Iranian Veterinary Surgery Association

Iranian Journal of Veterinary Surgery

Journal homepage: www.ivsajournals.com

Original Article

Investigating the Effect of Pentoxifylline and Zinc Oxide Combination on Experimental Full-Thickness Wound Healing in Rats

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ARTICLE INFO

Article History:

Received 9 August 2022
Revise 14 September 2022
Accepted 5 October 2022
Online 5 October 2022

Keywords:

Zinc oxide
Pentoxifylline
Wound healing
Rat

ABSTRACT

This paper aims to investigate the effect of topical application of pentoxifylline and zinc oxide combination on experimental full-thickness wound healing in rats. Forty-eight adult male Wistar rats weighing 250 to 300 g were randomly divided into four groups of 12: Control (C), Zinc oxide (Z), Pentoxifylline (P), and Zinc-Pentoxifylline (ZP). For topical use of pentoxifylline, the ointment was formulated as 5%. All rat were anesthetized by intraperitoneal administration of a combination of xylazine 2% and ketamine 10% and after transfer to the operating table, under aseptic conditions, using a sterile ruler and surgical razor, a 2×2 cm2 skin defect in the back was created. In order to manage the wound, each group received appropriate treatment. In the ZP group, the combination of pentoxifylline and zinc oxide was treated in a ratio of 1:1. On days 7, 14, and 21 after facilitation, a subgroup of each main group was sampled, then the samples were examined macroscopically and microscopically. At the end of this period, the highest percentage of the healing, wound closure, and keratinocyte migration and the lowest inflammation belonged to the PZ group. The rate of inflammation and the number of inflammatory cells at the end of the period were lower in the ZP group than Z group and there was a statistically significant difference ($p \leq 0.05$). Overall, this study showed that wound healing is performed with better speed and quality following topical application of a combination of zinc oxide and pentoxifylline. However, more studies are needed to confirm this conclusion.

Introduction

Wounds can be considered as a disorder in the continuity of the outer surface of the body or the surface of an internal organ.[1] Wound healing is a physiological response to injury that is essential in all tissue systems.[2] Wound healing begins immediately after forming an injury.[3] This process has four distinct and successive stages that, of course, have many overlaps: homeostasis, inflammation, proliferation, and tissue regeneration.[4] Proper wound healing in adults, with rapid homeostasis; proper inflammation; differentiation, proliferation, and migration of mesenchymal cells to the wound site; Proper

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https://doi.org/10.30500/ivsa.2022.355334.1313

regurgitation; rapid epithelialization (regrowth of epithelial tissue on the wound surface); and proper synthesis, crosslinking, and collagen alignment depend on the elastic strength of the repair tissue.[5] A deep understanding of the physiology of the natural wound healing process and the causes of treatment delays is a prerequisite for developing more effective therapeutic interventions.[6]

Pentoxifylline (PTX), a derivative of xanthine, has several medicinal properties in this regard and due to effects, such as vasodilation, increased flexibility of red blood cells and rheology, improved peripheral blood flow, improved oxygenation, and anti-thrombotic, fibrinolytic, anti-L properties. Modulating the immune system and its antioxidants can be potentially useful for healing all types of wounds.[7] Controlling inflammation plays an important role in wound healing, and over-activation of the inflammatory cascade disrupts this process. PTX has been shown to inhibit the synthesis of inflammatory mediators, reduce cytokine release, and suppress leukocyte function.[8] Few studies have evaluated the topical use of PTX and evaluated the effectiveness of this formulation and have shown that topical administration of this drug improves tissue healing properties and wound healing time.[9]

PTX appears to improve flap survival with its antithrombotic effect, improve blood rheology, and stimulate collagen growth. In an experimental study that examined the effect of PTX on bedsores healing, the results showed that PTX accelerates the healing of pressure sores.[10] The fibrinolytic effect and improvement of wound elastic properties have been proposed as PTX mechanisms in wound healing. Direct inhibition of fibroblast proliferation, decreased synthesis of type I and III collagens and glycosaminoglycans, and increased collagenase activity has been defined as PTX molecular activity.[11]

On the other hand, zinc deficiency causes tissue damage and delays wound healing and burns.[12] Zinc has the greatest effect on the skin by affecting the cell proliferation cycle.[13] Zinc has a significant effect on all stages of wound healing.[2] Various studies have shown that topical application of zinc oxide accelerates antibacterial action, and anti-inflammatory effects, increases wound contraction, and accelerates re-epithelialization and activation of metalloenzymes. Wound healing.[14] Topical application of zinc oxide to partially thick and full-thickness wounds of healthy pigs has been shown to improve wound healing by up to 30%.[15,16]

There was no published study evaluating the effectiveness of the topical application of pentoxifylline and zinc on wound healing. Therefore, this study aimed to evaluate and compare the effectiveness of pentoxifylline ointment alone or in combination with zinc oxide ointment on repairing the experimental defect of full-skin thickness in rats.

