Journal of Current Veterinary Research

ISSN: 2636-4026

Journal homepage: http://www.jcvr.journals.ekb.eg

Surgery

Comparative Evaluation of Propolis, Zinc Oxide Nanoparticles, and Mesenchymal Stem Cell-Derived Microvesicles on Full-Thickness Cutaneous Wounds in Donkeys

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ABSTRACT

Objective: This study aimed to determine the efficacy of mesenchymal stem cell-derived microvesicles (MVs), zinc oxide (ZnO) nanoparticles, and propolis for the treatment of full-thickness cutaneous wounds in donkeys.

Methods: Thirty healthy adult donkeys, both male and female, aged between 3-15 years, were selected for this study. The animals were randomly divided into five groups: a positive control group, a negative control group, an MVs-treated group, a ZnO nanoparticles-treated group, and a propolis-treated group. The intervention's effects were assessed at 21st and 42nd days post-wounding through histopathological evaluations while clinical observations was done every week. Clinical evaluation included monitoring wound closure, reduction in wound size, and absence of infection. Histopathological evaluation focused on key indicators of wound healing such as proliferation of fibrous connective tissue, collagen deposition, and epithelial migration.

Results: The results revealed that the MVs, ZnO nanoparticles, and propolis treatments significantly enhanced wound healing compared to the control groups. These treatments were particularly effective in promoting the proliferation of fibrous connective tissue, enhancing collagen deposition, and facilitating epithelial migration, which are critical factors in wound healing. Among the treatments, propolis, ZnO nanoparticles, and MVs demonstrated superior wound healing capabilities with minimal scar tissue formation.

Conclusion: The use of mesenchymal stem cell-derived microvesicles, zinc oxide nanoparticles, and propolis shows promising potential for accelerating the healing of full-thickness cutaneous wounds in donkeys, suggesting their potential application in veterinary and possibly human medicine. Further research is recommended to explore the underlying mechanisms and long-term benefits of these treatments.

Keywords: Donkey, Skin, Stem cells, Micro vesicles, Zinc Oxide nanoparticles, Propolis

INTRODUCTION

Wound healing is an intricate and multifaceted process encompassing several phases, including inflammation, proliferation, and remodeling (Mendonça and Coutinho-Netto, 2009; Velnar et al., 2009; Sorg et al., 2017; Wang, 2018). Donkeys, similar to horses, are prone to various injuries such as lacerations, abrasions, and surgical wounds (Köse et al., 2010; Nejash et al., 2017; Leise, 2018). Effective wound management in donkeys is paramount to preventing complications such as infection, delayed healing, and excessive scarring, which can significantly impact their health and welfare (Hanson, 2008). Comprehensive wound management in donkeys necessitates timely assessment, meticulous cleaning, and appropriate treatment to promote optimal healing and minimize complications (Theoret, 2004; Hanson, 2008). Factors influencing the selection of treatment modalities include wound size, location, and depth, as well as the overall health status of the animal (Guo and DiPietro, 2010).

Propolis, a natural resinous substance produced by bees, is renowned for its antimicrobial, anti-inflammatory, and wound-healing properties (Meto et al., 2016; El-Tayeb et al., 2019; Jaldin-Crespo et al., 2022). Historically, it has been utilized in wound care due to its efficacy in promoting tissue regeneration and inhibiting microbial growth (Jaldin-Crespo et al., 2022; Oryan et al., 2018). Zinc oxide nanoparticles have garnered attention for their notable antimicrobial, anti-inflammatory, and regenerative properties (Mandal et al., 2022). They are incorporated into wound dressings to enhance healing by promoting epithelialization, reducing inflammation, and preventing infection (Liang et al., 2021; Pino et al., 2023). Mesenchymal stem cells (MSCs) are well-recognized for their regenerative potential and immunomodulatory capabilities (Ding et al., 2011). MSC-derived microvesicles, which are small extracellular vesicles released by MSCs, contain bioactive molecules such as growth factors and cytokines that facilitate tissue repair and regeneration (Phinney and Pittenger, 2017; Keshtkar et al., 2018; Xunian and Kalluri, 2020; Aguiar Koga et al., 2023).

