Thesis for PhD degree in pharmacology

Title:
Study of the effects of quince (*Cydonia oblonga* Miller) seed mucilage on human skin fibroblasts proliferation, serum and wound fluid levels of growth factors in rabbit during wound healing process.

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To my mother
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Abstract

Introduction: Quince seed mucilage (QSM) has been used in Iranian traditional medicine for the treatment of wounds, respiratory and inflammatory diseases. Recent studies indicated that QSM accelerated wound healing. The present study was undertaken to investigate the healing efficiency of QSM formulated as 5%, 10%, and 20% creams in eucerin base, with especial attention on growth factors involving in wound healing. The effect of QSM at concentrations of 50, 100, 200 and 400µg/ml on human skin fibroblast proliferation was also evaluated.

Materials and methods: Full thickness wounds were created in Iranian male rabbits divided into five experimental groups (n=6), as negative control, eucerin and treatments. Negative control group did not receive any treatment. Eucerin group received topical eucerin. Treatment groups were treated topically by creams of QSM 5, 10 and 20% (w/w) in eucerin base. Treatments were applied twice a day from the beginning of the experiments to complete wound closure. The efficacy of treatment was evaluated based on wound contraction, hydroxyproline content, tensile strength of wound tissue. The levels of epidermal growth factor (EGF), transforming growth factor-β1 (TGF-β1), vascular endothelial growth factor (VEGF) and platelet derived growth factor (PDGF) were also determined in serum and wound fluid of tested animals. For investigation of the proliferative activity of QSM, human skin fibroblast cell line (HNFF-P18) was used in the experiment. Cell proliferation assay was measured by a MTT assay.

Results: QSM promoted wound healing activity significantly in full thickness wound model. High rate of wound contraction (P<0.01), high tensile strength (P<0.001), significant increase in the levels of EGF, TGF-β1, VEGF, PDGF (P<0.05) in wound fluid and hydroxyproline content (P<0.001) were observed in animals treated with QSM compared to control groups.

In vitro results showed that the cells treated with QSM at concentrations less than 400µg/ml increased their proliferative activity. The concentration of 50µg/ml was the most effective dose after 72 hours treatment.

Conclusion: The present study suggested that QSM enhances the wound repair process by increasing collagen production and the concentrations of some growth factors in wound site. Furthermore it stimulates the proliferation of fibroblasts.

Key words: wound healing, quince seed mucilage, growth factors, fibroblast proliferation, tensile strength.
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List of abbreviations

AAPH \hspace{1cm} 2, 20 -diphenyl-1-picrylhydrazyl
BMP \hspace{1cm} Bone morphogenic protein
COPD \hspace{1cm} Chronic obstructive pulmonary disease
DFU \hspace{1cm} Diabetic foot ulcer
DMEM \hspace{1cm} Dulbecco's modified Eagle's medium
DMSO \hspace{1cm} Dimethyl sulfoxide
DPBS \hspace{1cm} Dulbecco's Phosphate-Buffered Saline
DPPH \hspace{1cm} 2,2-diphenyl-1-(2,4,6-trinitrophenyl) hydrazyl
EC \hspace{1cm} Effective concentration
ECM \hspace{1cm} Extra cellular matrix
EGF \hspace{1cm} Epidermal growth factor
EGFR \hspace{1cm} Epidermal growth factor receptor
ELISA \hspace{1cm} Enzyme-linked immunosorbent assay
FBS \hspace{1cm} Fetal bovine serum
FDA \hspace{1cm} Food and drug administration
FGF \hspace{1cm} Fibroblast growth factor
GAG \hspace{1cm} Glycosaminoglycan
GC/FID \hspace{1cm} Gas chromatography/ Flame ionization detector
GM.CSF \hspace{1cm} Granulocyte- macrophage colony stimulating factor
HPLC/DAD \hspace{1cm} High performance liquid chromatography/ Diode array detector
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>HGH</td>
<td>Human growth hormone</td>
</tr>
<tr>
<td>HPLC/UV</td>
<td>High-performance liquid chromatography/Ultra violet</td>
</tr>
<tr>
<td>IC</td>
<td>Inhibitory concentration</td>
</tr>
<tr>
<td>IL-β</td>
<td>Interleukine-β</td>
</tr>
<tr>
<td>IGF-1</td>
<td>Insuline like growth factor-1</td>
</tr>
<tr>
<td>KDR</td>
<td>Kinase insert domain receptor</td>
</tr>
<tr>
<td>KGF</td>
<td>Keratinocyte growth factor</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloproteinase</td>
</tr>
<tr>
<td>MTT</td>
<td>3-(4,5-Dimethyl thiazol-2-yl)-2,5- diphenyltetrazoliumbromide</td>
</tr>
<tr>
<td>NRG</td>
<td>Neureguline</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered salin</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet derived growth factor</td>
</tr>
<tr>
<td>PDGFR</td>
<td>Platelet growth factor receptor</td>
</tr>
<tr>
<td>PVD</td>
<td>Peripheral vascular disease</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>TGF-β₁</td>
<td>Transforming growth factor- β₁</td>
</tr>
<tr>
<td>TGFR</td>
<td>Transforming growth factor receptor</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor necrosis- α</td>
</tr>
<tr>
<td>TNBS</td>
<td>2, 4, 6-trinitrobenzenesulfonic acid</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>VEGFR</td>
<td>Vascular endothelial growth factor receptor</td>
</tr>
<tr>
<td>Vis</td>
<td>Visible light</td>
</tr>
<tr>
<td>VU</td>
<td>Venus ulcer</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
<td>------------------</td>
</tr>
<tr>
<td>WB C</td>
<td>White blood cell</td>
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<td>Weight/Weight</td>
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1.1 INTRODUCTION

