ORIGINAL REPORT

Impact of Morphine Dependency on Secondary Intention Wound Healing in Rat

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Abstract- Wound healing has always been among important and crucial subjects in medicine. Morphine dependency has also been a social and health problem in the Middle East. This study was aimed to investigate the effects of morphine dependency on pro-inflammatory and fibroblast cell recruitment, as well as re-epithelialization and the revascularization processes involved in secondary intention wound healing in rats. A full-thickness wound (2×2 cm in diameters) was created on the dorsum of two groups of rats, a control group and a second group consisted of morphine dependent rats. During the first 14 days of post wounding the wound was excised consecutively at priorly planned days with peripheral margins of normal skin. The specimens were evaluated by two pathologists, who were blind to the study design, and the cellular population, re-epithelialization and revascularization were reported by them. Histological examination of the wound tissue showed evidence of increased population of fibroblasts and a plateau or decreased recruitment of macrophage and neutrophile cells. In the dependent group re-epithelialization was observed to be enhanced significantly in comparison to the control group while having an inhibitory effect on revascularization. The present study demonstrates that morphine dependency enhances re-epithelialization as well as tissue recruitment of fibroblasts; thereby probably enhancing secondary intention wound healing.

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Keywords: Wound healing; Morphine; Addiction; Rat

Introduction

Wound healing occurs in a biphasic manner (1). Upon tissue injury, pro-inflammatory cells, including neutrophils and macrophages are recruited to induce resolution of bacterial clearance. Moreover, the second phase of wound healing entails wound closure events including fibrin matrix formation and collagen deposition, epidermal migration (re-epithelialization), and formation of new blood vessels (1,2).

Morphine, the prominent alkaloid of opium, is among the most prescribed analgesic to control both chronic pain and post surgical pain. Furthermore, chronic morphine dependency is a common social and health problem in some countries in the Middle East including Iran. Many morphine dependent peoples require surgery or may suffer wound following various events.

Some data show that acute high dose morphine delays wound healing by generating excessive superoxide anions and impaired angiogenesis (3). However, the underlying mechanisms of wound healing complications among chronically morphine users have not been fully explored (2). On the other hand, *in vitro* studies have shown that morphine enhances proliferation and matrix accumulation in cultured renal fibroblasts and medullary interstitial cells. Morphine enhances accumulation of collagen type I in a dose-dependent manner (4-6).

Based on the various discouraging and promising results of other studies on this important subject, we decided to combine pathologic and clinical findings in order to evaluate the impact of morphine on wound healing. The present study focusing on the several factors has shown that morphine dependency plays a critical role in the first phase and early second phase of the secondary intention wound healing. Additionally, based on our clinical experience we noticed that chronic morphine usage can have an impact on the wound healing process. Furthermore in chronically morphine dependent patients we also observed a probable enhancement of surgical wound healing. Therefore,

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herein we would like to present our trial in a rat model to evaluate the impact of morphine dependency on the wound healing process.

Materials and Methods

One hundred and eight female adult rats weighing 215 ± 20 g were included in this study. They were collected from the Animal Laboratory Center of Kerman University of Medical Sciences. The animals were housed at a controlled temperature of 21 ± 0.5°C in wire-mesh cages (30×40×50 cm), with free access to food and water. They were randomly assigned to the control or morphine-addicted groups (54 rats in each group) (Figure 1).

Evaluation of the wounds was planned to be performed on the 2nd, 4th, 5th, 7th, 10th and 14th postoperative days, each of these two groups were subdivided into 6 subgroups with 9 members in each group (Figure 1).

Rats in the morphine group were receiving increasing doses of morphine orally in their drinking water (starting on a dose of 0.1 mg/kg and enhancing to a dose of 0.4mg/kg) for 21 consecutive days (7). At the end of this period all rats in this group received peritoneal injection of naloxon at a dose of 1 mg/kg in order to demonstrate withdrawal signs, confirming addiction to morphine. Afterwards animals were anesthetized by inhaling ether. Following their skin preparation, by shaving and disinfection using povidone iodine, a full-thickness skin with a diameter of 20×20 mm was excised from the dorsum of each animal. The created wound was left open for secondary intention healing. Rats were allowed to recover freely and no antibiotic was administered.

