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To Investigate the Underlying Mechanism of MEBO in Facilitating the Healing of Chronic Refractory Wounds Via the Ferroptosis-related P53/SLC7A11/GPX4 Signaling Pathway

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Abstract: Objective: To investigate the effects and underlying mechanisms of moist exposed burn ointment (MEBO) on chronic refractory wounds. **Methods:** Eighty specific pathogen-free (SPF) healthy male Sprague-Dawley rats weighing 180–220 g were selected and randomly assigned to four groups—acute wound group, chronic wound control group, MEBO group, and positive drug control group—based on a random number table, with 20 rats in each group. All animals were housed and maintained under standardized SPF conditions at the Animal Experimental Center of Youjiang Medical University for Nationalities. An acute full-thickness skin defect wound model was established in the acute wound group, while chronic refractory wound models were induced in the other three groups. During dressing changes, normal saline was applied to the acute wound group and the chronic wound control group based on wound area. The MEBO group received treatment with MEBO at a dosage of 200 mg/cm², and the positive drug control group was administered recombinant bovine basic fibroblast growth factor gel at a dose of 60 IU/cm². Following dressing changes, sterile gauze was used to cover and bandage the wounds in all groups. The wound healing rates in each experimental group were compared on days 3, 5, 7, and 14 after dressing application. Histopathological changes were assessed using H&E staining on days 0, 3, 5, 7, and 14, and semi-quantitative analyses of inflammatory cell infiltration and collagen fiber deposition were conducted. In addition, Western blotting was employed to evaluate the protein expression levels of P53, SLC7A11, and GPX4 in wound tissues on days 3, 5, 7, and 14 post-treatment. **Results:** The wound healing rates in both the acute wound group and the MEBO group were significantly higher than those in the chronic wound control group ($P < 0.05$). Additionally, the semi-quantitative scores of inflammatory cells in the acute wound group and the MEBO group were significantly lower than those in the chronic wound control group ($P < 0.05$). Moreover, the semi-quantitative scores of collagen fibers in both the acute wound group and the MEBO group were markedly higher compared to the chronic wound control group ($P < 0.05$). There were statistically significant differences in the time, between-group comparisons, and the interaction of P53 protein expression levels among the acute wound group, the chronic wound control group, and the positive drug control group ($P < 0.05$). Additionally, statistically significant differences in time, between-group effects, and interactions of P53 protein expression levels were observed between the chronic wound control group and the MEBO group ($P < 0.05$). There were significant differences in the expression levels of the SLC7A11 protein among the acute wound group, the chronic wound control group, and the positive drug control group with respect to time, group, and their interaction ($P < 0.05$). Additionally, significant differences were observed in SLC7A11 protein expression between the chronic wound control group and the MEBO group in terms of time, group, and their interaction ($P < 0.05$). There were significant differences in the time course and interaction of GPX4 protein expression among the acute wound group, the chronic wound control group, and the positive drug control group ($P < 0.05$). However, no significant difference was observed between the two groups ($P > 0.05$). Additionally, significant differences in GPX4 protein expression were found between the chronic wound control group and the MEBO group in terms of time, group, and their interaction ($P < 0.05$). **Conclusion:** MEBO may promote the healing of chronic refractory wounds by regulating the P53/SLC7A11/GPX4 signaling pathway to inhibit ferroptosis in wound tissues during the early stage of wound healing.

Keywords: Chronic Refractory Wounds; Ferroptosis; MEBO; P53; SLC7A11; GPX4.

1. Introduction

Chronic Refractory Wound (CRW) poses numerous challenges in clinical treatment because of its delayed healing, including a long treatment cycle, high treatment difficulty, and substantial costs. As China's population ages and the disease spectrum changes, the incidence of related CRW is expected to rise further. Modern studies have demonstrated that Moist Exposed Burn Ointment (MEBO) is rich in pharmacological components like β-sitosterol, baicalin, and berberine. These components possess anti - inflammatory, antibacterial, and muscle - promoting effects, which can effectively facilitate wound healing [1, 2]. Although MEBO has been extensively utilized in the treatment of CRW, its mechanism of action remains incompletely understood. Recent research has demonstrated that the level of ferroptosis

in wound tissue is intricately associated with wound repair. As the pivotal regulatory pathway of ferroptosis, the tumor protein P53 (P53)/solute carrier family 7 member 11 (SLC7A11)/glutathione peroxidase 4 (GPX4) signaling pathway mediates the accumulation of reactive oxygen species (ROS), lipid peroxidation, and anti - oxidative stress, and modulates the level of ferroptosis[3, 4].Therefore, an animal model of CRW was constructed to further explore the mechanism of MEBT/MEBO in promoting the repair of chronic refractory wounds based on the ferroptosis P53/SLC7A11/GPX4 signaling pathway, aiming to provide important experimental data and theoretical basis for the treatment of CRW.

2. Materials and Methods

2.1. Laboratory Animals

Eighty specific-pathogen-free (SPF) healthy male Sprague - Dawley rats, weighing 180 - 215 g, were purchased from Changsha Tianqin Biotechnology Co., LTD. The production license number of the company is SCXK (Xiang) 2022 - 0011, and the qualification certificate number is No.43072624010 0304272. This study was reviewed and approved by the Animal Experiment Ethics Committee of Youjiang Medical University for Nationalities (No.2024061 301).

