ORIGINAL ARTICLE

MODULATION OF TNF-α LEVEL ON BUCCAL WOUND HEALING OF ALBINO RATS THROUGH COCOA POWDER EXTRACT

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ABSTRACT

Cocoa powder has been used widely as a soothing rich chocolate drink, in baking and medical purposes for centuries. It is a rich source of polyphenols. The study was conducted on 48 Albino rats, randomly allocated into 4 groups. The control group was considered as group I in which rats were given normal saline along with standard diet while rats in the experimental group II, III and IV 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. Tissue samples were obtained by sacrificing the rats on 3rd, 7th and 14th day after the surgical procedure. Gene expression of Tumor necrosis factor alpha (TNF-) was evaluated through Real Time PCR (qPCR) and levels were measured quantitatively. The study concluded that cocoa powder extract in high dosage did have a positive effect in modulating the pro-inflammatory cytokines by decreasing gene expression of TNF-α and reducing the inflammatory process.

Key Words: *Wound healing, cocoa powder extract, polyphenols, Tumor necrosis factor alpha*

INTRODUCTION

Wound healing process is primarily dependent upon the delicate balance between the pro-inflammatory cytokines and their antagonists, which play an important part in cellular processes of wound healing .[1] Tumor necrosis factors, also referred to as the "TNF family", are a group of cytokines that cause cell death or apoptosis. Nineteen cytokines belonging to the TNF family have been discovered so far[2], but TNF-α, a pleiotropic pro-inflammatory cytokine, is clinically important among these cytokines. It is produced chiefly by activated macrophages and somewhat by T cells, mast cells, fibroblasts, adipose tissue, keratinocytes and neurons.[3] TNF-α is an acute-phase protein that increases vascular permeability by initiating a cascade of cytokines and, hence neutrophils and macrophages are recruited to the site of trauma or infection.[4] So inhibition or reduction of TNF-α reduces the leukocyte infiltration which in turn causes reduction in tissue damage mechanisms by these leukocytes, hence promoting wound healing.[5] TNF-α has been targeted therapeutically by handful of researchers 6 by using anti-TNF-α topical agents on human leg ulcers[7,8] and observed a remarkable acceleration in wound healing.

Theobroma cacao L. is a small evergreen tree from which cocoa beans are harvested. Theses beans are dried fermented and then roasted to obtain cocoa seeds or nibs, which are later on converted into cocoa powder and cocoa butter through various processes.[9] Cocoa beans contain high concentration of polyphenols especially the flavanols and it is the main focus of many researches .[10] These flavonoids are further subdivided into monomers mainly (-)-epicatechin, (+)-catechin and polymers derived from these monomers i.e. procyanidins B1 and B2. Polyphenols are present in fruits, vegetables, tea, cocoa and certain plants known for their antioxidant, anti-carcinogenic and anti-inflammatory properties.[11] In fact cocoa has the highest antioxidant capacity and polyphenols than other products like green tea, black tea, red wine etc.[12] As the beans pass through the various manufacturing processes to produce the end products like chocolates and cocoa powder, the level of polyphenol drops from 100% to 10%.[13] Cocoa powder contains up to 20mg/g polyphenols (catechin) whereas in chocolates the phenolic content varies from 5 and 8.4 mg/g according to the type of chocolate.[14] In cocoa powder, the flavonoid composition can be improved by enhancing the industrial processing of fresh cocoa beans. Hence by consuming these flavonoid-enriched samples, the bioavailability of flavanoids present in cocoa will eventually increase in the plasma and urine.[15]

Cocoa has numerous health benefits due to its polyphenolic content, but its notably positive role in immune system is creditable.[16] Flavonoids from cocoa down regulate inflammatory process by affecting the release of pro-inflammatory cytokines like TNF-α; however at the

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Received for Publication: May 20, 2018
Resived: June 21, 2018
Approved: June 23, 2018

same time stimulate the release of anti-inflammatory cytokines like transforming growth factor-β (TGF-β) and interleukin-4 (IL-4).[17] It was revealed in another article that cocoa polyphenols like procyanidins can modulate or change the activation of NF-□B (nuclear factor kappa-light-chain-enhancer of activated B-cells) in epithelial cells of intestine.[18] NF-□B is an important transcription factor which regulates the genes encoding cytokines (like TNF-α and interleukins) and adhesion molecules.[19] After 6 hours of receiving 40 gm of cocoa powder in healthy individuals, there was a decrease in serum concentration of some adhesion molecules and leukocytes also showed decrease activation of NF-κB.[20]

Few in vivo studies done on animal models suggested that cocoa indeed has anti-inflammatory effect.[21,22,23] It was also proven in a four weeks study that diet rich in cocoa reduced the concentration of TNF-α and reactive oxygen metabolites (ROM) in periodontal lesions as compared to periodontitis group which received no cocoa diet.[24] However, its action on soft tissue wound healing is not available yet. Hence variation of TNF-α level through different doses of cocoa powder extract was observed in the present study at wound sites in this hope that it might open new doors for alternative wound healing therapies, derived from natural sources.

