Wound healing potential by hyaluronate gel in streptozotocin-induced diabetic rats

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This study was conducted to investigate whether topical application of Hyaluronate gels could improve the impaired wound healing in streptozotocin-induced diabetic rats. Four groups of adult male Sprague Dawley rats, 2 cm full-thickness skin wound were experimentally created on the posterior neck area of streptozotocin-induced diabetic rats (STZ). Wounds of Group 1 animals were topically treated with the vehicle, gum acacia in normal saline, as a placebo control group. Group 2 animals served as reference standard and treated topically with Intrasite gel. Animals of Group 3, 4 were treated topically with new oral high molecular weight hyaluronic acid 240 mg/100 g gel, 0.8% hyaluronic acid gel respectively. Macroscopically, the reference standard gel and the three gels-treated wounds were significantly healed faster in comparison to placebo control wounds group. Wound closure was significantly accelerated by topical application of high molecular weight hyaluronic acid compared to reference standard gel, 0.8% hyaluronic acid gel. Furthermore, histological examination of healed wounds with reference standard gel, 0.8% hyaluronic acid gel, and hyaluronic acid 240 mg/100 g gel-treated wounds revealed comparatively increases in macrophages, fibroblast migration, collagen regeneration, and epithelization compared with the placebo control group. In conclusion, the new oral high molecular weight hyaluronic acid gel can improve the impaired healing of diabetic wounds and could be useful in treating oral ulcerations.

Key words: Wound, diabetes, hyaluronic acid gel, intrasite gel.

INTRODUCTION

Healing of wounds is a fundamental response to tissue injury, occurs by a process of connective tissue repair. A fibrous scar is the end product of this process, the predominant constituent is collagen. Collagen and other components of the ground substance are synthesized by the highly vascular granulation tissue that is formed within the wound space. Collagen provides strength and integrity to the dermis (Raghow, 1994), Diabetes mellitus is a condition which is known to be associated with a variety of connective tissue abnormalities. These abnormalities contribute to the impaired wound healing observed in diabetes. The collagen content of the skin is decreased as a result of reduced biosynthesis and/or accelerated degradation of newly synthesized collagen (Chithra et al., 1998). These qualitative (Schnider and Kohn, 1981) and quantitative (Spanheimer et al., 1988) abnormalities contribute to the impaired wound healing observed in diabetes. Diabetic wounds result in significant morbidity, prolonged hospitalization and enormous health-care expenses (Velander et al., 2008). Surgical treatment of diabetic wounds remains difficult and often insufficient, leading to high morbidity among those patients (Bowler, 2002). Better ways to treat diabetic wounds are needed to develop new therapeutic strategies.

Hyaluronic acid is an important physiological substance that plays a vital role in the healthy formation of connective tissue and is known as a Glycosaminoglycan. Gengigel® containing Hyaluronic acid has a number of significant applications in Dentistry, in the repair, healing and regeneration of gingival tissue as an integral element of the treatment of gingivitis and periodontitis (Brandimarte, 1973; Fornara, 1992). Gengigel offers to be an exciting new product development which demonstrates very effective and accelerated...
tissue healing properties, in addition to its antiinflammatory and anti-oedematous characteristics. Gengigel® can help as part of a periodontal regime to accelerate healing and help prevent the recurrence of gingivitis so slowing that the cyclical progression is leading to periodontitis. High molecular weight hyaluronic acid reduces inflammation in patients with periodontal disease by decreasing proliferation of epithelial cells such as fibroblasts and lymphocytes. Nevertheless, the efficacy of hyaluronic acid in reducing epithelial cells proliferation is depends on its molecular weight and concentration (Mesa et al., 2002). These findings are parallel with the previous data published by Laurent et al. (1995). They claimed that the hyaluronic acid was able to act as an anti-inflammatory agent by scavenging prostaglandin, metalloproteinases and other bio-active molecules, thus reducing the level of inflammatory mediators. Afta med a new formulation of oral high molecular weight hyaluronic acid 240 mg/100 g gel demonstrated by controled clinical trials, promotes healing of the ulcers and reduces thier numbers by cotroling the inflammatory processes and dehydrating the tissues. There were no data regarding the wound healing effect between gengigel 0.8% hyaluronic acid gel and Afta med high molecular weight hyaluronic acid 240 mg/100 g gel. This encouraged us to assess the rate of wound healing enclosure of hyaluronic acid gel macroscopically and microscopically in streptozotocin-induced diabetic rats.

MATERIALS AND METHODS

Intrasite gel

Intrasite gel was purchased from University Malaya Medical Center Pharmacy. Intrasite gel is an amorphous hydrogel which gently rehydrates necrotic tissue, and facilitate autolytic debridemen, while being able to loosen and absorb slough and exudates, cleaning the way for effective wound healing. Therefore, 0.2 ml of Intrasite gel was applied topically twice daily to the wound of Group 2 rats (Intrasite gel is a trademark for Smith and Nephew Ltd) (Williams, 1994).

