RESEARCH
www.commprac.com
ISSN 1462 2815

PIPERIN DERIVED SILVER NANOPARTICLES ENHANCED WOUND HEALING IN ZEBRAFISH

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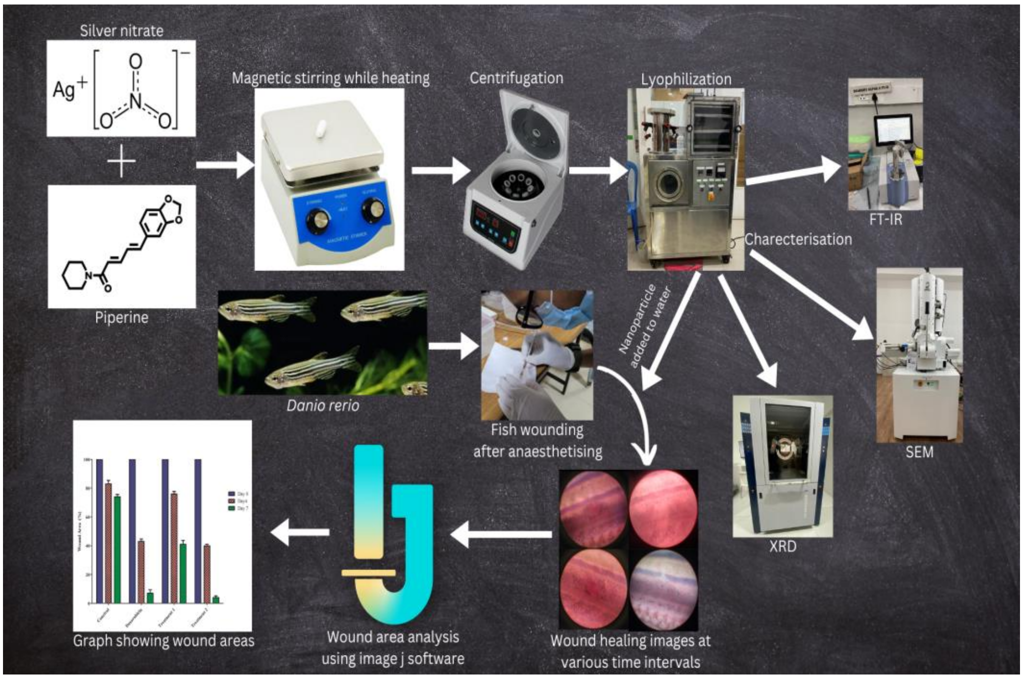
DOI: 10.5281/zenodo.10964787

Abstract

A successful chemical reduction approach was used to create silver nanoparticles (AgNPs) from piperin, which were then physico-chemically characterized and tested for their impact on zebrafish wound-healing activities. The produced AgNPs had an average diameter and zeta potential of 72.66 nm and 0.45 mv, respectively. They were flask- like and water soluble. Zebrafish were used to create a mechanical skin wound, and the influence of AgNPs on wound-healing activity using immersion in a solution of AgNPs and water (50 g/L). The zebrafish were monitored for 7 days post-wounding (dpw) using histological analysis, ocular measurement of the size of the wound, and wound healing percentage (WHP). In contrast to the controls, immersion application treatment showed clear and quicker wound closure at 4 and 7 dpw, which was supported by histological data at 0, 4 and 7 dpw. At 4 dpw, WHP was highest in the AgNPs immersion group (36.6%) than and/controls (18.2%), demonstrating that fish immersed in AgNPs solution benefited from WHP the most. According to these findings, Piperin derived AgNPs can speed up wound healing and change how specific genes involved to wound healing are expressed. However, more research needs to be done on fish to determine the precise mechanism of improved wound healing.

Keywords: Zebrafish, Wound Healing, Piperin, Nanoparticles, Good Health And Well -Being, Sustainable Development.

