

IN VITRO AND IN VIVO STUDY OF N-ACETYLCYSTEINE (NAC) AND ZINC OXIDE NANOPARTICLES (ZNONP) ON BONE-MARROW MESENCHYMAL STEM CELLS (BM-MSCS) IN ALBINO RATS.

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ABSTRACT:

Zinc oxide nanoparticle (ZnONP), one of the most important metal oxide nanoparticles, has a wide range of applications due to their unique physical and chemical properties. Zinc is an essential trace mineral found in all human tissues, including the brain, muscles, bones, and skin. Zinc helps the body's metabolism and is required for protein, nucleic acid synthesis, hematopoiesis and neurogenesis. ZnONP have medicinal applications for diabetes, cancer, drug delivery and inflammatory conditions. N-acetylcysteine (NAC), an antioxidant, has been shown to be cytoprotective after exposure to cellular-damaging chemicals such as reactive oxygen species, it is also a free radical scavenger that helps prevent damage in numerous tissues. MSCs contain anti-inflammatory and antioxidant characteristics as well as the ability to repair damage. Preconditioning MSCs with NAC has been demonstrated to be cytoprotective

This research aims to define the protective role of NAC and effectiveness of ZnONP on bone marrow mesenchymal stem cells (BM-MSCs). In comparison to ZnONP working individually on BM-MSCs, the synergistic effect of NAC and ZnONP has proven to be a promising approach. *In vitro* and *in vivo* evaluation was done using MTT, ELISA, nitrite determination and LDH. Glutathione (GSH), superoxide dismutase (SOD), catalase (CAT), and malodialdehyde (MDA) activities are measured to evaluate oxidative stress. The investigation has been done with NAC and ZnONP to study the impact on BM-MSCs. The NAC treatment promoted the efficacy of ZnONP. A significant increase was measured in 5mMZnONP+NAC and 10mMZnONP+NAC in CAT, GSH, SOD including ELISA parameters meanwhile a decrease in MDA, nitrite determination and LDH was observed. The findings suggest that combination of ZnONP and NAC increased BM-MSCs efficacy. Preconditioning of NAC improves protection and effectivity of ZnONP.

KEYWORDS: Bone-marrow Mesenchymal Stem Cells (BM-MSCs), N-acetylcysteine (NAC), Zinc-oxide Nanoparticles (ZnONP), *in vitro* analysis, *in vivo* analysis, antioxidant, preconditioning.

INTRODUCTION:

Nanomaterial through antibacterial, anti-inflammatory and angiogenic activities can activate a variety of supporting cellular and molecular mechanisms for the microenvironment of wound and may even shift the environment from non-healing to healing (Kushwaha *et al.*, 2022). Because of their widespread biological characteristics and potential applications in medicine, biomedical nanomaterials have recently gained attention. Metal oxide nanoparticles have intriguing broad implementations in biomedical areas, particularly for antibacterial, anticancer, cell imaging, bio sensing and other applications (Mishra *et al.*, 2017). Because of its physical and chemical properties ZnONP is one of the most important metal oxide nanoparticles, are widely used in a variety of sectors. (Smijis and Pavel, 2011). Nanotechnology, in its broadest sense, refers to the molecular-scale manipulation of matter. This is concerned with the development of constructs and possibly technology known as nanomaterials, which range in particle size from 1 to 100 nm (Nel *et al.*, 2006).

Zinc is an essential component that is found in all bodily tissues, notably the brain, skin, bone and muscle. As the primary component of different enzyme systems, zinc contributes to the body's metabolism and performs important functions in protein and nucleic acid production, hematopoiesis, and neurogenesis (Ruszkiewicz *et al.*, 2017; Kolodziejczak and Jesionowski, 2014). ZnONP are considerably cheaper as compared to various metal oxide NPs, having excellent medicinal applications in treatment for diabetes, cancer, drug delivery, and inflammatory conditions, as well as wound healing and bio imaging (Kim *et al.*, 2017; Zhang and Xiong, 2015). Antioxidants like NAC have been demonstrated to be cytoprotective after exposure to cellular-damaging substances like reactive oxygen species. NAC is a precursor to Glutathione (GSH), a cellular antioxidant that effectively removes oxygen species such hydrogen peroxide, superoxide, and lipid peroxides that can harm DNA

and cells. Numerous studies have shown that NAC provides strong defense against the genomic and cell damage brought on by stress (Albini *et al.*, 2001).