In each of the 6 subgroups on postoperative days of 2, 4, 5, 7, 10 and 14, the animals were re-anesthetized with ether and the wound was excised totally with a one millimeter margin of depth and also the surrounding normal skin. The animals were sacrificed at the end of experiment using intra cardiac injection of potassium chloride. The specimens were immersed in formalin (10%) and submitted for pathologic study. The pathologists were blind to the study design.

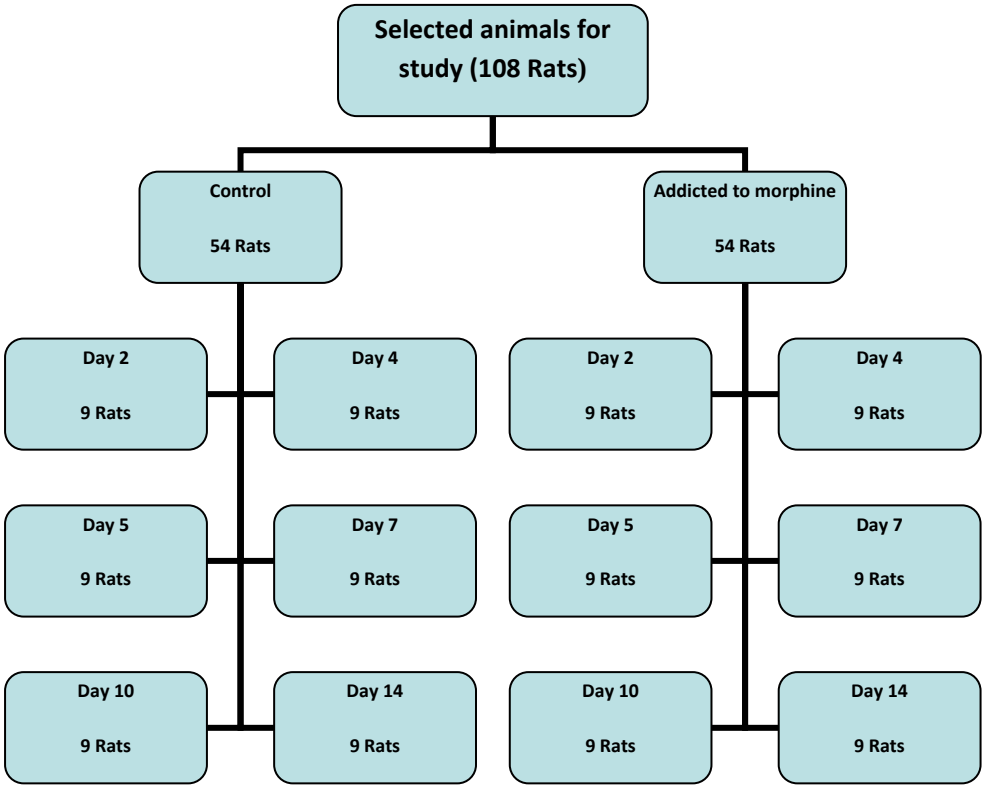

Figure 1. Arrangement of animals in the study frame

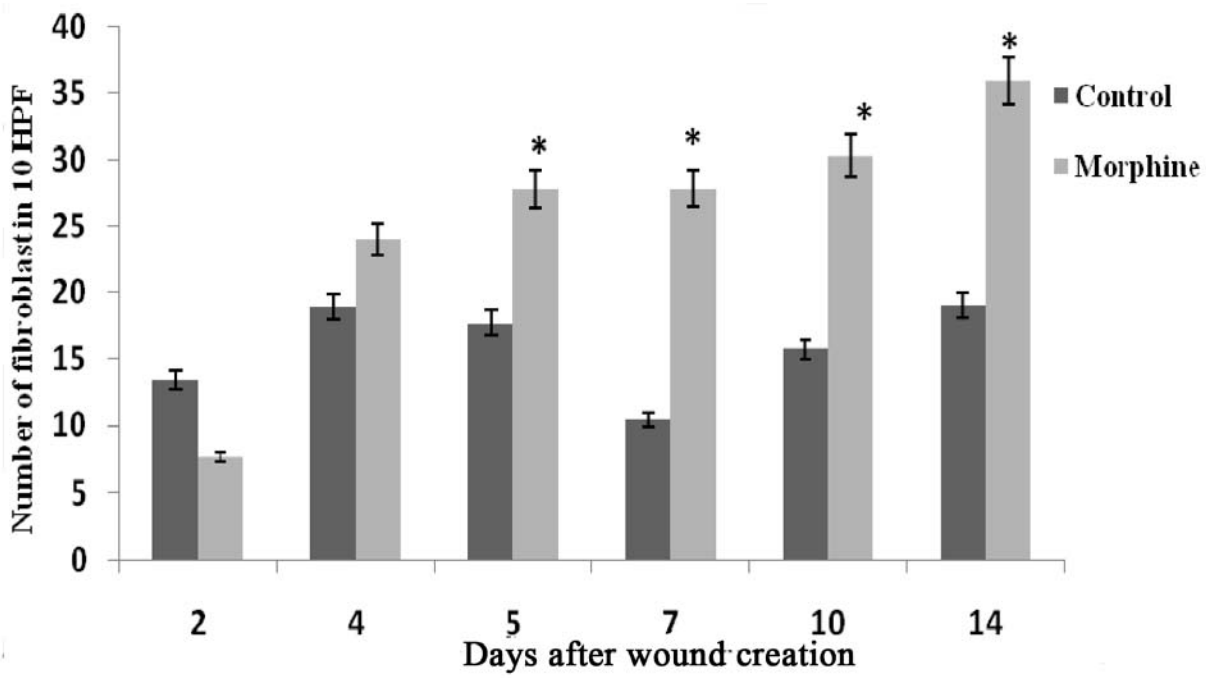

Figure 2. Number of fibroblast cells, in 10 high power field (HPF) of excised wound, in different post wounding days.

Histological examination

The specimens were studied histologically to evaluate wound healing parameters. Biopsies of formalin-fixed wound tissues were embedded in paraffin wax and sectioned at 3 and 5 µm thickness. Sectioned tissues were stained with hematoxylin and eosin and quantitative parameters of wound healing process were studied on ten-high power field in each of them. The quantitative parameters included: number of fibroblasts, polymorphonuclears (PMN), macrophages, cross sections of new vessels (angiogenesis), and scoring of re-epithelialization. Re-epithelialization was defined in three scores, i.e. ratio of thickness of re-epithelialized layer to adjacent normal epithelium surrounding the wound. Score 1: RLT (Re-epithelialized thickness of wound bed) = 1/3 NET (Normal epithelium thickness); Score 2: RLT = 2/3 NET, and Score 3: RLT = NET).