2.2. Main Instruments and Reagents

Moist burn ointment (batch number: 2101803A) was procured from Shantou Meibao Pharmaceutical Co., LTD. Recombinant bovine basic fibroblast growth factor gel (batch number: 092403A02) was obtained from Zhuhai Yisheng Bio - Pharmaceutical Co., LTD. Hydrocortisone injection (batch number: 20240302) was acquired from Jilin Huamu Animal Health Products Co., LTD. Western blot transfer solution powder, SDS - PAGE electrophoresis solution powder, and BCA protein concentration determination kit (product numbers: P0021B, P0014B, BL521A) were purchased from Shanghai Biyuntian Biotechnology Co., LTD. A 10% PAGE rapid preparation kit, 5x protein loading buffer, and protein pre - staining three - color marker (product numbers: PG112, LT101S, WJ101) were bought from Shanghai Yase Biomedical Technology Co., LTD. TBS powder (item number: AR0031) was sourced from Wuhan Doctor De Biological Engineering Co., LTD.; the β- actin primary antibody (product numbers: #4970) was obtained from Cell Signaling Technology; Primary antibodies against P53, SLC7A11, and GPX4 (product numbers: 10442 - 1 - AP, 68785 - 1 - PBS, 67763 - 1 - IG) were purchased from Wuhan Shawk Biotechnology Co., LTD. HRP - labeled goat anti - mouse/rabbit IgG and hematoxylin and eosin high - clear constant staining kits (item numbers: GB23301/GB23303, G1076) were procured from Wuhan Sevier Biotechnology Co., LTD.

The biomicroscope (model: BX53) was purchased from Olympus Corporation; the electrophoresis apparatus, vertical electrophoresis cell, and transfer electrophoresis cell (model: BV - 4) were purchased from Bio - Rad Laboratories. The chemiluminescence imager (model: Tanon - 4800) was purchased from Shanghai Tianneng Technology Co., LTD. The freezing microtome (model: NX50) was purchased from Thermo Fisher Scientific.

2.3. Experimental Grouping and Treatment

After a 1 - week adaptive feeding period, based on the effect size obtained from the preliminary experiment, 80 rats were randomly assigned to four groups using the random number table: the acute wound group, the chronic wound control group, the MEBO group, and the positive drug control group, with 20 rats in each group. These rats were housed in the SPF animal Experimental Center of Youjiang Medical University for Nationalities. The environmental conditions were maintained at a temperature of (22 ± 2) °C, a humidity of (60 ± 10) %, and a 12 - hour light - dark cycle.The acute wound group was not injected with hydrocortisone, while the remaining groups were injected with hydrocortisone injection (8mg/100g) to establish a chronic refractory wound model[5, 6].The dorsal skin on both sides of the spine was completely exposed, and circular marks with a diameter of 25mm were made on the skin on both sides of the spine using a circular stamp. Under sterile conditions, full - thickness skin defect wounds were created along the sides of the spine. In the acute wound group and the chronic wound control group, normal saline was used for wound dressing change. In the MEBO group, MEBO at a dosage of 200mg/cm² was applied for dressing change, and in the positive drug control group, Recombinant bovine basic fibroblast growth factor gel at a dosage of 60IU/cm² was used for dressing change. After dressing change, all the wounds were bandaged and fixed with sterile gauze twice a day [7].

2.4. Sample Handling

Five rats from each group were randomly selected on days 0, 3, 5, 7, and 14 after the establishment of the model. Under sterile conditions, the skin on the left and right sides of the wound was excised from the sub - deep fascia, and the complete wound and the surrounding tissue within 0.5 cm from the wound edge were collected. The tissue samples on the left side were frozen and embedded, while the tissue samples on the right side were placed into a 5 - ml centrifuge tube and rapidly frozen in a refrigerator at - 80 °C until subsequent unified detection.

2.5. Observation Indicators

2.5.1. Wound Healing Rate

The wound healing status was observed on days 3, 5, 7, and 14 post-modeling in each group. Five rats were randomly selected from each group, and the wounds were photographed using the same imaging device. The wound area was measured using ImageJ image analysis software, and the wound healing rate was calculated using the following formula: Wound Healing Rate = [(Wound area at initial modeling - Wound area at observation time point) / Wound area at initial modeling] × 100%.

2.5.2. Wound Histopathological Observation and Semi - Quantitative Analysis

The frozen embedded tissue blocks were removed and sectioned on a frozen microtome at a thickness of 6 μm. The sections were stained following the instructions of the hematoxylin and eosin staining kit. After dehydration, clearing, and sealing with neutral resin, the histopathological changes in the wound were observed under a microscope.

2.5.3. Inflammatory Cells and Collagen Fibers Were Semi-Quantified in the Wound

The sections stained with hematoxylin and eosin staining reagent were placed under a biomicroscope for image acquisition. Image J software was employed to conduct a semi - quantitative analysis of the inflammatory cells and collagen fibers in each field of view.