Graphical Abstract



INTRODUCTION

The aquatic organisms are under continuous threats and prone for environmental changes that vary from sub-lethal to fatal. As a result, they develop wounds due to external stimuli such as mechanical and or physical forces, microbial infections, abrasions etc., Such skin and tissue damages resulting in the creation of wounds. Wound healing is an essential process that requires complex enzymes and their physiological regulations. Every factor either environmental or physiological definitely affects the wound healing stages. There are four basic stages, namely, hemostasis, inflammation, proliferation and remodeling of the wounded sites[1,2]. These are achieved by the coordinated process between different cell types, cytokines, growth factors and extracellular matrix proteins (ECM).

Danio rerio, serve a better animal model due to their cost- effectiveness, easy breeding and maintenance[3]. When compared to the human reference genome, approximately 70% of human genes have at least one obvious zebrafish orthologue[4]. Except for a few variations, wound healing in zebrafish is similar to the mammalian wound healing process. The fish lack well- differentiated neutrophils and macrophages. Instead, they have myeloperoxidase and lysozyme filled cells for removal of foreign bodies and debris, the processes occurring before re-epithelization. Another, main difference is time of epithelialization. Usually, in zebrafish, it occurs after 10h after wounding (post wound hour, PWH); where, in mammals, it takes place after 48 pwh. Except, all of the other processes and stages are mostly similar to mammals and zebrafishes. Hence, they serve as excellent wound healing models for discovering new drugs. Neovascularization and the deposit of collagen beneath the re-epithelialized region will follow these. By day six after injury, the granulation tissue and inflammatory cells are significantly reduced, and dermal thickenings start to replace missing scales. The tissues are believed to be fully restored by day 30 after injury[5,6]. It's interesting to note that new research shows that the inflammatory response has little to no impact on the re-epithelialization of animals[7]. On the other hand, unlike mammals, zebrafish are less affected by neutrophils and chemotactic signals from macrophages when it comes to the re-epithelialization of wound debridement[8]. Zebrafish have a quick healing process that may be a result of the presence of living cells that can elongate and intercalate radially under the control of Rho/Rock and the TGF signalling pathway[9]. Due to the similarity in the structure of their skin, humans and zebrafish share the same fundamental concepts in their wound-healing mechanisms. In support of this assertion, the zebrafish's skin already consists of a basal layer that is adhered to the foundation membrane, an epidermis bilayer in the middle, and periderm at the superficial layer[10]. After the 25th day after conception, a process known as metamorphosis causes the epidermis' multilayer to form. In addition, as fibroblasts enter the dermis, basal keratinocytes that were previously producing collagen begin to produce scale-forming dermal papilla[11]. This demonstrates how human skin architecture is similar, opening the door to the prospect of improving this species as a model for cutaneous wound healing. Plant compounds are medicinally- invaluable since they possess anti-oxidative, antimicrobial and wound healing potential[12,13]. Herbal formulations have shown great results in recent times[14]. Their major drawback lies in their solubility and membrane permeability. Even extensively used plant compounds suffer due to the same problem, hence, they require an optimal vehicle for reaching the cell’s inner-side. Piperine is a bioactive alkaloid derived from black pepper (Piper nigrum) and long pepper (piper longum)[15]. Nowadays, different nanomaterials are used as excellent adjuvants for synthetic and or bioactive products.

Nanomaterials are extensively applied in biological fields for improving the human medical care and extended into different applications including, aquaculture, they have a great impact on society[12]. They are acting as immune-stimulators, adjuvants, antimicrobial agents and wound healing matrix. Among them, silver nanoparticles are used widely from house- hold goods to wound wrapping materials, these are known to have anti-microbial properties[3,16]. In this study, piperin derived silver nanoparticles were analysed for their wound healing potential using zebrafishes (*D. rerio*) as animal models.

MATERIALS AND METHODS

Piperin was purchased for the study (Sigma Adrich, USA). The source of the silver was a 1 mM silver nitrate solution in double-distilled water. A 1:9 mixture of silver nitrate and piperin (0.001M) was used. A magnetic stirrer was used to continually swirl the reaction mixture at 800 rpm while it was heated below the boiling point. Within a single hour, the mixture became reddish brown. Darkness pervaded the whole response. Centrifuging was done for 45 minutes at 15,000 rpm using the Ag/piperin suspension that had been obtained. Silver ions and compound residue were removed by washing the silver nanoparticle-containing pellet three to four times with deionized water. Lyophilization was done on the precipitated nanoparticles (NPs). In order to further characterise the lyophilized nanoparticles, they were kept in a cold, dry, and dark environment. UV-vis spectroscopy, X-ray diffraction (XRD), Fourier transform infrared (FTIR) spectroscopy, and scanning electron microscopy were used to analyze the produced AgNPs (SEM)(Chockalingam, Sasanka, Babu K, Ramanathan, & Ganapathy, 2020).

