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ORIGINAL ARTICLE

Morphine enhances tissue content of collagen and increases wound tensile strength

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Abstract
Purpose Morphine is a commonly prescribed analgesic for wound pain. Previous studies have shown that morphine enhances accumulation of collagen in cultured fibroblasts. Because fibroblasts are important for the remodeling of connective tissue in incisional wound, this study investigates the biological effects of morphine on cutaneous collagen content and wound tensile strength.
Methods A full-thickness incisional wound (2 cm in length) was created on the dorsum of mice followed by treatment with placebo or morphine (5 and 20 mg/kg/day, i.p.). Fourteen days later, tensile strength of the healed incisional wound was measured using a tensiometer. Protein expression of transforming growth factor (TGF)-β1 and matrix metalloproteinases (MMP)-2 in the incisional wound tissue was analyzed. Degree of tissue remodeling and levels of collagen were determined by histological examination and a dye-binding collagen assay, respectively.
Results Morphine enhanced the breaking strength of incisional wound 14 days after treatment (92 ± 10, 102 ± 10 and 134 ± 12 mg for control, morphine 5 mg/kg/day and morphine 20 mg/kg/day, respectively; $P = 0.03$, $n = 6$–7). Protein expression of TGF-β1 and MMP-2 was significantly enhanced in mice treated with morphine. Histological examination of the wound tissue showed evidence of increased thickness of the cutaneous fibrous layer and deposition of collagen in the high-dose morphine treatment group. Collagen assays also demonstrated that tissue concentrations of collagen were significantly increased in the wound tissue of morphine-treated animals on day 2 of drug treatment.
Conclusion The present study demonstrates that systemic administration of morphine enhances tissue collagen deposition in the cutaneous tissue, thereby increasing the tensile strength of the incisional wound.

Keywords Morphine · Fibroblast · Collagen · MMP-2 · TGF-β1

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Introduction

Surgical intervention usually creates excisional or incisional wounds, and both types of skin defects require medication for postoperative pain control. Among the commonly available analgesics, morphine has been one of the most effective drugs known for the relief of severe postoperative pain. The healing process of excisional and incisional wounds commonly involves three classic phases, namely, inflammation, proliferation, and remodeling. However, these two distinct types of wound defects are physiologically different in the process of healing. The closure of an excisional wound requires more complex biological responses involving formation of granulation tissue mediated through both epithelialization and angiogenesis [1]. On the other hand, the healing of a surgical incisional wound depends more heavily on the qualitative and quantitative formation of the extracellular matrix, especially tissue collagen content [2, 3], rather than

angiogenesis. Our recent data showed that high-dose morphine delays excisional wound healing by generating excessive superoxide anions and impaired angiogenesis [4]. Because of differences in the tissue regenerative process, the biological effect of morphine on an incisional wound may differ from its effects on an excisional wound. It has been previously reported that morphine or, more frequently, heroin addicts were associated with certain fibrotic pulmonary and renal complications [5, 6]. In vitro studies also showed that morphine enhanced proliferation and matrix accumulation in cultured renal fibroblasts and medullary interstitial cells [7–9]. Morphine, at concentrations of 10^{-8} to 10^{-4} M, promotes apoptosis of kidney fibroblasts and enhances accumulation of collagen type I in a dose-dependent manner. Although accumulated evidence has converged toward the essential role of morphine on modulation of tissue fibroblasts, there is a lack of integrated research investigating into the effects of morphine on tissue fibroblasts. Using a mouse model of incisional wound injury, we determined the molecular mechanisms and biological response of morphine on the repair of an incisional wound.