According to wound healing society wounds are physical injuries that result in an opening or break of the skin that causes disturbance in normal skin anatomy and function (1). Wounds represent a significant burden on the patients and health care professionals worldwide they not only affect physical and mental health of millions of patients but also impose significant costs on them. Current estimates indicate that worldwide nearly 60 million people suffer from chronic wounds (2). Impaired skin integrity a consequence of illness or injury may lead to acute loss of equilibrium such as water and electrolyte balance or may also cause disability or even death (3). Cutaneous injury can take many forms, surgical trauma, burns, and immunologically mediated injury, are just a few in a long list. Despite this, the general sequence of events that are activated in response to injury and subsequent wound repair show striking similarity irrespective of the initial insult. Unhealed wounds constantly produce inflammatory mediators that produce pain and swelling at the wound site. Wounds are a substrate for infection and prolong the recovery of injured patient. Chronic wounds may even lead to multiple organ failure or death of the patients (4).

About 2500 years ago, Hippocrates professed “let food be thy medicine and medicine be thy food” and, for centuries, plants have been widely used as food and remedies in both Western and Eastern cultures (5). In addition Plant-based medicines enjoy a respectable position today, especially in the developing countries, where modern health service is limited. Indigenous remedies which are more effective, safe and inexpensive are gaining popularity among both rural and urban areas. Many medicinal plants and seeds have been reported to posses wound healing activity and found useful in the treatment of wounds, one of these medicinal plants is Cydonia oblonga Miller (Quince), Previous studies on full thickness wound model in rabbit showed the wound healing effects of quince seed mucilage (6), but it's mechanism of action have not been
evaluated. Because of the vital role of fibroblasts and growth factors in wound healing process it has been suggested that QSM may affect fibroblasts growth and proliferation during wound healing process or it may also enhance the production of some growth factors in different phases of wound healing. In the present study detailed evaluation of the wound healing activity of QSM with special attention on growth factor involving in wound healing and fibroblast proliferation carried out using *in vivo* and *in vitro* models respectively.

### 1.2 SKIN AND WOUNDS

The integument or skin is the largest organ of the body, making up 16% of body weight, with a surface area of 1.8m (7). There are three structural layers to the skin: the epidermis, the dermis and subcutis (figure 1.1). Hair, nails, sebaceous, sweat and apocrine glands are regarded as derivatives of skin (8).

**Figure 1.1 Skin structure**

The epidermis is the outer layer, serving as the physical and chemical barrier between the interior body and exterior environment; the dermis is the deeper layer providing the structural support of the skin, below which is a loose connective tissue layer, the subcutis or hypodermis which is an important depot of fat (9).

The main cells of the epidermis are the keratinocytes, which synthesize the protein keratin. Protein bridges called desmosomes connect the keratinocytes, which are in a constant state of transition from the deeper layers to the superficial. The four separate layers of the epidermis are formed by the differing stages of keratin maturation. Moving from the lower layers upwards to the surface, the four layers of the epidermis are: stratum basale (basal or germinativum cell layer), stratum spinosum (spinous or prickle cell layer), stratum granulosum (granular cell layer), stratum corneum (horny layer), stratum lucidum (only in thick skin). In addition to the keratinocytes, melanocytes, langerhan’s cell and merkel cell are also present within the epidermis (10).

The dermis is the thickest layer of the skin, is divided into 2 layers, and is composed of sparsely populated cells when compared with the epidermis. The main function of the dermis is to provide strength, support, blood, and oxygen to the skin. Major proteins found in the dermal layer include collagen (which provides strength) and elastin (which gives skin its recoil). They are synthesized and secreted by fibroblasts (10). Fibroblasts are the main type cell within the dermis that synthesize the precursors of extra cellular matrix such as fibers and glycosaminoglycans to maintain the structural integrity of connective tissue (11). Under the dermis is a layer of loose connective tissue, called subcutaneous tissue, or hypodermis, which attaches the dermis to underlying structures. This layer consists of adipose and connective tissue, blood and lymphatic vessels, and nerves. Its function is to provide blood supply to the dermis for regeneration (9).
The skin has several functions, the most important being to form a physical barrier to the environment, allowing and limiting the inward and outward passage of water, electrolytes and various substances while providing protection against micro-organisms, ultraviolet radiation, toxic agents and mechanical insults. Several problems such as loss of homeostatic function can arise when this physical barrier is compromised through a wound. A wound is a breach in the epidermis or dermis resulting from trauma or pathological change that initiates a process of repair (12). Therefore it is necessary that any wound sustained by the skin be healed to restore homeostasis.

