Clinical and Experimental Health Sciences

Evaluation of the Clinical Effect of Hyaluronic Acid Mouthwash on Palatal Secondary Wound Healing in Diabetic Rats

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ABSTRACT

Objective: The objective of this study was to investigate clinical effect of topically administered hyaluronic acid (HA) mouthwash on healing of secondary palatal wound in diabetic (D) rats.

Methods: 60 Wistar albino male rats were divided into D and non-diabetic (ND) groups. Diabetes was induced to 30 randomly selected rats by initially administering 110 mg/kg of nicotinamide intraperitoneally, followed by 15 min of intraperitoneal injection of 65 mg/kg of streptozotocin solution. 5 mm excisional wounds were made in the centre of the palate. After that, 6 animals from each group were sacrificed. Then, both groups were subdivided into two groups: 0.12% HA mouthwash and saline (S) (n=12 per group), depending on the agent to be administered to the wound area (WA). On days 7 and 14, six rats from each group were sacrificed, and the WAs were measured through photographic measurements utilizing Image J software.

Results: The WA decreased with time in each group ($p<0.05$). A significant difference was detected in the intergroup comparison of the WA on days 7 and 14 ($p<0.05$). On days 7 and 14, the smallest WA was observed in the ND-HA group, while the largest was in the D-S group ($p<0.05$). On day 14, the WA of both HA groups was similar ($p>0.05$) and smaller than that of both S groups ($p<0.05$).

Conclusion: Topical application of HA mouthwash effectively improved secondary wound healing and reduced WA in D and ND rats. Thus, topical application of HA can be used in diabetic palatal secondary wound healing.

Keywords: Wound healing, diabetes mellitus, hyaluronic acid, rat, palate

1. INTRODUCTION

The loss of normal anatomical structure and functional continuity is referred to as a wound (1). A complex series of biochemical and cellular processes known as wound healing are required in order to repair tissue damage and preserve tissue integrity. It can be divided into four overlapping phases: hemostasis, inflammation, proliferation, and remodeling (2,3). Clot formation, inflammation, re-epithelialization, angiogenesis, formation of granulation tissue, wound contraction, formation of scar tissue, and tissue remodeling are all normal stages of the wound healing process (4). Many local and systemic factors can affect one or more phases of wound healing and lead to inappropriate or impaired tissue repair (5).

Diabetes mellitus (DM) is a chronic metabolic disease characterized by hyperglycemia resulting from defects in insulin secretion, impaired insulin action, or both (6). DM is one of the well-known systemic diseases that impairs wound healing by disrupting one or more biological mechanisms involved in the healing process (7). One of the well-known complications of DM is impaired wound healing, which leads to chronic wounds. Chronic wounds have disruption of wound healing phases and therefore wound does not heal within a normal time frame (5). In addition, as a result of hyperglycemia, there is a decrease in endothelial cell proliferation, angiogenesis, the formation of granulation tissue, and collagen synthesis (8). Due to all of these factors, DM increases the risk of infection in wounds and delays wound healing. In the case of oral wounds, DM patients have increased susceptibility to bacterial invasion and wound infection; as a result, bacteraemia can cause systemic inflammation and sepsis. Moreover, oral infections and poor wound healing in DM patients might impair the body's natural ability to heal (9).

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Hyaluronic acid (HA) is a non-sulfated polysaccharide that is a member of the glycosaminoglycan family and is an essential part of the extracellular matrix of skin, connective tissue, and synovial joints (10). Bacteriostatic, anti-inflammatory, anti-oedematous, osteoinductive, and pro-angiogenic characteristics of HA's highly biocompatible and non-immunogenic nature promote wound healing (11). The effect of HA is related to its molecular weight. In comparison to lower molecular weight HA, higher molecular weight HA could be more effective for increasing tissue regeneration, the anti-inflammatory response, and accelerating wound healing (12). HA promotes wound healing by reducing the negative effects of inflammation during the stages of granulation tissue formation and remodeling and increasing cell proliferation, reepithelialization, angiogenesis, regeneration, and inflammatory response (13,14,15,16). Additionally, higher molecular weight HA accelerated the healing of diabetic (D) wounds and protected against infection by enhancing the antioxidant defence system (17).

The success of surgical procedures depends on the healing of the wound. In recent years, it has been demonstrated that topically applied HA promotes clinically beneficial wound healing outcomes in both animal studies and clinical trials on secondary wound healing (11,18,19). To the best of our knowledge, based on findings of previous research, topical application of higher molecular weight HA demonstrated a positive effect and enhanced D dorsal secondary wound healing (12,17). However, there is no study on the efficacy of higher molecular weight HA on the healing of secondary palatal wound in diabetic (D) rats. Thus, the objective of this study was to investigate clinically the effectiveness of topically administered HA mouthwash in palatal wound healing in D and non-diabetic (ND) rats by assessing the wound closure.