NAC has demonstrated to improve collagen expression, wound healing, cell migration, and proliferation in addition to that NAC promotes angiogenesis and epidermal maturity, which speeds up the healing of wounds (Tsai *et al.*, 2014). According to studies, ZnONP cytotoxic effects on cancerous cells depend on their size, with smaller nanoparticles showing higher toxicity (Nair *et al.*, 2009). Despite all these advantages, NAC therapeutic effects are constrained by its low bioavailability (6 - 8%). This occurs due to drug joining with plasmatic proteins and develop disulfide bridges once it reaches the bloodstream (Navath *et al.*, 2008). When combined, they offer a promising way to boost NAC and ZnONP protective effects on BM-MSCs. MSCs exhibit anti-inflammatory and antioxidant properties, as well as the potential to repair damage. It has been demonstrated that preconditioning MSCs with NAC has cytoprotective effects (Ma *et al.*, 2019).

The purpose of this study was to analyze protective role of NAC and effectiveness of ZnONP on BM-MSCs. The current work examines efficacy of the preconditioning of NAC and ZnONP on BM-MSCs including *in vitro* and *in vivo* study of albino rats.

MATERIALS AND METHODS:

All study had been done after receiving consent from the Institutional Animal Ethics Committee Institute of Molecular Biology and Biotechnology (IMBB), The University of Lahore, Lahore, Pakistan.

Chemicals:

N-Acetyl-L-cysteine (NAC), ZnONP (<100 nm, purity 99,9%), and all other compounds were purchased from Sigma-Aldrich (St. Louis, Missouri, USA).

ZnONP solution preparation:

ZnONP from Sigma Aldrich (Cat. no. 544906; size: <100 nm, purity: 99,9%) were used. Stock solutions were prepared by sonicating culture media for two hours at dosage level of 10 mg mL⁻¹. Prior to cell incubation, the stock suspensions were diluted in complete culture media supplemented with 5% fetal bovine serum, 2mM L-glutamine and 1% penicillin/streptomycin to produce the varied concentrations employed in this work. Prior to each delivery, ZnONP suspensions were vortexed for 1 minute.

Primary culture of rat BMSCs:

Rats weighing 100 to 150 g were euthanized via cervical dislocation. Following ethanol disinfection, the tibia and femur were separated via incision. After the undesirable muscles were removed, the femur was maintained in ice-cold Dulbecco Modified Eagle Medium (DMEM) without fetal bovine serum (FBS). A syringe needle was used to vigorously wash the bone marrow out. After being centrifuged for 5 min at 1500 rpm, the cells were separated. A T25 flask was seeded with the isolated cell suspension, and BM-MSCs was cultured in DMEM with 10% FBS at 37°C with 5% CO₂. The BM-MSCs were maintained in complete medium for further experiments. For experiment BM-MSCs at p2 were used.

Experimental Design:

Twenty male SD rats were randomly categorized into four groups (n = 5 in each group) as shown in Table 1.

Cytotoxicity assay:

By using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium) test, cytotoxic potential of NAC and ZnONP was evaluated. In 96-well plates, 1 × 10⁵ BM-MSCs cells were plated in 0.2 ml of medium each well. After incubation, the media from the wells was carefully removed for the MTT assay. After two to three PBS washes in each well, 200µl of MTT (5 mg/ml) was added to incubate cells for 4 hours after being exposed to ZnONP at various concentrations (5, 10, 20, 25, 50, and 70 µg/ml) for 1 hour. Using a micropipette, 1 ml of DMSO (a solubilizing reagent) into each well was added and incubated for 45 seconds. The appearance of purple color because of formazan crystal production indicated the presence of viable cells. The values were measured at 595 nm (Senthilraja and Kathiresan, 2015).

Evaluation of oxidative parameters:

Medium of treated groups was used to determine oxidative stress parameters and centrifugation were done at 12,000xg for 5 min to remove cell debris. Medium was used to determine oxidative stress parameters for instance, the levels of glutathione (GSH), catalase (CAT), superoxide dismutase (SOD), and lipid peroxidation (malondialdehyde; MDA). Oxidative stress estimation was done using previously published protocol of Ali F *et al.*, 2017.

Enzyme linked immunosorbent assay:

ELISA assays for VEGF, IGF-1, SDF-1, CXCR-4, THY-1, and β-actin were carried out according to Ali *et al.*, 2021 protocol. ELISA was performed via using 10µl protein from healed wound skin area and 90µl dilution buffer by adding into 96 wells microtiter plate. Then samples were incubated at 37°C for overnight at room temperature and washing was done by 100µl dilution buffer. Primary antibodies VEGF, IGF-1, SDF-1, CXCR-4, THY-1, and β-actin with volume of 100µl were added in all samples. After incubation for several hours, solution was discarded after 3 times washing. 10% BSA was added as blocking agent. Secondary antibody (Anti-Mouse and Anti-Rabbit) 100µl were added in all samples. After incubation at 37 °C for 2 hours washing was done with dilution buffer. TMB was used as a chromogenic substrate with volume of 100µl. A stop solution was used, and plate reading was done at 450 nm.