The objective of this study is to evaluate and compare the efficacy of propolis, zinc oxide nanoparticles, and mesenchymal stem cell-derived microvesicles in promoting the healing of full-thickness cutaneous wounds in donkeys.

MATERIALS AND METHODS

Animal Model and Sample Size

Healthy adult donkeys of both sexes, aged 3-15 years, were selected for this study. The sample size was determined based on statistical power calculations, considering expected effect size, variability, and significance level. A total of 30 donkeys were randomly allocated into five groups (n=6 per group) to ensure adequate statistical power. All donkeys included in the study were apparently healthy, with no pre-existing medical conditions affecting wound healing.

Induction of Full-Thickness Cutaneous Wounds

Full-thickness cutaneous wounds were induced under sterile conditions using a standardized procedure (Gadallah et al., 2023). The dorsal aspect of the chest between thoracic vertebrae 10 and 15 was shaved, cleaned, and marked for consistency. Wounds of standardized dimensions (3 cm x 3 cm) were created using a sterile surgical scalpel, ensuring uniform depth and size across all animals (Sadek et al., 2020) (Fig. 1)

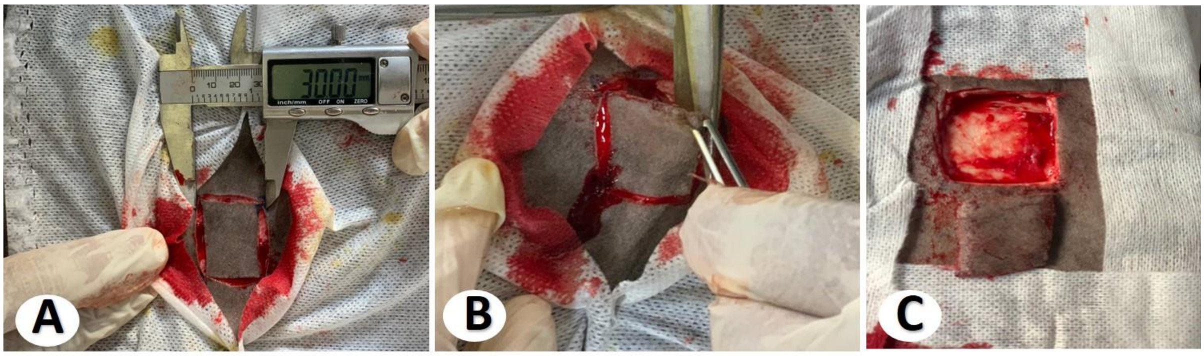

Figure 1. Surgical operation for induction of skin wounds; A) Incision of the skin after marking of the wound area 3 x3 cm. B) Dissection of the skin covering the area using scissors. C) The wound area after skin removal.

Treatment Groups and Administration Protocols

Propolis Group (A): Donkeys in this group received topical application of propolis extract (10% w/v) (Abdel-Wahed et al., 2011) onto the wound bed once daily for the duration of the study. **Zinc Oxide Nanoparticles Group (B):** Donkeys in this group received a topical application of zinc oxide nanoparticles suspension (5% w/v) onto the wound bed once daily. **Mesenchymal Stem Cell-Derived Microvesicles Group (C):** Donkeys in this group received intradermal injections of mesenchymal stem cell-derived microvesicles solution (1x10^9 particles/mL) around the wound margins at designated time points at 24 hours after wounding and was repeated at the 7th day post-wounding.
Control Positive Group (D): Donkeys in this group received a topical application of povidone iodine (Betadine®) (5% w/v) onto the wound bed once daily. **Control Negative Group (E):** Donkeys in this group received a topical application of normal saline onto the wound bed once daily (Bancroft and Gamble, 2008)

Evaluation and Outcome Measures

Wound Area Reduction: Changes in wound surface area were clinically evaluated for 42 days using a digital caliper after cleaning the wound with saline to visualize wound margins.