2. METHODS

The protocol of present study was approved by the Istanbul University Animal Experiments Local Ethics Committee on March 26, 2021 (Protocol no: 2021/08). Our protocol and the manuscript were created according to the ARRIVE Guidelines, Animal Research: Reporting of in Vivo Experiments.

2.1. Animals

60 male Wistar albino rats that were 3 months old and weighed 250–300 g were obtained from the Istanbul University Aziz Sancar Institute of Experimental Medicine, where the experiment was conducted. Female rats were not included in the study as hormonal changes may affect wound healing. Rats were placed in standard experimental cages with a maximum of 3 rats in a cage and were kept under standard conditions with a 12-h light and dark cycle, a temperature of 22 ± 1°C, and a relative humidity of 40–60%. Animals were fed a standard diet of pellets and water ad libitum. Sixty animals were randomly divided into two main groups: an ND group (n=30) and a D group (n=30) via a computer-generated randomization table.

2.2. Induction of Diabetes

After 30 rats were fasted overnight, a single dose of 65 mg/kg streptozotocin (STZ) (ChemCruz, Santa Cruz Biotechnology, Dallas, TX) dissolved in distilled water was injected intraperitoneally, 15 min after 110 mg/kg nicotinamide (Acros Organics BV, Geel, Belgium) was administered intraperitoneally to induce diabetes. In order to prevent hypoglycemia due to massive pancreatic insulin release, 6 h after STZ administration, a 5% glucose solution was given to rats in the first 24 h. Following 72 h of STZ injection, blood was withdrawn from the animals' tail veins, and fasting blood glucose was measured using glucose reagent strips and a glucose meter (eBsensor Blood Glucose Monitoring System, Visgeneer Inc., Taiwan*)*. The rats were classified as D when their fasting blood glucose levels were above 200 mg/dl and were utilized for this study (20).

2.3. Wound Creation

The animals were anesthetized intraperitoneally with 100 mg/kg ketamine hydrochloride (Ketalar, Eczacıbaşı, Türkiye) and 10 mg/kg xylazine hydrochloride (Rompun, Bayer, Germany). All surgical procedures were performed by an experienced researcher (EA). After general anesthesia, each rat was stabilized, and the mouth was opened using a retractor. A circular excisional wound with a diameter of 5 mm was made in the centre of the palatal mucosa using a disposable punch biopsy tool (Kai Medical, Kai Industries Co., Ltd., Seki, Japan). Following bleeding control, the wounds were left for secondary healing. On day 0, after wound induction, six animals from the ND and D groups were sacrificed immediately. Then, the ND and D groups were subdivided into two groups according to the agents with a computer-generated randomization table: ND with the HA (ND-HA) group (n=12), ND with the saline (ND-S) group (n=12), D with the HA (D-HA) group (n=12), and D with the saline (D-S) group (n=12). Six animals from each group were sacrificed with decapitation on days 7 and 14 after surgery.

2.4. Hyaluronic Acid and Saline Application

Without touching the wound, according to the treatment group, 1 ml of saline or higher molecular weight (1000-1800 kDa) 0.12% HA (Gengigel® First-aid, Ricerfarma SRL, Milano, Italy) was applied directly to the wound for 1 minute using a syringe (Beybi, Istanbul, Türkiye) with a blunt cannula. This process was repeated twice a day, in the morning and evening, for one week. After 2 hours of HA or saline application, animals were fed a standard diet of pellets and water ad libitum.

2.5. Clinical Evaluation

All animals were sacrificed on days 0, 7, and 14 after surgery. Maxillae were separated, and the palate specimens were photographed at a constant distance and magnification using a Nikon F-3 camera (Nikon Corp., Tokyo, Japan). Using

ImageJ software (National Institutes of Health, Bethesda, Maryland, USA, https://imagej.nih.gov/ij), the photographs of the wound area (WA) were analyzed, and calibrated with a 10-mm-long periodontal probe (Williams probe, Hu-Friedy Manufacturing Inc., Chicago, IL, USA). All measurements were carried out by the same blinded researcher (HSY).

