Milk-Based Ointment Accelerates Wound Healing: A Comparative Study in Rabbits

Chiranjeev Singh [1*], Kunal Chandrakar [1]

Abstract

Background: Wound healing is a multi-stage process that restores tissue integrity following injury. While chemical treatments like phenytoin aid in healing, traditional remedies, such as milk-based applications, remain underexplored. Milk, particularly its proteins, is known for promoting tissue repair and collagen synthesis. This study evaluated the wound-healing efficacy of a milk-based ointment (MO) on open skin wounds in rabbits. Methods: Male New Zealand white rabbits were randomly assigned to six groups, including negative controls, placebo (eucerin), and 1% phenytoin, alongside three MO groups (2%, 5%, 10% concentrations). The treatments were applied twice daily for 14 days. Wound healing was assessed through macroscopic analysis, tensile strength measurement, hydroxyproline levels, and histological examination. Results: The 5% MO-treated group exhibited the fastest wound healing, achieving full closure in 15 days, outperforming both controls and the phenytoin-treated group (17 days). Hydroxyproline levels and tensile strength were significantly higher in the 5% MO group, indicating enhanced collagen production and stronger tissue regeneration. Histological analysis further confirmed accelerated epithelialization and granulation in MO-treated groups. Conclusion: The study demonstrated that milk-based ointments, particularly at a 5% concentration, significantly enhance wound healing, suggesting their potential as cost-effective, biocompatible alternatives to conventional chemical treatments like phenytoin. Further research is needed to explore clinical applications in humans.

Keywords: Milk-based ointment, Wound healing, Tissue regeneration, Collagen production, Hydroxyproline

Significance | This study demonstrated that a 5% milk-based ointment significantly accelerated wound healing and improved tissue strength compared to conventional treatments in rabbits.

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Introduction

Wound healing is a complex process that involves the restoration of tissue integrity following physical, chemical, or biological damage (Dong & Guo, 2021). It typically occurs in four overlapping stages: hemostasis, inflammation, proliferation, and remodeling (Peña & Martin, 2024). The first stage, hemostasis, is initiated almost immediately after injury, with platelets aggregating at the site to form a clot that stops bleeding. This clot is made up of fibrin and platelet clusters, providing a temporary barrier to protect the wound (Nabeesab Mamdapur et al., 2019).

The next phase, inflammation, is the body's innate immune response to injury. Once hemostasis is achieved, the blood vessels in the wound area dilate, allowing for an influx of immune cells, growth factors, and enzymes (Mansouri, 2023). These components are crucial for clearing debris and preventing infection. During the proliferative phase, fibroblasts produce collagen and other extracellular matrix components, forming granulation tissue that supports new blood vessel growth, a process known as angiogenesis (Huang et al., 2022). This newly formed tissue is essential for wound closure and the restoration of skin integrity. The final stage, remodeling, involves the reorganization of collagen fibers and the

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maturation of the tissue, resulting in a stronger, more resilient scar (Forcina et al., 2020).

Wound healing has long been a subject of medical research, as delayed healing can lead to complications such as infection and chronic wounds. Although many synthetic and chemical treatments have been developed to accelerate wound healing, their effectiveness is often limited by side effects (Wang et al., 2024). In contrast, traditional remedies, such as the use of milk in wound care, have been employed for centuries. Breast milk, for example, is commonly applied to treat nipple soreness in lactating women, due to its nutrient-rich and bioactive properties (Kazimierska & Kalinowska-Lis, 2021).

Milk, particularly its proteins, has been recognized for its potential benefits in tissue regeneration (Figure 1). Whey protein, a major component of milk, contains essential amino acids that promote collagen synthesis and tissue repair (Minj & Anand, 2020). The dual hydrophilic and hydrophobic nature of milk proteins allows them to be absorbed into the skin, making them an attractive candidate for topical treatments (Kazimierska & Kalinowska-Lis, 2021). Despite these known benefits, little research has been conducted on the effects of cow's milk on skin wounds. To address this gap, a recent study explored the impact of 1.5% fat cow's milk on the healing of open skin wounds in rabbits (Ceniti et al., 2024).