Lactate Dehydrogenase Assay (LDH):

In cell-free culture supernatants, LDH release was quantified using an enzymatic redox process, in which tetrazolium salt is converted to the colorful product, formazan. The process was carried out with a Cytotoxicity Detection Kit (LDH) (Roche, Switzerland) in accordance with the manufacturer's instructions. Supernatants from each culture solution were added to 96-well plates for the analysis, and each well was incubated with 100 μ L of a premixed test solution. At room temperature, the color response was established for 30 minutes. At 490 nm, the light absorption was measured.

Determination of Nitrite:

Griess reagent nitrite determination kit (ThermoFisher G-7921) was used to measure the amount of nitric oxide (NO) released according to manufacturer's instructions. To measure the amounts of NO, the medium from all experimental groups were collected.

Burn experimental model:

The rats were given anesthesia via intraperitoneal injection (IP) of ketamine and xylazine hydrochloride for second degree burn wounds. Later rats were placed on their backs and wounds were created. For burn experiments on rats same protocol was used as Ali *et al.*, 2023.

Measurement of wound area:

For wound contraction assay on Day 7, 14 and 21 reductions in wound surface were measured through wound contraction assay and later was calculated by standard graph paper (Masson *et al.*, 2020).

Statistical Evaluation:

For conducting statistical analyses and creating graphics, GraphPad Prism software was used. Data were expressed as mean $\pm$ standard deviation (SD). One-way analysis of variance (ANOVA) was used to compare the results among the experimental groups. A *p*-value < 0.05 was considered statistically significant.

RESULTS:**NAC induce BM-MSCs proliferation:**

Figure 1 shows results of MTT, there was increase in proliferation of BM-MSCs treated with 10mM NAC in comparison to 5mM, 20mM, 30mM NAC. There was progressive increase in number of viable BM-MSCs at concentration of 10mM NAC. The cell proliferation of BM-MSCs increased with preconditioning of NAC. The observed values are 10mM NAC 0.51 ± 0.0030 , 5mM NAC 0.46 ± 0.0012 , 20mM NAC 0.41 ± 0.0017 , 30mM NAC 0.42 ± 0.0026 , control 0.69 ± 0.0051 .

Effect of ZnONP on BM-MSCs cell viability:

ZnONP at a concentrations of 10 μ g/ml, 20 μ g/ml, 50 μ g/ml, 80 μ g/ml, 100 μ g/ml, 200 μ g/ml, 300 μ g/ml, 400 μ g/ml and 500 μ g/ml were analyzed for 1 h on BM-MSCs (Figure 2). Lower concentrations of ZnONP induce decline in rate of viable cells in comparison to higher ones. 500 μ g/ml (1.97 ± 0.140) increased cell viability in BM-MSCs. This result data suggest that ZnONP had a concentration-dependent effect on BM-MSCs cell growth. Whereas other groups are as follows control 2.82 ± 0.037 , 10 μ g/ml 1.37 ± 0.1710 , 20 μ g/ml 1.48 ± 0.203 , 50 μ g/ml 1.61 ± 0.192 , 80 μ g/ml 1.30 ± 0.140 , 100 μ g/ml 1.26 ± 0.0691 , 200 μ g/ml 1.44 ± 0.0311 , 300 μ g/ml 1.56 ± 0.123 , 400 μ g/ml 1.64 ± 0.137 , 500 μ g/ml 1.97 ± 0.030 .

BM-MSCs preconditioning with NAC before addition of ZnONP increase the cell viability number specially in 5mMNAC+ZnONP (1.13 ± 0.043) in addition to 10mM+NAC (0.78 ± 0.073) (Figure 3). While in control 1.66 ± 0.121 were observed.

Evaluation of ZnONP and NAC in oxidative stress:

Figure 4 shows activities of CAT, GSH, SOD and MDA measured in BM-MSCs treated with ZnONP, 5mMNAC+ZnONP and 10mMNAC+ZnONP. Preconditioning of BM-MSCs with NAC leads to less cellular damage in comparison with ZnONP treated group. Preconditioned groups treated with NAC before ZnONP shows effective results. BM-MSCs treated with ZnONP represents lower levels in GSH, SOD and CAT except MDA. 5mMNAC+ZnONP and 10mMNAC+ZnONP showed significant efficacy in GSH, SOD and CAT. 5mMNAC+ZnONP level were observed to be increased in CAT, SOD and GSH. Meanwhile for CAT observed measurements are control 1.77 ± 0.034 , ZnONP 1.24 ± 0.035 , 5mMNAC+ZnONP 1.56 ± 0.065 , 10mMNAC+ZnONP 1.46 ± 0.038 .