Materials and methods

Mouse model of incisional wound injury

Mice (C57BL/6J, 8–10 weeks old) were obtained from the Animal Center of the National Cheng Kung University (Tainan, Taiwan). The animals were housed at a controlled temperature of 21 ± 0.5°C in wire-mesh cages, with free access to food and water. Animals were anesthetized by intramuscular injection of ketamine (30 mg/kg). After being prepped with iodine, a full-thickness incisional wound (approximately 2 cm in length) was created by surgical scissors on the dorsum, and the wound was closed by interrupted suture using a 4°nylon thread (Fig. 1). Mice were allowed to recover freely after the procedure. Following the surgical procedure, animals were randomly assigned to the control or morphine-treated group and received intraperitoneal injection of normal saline or morphine (5 and 20 mg/kg/day; National Bureau of Controlled Drugs, Department of Health, Taipei, Taiwan), respectively, for 14 days, as described in our previous study [4, 10, 11]. Parenteral administration of morphine up to 20 mg/kg in mice has been demonstrated to produce a significant analgesic effect with clinically compatible serum concentrations of morphine and its metabolites (morphine-3-gluronide) [12–14]. All procedures were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee (The National Cheng Kung University, Tainan, Taiwan).

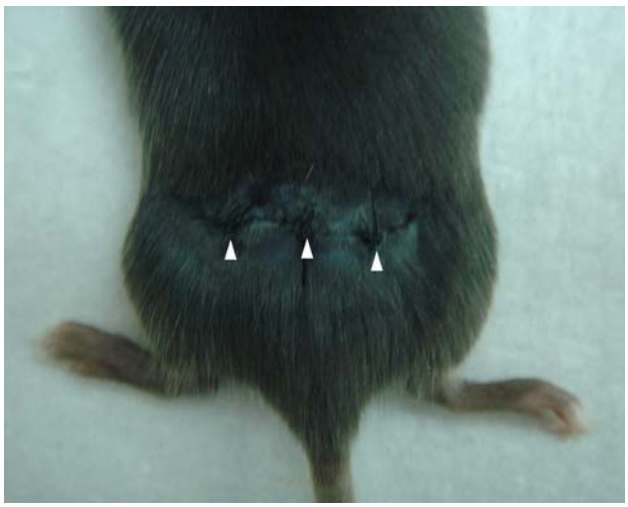

Fig. 1 Creation of an incisional wound on the dorsum of an experimental mouse. The length of the wound was approximately 2 cm, and the wound was closed by interrupted suture using a 4°-nylon thread (*arrowheads*)

Measurement of tensile breaking strength in incisional wounds

At the end of the treatment, mice were killed by injection of pentobarbital (250 mg/kg, i.p.) An incisional wound (0.5 cm in width) was excised from the dorsum of the mice and mounted longitudinally to a handheld tensiometer (Omega D670-44; Stanford, CA, USA). The tensile breaking strength of each isolated incisional wound strip was measured after consistent tension (displacement rate of 1 mm/min generated by an infusion pump; Perfusor Compact S, B. Braun, Germany) was applied to the wound tissues. Tension and time required for tissues to break apart were both recorded.

Western blot analysis

Soluble protein extracted from wound tissue was analyzed for expression of transforming growth factor (TGF)-β1 and matrix metalloproteinase (MMP)-2 by Western blotting [15]. Mouse monoclonal anti-TGF-β1 (BD Transduction Labs, San Jose, CA, USA) or anti-MMP-2 (BD Transduction Labs) antibodies were used. Protein levels were quantified by scanning densitometry (Scion Image, Frederick, Maryland).

Collagen assay

Concentrations of acid-soluble collagens (type I–IV) in the incisional wound tissue were quantified by a dye-binding method (Sircol Assay; Biocolor, UK), as previously described [16].

Histology examinations

Biopsies of formalin-fixed wound tissues were embedded in paraffin wax and sectioned at 5 μm thickness. Sectioned tissues were stained with hematoxylin and eosin and Masson's trichrome stains [17, 18].

Statistical analysis

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

Results

Morphine enhanced the breaking strength of the incisional wound in a dose-dependent manner following 14-day systemic administration (92 ± 10, 102 ± 10, and 134 ± 12 mg for control, morphine 5 mg/kg/day, and morphine 20 mg/kg/day, respectively; $P = 0.03$, $n =$ 6–7; Fig. 2a). The duration of breaking of the isolated wound tissue was similar among the three treatment groups (Fig. 2b). Protein expression of TGF-β1 and MMP-2 was significantly enhanced in mice treated with high-dose morphine (Fig. 3). Histological examination of the wound tissue showed evidence of increased thickness of the cutaneous fibrous layer with deposition of collagen in the high-dose morphine-treated group (Fig. 4). Collagen assays demonstrated that tissue levels of soluble collagen were significantly increased in the healing wound tissue of morphine-treated animals on day 2 after drug treatment, but the levels were similar on days 7 and 14 (Fig. 5).

