

The Effect of Diclofenac Sodium on Blood Vessel Formation

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Abstract

The blood vessel formation known as angiogenesis is an identifying characteristic of nearly all neoplastic and non-neoplastic degenerative diseases. The angiogenesis involves in normal physiology, in the progression of various diseases, as well as in the development of progressive arthritis and tumors. It is also deduced by inflammatory mediators that are included in cancer and are furthered by cyclooxygenases in order to contribution in endothelial cell spreading and cell migration. The effect of diclofenac sodium on angiogenesis has been explored using in vitro Chorioallantoic membrane assay. Structural changes in CAMs were carefully assessed by an inventive image probing system of scanning probe image processor (SPIP). About the number of fourteen parameters of 3D surface roughness was also quantified. The anti-angiogenic effect was observed at the sixth day of incubation by utilizing diclofenac sodium on Chorioallantoic membrane (0.7% of diclofenac sodium concentration). As a result, there have been changes in series of parameters containing the structure of CAMs, decrease in surface roughness, thinning of primary, secondary and tertiary blood vessels, enhancement of kurtosis of the 3D surface, and reduction of Abbott curve. The influential values for the local use of diclofenac sodium would demonstrate anti-angiogenic activity same as the in-vitro condition which illustrates its clinical efficacy.

Keywords: Angiogenesis; Diclofenac Sodium; Chorio-Allantoic Membrane (CAM) Assay

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1. Introduction

Angiogenesis means the formation of new capillary blood vessels that is extremely important for reproduction and cure. It is controlled by the body by making a natural balance between inhibitory and growth factors in healthy tissues. Angiogenesis will be disordered when disturbing this balance. Abnormal vasculature growth may cause grave situations such as cancer, diabetic ulcers, skin diseases, and many others. According to the initial hypothesis of Folkman in 1995, angiogenesis can limit the growth of solid tumors at 2-3 mm in diameter [1]. The main step of tumor growth is to extract blood supply from adjoining tissues which is totally documented [2]. Angiogenesis could be stimulated by inflammation which can also be facilitated by angiogenesis. Inflammatory

mediators as a source of pain may sensitize sensory nerves which can cause initiate angiogenesis and neurogenic inflammation (Bonnet and Walsh, 2004). Inhibition of angiogenesis by the help of NSAIDs is found to be multifactor, mainly as a promoting factor to cure the ulcer, including local changes in angiogenic growth factor expression, inhibition of cell migration, alteration in key regulators and mediators of vascular endothelial growth factor, recruitment of inflammatory cells and platelets, increased endothelial cell apoptosis (Klagsbrun, 1991). A dense capillary network is provided for the operation of chicken Chorioallantoic membrane. Histological aspects of the angiogenic process in vivo have extensively utilized CAM. It should be noted that the tumor begins to grow rapidly after 72 hours in the avascular situation by passing into new

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vasculatures. Therefore, CAM as a contributor factor could help with the inhabitation of vascular growth by implanting tumors on which and by comparing tumor growth and vascularization with or without the administration of an anti-angiogenic factor.

Tumor's invasion launched for blood vessels and the chorionic epithelium was also examined by the use of tumor cells/CAM model. The cells invade the epithelium and the mesenchymal connective tissue below, where they are found in the form of a dense bed of blood vessels, which is a target for intravasation (Ribatti and Domenico, 2010). Chorioallantoic Membrane (CAM) assay is an important model for investigation of vasculogenesis and angiogenesis which has been a favourite system in studying tumor angiogenesis and metastasis for a long time [3].

In accordance with the novel approach to analyse angiogenesis [4], an *in vivo* angiogenesis model system was developed by adaption of the CAM assay that is precisely quantitative, responsive to high-throughput screening, and applicable for the testing of systemic and/or topical administration of experimental agents. The present study was aimed at evaluating the effect of 0.7% diclofenac sodium concentration on angiogenic mechanism taking advantage of chicken Chorioallantoic membrane (CAM) assay.

2. Materials and Methods

Firstly, laid fertile broiler chicken eggs of a local hatchery must be incubated at 37°C and relative humidity of 55-60%. Eggs should be windowed in disinfected form on day five of incubation according to Ejaz et al. (2005). The modest window of almost 2cm in diameter was prepared by eliminating the shell and inner shell membrane which cause the removal of about 4-5 ml of albumin. Eggs were then kept in an incubator after sealing the windows by Para film.