The milk-based ointment (MO) containing casein, particularly at a 5% concentration, might significantly enhance wound healing compared to untreated controls and conventional treatments like phenytoin. The study aims to determine the wound healing treatment using MO to find faster healing, higher tensile strength, and increased tissue hydroxyproline levels. MO has the potential to serve as an effective, biocompatible, and cost-efficient alternative for wound management.

2.Materials and Methods

2.1. Animals

The study was conducted on male New Zealand white rabbits weighing between 1.8–2.8 kg. The rabbits were housed individually in designated enclosures and provided with a standardized diet, including fresh vegetables and unrestricted access to water (Surendar et al., 2024). They were kept under a 12-hour light/dark cycle with a controlled temperature of 23 ± 3 °C and a humidity level of 45%–55%. The research was blinded, with those administering medications and assessing wound areas unaware of the lotion ingredients. Additionally, the histologist examining the tissue samples was unaware of the specific treatments. This study prioritized animal welfare, providing appropriate housing, diet, and care, while minimizing discomfort and ensuring ethical treatment throughout. This study received ethical approval, ensuring proper care, housing, and welfare for New Zealand white rabbits throughout the research process.

2.2. Preparation of 1.5% Fat Milk

The milk used in this study was low-fat cow's milk (1-liter container) from the Iranian Dairy Industry Co., which had been pasteurized and homogenized for long-term preservation.

2.3. Preparation of Milk Proteins

Milk lyophilized flour was prepared using a freeze-drying method, which removes water from delicate biological materials to preserve their integrity. One liter of 1.5% fat milk was divided into four 250 mL bottles and freeze-dried. The bottles were wrapped in aluminum foil to protect the milk from light. Once fully dehydrated, the milk protein was extracted, ground into fine flour using a mortar, and stored for later use (Kazimierska & Kalinowska-Lis, 2021).

2.4. Preparation of Milk Ointment (MO)

The dehydrated milk protein was mixed with eucerin to create low-fat milk ointments (MO) at concentrations of 2%, 5%, and 10% (w/w). The milk protein was thoroughly blended with the eucerin base to ensure homogeneity, forming ointments for wound-healing evaluation (Minj & Anand, 2020). The dose-response relationship between milk protein concentration and wound healing was assessed.

2.5. Preparation of 1% Phenytoin Ointment

Phenytoin was used as the active control treatment. Phenytoin granules were obtained from DarouPakhsh Pharmaceuticals, and a 1% (w/w) ointment was formulated with eucerin following the same process as MO preparation.

2.6. Experimental Design

The animals were randomly assigned to six groups (n = 6 per group). Group 1 received no treatment (negative control), Group 2 received eucerin (placebo), and Group 3 received 1% phenytoin ointment (positive control). Groups 4, 5, and 6 were treated with 2%, 5%, and 10% MO, respectively. Treatments were applied twice daily. To prevent wound infection, the animals' bedding was changed regularly (Peña & Martin, 2024).

2.7. Histological Studies

Fourteen days post-injury, tissue samples were collected for microscopic analysis. The samples were preserved in buffered formalin, sectioned at 5 µm, and stained with Hematoxylin and Eosin. Epithelialization, granulation tissue formation, vascularization, and inflammatory cell presence were assessed to evaluate the wound healing process (Dong & Guo, 2021).

2.8. Tensile Strength of Tissue

At the end of the treatment period, 5 × 20 mm skin sections were excised from all animals. A tensiometer was used to measure tissue tensile strength at the point of rupture. The tensile strength was recorded in milligrams (Huang et al., 2022).

2.9. Measurement of Tissue Hydroxyproline

After treatment, tissue samples were taken from the wound sites for hydroxyproline analysis. The hydroxyproline concentration was measured using a kit from Chondrex, following the supplier's

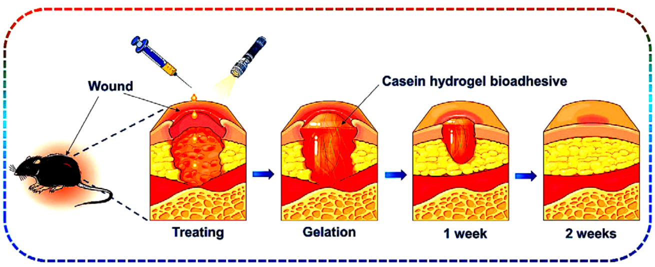

Figure 1. Biocompatibility, biodegradability, and injury repair properties of casein-based Milk Ointment (MO) evaluated using in vitro and in vivo methods, demonstrating its potential as a first-aid resource for wound management.