Zebrafish of the wild kind were purchased from a commercial shop in Chennai. Fish were kept in typical laboratory settings at 28 1 °C with 12 hours of light and 12 hours of darkness (10 fish/3.5 L tank). Three times a day, 144 brine shrimp (artemia) at 4% of body weight were fed to the fish. Fish of 6-7 months old that were healthy and of a consistent size were used in the tests(Prathap & Lakshmanan, 2022). All fish were put to sleep by being submerged in 0.2% Tricaine (ethyl 3- aminobenzoate methane-sulfonate). The zebrafish were separated into four groups of 6 (n = 24) to calculate the wound healing percentage. Following anaesthesia, a single wound was created by a sharp scalpel, posterior to the abdomen region, close to the lateral line of the zebrafish. Different concentrations of nanoparticles were prepared by dissolving in DMSO (0.01%). The group detail, group I – negative control, group II – positive control (donorubicin, 0.001M) and different concentrations of NPs (7 and 70µg/ ml respectively) respectively. The concentration was selected using acute toxicity study (data not shown). Using a digital camera attached to a stereo microscope, the wounds of 12 fish per 178 groups of zebrafish on days 1, 4 and 7 post-weaning were photographed (Nikon-SMZ179 100, Japan)(Akshaya & Ganesh, 2022). After that, Image J software was used to estimate the wound area (Ver 1.48, USA). After taking a photo of the caudal fin, each fish was uniquely recognized by comparing its pigmentation pattern to the pattern on the fin for optimal identification. Each fish had their wound areas assessed at various times(BABU & MOHANRAJ, 2020; Kumaresan et al., 2022). The distinction in skin tone between wounded and uninjured areas allowed for the identification of each wounded location. Each wound was deemed to be healed when the region that had been injured could no longer be distinguished (i.e., had fully regenerated and pigmented). By comparing the size of the wound on days 1, 4 and 7, expressing the difference as a percentage, the wound healing effect of NPs was also calculated (as WHP)(Varshan & Prathap, 2022).

RESULTS

SEM analysis

The emission of absorbed electrons are represented as a image in SEM and due to its wavelength at nano level, it is used to know the morphology, size and dispersion pattern of the nanoparticles. Figure. 1 represented SEM image of synthesized piperin derived ZnO-Ag nanoparticles. The flake- like structures seem to be ZnO in nanocomposite and Ag is seen as scattered spherical particles throughout the flakes. The block spherical circles indicated the Ag nanoparticles.

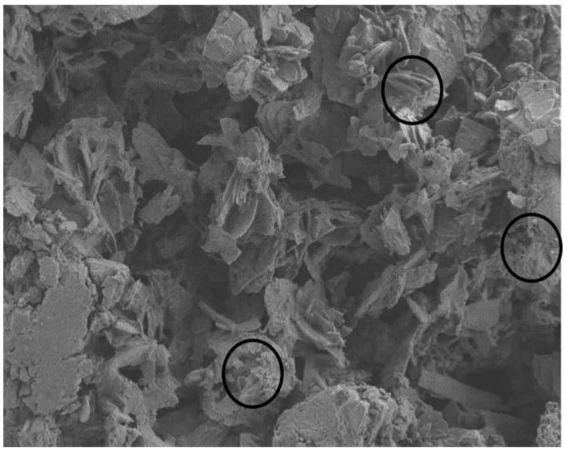


Figure 1: SEM image of the Piperin derived ZnO- Ag nanoparticles

Ag particles seen as spherical structures spread (as indicated by spherical shapes) throughout the flakes in ZnO- Ag nanocomposite.

