HISTOLOGICAL EFFECTS OF CARBONATED DRINKS ON ORAL SUBMUCOSAL HEALING IN ALBINO WISTA RATS

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ABSTRACT

Objective: In this study the effects of carbonated drinks on the healing process of oral submucosa of albino wistar rats were evaluated.

Design: This study comprised of 24 adult albino wistar rats which were randomly assigned to a experimental group 1 and an experimental group 2. A circular wound of 3.0 mm was created on the buccal mucosa of each albino wistar rat at day 0. Animals in control group were fed with chow pellet and water while those in experimental group were fed with chow pellet and a commercially available carbonated drink instead of water. 6 animals from each group were sacrificed by decapitation under deep anesthesia at day 7 and 21. The tissue dissected from buccal area was serially sectioned and stained with Haematoxylin & Eosin stains. Wound site was histologically assessed for differences in the healing pattern of submucosa; inflammatory cell count and neovascularization between two groups.

Results:There was a marked difference in the healing pattern between the two groups. Animals in group-1 showed a normal healing pattern with formation of a fibrous connective tissue at the end of day-21. In the group-2, there is altered healing phenomenon at the end of the experiment with a subsequent delayed inflammatory reaction at day-21

Conclusion: These findings suggest that consumption of carbonated drinks can disrupt oral wound healing. The contents in carbonated drinks have a proinflammatory action on the soft tissue.

INTRODUCTION

The mucous membrane which lines the oral cavity is called oral mucosa[1]. Histologically the buccal mucosa is made up of a thick (up to 500 µm 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[2].

In normal oral mucosa the epithelium, connective tissue and submucosa maintain a steady-state equilibrium, which forms a protective barrier against the external environment[3]. Once the barrier is broken, wound healing is initiated[4]. The phases involved in wound healing processes are: (1) hemostasis, (2) inflammatory phase, (3) cellular migration and proliferative phase, (4) reparative phase, and (5) remodeling[5].

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[6], infection[7] and/or foreign body[8]. Systemic factors affect the overall health and status of the individual. These factors include age[9] sex hormones[10], stress[11], obesity[12], alcoholism[13], smoking[14], nutritional status[15], medications; glucocorticoids, non-steroidal anti inflammatory drugs[16].

Fahim *et al*[17], explained the effects of carbonated drinks on regenerating epithelium. This study is the continuation of the said experiment and illustrates the effect of carbonated drinks on healing submucosa.

MATERIALS AND METHODS

An experimental animal study was conducted at the Experimental 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 day 7 and 21 after infliction of wound. The study protocol was approved by the Advanced Studies and Research Board of University of Health Sciences, Lahore and Ethical Committee of PGMI Lahore

Animals:

Twenty four albino Wistar rats 55 - 60 days old of either sex, weighing (180 - 250 g) were procured from National Institute of Health Islamabad. They were

individually housed in a climate-controlled environment and provided with food and water *ad libitum*. All animals used in this study were handled with the international, natural and institutional guidelines for care and use of laboratory animals in biomedical research as promulgated by the Canadian Council of Animal Care.[24] They were housed in cages with wire bar lids used to hold the water bottle and feed to prevent contamination with urine or feces. Animals were placed in their respective cages which were labeled by tags. Bedding was placed directly into the cage to allow the absorption of the urine. They were kept in well ventilated room at ambient temperature of 28.0±2.0ºC and humidity (60±10%) under 12 hr light/dark cycles.

Rats were divided into two equal groups by using random number generator. Animals in experimental 1 group were fed with chow pellet and water while those in experimental group 2 were fed with chow pellet and a commercially available carbonated drink (diet coke) instead of water, in the bottles housed in the animal cage for expediency and liberty of their drinking.

Extraction Wound Model:

On day 0, all animals were anesthetized with ketamine (100 mg/kg body weight) and xylazine (10 mg/kg body weight) by an intra-peritoneal injection[17-19]. A uniform piece of tissue was removed from the left buccal mucosa of the rest of the rats of both groups, using a disposable punch biopsy tool of 3.0 mm circumference. The cut was made deep to the level of the dermis[17, 19]. 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.