Histological Analysis: Biopsy samples were collected on the 21st and 42nd days post-wounding for histological evaluation of wound healing parameters. Specimens were fixed in 10% neutral buffered formalin, trimmed, washed in water, dehydrated in ascending grades of ethyl alcohol, cleared in xylene, and embedded in paraffin. Thin sections (4-6 µm) were processed and stained with Hematoxylin and Eosin (HandE) and Masson's trichrome (MTC) (Bancroft and Gamble, 2008).

Ethical Considerations and Approvals:

Ethical approval for the study protocol is obtained from the Institutional research ethics Committee (Assiut University) ref. number (04-2023-100071).

Statistical analysis

The obtained values were reported as mean, and standard deviations. A one-way ANOVA was used to carry out statistical analysis at $p \leq 0.05$ was considered statistically significant. All obtained values were analyzed using the SPSS software (version 20.0; IBM, America).

RESULTS

Clinical Evaluation of Wounds

In all experimental groups, the wound surface area gradually decreased throughout the observation period. By the end of the observation period, the MVs group exhibited almost complete closure of the wound area compared to the other experimental groups, which demonstrated varying degrees of wound healing (Fig. 2). At multiple time points post-wounding (7, 14, 21, 28, 35, and 42 days), the wound surface area was significantly smaller in the MVs group than in the other experimental groups (Fig. 3).

Histological Examination

At 21st days post-wounding:

In the propolis, ZnO nanoparticles, and MVs groups, the defect showed the formation of well-organized fibrous connective tissue with congested newly formed blood vessels covered by a scab.

In the control positive group, the defect displayed well-organized fibrous connective tissue infiltrated by a high number of inflammatory cells, mainly neutrophils and lymphocytes.

In the control negative group, the defect exhibited well-organized fibrous connective tissue without any newly formed blood vessels (Fig. 4).

At 42nd days post-wounding:

The propolis group showed well-organized fibrous connective tissue with mononuclear inflammatory cell infiltration, hemorrhage, newly formed blood vessels, and a scab.

The ZnO nanoparticles group exhibited dense, well-organized fibrous connective tissue with hemorrhage covered by epithelial layers (epidermis).

The MVs group demonstrated well-organized fibrous connective tissue with hemorrhage, congestion of newly formed blood vessels, covered by dermis and epidermis.

The control positive group showed dense organized fibrous connective tissue with mononuclear inflammatory cell infiltration and hemorrhage, but without epithelialization.

Similar to the control positive group, the control negative group had less dense fibrous connective tissue and more severe hemorrhage (Fig. 5).

There was a significant difference ($P \leq 0.05$) in the percentage of fibrosis in the wound defect among all experimental groups at 21st and 42nd days post-wounding. The control negative group exhibited the lowest percentage of fibrosis at 21st days post-wounding, while the MVs group had the lowest percentage of fibrosis by the end of the observation period (Table 1).

