancerous cells (Nishida *et al.*, 2006). Thus, angiogenesis plays an important role in cancer development and progression.

Chorioallantoic membrane (CAM) of chick embryo has gained immense importance in vascular biology, biomedicine, tumor progression and metastasis, screening and development of new drugs, wound healing etc. (Chen *et al.*, 2021). It serves as a preclinical model for angiogenic and anti-angiogenic effects of potential therapeutic drugs. CAM is the heavily vascularized outermost extra-embryonic membrane in developing chick embryos. The exchange of gases, and transport of molecules between embryo and its surrounding environment takes place through CAM (Metcalf and Stock, 1993). CAM assay is a simple, quick and economic technique without any ethical issues (Rabatti, 2018).

Ranitidine (a member of class histamine-2 blocker drugs) was sold over the counter as an antacid under brand names like Zintec, Rantac, Aciloc etc. (McGwin, 2021). It downregulates the amount of acid production in stomach and is thus used to treat indigestion, heartburns, stomach ulcers, reflux diseases etc. It is also recommended for patients with Zollinger-Ellison syndrome wherein stomach produces excess acid. Ranitidine is thought to be contaminated with N-Nitrosodimethylamine (NDMA), an impurity whose concentration increases concurrently with storage time (McGwin, 2021). NDMA is usually present in some food items and in water as well. However, the very low quantities of NDMA from food and water are easily ingested by human body without much medical issues. Exposure to persistently higher concentrations of NDMA, through drugs, may increase the risk of developing cancer (Li *et al.*, 2021). Since 2019, the sell of Ranitidine is kept on hold by the FDA, USA due to its possible contamination with NDMA. The health

ministries of many other countries have also banned use of Ranitidine on similar lines. In spite of these, there are hardly any reports which have evaluated the angiogenic and carcinogenic activity of the drug. To the best of our knowledge, the present study is the first ever report that shares the preliminary observations on the angiogenic potential of Ranitidine using CAM assay.

Materials and Methods

Ranitidine dose preparation:

Ranitidine tablets were purchased from a local pharmacy shop. As per the description written on the strip, each Ranitidine tablet contained 168 mg of Ranitidine HCl equivalent to 150 mg of Ranitidine. The tablets were dissolved and diluted with 1X PBS (pH 7.4) so as to prepare 1 µg/10 µl, 5 µg/10 µl and 10 µg/10 µl doses.

CAM assay:

CAM Assay was carried out according to Surekha *et al.* (2013). Fresh fertilized egg of *Gallus domestics* were purchased from a local poultry and incubated at 37 °C and 65% relative humidity. On day 3, a window (1 cm x 1 cm) was made on the egg shells, sealed with sterile tape and incubated further till day 9. Embryos were observed and the eggs with dead embryos were discarded. Sterile Whatman filter paper ring (1.5 cm diameter) was placed on Chorioallantoic Membrane (CAM) carefully. After resealing of windows, the eggs were incubated further. On the next day (Day 10), eggs were randomly distributed into control and treatment groups, and test sample (1XPBS, pH 7.4 in controls and various doses like either 1 µg, 5 µg or 10 µg of Ranitidine in treated groups) was applied in 10 µl volume inside the Whatman filter paper ring. All eggs were incubated for next 48 h. The embryos were removed from the incubator and ice cold 4% ice cold paraformaldehyde, prepared in PBS, was injected and spread over the membrane. The windows were resealed and kept at 4°C overnight for proper perfusion of the fixative. Next day the CAM were excised, washed with 1X PBS and observed under Leica DM 1000 microscope (DST-FIST

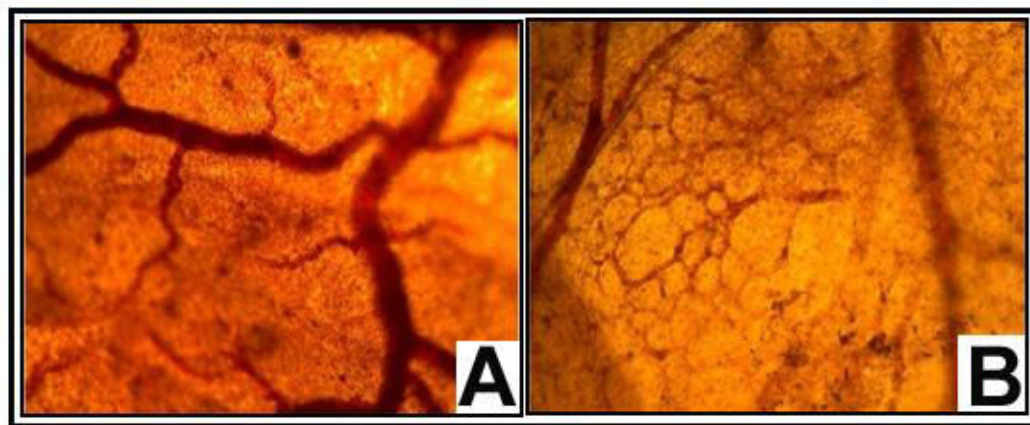


Fig. 1: Effect of 1µg/10 µl Ranitidine on angiogenesis in developing CAM in chick embryos; A: Control CAM, treated with 1X PBS; B: 1 µg/10 µl Ranitidine treated CAM, note a network of fine capillaries in B as compared to A.