Statistical analysis

Results are presented as the mean±SEM. The data was compared by an unpaired t-test or analysis of variance (ANOVA). Statistical significance was accepted at a level of $P<0.05$.

Results

In this study, we evaluated the histological parameters involved in secondary intention wound healing and the probable impacts of morphine dependency on this complicated process in rats. The results of histological examination of the wound tissue are as follows:

1- Increased fibroblast count in the wound of morphine dependent rats, comparing to control group, especially after the 7th post operative day (10.44±1.22 in control group and 27.83±3.13 in morphine addicted group, $P<0.001$) (Figure 2).

2- Decreased neutrophil count in the wound of morphine dependent rats comparing to control group after the 5th post operative day (20.25±4.12 in control group and 8.87±1.13 in morphine addicted group, $P<0.05$) (Figure 3).

3- Decreased macrophage count in the wound of morphine dependent rats comparing to control group from the 2nd post operative day on (44.66±4.34 in control and 23.22±3.89 in morphine addicted group, P<0.001) (Figure 4).

4- The rate of angiogenesis in the wound of morphine dependent group was lower than control group (11.44±1.65 in control group and 6.94±0.78 in morphine addicted group, $P<0.01$) (Figure 5).

5- The rate of epithelialization in the wound of morphine dependent group was higher than control group on the 5th post operative day (1.55±0.12 in control group and 2.77±0.85 in morphine addicted group, $P<0.001$) (Figure 6).