GSH measurements for experimental groups are as followed control 1.94 ± 0.0514 , ZnONP 1.23 ± 0.0315 , 5mMNAC+ZnONP 1.50 ± 0.049 , 10mMNAC+ZnONP 1.42 ± 0.087 . SOD measurements are as follows control 1.90 ± 0.098 , ZnONP 1.06 ± 0.086 , 5mMNAC+ZnONP 1.54 ± 0.052 , 10mMNAC+ZnONP 1.389 ± 0.07167 .

MDA assessment was observed as values are control 1.19 ± 0.042 , ZnONP 1.48 ± 0.092 , 5mMNAC+ZnONP 1.03 ± 0.035 , 10mMNAC+ZnONP 1.14 ± 0.024 .

ELISA analysis of ZnONP and NAC on BM-MSCs:

To monitor the effect of ZnONP and NAC on BM-MSCs release of growth factors (IGF-1, VEGF), progenitor cell mobilization (SDF-1, CXCR-4), β -actin, THY-1 was observed (Figure 5). ELISA activity data suggest pretreatment with NAC before addition of ZnONP showed increase in BM-MSCs cells in contrast to ZnONP treated group.

The levels of progenitor cell mobilization marker SDF and CXCR-4 showed significant results in ZnONP and 10mMNAC+ZnONP while 5mMNAC+ZnONP level were found to be increased. For growth factors (VEGF, IGF-1) 10mMNAC+ZnONP found to be highly significant, however ZnONP level were decreased in comparison to control and NAC+ZnONP treated groups (Table 2).

β -actin level showed increase in 5mMNAC+ZnONP while ZnONP found to be significant low. In THY-1 ZnONP were significantly lower in comparative to 5mMNAC+ZnONP and 10mMNAC+ZnONP. 5mMNAC+ZnONP levels were found to be elevated (Table 2). It has been found that 5mMNAC+ZnONP increased and ZnONP decreased in IGF-1, VEGF, SDF-1, CXCR-4, β -actin, and THY-1.

LDH Assay to evaluate effects of ZnONP and NAC:

LDH release was found to be significantly low in 5mMNAC+ZnONP (0.076 ± 0.0072). While other values are as follows control 0.11 ± 0.001 , ZnONP 0.09 ± 0.00033 , 10mMNAC+ZnONP 0.088 ± 0.00030 (Figure 6).

Nitrite Determination:

BM-MSCs treated with ZnONP (1.36 ± 0.098) represents high amount of nitric oxide in comparison to untreated cells and treated ones. In 5mMNAC+ZnONP (0.59 ± 0.027) significantly low rate of nitric oxide were observed whereas other group values are control 1.10 ± 0.060 , and 10mMNAC+ZnONP 0.940 ± 0.087 (Figure 7).

***In vivo* analysis:**

Impact of ZnONP and NAC on Nitrite levels:

Nitric oxide measurement was increased in ZnONP (0.135 ± 0.0010) on BM-MSCs (Figure 8). In addition to that Nitric oxide (NO) level found to be significantly decreased in 5mMNAC+ZnONP (0.090 ± 0.0054). In 10mMNAC+ZnONP (0.124 ± 0.0009) and control (0.130 ± 0.0168) measurement were observed.

Role of ZnONP and NAC in oxidative stress:

To analyse *in vivo* effects of NAC and ZnONP on BM-MSCs oxidative stress parameters CAT, GSH, SOD and MDA activities were assessed in all treated groups (Figure 9). ZnONP showed significantly increased MDA level and decreased CAT, GSH and SOD levels. A reduced level of MDA was found in 5mMNAC+ZnONP and 10mMNAC+ZnONP, while in CAT, GSD and SOD NAC preconditioned groups showed significant increase.

The values for CAT are as follows CAT control 1.77 ± 0.032 , ZnONP 1.25 ± 0.033 , 5mMNAC+ZnONP 1.57 ± 0.074 , 10mMNAC+ZnONP 1.43 ± 0.038 . GSH levels were measured as control 1.94 ± 0.040 , ZnONP 1.21 ± 0.022 , 5mMNAC+ZnONP 1.51 ± 0.035 , 10mMNAC+ZnONP 1.43 ± 0.099 .