Grant). The observations were recorded using Future Winjoe Camera (procured through DBT Star Scheme).

Results

Effect of Ranitidine on angiogenesis:

At the end of the treatment period, the vasculogenesis from control and treated CAM was observed and compared. Ranitidine, at all the tried doses, showed clear differences in the overall angiogenesis as compared to the respective controls.

Effect of 1µg/10µl Ranitidine on angiogenesis in CAM:

The present study evaluated the effects of Ranitidine on angiogenesis in a dose dependent manner. The major blood vessels sprouting into a few small sized blood vessels indicated the levels of normal angiogenesis pattern in the control CAM on day 12 (Fig. 1A). In contrast, when the CAM were treated with 1 µg of Ranitidine (Fig. 1B), the formation of blood vessel network was found to be considerably enhanced as compared to the respective control treated with only 1X PBS (Fig. 1A). A microscopic network of thin new capillaries was evident in treated CAM as shown in the Figure 1. The number of capillaries enhanced visibly as compared to the controls.

Effect of 5 µg/10 µl Ranitidine on angiogenesis in CAM:

In another set of experiments, when the CAM were treated with 5 µg of Ranitidine, there was a significant increase in the capillary network with elevated number of capillaries as compared to the respective control (Figs. 2A, B). The visual network of capillaries was noticeably higher at 5 µg/10 µl (Figure 2B) than 1µg/10 µl Ranitidine (Fig. 1B). Thus, with increased dosage, Ranitidine exhibited relatively higher potential of angiogenesis.

Effect of 10 µg/10 µl Ranitidine on angiogenesis in CAM:

When the 10 µg concentration of Ranitidine was used for treatment, the CAM exhibited greater amount of angiogenesis (Fig. 3B) as compared to the controls (Fig. 3A) and lower doses of Ranitidine (Figs. 1B, 2B). Thus, Ranitidine showed higher angiogenic potential at the highest tried dose i.e. 10 µg/10 µl.

Discussion

The process of *de novo* formation of blood vessels is known as neoangiogenesis whereas formation of new blood vessels from the pre-existing ones is known as angiogenesis. In cancers, there is enhanced angiogenesis so as to support the

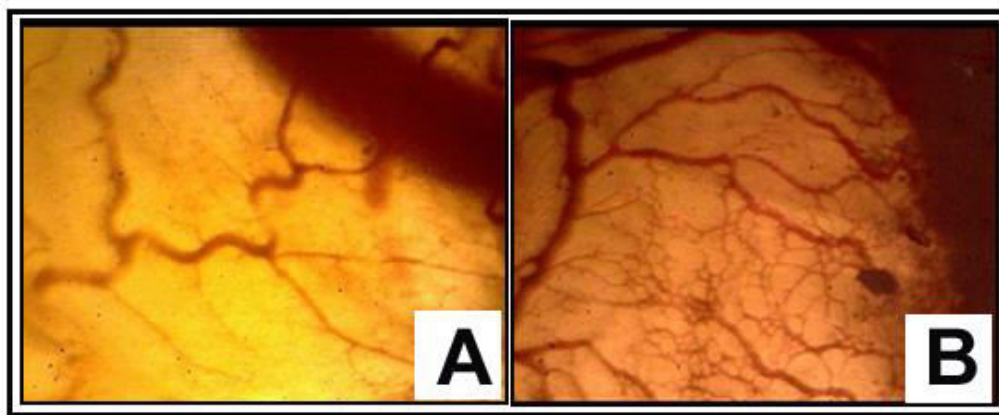


Fig. 2: Effect of 5 µg/10 µl Ranitidine on angiogenesis in developing CAM in chick embryos; A: Control CAM, treated with 1X PBS; B: 5 µg/10 µl Ranitidine treated CAM, note a network of fine capillaries in B as compared to A.