Diclofenac sodium concentration was diluted 0.7% by the use of distilled water and the pH was then adjusted in the range of 6.5 - 7.5 through a pH meter.

This dilution was filtered through a syringe filter (0.2 µm) to reduce the risk of contamination.

The number of twenty day six chicken Chorioallantoic membranes (CAMs) were employed in this research dividing into two groups so that both A and B groups contain ten eggs. On the sixth day of incubation, group A was selected as control while by applying distilled water and group B just get 0.7% of diclofenac sodium concentration. All eggs were then maintained in the incubator for farther 24 hours after sealing the openings with sterile Para-film tape.

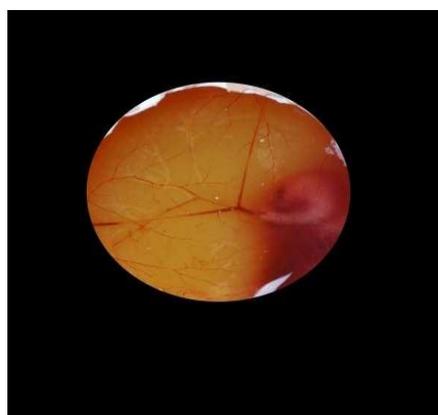
Control and treated CAMs were monitored by recording regular images on day seven of incubation that is 24 hours after dispensing diclofenac sodium. Using Adobe Photoshop 6.0, the contrast of vasculature and other tissues can be modified providing us with discrimination of anatomical structures for each image. SPIP software (IBM Denmark) which is a program to process images on a special algorithm was then used for automatically quantification of surface roughness and other associated parameters for precise assay on the anti-angiogenic response [6].

Afterwards, all data was statistically analysed. In this regards, any difference of control and treated parameters was evaluated by the analysis of variance (ANOVA) with a statistical significance of $P < 0.05$. As well, post hoc Student's *t*-test was also done with the significance of $P < 0.0$ [7].

3. Results and Discussion

The vascular structure of CAMs has significantly changed by the 0.7% of diclofenac sodium concentration causing anti-angiogenic activities which brought about primary and secondary blood vessels' thinning as well as tertiary blood vessels' fading. It caused a pronounced decrease in the total vascular network of CAM (Figure 1).

The CAM vasculature's diameter was determined by SPIP. The results showed a significant decrease in the diameter of primary, secondary and tertiary vasculature in treated groups in comparison with the control group (Figure 2).