SOD levels across all treated groups are as follows; control 1.90 ± 0.098 , ZnONP 1.06 ± 0.083 , 5mMNAC+ZnONP 1.54 ± 0.050 , 10mMNAC+ZnONP 1.38 ± 0.072 . For MDA the observed values were control 1.16 ± 0.046 , ZnONP 1.47 ± 0.092 , 5mMNAC+ZnONP 1.04 ± 0.037 , 10mMNAC+ZnONP 1.13 ± 0.023 .

ELISA responses to ZnONP and NAC:

The level of SDF-1, CXCR-4, VEGF, IGF-1, β -actin and THY-1 were determined by ELISA. As shown in Figure 10, ZnONP showed significantly decreased levels in progenitor cell mobilization (SDF-1, CXCR-4), growth factors (IGF-1, VEGF) and β -actin, THY-1 while 5mMNAC+ZnONP and 10mMNAC+ZnONP were found to be elevated.

The level of SDF showed significant results in ZnONP, 10mMNAC+ZnONP and CXCR-4 showed significant result in 10mMNAC+ZnONP, 5mMNAC+ZnONP, ZnONP. In VEGF ZnONP and 10mM+ZnONP were found to be significant. β -actin and THY-1 levels were found to be significant in ZnONP (Table 3).

Wound healing examination:

Figure 11 shows wound reduction post transplantation at Day 0, 7, 14 and 21. In all groups 5mM NAC+ZnONP showed higher rate of wound closing in comparisons to ZnONP group and 10mMNAC+ZnONP group.

Percentage of wound contraction following treatment of ZnONP and NAC:

The percentage of wound contraction for the groups after 7, 14 and 21 days are represented in which highest rate of wound contraction in 5mMNAC+ZnONP group were observed. Healing of wound activity after Day 7, 14 and 21 days is represented in the following graph (Figure 12). The highest rate of wound healing was seen after 21 days of treatment for all the models as values comprises of Day 7 1.54 ± 0.115 , Day 14 1.61 ± 0.12 , Day 21 1.61 ± 0.125 .

DISCUSSION:

The study successfully demonstrated the process of action of ZnONP on BM-MSCs. These nanoparticles generate oxidative stress in cells in concentration dependent manner once they have entered the cells. The affectivity of ZnONP in combination with NAC increases cell viability.

Globally, nanoparticles are receiving a lot of attention, and deliberate exposure in the form of nanomedicines for societal welfare is rising quickly. Due to nanoparticle appealing qualities, they have some uses in the medical profession, but if they are used improperly or without understanding their possible side effects may lead to unfavorable impacts. Benefits of employing *in vitro* model systems for predicting nanoparticle toxicity include

the ability to quickly and effectively evaluate how the particles interact with biological systems. Whereas *in vitro* research may also be used to construct mechanism-driven assessments, as well as to define concentration-effect connections and effect-specific limitations (Arora *et al.*, 2012).

Since BM-MSCs are the most often researched stem cells applied in applications for tissue engineering, particularly tissue regeneration, they were used as an *in vitro* system in our research effort. This investigation examined the impact of ZnONP on rat bone marrow MSCs *in vitro*, with addition of using NAC as a preconditioning antioxidant. Conventional MTT assay was used to determine NAC and ZnONP effectivity. NAC MTT data shows significant results at 5mM and 10mM, while in case of ZnONP result data suggest efficacy in concentration dependent manner. Lower concentrations of ZnONP demonstrate decrease in cell viability while increases in cell viability were observed at higher concentration of ZnONP. According to Kim *et al.*, 2019 ZnONP exhibits decreased in cell viability rate level at lower concentration mainly at 10-20µg/ml. Since they are tiny and have a relatively large surface area, nanoparticles tend to have size dependency. Nanomaterials cause cell-specific reactions that have varying levels of effects on cell types. The findings showed that intracellular reactions are significantly influenced by particle sizes as well as target cell types (Sahu *et al.*, 2016). ZnONP protection effects against kidney damage in rats has been reported by Barakat *et al.*, 2020 in which treatment with ZnONP showed renoprotective effectiveness through ZnONP anti-inflammatory, antioxidant and anti-apoptotic aspects. Herein, in our research we discovered that ZnONP cause cell viability in concentration dependent manner where BM-MSCs were subjected to different concentrations of ZnONP. Cell viability is significantly influenced by exposure duration and ZnONP content, both of which are critical variables. Exposure of ASCs to ZnONP for 24, 48, and 72 hours demonstrate substantial lower cell number at lower concentrations of ZnONP (Orazizadeh *et al.*, 2015). By releasing thiols from proteins by disulfide cleavage, NAC, a thiol and membrane-permeable cysteine precursor, can increase the intracellular cysteine pool and improve GSH-dependent detoxification. N-acetylcysteine prevents redox-sensitive nuclear factor kappa B from activating, which causes the release of several inflammatory cytokines and promotes the expression of proinflammatory genes during oxidative stress (Valencia *et al.*, 2024).