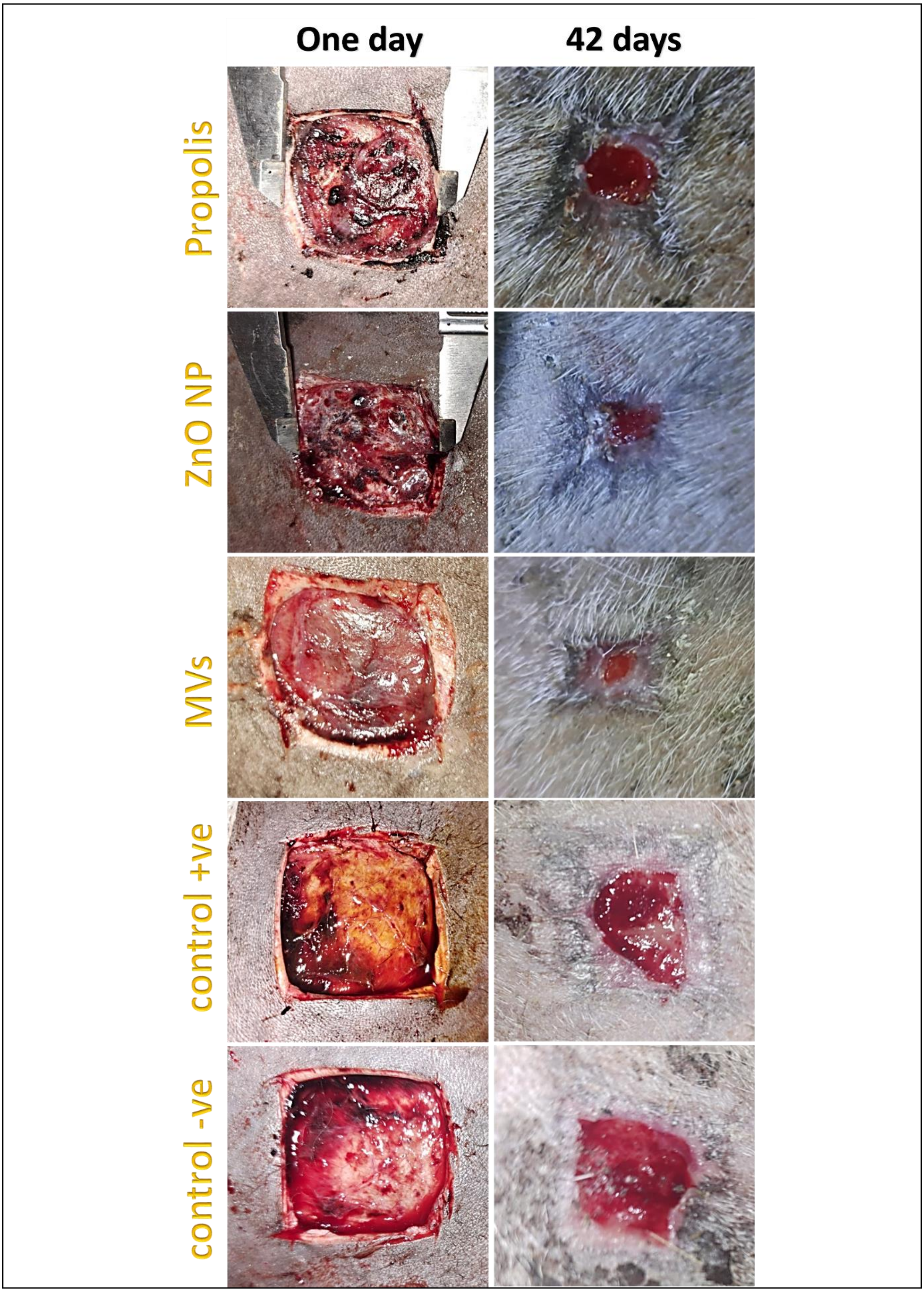

Figure 2. Showing the cutaneous wound area of all experimented groups at one day and at the end of observation period (42 days).

