ORIGINAL ARTICLE

INFLAMMATORY CHANGES IN BUCCAL WOUND HEALING OF ALBINO RATS THROUGH COCOA POWDER EXTRACT

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ABSTRACT

Cocoa or cacao, derived from Theobroma cacao L., is considered as the "Super Fruit" due to its high polyphenol content and antioxidant capacity. It has numerous health benefits besides its common use in chocolate drinks and baking. The healing effects of cocoa powder extract were studied on albino rats comprising of 4 groups with 12 rats in each group. Group A was the control group and rats were given normal saline along with standard diet while rats in the experimental group B, C and D were given low, medium and high dosage of cocoa extract respectively dissolved in water by oral gavage daily along with standard rat chow diet. A 3mm surgical excisional wound was created on both left and right buccal mucosa of rats of all four groups with the help of punch-biopsy procedure. Rats were then sacrificed on 3rd, 7th and 14th day after the surgical procedure. Samples were obtained for histological analysis to compare the regenerated epithelial thickness, number of inflammatory cells and number of capillaries among the four groups. Histological changes indicated accelerated wound healing in cocoa groups as compared to the control group.

INTRODUCTION

Wound healing is a body's reaction to injury in an effort to re-establish normal structure and function. Impaired or delayed wound healing leads to extensive morbidity and cost of treatment and it has been observed that these factors are aggravated in elderly people. It is associated with excessive leukocytosis and increased local and systemic tumor necrosis factor alpha (TNF-α) levels[1].

Biological effects of cocoa are mainly credited to the high content of antioxidant polyphenols[2]. Cocoa has an effective antioxidant capacity compared to other foods traditionally considered high in antioxidants[3,4]. Cocoa intake also reduces some of the adhesion molecules involved in the recruitment of inflammatory cells.[5]

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At present no human interventional studies are available regarding the use of cocoa products in inflammatory conditions but some studies done on animal models suggested that cocoa indeed has anti-inflammatory effect. For instance, oral administration of cocoa in mice prevented ear edema.[6] Furthermore, the severity of paw edema in rats was also decreased after a week of cocoa treatment (4.8g/kg/day).[7,8] It also reduced the oxidative stress associated with chronic inflammatory diseases such as adjuvant arthritis and collagen induced arthritis in rats.[9] Cocoa enriched diet also reduced alveolar bone loss, gingival oxidative stress and inflammatory cell infiltration in periodontal lesions in rats. TNF-α and reactive oxygen metabolites (ROM) levels were also markedly decreased in cocoa group.[10] Similar results were recently observed where high dosage of cocoa extract successfully reduced the TNF-α levels in buccal wounds of albino rats.[11] However the present study focuses on the inflammatory changes in buccal wound healing of Albino rats through different doses of cocoa powder extract.

MATERIALS AND METHODS

An experimental animal study was conducted at the animal house and histology laboratory of Postgraduate Medical Institute (PGMI). The study protocol was approved by the Advanced Studies and Research Board of University of Health Sciences, Lahore and Ethical Committee of Postgraduate Medical Institute, Lahore. The agent used in this study was cocoa powder extract which was prepared at PCSIR (Pakistan Council for Scientific and Industrial Research). Natural Forastero

cocoa powder was used in this study and the extract was prepared using the technique mentioned in previous study.[12]

Forty eight healthy albino Wistar rats of either sex, weighing between 250-300 grams were obtained from animal house of Agricultural University, Faisalabad. They were kept in iron cages under optimum temperature (24+2 oC) in hygienic conditions in animal house of PGMI, Lahore. After acclimatization of rats for a period of one week, rats were randomly divided into four equal groups. Rats in control group A were given normal saline by oral gavage along with standard diet throughout the experimental period, whereas rats in the experimental group B received 2.4 g/Kg (low dose) cocoa extract, group C received 4.8g/Kg (medium dose) cocoa extract[8] while group D rats received 10g/Kg (high dose) cocoa extract[4] daily by oral gavage along with standard diet throughout the experimental period.