Materials and Methods

Animals

Forty-eight adult male Wistar rats weighing 250 to 300 g were randomly selected and divided into four groups of twelve. Then, to facilitate the work, each group of twelve was divided into three subgroups of four. The four main groups were the control group (C), the group receiving zinc oxide ointment (Z), the group receiving pentoxifylline ointment (P), and the group receiving zinc oxide and pentoxifylline (ZP) combination ointment.

Preparation of Pentoxifylline 5% Ointment

Pentoxifylline 5% ointment was developed and used based on research conducted by Najafi et al.[17] According to this study, first osrine (79 g) and sanitary paraffin (8 g) were combined and placed in a laboratory bin until a uniform mixture was reached. Then, pentoxifylline powder (prepared from 400 mg tablets) was completely dissolved in distilled water using a magnetic stirrer and a uniform solution of the drug was prepared. Because pentoxifylline is a light and unstable compound, all preparation steps were performed in containers covered with aluminum foil. Finally, to make 5% pentoxifylline ointment with suitable consistency, the prepared drug solution was gradually added to the ointment base composition and mixed gently.

Full-Thickness Wounds

On the day of the experiment, all animals were anesthetized after transfer to the operating room by intraperitoneal administration of a combination of xylazine 2% (10 mg/kg) and ketamine 10% (100 mg/kg) and transported to the surgical site. After placing the animals in a truncated position, using a ruler and surgical razor, under aseptic conditions; A 2×2 cm2 thick skin defect was created in the skin of the animal's back and between the neck and the pelvis. To manage the defect, the wound of the control group (C) was washed only with normal saline and a sterile swab, and no ointment was applied to the wound. In the zinc

(Z) group, after cleansing and washing the wound using normal saline and sterile swab, a thin layer of zinc oxide ointment, in the pentoxifylline group, a thin layer of pentoxifylline ointment, and in the target group, a mixture of both ointments in the proportion used 1 to 1. At the end of the study, all rats were kept in separate cages under separate temperature and humidity conditions with a 12-hour automatic light-dark cycle and free access of animals to water and food at all stages.

Sampling

For macroscopic examination of the wounds, on days 7, 14, and 21 after surgery, a quadruple subgroup of each major group was over-facilitated by intraperitoneal injection of a combination of 10% ketamine and 2% xylazine. The wound site of each animal was isolated on the day of sampling, imaging, and incision by making a full-thickness around the skin defect and immersed in formalin. Slides related to each sample were prepared and hematoxylin-eosin (H&E) staining was performed.

Macroscopic and Microscopic Evaluation

The images were examined with the Image J software and the area of each wound was measured. Then, using the following relationship, the percentage of wound contraction (percentage of wound healing) and the area of each wound were measured:

Percentage of wound contraction= [(Secondary wound area - Primary wound area)/ Primary wound area] × 100

Version 24 of SPSS software (IBM Corporation, NY, USA) was used to analyze the data. The one-way analysis of variance (ANOVA) and Bonferroni post hoc test were used to compare the obtained data between the study groups. The results were presented as mean ± standard deviation and $p \leq 0.05$ was considered significant.

Results

Wound Healing Percentage

On the seventh day after wounding, the highest percentage of recovery belonged to the ZP group, which was significantly higher than the P group ($p = 0.001$). The lowest wound healing rate on this day belonged to the P group, which had a statistically significant difference from the C group ($p = 0.001$), the P group ($p = 0.005$), and the ZP group ($p = 0.001$). The lowest rate of wound healing on the fourteenth day after wounding belonged to the Z group, which was significant compared to the C group ($p = 0.001$), the P group ($p = 0.001$), and the ZP group ($p = 0.001$). At the end of the study, the wound healing percentage in the ZP group was superior to the other groups, so it was significant compared to the C group ($p = 0.001$) and Z group ($p = 0.03$) (Figure 1 and Table 1).