2.6. Statistical Analysis

The primary outcome of this study was WA measurements. The sample size was determined using data from research with a comparable design. (21). A sample of 5 rats per group would have 80% power to detect a difference of 1 mm^2 WA between the HA and control groups, assuming that the standard deviation is 0.56. 6 rats were enrolled in each group, considering possible dropouts. Statistical analyses were performed using statistical software (IBM SPSS version 24, Chicago, IL, USA). Mean and standard deviation are used to present the results. To examine the distribution of variables, the Shapiro-Wilk test was used. For intragroup multiple comparisons, the Friedman test was applied since the data were not normally distributed. If significance was found, pairwise comparisons using the Wilcoxon Signed Rank test were carried out. Intergroup comparisons among groups were performed by the Kruskal-Wallis test. If significance was found, the Mann-Whitney U test was used for pairwise comparisons between groups. $p<0.05$ was considered statistically significant.

3. RESULTS

Clinical images of the WA of all groups on days 0, 7, and 14 are presented in Figure 1. Clinical examination of the wounds revealed that all groups' wounds gradually healed over time. Slow wound healing was observed at 7th day postoperatively in the ND-S, D-HA, and D-S groups.

Table 1 shows the mean WA measurements of all groups on days 0, 7, and 14 post-surgery. The mean WA between the ND and D groups at baseline (day 0) was not found to be statistically different (19.40±1.34 mm^2 and 21.65±2.89 mm^2, respectively) ($p>0.05$). There was a significant decrease in the mean WA of all groups on day 14 compared to the baseline ($p<0.05$).

Table 1. *Inter and intra group comparison of wound area (mm^2)*

	Groups													
	ND (a) (n=6) Mean±SD	D (b) (n=6) Mean±SD	ND-HA (c) (n=12) Mean±SD	ND-S (d) (n=12) Mean±SD	D-HA (e) (n=12) Mean±SD	D-S (f) (n=12) Mean±SD	$p^{*(a-b)}$	$p^{\#(c-d-e-f)}$	$p^{*(c-d)}$	$p^{*(c-e)}$	$p^{*(c-f)}$	$p^{*(d-e)}$	$p^{*(d-f)}$	$p^{*(e-f)}$
Day 0	19.40±1.34	21.65±2.89					0.578							
Day 7			14.00±0.68	17.95±0.36	16.95±0.40	20.25±0.61		0.000	0.004	0.004	0.004	0.004	0.004	0.004
Day 14			4.64±2.16	12.62±0.95	6.18±1.68	15.20±1.58		0.000	0.004	0.332	0.004	0.004	0.023	0.004
p^{**}			0.002	0.002	0.002	0.009								
$p^{\#\#(0-7)}$			0.026	0.026	0.026	0.459								
$p^{\#\#(0-14)}$			0.026	0.026	0.026	0.026								
$p^{\#\#(7-14)}$			0.026	0.026	0.026	0.026								

*SD; Standart deviation, ND; Non-diabetic, D; Diabetic, HA; Hyaluronic acid, S; Saline, * Mann Whitney U test, # Kruskal Wallis test, ** Friedman test, ## Wilcoxon test, p<0.05.*

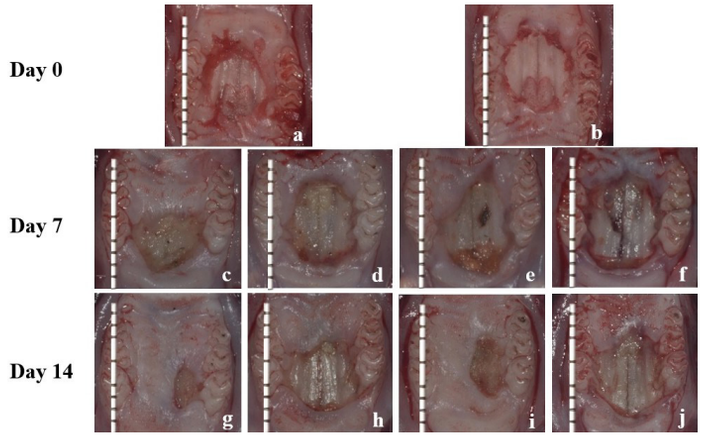

Figure 1. *Representative clinical images of the palatal wound area in (a) non-diabetic group and (b) diabetic group at day 0, (c) non-diabetic HA group, (d) non-diabetic saline group, (e) diabetic HA group and (f) diabetic saline group at day 7, (g) non-diabetic HA group, (h) non-diabetic saline group, (i) diabetic HA group and (j) diabetic saline group at day 14 after surgery.*