Discussion

This study provides supporting evidence that high-dose morphine enhances tissue concentration of collagen during the regenerative process of incisional wounds with upregulated expression of TGF-β1 and MMP-2, the crucial proteins involved in tissue remodeling, thereby strengthening tensile force of the remodeled incisional wound.

Morphine is one of the most effective agents for the relief of severe wound pain, and it has also become a common drug of abuse for more than a century. Morphine—or more frequently heroin (diacetylmorphine)—addicts are associated with certain pulmonary and renal complications. Although it is an uncommon complication, pulmonary fibrosis has been reported in patients with morphine or heroin drug abuse [6]. Heroin-associated nephropathy, first described in the 1970s, presents as nephrotic syndrome and progresses rapidly to end-stage renal failure. Biopsy examination of the kidney in these patients usually shows focal segmental glomerulosclerosis [5]. After examination of 851 consecutive judicial autopsies of heroin addicts, interstitial nephritis and glomerular sclerosis were found in about 20% of these patients [5]. Singhal et al. [7] demonstrated that morphine, at a concentration of 10^{-12} M, enhanced proliferation of cultured kidney fibroblasts and might therefore be responsible for the development of renal interstitial scarring in patients with heroin addiction. Based on these previous clinical and laboratory findings, the present study further investigated the effects of morphine on the modulation of cutaneous fibroblasts during wound injury.

The proliferative phase of wound healing initiates on day 4 after tissue injury [19]. Fibroblasts, the most important cellular components during this phase, reach their peak biological activity approximately 3–21 days

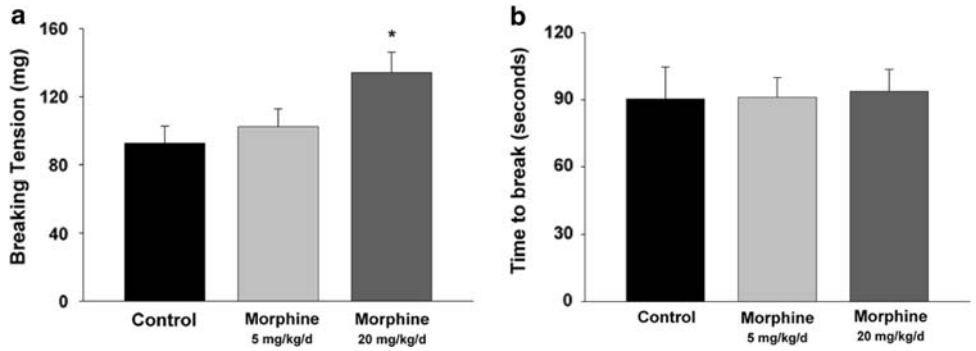

Fig. 2 **a** Wound breaking tension (in mg) following treatment with placebo (control) or morphine (5 or 20 mg/kg/day) for 14 consecutive days. Breaking tension was significantly enhanced in animals treated with high-dose morphine (20 mg/kg/day), but there were no differences in the time interval required to reach maximal breaking tension (**b**). Data were analyzed by one-way analysis of variance (ANOVA) and are presented as mean ± SEM. *$P < 0.05$ compared with control. $n = 6$–7 different animals in each group