2.5.4. The Protein Expressions of P53, SLC7A11, and GPX4 in the Wound Tissue Were Detected by Western Blot

On days 0, 3, 5, 7, and 14 after modeling, 50 mg of wound tissue was collected from the rats in each group. A steel ball, high - efficiency RIPA lysate, and PMSF protease inhibitor were added to a 2 - ml grinding tube, which was then placed in a low - temperature grinder (12000 HZ, grinding for 30 s, pausing for 15 s, and repeating this cycle 3 times). Subsequently, the supernatant was obtained by centrifugation at 12000 r/min for 5 min at 4 °C. The protein concentration was determined using the BCA Protein Concentration Kit. Samples for 10% PAGE gel electrophoresis were prepared (stacking gel:80V, 30min; separating gel: 120V, 60min). After electrophoresis, the membranes were blocked with skim milk powder and incubated with primary antibodies (β - actin 1:3000, P53 1:1000, SLC7A11 1:1000, GPX4 1:1000) at 4 °C overnight. Then, the membranes were incubated with an HRP - conjugated secondary antibody (1:4000) at room temperature for 2 h. ECL developer solution was added to visualize the bands. The gray value of the bands was analyzed using ImageJ software.

2.6. Statistical Analysis

SPSS 25.0 and GraphPad Prism 10.0 software were employed for statistical analysis and graphing. Measurement data were presented as the mean ± standard deviation ($\bar{x}$±s). Repeated - measures analysis of variance was utilized for comparisons among multiple groups, and the Least Significant Difference (LSD) method was applied for pairwise comparisons between groups. A P - value less than 0.05 was regarded as statistically significant.

3. Results

3.1. Comparison of the Wound Healing Rates of Rats in Each Group

The wound healing rates in the acute wound group and the MEBO group were higher than those in the chronic wound control group at each time point ($P < 0.05$). Refer to Figure 1 and Tables 1 - 2.

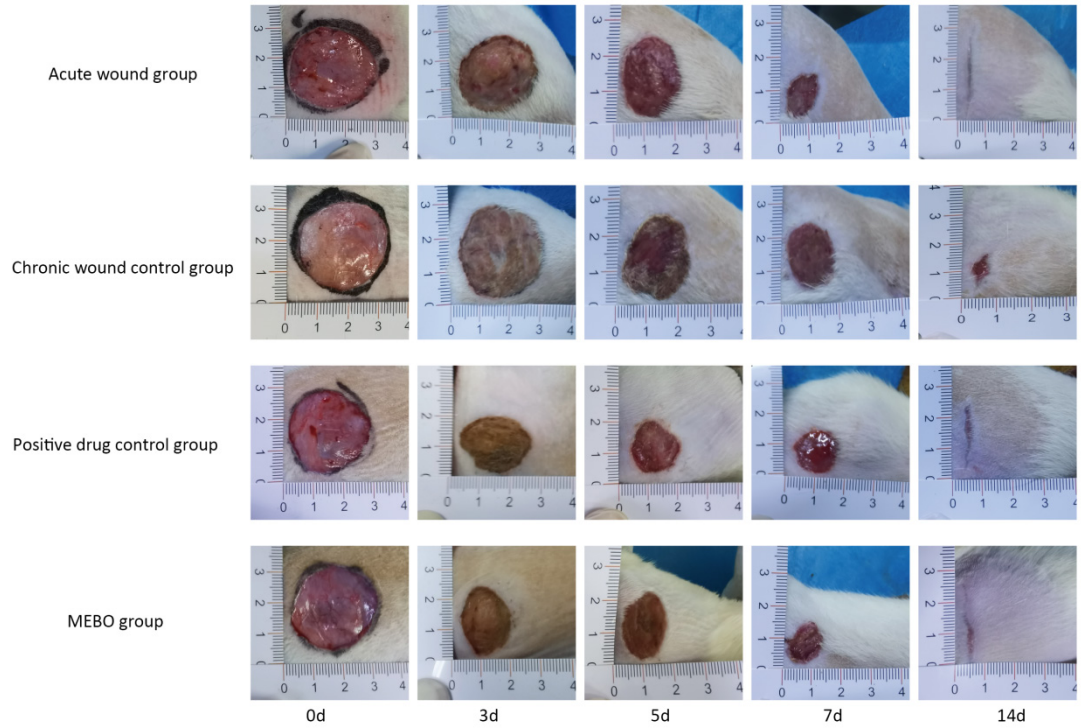


Fig 1. Wound healing

Table 1. Comparison of wound healing rates among the acute wound group, the chronic wound control group, and the positive drug control group

Groups	Cases	3d	5d	7d	14d
Acute wound group	5	$19.68±4.86^{bcde}$	$38.34±3.79^{acde}$	$67.69±4.27^{abde}$	$97.83±0.98^{abce}$
Chronic wound control group	5	$6.59±2.03^{bcd}$	$30.96±2.90^{acd}$	$56.39±1.30^{abd}$	$95.43±0.53^{abc}$
Positive drug control group	5	50.18±2.80	65.59±2.19	77.39±3.62	97.77±0.96
F_{time},P_{time}		$F_{time}=2980.95$,$P_{time}<0.01$			
F_{group},P_{group}		$F_{group}=180.15$,$P_{group}<0.01$			
$F_{time×group}$,$P_{time×group}$		$F_{time×group}=97.45$,$P_{time×group}<0.01$			

Compared with the 3d of this group, $^{a}P<0.05$; compared with the 5d of this group, $^{b}P<0.05$; compared with the 7d of

this group, $^{c}P<0.05$; compared with the 14d of this group, $^{d}P<0.05$; compared with the chronic wound control group,$^{e}P<0.05$

Table 2. Comparison of wound healing rates between the chronic wound control and MEBO groups.