The animals were anesthetized intraperitoneally with Ketamine (50 mg/ml) and Xylazine (23.32 mg/ml).[13] An adequate and uniform piece of tissue (deep up to the level of the dermis) was removed from the buccal mucosa of rats using a disposable punch biopsy tool of 3.0 mm circumference.[14] The wound was left open for healing. All the animals were visually monitored every day to check for possible signs of infection. On the 3rd, 7th and 14th day after infliction of wound, four animals from each group (n=4) were randomly selected, placed in a chloroform chamber and sacrificed under deep anesthesia. The whole cheek was dissected out, washed with saline for further treatment. The tissue was processed and Hematoxylin and Eosin stain was used for histological analysis of buccal mucosa. Microscopic study was carried out with light microscope (Olympus CH2) under 100X and 400X magnifications using an eyepiece micrometer scale. The histological architecture and extent of wound healing in the four experimental groups was observed and compared with each other.

STATISTICAL ANALYSIS

Data was entered and analyzed by using SPSS 20.0 (Statistical Package for Social Sciences). The one way ANOVA was applied to determine the mean difference in thickness of regenerated epithelium, number of inflammatory cells and number of blood vessels. Post Hoc Tukey test was used for multiple comparisons. P-value < 0.05 was considered significant.

RESULTS

Regenerating epithelium thickness was measured in all the study groups on days 3, 7 and 14. On day 3 just a two cell thick layer of squamous epithelium was seen at the wound margins of all the four groups (table 1) (figure 2). None of the epithelial layers were appreciated; hence there was no significant difference in epithelial thickness among study groups on 3rd day (table 1.1). On day 7, there was a prominent increase in thickness of stratified squamous epithelium in all the study groups (table 1). However, the mean epithelium thickness in group A was significantly less as compared to group D showing signs of delayed wound healing (table 1.1) (figure 3 and 5). On day 14, the buccal epithelium had attained its full thickness with stratum basal, stratum spinosum, stratum granulosum and stratum corneum clearly visible (figure 6) (table 1). Nevertheless the mean epithelium thickness in group A was significantly less as compared to groups C and D (table 1.1), showing accelerated wound healing in groups C and D as compared to group A.

Histological changes in the connective tissue of all the four groups were observed as well. On day 3, in group A, profound inflammatory cell infiltrate was visible in the lamina propria (figure 1) as compared to other experimental groups (table 2). On further comparison using Tukey test it was found that there was significant difference only in group-A vs. group-D (figure 1 and 2) (table 2.1). Few newly formed capillaries were also visible at wound sites in all the study groups on day 3 (Table 3) with group A (figure 1) having the lowest mean number of capillaries as compared to groups B, C and D (table 3.1)(figure 2).

On day 7, the inflammatory cell infiltrate had decreased considerably as compared to day 3 in all the study groups. However group D (table 2) (figure 5) showed the lowest mean inflammatory cell count as compared to groups A (figure 3) and B (figure 4) (table 2.1). Angiogenesis was at its peak on day 7 in all the study groups, showing large number of newly formed capillaries in groups C and D (figure 5) as compared to groups A and B (figure 3 and 4) (table 3). On further comparison using Tukey test it was found that there

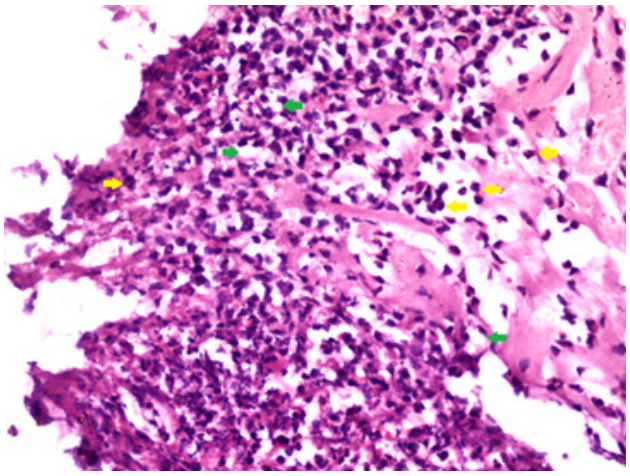

Fig 1: Photomicrograph of histologic section of wound area on day-3 of control group A showing increased inflammatory cells (yellow arrow) and lower regenerating capillaries (green arrows) in the connective tissue. 400X, H&E stain.