Figure 3. Number of nutrophil cells, in 10 high power field (HPF) of the excised wound, in different post wounding days.

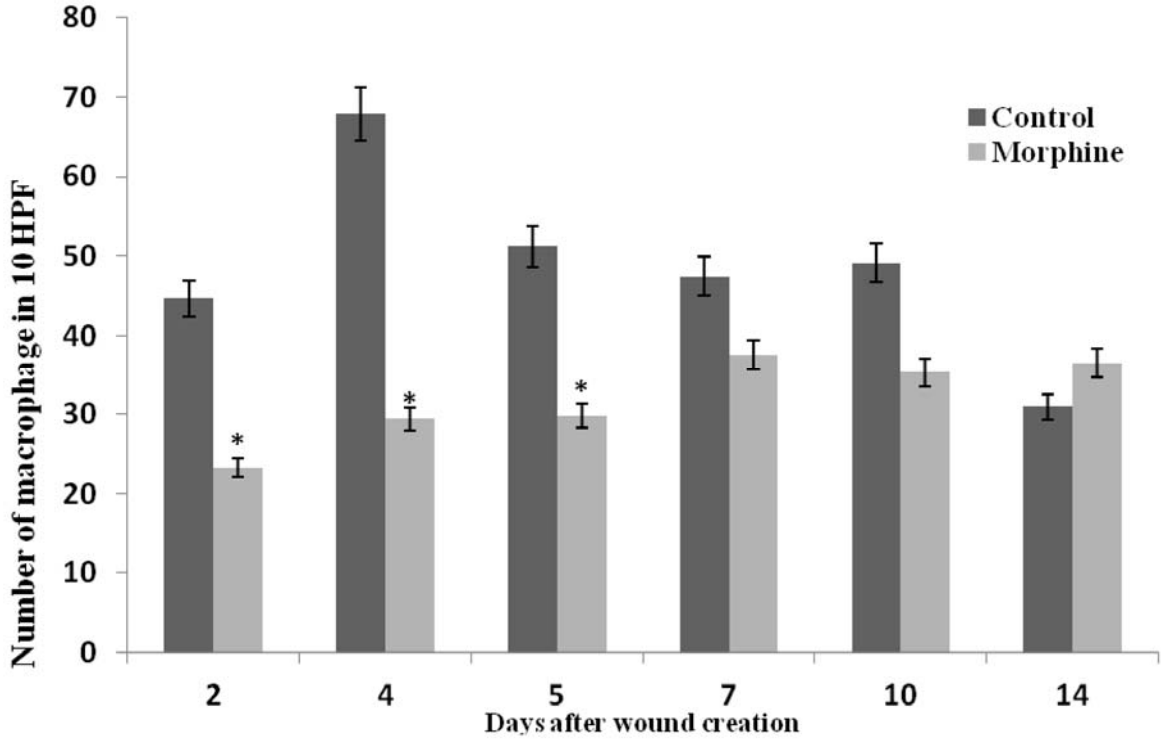

Figure 4. Number of macrophage cells, in 10 high power field (HPF) of the excised wound, in different post wounding days.