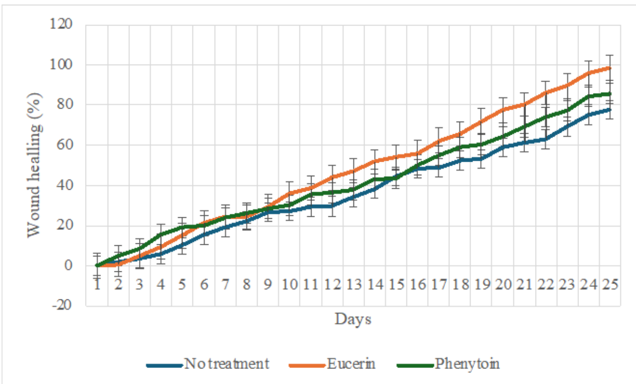

Figure 2. Macroscopic analysis of wound healing progression in various treatment groups, including adverse control, mock, and beneficial control categories, showing differential healing rates across the groups.

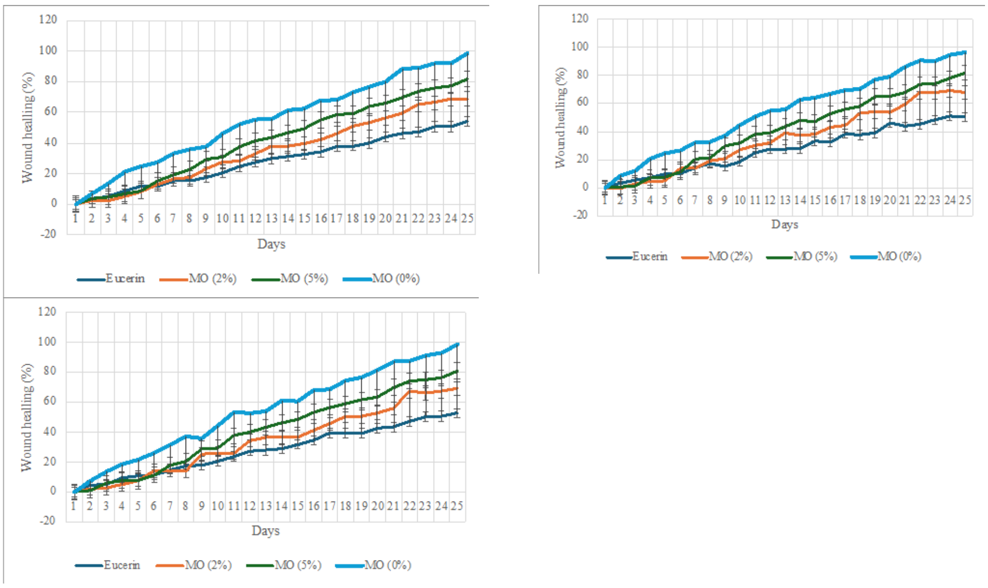

Figure 3. Comparative wound healing analysis between (a) normal untreated group, (b) adverse control group, and (c) mock group treated with eucerin, showing variations in healing times and outcomes.

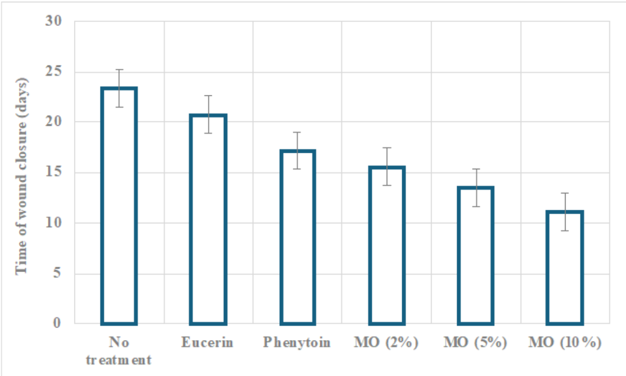

Figure 4. Statistical analysis of wound healing time among different treatment groups, illustrating the enhanced healing efficacy of the 5% MO compared to other concentrations and the phenytoin group.

