Investigation of Diclofenac Sodium on Angiogenesis through Blood Assay

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Abstract

Angiogenesis, the formation of new blood vessels, is a hallmark of almost all neoplastic and non-neoplastic degenerative diseases. This process supports normal physiology as well as it contributes in progression of different diseases. Angiogenesis contributes in growth of tumor and progressive arthritis. Inflammatory mediators are involved in cancer induced angiogenic process. Cyclo-oxygenases promote these mediators which help in cell migration and endothelial cell spreading. To explore the role of diclofenac sodium in angiogenesis we have used in vitro Chorioallantoic membrane assay. A novel image probing system SPIP (scanning probe image processor) was utilized for assessment and quantification of structural changes in CAMs. Fourteen parameters of 3D surface roughness were also evaluated for quantification. Application of diclofenac sodium on Chorioallantoic membrane at day six of incubation (0.7% concentration of diclofenac sodium) showed anti-angiogenic effect. Results showed marked changes in architecture of CAMs, thinning of primary, secondary and tertiary blood vessels, reduction in surface roughness parameters, increase in kurtosis of surface and decrease in Abbott curve. The substantial quantities of diclofenac sodium use locally may exhibit anti-angiogenic activity in the same manner those seen in in-vitro and explain its clinical efficacy.

Keywords: Diclofenac sodium; Angiogenesis; Chorio-allantoic Membrane (CAM) assay

1. Introduction

Angiogenesis, the growth of new capillary blood vessels in the body, has much more importance in healing and reproduction. The body controls angiogenesis as there is a natural balance between growth and inhibitory factors in healthy tissues. When this balance is disturbed, the result is either too much or too little angiogenesis. Abnormal blood vessel growth causes serious conditions like cancer, skin diseases, diabetic ulcers and many others. Folkman first hypothesized in 1971 that solid tumors remain growth restricted to 2-3 mm in diameter until the onset of angiogenesis [1]. Tumors derive blood supply from adjacent tissues, are an important step in tumor growth, and are now well documented [2]. Inflammation can stimulate angiogenesis and angiogenesis can facilitate inflammation. The sensitization of sensory nerves by inflammatory mediators is also a source of pain, and sensitized nerves can cause neurogenic inflammation and initiate angiogenesis [3]. Inhibition of angiogenesis caused by NSAIDs is a contributing factor in ulcer healing, a mechanism by which NSAIDs inhibit angiogenesis is appearing to be multifactor and includes local changes in angiogenic growth factor expression, alteration in key regulators and mediators of vascular endothelial growth factor, increased endothelial cell apoptosis, inhibition of cell migration, recruitment of inflammatory cells and platelets [4]. Function of chicken Chorioallantoic membrane is supported by presence of dense capillary network. CAM has been broadly used to study the morpho-functional aspects of the angiogenesis process in vivo due to its extensive vascularization. Tumors remain avascular for 72 h, after which they are penetrated by new blood vessels and begin a phase of rapid growth. The CAM may also use to verify the ability to inhibit the growth of capillaries by implanting tumors onto the CAM and by comparing

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tumor growth and vascularization with or without the administration of an anti-angiogenic molecule. Other studies using the tumor cells/CAM model have focused on the invasion of the chorionic epithelium and the blood vessels by tumor cells. 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 [5-7]. Chorioallantoic Membrane (CAM) assay is a valuable model for evaluating angiogenesis and vasculogenesis and it has long been a favored system for the study of tumor angiogenesis and metastasis [8-9]. By utilizing a novel approach to quantify angiogenesis [9-11], we have adapted the CAM assay to create an in vivo angiogenesis model system that is rigorously quantitative, amenable to high-throughput screening, and applicable for the testing of systemic and/or topical administration of experimental agents. Here we report on the effect of 0.7% concentration of diclofenac sodium on angiogenesis using chicken Chorioallantoic membrane (CAM) assay.