Groups	Cases	3d	5d	7d	14d
Chronic wound control group	5	6.59 ± 2.03^{bcd}	30.96 ± 2.90^{acd}	56.39 ± 1.30^{abd}	95.43 ± 0.53^{abc}
MEBO group	5	50.74 ± 2.33^{bcde}	62.12 ± 1.34^{acde}	79.12 ± 0.94^{abde}	97.26 ± 0.33^{abce}
F_{time},P_{time}		$F_{time}=2842.23,P_{time}<0.01$			
F_{group},P_{group}		$F_{group}=2606.14,P_{group}<0.01$			
$F_{time\times group},P_{time\times group}$		$F_{time\times group}=264.34,P_{time\times group}<0.01$			

Compared with the 3d of this group, $^{a}P<0.05$; compared with the 5d of this group, $^{b}P<0.05$; compared with the 7d of this group, $^{c}P<0.05$; compared with the 14d of this group, $^{d}P<0.05$; compared with the chronic wound control group, $^{e}P<0.05$

3.2. The Wound was Examined Via Histopathology

On the third day after dressing change, a large number of inflammatory cell infiltrations were observed in the wound tissues of rats in the acute wound group, chronic wound control group, positive drug control group, and MEBO group. On the 5th day after dressing change, the inflammatory cells in the wound tissues of rats in the acute wound group, positive drug control group, and MEBO group began to decrease, while a large number of inflammatory cell infiltrations were still seen in the wound tissues of rats in the chronic wound control group. On the 7th day after dressing change, the inflammatory cells in the wounds of rats in the acute wound group, positive drug control group, and MEBO group were significantly reduced, and fibroblasts and capillary structures were observed. There were still inflammatory cell infiltrations and a small number of fibroblasts in the wound tissues of rats in the chronic wound control group. On the 14th day after dressing change, the number of fibroblasts in the wound tissues of rats in the acute wound group, positive drug control group, and MEBO group increased significantly, were arranged regularly, and skin appendages such as hair follicles and sebaceous glands could be seen. Although there were also structures such as hair follicles and sebaceous glands with increased fibroblasts, there was still a little inflammatory cell infiltration in the chronic wound control group. See Figure 2.

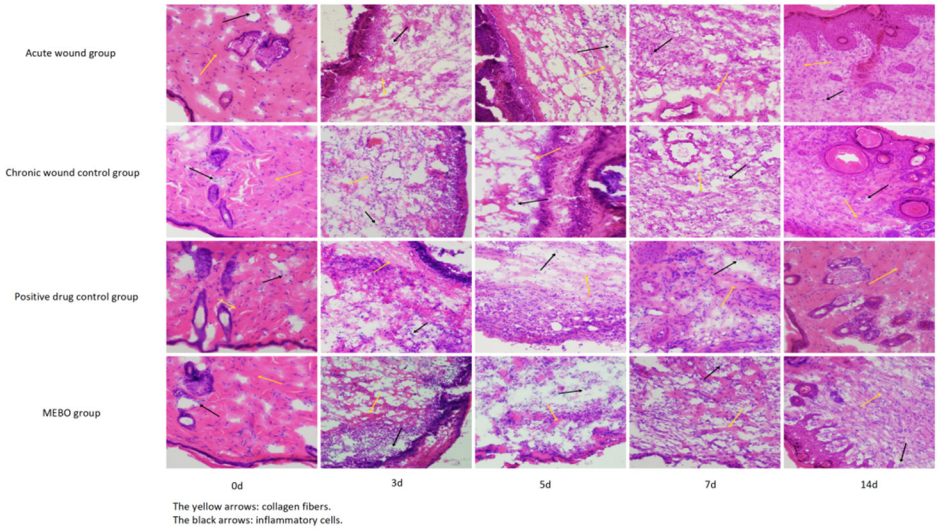


Fig 2. HE staining of wound tissue (200x)

3.3. Semi - Quantitative Comparison of Inflammatory Cells and Collagen Fibers in the Wounds of Each Group

Compared with the chronic wound control group, the number of inflammatory cells in the acute wound group decreased at each time point ($P < 0.05$), and the amount of collagen fibers in the acute wound group increased on the 5th, 7th, and 14th days after dressing change ($P < 0.05$), but there was no significant difference on the 0th and 3rd days ($P > 0.05$). Compared with the chronic wound control group, there was no significant difference in the number of inflammatory cells and the amount of collagen fibers in the wounds of the MEBO group on the 0th day after dressing change ($P > 0.05$). Compared with the chronic wound control group, the number of inflammatory cells in the wounds of the MEBO group decreased ($P < 0.05$) and the amount of collagen fibers increased ($P < 0.05$) at the other time points. See Tables 3 through 6.