NAC raises intracellular glutathione levels, which makes it a significant antioxidant and free radical scavenger (Sun, 2010). NAC's thiol groups serve as a metal chelating site and a free radical reducer (Flora and Pachauri, 2010).

NAC may have a protective impact on metal nanoparticle toxicity through a number of ways. The most proposed way to lessen metal nanoparticle toxicity through preventing reactive oxygen species formation is NAC antioxidant qualities. The toxicity of metal nanoparticles may be decreased by NAC interaction with metal ions prior to uptake in the extracellular area. By conserving ions to prevent toxicity, NAC pre-incubation inhibits the production of reactive oxygen species and preserves the cell's metabolic function (Gholinejad *et al.*, 2018).

In addition to that BM-MSCs pretreatment with NAC led to a substantial drop increase in BM-MSCs rate specifically at concentration of 5mM. A study of zinc oxide nanoparticle on an invertebrate model also demonstrates that pre-treatment with NAC causes the drop in ROS, which ultimately causes a reduction in the number of cells dying (Mir *et al.*, 2020). NAC reported to be a strong antioxidant utilized to defend cells, a mucolytic, anti-inflammatory and hepatoprotective substance (Bulut and Sayar, 2015). Wu *et al.*, 2013 has demonstrated the effect of NAC (5mM) and ZnONP in nematode test species in which NAC successfully increased ZnONP effects. Similarly Kiss *et al.*, 2021 reported the same result data in which NAC have shown protective function in presence of ZnONP.

Pre-treatment with NAC also manifests notable effectivity in oxidative stress assays mainly in GSH, Catalase and SOD. BM-MSCs pre-treated with NAC exhibits higher levels of effectiveness in comparison to groups treated with ZnONP alone. Similar results were reported in ELISA assay analysis. Our results demonstrate that NAC could increase efficacy of zinc oxide nanoparticles in BM-MSCs and ZnONP effectiveness on BM-MSCs are mainly concentration reliant. Hamed *et al.*, 2015 suggests that against MDR pathogens, ZnONP has shown strong antibacterial action. Where they concluded that NAC and ZnONP have a potent synergistic effect against MDR bacteria, which may signal a paradigm shift in the treatment of various diseases.

In this investigation, it was evaluated that ZnONP have an effect on BM-MSCs that is concentration based. While cells treated with ZnONP showed less cell proliferation, comparatively cells preconditioned with NAC showed improved proliferative capacity. The main purpose of adding NAC was to examine its adjuvant effect on ZnONP at higher concentrations, which showed that NAC could increase ZnONP efficacy.

CONCLUSION:

Pretreatment with NAC before ZnONP administration increases cell viability in BM-MSCs. ZnONP exhibits a promising efficacy in BM-MSCs which could serve as potential for new developing therapies.

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Authors Contribution:

Nida Sadaqat executed the experiment and analyzed the primary culture, *in vitro* and *in vivo* assays. Fatima Ali designed the study and provided guidance in sample analysis and statistical analysis. Muhammad Atif analyzed the primary culture cells. Tahir Maqbool helped in editing.

Statement of conflict of interest

The authors have declared no conflict of interest.

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Tables and Figure Legends:

Table:

- **Table 1:** Rats were divided into four experimental groups.
- **Table 2:** *In vitro* ELISA values of ZnONP and NAC on BM-MSCs.
- **Table 3:** *In vivo* ELISA values of ZnONP and NAC on BM-MSCs.

Figures:

- Fig 1- Graphical representation of selection of NAC. All values are expressed as mean ± SEM. p value significance as ^{ns} p> 0.05, *p < 0.05, **p < 0.001, ***p <0.0001.
- Fig 2- Graphical representation of MTT result data of ZnONP on BM-MSCs. All values are expressed as mean ± SEM. p value significance as ^{ns} p> 0.05, *p < 0.05, **p < 0.001, ***p <0.0001
- Fig 3- Graphical representation of BM-MSCs preconditioning with NAC and ZnONP. All values are expressed as mean ± SEM. p value significance as ^{ns} p> 0.05, *p < 0.05, **p < 0.001, ***p <0.0001.
- Fig 4- Graphical representation of oxidative stress analysis of BM-MSCs with NAC and ZnONP including GSH, CAT, SOD and MDA. All values are expressed as mean ± SEM. p value significance as ^{ns} p> 0.05, *p < 0.05, **p < 0.001, ***p <0.0001.
- Fig 5- Graphical representation of assessment of ELISA markers on BM-MSCs. All values are expressed as mean ± SEM. p value significance as ^{ns} p> 0.05, *p < 0.05, **p < 0.001, ***p <0.0001.