Wound Opening or Closing

By examining the appearance of the wounds with the ocular index at the end of the period, all the wounds in the C groups and the Z group were still open. In the P

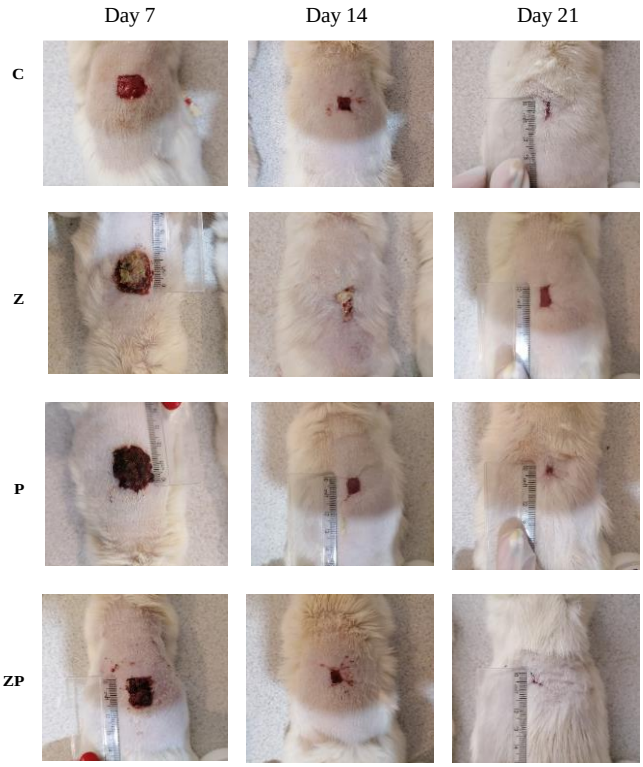

Figure 1. Microscopic images of the wound site at the time evaluated and in different groups.

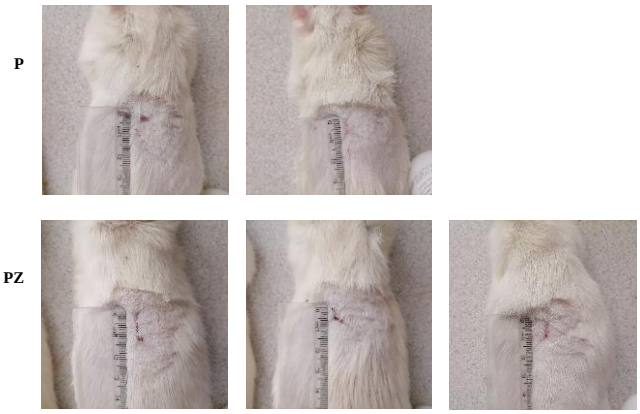

Figure 2. Images of closed wounds of pentoxifylline and zinc-pentoxifylline group on the 21st day.

group, 50% of the wounds (two out of four) were completely closed. In the target group, the ZP group, 75% (three out of four) were closed (Figure 2).

Qualitative Histopathologic Examination

On microscopic examination of the sections on the seventh day, the rats of the C group had a large number of inflammatory cells and fibrin filaments on the surface of the scab. Immature fleshy tissue with numerous fibroblasts and new and small blood vessels was observed in the deep parts of the wound site. Also, keratinocytes on the edge of the wound proliferated and a small number of them began to migrate to the wound. In the C group at the wound edges, keratinocyte proliferation and migration to the wound surface were more significant than in other groups. Inflammation was more severe in the P group, with a thick layer of an inflammatory exudate containing pink fibrin filaments with red blood cells and inflammatory cells visible, and a thick scab on the wound surface of all pentoxifylline rats.) Was seen. In the P and Z groups, inflammation was observed in two rats. The other two rats had more limited inflammation and inflammatory exudate (Figure 3).

Microscopic examination of the skin sections of C rat on the fourteenth day showed immature fleshy bud tissue, which consisted of abundant fibroblasts and newly formed capillaries. A large number of neutrophils were also observed among fibroblasts. Inflammation in the P group was reduced to the border of similarity to the C group compared to the seventh day. The repair process was similar in the Z and ZP groups (Figure 4).

Table 1. Mean ± standard error of wound healing rate (percentage) in full-thickness experimental wound in treated rats in the study groups (n = 5).

Parameter	Group / Time	Day 7	Day 14	Day 21
Wound healing percentage	Control (C)	42.34 ± 4.27 P	84.76 ± 1.96 Z	93.83 ± 1.27 P, PZ
	Pentoxifylline (P)	14.91 ± 7.37 C, PZ	86.60 ± 1.22 Z	97.64 ± 0.25 C
	Zinc oxide (Z)	28.49 ± 10.51 P	72.78 ± 1.23 C, P, PZ	96.02 ± 0.52 PZ
	Pentoxifylline + Zinc oxide (PZ)	42.64±4.79 P	85.91 ± 0.82 Z	99.30 ± 0.36 C, Z