Tissue Sampling, Processing and Staining:

On day 7, and 21, six animals (n = 6) from each group were placed in a carbon dioxide plus chloroform chamber and euthanized under deep anesthesia[20]. The whole left cheek was dissected out and washed with saline for further treatment. The tissue was fixed in neutral 10% buffered formalin for 48 hours at room temperature. Sections made were cut and placed inversely (buccal area faced towards cassette) in a single tissue cassette after labeling its identification. The tissue pieces were processed for 18 hours. The wax blocks containing samples were kept in refrigerator for approximately 15 minutes prior to sectioning. Blocks were mounted on rotary microtome ("Jung Histocut 820" Leica) and serial sections of thickness 3-5µm were obtained. Slides were stained using haematoxylin and eosin stains for routine histological study of the buccal lamina propria.

A manual count was performed for the assessment of inflammatory cell infiltration and neovascilarization[21]. 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. Three standard and representative areas were selected from each wound for the assessment. 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 100,000µm^2. Inflammatory cell infiltration into the fibrin scab over the surface of the wound was not included in these measurements collected from within the wound bed.

Neovascularization/capillary in-growth was also assessed in these same standardized wound areas within the wound bed of hematoxylin and eosin stained sections. The area occupied by capillaries was expressed as µm^2 capillary area per 100,000 µm^2 of tissue.

However to check the **histology of normal mucosa**, right cheeks of two rats from both groups were also dissected and processed. 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. A p-value of equal to or less than 0.05 was considered statistically significant.

Micrometry

Slides were studied under light microscope (Olympus CH2) with 40X, 100X, 200X and 400X magnifications to see the histological architecture and compared.

STATISTICAL ANALYSIS

The observations were recorded in MS Word® and Excel(R) data sheet and summarized in tabulated form. The data was entered and analyzed using SPSS (Statistical Package for Social Sciences) version 20.0. Data was analyzed by using two-paired student's t-test for quantitative differences between control group and experimental group at the 5% level of significance. A p-value of equal to or less than 0.05 was considered statistically significant.

RESULTS

QUANTITATIVE MORPHOMETRIC ANALYSIS:

Fahim et al[17], showed the normal (baseline) epithelium to be stratified squamous keratinized, with four basic layers. The epithelial thickness was measured to be 295 ± 5.00 µm. Thickness of regenerating epithelium of

groups 1 and 2 was measured and compared at day 7 and 21.

The present study deals with the comparison of inflammatory cell count and newly formed capillaries at day 7 and 21 in the healing submucosa of groups 1 and 2 (Table 2).

The normal unwounded mucosa reveals stratified squamous epithelium and lamina propria which displayed fine network of collagen bundles. Fibroblasts and blood vessels were also present (figure 3).

On **post wound day 7**, the connective tissue of **group-1** showed evidence of angiogenesis; a large number of newly forming capillaries 27.33±2.07 (figure 2)(table 2), were seen forming the granulation tissue along with the chronic inflammatory cells 123.33±9.90 (figure1 and 4) (table 2); macrophages with large nucleus, lymphocytes and few neutrophils were also present. The granulation tissue also contained fibroblasts forming collagen fibers.

In contrast to which, **group-2** at **day 7**, showed abundant inflammatory cells 175.67±14.71 (figure1 and 5) (table 2) beneath the epithelium. The subepithelial tissue consisted of numerous macrophages and lymphocytes. There were very few to nil newly formed capillaries 5.67±1.03 (figure2 and 5) (table 2). The difference in values of a number of inflammatory cells and number of capillaries in the two groups was statistically significant ($p<0.005$ table 2). There were only a few fibroblasts in the connective tissue with an extremely sparse extracellular matrix (figure 5). In a few instances, the inflammatory cells reached up to the epithelium. In these samples, there was no indication of collagen fiber formation. There was perivascular cuffing surrounding the newly formed vessels (figure 5).

On **post wound day 21**, fine collagen bundles were seen in the connective tissue of **group-1**(figure 6). There was an insignificant amount of inflammatory cells underneath the epithelium 6.00±1.79 (figure1) (table 2). Only a few capillaries were seen 9.33±0.82 (figure2, 4).