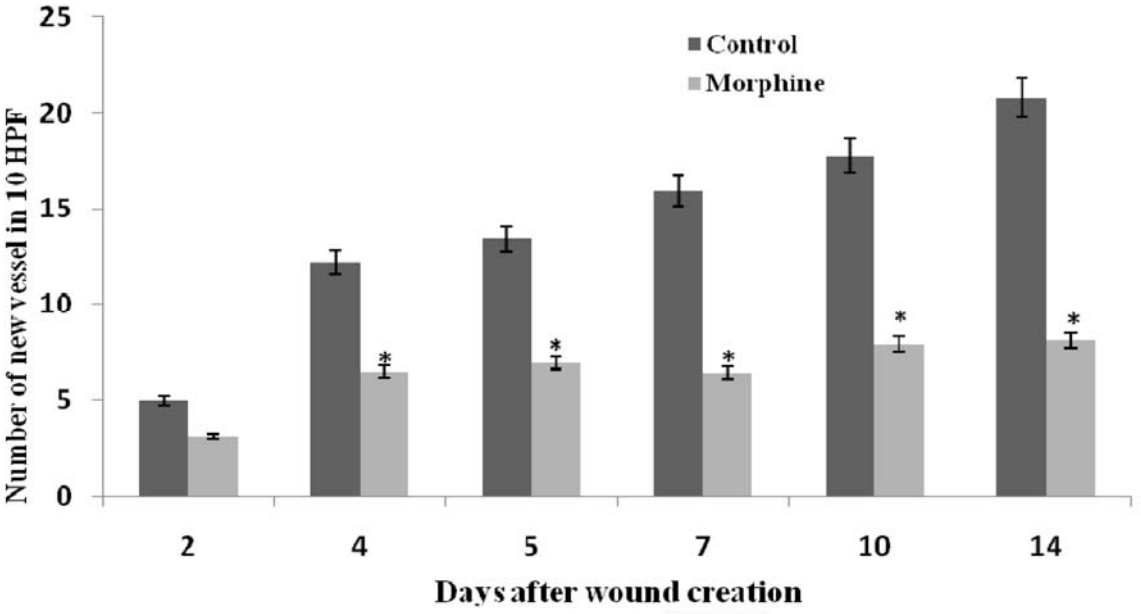

Figure 5. Number of new vessels, in 10 high power field (HPF) of the excised wound, in different post wounding days.

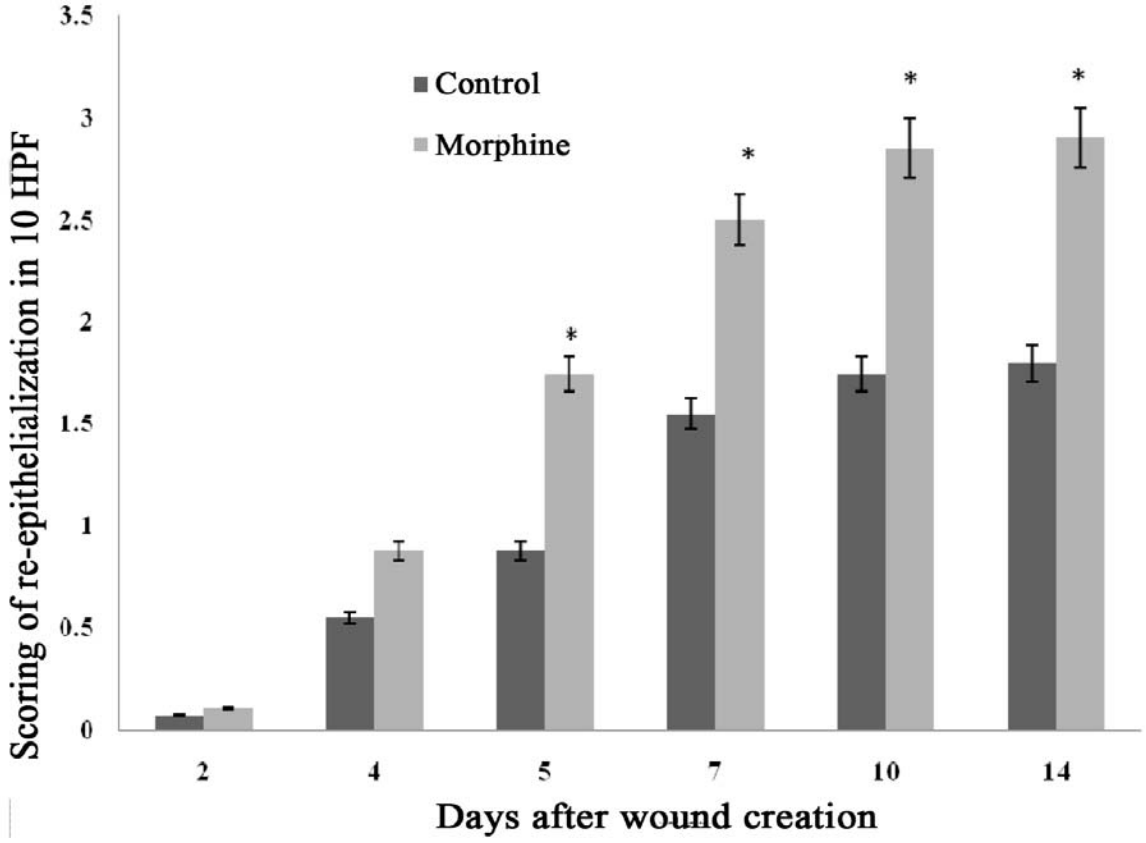

Figure 6. Scoring of re-epithelialization, in 10 high power field (HPF) of the excised wound, in different post wounding days.