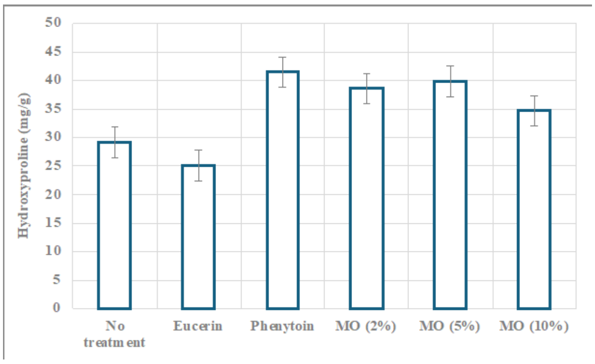

Figure 5. Hydroxyproline concentration analysis across treatment groups, revealing significant increases in collagen production in tissues treated with MO and phenytoin compared to controls.

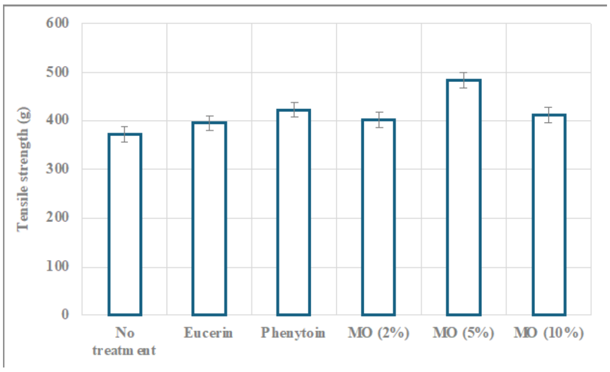

Figure 6. Tensile strength analysis of wound tissues, demonstrating the superior mechanical strength in wounds treated with 5% MO compared to other groups.

protocol. Specimens were dehydrated at 60-70 °C, hydrolyzed in 6N HCl at 130 °C for 4 hours, neutralized, oxidized with chloramine-T, and reacted with Erlich's reagent at 60 °C. The color intensity was measured at 557 nm using a spectrophotometer. The hydroxyproline concentration was determined by comparing the results with a standard curve (Forcina et al., 2020).

2.10. Statistical Analysis

The data were presented as means ± standard error of the mean (SEM). Statistical analysis was performed using one-way ANOVA followed by Tukey's post-hoc test. A p-value of less than 0.05 was considered statistically significant. Analyses were performed using SPSS 19.0 (Wang et al., 2024).

3. Results

3.1. Macroscopic Analysis of the Wound

The macroscopic analysis revealed significant differences in wound healing times between the various treatment groups. Wounds in the adverse control and mock categories fully healed within 21 days. In contrast, the favorable control group, treated with phenytoin, achieved complete wound closure after 17 days (Figure 2). The groups treated with milk-based ointment (MO) exhibited notable variation in healing times depending on the concentration used. Specifically, the 2% MO group healed after 17 days, the 5% MO group after 15 days, and the 10% MO group after 16 days. Statistical analysis confirmed that the differences in healing times between the MO-treated groups and the controls were significant ($p < 0.05$) (Figs. 3(a), 3(b), 3(c)).

Among the experimental groups, the 5% MO-treated wounds showed the most significant improvements. This group not only demonstrated faster healing compared to controls but also outperformed the phenytoin-treated group in terms of the percentage of healed wounds ($p < 0.01$). The difference became apparent from the fourth day of treatment and persisted throughout the study (Figure 4). Interestingly, although the 5% MO group healed slightly faster than the 10% MO group, the difference in healing time was not statistically significant.

3.2. Tissue Hydroxyproline Data

Tissue hydroxyproline levels, an indicator of collagen production, were measured to assess tissue regeneration. The MO-treated groups, particularly the 5% and 10% concentrations, demonstrated significantly higher hydroxyproline levels compared to the adverse control and mock categories ($p < 0.05$). The phenytoin-treated group and the 5% MO group exhibited the highest hydroxyproline levels, with values of 41.7 ± 2.04 and 42 ± 3.6, respectively (Figure 5). However, there was no significant difference between the phenytoin and 5% MO groups in terms of hydroxyproline levels.