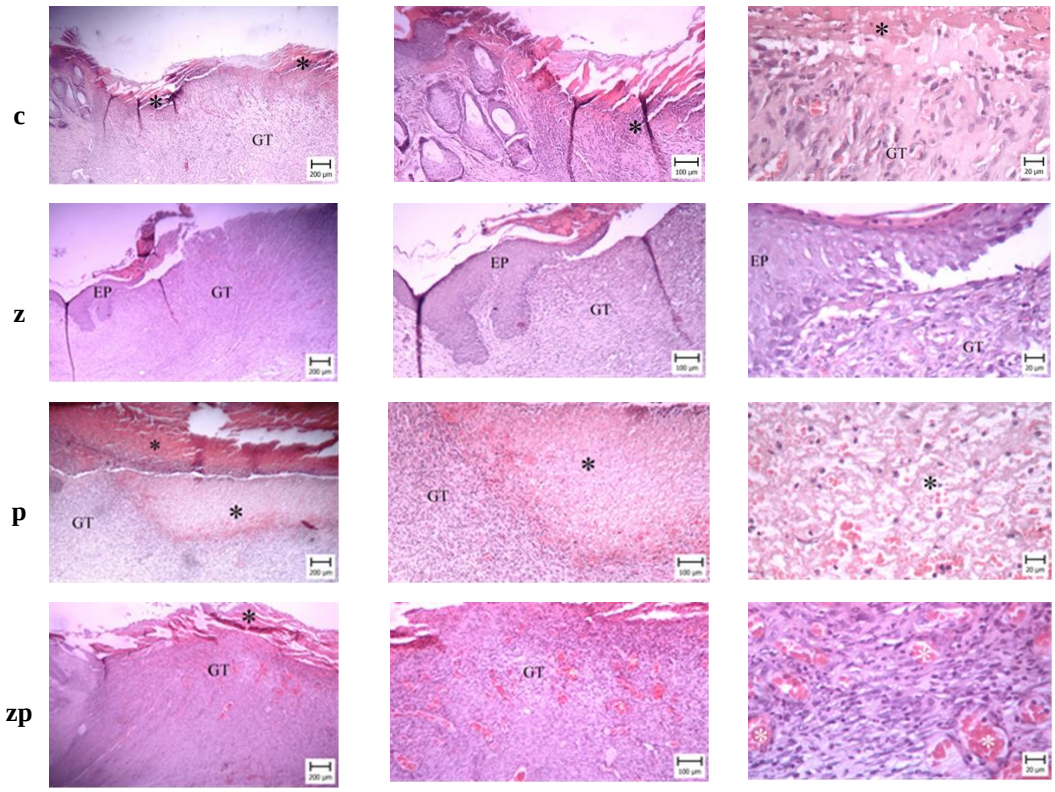

Figure 3. Microscopic examination of rat skin on the seventh day. Black Star: Inflammatory scab and exudate, GT: Flesh bud tissue. (H&E).