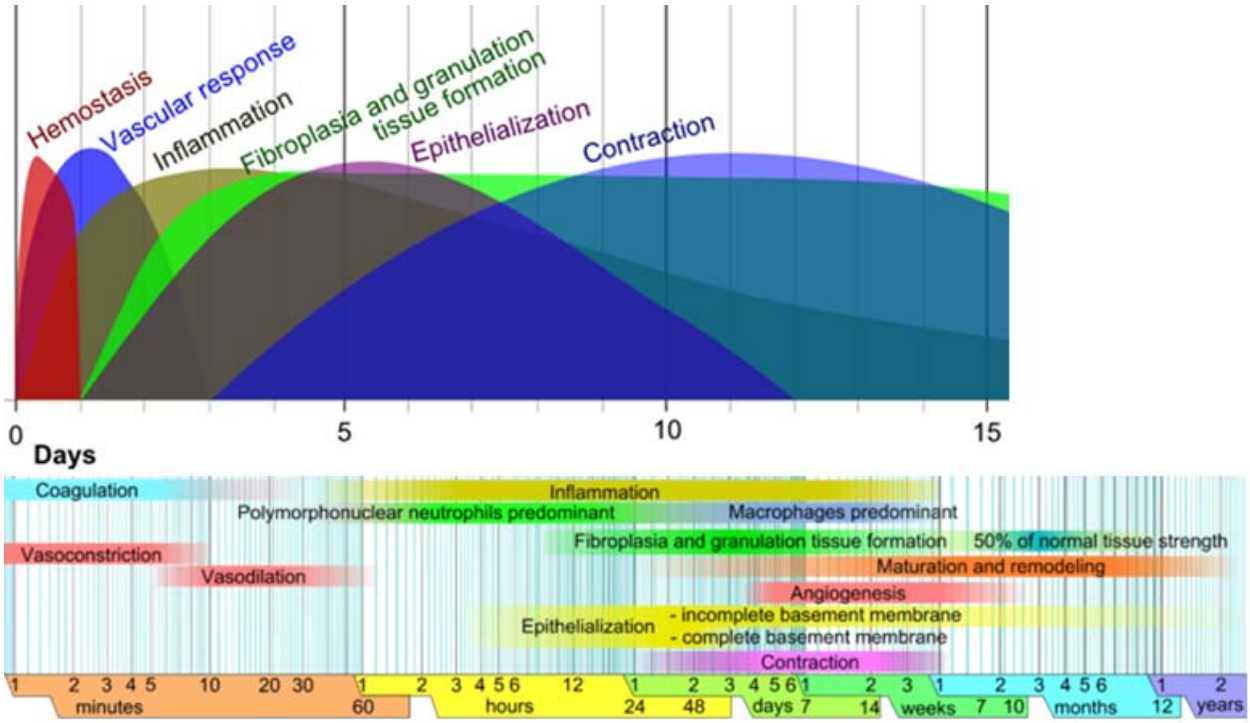

Figure 7. Approximate times of the different phases of wound healing, with faded intervals marking substantial variation, depending mainly on wound healing conditions (19).

Discussion

This study showed that, in the process of secondary intention wound healing, although pro-inflammatory cells had not increased (or even decreased in some cases) in the morphine dependent rats, recruitment of fibroblasts and re-epithelialization increased significantly in comparison to the control group (Figures 2-6).