- Fig 6- Graphical representation of LDH assay. All values are expressed as mean $\pm$ SEM. p value significance as ^{ns} $p > 0.05$, * $p < 0.05$, ** $p < 0.001$, *** $p < 0.0001$.
- Fig 7- Graphical representation of nitrite determination assay. NO value found to be lower in NAC+ZnONP groups. All values are expressed as mean $\pm$ SEM. p value significance as ^{ns} $p > 0.05$, * $p < 0.05$, ** $p < 0.001$, *** $p < 0.0001$.
- Fig 8- Graphical representations of *in vivo* nitrite determination assay. NO value found to be lower in NAC+ZnONP groups. All values are expressed as mean $\pm$ SEM. p value significance as ^{ns} $p > 0.05$, * $p < 0.05$, ** $p < 0.001$, *** $p < 0.0001$.
- Fig 9- Graphical representation of *in vivo* oxidative stress (CAT, GSH, SOD and MDA). NAC+ZnONP groups show high values in SOD, CAT, GSH except MDA. All values are expressed as mean $\pm$ SEM. p value significance as ^{ns} $p > 0.05$, * $p < 0.05$, ** $p < 0.001$, *** $p < 0.0001$.
- Fig 10 - Graphical representation of *in vivo* ELISA (SDF-1, CXCR-4, VEGF, IGF-1, β -actin, THY-1). All values are expressed as mean $\pm$ SEM. p value significance as ^{ns} $p > 0.05$, * $p < 0.05$, ** $p < 0.001$, *** $p < 0.0001$.
- Fig 11- Representative photographs of *in vivo* effect of ZnONP and 5mMNAC+ZnONP, 10mMNAC+ZnONP on wound healing process Day 0, 7, 14 and 21.
- Fig 12 - Graphical representation of *in vivo* wound healing measurements. All values were expressed as mean $\pm$ SEM. p value significance as ^{ns} $p > 0.05$, * $p < 0.05$, ** $p < 0.001$, *** $p < 0.0001$.

Graphs

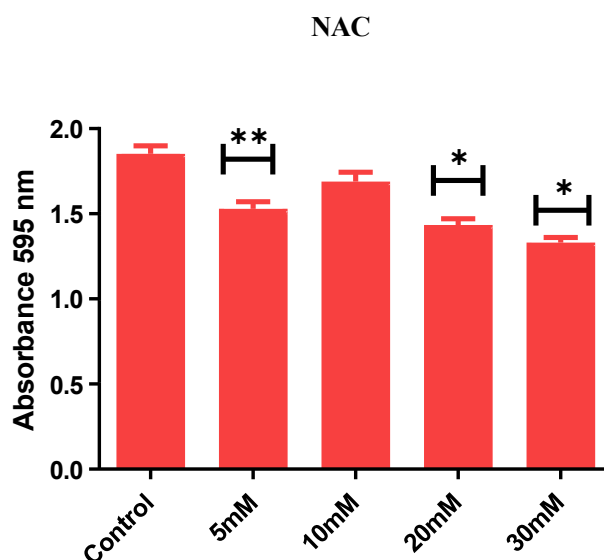


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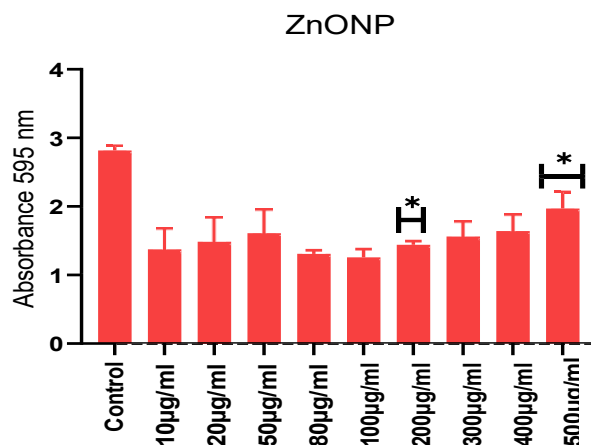