In **group-2** however, Connective tissue showed fibroblasts, collagen fibers were also present. There was slight verification of collagen bundle formation. Inflammatory cells were still present, indicating signs of inflammation 26.67±4.76 (figure1 and 7) (table 2). There were quite a few newly formed capillaries in the connective tissue, indicating continuing angiogenesis 16.00±2.37 (table 2) (figure2 and 5). The difference in inflammatory cell count and capillary count in the two groups was statistically significant ($p <0.005$ table 2).

Table 1: Detail of Animal Groups:

Groups	Experimental Day	Number of Animals	Remarks
Experimental Group 1	7	6	Food with Water
	21	6	
Experimental Group 2	7	6	Food with Carbonated Drink
	21	6	

Table 2: Comparison of Lamina Propria between Group 1 and 2, on Day 7 and Day 21

Experimental Day	Experimental Group 1, Mean ± SD		Experimental Group 2, Mean ±SD		Number of Animals in Each Group (N)	P-value
	Number of inflammatory Cells	Number of Newly Formed Capillaries	Number of inflammatory Cells	Number of newly formed Capillaries		
Day 7	123.33± 9.90	27.33±2.07	175.67±14.71	5.67±1.03	6	<0.001
Day 21	6.00±1.79	9.33±0.82	26.67±4.76	16.00±2.37	6	<0.001

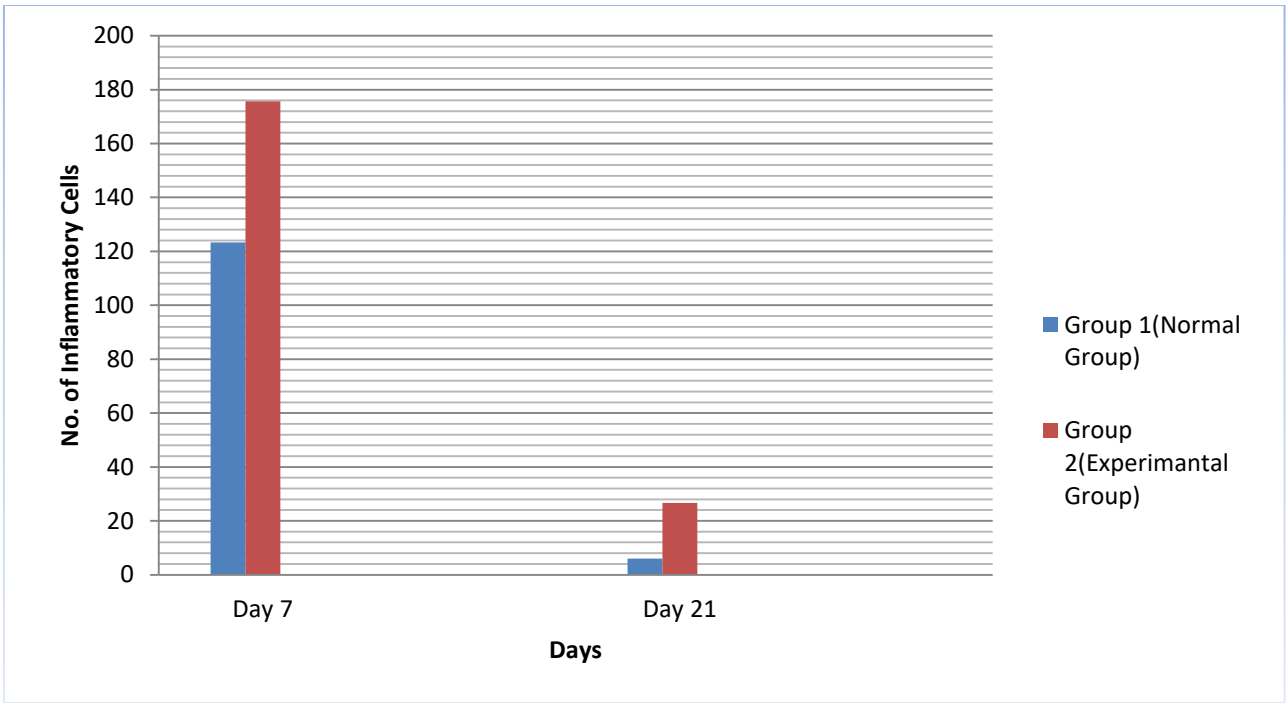

Fig 1: Graph showing the number of Inflammatory cells in both groups at days 7 and 21.