Wound healing is an intricate process in which the skin (or another organ-tissue) repairs itself after surgery and/or injury. In normal skin, the epidermis and dermis exists in steady-state equilibrium, forming a protective barrier against the external environment. Once this protective barrier is broken, the normal process of wound healing is immediately set in motion. The classic model of wound healing is divided into three or four sequential, of course overlapping phases including: 1- hemostasis (not considered a phase by some authors), 2- inflammatory, 3- proliferative and 4- remodeling (8,9). Upon injury to the skin, a set of complex biochemical and cellular events take place in a closely orchestrated cascade to repair the damaged tissue (Figure 7). This sequence of events is fluid and overlapping, and in most circumstances spans the time from injury to resolution of acute wounds (10). In the inflammatory phase, bacteria and debris are phagocytosed and removed, and factors are released that cause the migration and division of cells involved in the proliferative phase.

The proliferative phase is characterized by angiogenesis, collagen deposition, granulation tissue formation, epithelialization, and wound contraction (11). In angiogenesis, new blood vessels are formed by vascular endothelial cells (12). In fibroplasia and granulation tissue formation, fibroblasts grow and form a new, provisional extracellular matrix by excreting collagen and fibronectin (13). Concurrently, re-epithelialization of the epidermis occurs, in which epithelial cells proliferate and 'crawl' on the wound bed, providing cover for the new tissue (14). In contraction, the wound is made smaller by the action of myofibroblasts, which establish a grip on the wound edges and contract themselves using a mechanism similar to that in smooth muscle cells. When the cells' roles are close to complete, unneeded cells undergo apoptosis (11). However, this process is not only complex but also fragile, and susceptible to interruption or failure leading to the formation of non-healing chronic wounds. Contributing factors to this failure include diabetes, venous or arterial diseases, aging, and infection (14).

Opium dependency is a commonly encountered problem in surgical patients in Iran. During many years of clinical surgery, we noticed that patients having morphine dependency may experience a better tolerance of major surgeries and their postoperative course. Moreover, previous studies have shown variable results in this regard. Therefore, the study carried out in this manuscript focused on the several factors contributing to the first phase and early second phase of secondary intention wound healing in morphine dependent rats in comparison to a control group.

In vitro studies have shown that morphine enhances proliferation and matrix accumulation in cultured fibroblasts and interstitial cells (15-17). Moreover, some recent *in vivo* studies have demonstrated enhancement of tissue collagen deposition in the cutaneous tissue and increasing the tensile strength of the incisional wound following systemic administration of morphine (18). Pro-inflammatory cells and fibroblasts are mainstays for early phase of wound healing and later phase of maturation and remodeling, as well as re-epithelization and re-vascularization are crucial in secondary intention wound healing (10). This study investigated the impact of morphine addiction on pro-inflammatory and fibroblast cell recruitment; as well as the re-epithelization and revascularization of the secondary intention wound healing in the rat. Fibroblast recruitment and re-epithelization are increased significantly in morphine dependent rats in comparison to non-dependent rats.

Creation of a full-thickness wound in the rat represents a clinically compatible experimental model in the study of secondary intention wound healing. All wounds need to progress through a series of cellular events that lead to a successfully re-established tissue integrity (10). Therefore we selected pro-inflammatory cell types and fibroblasts, as well as re-epithlization and re-vascularization as criteria, to study the healing process during the first 2 weeks of post wounding (Figure 7) in a morphine addicted state.

Our study shows that morphine dependency enhances re-epithelization and recruitment of fibroblasts during the first 14 days after creation of a tissue loss wound; but it does not enhance macrophage accumulation and also leads to a decreased presentation of neutrophils. These findings are in accordance with a study by Pei-Jung Chang and co workers (18).

Although pro-inflammatory cells are essential in the early stage of wound healing, lack of their over expression, as the effect of morphine dependency in this study, may provide an appropriate environment for the physiological effect of other cellular and/or biochemical factors in the process of wound healing. Moreover, re-epithelialization is an essential requirement for proper wound healing. This study shows that morphine, in low but an increasing dose may provide an appropriate condition to enhance re-epithelialization. This study has focused on cellular and morphologic events as factors involved in the mechanism of wound healing, but the impact of morphine on biochemical and molecular basis of the mechanics of wound healing needs to be further investigated. In conclusion, the present study demonstrates that morphine dependency enhances reepithelialization as well as tissue recruitment of fibroblasts; thereby probably enhancing secondary intention wound healing by means of re-epithelization and fibroplasia and granulation tissue formation.

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