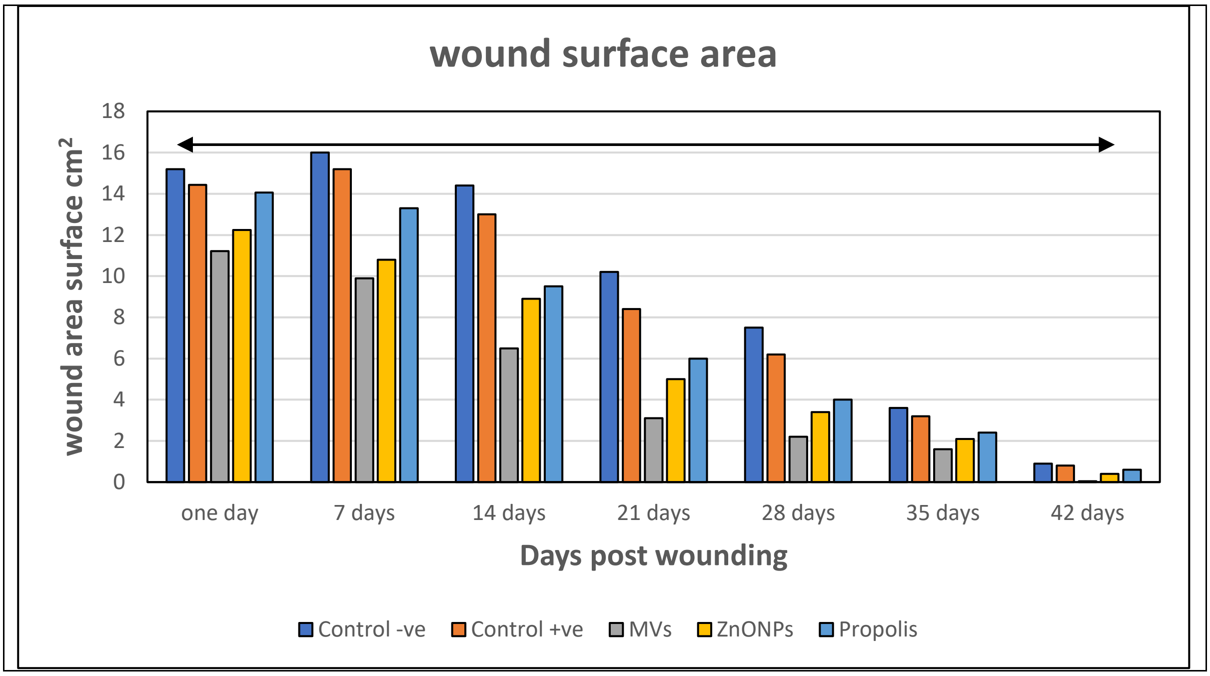

Figure 3. The mean of wound surface area all experimented groups. (↔) Significant difference between groups.