Table 3. Semi - quantitative comparison of inflammatory cells in the wounds of the acute wound group, the chronic wound control group, and the positive drug control group

Groups	Cases	0d	3d	5d	7d	14d
Acute wound group	5	53.35 ± 1.67^{bcdef}	118.79 ± 5.13^{acdef}	100.98 ± 4.24^{abef}	98.17 ± 3.95^{abef}	84.71 ± 2.10^{abcdf}
Chronic wound control group	5	59.46 ± 1.59^{bcde}	180.13 ± 3.50^{acde}	152.55 ± 10.30^{abde}	128.89 ± 4.62^{abce}	99.54 ± 4.61^{abcd}
Positive drug control group	5	59.06±2.45	135.68±1.39	120.49±2.58	98.00±2.51	86.05±0.80
F_{time},P_{time}		$F_{time}=1169.97,P_{time}<0.01$				
F_{group},P_{group}		$F_{group}=275.02,P_{group}<0.01$				
$F_{time\times group},P_{time\times group}$		$F_{time\times group}=54.04,P_{time\times group}<0.01$				

Compared with the 0d of this group, $^{a}P<0.05$; compared with the 3d of this group, $^{b}P<0.05$; compared with the 5d of this group, $^{c}P<0.05$; compared with the 7d of this group, $^{d}P<0.05$; compared with the 14d of this groucompared, $^{e}P<0.05$; with the chronic wound control group, $^{f}P<0.05$

Table 4. Semi - quantitative comparison of inflammatory cells in the chronic wound control group and the MEBO group

Groups	Cases	0d	3d	5d	7d	14d
Chronic wound control group	5	59.46 ± 1.59^{bcde}	180.13 ± 3.50^{acde}	152.55 ± 10.30^{abde}	128.89 ± 4.62^{abce}	99.54 ± 4.61^{abcd}
MEBO group	5	60.89 ± 2.04^{bcde}	124.72 ± 2.29^{acdef}	108.37 ± 3.18^{abdef}	88.91 ± 0.53^{abcf}	85.19 ± 0.90^{abcf}
F_{time},P_{time}		$F_{time}=701.04,P_{time}<0.01$				
F_{group},P_{group}		$F_{group}=565.07,P_{group}<0.01$				
$F_{time\times group},P_{time\times group}$		$F_{time\times group}=76.26,P_{time\times group}<0.01$				

Compared with the 0d of this group, $^{a}P<0.05$; compared with the 3d of this group, $^{b}P<0.05$; compared with the 5d of this group, $^{c}P<0.05$; compared with the 7d of this group, $^{d}P<0.05$; compared with the 14d of this groucompared, $^{e}P<0.05$; with the chronic wound control group, $^{f}P<0.05$

Table 5. Semi - quantitative comparison of collagen fibers in the wounds of the acute wound group, the chronic wound control group, and the positive drug control group

Groups	Cases	0d	3d	5d	7d	14d
Acute wound group	5	65.35 ± 2.89^{bcde}	11.02 ± 1.40^{acde}	17.76 ± 1.26^{abdef}	33.09 ± 2.27^{abcef}	49.09 ± 3.11^{abcdf}
Chronic wound control group	5	64.55 ± 2.49^{bcde}	8.91 ± 1.13^{acde}	13.03 ± 2.41^{abde}	28.64 ± 2.61^{abce}	40.17 ± 2.75^{abcd}
Positive drug control group	5	66.04±2.69	19.89±3.08	32.24±3.23	41.20±3.23	52.09±1.86
F_{time},P_{time}		$F_{time}=953.86,P_{time}<0.01$				
F_{group},P_{group}		$F_{group}=172.92,P_{group}<0.01$				
$F_{time\times group},P_{time\times group}$		$F_{time\times group}=9.70,P_{time\times group}<0.01$				

Compared with the 0d of this group,$^{a}P<0.05$; compared with the 3d of this group,$^{b}P<0.05$; compared with the 5d of this group, $^{c}P<0.05$; compared with the 7d of this group, $^{d}P<0.05$; compared with the 14d of this groucompared, $^{e}P<0.05$; with the chronic wound control group, $^{f}P<0.05$

Table 6. Semi - quantitative comparison of collagen fibers in the wounds of the chronic wound control group and the MEBO group

Groups	Cases	0d	3d	5d	7d	14d
Chronic wound control group	5	64.55 ± 2.49^{bcde}	8.91 ± 1.13^{acde}	13.03 ± 2.41^{abde}	28.64 ± 2.61^{abce}	40.17 ± 2.75^{abcd}
MEBO group	5	66.75 ± 2.64^{bcde}	20.72 ± 3.28^{acdef}	29.69 ± 2.87^{abdef}	41.69 ± 2.55^{abcef}	51.01 ± 4.62^{abcdf}
F_{time},P_{time}		$F_{time}=873.84,P_{time}<0.01$				
F_{group},P_{group}		$F_{group}=67.53,P_{group}<0.01$				
$F_{time\times group},P_{time\times group}$		$F_{time\times group}=15.29,P_{time\times group}<0.01$				

Compared with the 0d of this group, [a]P<0.05; compared with the 3d of this group, [b]P<0.05; compared with the 5d of this group, [c]P<0.05; compared with the 7d of this group, [d]P<0.05; compared with the 14d of this groucompared, [e]P<0.05; with the chronic wound control group, [f]P<0.05

3.4. The Protein Expression Levels of P53, SLC7A11, and GPX4 in the Wound Tissue of Each Group Were Compared

In the acute wound group, the expression of P53 protein decreased to the lowest value on the 7th day after dressing change (P < 0.05). In contrast, in both the chronic wound control group and the MEBO group, it decreased to the lowest value on the 5th day after dressing change (P < 0.05). The expression level of P53 protein in the MEBO group was lower than that in the chronic wound control group on days 0, 3, 5, and 7 after dressing change (P < 0.05).