2. Materials and Methods

Laid fertilized broiler chicken eggs obtained from a local hatchery were incubated at 37°C and relative humidity of 55-60%. On day five of incubation, eggs were windowed aseptically as described by [9]. Briefly a small window (approximately 2cm in diameter) was made by removing the shell and inner shell membrane. About 4-5 ml of albumin was removed, windows were then sealed with Para film and eggs were returned to incubator.
0.7% concentration of diclofenac sodium was prepared using distilled water. The pH of the dilution was then checked with the help of pH meter and was adjusted in the range of 6.5-7.5. This dilution was filtered through syringe filter (0.2 μm) to reduce the risk of contamination.
Twenty chicken Chorioallantoic membranes (CAMs) of day six were used for the present study.

Eggs were divided in two groups A and B containing ten eggs in each. On day six of incubation, Group A received distilled water and kept as control while group B received 0.7% concentration of diclofenac sodium. Windows were sealed again with sterile Para-film tape and eggs were kept in incubator for further 24 hours.
Serial images of control and treated CAMs were recorded 24 hours after the administration of diclofenac sodium (on day seven of incubation). The contrast between blood vessels and other tissues was adjusted by using Adobe Photoshop 6.0, making it possible to discern the anatomical structures on every image. These images were then imported to SPIP software (IBM Denmark), an image processing program that works on specific algorithm [8,10], for automatic measurement of surface roughness and related parameters for detailed evaluation of the anti-angiogenic response. The collected data from the study was analyzed by appropriate statistical procedure. Analysis of variance (ANOVA) was performed to evaluate different parameters between controlled and treated samples; statistical significance was set at $P < 0.05$. Post hoc Student's t- test was also performed when significance was found $P < 0.0$ [10-13].

3. Results and Discussion

Application of the 0.7% concentration of diclofenac sodium caused marked changes in vascular architecture of the CAMs. Anti-angiogenic activities were observed after application of 0.7% concentration of diclofenac sodium, which resulted in thinning of primary and secondary blood vessels, and fading of tertiary blood vessels. This shows a marked reduction in the complete vascular network of CAM (Figure 1).
SPIP was utilized for computerized quantification of the diameter of CAM vasculature. A significant reduction in diameter of primary, secondary and tertiary blood vessels was evident among all treated groups as compared to control group (Figure 2).
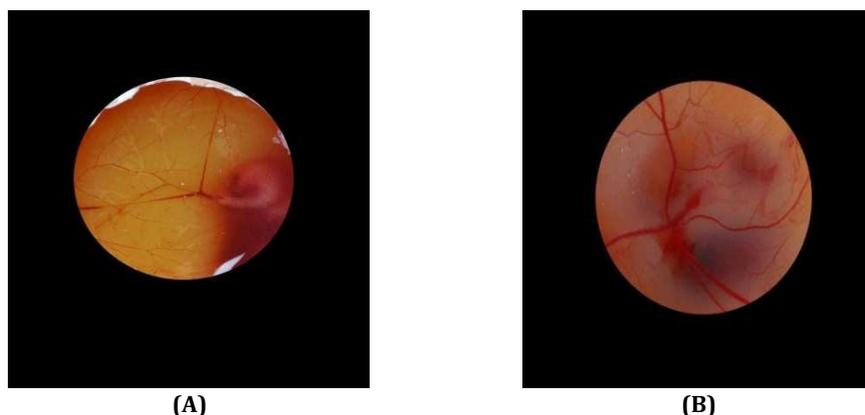