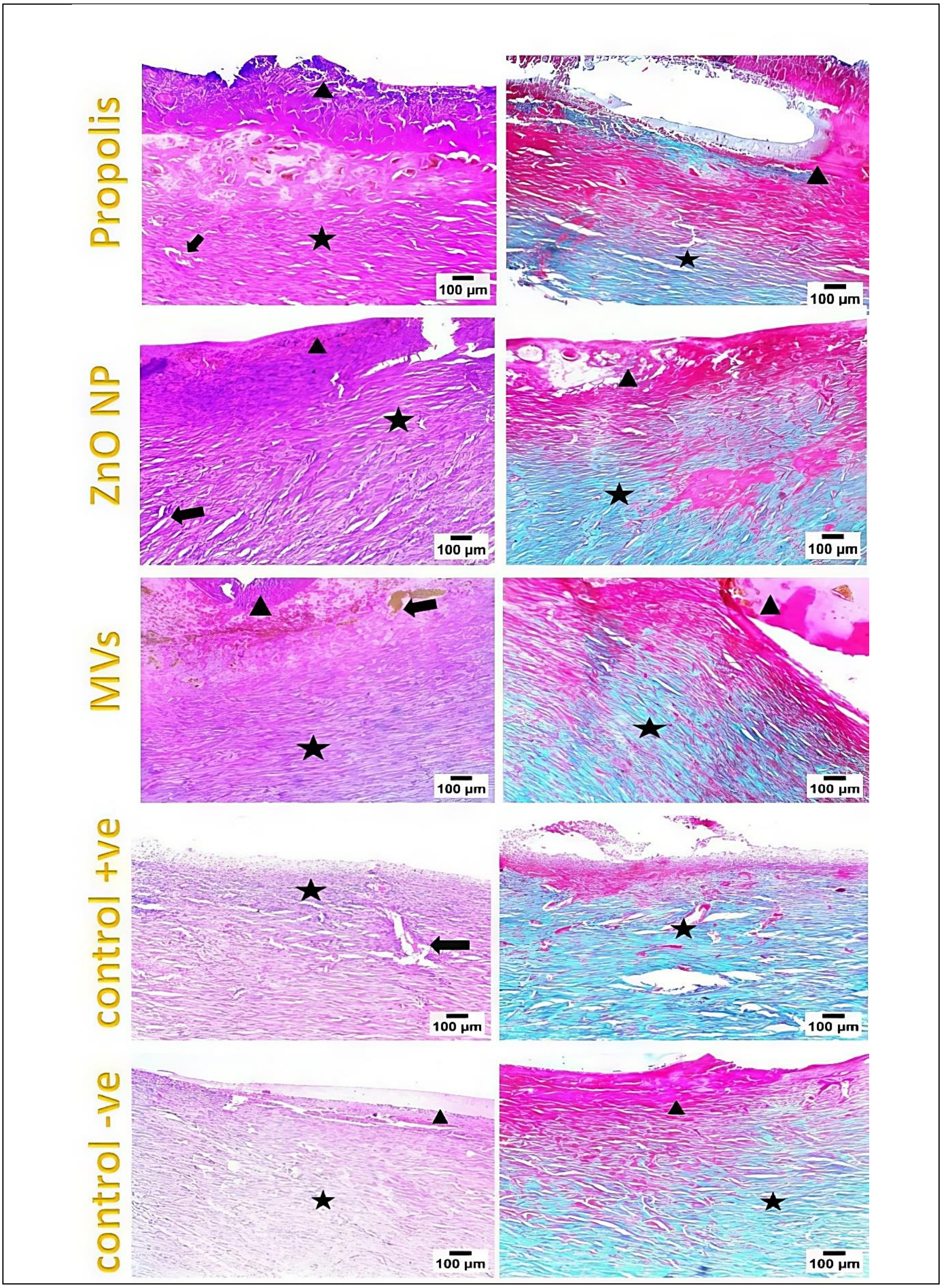

Figure 4. Showing the histological findings stained with Hematoxylin and Eosin stain (Left) and and Masson's trichrome (Right) at 21 days post- wounding of all groups. The stars referred to fibrous connective tissue, the arrows referred to newly formed blood vessels and the arrowhead referred to the scab.