The expression level of SLC7A11 protein reached its peak on the 3rd day after dressing change in the acute wound group and the MEBO group (P < 0.05), while in the chronic wound control group, it reached the peak on the 5th day after dressing change (P < 0.05). The expression level of SLC7A11 protein in the MEBO group was higher than that in the chronic wound control group on the 3rd day after dressing change (P < 0.05), and lower than that in the chronic wound control group on the 5th and 7th days after dressing change (P < 0.05).

The expression levels of GPX4 protein in the acute wound group, the chronic wound control group, and the MEBO group all reached their peaks on the fifth day after dressing change (P < 0.05). The expression level of GPX4 protein in the MEBO group was higher than that in the chronic wound control group on the 5th day after dressing change (P < 0.05), but there was no significant difference on the 7th and 14th days after dressing change (P > 0.05). See Figure 3 and Tables 7 through 12.

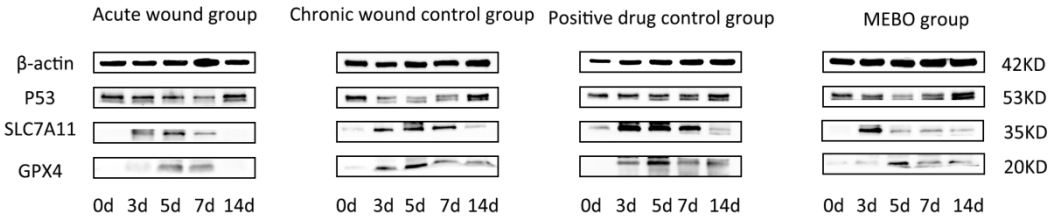


Fig 3. The protein expression of P53, SLC7A11, and GPX4 in the wound

Table 7. Comparison of P53 protein expression among the acute wound group, the chronic wound control group, and the positive drug control group

Groups	Cases	0d	3d	5d	7d	14d
Acute wound group	5	1.20 ± 0.05^{bcdef}	0.98 ± 0.15^{acdef}	0.77 ± 0.09^{abdef}	0.36 ± 0.07^{abcef}	1.50 ± 0.06^{abcdf}
Chronic wound control group	5	1.04 ± 0.13^{bcde}	0.54 ± 0.06^{acde}	0.38 ± 0.07^{abde}	0.66 ± 0.12^{abce}	0.87 ± 0.04^{abcd}
Positive drug control group	5	0.92±0.07	0.74±0.06	0.63±0.08	0.52±0.11	0.79±0.04
F_{time},P_{time}		$F_{time}=192.06$,$P_{time}<0.01$				
F_{group},P_{group}		$F_{group}=32.42$,$P_{group}<0.01$				
$F_{time\times group}$,$P_{time\times group}$		$F_{time\times group}=43.26$,$P_{time\times group}<0.01$				

Compared with the 0d of this group, [a]P<0.05; compared with the 3d of this group, [b]P<0.05; compared with the 5d of this group, [c]P<0.05; compared with the 7d of this group, [d]P<0.05; compared with the 14d of this groucompared, [e]P<0.05; with the chronic wound control group, [f]P<0.05

Table 8. Comparison of P53 protein expression between the chronic wound control group and the MEBO group

Groups	Cases	0d	3d	5d	7d	14d
Chronic wound control group	5	1.04 ± 0.13^{bcde}	0.54 ± 0.06^{acde}	0.38 ± 0.07^{abde}	0.66 ± 0.12^{abce}	0.87 ± 0.04^{abcd}
MEBO group	5	0.80 ± 0.05^{bcdef}	0.48 ± 0.09^{ace}	0.29 ± 0.05^{abdef}	0.51 ± 0.04^{acef}	1.02 ± 0.10^{abcdf}
F_{time},P_{time}		$F_{time}=114.96$,$P_{time}<0.01$				
F_{group},P_{group}		$F_{group}=9.08$,$P_{group}<0.01$				
$F_{time\times group}$,$P_{time\times group}$		$F_{time\times group}=8.52$,$P_{time\times group}<0.01$				

Compared with the 0d of this group, [a]P<0.05; compared with the 3d of this group, [b]P<0.05; compared with the 5d of this group, [c]P<0.05; compared with the 7d of this group, [d]P<0.05; compared with the 14d of this groucompared, [e]P<0.05; with the chronic wound control group, [f]P<0.05

Table 9. Comparison of SLC7A11 protein expression among the acute wound group, the chronic wound control group, and the positive drug control group

Groups	Cases	0d	3d	5d	7d	14d
Acute wound group	5	0.10 ± 0.06^{bcd}	1.21 ± 0.03^{acdef}	1.04 ± 0.09^{abde}	0.30 ± 0.07^{abcef}	0.07 ± 0.03^{bcd}
Chronic wound control group	5	0.12 ± 0.04^{bcd}	0.73 ± 0.08^{ace}	0.98 ± 0.10^{abde}	0.64 ± 0.10^{ace}	0.13 ± 0.06^{bcd}
Positive drug control group	5	0.21±0.09	1.24±0.15	1.00±0.09	0.57±0.15	0.16±0.05
F_{time},P_{time}		$F_{time}=538.18,P_{time}<0.01$				
F_{group},P_{group}		$F_{group}=6.63,P_{group}<0.05$				
$F_{time\times group},P_{time\times group}$		$F_{time\times group}=21.82,P_{time\times group}<0.01$				