(A) **(B)**

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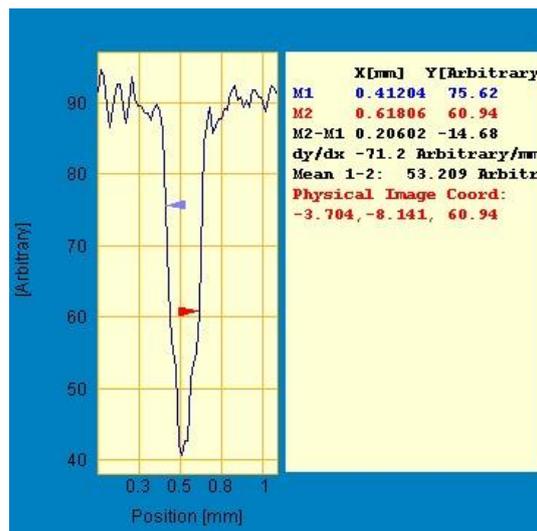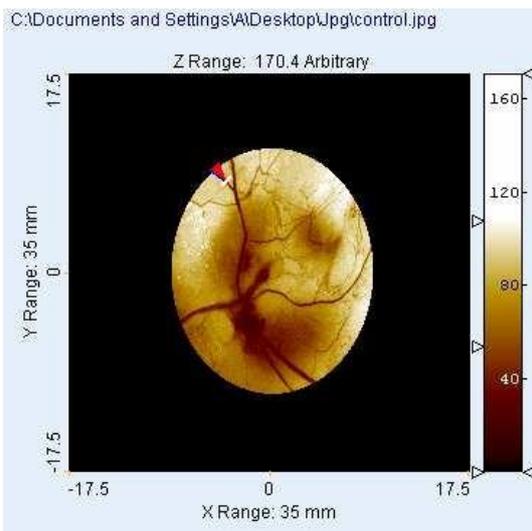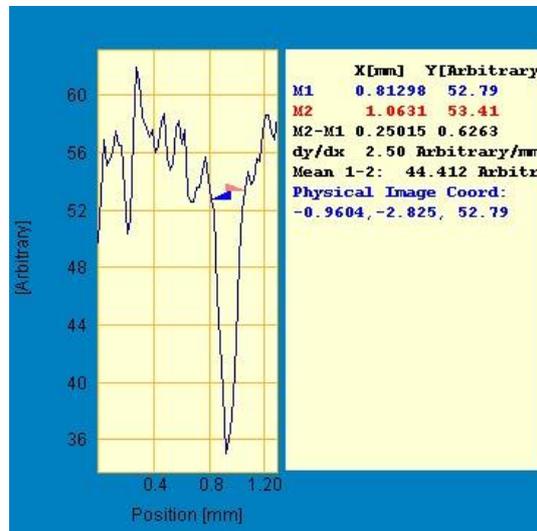