XRD analysis

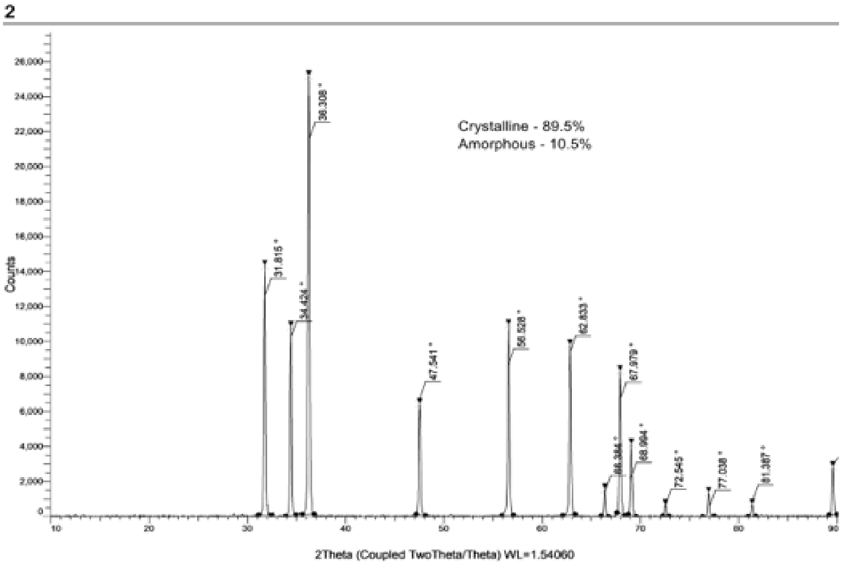


Figure 2: XRD analysis of synthesized piperin derived Ag nanoparticles

The crystalline nature of ZnO-Ag was analysed using XRD pattern analysis, as depicted in Fig. 2. A number of modest, identifiable diffraction peaks at 36.532, 34.512, 47.712, 56.909 and 67.919 were visible in the XRD spectrum. These correspond to the primitive structures of Zn and Ag nanoparticles were 101, 002, 102, 110 and 201 respectively. The Joint Committee on Powder Diffraction Standards database was used to match the XRD pattern of the nanoparticles (JCPDS Card No. 36-1451) (Gauri et al., 2016). The mean grain size of ZnO- Ag formed in the bioreduction process was measured using the Debye-Scherrer formula D= kλ/βcosθ, where D is the average crystalline size (Å), k is a constant 1, 'λ' is the wavelength of X-ray source (0.1541nm), β is the angular line full width at half maximum (FWHM) intensity in radians and 'θ' the Bragg's angle [29,30]. XRD pattern revealed the nature of the NP. They were predominately in crystalline structure (89.59%) than amorphous (10.41%).