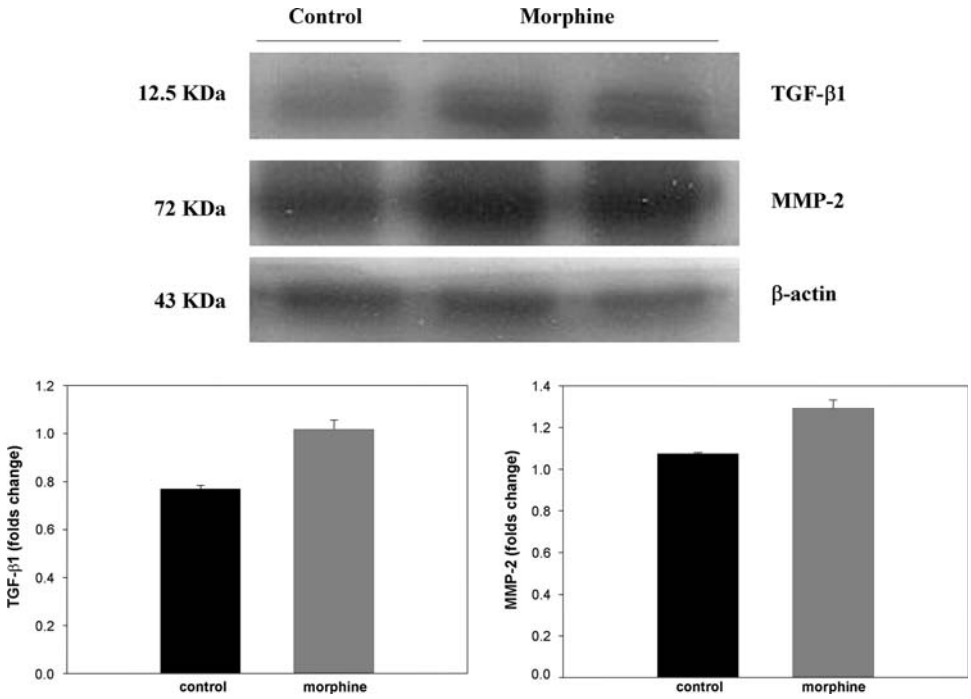

Fig. 3 Western blot analysis for protein expression of transforming growth factor $\beta 1$ (*TGF-$\beta 1$*) and matrix metalloproteinases (*MMP-2*) obtained from the wound homogenates isolated from control and morphine-treated (20 mg/kg/day) mice. Wound tissues were obtained at day 14 after creation of incisional wound. $P < 0.05$ control versus morphine in both analyses. Statistical analysis (unpaired *t* test) was performed by comparing the relative density of bands quantified by scanning densitometry (Scion Image) in the two groups. Data are shown as mean ± SEM. $n = 4$ different animals in each group

after injury; however, wound strength returns to 80–90% of the baseline level only after 6 weeks [19]. Creation of a full-thickness incisional wound in mice or rats represents a clinically compatible experimental model in studying wound tensile strength [20]. Three days after injury, breaking strength and tissue levels of collagen in the healing incisional wound were significantly increased in comparison to an open wound [21]. Incisional wound breaking strength has been recognized as the most clinically relevant outcome measure for acute surgical wounds [22]. In the present study, an incisional wound was created on the dorsum of mice and closed by suture material. Wound tensile strength was assessed 14 days after treatment with different doses of morphine by intraperitoneal injection. High daily dose of morphine injection (20 mg/kg/day) was defined in accordance with the previously reported model of morphine dependence [23, 24] and in our previous study [10, 11]. Breaking strength was significantly increased in the wounds of mice treated with high-dose morphine (20 mg/kg/day), but the time intervals of the breaking apart of tissue were similar among the three treatment groups. Accordingly, we measured the tissue content of collagen in the incisional wound strips. Serial analysis of soluble collagen at the tissue level using the dye-binding method showed that the level of collagen was significantly enhanced 2 days after injury and was significantly higher in the tissue of morphine-treated animals. Tissue collagen content declined to lower levels on days 7 and 14 after operation. There was no difference between the two groups at these time points. These findings suggest that high-dose morphine enhanced biosynthesis of collagen in wound tissue in the early phase of a full-thickness skin incisional injury and consequently resulted in increased tensile strength of the wound. Nevertheless, increased accumulation of dense connective tissue was still observed in the subcutaneous layer of the incisional wound of morphine-treated mice, suggesting the differences in collagen turnover and deposition of other extracellular matrix following treatment with high-dose morphine.