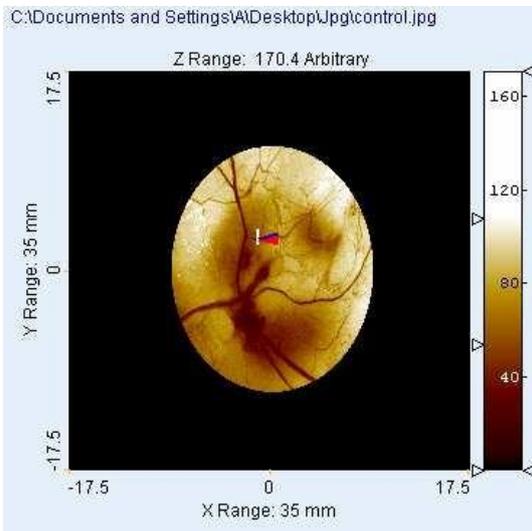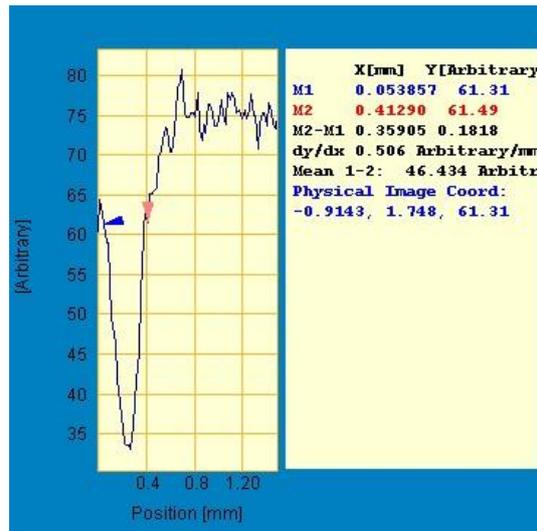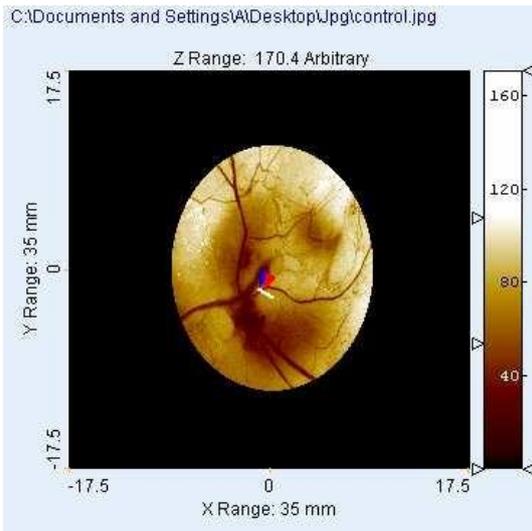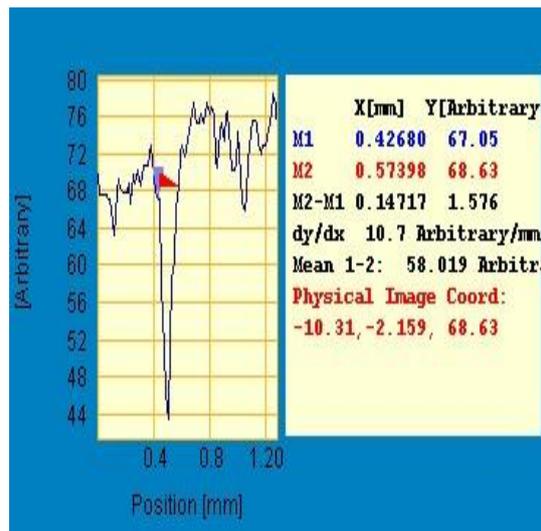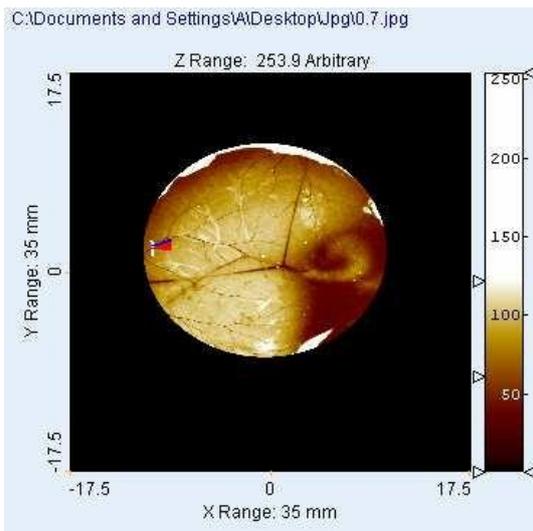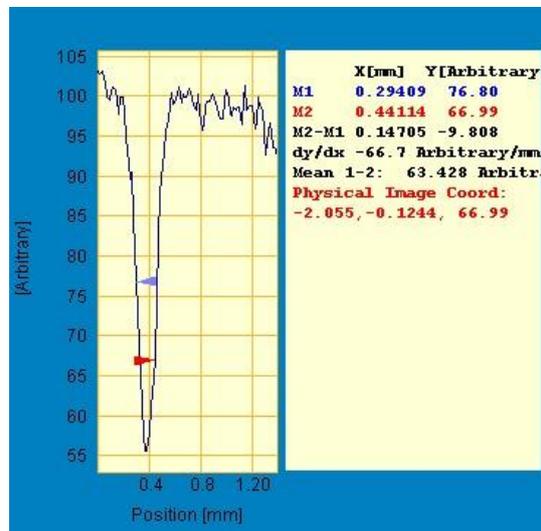