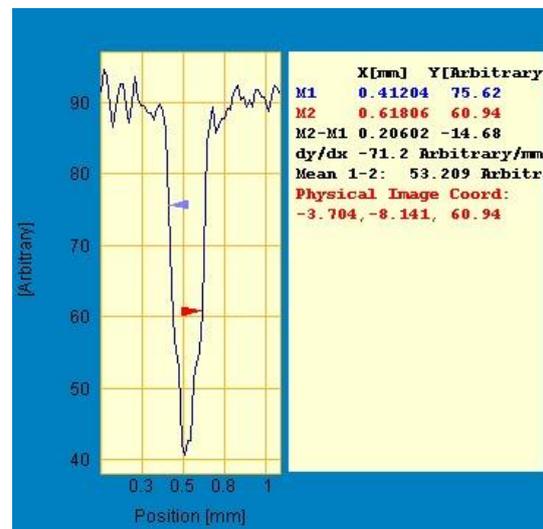
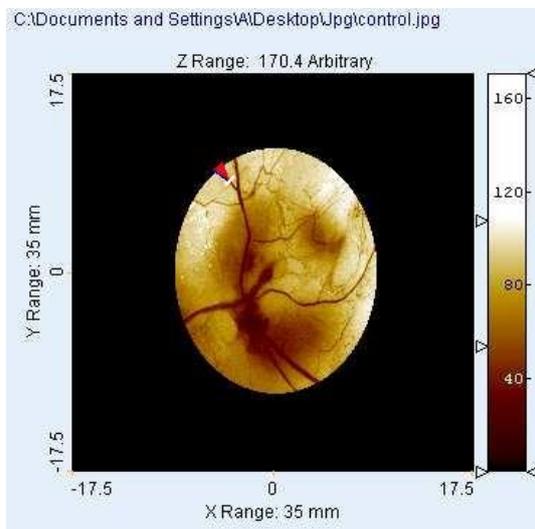
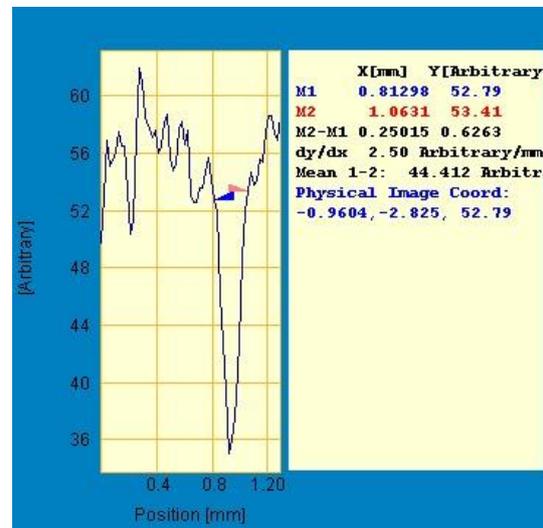
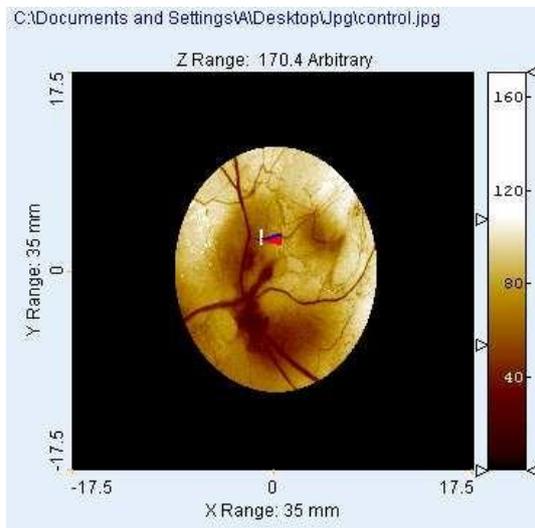
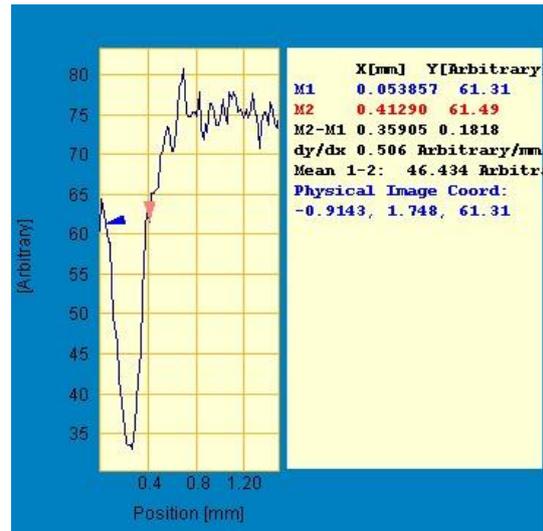
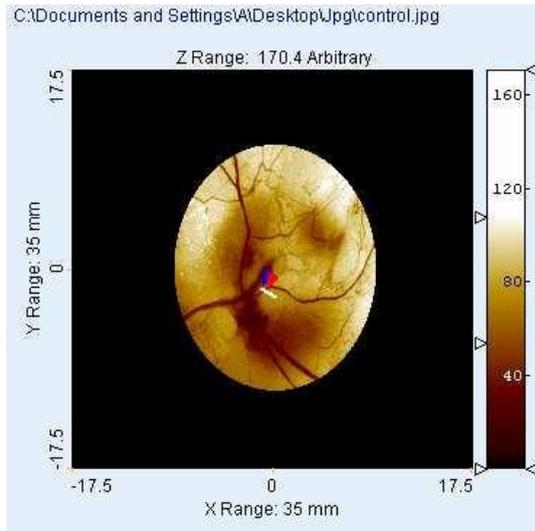


(A)



(B)

Figure 1. Macroscopic evaluation of chicken chorio-allantoic membrane at day 6 of incubation. Note the well defined architecture of CAM blood vessels consisting of primary, secondary and tertiary blood vessels in control group with well-developed area of CAM (A), while CAM treated with diclofenac sodium resulted in extensive decrease in CAM blood vessels and reduction in total area of CAM representing extensive anti-angiogenic activities.