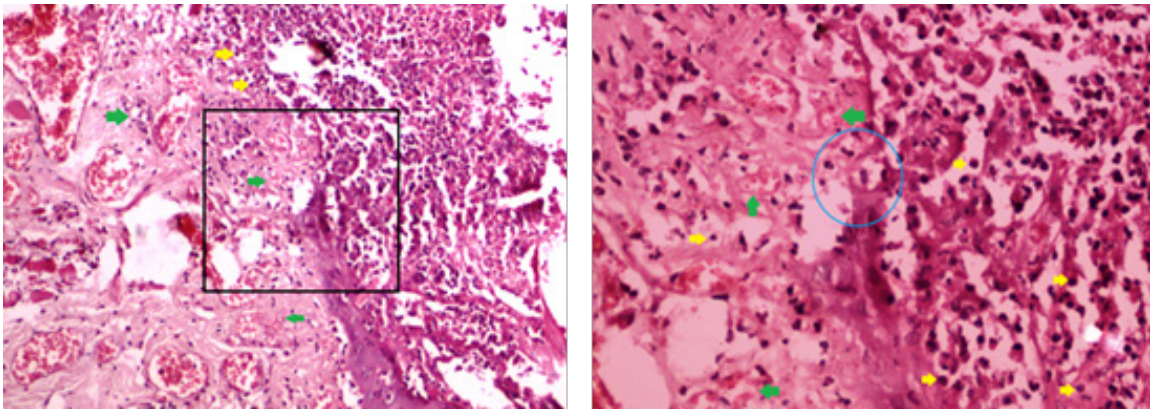

Fig 2: A) Photomicrograph of histologic section of wound area on day-3 of group D (100X, H & E stain); B) Magnified photomicrograph (400 X, H & E stain) showing decreased inflammatory cells (yellow arrow) and increased regenerating capillaries (green arrows) in the underlying connective tissue of wound site. Regenerating epithelium (E) (blue circular area) is also visible at wound margin but not completely covering the wound surface.

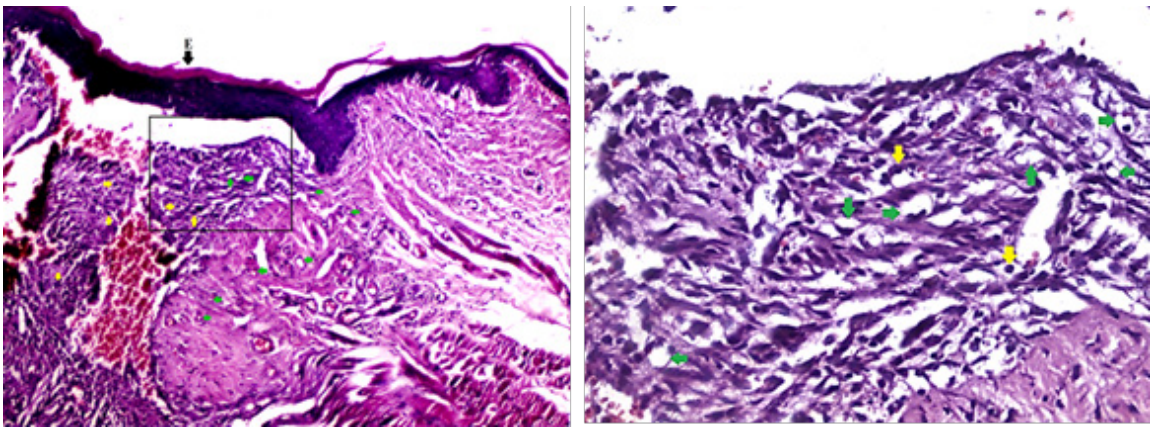

Fig 3: A) Photomicrograph of histologic section of wound area on day-7 of control group A (100X, H&E stain); B) Magnified photomicrograph (400X, H and E stain), showing inflammatory cells (yellow arrow) and regenerating capillaries (green arrows) in the underlying connective tissue of wound site. Thin layer of stratified squamous epithelium (E) is visible covering the wound surface in figure A.