Significant differences were found in the intergroup comparison of the WA on days 7 and 14 ($p<0.05$). On days 7 and 14, the smallest WA was observed in the ND-HA group (14.00±0.68 mm^2 and 4.64±2.16 mm^2, respectively), while the largest WA was observed in the D-S group (20.25±0.61 mm^2 and 15.20±1.58 mm^2, respectively) ($p<0.05$). On day 7, the WA in the ND-HA group was found to be significantly smaller than the D-HA group ($p<0.05$). However, there was no statistically significant difference in WA between the ND-HA and D-HA groups on day 14 ($p>0.05$). Additionally, on days 7 and 14, the WA in the D-S groups was detected to be larger than the ND-S group ($p<0.05$). Furthermore, the WAs of the ND-HA and D-HA groups were significantly smaller than the ND-S and D-S groups on day 14 ($p<0.05$).

4. DISCUSSION

HA promotes wound healing by reducing inflammation, increasing collagen formation, and accelerating angiogenesis (12,14,16). However, the benefits of HA are still controversial, and there is no data about using HA mouthwash in D palatal secondary wound healing. Therefore, the goal of this study was to investigate the clinical effects of HA mouthwash application on WA in D rats. Based on the findings of this study, evaluating HA mouthwash clinically and the decrease in WA, no significant difference was observed between the D and ND HA groups on day 14 after surgery, suggesting that applying HA mouthwash to palatal secondary wound healing was efficient in both the D and ND groups.

In terms of the healing process, wound healing consists four distinct but overlapping phases (2). During the first week following surgery, the inflammatory phase of oral wound healing occurs. The first fibroblasts appear at the wound site between the end of the inflammatory phase and the beginning of the proliferative phase (24–48 hours post-injury). After reaching a peak on day 7, they gradually return to normal levels by day 14 (22). Accordingly, fibroblast migration along the fibrin network causes reepithelialization to begin at the edges of the wound (23). Hence, it is possible to see that the wound clinically heals. Therefore, in our study, the WA assessment was done on days 7 and 14.

According to our study, the ND-HA and D-HA groups showed greater clinical wound healing compared to the ND-S and D-S groups on days 7 and 14. The WA in the HA groups was smaller than the saline groups on day 14. The wound closure in ND and D rats was similar on the 14th day following the application of HA. In this study, HA significantly accelerated the clinical healing of wounds in ND and D rats. There are several studies on humans that evaluated the topical HA applied to the donor area following a free gingival graft promotes palatal wound healing (11,24) and on animals that examined the efficiency of HA in secondary wound healing of oral mucosa or skin (12,19,21,25). Chen et al (12), in a study evaluating cutaneous wound healing in D rats, reported that the higher molecular weight of HA significantly accelerated wound healing and reduced the time required for healing compared to the untreated D control group. Lee et al (25) compared HA gel and film in secondary wound healing by creating wounds on the tongue surfaces of systemically healthy rats. Although no significant difference was found between the groups on day 3, they observed that HA gel and film accelerated clinical wound healing on day 7 than the untreated control group. Hammad et al (21) reported that HA gel facilitated clinical palatal wound closure and reduced palatal WA on days 7 and 14 compared to chlorhexidine digluconate gel, allantoin gel, and placebo gel in systemically healthy rats. Taşkan et al (19) discovered that in the HA gel-applied group, the palatal WA was smaller on days 3, 7, 14, and 21 as than the placebo group. This is the first study that was conducted to investigate efficacy of the 0.12% HA mouthwash in the secondary healing process of palatal wounds on the D and ND rats by assessing the wound closure clinically. The results of our study on days 7 and 14 were consistent with the results of the abovementioned studies reporting favouring the ND-HA group. Moreover, our study demonstrated that HA mouthwash could increase palatal wound healing in D rats. According to the findings of previous studies and our study, HA application leads to improved secondary wound healing in ND and D rats by enabling polymorphonuclear leukocytes and macrophages to migrate and adhere to the inflamed area, inducing the production of pro-inflammatory cytokines by fibroblasts and keratinocytes that support the inflammatory response, promoting angiogenesis, stabilizing the granulation tissue, and re-establishing the epithelium (26).

One of the limitations of the present study was the short follow-up period (14 days) in palatal secondary wound healing in ND and D rats. Another limitation was that this study only used one HA concentration. Therefore, further investigations with a longer follow-up period in different concentrations of HA and various wound types on D rats are needed before such firm conclusions can be obtained.

5. CONCLUSION

Within the limitations of this study, topical application of HA mouthwash demonstrated a positive effect that improved secondary wound healing and reduced WA in D and ND rats. Thus, topical application of HA can be used in diabetic palatal secondary wound healing.

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