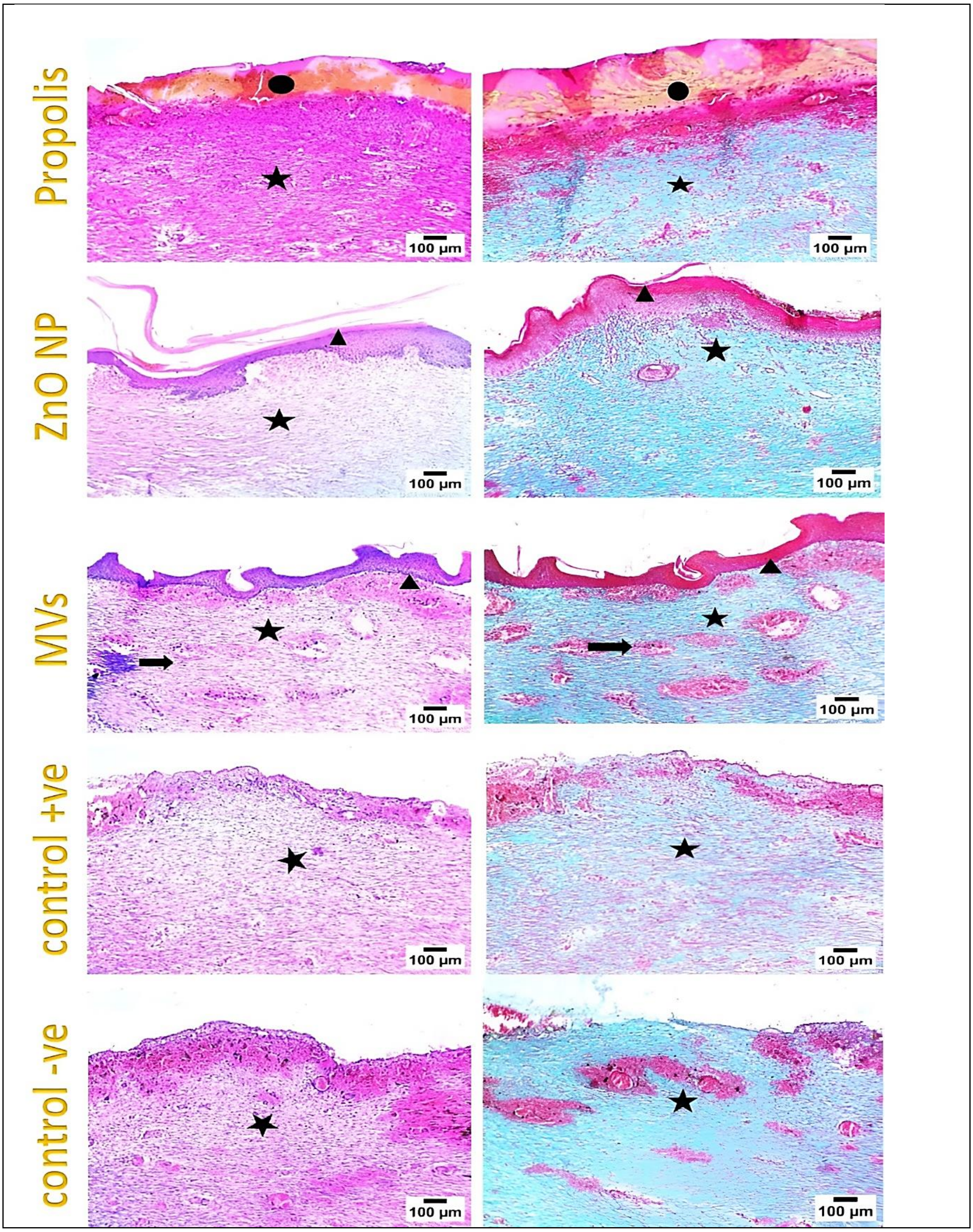

Figure 5. Showing the histological findings stained with Hematoxylin and Eosin stain (Left) and and Masson's trichrome (Right) at 42 days post- wounding of all groups. The stars referred to fibrous connective tissue, the arrows referred to newly formed blood vessels, the arrowhead referred to the epidermis the circles referred to the scab.

Table 1. Showing the fibrosis percent in the wound defect of each group at 21st and 42nd day post- wounding:

Groups \ Fibrosis %	Period of observation	
	21 days	**42 days**
Propolis	36.24 ± 1.3	30.25 ± 1.8
ZnO nanoparticles	36.78 ± 1.9	28.95 ± 1.1
MVs	33.11 ± 1.4	23.46 ± 1.9
Control +ve	42.19 ± 1.8	39.88 ± 1.4
Control -ve	12.69 ± 1.3	36.77 ± 1.2
P-value	**0.001**	**0.000**

DISCUSSION

Wound healing is an essential process for the well-being of animals, yet it is often accompanied by a variety of complications (Du Cheyne et al., 2021). In equines, skin wounds are frequently encountered and require costly and extensive treatment due to several factors (Theoret and Wilmink, 2013). These include the inability to achieve primary closure in many cases, attributed to significant tissue loss, excessive tension on the skin, severe contamination, or an excessive amount of time that has passed since the injury occurred (Theoret et al., 2001; Ribeiro et al., 2024).