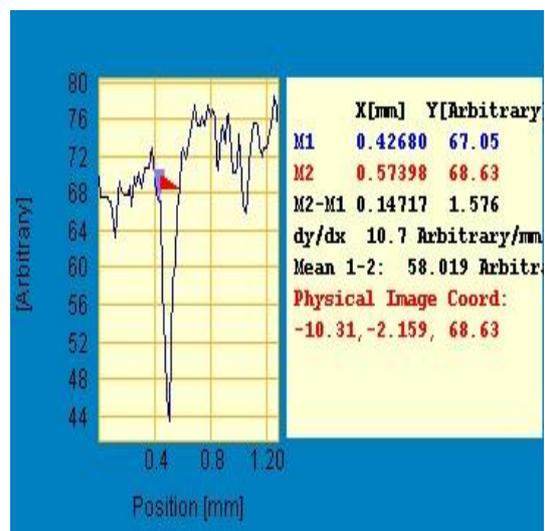
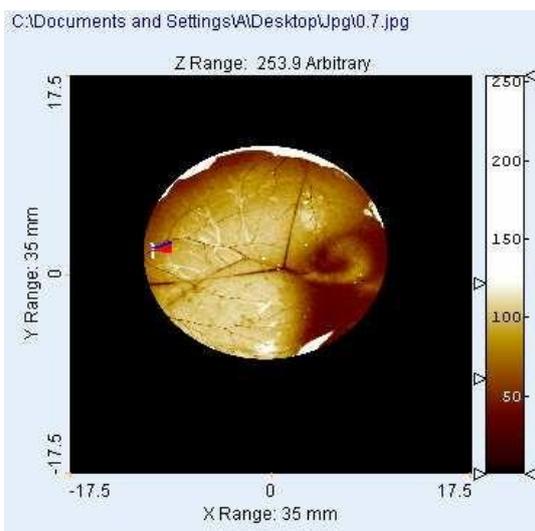
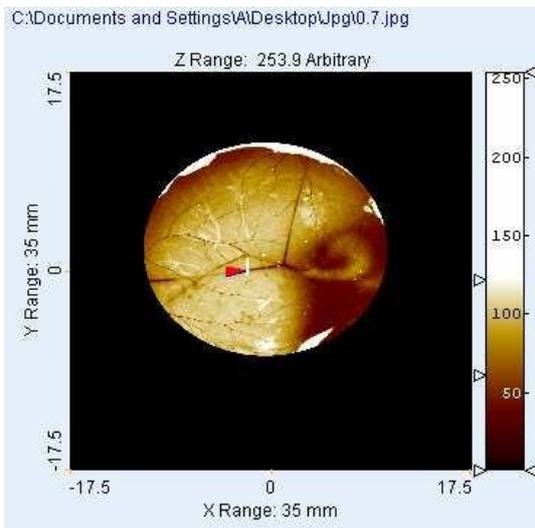
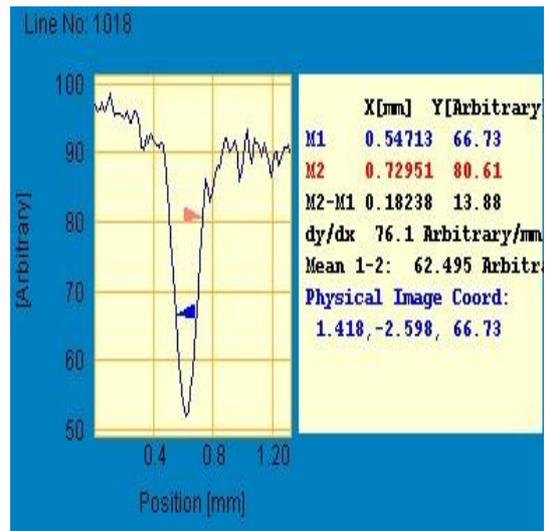