Connective tissue fibroblasts are the key players responsible for the tissue remodeling and healing process. Fibroblasts serve as potent antigen-presenting cells, release essential cytokines and growth factors, secrete extracellular matrix, increase tension in regenerating tissue, and promote angiogenesis [25]. The composition of the extracellular matrix, such as vimentin, fibronectin, collagen, and other proteoglycans determines the physical properties of connective tissues [26, 27]. Collagens, fibronectin, proteoglycans, and other extracellular matrix are synthesized by fibroblasts through the release of TGF-$\beta 1$ [28]. Therefore, TGF-$\beta 1$ has been implicated as one of the most important cytokines released by tissue fibroblasts that are involved in all stages of tissue repair and wound healing [29]. In addition, administration of TGF-$\beta 1$ enhances the repair of injured tissue, including incisional and excisional wounds, punch wounds, and ulcers [29]. On the other hand, MMPs regulate the three phases of wound healing, namely reepithelization, inflammation, and resolution [30]. During the process of wound healing, MMPs cleave cell–matrix contacts to promote reepithelization, activate inflammatory

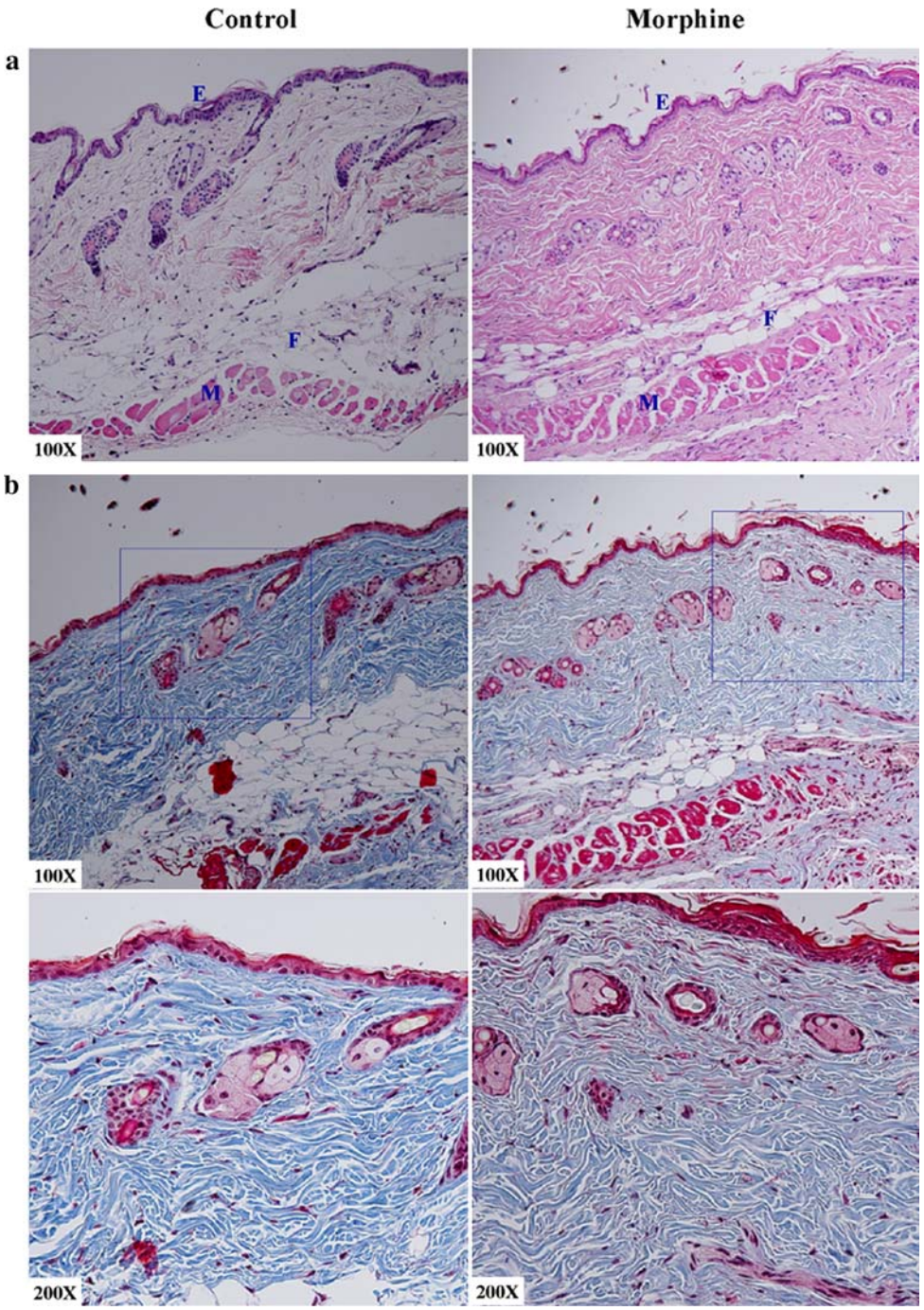

Fig. 4 Representative hematoxylin and eosin (H&E) (**a**) and Masson's trichrome (**b**) -stained histological sections of incisional wound tissue of control and morphine-treated animals under light microscopy. Significantly increased deposition of connective tissue was seen in between dermal epithelium (*E*) and muscular layer (*M*) in the morphine-treated animals. *F*, subcutaneous adipose tissue. The *bottom panels* represent the magnified histological sections in the windows of the *upper panels*. Experiments were performed on three different mice in each group

cytokines and proteolytically degrade remodeling proteins [30]. Among these metalloproteinases, expression of MMP-2 has been found in the tissue fibroblasts and endothelial cells during all phases of wound healing [31], and expression of MMP-2 is a clinically reliable indicator in the progress of wound healing [32]. We therefore examined the expression of the two major regulatory molecules, TGF-β1 and MMP-2, in the healing incisional wound. Consistent with the functional analysis of wound tension and tissue levels of collagen, expression of both TGF-β1 and MMP-2 was