

Histologic Effects of Carbonated Drinks on Regenerating Buccal Mucosa of Albino Rats

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Abstract

Background: To assess the effect of carbonated-drinks on regenerating buccal mucosa.

Methods: A circular wound of 3.0mm was created on the buccal mucosa of 24 Wistar rats and they were divided into two groups. Animals in group-1 were fed with chow pellet and water while those in group-2 were fed with a randomly selected carbonated-drink instead of water. Six animals from each group were sacrificed at 3 and 14 days. Wound site was histologically assessed for differences in the regenerating epithelium and submucosa; inflammatory cell count and neovascularization between two groups.

Results: There was marked difference in the pattern of regenerating epithelium between the groups. Animals in group-1 showed a normal regeneration of stratified squamous epithelium at day-3 with significant inflammatory reaction in the submucosa. In group-2 however, there was a delay in epithelial regeneration; with only a two-cell thick layer of epithelium covering the underlying tissue and an altered healing phenomenon at the end of the experiment.

Conclusion: Consumption of carbonated-drinks can disrupt oral wound healing process. Studies suggest that low pH of drinks could have detrimental effects on the oral microbiota, inhibiting neutrophil recruitment in the wound area; thus delaying epithelial regeneration at day-3 and subsequent wound healing of the buccal mucosa.

Key Words: Carbonated drinks, Buccal mucosa, Wistar rats.

Introduction

In normal oral mucosa, epithelium and submucosa maintain a steady-state equilibrium, which forms a protective barrier against the external environment. Once the barrier is broken, wound healing is initiated. Local factors influence the characteristics of wound directly. Histologically the buccal mucosa is made up of a thick (up to 500 μ m in humans) non-keratinized

stratified squamous epithelium supported by a lamina propria containing long slender papillae, dense fibrous connective tissue with collagen and elastic fibers and a rich vascular supply giving off anastomosing capillary loops into papillae. The submucosa which is rigidly attached to underlying buccinator muscle by fibers has a dense connective tissue with fat, minor salivary glands and sebaceous glands.¹

In normal oral mucosa the epithelium, connective tissue and submucosa maintain a steady-state equilibrium, which forms a protective barrier against the external environment. Once the barrier is broken, wound healing is initiated. The phases involved in wound healing processes are hemostasis, inflammatory phase, cellular migration, proliferative phase, reparative phase and remodeling.²

Wound healing being a physiological event can be affected by a variety of local or systemic factors. Local factors influence the characteristics of the wound directly. These include oxygenation, infection and/or foreign body. Systemic factors affect the overall health and status of the individual. These factors include age sex hormones, stress, obesity, alcoholism, smoking, nutritional status, medications; glucocorticoids, non-steroidal anti-inflammatory drugs.³

Materials and Methods

An experimental animal study was conducted at the Research Laboratory of Post Graduate Medical Institute (PGMI), Lahore to observe histological changes in soft tissue wound on the left buccal mucosa of adult male albino Wistar rats on 3rd and 14th days after infliction of wound. A randomly selected carbonated drink was used for this study. Study was conducted on 24 adult male albino Wistar rats in the animal house, Anatomy department, PGMI, Lahore. Twenty four healthy male adult albino Wistar rats, 55-60 days old, weighing between 170-230g was procured from National Institute of Health, Islamabad. All animals used in this study were handled with the international, natural and institutional guiding principles for the care and use of laboratory animals in