FTIR Analysis

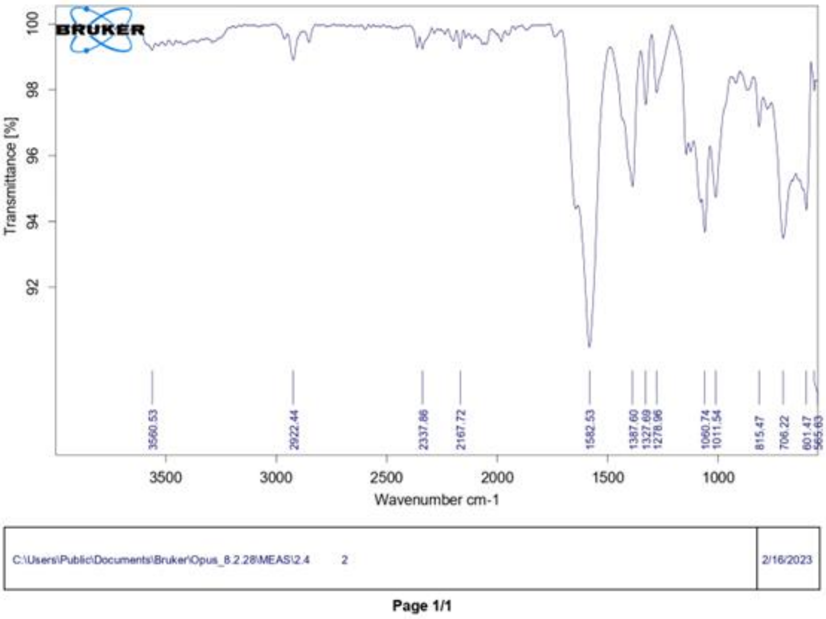


Figure 3: FTIR analysis of synthesized piperine derived Ag nanoparticles

Wound Healing Efficiency

Piperin derived AgNPs' ability to heal wounds was assessed using time-series wound size visual observations (on 0, 4 and 7 dpw) and WHP calculations on 0, 4 and 7 dpw. The first visual evaluation of the wound size was performed at 1-day post-wounding since a wound with clear borders had been noticed; additional inspections were performed at 4 and 7 days post-wounding. At 4 and 7 dpw, immersion treatment with Piperin AgNPs showed apparent and quicker wound closure than the control (Fig. 4). At 7 dpw, there was no sign of a wound in the piperin derived AgNP-treated groups.

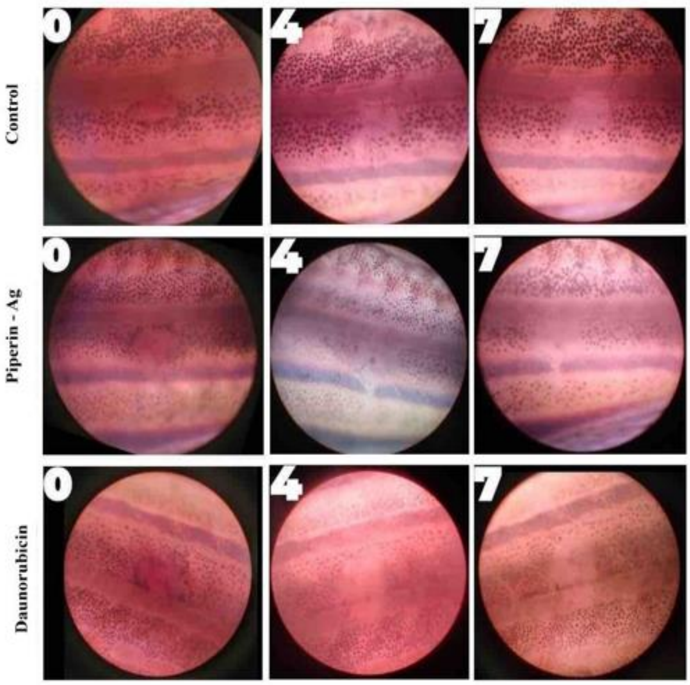


Figure 4: Wound Healing effects of Piperin derived AgNps on Zebrafish

The respective post wound days were indicated as numerical (n=6).