Hyaluronic acid gels

Afta med high molecular weight hyaluronic acid 240 mg/100 g gel and Gengigel 0.8% hyaluronic acid gel were purchased from the dental suppliers of the Phamaniaga Manufacturing Bhd Kuala Lumpour, Malaysia.

Lignocaine HCl (2%, 100 mg/5 ml)

The local anesthesia was purchased from Experimental Animal House, Faculty of Medicine, University Malaya. There was 1 ml of Lignocaine injected subcutaneous.

Experimental animals

Sprague Dawley healthy adult male rats were obtained from the experimental animal house, Faculty of Medicine, University of Malaya. The rats were divided randomly into 4 groups of 6 rats each. Each rat that weighted between 220 - 250 g was housed separately (one rat per cage). The animals were controlled on standard pellet diet and tap water. The study was approved by the Research Committee on the Ethical Use of Animal in Research (UiTM Care) Universiti Teknologi MARA Ethic No. 600-FF (PT,5/2) 2007/2009. All animals received human care according to the criteria outlined in the “Guide for the Care and Use of Laboratory Animals” prepared by the National Academy of Sciences and published by the national Institute of health.

Diabetic animals

Diabetes Melitus induced in all groups by a single injection of streptozotocin (STZ; 50 mg/kg, i.p.) prepared in citrate buffer (0.1 M, pH 4.5) after overnight fasting (Junod et al., 1969). Blood was drawn from the tail 24 h after the injection and the glucose level was estimated using Glucometer ( Ames., Bayer, Diagnostic). Wounds were made on the rats showing elevated blood glucose (> 250 mg/dl). Blood glucose levels were estimated at the time of creation of the wounds.

Experimentally induced excision wounds

Wounds were created on the 7th day after induction of diabetes in all rats. The animals were anesthetizied by diethyl ether. The skin shaved by electrical clipper, disinfected with 70% alcohol and injected with 1 ml of Lignocaine HCl (2%, 100 mg/5 ml). An area of uniform wound 2.00 cm in diameter (circular area = 3.14 cm²) using circular stamp, was excised from the nape of the dorsal neck of all rats with the aid of round seal as described by Morton and Melone (1972) with slight modification. Avoid incision of the muscle layer and tension of skin was kept constant during the procedure. The wound area was measured immediately under light diethyl ether anesthesia by placing a transparent tracing paper over the wound and tracing it out. The tracing paper was placed on 1 mm² graph sheet, and traced out. The squares were counted and the area was recorded as described by Chah et al. (2006) with slight modification.

Topical application of vehicles

Wounds of Group 1 animals were dressed with 0.2 ml of vehicle, sterilized distilled water was given as a placebo control group twice daily (Chah et al., 2006). However, wounds of Group 2 rats were dresses topically with 0.2 ml of Intrasite gel as a reference, twice daily. Moreover, 0.2 ml of each Afta med high molecular weight hyaluronic acid 240 mg/100 g gel and Gengigel 0.8% hyaluronic acid gel were applied topically twice daily to the wound of Group 3 and 4, respectively. The wound was observed daily until complete wound-healing enclosure completely occurs. The wound closure area of each animal was assessed by tracing the wound on days 1, 5, 10, 15 and 20 post-wounding surgery using transparency paper and a permanent marker under light diethyl ether anesthesia as described by Nayak and Pinto-Pereira (2006) with slight modification. The wound areas recorded were measured using a graph paper. The percent of wounds healing on these days were determined (Chah et al., 2006). Number of days required for falling of scar without any residual raw wound gives the period of epithelization.

Histological evaluation of healed wounds

The skin specimen from wounds healed areas was fixed in 10%
Table 1. Time required for wound healing by Afta med hyaluronic acid 240 mg/100 g gel and Gengigel 0.8% hyaluronic acid and its effect on percentage wound healing in rats.

<table>
<thead>
<tr>
<th>Animal groups</th>
<th>No of animals</th>
<th>Type of dressings 0.2 ml/wound</th>
<th>Healing time (days) (Mean ± S.E.M)</th>
<th>Percentage (%) wound healing (Mean ± S.E.M) on day post surgery</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>Group 1</td>
<td>6</td>
<td>deH2O (negative control)</td>
<td>25.17 ± 0.6a</td>
<td>0.00</td>
</tr>
<tr>
<td>Group 2</td>
<td>6</td>
<td>Intrisite gel (positive control)</td>
<td>16.17 ± 0.6a</td>
<td>0.00</td>
</tr>
<tr>
<td>Group 3</td>
<td>6</td>
<td>Gengigel 0.8% hyaluronic acid gel</td>
<td>17.00 ± 0.58b</td>
<td>0.00</td>
</tr>
<tr>
<td>Group 4</td>
<td>6</td>
<td>Afta med hyaluronic acid 240 mg/100 g gel</td>
<td>15.00 ± 0.6b</td>
<td>0.00</td>
</tr>
</tbody>
</table>

Buffered formalin and processed by paraffin tissue processing machine. The healed skin was assessed by taking a 5 μ section and stained with hematoxylin and eosin. Three separated sections of each wound were examined by light microscopy. The number of fibroblasts and blood vessels were counted in 5 high-power fields (×100) over 3 separated sections. The stained sections of collagen were displayed at 40 × magnification. The collagen stained area was measured using plain meter grid in 0.15 mm².