METHODOLOGY

An experimental animal study was conducted at the animal house of Postgraduate Medical Institute (PGMI) and Resource laboratory of University of Health Sciences (UHS). Approval for this study protocol was obtained by the Ethical Committee of Postgraduate Medical Institute, Lahore and Advanced Studies and Research Board of University of Health Sciences, Lahore. The agent used in this study was cocoa powder extract which was prepared at PCSIR (Pakistan Council for Scientific and Industrial Research). The extract was prepared using Natural Forastero cocoa powder, though a technique mentioned in earlier study.[25]

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 under optimum temperature of 24+2 oC in clean and hygienic iron cages in animal house of PGMI, Lahore. Diseased rats or rats that died during the experiment were excluded from the study. After acclimatization of rats for a period of one week, rats were then allocated into four groups using simple random sampling technique. Rats in all four groups received surgical wounds on buccal mucosa. Rats in control group I were given normal saline by oral gavage along with standard diet throughout the experimental period, whereas rats in the experimental group II received 2.4 g/Kg (low dose) cocoa extract, group III received 4.8g/Kg (medium dose) cocoa extract 22 while group IV rats received 10g/Kg (high dose) cocoa extract[26] daily by oral gavage along with standard diet throughout the experimental period.

The animals were sedated intraperitoneally with Ketamine (50 mg/ml) and Xylazine (23.32 mg/ml).[27] An adequate and uniform piece of tissue (deep up to the level of the dermis) was excised from the buccal mucosa with the help of punch biopsy tool of 3mm circumference.[28] The wound was left open to heal properly. All animals were visually monitored every day to check for possible signs of infection. On the 3rd, 7th and 14 day after infliction of wound, four animals from each group (n=4) were randomly selected, heavily sedated in a chloroform chamber and sacrificed. The required cheek sample was dissected, washed with saline and were immediately placed in individual sterile tubes. These were then placed in an ice box in order to preserve the RNA in intact tissues. Samples were then transported to resource lab for Real time PCR analysis.

Extraction of total RNAs and Real Time Polymerase Chain Reaction (qPCR) analysis:

The total RNA was extracted from buccal mucosa of rats (approximately 30 mg) using FavorPrep Tissue Total RNA Extraction Mini Kit. RNA quantity and quality was determined by using Nanodrop ND-1000 spectrophotometer from Forman Christian College University, Lahore. After the RNA isolation, first strand cDNA was prepared by Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) by using RevertAidTM First Strand cDNA synthesis Kit Fermentas. cDNA were prepared from the RNA by maintaining a constant concentration of 2 µg of RNA by calculation. The prepared cDNA were confirmed for their activity by GAPDH PCR reaction. These verified cDNA were then used for the primer optimization.

The primer used for amplification of TNF- α was obtained from previous article (29) and this primer was checked by NCBI (National Center for Biotechnology Information) BLAST (Basic Local Alignment Search Tool).

TNF- α F 5'-CCTCTTCTCATTCCTGCTCGT-3'
TNF-α R 5'-TGAGATCCATGCCATTGGCC-3'
(amplified band/base pair 266)

To determine the optimal cycle number and annealing temperature, confirmed cDNA were then used for the primer optimization with gene specific primers of TNF-α by applying a temperature gradient PCR. The optimized temperature for the primer was 60 °C.

2 µl of cDNA were amplified by the regular PCR with the optimized conditions, using the synthesized forward and reverse gene specific TNF-α primers. Cellular gene expression analysis was done by using specific-primers of cellular genes on BIO-RAD iQ™5 Multicolor Real Time PCR Detection System using SYBR Green mix (Fermentas). Each real time PCR assay was performed in triplicate.

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 TNF-α level among the study groups. Post Hoc Tukey test was used for multiple comparisons. Confidence level was taken as 80% and P-value < 0.05

was considered significant.

RESULTS

A decreased expression of TNF-α was observed in experimental groups II, III and IV on 3rd, 7th and 14th day as opposed to group I (table 1). On both 3rd day and 7th day, mean gene expression of TNF-α was highest in group-I, with group IV showing the lowest gene expression. Mean TNF-α was statistically significant in different groups (table 1) on both days. These results were further confirmed by Tukey test with group I showing significantly increased TNF-α level as compared to other three experimental cocoa groups. Moreover there were also significant difference of TNF-α levels in low dose cocoa group (II) versus medium dose (III) and high dose (IV) cocoa group on both 3rd and 7th day (p-value < 0.001) (table 2). Therefore medium dose and high dose cocoa groups showed the maximum decrease in gene expression of TNF-α as compared to control and low dose cocoa group through cocoa powder extract.