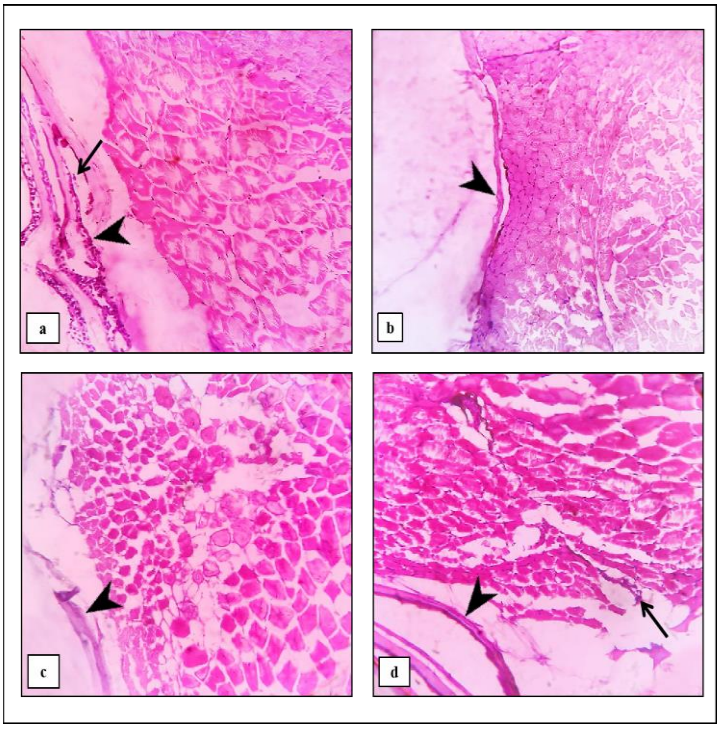


Figure 5: Photomicrographs showing the histopathology of wounded skin tissues of adult zebrafish. a) Control, b) Daunorubicin, c (7µg/ ml) and d (70µg/ ml) Piperin- derived Ag, stained with Haematoxylin & Eosin. Magnification: 40X. Thick black arrow: development of epithelial layer; thin black arrow: thin epithelium with immune cell infiltration

The histological pictures of the AgNPs direct application group and the AgNPs immersion group at 7 dpw were compared with those of the untreated control zebrafish (Fig. 3a in a transverse section through the mechanical -wounded tissues (skin and muscle). In the AgNPs-treated groups compared to the controls, H&E stained sectioning revealed greater epidermis and dermis that had recovered. With a thin layer of epithelium (neo-epithelium), the wound edge distance was longer in the injured tissues that had not been treated (Fig. 4 b and c). The wound cavity was deeper in the untreated fish (control group) than it was in the fish treated with AgNPs. In contrast to the control, it was clear that epithelial cells had re-surfaced the wound in the AgNPs-treated fish, resulting in a thick epithelium with a densely packed dermal layer and well-forming granulation of tissues.

The skin area that has a wound (Arrow-heads) exhibits the elimination of skin tissue layers like the epidermis and dermis, together with the outer scales as a result of the wound's development. A thin, recently developed epidermal layer with disorganized stratified epithelialization is seen in daunorubicin and piperin derived AgNPs groups. Granulation of the skin wound was also evident, along with the development of new connective tissue, inflammatory cell infiltration (Arrows), and microvasculature. AgNPs' influence on adult zebrafish wound healing. And area reduction (Figure. 6). Illustrations of the zebrafish wound-healing process at 4 and 7 dpw, images show that zebrafish in AgNPs-treated groups had considerably less wounds than those in the control groups.

The graph shown below represents the wound areas in each group on day 0, day 4 and day 7. The percentage values of the wound areas shown in the graph are obtained after analysis of each of the images using ImageJ software. Negative control group showed an area reduction of 26% after 7 days, whereas the positive control group with Daunorubicin showed a reduction of 93%. Group 1 and group 2 showed a wound area reduction of 59% and 96% respectively.

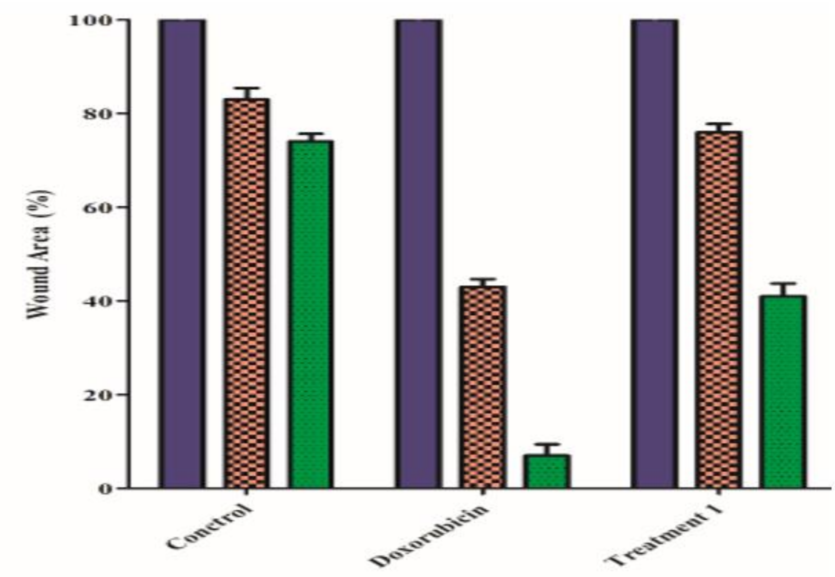


Figure 5: Effects of AgNPs on adult zebrafish wound healing and Wound area reduction

Bars represent the mean ± SD (n = 6).