Compared with the 0d of this group, $^{a}P<0.05$; compared with the 3d of this group, $^{b}P<0.05$;compared with the 5d of this group, $^{c}P<0.05$;compared with the 7d of this group, $^{d}P<0.05$;compared with the 14d of this groucompared, $^{e}P<0.05$; with the chronic wound control group, $^{f}P<0.05$

Table 10. Comparison of SLC7A11 protein expression in wounds of chronic wound control and MEBO groups

Groups	Cases	0d	3d	5d	7d	14d
Chronic wound control group	5	0.12 ± 0.04^{bcd}	0.73 ± 0.08^{ace}	0.98 ± 0.10^{abde}	0.64 ± 0.10^{ace}	0.13 ± 0.06^{bcd}
MEBO group	5	0.09 ± 0.02^{bcf}	0.89 ± 0.05^{acdef}	0.28 ± 0.11^{abef}	0.18 ± 0.02^{bf}	0.12 ± 0.02^{bc}
F_{time},P_{time}		$F_{time}=211.08,P_{time}<0.01$				
F_{group},P_{group}		$F_{group}=100.43,P_{group}<0.01$				
$F_{time\times group},P_{time\times group}$		$F_{time\times group}=70.80,P_{time\times group}<0.01$				

Compared with the 0d of this group, $^{a}P<0.05$; compared with the 3d of this group, $^{b}P<0.05$; compared with the 5d of this group, $^{c}P<0.05$; compared with the 7d of this group, $^{d}P<0.05$; compared with the 14d of this groucompared, $^{e}P<0.05$; with the chronic wound control group, $^{f}P<0.05$

Table 11. Comparison of GPX4 protein expression among the acute wound group, the chronic wound control group, and the positive drug control group

Groups	Cases	0d	3d	5d	7d	14d
Acute wound group	5	0.10 ± 0.12^{bcde}	0.38 ± 0.12^{acdef}	1.27 ± 0.14^{abdef}	0.73 ± 0.09^{abcef}	0.19 ± 0.11^{abcdf}
Chronic wound control group	5	0.10 ± 0.04^{bcde}	0.69 ± 0.10^{ade}	0.84 ± 0.05^{ade}	0.53 ± 0.09^{abce}	0.32 ± 0.07^{abcd}
Positive drug control group	5	0.03±0.04	0.67±0.13	1.14±0.07	0.60±0.10	0.47±0.07
F_{time},P_{time}		$F_{time}=338.07,P_{time}<0.01$				
F_{group},P_{group}		$F_{group}=2.53,P_{group}>0.05$				
$F_{time\times group},P_{time\times group}$		$F_{time\times group}=20.22,P_{time\times group}<0.01$				

Compared with the 0d of this group, $^{a}P<0.05$; compared with the 3d of this group, $^{b}P<0.05$; compared with the 5d of this group, $^{c}P<0.05$; compared with the 7d of this group, $^{d}P<0.05$; compared with the 14d of this groucompared, $^{e}P<0.05$; with the chronic wound control group, $^{f}P<0.05$

Table 12. Comparison of GPX4 protein expression in wounds of chronic wound control and MEBO groups

Groups	Cases	0d	3d	5d	7d	14d
Chronic wound control group	5	0.10 ± 0.04^{bcde}	0.69 ± 0.10^{ade}	0.84 ± 0.05^{ade}	0.53 ± 0.09^{abce}	0.32 ± 0.07^{abcd}
MEBO group	5	0.12 ± 0.04^{bcde}	0.25 ± 0.05^{acdf}	1.05 ± 0.16^{abdef}	0.57 ± 0.06^{abce}	0.34 ± 0.06^{acd}
F_{time},P_{time}		$F_{time}=126.52,\ P_{time}<0.01$				
F_{group},P_{group}		$F_{group}=8.07,P_{group}<0.05$				
$F_{time\times group},P_{time\times group}$		$F_{time\times group}=19.56,P_{time\times group}<0.01$				

Compared with the 0d of this group, [a]P<0.05; compared with the 3d of this group, [b]P<0.05; compared with the 5d of this group,[c]P<0.05; compared with the 7d of this group, [d]P<0.05; compared with the 14d of this groucompared, [e]P<0.05; with the chronic wound control group, [f]P<0.05

4. Discussion

REDOX imbalance and pathological inflammation in wound tissue are crucial factors contributing to the formation of chronic refractory wounds. The stability of the oxidation and antioxidant system serves as an essential link in wound repair. An excessive oxidative stress response and a decreased antioxidant capacity accelerate tissue cell damage and the pathological inflammatory response, which is unfavorable for tissue regeneration and ultimately results in delayed wound healing[8].Recent studies have demonstrated that the abnormal levels of ROS and lipid peroxidation in chronic refractory wounds exacerbate the local ferroptosis level of the wounds, facilitate the accumulation of inflammatory factors, induce cytotoxicity, exacerbate tissue damage and trigger an inflammatory storm, thereby impeding wound healing[9].Cao et al. further confirmed that in chronic refractory wounds, the intracellular levels of Fe^{2+} and lipid peroxidation increased, while the level of Glutathione (GSH) decreased. Moreover, the application of a ferroptosis inhibitor could effectively promote wound healing[10].