upregulated in the wound tissue of mice treated with morphine, suggesting that the process of tissue remodeling in the incisional wound was enhanced after administration of high-dose morphine. Nevertheless, in the present in vivo experimental study design we were not able to establish a direct causal relationship between wound healing and regulation of TGF-β1 and MMP-2 following morphine treatment.

We have recently demonstrated that high-dose morphine (20 mg/kg) delayed closure of excisional wounds in mice and that this detrimental effect on repairing skin-denuded

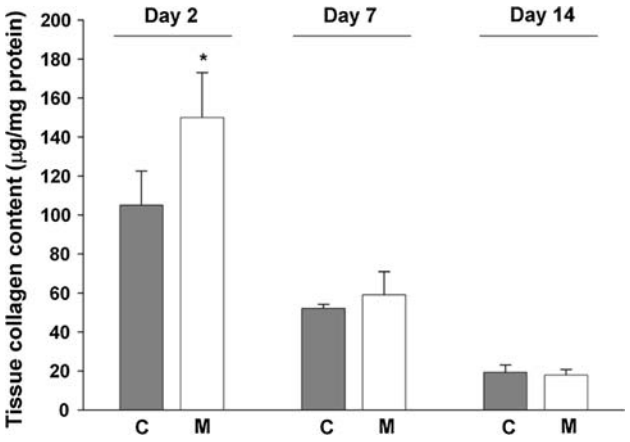

Fig. 5 Analysis of collagen levels in the incisional wound isolated from control (*C*) and morphine-treated (*M*) mice using the standard dye-binding method (Sircol assay). Higher tissue content of collagen in the wounds was found at day 2 after skin injury. There was a statistically significant increase in collagen deposition in animals treated with high-dose morphine (20 mg/kg/day). Data were analyzed by one-way ANOVA and are presented as mean ± SEM. $*P < 0.05$ control versus morphine. $n = 3$–5 different animals in each group

wound was associated with increased superoxide anion production and impaired mobilization of endothelial progenitor cells into the systemic circulation [4]. Delayed excisional wound closure following treatment with high-dose morphine has therefore been suggested to be the result of impaired angiogenesis [4, 33]. However, in the present incisional wound injury model we found that high-dose morphine actually enhanced tissue synthesis of collagen and wound tensile strength. These results demonstrate the diverse biological effects of morphine on tissue remodeling, and these effects can be tissue specific.

Adequate tissue tensile strength is essential for prevention of fascial dehiscence and wound herniation after surgical incisional injury. However, excessive collagen deposition in the incisional wound results in hypertrophic scars and keloids [34]. Furthermore, increased expression of opioid receptors was also detected in the human hypertrophic scar [35]. Therefore, modulation of the wound healing process by exogenous or endogenous opioids involves multifactorial physiological responses, and the biological effects of morphine on the healing of incisional wound require further clinical investigation.

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