The graph shown below represents the wound areas in each group on day 0, day 4 and day 7. The percentage values of the wound areas shown in the graph are obtained after analysis of each of the images using ImageJ software. Negative control group showed an area reduction of 26% after 7 days, whereas the positive control group with Daunorubicin showed a reduction of 93%. Group 1 and group 2 showed a wound area reduction of 59% and 96% respectively. The images used to obtain the percentage values are given below.

DISCUSSION

The quest for effective wound healing therapies has led researchers to explore novel compounds and innovative approaches. Two notable studies by Saeed Ali Alsareii et al. and Seung Beom Seo et al. have contributed significantly to this field by investigating the wound healing potential of piperine and silver nanoparticles (AgNPs), respectively. Our study builds upon their findings, focusing on the synergistic effects of piperine-coated AgNPs in promoting wound healing using zebrafish models. Alsareii et al. demonstrated the potential of piperine as a wound healing agent through their research involving a piperine-containing hydrogel applied to rat excision wounds. In line with their findings, our investigation explored the wound healing effects of piperine-coated AgNPs using zebrafish models.

While Alsareii et al. observed enhanced wound healing in rat models with their piperine hydrogel, our study provides complementary insights into the potential mechanisms underlying piperine's wound healing effects in a different biological context. Similarly, Seo et al. delved into the wound-healing potential of AgNPs in zebrafish. Their research demonstrated accelerated wound closure with AgNPs, which aligns with our findings of enhanced wound healing with our piperine-coated AgNPs intervention. Despite using distinct nanoparticles, both investigations underscored the efficacy of nanomaterials in promoting wound healing processes. Furthermore, both studies observed gene expression changes associated with wound healing, suggesting a shared mechanism driving these effects.

The synergy between our results and those of Seo et al. reinforces the potential of nanoparticle-based interventions for facilitating wound healing. It also provides a robust foundation for future mechanistic inquiries in this area. By leveraging different biological models and approaches, we can gain a more comprehensive understanding of the mechanisms underlying the therapeutic effects of these interventions. Piperine, a bioactive compound found in black pepper, possesses anti-inflammatory, antioxidant, and antimicrobial properties, all of which are crucial for the wound healing process.

Coating AgNPs with piperine not only enhances the biocompatibility and stability of the nanoparticles but also synergistically harnesses the therapeutic benefits of both compounds. However, further investigation is warranted to elucidate the precise mechanisms underlying the wound healing effects of piperine-coated AgNPs, including their interactions with cellular signaling pathways involved in tissue repair and regeneration. In conclusion, the collective findings from Alsareii et al., Seo et al., and our study highlight the promising translational applications of piperine and AgNPs for wound healing across different species and contexts. By unraveling the mechanisms underlying these effects, we can pave the way for the development of novel therapeutic strategies to improve wound management and patient outcomes.

CONCLUSION

Research on silver nanoparticles (AgNPs) in wound healing, alongside piperine-coated AgNPs, highlights the importance of understanding molecular mechanisms and temporal expressions. AgNPs show promise for wound care, but further research is crucial to fully utilize their therapeutic benefits.

Investigating AgNPs' roles, mechanisms, and safety in wound healing across diverse models is essential. Zebrafish studies indicate AgNPs' potential, prompting broader investigations. Clarifying AgNPs' significance in wound healing across species is pivotal for clinical applications. In conclusion, while AgNPs offer promise, ongoing research is vital to optimize their efficacy and safety in diverse biological contexts, potentially revolutionizing wound healing treatments.

References

1) Gosain A, DiPietro LA. Aging and Wound Healing. World J Surg. 2004 Feb 17;28(3):321–6.