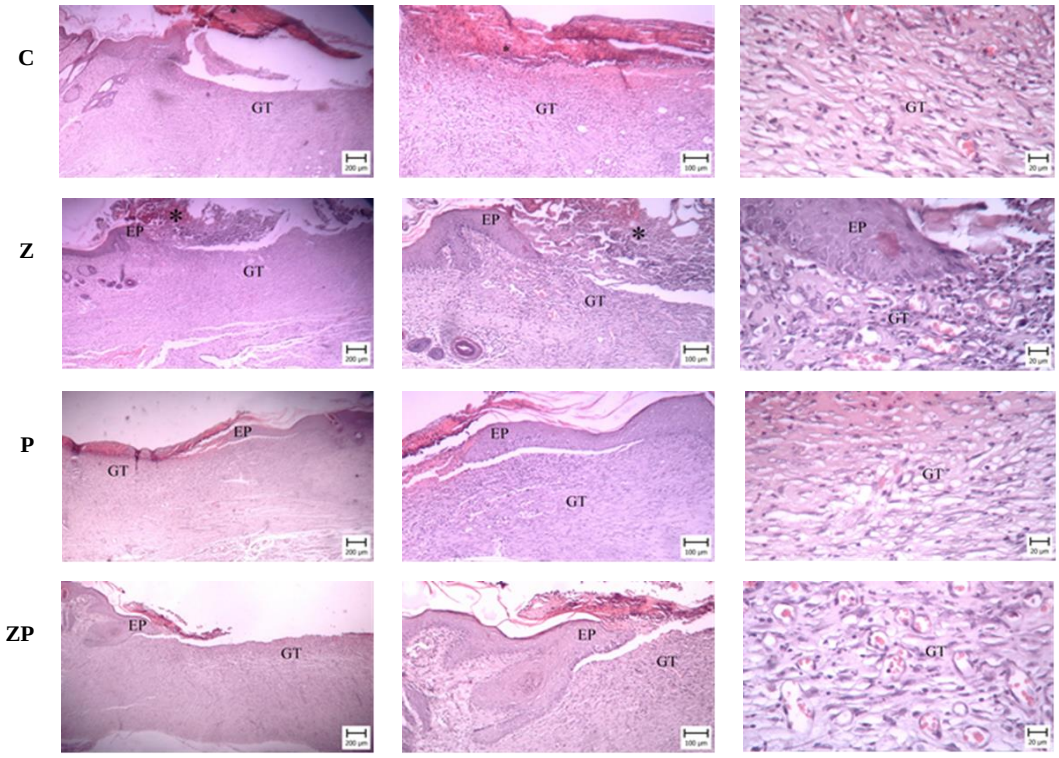

Figure 4. Microscopic examination of rat skin on the fourteenth day. Black Star: Inflammatory scab and exudate, EP: GT epithelial tissue: Flesh bud tissue. (H&E).

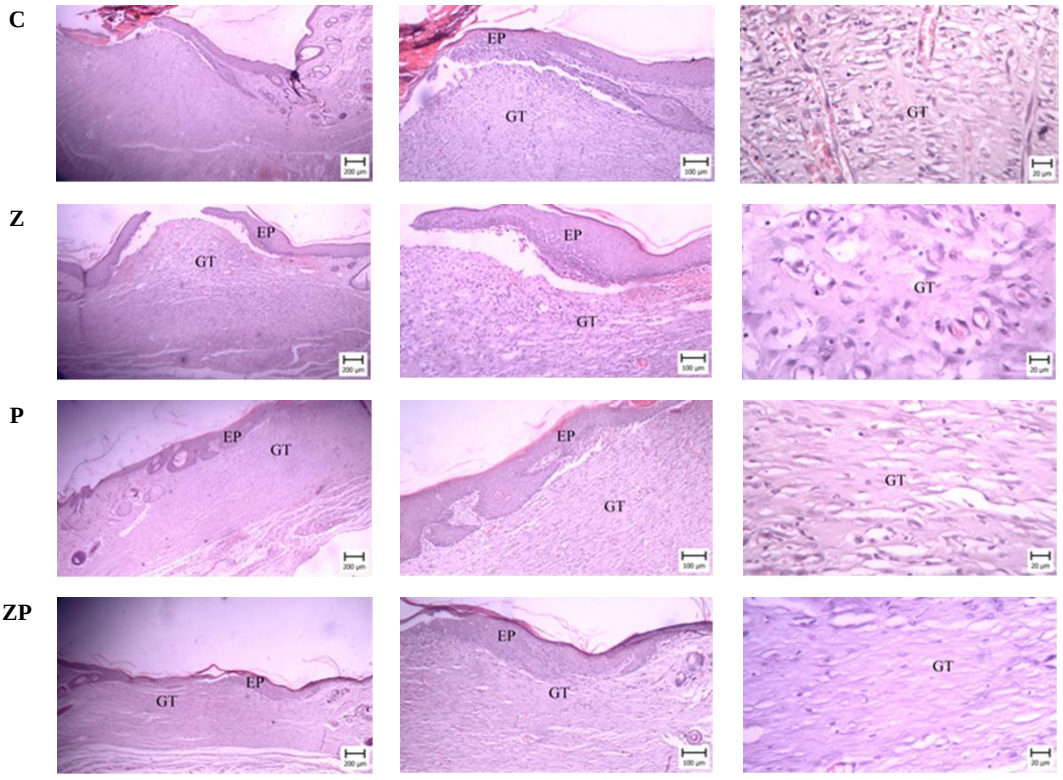

Figure 5. Microscopic examination of rat skin on day 21. Black Star: Inflammatory scab and exudate, EP: GT epithelial tissue: Flesh bud tissue. (H&E).

On the 21st day, mature fleshy bud tissue was observed at the site. The number of fibroblasts in this tissue decreased and the number of pink filaments increased. This was more evident in the Z and ZP groups. The wound surface in three rats of the ZP group and two rats of the P group was completely covered

with keratinocytes, and in other cases, only a small part of the wound surface remained without epithelium (Figure 5).

Quantitative Histopathologic Examination

Number of Blood Vessels. Examining the number of blood vessels in the wound, it was observed that on the seventh day after the defect, the highest amount of blood vessels belonged to the ZP group, so this amount compared to the C groups (p = 0.004) and the Z group (p = 0.005) had a statistically significant difference. On the fourteenth day after the defect, the highest number of blood vessels counted at the wound site belonged to the ZP group, but no statistically significant differences were observed between other groups. On the 21st day after the defect was created, the lowest number of counted vessels belonged to the ZP group, but there was no statistically significant difference between the other groups (Table 2).