biomedical research as promulgated by the National Research Council.⁴ Animals were tagged and were housed in cages with wire bar lids to hold the water bottle and feed, to avoid contamination with urine and faeces. Bedding was located directly into the cage to allow the absorption of urine. They were kept in a well-ventilated room at ambient temperature of $28.0 \pm 2.0^{\circ}\text{C}$ and humidity ($60 \pm 10\%$) under 12 hour light/dark cycles and well provided with food and water ad libitum. The rats were divided into two equal groups i.e., Group 1 and Group 2 (Table 1). Simple water to Group 1 and carbonated drink to group 2 was administered, in the bottles housed in the animal cage for expediency and liberty of their drinking. The pH of the carbonated drink was measured using a Vermier pH sensor every 12 hours. The average pH of carbonated drink, tested by the pH sensor was 2.49. On day 0, a uniform piece of tissue was removed from the left buccal mucosa of all the rats of both groups, using a disposable punch biopsy tool of 3.0 mm circumference after anesthetization with ketamine (100 mg/kg body weight) and xylazine (10 mg/kg body weight) by an intraperitoneal injection.⁵ The cut was made deep to the level of the dermis⁴. The wound was left open for healing and all the animals were returned to their cages to recover from anesthesia. All the animals were visually monitored every day to check for probable signs of contagion. On day 3 and 14, six animals ($n = 6$) from each group were placed in a carbon dioxide plus chloroform chamber and euthanized under deep anesthesia.⁵ The whole left cheek was dissected out and washed with saline for further treatment.

Table 1:Detail of animal groups

Groups	Sub groups	Number of animals	Remarks
Experimental group 1	1a (day 3)	6	Food with water
	1b (day 14)	6	
Experimental group 2	2a (day 3)	6	Food with Carbonated Drink
	2b (day 14)	6	

Microscopic study was carried out and observations were made separately on the left mucosa. Thickness of the regenerating epithelium from the base of the stratum basale till the outermost layer of the epithelium was measured using an eyepiece micrometer scale which was calibrated against the stage micrometer. Based on the features of acute and chronic inflammatory cells, the density of the infiltrating cells was determined by manually counting

the leukocytes within a standardized microscopic area. Two areas were chosen at each margin of the wounded area and one area was selected in the central wound bed. The results were expressed as the total number of infiltrating leukocytes per $100,000\mu\text{m}^2$. Inflammatory cell infiltration into the fibrin scab over the surface of the wound was not included in these measurements. Neovascularization/capillary in-growth was also assessed in these same standardized wound areas within the wound bed. The area occupied by capillaries was expressed as μm^2 capillary area per $100,000 \mu\text{m}^2$ of tissue. Data was analyzed by using two-paired student's t-test for quantitative differences between experimental group 1 and experimental group 2 at the 5% level of significance.

Results

On day 3, essentially all of the wounded area was covered by stratified squamous epithelium in group-1 animals (water). An epithelium of the thickness of $54.17\mu\text{m} \pm 3.77$ was clearly visible in group-1a with a single layer of stratum basale; 2-3 layers of polyhedral stratum spinosum and 1-2 layers of the stratum granulosum on the top (Table 2). There was no evidence of keratin formation on day 3 (Figure 1). Two cell thick layer of squamous epithelium ($5.00\mu\text{m} \pm 0.0$) (Table 2) was seen in group-2a animals (carbonated drink). None of the epithelial layers were appreciated in group-2a. There was an apparent delay in the formation of basement membrane and the epithelium (Figure 2). Regeneration of epithelium in group 1a was faster than that in experimental group 2a. Difference between the epithelial thickness of the two groups was statistically significant ($p < 0.001$ Table 2). Connective tissue of two groups presented differences as well; in group-1a, abundance of inflammatory cells was visible in the lamina propria, a large number of macrophages containing large nuclei surrounded by a rim of cytoplasm were present. Neutrophils with multi lobulated nuclei were also recognized in the wounded area, so were a few lymphocytes accompanied by a very scarce, but noticeable granulation tissue beneath the epithelium (Figure 3). The total count of inflammatory cells was 96.33 ± 8.24 (Table 2). Newly forming capillaries, fibroblasts and scattered collagen fibers were also visible. In group-2a, the epithelium directly covered the wounded area. Only a few inflammatory cells (1.00 ± 0.63) were visible underneath regenerating epithelium (Table 2). There was no sign of angiogenesis or any appreciable granulation tissue (Figure 2). The difference between inflammatory cell