2) de Lima Lima T de P, Passos MF. Skin wounds, the healing process, and hydrogel-based wound dressings: a short review. J Biomater Sci Polym Ed [Internet]. 2021 Jul 20 [cited 2023 Aug 22]; Available from: https://www.tandfonline.com/doi/abs/10.1080/09205063.2021.1946461

3) Chokkattu JJ, Mary DJ, Shanmugam R, Neeharika S. Embryonic Toxicology Evaluation of Ginger- and Clove-mediated Titanium Oxide Nanoparticles-based Dental Varnish with Zebrafish. J Contemp Dent Pract. 2022 Nov 1;23(11):1157–62.

4) Howe K, Clark MD, Torroja CF, Torrance J, Berthelot C, Muffato M, et al. The zebrafish reference genome sequence and its relationship to the human genome. Nature. 2013 Apr 17;496(7446):498–503.

5) Skin and scale regeneration after mechanical damage in a teleost. Mol Immunol. 2018 Mar 1;95:73–82.

6) Naomi R, Bahari H, Yazid MD, Embong H, Othman F. Zebrafish as a Model System to Study the Mechanism of Cutaneous Wound Healing and Drug Discovery: Advantages and Challenges. Pharmaceuticals. 2021 Oct 18;14(10):1058.

7) Richardson RJ. Parallels between vertebrate cardiac and cutaneous wound healing and regeneration. npj Regenerative Medicine. 2018 Nov 7;3(1):1–9.

8) Inflammation and wound repair. Semin Immunol. 2014 Aug 1;26(4):315–20.

9) Richardson R, Hammerschmidt M. The role of Rho kinase (Rock) in re-epithelialization of adult zebrafish skin wounds. Small GTPases [Internet]. 2016 Nov 1 [cited 2023 Aug 22]; Available from: https://www.tandfonline.com/doi/abs/10.1080/21541248.2016.1219208

10) Adult Zebrafish as a Model System for Cutaneous Wound-Healing Research. J Invest Dermatol. 2013 Jun 1;133(6):1655–65.

11) Nasim I, Jabin Z, Kumar SR, Vishnupriya V. Green synthesis of calcium hydroxide-coated silver nanoparticles using and Linn. leaf extracts: An antimicrobial and cytotoxic activity. J Conserv Dent. 2022 Aug 2;25(4):369–74.

12) Alsareii SA, Ahmad J, Umar A, Ahmad MZ, Shaikh IA. Enhanced In Vivo Wound Healing Efficacy of a Novel Piperine-Containing Bioactive Hydrogel in Excision Wound Rat Model. Molecules. 2023 Jan 5;28(2):545.

13) Silver nanoparticles enhance wound healing in zebrafish (Danio rerio). Fish Shellfish Immunol. 2017 Sep 1;68:536–45.

14) Akshaya, A., & Ganesh, K. (2022). Knowledge, prevalence, and impact on social distancing on coronavirus (COVID-19) infection spread in young, adult, and aged population: a survey. *ECS Transactions, 107*(1), 14063.

15) BABU, B. V., & MOHANRAJ, K. G. (2020). Knowledge, frequency and health hazards of overuse of mobile phone among Chennai population-A questionnaire based survey. *International Journal of Pharmaceutical Research (09752366), 12*(4).

16) Chockalingam, S., Sasanka, K., Babu K, Y., Ramanathan, V., & Ganapathy, D. (2020). Role of Bruxism in Prosthetic Treatments-A Survey. *Indian Journal of Forensic Medicine & Toxicology, 14*(4).

17) Kumaresan, A., Suganthirababu, P., Srinivasan, V., Vijay Chandhini, Y., Divyalaxmi, P., Alagesan, J., . . . Prathap, L. (2022). Prevalence of burnout syndrome among Work-From-Home IT professionals during the COVID-19 pandemic. *Work, 71*(2), 379-384.

18) Prathap, L., & Lakshmanan, G. (2022). Evaluation of incidence of various types of coronoid process in South Indian population. *Journal of Pharmaceutical Negative Results*, 1387-1390.

19) Varshan, I., & Prathap, L. (2022). Evaluation of mandibular condylar morphology using orthopantomogram in South Indian population. *Journal of Advanced Pharmaceutical Technology & Research, 13*(Suppl 2), S530-S533.