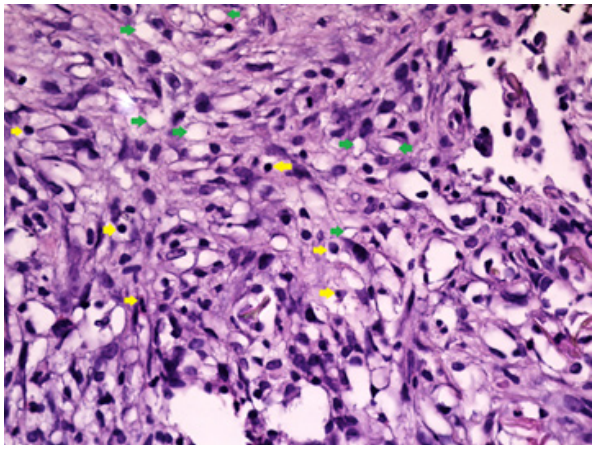

Figure 4: Photomicrograph of histologic section of wound area on day-7 of group B (given low dose cocoa) showing inflammatory cells (yellow arrow) and regenerating capillaries (green arrows) in the connective tissue. 400 X, H and E stain.

was significant difference in capillary count in group A vs. group C, group A vs. group D (table 3.1).

On day 14, the healing process was complete in all the study groups. Inflammatory cells had started to clear out with very few cells still visible in the connective tissue. The mean inflammatory cell count in all the study groups had no significant difference (table 2) (figure 6). There was also visible decrease in number of newly formed capillaries in the connective tissue from day 7 to the day 14 in all the four groups, again with no significant difference among the study groups (table 3) (figure 6).

DISCUSSION

The most significant finding of this study was that cocoa powder extract did accelerate the healing process in excisional wound in buccal mucosa of albino rats as compared to control group animals. On 3rd day, epithelium had started to regenerate in all four groups as part of the normal physiological process of wound

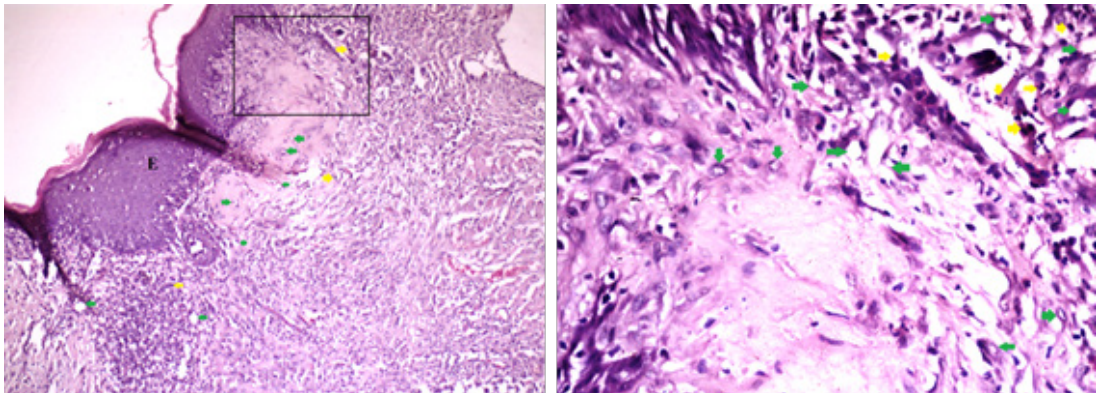

Fig 5: A) Photomicrograph of histologic section of wound area on day-7 of group D (100X, H & E stain); B) magnified photomicrograph (400 X, H & E stain) showing inflammatory cells (yellow arrow) and regenerating capillaries (green arrows) in the underlying connective tissue of wound site. Thick layer of stratified squamous epithelium (E) is visible covering the wound surface in figure A.