Blood Vessel Diameter. The largest diameter of blood vessels measured on the seventh day after the defect belonged to the P group, which was significant compared to the C group (p = 0.005). There was no statistically significant difference between the measured vessel diameters on the fourteenth day after the defect. However, on the 21st day after wounding, the largest diameter of vessels belonged to the C group, so there was a statistically significant difference between the groups of the P (p = 0.004), Z (p = 0.004), and ZP (p = 0.005) (Table 3).

Number of Fibroblasts. The highest number of fibroblasts counted on the seventh day after wounding belonged to the P group, which was significantly different from the C group (p = 0.002). On the fourteenth day, the highest number of fibroblasts was observed in the Z group which was significantly different from the C group (p = 0.018). Which had a statistically significant difference compared to the Z group (p = 0.001) (Table 4).

Number of Inflammatory Cells. The highest rate of inflammation at the wound site on the seventh day after the defect belonged to the ZP group, which was statistically significant compared to the C group (p = 0.006). On the fourteenth day after wounding, the highest number of registered inflammatory cells belonged to the Z group, which was significantly different from the C group (p = 0.028). On the 21st day after wounding, the lowest number of recorded inflammatory cells belonged to the ZP group, which was statistically significant compared to the Z group (p = 0.03) (Table 5).

Migration of Keratinocytes. The highest rate of keratinocyte migration on the seventh day after wounding belonged to the P group, which was only statistically significant compared to the Z group (p = 0.019). In the evaluation of the fourth day, the highest migration was related to the ZP group, which was statistically significant compared to the C (p = 0.006) and Z (p = 0.001) groups. At the 21st day assessment, the highest rate of keratinocyte migration was still in the ZP group, which was significantly higher than the C (p = 0.005) and Z (p = 0.006) groups (Table 6).

Table 2. Mean ± standard error of number of blood vessels in experimental full-thickness skin wounds in treated rats in the study groups (n = 5).

Parameter	Group / Time	Day 7	Day 14	Day 21
Number of vessels	Control (C)	7.75 ± 0.43 PZ	9.55 ± 1.59	6.50 ± 0.51
	Pentoxifylline (P)	13.87 ± 1.27 PZ	8.00 ± 1.25	5.10 ± 1.47
	Zinc oxide (Z)	7.82 ± 0.80 PZ	11.25 ± 2.07	7.75 ± 2.01
	Pentoxifylline + Zinc oxide (PZ)	21.25 ± 4.07 C, P, Z	12.67 ± 3.24	4.6 ± 0.39

Table 3. Mean ± standard error of blood vessel diameter (mm) in experimental wounds of full skin thickness in treated rats in the study groups (n = 5).

Parameter	Group / Time	Day 7	Day 14	Day 21
Diameter of vessels	Control (C)	0.49 ± 0.04 P, Z, PZ	0.89 ± 0.04	0.94 ± 0.10 P, Z, PZ
	Pentoxifylline (P)	0.76 ± 0.07 C, PZ	0.92 ± 0.07	0.50 ± 0.01 C
	Zinc oxide (Z)	0.67 ± 0.02 C	0.98 ± 0.07	0.57 ± 0.05 C
	Pentoxifylline +Zinc oxide (PZ)	0.68 ± 0.03 C, Z	0.97 ± 0.05	0.58 ± 0.02 C

Table 4. Mean ± standard error of number of fibroblasts in experimental full-thickness skin lesions in treated rats in the study groups (n = 5).

Parameter	Group / Time	Day 7	Day 14	Day 21
Number of fibroblasts	Control (C)	54.35 ± 9.32 P, PZ	36.35 ± 1.58 Z	21.50 ± 2.82 Z
	Pentoxifylline (P)	91.00 ± 6.11 C	45.00 ± 2.59	24.40 ± 1.48 Z
	Zinc oxide (Z)	74.05 ± 6.31	62.05 ± 6.50 C	40.35 ± 0.90 C, P, PZ
	Pentoxifylline + Zinc oxide (PZ)	90.60 ± 1.26 C	47.30 ± 7.23	17.65 ± 1.44 Z

Table 5. Mean ± standard error of number of inflammatory cells in experimental wounds of full skin thickness in treated rats in the study groups (n = 5).