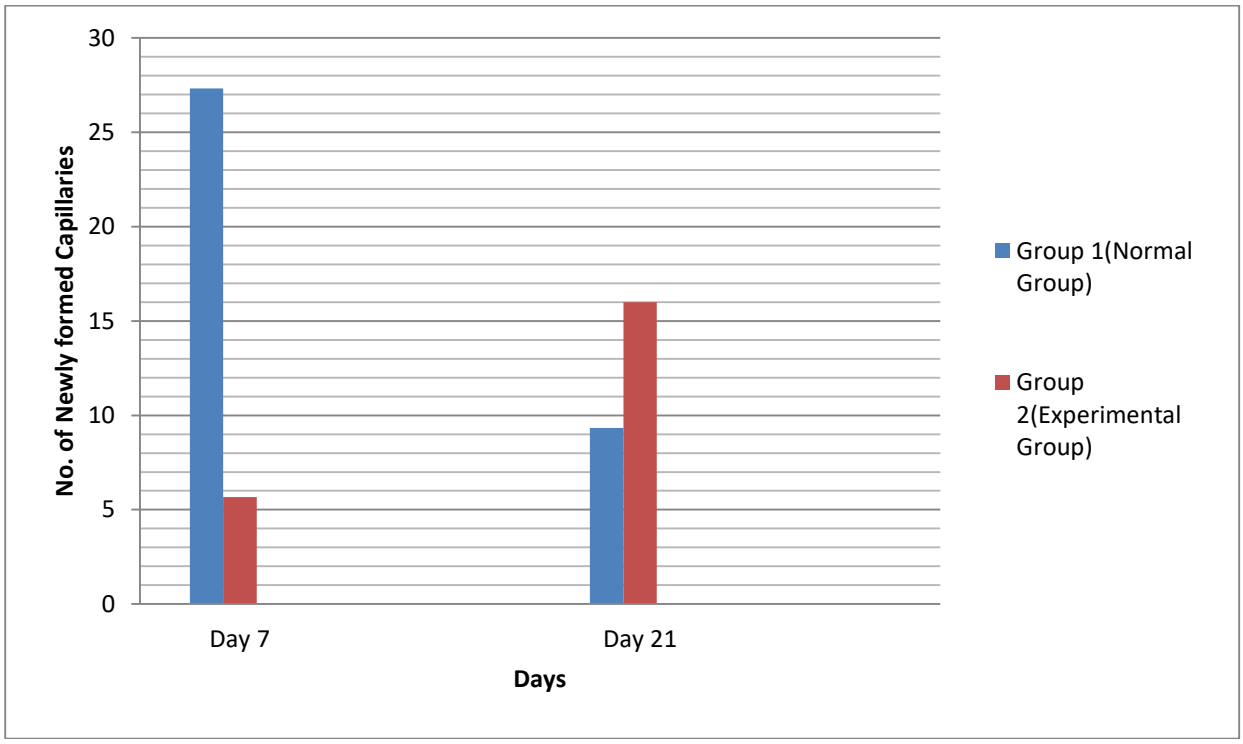

Fig 2: Graph showing the number of new capillaries in both groups at days 7 and 21

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Fig. 3 – Section of left buccal mucosa of rat showing normal epithelium with stratum basale (red arrow), stratum spinosum (blue arrow), stratum granulosum (green arrow) and stratum corneum (yellow arrow). The lamina propria consists of blood vessels

(BV), fibrobasts (F) and collagen fiber ©. H & E stain: photomicrograph approx. 200x.

Fig. 4 – Section of left buccal mucosa of group 1 on post-wound day 7 showing epithelium with stratum basale (red arrow), stratum spinosum (blue arrow), and stratum granulosum (green arrow). The lamina propria contains fibroblasts (F), collagen fibers (C), blood vessels (BV), and inflammatory cells (L) forming the granulation tissue. H & E stain: photomicrograph approx. 200x.

Fig. 5 – Section of left buccal mucosa of group 2 on post-wound day 7 showing epithelium with stratum basale (red arrow), stratum spinosum (blue arrow), and stratum granulosum (green arrow). The lamina propria consists of inflammatory cells (IL) and collagen fibers (C). H & E stain: photomicrograph approx. 200x.