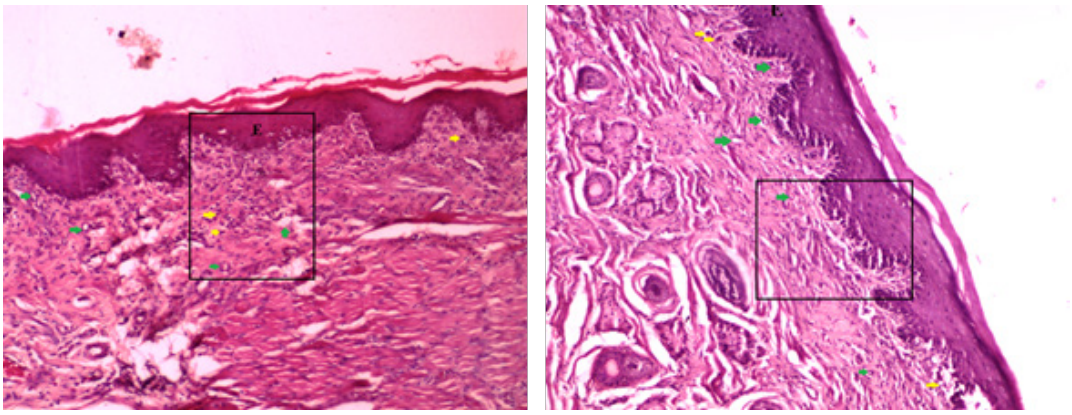

Fig 6: Photomicrograph of histologic section of wound area on day-14 of control group A (A) and group- C (B) showing completion of healing process. Some inflammatory cells (yellow arrow) and capillaries (green arrows) are visible in the underlying connective tissue of wound site. In control group A (A), thin layer of stratified squamous epithelium (E) with superficial keratin layer is covering the wound surface; whereas, thicker layer of epithelium is visible in group-C (B)100X, H&E stain.. 100X, H & E stain.

TABLE 1: DESCRIPTIVE STATISTICS OF REGENERATED EPITHELIUM THICKNESS AND COMPARISON IN DIFFERENT STUDY GROUPS

	Days	Study groups	Mean	S.D	Low/er Limit	Upper Limit	p-value
Regenerated Epithelium Thickness	3rd day	Group-A	1.75	2.36	-2.01	5.51	0.972
		Group-B	2.50	2.89	-2.09	7.09	
		Group-C	2.50	2.89	-2.09	7.09	
		Group-D	2.25	2.06	-1.03	5.53	
	7th day	Group-A	146.25	7.50	134.32	158.18	0.012
		Group-B	188.75	8.54	175.16	202.34	
		Group-C	171.25	10.31	154.85	187.65	
		Group-D	167.50	25.00	127.72	207.28	
	14th day	Group-A	233.75	11.09	216.11	251.39	0.005
		Group-B	238.75	8.54	225.16	252.34	
		Group-C	257.50	28.72	211.80	303.20	
		Group-D	285.00	12.91	264.46	305.54	

TABLE-1.1: MULTIPLE / PAIRED WISE COMPARISON USING POST HOC TUKEY TEST

	(I) Group	(J) Group	Mean Difference (I-J)	p-value
Regenerated Epithelium Thickness- 7th Day	Group-A	Group-D	-42.50000*	0.007
Regenerated Epithelium Thickness- 14th Day	Group-A	Group-C	46.25000*	0.012
		Group-D	51.25000*	0.006

TABLE 2: DESCRIPTIVE STATISTICS NUMBER OF INFLAMMATORY CELLS AND COMPARISON IN DIFFERENT STUDY GROUPS

	Days	Study groups	Mean	S.D	Lower Limit	Upper Limit	p-value
Number of Inflammatory Cells	3rd day	Group-A	412.50	62.92	312.39	512.61	0.026
		Group-B	385.00	62.45	285.63	484.37	
		Group-C	291.25	83.50	158.38	424.12	
		Group-D	268.75	55.43	180.54	356.96	
	7th day	Group-A	237.50	47.87	161.33	313.67	0.003
		Group-B	222.50	38.62	161.04	283.96	
		Group-C	170.00	29.44	123.16	216.84	
		Group-D	128.75	8.54	115.16	142.34	
	14th day	Group-A	16.75	2.75	12.37	21.13	0.58
		Group-B	13.50	2.65	9.29	17.71	
		Group-C	12.50	6.45	2.23	22.77	
		Group-D	13.75	4.79	6.13	21.37	