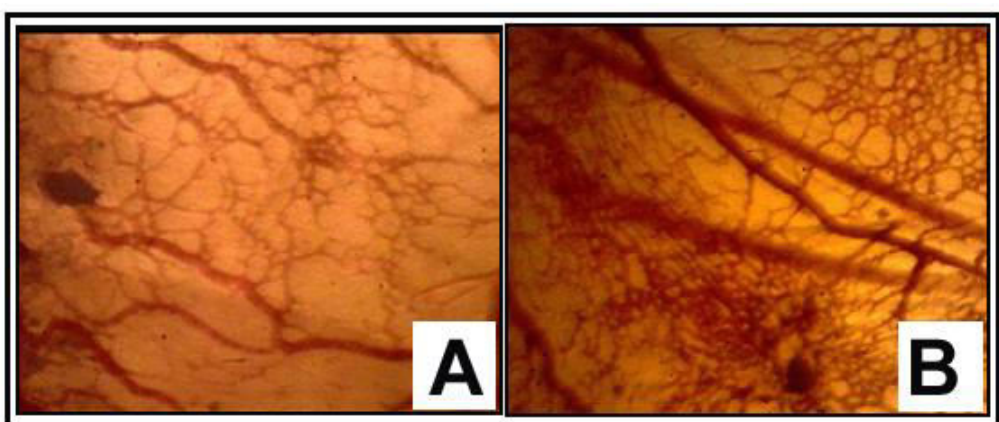


Fig. 3: Effect of 10 µg/10 µl Ranitidine on angiogenesis in developing CAM in chick embryos; A: Control treated with 1X PBS; B: 10 µg/10 µl Ranitidine treated, note enhanced network of capillaries in treated CAM as compared to the control.

developing new tissue. One of the major effects of carcinogen is to increase angiogenesis to suffice the need of the growing tissue (Nishida *et al.*, 2006). Being a rich vascular system, chorioallantoic membrane (CAM) of chick embryo is a well-known experimental model for angiogenesis and cancer progression studies (Burggren and Rojas, 2020; Chen *et al.*, 2021). Using CAM assay, one can identify the potential carcinogen by analyzing its proangiogenic activity. As compared to other available angiogenesis models, CAM shows rapid vascularization and is easy to monitor the morphological changes, during blood vessel formation, without disturbing and terminating the process. CAM assay has gained popularity due to its low cost, reliability and reproducibility. The only limitation this assay is

the non-specific inflammatory reactions that may take place in tumor studies (Metcalf and Stock, 1993; Chen *et al.*, 2021).

The present study clearly demonstrated the ill effects of Ranitidine on angiogenesis pattern in the developing chick embryo chorioallantoic membranes. Ranitidine significantly affected chick embryo angiogenesis, within 48 h, in a dose dependent manner. Our study thus identified Ranitidine to be a proangiogenic drug and showed higher angiogenic potential with increasing concentration. Ranitidine is an oral drug that blocks the production of acid in the stomach. It belongs to a class of drugs called H₂ (histamine-2) blockers. N-Nitrosodimethylamine is the main contaminant of many drugs, including Ranitidine, which is supposed to be the key element in

inducing cancer in a number of animal species (McGwin, 2021). In fact, air, soil and water are supposed to contain NDMA and other related nitrosoamines, formed by interactions between secondary or tertiary amines with the oxidizing agents. A couple of studies point at the NDMA as a potent carcinogen (Li *et al.*, 2021). Since Ranitidine also contains NDMA and increased angiogenesis is a prerequisite for the developing cancer, the present study was undertaken to assess the angiogenic potential of Ranitidine. The CAM, treated with Ranitidine, were found to be enriched with new capillaries as compared to the control CAM which were treated with only 1X PBS. Also, the angiogenic effect of Ranitidine was increasing in a dose dependent manner. One of the mechanisms by which NDMA may cause cancer is through metabolic activation. It interacts covalently with the DNA and causes promutagenic adducts of DNA. The DNA repair systems usually identify these changes and revert back the DNA damages during replication. However, if the DNA repair systems fail to identify and repair or are unable to deal with the extent of DNA damages, the changes may remain as it is. This may result in point mutations during next cycle of DNA replication (Adamson and Chabner, 2020). The present study suggests Ranitidine to be an angiogenic drug thus, could be a potential carcinogen which may be due to NDMA content.

Conclusion

The present study concludes that the excess use of Ranitidine may lead to disruption in normal angiogenic process, and act as a proangiogenic and potential carcinogenic molecule, too. The present study thus highlights precocious use of Ranitidine, a suspected carcinogen. A further detailed study for analyzing the rate of angiogenesis using statistical tools and potential carcinogenic activity needs to be performed. Further studies are essential to see if it also acts as a carcinogen. Our preliminary work indicated that Ranitidine is a proangiogenic molecule and a detailed study for molecular elucidation of the angiogenic potential of Ranitidine is in progress.

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