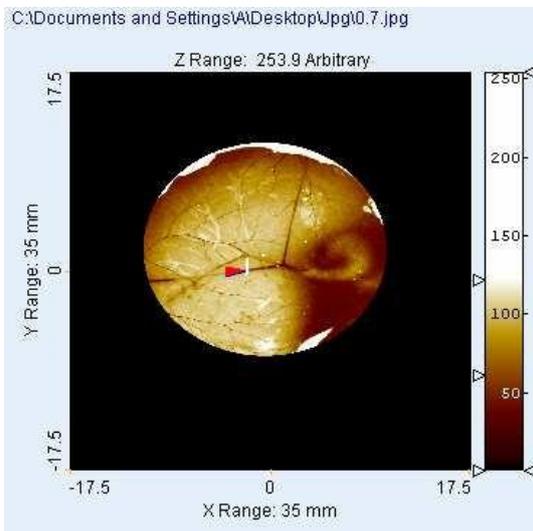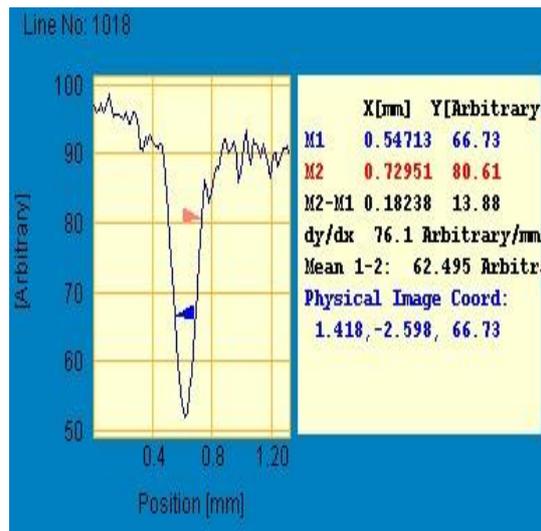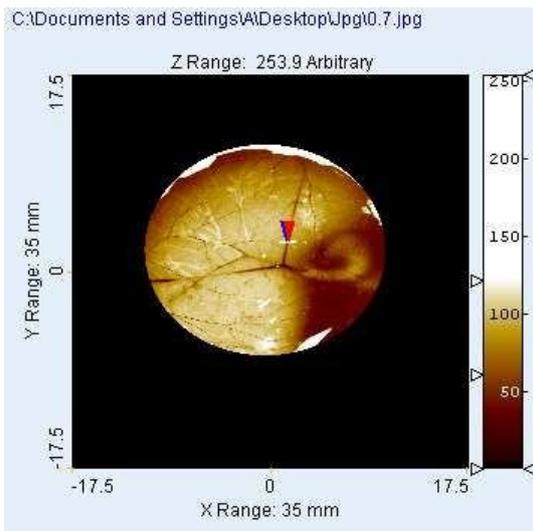
Figure 2. Diameter of blood vessels on CAM of control (A), 0.7% (B).