3.3. Tensile Strength of Cells

The tensile strength of the healing tissues was evaluated to measure the durability and quality of wound repair. The adverse control and mock categories showed the lowest tensile strength, indicating weak tissue regeneration. In contrast, tensile strength improved significantly in the groups treated with phenytoin and various concentrations of MO. Notably, the 5% MO group exhibited the highest tensile strength at 481 ± 29 g, which was statistically superior to all other groups, including the phenytoin-treated group ($p < 0.01$) (Figure 6). This suggests that the 5% MO formulation not only accelerates healing but also promotes the development of stronger, more resilient tissue.

3.4. Histological Research

Histological analysis further supported the superior wound healing effects of MO (data not shown). In the MO-treated groups, epithelialization, the process of skin layer regeneration, was observed as early as 14 days after wounding. Granulation tissue formation and neovascularization, essential steps in wound healing, were more pronounced in the MO-treated groups compared to the controls. By the 14th day, wounds in the 5% and 10% MO groups had fully re-epithelialized, with mature granulation tissue and minimal inflammation. In contrast, the control groups showed delayed fibroblast proliferation and less collagen deposition.

Overall, the results suggest that MO, especially at a 5% concentration, significantly enhances wound healing through faster tissue regeneration, increased collagen production, and stronger tissue formation compared to both untreated controls and conventional treatments like phenytoin.

Discussion

The results of this study highlight the potential of milk-based ointment (MO) as an effective and natural wound-healing agent. The macroscopic analysis clearly demonstrated that wounds treated with MO, particularly at a 5% concentration, healed faster than those treated with either eucerin or phenytoin. The 5% MO-treated wounds healed in just 15 days, outperforming both the 2% and 10% MO groups, as well as the phenytoin group, which required 17 days for complete healing. This is consistent with previous research indicating that casein, a key protein in milk, promotes cell proliferation and tissue repair (Kazimierska & Kalinowska-Lis, 2021).

The enhanced wound healing observed in the 5% MO group can be attributed to several factors. First, the significant increase in hydroxyproline levels in the MO-treated groups suggests improved collagen production, a critical component of tissue repair. Collagen provides structural integrity to the skin, and its increased production correlates with faster and stronger wound healing (Peña & Martin, 2024). The hydroxyproline levels in the 5% MO and phenytoin-treated groups were nearly identical, further supporting the efficacy of milk proteins in promoting collagen synthesis (Figure 5). Similar findings have been reported in other studies exploring the use of milk derivatives for skin regeneration (Ceniti et al., 2024).

Tensile strength data further corroborate these findings, as the 5% MO-treated wounds exhibited the highest tensile strength among all groups. This suggests that not only does the 5% MO accelerate wound closure, but it also enhances the quality of the regenerated tissue, making it more resilient to future damage. The significant difference in tensile strength between the 5% MO and phenytoin groups ($p < 0.01$) indicates that MO may be more effective in promoting robust tissue regeneration than conventional chemical treatments (Huang et al., 2022).

Histological analysis also provided important insights into the mechanisms underlying MO's wound-healing effects. The early epithelialization observed in the 5% and 10% MO-treated groups indicates that MO promotes faster skin regeneration compared to the controls. Additionally, the increased granulation tissue formation and neovascularization suggest that MO enhances the overall healing process by facilitating both structural and vascular components of tissue repair. This aligns with previous studies suggesting that bioactive components in milk can stimulate angiogenesis and fibroblast activity, both of which are essential for effective wound healing (Sierawska et al., 2022).

Conclusion

In conclusion, the findings from this study indicate that milk-based ointments, particularly at a 5% concentration, are highly effective in promoting wound healing. The significant improvements in healing time, collagen production, tensile strength, and tissue regeneration suggest that MO could serve as a promising alternative to conventional chemical-based therapies such as phenytoin. Given the biocompatibility, biodegradability, and cost-effectiveness of milk proteins, further research should explore their potential for broader clinical applications in human wound care (Wang et al., 2024). The promising results of this study pave the way for the development of new, natural wound treatments that could benefit both humans and animals alike.

Author contributions

CS and KC contributed to conceptualization, fieldwork, data analysis, drafting the original manuscript, editing, funding acquisition, and manuscript review. Both CS and KC were involved in research design, methodology validation, data analysis, visualization, and manuscript review and editing. Additionally, CS took the lead in methodology validation, investigation, funding acquisition, supervision, and final revisions. All authors have reviewed and approved the final version of the manuscript.

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Competing financial interests

The authors have no conflict of interest.

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