1.2.1. CLASSIFICATION OF WOUNDS

Wounds classified as open and closed wounds on the basis of underlying cause of wound creation and as acute and chronic wounds on the basis of physiology of wound healing (13).

Open wounds

An open wound is a break in the skin's surface resulting in external bleeding. Open wounds can be further classified into various types according to the objects causing wounds:

- **Incised wounds:** Incision tends to have smooth edges and resemble a surgical or paper cut.
- **Tear wounds or laceration wounds:** Laceration is cut skin with jagged, irregular edges. This type of wound is usually caused by forceful tearing away of skin tissue.
- **Abrasion or Superficial wounds:** with an abrasion top layer of skin is removed by little or no blood loss. Abrasions tent to be painful because the nerve endings are abraded along with the skin.
- **Puncture wounds:** Punctures are usually deep, narrow wounds in the skin and underlying organs such as a stab wound from a nail or a knife. The entrance is usually small and the risk of infection is high.
چکیده
مقدمه: موسیلاژ به دانه در طب سنتی ایران برای درمان زخم‌ها، بیماری‌های تنفسی و بیماری‌های التهابی به کار رفته است. مطالعات انجام شده در سال‌های اخیر نشان داده که این ماده باعث افزایش سرعت ترمیم زخم می‌شود. هدف از انجام این مطالعات بررسی دقت اثر موسیلاژ به دانه (که به صورت مسکه‌های ۱۰، ۲۰ و ۵٪ در پابی اورسین قلموم شده است) در ترمیم زخم مهربان با توجه ویژه به فاکتورهای رشد در درون زخم است. همچنین اثر موسیلاژ به دانه بر سرعت رشد و تکثیر فیبروبلاست‌های پوستی انسانی در محیط کشت برسی گردید.

روش کار: در مطالعه in vivo، خرگوش‌های نر ایرانی با وزن گرم ۲۴۰۰-۱۸۰۰ مورد استفاده قرار گرفتند. زخم‌ها با ضخامت کامل پوست در ابعاد ۲۰×۲۰مم در ناحیه شست هر یک از حیوانات اجاد شدند. حیوانات به گروه (n=۶) تقسیم شدند: گروه کنترل منفی که هیچ درمانی دریافت نکرده. گروه درمان شده با اسیرین که روزانه دو بار با اسیرین درمان می‌شدند و گروه‌های درمان شده با موسیلاژ که روزانه دو بار با کرمه‌ای ۵، ۱۰ و ۲۰٪ به هنگام اسیرین درمانی در اسیرین درمان شدند. درمان آنها از روز شروع مطالعه تا کامل زخم‌ها ادامه یافت. اثر درمانی موسیلاژ بر اساس اندیشه گیری پارامترهای مانند: سرعت بستر شدن زخم، محتوای هیدروکسی پروپون، استحکام کشته بافت زخم، شده و همچنین غلظت فاکتورهای رشد مدل گروه‌ها (EGF، TGF-β، VEGF، PDGF) و فاکتور رشد مشتاق از پلاکت‌ها در خون و مایع زخم به دست آمده از حیوانات مورد مطالعه، در میزان تکثیر فیبروبلاست‌های پوستی انسانی (HIFN-P18) در مورد استفاده قرار گرفت. میزان تکثیر سلولی با استفاده از روش MTT اندازه‌گیری شد.

نتایج: نتایج مطالعه in vivo نشان داد که موسیلاژ به دانه در سرعت ترمیم زخم را به طور قابل توجهی افزایش می‌دهد.

نتایج مطالعه in vitro نشان داد که موسیلاژ به دانه در غلظت‌های کمتر از ۴۰۰ μg/ml تکثیر فیبروبلاست‌های پوستی را افزایش می‌دهد. میزان تکثیر در این مطالعه در گروه ۵۰ μg/ml بود.

نتیجه‌گیری: نتایج به دست آمده از این مطالعه نشان می‌دهد که موسیلاژ به دانه به وسیله افزایش تولید کلاران و غلظت بخصوص از فاکتورهای رشد در محل زخم سرعت ترمیم زخم را در گروه‌ها افزایش می‌دهد. همچنین موسیلاژ باعث افزایش سرعت تکثیر فیبروبلاست‌ها می‌شود.

کلمات کلیدی: ترمیم زخم، موسیلاژ به دانه، فاکتورهای رشد، تکثیر فیبروبلاست‌ها، استحکام کشته