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

For more accuracy, the 3D surface roughness of control and treated CAMs was measured. The average roughness values of control were more as compared to treated CAM. This shows that surface roughness, representing neo-vascularization, of treated CAMs was significantly (P < 0.05) less than that of control CAMs. Twelve parameters of surface roughness of CAMs were calculated to quantify angiogenesis (Table 1). These parameters explain the differences in surface roughness between control and treated CAMs. The Abbott curve, a graphical representation of roughness, was also measured to evaluate even minute differences in the height of blood vessels on the surface of CAMs. The heights of the Abbott curve for control and treated CAMs were 213 nm and 164 nm respectively (Figure 3).

4. Conclusion

All the parameters evaluated demonstrate the anti-angiogenic effect diclofenac sodium in chicken Chorioallantoic membrane. Our results showed that inhibition of angiogenesis by diclofenac sodium may be due to suppression of alphaVbeta3 integrin mediated and Cdc42/ Rac-dependent endothelial cell spreading, migration and angiogenesis. It is recommended that this area of research for diclofenac sodium should continue to be explored, as this can lay the foundation for the development of strategies for the prevention and therapy of several types of angiogenesis dependent diseases
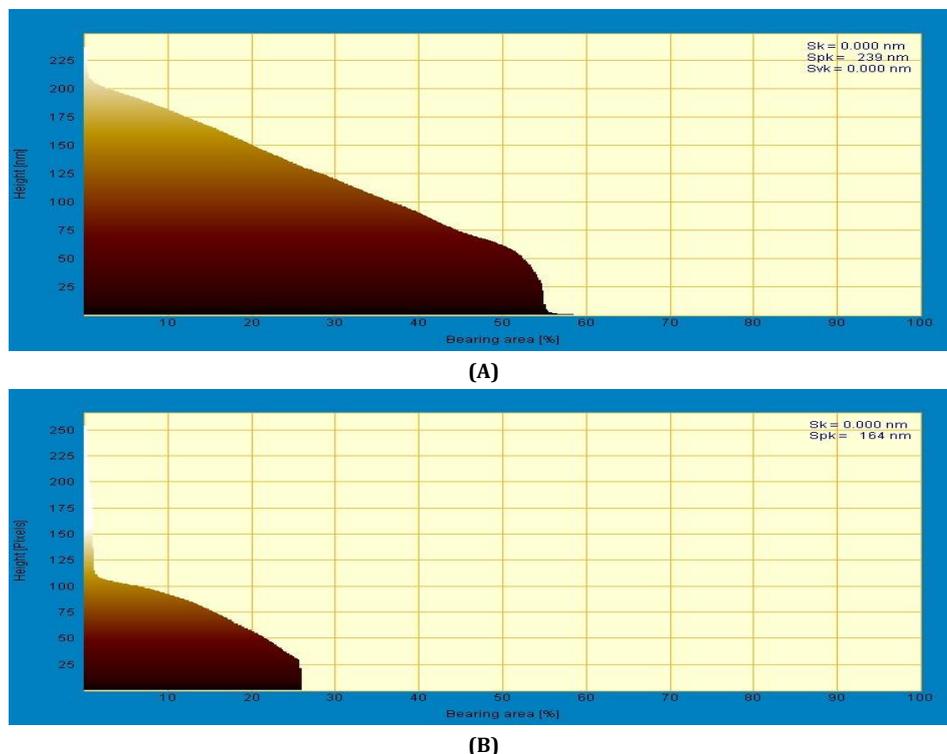

(A)

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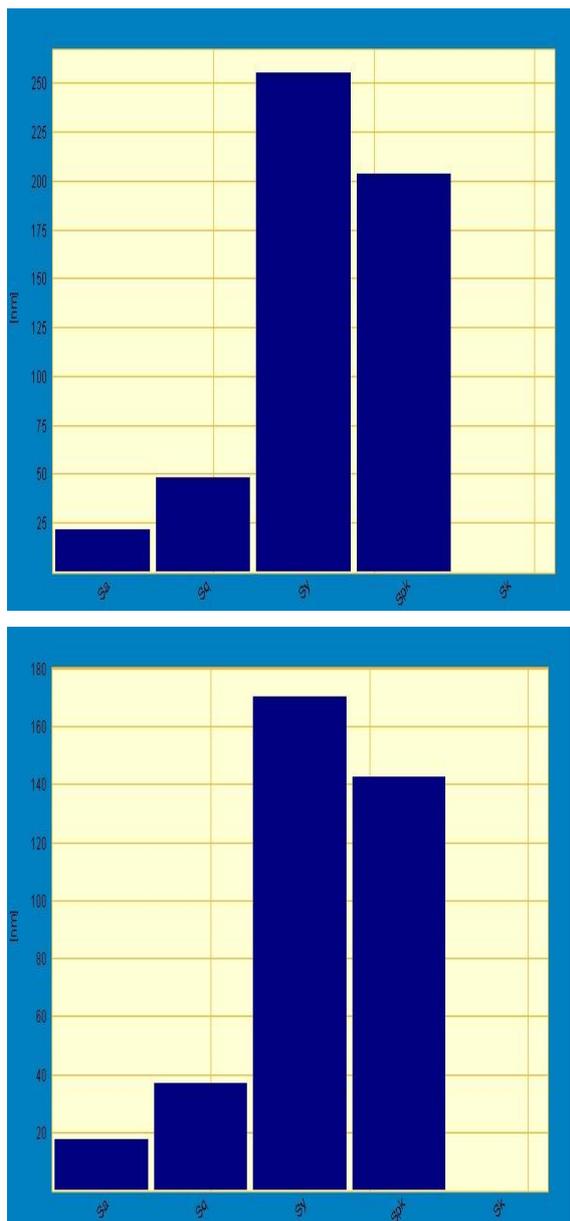
Figure 4. A graphical representation of various surface roughness parameters of control (A) and 0.7% treated (B) CAMs