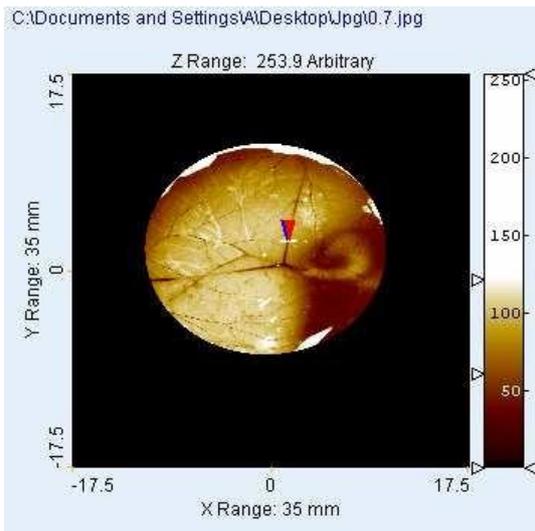
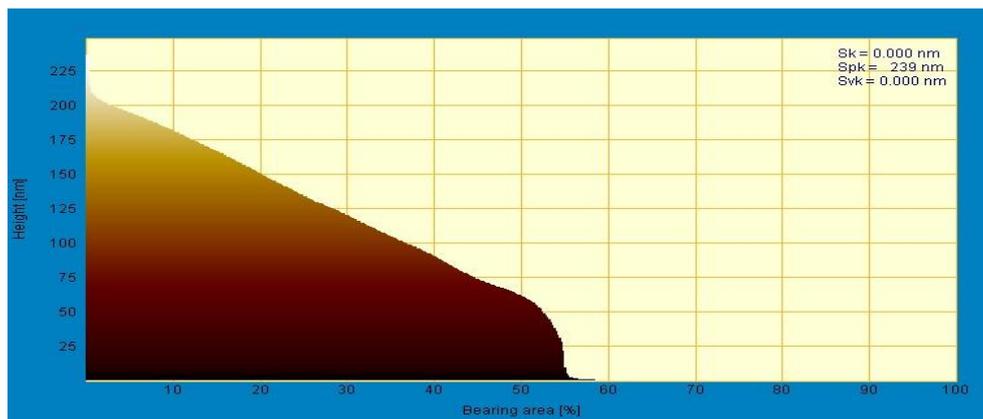
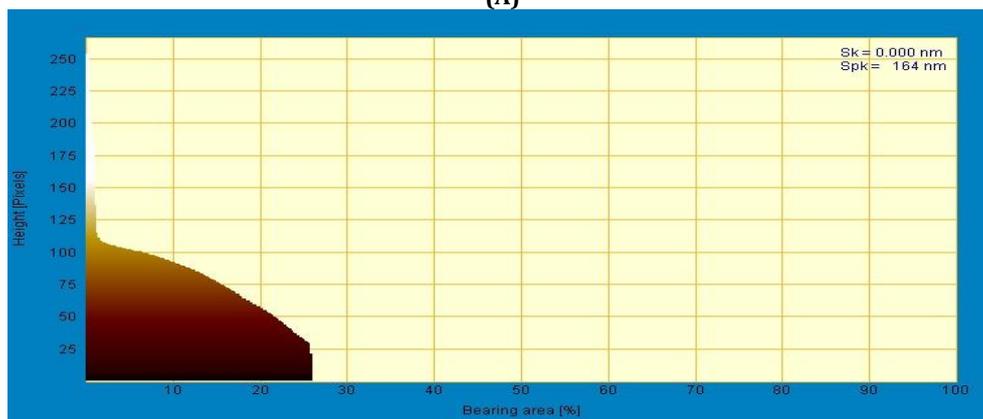