count and the capillary count between the two groups was statistically significant ($p < 0.001$ table 2). On day 14, the buccal epithelium was seen attaining its full thickness. There was regular stratification of epithelial cells in group-1b. The epithelium also demonstrated signs of keratinization. There were 5-6 layers of polyhedral cells forming stratum spinosum over a layer of tall columnar basal cells. Stratum granulosum was 2-3 cell layers thick (Figure 4). The thickness of the epithelium from basement membrane up to the outer keratin layer was 252.33 ± 8.16 (Table 3). In contrast to this, group-2b showed a hypertrophic and hyperkeratotic stratified keratinized epithelium. There were 8-9 layers of polyhedral cells present over the basal layer. Stratum granulosum consisting of 3-5 layers was prominent in the epithelium with abundant keratohyalin granules. Acanthosis was easily appreciated. Rete ridges reached up to the cornified layer in some instances (fig 5). The thickness of epithelium reached up to $371 \mu\text{m} \pm 18.35$ (table 3). The difference between the values of the two groups was statistically significant ($p < 0.001$; Table 3).

The number of newly formed capillaries in the connective tissue of group-1b decreased significantly from day-7 to the day-14 (14.17 ± 1.94) (Table 3). Fibroblasts started forming collagen. In fact, this tissue was hallmarked by excessive fibroblastic activity; criss-cross arrangement of collagen fibers. Inflammatory cells started to clear out with very few

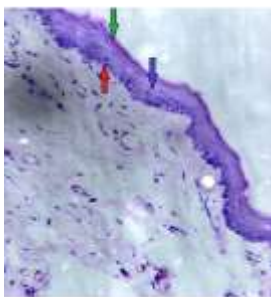


Fig 1: Left buccal mucosa of group -1a, on post wound day 3, showing epithelium with stratum basale (red arrow), stratum spinosum (blue arrow) and stratum granulosum (green arrow)

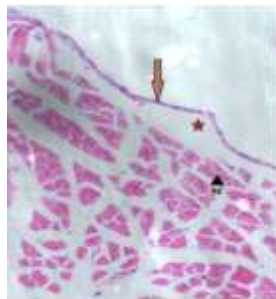


Fig. 2. Left buccal mucosa of group-2a on post wound day-3, showing a thin, cell layer thick epithelium covering the wounded area (brown arrow). Empty space shows lack of connective tissue (red star). The epithelium directly overlies skeletal muscle fibers of cheek (M).

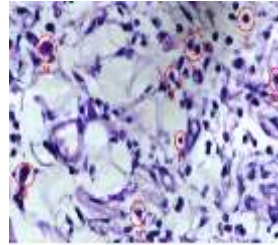


Fig. 3. Left buccal mucosa of group-1a on post wound day-3 showing connective tissue consisting of inflammatory cells; neutrophils (N), macrophages (M) and lymphocytes (L) in a scarce granulation tissue

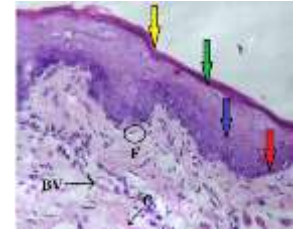


Fig. 4. Left buccal mucosa of group-1b on post wound day-14 showing stratification and keratinization of the epithelium. Epithelium consists of stratum basale (red arrow), stratum spinosum (blue arrow), stratum granulosum (green arrow) and stratum corneum (yellow arrow). Lamina propria consists of fibroblasts (F), collagen fiber bundles (C) and blood vessels (BV).

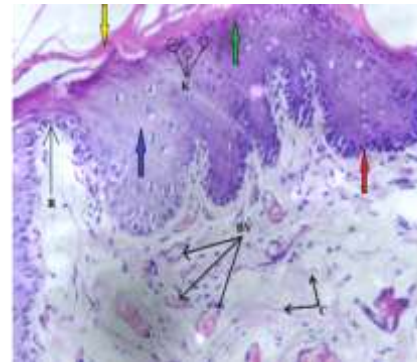


Fig. 5. Buccal mucosa of group-2b on post wound day-14 showing epithelium with stratum basale (red arrow), hypertrophic acanthotic stratum spinosum (blue arrow), stratum granulosum (green arrow) with prominent keratohyaline granules (K) and hyperkeratotic stratum corneum (yellow arrow). Rete ridges are visible reaching up to the corneal layer (R). blood vessels (BV) and scattered collagen fibers (C) are visible in lamina propria.