Parameter	Group / Time	Day 7	Day 14	Day 21
Number of inflammatory cells	Control (C)	208.90 ± 26.37 P, PZ	168.00 ± 11.34 Z, PZ	125.60 ± 16.65 Z
	Pentoxifylline (P)	326.60 ± 27.31 C	187.55 ± 6.64	124.75 ± 2.85 Z
	Zinc oxide (Z)	291.65 ± 32.11	222.40 ± 5.07 C	153.60 ± 1.64 C, P, PZ
	Pentoxifylline + Zinc oxide (PZ)	358.85 ± 6.55 C	208.65 ± 10.16 C	123.35 ± 3.67 Z

Table 6. Mean ± standard error of migration of keratinocytes (mm) in experimental full-thickness skin wounds in treated rats in the study groups (n = 5).

Parameter	Group / Time	Day 7	Day 14	Day 21
Migration of keratinocytes	Control (C)	15.52 ± 1.15	17.10 ± 2.49 PZ	21.65 ± 2.61 PZ
	Pentoxifylline (P)	20.95 ± 2.83 Z	24.70 ± 2.38 Z	32.75 ± 2.61
	Zinc oxide (Z)	11.55 ± 1.51 P, PZ	13.17 ± 0.54 P, PZ	21.75 ± 3.49 PZ
	Pentoxifylline + Zinc oxide (PZ)	20.32 ± 1.60 Z	28.27 ± 1.37 C, Z	37.97 ± 1.92 C, Z

Discussion

The potential effect of pentoxifylline on the healing of various injuries and wounds has been evaluated. Although data on the effect of PTX on flap survival are inconsistent, and there is little evidence of the beneficial and promising effects of PTX in healing pressure sores, skin lesions, and burns, oral or injectable PTX has beneficial effects on improving colorectal anastomosis. It has been shown to cause radiation-induced skin/soft tissue damage, venous ulcers, recurrent stomatitis, and cutaneous/mucosal leishmaniasis.[18] Studies in 150 patients have shown that receiving 400 mg of PTX twice daily with topical use of honey is an ideal measure for the treatment of radiation burns.[19] Due to the inhibitory effects of pentoxifylline on the proliferation and some biosynthetic activities of human skin-derived fibroblasts, it has a potential adjuvant role for colloids, hypertrophic scars, scleroderma, and other fibrotic conditions.[20] Pentoxifylline accelerates the healing of acetic acid-induced gastric ulcers in rats. This effect may be due in part to reduced neutrophil infiltration and inhibition of TNF production by inflammatory cells. Inhibition of the early phase of the inflammatory response may accelerate wound healing. Karasoy *et al*. examined the effects of systemic administration of pentoxifylline on normal and diabetic wound healing and found that pentoxifylline significantly reduced the time required for complete epithelialization as well as increased wound tensile strength in rats with normal blood sugar. [21] According to the hypothesis, a significant difference was recorded between healthy and diabetic rats in terms of recovery time and scar tissue strength. The results of this study are consistent with the data obtained from macroscopic studies of the present study, including wound area, percentage of healing rate, number of closed wounds, and microscopic results such as epithelialization rate and keratinocyte migration, in pentoxifylline-receiving experimental groups. Velaei *et al*. examined the effect of pentoxifylline administration on the healing process of experimental pressure ulcers

in ten adult male rats and showed the administration of pentoxifylline to rats with experimental pressure ulcers in the skin. This caused a significant acceleration of their wound healing process in comparison with the control group.[10] According to a study conducted by Babaei *et al*. to investigate the effect of systemic PTX on diabetic wound healing, there was a significant improvement in all biomechanical parameters. Histologically, PTX also reduced inflammation by day 7.[22]

Quantitative studies showed a significant increase in the number of fibroblasts in the control group on the third day after surgery and the number of neutrophils and macrophages on the third and seventh days. Also, on the seventh day after surgery, fibroblasts were significantly increased in the experimental group. Evaluation of angiogenesis and the number of blood vessels in the third and seventh days after surgery showed a significant increase in the control group. On the seventh day after surgery, a slight examination of the thickness of the epithelium showed a significant increase in the experimental group.[22] The trend of decreasing inflammation and inflammatory cells in the experimental group receiving pentoxifylline and increasing the same parameters in the control group of this study is consistent with the research of Babaei et al. Examination and counting of blood vessels in this study showed that the highest rate of angiogenesis in the first week belonged to the pentoxifylline-zinc group, which is not consistent with this study. Due to the close degree of angiogenesis in the pentoxifylline group and the control group, this effect can be attributed to the synergy of zinc and pentoxifylline on angiogenesis.