Figure 2. Diameter of blood vessels on CAM of control (A), 0.7% (B)



(A)



(B)

Figure 3. Abbott curve of the blood vessels on CAM of control (A) and treated (B) eggs showing less height of blood vessels on the CAM of treated sample (B) than control (A).

Table 1. Roughness of control and treated CAMs

No.	Parameter (nm)	Control	0.7% Concentration
1	Sa	25.33±1.45	18.25±2.38
2	Sq	50.21±1.11	39.37±2.77
3	Ssk	1.55±0.22	1.37±0.19
4	Sku	2.83±0.14	3.35±0.10
5	Sdr	1.52±0.07	1.16±0.01
6	Sci	1.12±0.32	0.74±0.02
7	Sy	254.2±4	169.1±5.13
8	Sz	266.3±2.97	203.2±4.43
9	Ssc	1.12±0.13	0.9±0.27
10	Sdq	0	0
11	Spk	239.5±9.3	145.1±2.1
12	Svk	0.76±0.03	0.42±0.07
13	Stdi	0.831±0.10	0.756±0.09
14	Sk	0.42±0.06	0.20±0.07

Sa: average roughness; **Sq:** root mean square deviation; **Ssk:** skewness of the surface; **Sku:** kurtosis of the surface; **Sdr:** developed surface area ratio; **Sci:** core fluid retention; **Sy:** lowest valley; **Sz:** maximum height of the surface; **Ssc:** arithmetic mean summit; **Sdq:** root mean square slope; **Spk:** reduce summit height; **Svk:** reduce valley depth; **Sti:** texture index; **Sk:** core roughness depth

The 3D surface roughness parameters of control and treated CAMs was determined for more details. The control CAMs had more average roughness

values than treated CAMs which represented significantly more neo-vascularization ($P < 0.05$) in comparison of treated CAMs. Table 1 demonstrates quantification of twelve surface roughness parameters of CAMs which are different for control and treated groups to recognize the angiogenesis. The graphical representation of roughness parameters was also investigated as Abbott curve to measure any specific changes in the height of blood vessels on the surface of CAMs. The heights of the Abbott curve were 213 nm and 164 nm for control and treated CAMs respectively (Figure 3).

4. Conclusion

Anti-angiogenic effect of diclofenac sodium was confirmed by the evaluation of the whole parameters in chicken Chorioallantoic membrane. All things considered, diclofenac sodium can inhibit the angiogenic process possibly because of suppression of alphaVbeta3 integrin-mediated and Cdc42/ Rac- dependent endothelial cell spreading, migration, and angiogenesis.

Further studies on diclofenac sodium are somehow necessary, particularly in order to find out principles of outlining strategies for the cure and prevention of several types of diseases related to angiogenesis.

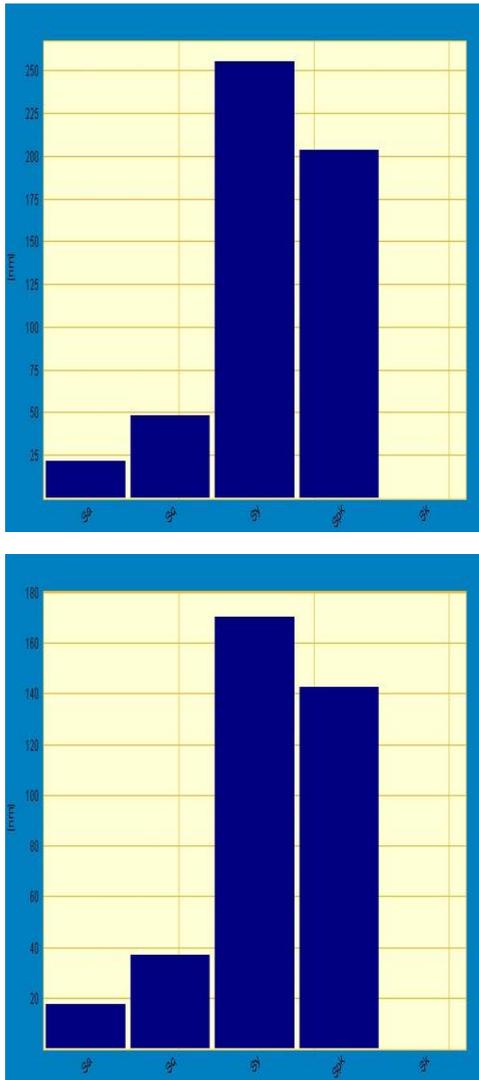


Figure 4. A graphical representation of various surface roughness parameters of control (A) and 0.7% treated (B) CAMs

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