References

1. Bagchi, D; C.K. Sen, M. Bagchi and M. Atalay (2004). Antiangiogenic, antioxidant and anticarcinogenic properties of a novel anthocyanin rich berry extract formula. Biochemistry. 69(1): 75-80.

2. Ejaz, S; K.B. Seok and L.C. Woong (2004). A novel image probing system for precise quantification of angiogenesis. Tumori. 90: 611-617.

3. Ejaz, S; K.B. Seok and L.C. Woong (2005). Toxicological effects of mainstream whole smoke solutions on embryonic movements of the developing embryo. Drug Chem. Toxicol. 28: 114.

4. Folkman, J (1995). Angiogenesis in cancer, vascular, rheumatoid and other diseases. Nat. Med. 1: 27-31.

5. Folkman, J (2001). A new family of mediators of tumor angiogenesis. Cancer Invest. 19: 754- 755.

6. Garnaes, J; P.E. Hansen, N. Agersnap, J. Holm, F. Borsetto and A. Kuhle (2006). Profiles of a high-aspect- ratio grating determined by spectroscopic and atomic-force microscopy. Appl. Opt. 45: 3201-3212.

7. Haas, T.L and J.A. Madri (1999). Extracellular matrix-driven matrix metalloproteinase production in endothelial cells: implications for angiogenesis. Trends Cardiovasc. Med. 9: 70-77.

8. Lin, J.Y and C.Y. Tang (2008). Strawberry, loquat, mulberry and bitter melon juices exhibit prophylactic effects on LPS-induced inflammation using murine peritoneal macrophages. Food Chemistry. 107: 1587-1596.

9. Melkonian, G; N. Munoz, J. Chung, C. Tong, R. Marr and P. Talbot (2002b). Capillary plexus development in the day five to day six chick chorioallantoic membrane is inhibited by cytochalasin D and suramin. J. Exp. Zool. 292: 241-254.

10. Ribatti, D; B. Nico, A. Vacca, L. Roncali, P.H. Burri and V. Djonov (2001). Chorioallantoic membrane capillary bed: a useful target for studying angiogenesis in vivo. Anat. Rec. 264: 317-324.

11. Roy, S; S. Khanna, H.M. Alessio, J. Vider, D. Bagchi, M. Bagchi and C.K. Sen (2002). Antiangiogenic property of edible berries. Free Radic. Res. 36(9): 1023-1031.

12. Seeram, N.P (2008). Berry fruits for cancer prevention: current status and future prospects. J. Agric. Food Chem. 56(3): 630-635.

13. Stone, S.Z; M. Bagchi and D. Bagchi (2007a). health benefits of edible berry anthocyanins: novel antioxidant and antiangiogenic properties. ACS Symposium Series. 22: 337- 351.