TABLE-2.1: MULTIPLE / PAIRED WISE COMPARISON USING POST HOC TUKEY TEST

	(I) Group	(J) Group	Mean Difference (I-J)	p-value
Number of Inflammatory Cells-3rd Day	Group-A	Group-D	47.30*	0.044
Number of Inflammatory Cells-7th Day	Group-A	Group-D	24.29*	0.004
	Group-B	Group-D	24.30*	0.001

TABLE 3: DESCRIPTIVE STATISTICS OF NUMBER OF CAPILLARIES AND COMPARISON IN DIFFERENT STUDY GROUPS

	Days	Study groups	Mean	S.D	Lower Limit	Upper Limit	p-value
Number of Capillaries	3rd day	Group-A	4.50	0.58	3.58	5.42	0.001
		Group-B	7.00	1.41	4.75	9.25	
		Group-C	9.00	1.83	6.09	11.91	
		Group-D	9.50	1.00	7.91	11.09	
	7th day	Group-A	9.00	1.83	6.09	11.91	0.007
		Group-B	14.00	2.16	10.56	17.44	
		Group-C	16.25	2.63	12.07	20.43	
		Group-D	18.50	5.07	10.44	26.56	
	14th day	Group-A	4.75	1.50	2.36	7.14	0.540
		Group-B	3.75	1.26	1.75	5.75	
		Group-C	4.00	0.82	2.70	5.30	
		Group-D	3.50	1.29	1.45	5.55	

TABLE-3.1: MULTIPLE / PAIRED WISE COMPARISON USING POST HOC TUKEY TEST

Time of Sacrifice	(I) Study Groups	(J) Study Groups	Mean Difference (I-J)	p-value
Number of Capillaries 3rd Day	Group-A	Group-C	-4.50000*	.002
		Group-D	-5.00000*	.001
	Group-C	Group-A	4.50000*	.002
Number of Capillaries 7th Day	Group-A	Group-C	-7.25000*	.032
		Group-D	-9.50000*	.006

healing. On day-7, the thickness of the buccal epithelium was increased in all of the groups with stratum basale, stratum spinosum, stratum granulosum and stratum corneum easily appreciated. Full thickness of the epithelium was not yet attained and there was thin superficial layer of keratin in all the samples. However there was a significant increase in epithelial thickness in experimental group D (given high dose cocoa extract) as compared to control group A. On 14th day of healing, a layer of stratified squamous keratinized epithelium including all four layers were visible in all four groups however the epithelium thickness was more in groups C and D than in group A. During the course of skin wound healing, fibrogenic growth factors such as TGF-β1 cause an increase in collagen production and epithelial migration[15], thus promoting re-epithelialization of skin wounds.[16] TNF-α plays an inhibitory role on TGF- β1 as well as collagen gene expression[17] ultimately leading to decreased epithelial and collagen regeneration.[18] Previously it was observed that high dose cocoa caused the maximum decrease in TNF-α level in buccal wound of rats; hence there might have been minimal inhibition on TGF-β stimulation leading to acceleration of the epithelial regeneration process in group D as compared to groups A and B.[11] The epithelial regeneration was somewhat accelerated in groups B, C and D which were given low dose, medium dose and high dose cocoa extract respectively on days 7 and 14 of wound healing, but the thickness of epithelium was greater in group D which can be linked to the significant decrease in TNF-α level in group D as compared to control group A.

Reduced lymphocytic infiltration on days 3 and 7 in medium and high dose cocoa groups might be due to the reduced production of reactive oxygen species (ROS) and pro-inflammatory cytokines by cocoa intake. ROS is produced mainly by neutrophils and macrophages at wound sites and protect the host against bacterial and fungal infection.[19] But excessive production of ROS might lead to dysruption of cell membrane, apoptosis and tissue necrosis, hence prolonging the inflammatory phase leading to delayed wound healing in skin and mucosa[20]. Cocoa enriched diet reduced the production of pro-inflammatory cytokines and leukocytic infiltration in experimental periodontitis by reducing oxidative stress.[10] It was also observed that diets containing high percentages of cocoa had more antioxidant effects and greatly diminished serum TNF-α levels than low percentage cocoa diets.[10] TNF-α levels were also greatly diminished in buccal wounds of rats who were given medium to high dosage of cocoa extract[11] thus further authenticating our results observed in groups C and D given medium dose and high dose cocoa extract respectively. Meanwhile, no changes were observed in group B animals given low dose cocoa proving the point cocoa extract and flavonoids down-regulate inflammatory process in a dose dependent manner[12] we report the effects of a cocoa extract on the secretion and RNA expression of various proinflammatory mediators by macrophages. Monocyte chemoattractant protein 1 and tumor necrosis factor alpha (TNFalpha.