**Statistical analysis**

All values are reported as mean ± S.E.M. and the statistical significance of differences among groups were assessed using one-way ANOVA. A value of p < 0.05 was considered significant.

**RESULTS**

**Wound healing activity in diabetic rats**

The rate of wound healing activity were evaluated by a blind observer unaware of the experimental protocol. Wounds dressed with intraSite gel as a reference, Afta med high molecular weight hyaluronic acid 240 mg/100 g gel and Gengigel 0.8% hyaluronic acid gel showed considerable signs of dermal healing in diabetic rats and significantly (p<0.05) healed earlier compared to wounds dressed with a placebo control group (sterilized distilled water) (Table 1, Figures 1a and b). Throughout the experiment, the percentage healing in placebo control-treated group was significantly lower than those of intraSite gel, Afta med high molecular weight hyaluronic acid 240 mg/100 g gel and Gengigel 0.8% hyaluronic acid gel-treated groups (Table 1).

Histologically, gel-treated groups contained comparatively less scar at wound closure (Figures 2a and b), and the granulation tissue in healed wound contained comparatively few inflammatory cells, and more collagen and proliferating blood capillaries compared with placebo control-treated group (Figures 3a and b). Vascularization was enhanced in the gel-treated groups and significantly greater than the placebo control-treated group.

**DISCUSSION**

Gingival gel based on hyaluronic acid, providing active protection from gum disease and support for the natural healing of inflamed and damaged tissue in the mouth (Pirnaza et al., 1999). Hyaluronic acid is a natural substance found in the connective tissues of the body (Moseley et al., 2002). When applied to gums; it stimulates the production of healthy new tissue. Fibroblasts are the major components which secrete hyaluronic acid into the extracellular matrix (Mesa et al., 2002). Cutaneous wound repair is accompanied by an ordered and definable sequence of biological events starting with wound closure and progressing to the repair and remodeling of damaged tissue (Phillips et al., 1991).

In spite of tremendous advances in the pharmaceutical drug industry, the availability of drugs capable of stimulating the process of wound repair is still limited (Udupa et al., 1995). Moreover, the management of chronic wounds is another major problem due to the high cost of therapy and the presence of unwanted side effects (Porras-Reyes et al., 1993; Suh et al., 1998).

It is consented that Reactive Oxygen Species (ROS) are deleterious to wound healing process due to the harmful effects on cells and tissues. Absorbable synthetic biomaterials are considered to be degraded via ROS (Aliyeva et al., 2004). Free-Radical-Scavenging Enzymes (FRSE) are a cytoprotective enzymatic group that has an essential role in the reduction, de-activation and removal of ROS as well as regulating wound healing process.

In the present study, topical application of gel-treated groups significantly accelerated the rate of wound healing.
and histologically, healed wound contain comparatively less inflammatory cells, more collagen and angiogenesis. Wound healing effects may be due to up-regulation of collagen expression (Bonte et al., 1993) and increase in tensile strength of the wounds (Suguna et al., 1996). In response to tissue loss, fibroblasts proliferate and migrate into the defect until the wound is populated by fibroblasts and extracellular matrix (Clark, 1993).
Similarly, enhanced healing activity has been attributed to increase collagen formation and angiogenesis. (Trabucchi et al., 1986; Shukla et al., 1999). Collagen played a central role in the healing of wounds and it is a principal component of connective tissue and provides a structural framework for the regenerating tissue. Angiogenesis in granulature tissues improves circulation to the wound site thus providing oxygen and nutrients essential for the healing process (Szabo et al., 1995) that include-re-epithelization. Stimulated epithelial cell proliferation and angiogenesis are important for wound healing process (Buntrock et al., 1982). With the consistence of the present study, Habibipour et al. (2003) showed that histological analysis of the treated healed wound group contained a large amount of fibroblast proliferation, collagen synthesis, and neovascularization, which resulted in an increased wound tensile strength and accelerated healing wound. In contrary to their results, Nascimento and Costa (2006) demonstrated that histology of wound healed in overweight rats induced by a high-fat diet increased the inflammatory infiltrate and delayed myofibroblastic differentiation, collagen deposition epithelial and connective tissue cells proliferation and angiogenesis.

**Conclusion**

These results suggest that the new oral high molecular weight hyaluronic acid gel can improve the impaired healing of diabetic wounds and could be useful in treating oral ulcerations.

**ACKNOWLEDGMENT**

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