However, the slope of the decreasing trend in the number of vessels in the pentoxifylline-zinc group is significantly greater than the slope of the decrease in the number of vessels in the other groups. Why this needs further investigation. Najafi *et al*. designed an experiment to evaluate the effect of topical pentoxifylline ointment on bed sores in hospitalized patients, during which the control group and the experimental group twice a day, respectively, without active ingredient ointment and ointment, respectively. 5% received pentoxifylline at the wound site. Similar to the present study, the severity and extent of wounds were significantly reduced in the pentoxifylline ointment group.[17]

Gray reported in 2003 that consuming too much natural zinc could cause iron and copper deficiency anemia, which delayed healing by reducing oxygen supply to the wound site.[23] A 2004 study by Lim *et al*. found that high-dose zinc delayed recovery.[24] Routine use of zinc supplementation is not permitted in all patients with ulcers. However, those patients who are at risk for abnormal zinc status, including those with gastrointestinal upset, malabsorption, diabetics, malnutrition, burns, and acute catabolism, may be able to heal if zinc supplementation is provided.[25] Topical application of zinc for 12 days, regardless of the nutritional status of the rat, is effective in treating full-thickness skin lesions.[26]

Research by Yadav *et al*.[27] on the effectiveness of the topical application of zinc nanoparticles on wound healing of full-thickness rat skin. Evaluated. Faster wound contraction and re-epithelialization in the experimental group compared to the control group confirmed the vital role of biosynthesized nanoparticles in skin wound healing. These particles also significantly accelerated the healing process at various stages by reducing proinflammatory cytokines. The antioxidant effect of these compounds also contributed to fibroblast proliferation, angiogenesis, collagen production, granulation tissue formation, and wound maturation. It is claimed that these results were probably due to the synergistic effect of phenolic compounds with zinc. In the present study, the effect of zinc oxide in combination with pentoxifylline has achieved very different and better results compared to the exclusive use of this compound.

Lotfi *et al*. in 2020, by examining and comparing the effectiveness of honey alone or in combination with zinc oxide powder on repairing the experimental defect of full skin thickness in rats, showed that the group receiving the combination of zinc oxide and honey compared to other groups in the repair process wounds heal better and this combination accelerates the wound healing process. The combination of these two substances also increases the formation of fleshy bud tissue, increases wound shrinkage, reduces inflammation, and reduces wound scarring.[28]

According to the present study, during the experimental study of Kaufman *et al*.[29] In 2014, the effect of topical gluconate on wound healing, analgesia, and bacterial growth of full-thickness skin wounds of 98 rats was investigated. In this study, in addition to the control group and the zinc gluconate group, a group was treated with corticosteroids. A comparison of wound healing factors on days 4 and 21 after surgery showed that although the corticosteroid group was delayed in wound healing, there was no significant

difference between the control group and the gluconate-receiving group and they recovered similarly. The bacterial load of the wound in this group was quite similar to the control group. In 2018, Zhai *et al*. created a unique bandage by incorporating zinc nanoparticles into creatine-chitosan hydrogels, which reduced the activity of gram-negative and gram-positive bacteria due to the presence of zinc nanoparticles compared to the group that used conventional bandages.[30] In 2017, Xu *et al*. examined the effect of chemical structures, including organic cations, alkyl side chains, and zinc atoms on antimicrobial activity against Escherichia coli, *Staphylococcus aureus*, and *Candida albicans*. This study examined wound healing in rats infected with *Staphylococcus aureus* and showed that zinc ion could potentially be used as an antimicrobial wound dressing in the clinical setting.[31]

In the present study, the group treated with the combination of zinc oxide and pentoxifylline showed better results than the other groups in the wound healing process. Also, based on the results of histopathology, the overall outcome of healing in this group in the second and third weeks was better than in other groups and the restorative tissue was better organized than in other groups. The combination of these two substances increased the formation of fleshy bud tissue, increased keratinocyte migration, increased wound contraction, somewhat increased angiogenesis, and decreased inflammatory cells, especially in the third week of wound healing.

Based on previous studies and the results of the present study and clinical and histopathological evidence, it seems that the combined use of zinc and pentoxifylline topically improves the quality of wound healing in male rats. The use of this compound has significantly improved wound healing parameters compared to the separate topical application of these substances. However, further experimental and case studies in this area are recommended before the clinical use of this method.

Acknowledgment

The project was approved by the local Committee of the Institutional Animal Care and Use of Shahid Chamran University of Ahvaz, Iran. There is no funding for this paper. The authors would like to thank members of surgery division, veterinary hospital, Faculty of veterinary medicine, Shahid Chamran University of Ahvaz, Iran.

Conflict of Interest

The authors declare no conflict of interest related to this report.

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