Fig. 6 – Section of left buccal mucosa of group 1 on post-wound day 21 showing stratified squamous epithelium keratinized with stratum basale (red arrow), stratum spinosum (blue arrow), stratum granulosum (green arrow), and stratum corneum (yellow arrow). The connective tissue shows collagen fiber bundles (C) and blood vessels (BV). H & E stain: photomicrograph approx. 200x

Fig. 7 – Section of left buccal mucosa of group 2 on post-wound day 21 showing acanthotic epithelium with stratum basale (red arrow), hypertrophic stratum spinosum (blue arrow), stratum granulosum (green arrow) with prominent keratohyaline granules (K), and hyperkeratotic stratum corneum (yellow arrow). Irregular rete ridges (R) are present. The lamina propria consists of fibroblasts (F), collagen bundles (C), blood vessels (BV), and inflammatory cells (IL). H & E stain: photomicrograph approx. 200x

DISSCUSSION

On **day-7,** in the **group-1**, there was presence of immense fibroblast proliferation, collagen fiber synthesis and neovasculization was seen at its peak. A large number of newly formed capillaries were also visible in the subcutaneous area. Kurkinene et al and Woodley et al[22, 23,] concurred in their studies that fibroblasts in the wound edges began to proliferate and by approximately day 4, started to migrate into the provisional matrix of the wound clot, where they laid down a collagen-rich matrix, including collagens, proteoglycans, and elastin. By the end of the first week of wound healing, fibroblasts were the dominant cells in the wound[24, 25]. Tonnesen et al[26] studied that during wound healing, angiogenic capillary sprouts invaded the fibrin/fibronectin-rich wound clot and within a few days, structured into a microvascular network throughout the granulation tissue. Maximum neovasculazition could be seen on the fifth day of wound healing. As collagen accumulated in the granulation tissue to reinstate tissue strength, the density of blood vessels gradually diminished[27, 28].

In contrast to these findings, the subcutaneous tissue in **group-2** contained mostly inflammatory cells. A large number of macrophages were visible along with some lymphocytes and plasma cells. Neovascularization was extremely scarce and few samples showed no newly formed capillaries while only a few scattered fibroblasts were visible beneath the epithelium (figure 4). Doyle et al[29] conducted a study to assess the effects of energy beverages on mesenchymal cells. Their results revealed a decrease in lamellipodia formation and a decreased proliferative activity of fibroblasts leading to delay in wound closure. A study conducted by Suragimath et al[30] supported the delay in fibroblast proliferation. Since the optimal pH required for viability and activity of the fibroblasts has been reported to be 7.2 to 7.5, a pH below 3.0 created by carbonated drinks would hinder fibroblast proliferation, thus delaying the normal wound healing process[31].

On **day 21**, in the subepithelial tissue in **group-1** a few inflammatory cells were seen scattered, along with only 3-4 capillaries. Very finely aligned collagen bundles were also visible. A study done by Broadly et al[32] supports that wounds regain 78% of their normal strength by 21 days in rats. The newly formed collagen fibers assemble and cross-link to form collagen bundles which provide strength and turgor to the healing tissue decreasing the fibroblast density from the connective tissue. However, classic studies by Levenson et al[33] using a rat model, demonstrated that wounds never achieve more than 80% of normal pre wound tensile strength.

Whereas, in **group-2** relatively more inflammatory cells and capillaries were visible, suggesting that the inflammation has not yet subsided. The subepithelial tissue showed inflammation and much fibroblastic proliferation. A study done by Sirsat[34] supports this finding and stated that stress or irritation caused by diet can lead to mucosal changes and hinder with the normal healing pattern. A previous study conducted by Suragimath et al[30] on the effects of carbonated drinks on palatal wound healing showed a disruption in wound healing by appearance of necrosis in the area. However, in the present study no signs of necrosis were seen in any of the animals, only epithelial changes were seen.

Results suggest that changes in lamina propria seen in the experimental group-2 could be a result of constant irritation caused by the acidic and fizzy nature of carbonated drinks. Carbonated drinks must be avoided, especially in case of wound in the oral cavity.

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