cells still visible in the connective tissue (13.00 ± 2.00) (table 3). In group-2b however, there were abundant inflammatory cells in the connective tissue (37.83 ± 4.67) (table 3). Neovascularization was also visible. New capillaries were found scattered throughout the subepithelial tissue (8.17 ± 1.60) (table 3). The difference between inflammatory cell count and capillary count in two groups was statistically significant ($p < 0.001$ table 3). The newly formed vessels displayed perivascular cuffing, indicating the persistence of inflammation. Fibroblasts and collagen fibers were also appreciated (Figure 5).

Table 2: Comparison of thickness of epithelium (µm), number of inflammatory cells and number of newly formed capillaries between two groups on Day-3

Parameter	Experimental group 1 (mean ± S.D)	Experimental group 2 (mean ± S.D)	Number of animals (N)	p- value
Thickness of epithelium (µm)	54.17 ± 3.76	5.24 ± 3.51	6	<0.001
Number of inflammatory cells	96.33 ± 8.29	1.00 ± 0.63	6	<0.001
Number of newly formed capillaries	4.00 ± 1.41	0.00 ± 0.00	6	<0.001

Table 3: Comparison of thickness of epithelium (µm), number of inflammatory cells and number of newly formed capillaries between two groups on Day-14

Parameter	Experimental group 1 (mean ± S.D)	Experimental group 2 (mean ± S.D)	Number of animals (N)	p- value
Thickness of epithelium (µm)	252.33 ± 8.16	371.67 ± 18.35	6	<0.001
Number of inflammatory cells	13.00 ± 2.00	37.83 ± 4.67	6	<0.001
Number of newly formed capillaries	14.17 ± 1.94	8.17 ± 1.60	6	<0.001

Discussion

Results of present showed remarkable differences between the healing patterns of the two groups. On **day-3**, the epithelium had covered the wounded area in experimental group-1. The epithelium was rather thin, non-keratinized, consisting of a basal layer and only a few layers of stratification. According to Chen *et al.* (2010), mucosal epithelium regenerated and covered the wound area completely within the first 24 hrs of wound.⁶ Faster keratinocyte proliferation and migration allowed faster stratification and regeneration of the oral epithelium to its normal thickness. The underlying granulation tissue was seen filled with inflammatory cell infiltrate. The granulation tissue formed in the open wound allowed the reepithelialization phase to take place, as epithelial cells migrated over the new tissue to form a barrier between the environment and the wound.⁷ A study done by Kim *et al.* (2001) stressed on the fact that formation of granulation tissue in earlier stages of wound healing played a key role in aiding epithelial cell migration and proliferation.⁸

In the experimental group-2 however, there was scarce formation of granulation tissue. Only a two cell layer thick squamous cell epithelium covered the wounded area. These findings suggested that there had been a delay in keratinocyte proliferation and migration over the wounded area and the epithelium had not yet undergone stratification which was visible in experimental group-1. Scarcity in the formation of granulation tissue could be the reason of slow keratinocyte proliferation and differentiation in group-2. A recent study done by Suragimath *et al.* (2010) evaluated the effects of carbonated drinks on palatal mucosal wound healing. They studied that the wounded mucosa showed signs of necrosis on day-3 which differs from the findings of the present study.⁹ Since Suragimath *et al.* (2010) did not explain the exact cause of necrotic tissue in their study; thus the results of the two studies do not contradict each other owing into account the difference in wound area could give different results.