On day 14 however, inflammation had subsided considerably in all the study groups and inflammatory cells were scarce. Henceforth the healing process was complete. Numerous studies have shown that on 14 and 21 days of wound healing, the density of inflammatory cells gradually decreased and reached the physiological levels by 21 days.[21] Similarly in the present study there was no difference in cell density between control and experimental groups.

Angiogenesis is essential for formation of granulation tissue and it is promoted by vascular endothelial growth factor (VEGF).[22] It is a proven fact that TNF-α inhibits angiogenesis through reduction of expression of VEGF at wound sites.[18] Medium and high dose cocoa diet successfully decreased the gene expression of TNF-α in buccal wound of albino rats[11] which might be the source for increased regeneration of blood capillaries in wound healing of group C (medium dose cocoa group) and D (high dose cocoa group) animals on day 3 as compared to control group A. On day 7, neovasculization was at its peak and there was again a marked increase in number of blood capillaries in groups C and D as compared to group A. Maximum neovasculization is usually visible on the 5th day of wound healing. The density of blood vessels gradually diminishes later on as collagen fibers begin to accumulate in the granulation tissue to restore tissue strength.[23] Henceforth by day 14 in the present study, healing was complete in all the study groups and the number of capillaries was nearly the same with no significant difference. It was demonstrated in a previous study that during healing of cutaneous wounds, VEGF proteins were induced within

24 hours of injury, which reached to a maximum level at 2–3 days and declined to basal level after 7 days of skin injury[24]; and thus the number of capillaries was decreased on day 14 of wound healing. Similar results were observed in the present study.

Numerous data is available on the anti-inflammatory effects of high intake of cocoa in *in-vitro* studies and present study has shown that high doses of cocoa can have anti-inflammatory effects by modulating the pro-inflammatory cytokines like TNF-α *in-vivo*.

REFERENCES

1 Bruunsgaard H. Effects of tumor necrosis factor-alpha and interleukin-6 in elderly populations. Eur Cytokine Netw. 2002;13(4):389–91.

2 Ramiro-Puig E, Castell M. Cocoa: antioxidant and immunomodulator. Br J Nutr. 2009;101(7): 931–40.

3 Lee KW, Kim YJ, Lee HJ, Lee CY. Cocoa has more phenolic phytochemicals and a higher antioxidant capacity than teas and red wine. J Agric Food Chem. 2003;51(25): 7292–5.

4 Vinson JA, Proch J, Bose P, Muchler S, Taffera P, Shuta D, et al. Chocolate is a powerful ex vivo and in vivo antioxidant, an antiatherosclerotic agent in an animal model, and a significant contributor to antioxidants in the European and American diets. J Agric Food Chem. 2006;54(21):8071–6.

5 Monagas M, Khan N, Andres-Lacueva C, Casas R, Urpi-Sarda M, Llorach R, et al. Effect of cocoa powder on the modulation of inflammatory biomarkers in patients at high risk of cardiovascular disease. Am J Clin Nutr. 2009;90(5):1144–50.

6 Lee KW, Kundu JK, Kim SO, Chun K-S, Lee HJ, Surh Y-J. Cocoa polyphenols inhibit phorbol ester-induced superoxide anion formation in cultured HL-60 cells and expression of cyclooxygenase-2 and activation of NF-kappaB and MAPKs in mouse skin in vivo. J Nutr. 2006;136(5):1150–5.

7 Ramos-Romero S, Ramiro-Puig E, Pérez-Cano FJ, Castellote C, Franch A, Castell M. Anti-inflammatory effects of cocoa in rat carrageenin-induced paw oedema. Proc Nutr Soc. 2008;67(OCE1):E65.