Ferroptosis, an iron-dependent programmed cell death mode distinct from apoptosis, autophagy, and necrosis, is extensively involved in the occurrence and development of a wide range of diseases[11-13].Abnormal iron metabolism, lipid peroxidation, and excessive oxidative stress are the core mechanisms underlying the occurrence and development of ferroptosis. Meanwhile, the P53/SLC7A11/GPX4 pathway serves as a key pathway for regulating ferroptosis[14, 15].The P53 protein is a crucial intracellular transcription factor that possesses multiple functions, including the regulation of the cell cycle, the facilitation of DNA repair, and the induction of apoptosis. Moreover, P53 can directly interact with its downstream target, SLC7A11, and down - regulate the expression of SLC7A11[16].SLC7A11 is one of the subunits of the cystine/glutamate antiporter. When its expression is inhibited, it impedes the transfer of extracellular cystine into the cell, impacts GSH synthesis, and results in the reduction of GPX4 activity[17, 18].Therefore, when the body is stimulated by injury, the accumulation of Fe^{2+} and ROS in the body and the activation of the P53 expression level increase. This can inhibit the expression of SLC7A11 and affect the activity of GPX4, preventing the body from timely catabolizing peroxides. As a result, the body's sensitivity to ferroptosis increases, inducing the occurrence of ferroptosis. Studies have demonstrated that the early reduction of ROS levels to alleviate oxidative stress is conducive to promoting the expression of various cytokines, including vascular endothelial growth factor (VEGF), fibroblast growth factor-2 (FGF-2), phosphorylated extracellular signal-regulated kinase (p-ERK), and phosphorylated protein kinase B (p-Akt), as well as facilitating the proliferation and migration of cells and vascular endothelium[19, 20].In addition, reducing the ferroptosis level in the tissue facilitates the up - regulation of M2 phenotype macrophages around the wound, thereby promoting wound healing[21].Cen Lijun et al. measured the expression levels of malondialdehyde, superoxide dismutase, and Nuclear factor-erythroid 2-related factor 2 (Nrf2) in wound tissue by constructing a rat model of chronic refractory wound. Their study confirmed that MEBO can alleviate oxidative stress injury and accelerate wound healing in chronic refractory wounds of rats by activating the Nrf2 axis [22]. MEBO can also activate autophagy by regulating autophagy-related signaling factors, which in turn promotes chronic wound healing[23]. Moreover, it plays an important role in regulating the content of matrix metalloproteinases and maintaining the homeostasis of the extracellular matrix[24].

In this study, a chronic refractory wound model in rats was established, and the wound - healing rates of each group were compared to verify that MEBO has the effect of promoting the healing of chronic refractory wounds in rats. Additionally, by observing the pathological changes of wounds in each group under a microscope, it was found that both the MEBO group and the positive drug control group could effectively reduce inflammatory cells and promote the proliferation of collagen fibers. However, the MEBO group could more effectively reduce the infiltration of inflammatory cells in the wounds. The collagen fiber content in the positive drug control group and the MEBO group was higher than that in the acute wound group and the chronic wound control group. However, on the 5th day after dressing change, the collagen fiber content in the positive drug control group was higher than that in the MEBO group. This might be associated with the difference in the action mechanisms between the two drugs. Compared with MEBO, Recombinant bovine basic fibroblast growth factor gel is rich in basic fibroblast growth factor, which up - regulates the expression of proliferating cell nuclear antigen, CD31, and vascular endothelial growth factor, thereby promoting cell proliferation[25].

In this study, Western blot results indicated that the ferroptosis level of the wound exhibited abnormal fluctuations during the wound - healing process, which suggests a correlation between the ferroptosis level and the wound - healing process. The expression of P53 in the wounds of each group initially decreased and then increased as the dressing - change time progressed. This implies that the expression of P53 was inhibited in the early stage of wound healing, and this inhibition was gradually alleviated as the wound healed, with high expression observed on the 14th day. In contrast, the expressions of SLC7A11 and GPX4 proteins showed an opposite trend. Compared with the chronic wound control group, the expression of P53 protein in the MEBO group was lower on days 0, 3, 5, and 7 after dressing change, and it reached the lowest value on day 5 after dressing change. This suggests that MEBO has a stronger inhibitory effect on P53 in the early stage of chronic wound healing. However, the level of P53 protein in the MEBO group was higher on the 14th day after dressing change, which may be related to the complex biological role of P53 protein. This indicates that MEBO may up - regulate the expression level of P53, regulate the cell cycle, and promote cell DNA repair in the later stage of wound healing[26].When compared with the SLC7A11 protein and GPX4 protein, it was observed that the SLC7A11 protein and GPX4 protein in the MEBO group did not reach their peaks. Recent research has revealed that Nrf2 is one of the primary transcription factors regulating ferroptosis. Moreover, the up - regulation of Nrf2 can maintain the activity of GPX4 and the level of GSH in cells and inhibit ferroptosis[27]. Therefore, the asynchronous peaks of SLC7A11 and GPX4 may be attributed to this, and this

phenomenon will be further investigated in the future.

In summary, this study preliminarily suggests that MEBO may inhibit the level of P53, up - regulate the expressions of SLC7A11 and GPX4, and reduce the level of ferroptosis in cells to promote wound healing at the early stage of the wound. However, this study failed to detect the content of Fe^{2+}, lipid peroxide, and ferroptosis markers in the wounds, and further studies will be carried out in the future. Meanwhile, the ferroptosis experiment of MEBO on fibroblasts/endothelial cells and the study of mRNA and protein expression of related pathways will be further optimized to clarify the direct drug targets.

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