There was an abundance of inflammatory cells present beneath the regenerating epithelium in experimental group-1. Fibroblasts were seen proliferating in the subcutaneous region. Abundant macrophages and neutrophils were present in the granulation tissue. According to Li *et al.* (2007) neutrophils and monocytes were the first inflammatory cells to arrive at the wound site for removal of foreign particles and bacteria.¹⁰ Neutrophils were recruited by chemotactic factors released during hemostasis.¹¹ Park and Barbul (2004) investigated the role of immune regulation in wound healing confirming the arrival of neutrophils within first 24 hours of wound.¹² After the first 24 hours, neutrophils were either extruded with eschar or were phagocytosed by macrophages in the next two days.¹³ Macrophages migrated into the wound 48 to 96 hours after injury to the tissue. So by the third day, wounded area was flooded with macrophages.¹⁴ In contrast to this, there were few to none inflammatory cells found on day-3 in experimental group-2. There seemed to be a delay in recruitment of neutrophils to the site of injury. A study by Devalaraja *et al.* (2000) described the importance of chemokines in wound healing. Any defect in chemokines could inhibit neutrophil and leukocyte recruitment and hence resulted in delayed wound healing.¹⁵ As stated by Kahle *et al.* (2013), bacteria acted as chemotactic agents for neutrophils.¹⁶ Some bacterial products like formylmethionine directly acted as a chemoattractant for neutrophils by production of interferon-α (IFN-α).¹⁷ Indirectly, oral bacteria like *Streptococcus pyogenes* and *Lactobacillus rhamnosus* recruited monocytes and macrophages by enhancing the mRNA expression of CCL3/macrophage-

inflammatory protein-1 α (MIP-1 α) and inflammatory chemokine ligands CCL2/monocyte chemoattractant protein-1(MCP-1). The expression of these chemokines was dependent on bacteria-induced IFN- α / β production. Bacteria could stimulate efficient inflammatory chemokine gene expression, particularly those that recruit T-helper cells(Th1) to the site of inflammation.¹⁸

A low pH can have detrimental effects on oral microbiota and the commercially available carbonated drinks lower the pH of oral cavity to 2.3-2.45.^{19,20} The fizziness of the drink can dislodge the debris and thus help in keeping the wound clean. Since low pH of a tissue inhibits bacterial proliferation, the reason for lack of neutrophils at this stage might be explained by the absence of chemotactic attractants by bacteria in the wounded tissue.

On day-14 all four layers of a keratinized stratified squamous epithelium were appreciated in both groups, however the number of layers of cells and amount of keratin in the epithelium was more in group-2 than in group-1 showing signs of hyperplasia. In the subcutaneous tissue of group-1, inflammation was seen to have subsided; only a few inflammatory cells were visible along with fewer capillaries. Collagen fibers, forming bundles were fairly abundant in the tissue demonstrating the healing process. Fibroblast proliferation was the hallmark of this stage of wound healing. Singer and Clark (1999) confirmed in their study that within two weeks, inflammation in the healing wound subsided and fibroblasts took the shape of myofibroblasts; producing collagen to replenish the strength of the injured tissue.²¹Type-1 collagen fibers were seen organized in the form of collagen bundles which replaced granulation tissue matrix and provided structure to the wound.²²

In group-2 however, there were fewer collagen fibers and not many bundles were visible. A relatively high number of macrophages were still visible in the tissue along with many capillaries. These features concluded that the wound healing process was delayed. Any persistent stress to the wound tissue can lead to a prolonged inflammatory reaction characterized by an abundance of macrophages, which can delay healing. An excessive influx or activation of infiltrating inflammatory cells into the damaged tissue can have profound effects on downstream cellular migration, proliferation, differentiation and eventually on healing response.²³ According to Guo and DiPietro (2010) wounds that exhibited delayed or impaired healing generally failed to advance through the normal stages of healing. Such wounds customarily entered a state of pathologic inflammation due to a postponed or uncoordinated healing process.³

Conclusion

Consumption of carbonated drinks can disrupt oral wound healing. Low pH of carbonated drinks can have detrimental effects on the oral microbiota, inhibiting neutrophil recruitment in the wound area; thus delaying epithelial regeneration and subsequent wound healing of the buccal mucosa.

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