8 Castell M, Franch A, Ramos-Romero S, Ramiro-Puig E, Pérez-Cano F. J. CC. Effect of a diet rich in cocoa flavonoids on experimental acute inflammation. In: B. KR, editor. Flavonoids: Biosynthesis, Biological Effects and Dietary Sources. New York: Nova Science Publishers; 2009. p. 213–229.

9 Ramos-Romero S, Perez-Cano FJ, Ramiro-Puig E, Franch A, Castell M. Cocoa intake attenuates oxidative stress associated with rat adjuvant arthritis. Pharmacol Res. 2012; 66(3):207–12.

10 Tomofuji T, Ekuni D, Irie K, Azuma T, Endo Y, Tamaki N, et al. Preventive Effects of a Cocoa-Enriched Diet on Gingival Oxidative Stress in Experimental Periodontitis. J Periodontol. 2009;80(11):1799–808.

11 Shahbaz M, Zaheer N, Zaheer U, Akhtar J. Modulation of TNF- level on buccal wound healing. 2018;38(2):182–186.

12 Ramiro E, Franch À, Castellote C, Pérez-Cano F, Permanyer J, Izquierdo-Pulido M, et al. Flavonoids from Theobroma cacao down-regulate inflammatory mediators. J Agric Food Chem. 2005;53(22):8506–11.

13 Suragimath G, Krishnaprasad KR, Moogla S, Sridhara SU, Raju S. Effect of carbonated drink on excisional palatal wound healing: a study on Wistar rats. Indian J Dent Res. 2010;21(3):330–333.

14 Logan R, Goss A. Biopsy of the oral mucosa and use of histopathology services. Aust Dent J [Internet]. 2010;55:9–13. Available from: http://doi.wiley.com/10.1111/j.1834-7819.2010.01194.x

15 Faler BJ, Macsata RA, Plummer D, Mishra L, Sidawy AN. Transforming growth factor-beta and wound healing. Perspect Vasc Surg Endovasc Ther. 2006;18(1):55–62.

16 Mustoe TA, Pierce GF, Thomason A, Gramates P, Sporn MB, Deuel TF. Accelerated healing of incisional wounds in rats induced by transforming growth factor-beta. Science. 1987; 237(4820):1333–36.

17 Buck M, Houglum K, Chojkier M. Tumor necrosis factor-alpha inhibits collagen alpha1(I) gene expression and wound healing in a murine model of cachexia. Am J Pathol [Internet]. 1996;149(1):195–204. Available from: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC1865213/

18 Mori R, Kondo T, Ohshima T, Ishida Y, Mukaida N. Accelerated wound healing in tumor necrosis factor receptor p55-deficient mice with reduced leukocyte infiltration. FASEB J Off Publ Fed Am Soc Exp Biol. 2002;16(9):963–74.

19 Kanta J. The role of hydrogen peroxide and other reactive oxygen species in wound healing. Acta Medica (Hradec Kralove) [Internet]. 2011;54:97–101. Available from: http://www.ncbi.nlm.nih.gov/pubmed/22250477

20 Schreml S, Szeimies RM, Prantl L, Landthaler M, Babilas P. Wound healing in the 21st century. J Am Acad Dermatol. 2010;63(5):866–81.

21 Fronza M, Caetano GF, Leite MN, Bitencourt CS, Paula-Silva FWG, Andrade TAM, et al. Hyaluronidase modulates inflammatory response and accelerates the cutaneous wound healing. PLoS One. 2014;9(11):e112297.

22 Ferrara N. Vascular endothelial growth factor: basic science and clinical progress. Endocr Rev. 2004;25(4):581–611.

23 Machado MJC, Watson MG, Devlin AH, Chaplain MAJ, McDougall SR, Mitchell CA. Dynamics of angiogenesis during wound healing: a coupled in vivo and in silico study. Microcirculation. 2011;18(3):183–97.

24 Shukla A, Dubey MP, Srivastava R, Srivastava BS. Differential expression of proteins during healing of cutaneous wounds in experimental normal and chronic models. Biochem Biophys Res Commun. 1998;244(2):434–9.

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3 Usman Zaheer:	Data collection, data analysis and drafting the article.
4 Asim Riaz:	Manuscript editing, data interpretation.
5 Asad Aizaz Chatha:	Literature search.
6 Uzma Waseem:	Data collection, manuscript review.