By day 14, TNF-α level had considerably increased in all the experimental groups as compared to that observed in 3rd and 7th day of wound healing with a mean gene expression of 100±00 in group-I, 88.50±2.38 in group II, 72.75±6.70 in group III and 59.50±15.00 in group IV (table 1). However using pair wise comparison there was still a significant difference of TNF-α levels in control group versus medium (p-value = 0.003) and high dose (p-value = 0.00) cocoa groups. Whereas only group II versus group IV showed significant difference in TNF-α levels (p-value = 0.002) (table 2). The difference in mean TNF-α level were almost the same in groups I vs. II and II vs. III (table 2).

TABLE 1: DESCRIPTIVE STATISTICS OF TNF-α AND COMPARISON IN DIFFERENT STUDY GROUPS

	Days	Study groups	Mean	S.D	Lower Limit	Upper Limit	p-value
TNF-α	3rd day	Group-I	100.00	0.00	100.00	100.00	< 0.001
		Group-II	82.00	7.07	70.75	93.25	
		Group-III	53.25	7.41	41.46	65.04	
		Group-IV	39.75	8.96	25.50	54.00	
	7th day	Group-I	100.00	0.00	100.00	100.00	< 0.001
		Group-II	75.50	9.47	60.43	90.57	
		Group-III	43.25	4.65	35.86	50.64	
		Group-IV	34.00	11.58	15.58	52.42	
	14th day	Group-I	100.00	0.00	100.00	100.00	< 0.001
		Group-II	88.50	2.38	84.71	92.29	
		Group-III	72.75	6.70	62.09	83.41	
		Group-IV	59.50	15.00	35.63	83.37	

DISCUSSION

The present study involved administering various doses of cocoa extract to adult albino Wistar rats and observing their effects on wound healing. Albino rats were preferred over human subjects due to ethical issues and rodent models comprise a large portion of preclinical wound healing research.[30] On 3rd, 7th and 14th day of wound healing, significant increase in gene expression of TNF-α at surgical excisional wound sites was constantly observed in control group I as compared to experimental groups II (given low dose cocoa), III (given medium dose cocoa and IV (given high dose cocoa). Surgery causes trauma, which results in stress, inflammation, and immunosuppression and cytokines are the key mediators in this response.[31] Following surgery, inflammatory process begins immediately and pro-inflammatory cytokines like Interleukins (IL), TNFα and transforming growth factors (TGF) are the first to be released .[32,33] Surgery related immunosuppression is also linked with high levels of pro-inflammatory cytokine, like TNF-α, IL-1, IL-6 and TGF-β.[34] So decreasing the TNF-α level might be helpful in lowering the rate of delayed wound healing and acute trauma-induced immunosuppression but very few in vivo studies have been done in this regard. It was assumed in an in vitro study that epicatechin monomers from cocoa combine with NF-κB (involved in expression of various cytokines including TNF-α) and inhibit stimulation of T lymphocytes consequently preventing the release of cytokines.[35] Subsequently it was further observed in in vitro studies 25 that cocoa extract inhibited the release of TNF-α from rat macrophages and stimulated human

TABLE 2: MULTIPLE / PAIRED WISE COMPARISON USING POST HOC TUKEY TEST

Days of Sacrifice	(I) Study Groups	(J) Study Groups	Mean Difference (I-J)	p-value
TNF-α at 3rd day	Group-I	Group-II	18.00000*	0.013
		Group-III	46.75000*	< 0.001
		Group-IV	60.25000*	< 0.001
	Group-II	Group-III	28.75000*	< 0.001
		Group-IV	42.25000*	< 0.001
TNF-α at 7th day	Group-I	Group-II	24.50000*	.004
		Group-III	56.75000*	< 0.001
		Group-IV	66.00000*	< 0.001
	Group-II	Group-III	32.25000*	< 0.001
		Group-IV	41.50000*	< 0.001
TNF-α at 14th day	Group-I	Group-III	27.25000*	0.003
		Group-IV	40.50000*	.000
	Group-II	Group-IV	29.00000*	.002

peripheral blood mononuclear cells (PBMC).[36] Hence in the current in vivo study, the cocoa powder extract successfully reduced the TNF-α level in surgical excisional wound sites which might be due to decrease secretion of NF-κB by T-lymphocytes or decreased secretion of pro-inflammatory cytokines by PBMCs and macrophages. Gene expression of TNF-α was significantly decreased at surgical excisional wound sites in experimental groups III (given medium dose cocoa) and IV (given high dose cocoa) in comparison to group I (control) and experimental group II (given low dose cocoa). The same results were obtained on 7th day and 14th day of wound healing with groups III and IV showing the maximum decrease in gene expression of TNF-α as compared to groups I and II. These results are somewhat similar to earlier studies performed using higher dosage of cocoa[21,22,37] stating that cocoa extract or flavonoids alone decrease the production of reactive oxygen species (ROS) and various pro-inflammatory cytokines in a dose-dependent manner.

CONCLUSION

Cocoa extract and products derived from cocoa are rich in flavonoids. They have the potential to positively modify the release of cytokines involved in several acute and chronic inflammatory processes. Although the exact role and mechanism of cocoa in this regard remains to be ascertained, but it can be safely said that cocoa can be considered as a functional food.

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