Exosomes (MVs) represent one of the numerous secretory factors secreted by mesenchymal stem cells (MSCs), standing as the most potent components significantly contributing to the crucial process of intercellular communication. As a result, it has been posited that the therapeutic benefits derived from stem cell therapy may be attributed, in part, to the exosomal secretions of these cells. Therefore, it is conjectured that administering these beneficial exosomal secretions directly could potentially mitigate the constraints and hazards commonly associated with stem cell therapy (Bian et al., 2022; Prasai et al., 2022; Gadallah et al., 2023).

The present study exhibited enhanced and accelerated wound healing in wounds treated with MVs as compared to those in the control group. Wounds treated with MVs demonstrated a more rapid rate of wound contraction than observed in the control group. Furthermore, the percentage of wound contraction in the MVs group surpassed that in the control group throughout the duration of the study, starting from 7 days post-wounding and extending to the end of the observation period. These data agreed with (Sadek et al., 2020; Bahr et al., 2021; Gadallah et al., 2023). Also, ZnO NPs and Propolis groups showed accelerated wound healing compared to control groups. These groups showed clean wound defect without any signs of infection, this can be attributed to the antimicrobial effect of ZnO NPs and Propolis (Kaushik et al., 2019; Batool et al., 2021; Abd Elkawi et al., 2022).

The histopathological examination revealed better wound healing quality of MVs and ZnO NPs groups compared to other groups, both groups showed high proliferation of epidermal cells with migratory islets of epidermal cells to the surface and the dermis is mainly hypercellular condensed immature collagen at 21st days post-wounding and thickening of the epidermal layer with a thin layer of keratin and increased production of collagen in the dermis layer at 42nd days post-wounding with minimal fibrosis percent. On the other hand, betadine and saline treated control groups showed highly fibrous tissue proliferation with congestion and hemorrhages at 21st days post-wounding and showed mature hemorrhagic granulation tissue at 42nd days post-wounding. These data are similar to that reported by (El-Tookhy et al., 2017; Bahr et al., 2021; Gadallah et al., 2023).

In the course of this study, wounds treated with propolis demonstrated satisfactory healing parameters, marked by the lack of inflammatory indicators, exudation, and infection. Concurrently, the processes of granulation and epithelial tissue regeneration were observed to be moderate. The efficacious role of bee propolis during the healing process remains a subject of debate, primarily due to its antimicrobial properties and its activity in stimulating metabolic processes rather than promoting direct tissue regeneration (Abdel-Wahed et al., 2011; Abu-Ahmed et al., 2013; Saber et al., 2024). The histologic features following propolis, ZnO NPs and MVs dressing proved excellent characterized by thick vascular granulation tissues, more fibroblast and collagen deposition and epithelial migration.

CONCLUSIONS

The findings of this study demonstrate that propolis, zinc oxide nanoparticles (ZnO NPs), and mesenchymal stem cell-derived microvesicles (MVs) significantly enhance wound healing with minimal scar tissue formation. The clinical evaluation revealed that MVs were particularly effective in reducing wound surface area and promoting rapid wound contraction. Histopathological analysis corroborated these findings, showing superior quality of wound healing in the MVs and ZnO NPs groups, characterized by organized fibrous connective tissue, increased collagen deposition, and enhanced epithelial migration. Based on these results, it is evident that propolis, ZnO NPs, and MVs present valuable therapeutic options for the management of full-thickness cutaneous wounds in donkeys. Their ability to accelerate wound healing and minimize scarring makes them suitable candidates for use as wound dressings in equine medicine. The antimicrobial properties of ZnO NPs and propolis further contribute to their effectiveness by preventing infection and promoting a clean wound environment. Further research should focus on optimizing dosing regimens and exploring the molecular mechanisms underlying their wound healing effects to further enhance their therapeutic potential.

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