Fig 2- Graphical representation of MTT result data of ZnONP on BM-MSCs. All values are expressed as mean $\pm$ SEM. p value significance as ^{ns} $p > 0.05$, * $p < 0.05$, ** $p < 0.001$, *** $p < 0.0001$

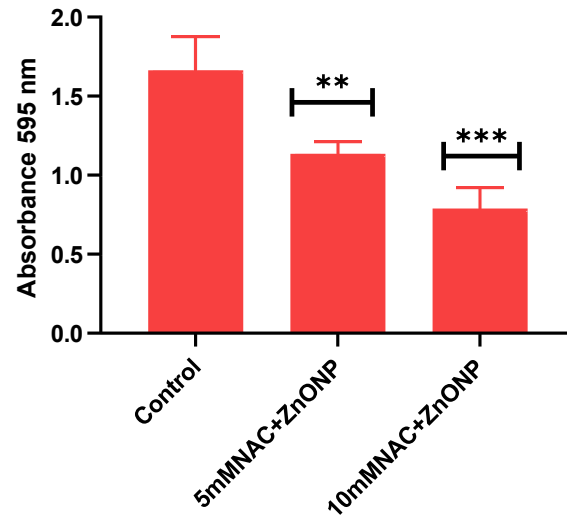


Fig 3- Graphical representation of BM-MSCs preconditioning with NAC and ZnONP. All values are expressed as mean $\pm$ SEM. p value significance as ^{ns} $p > 0.05$, * $p < 0.05$, ** $p < 0.001$, *** $p < 0.0001$.

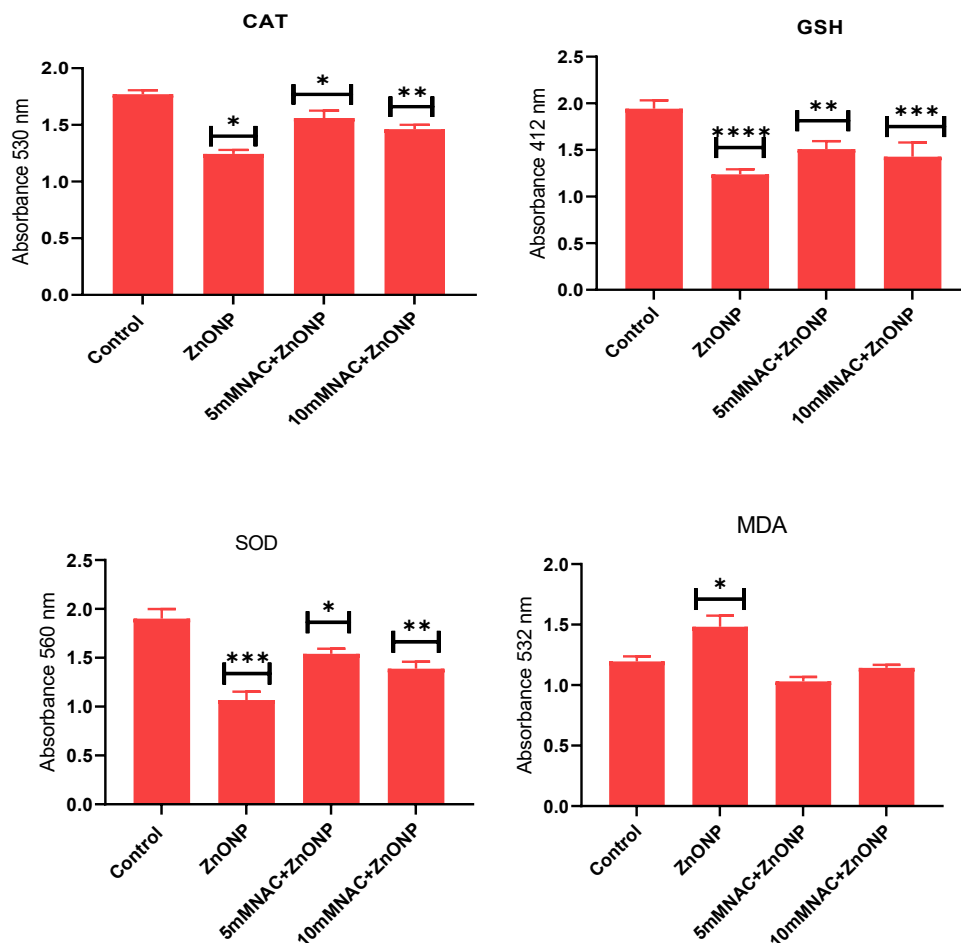


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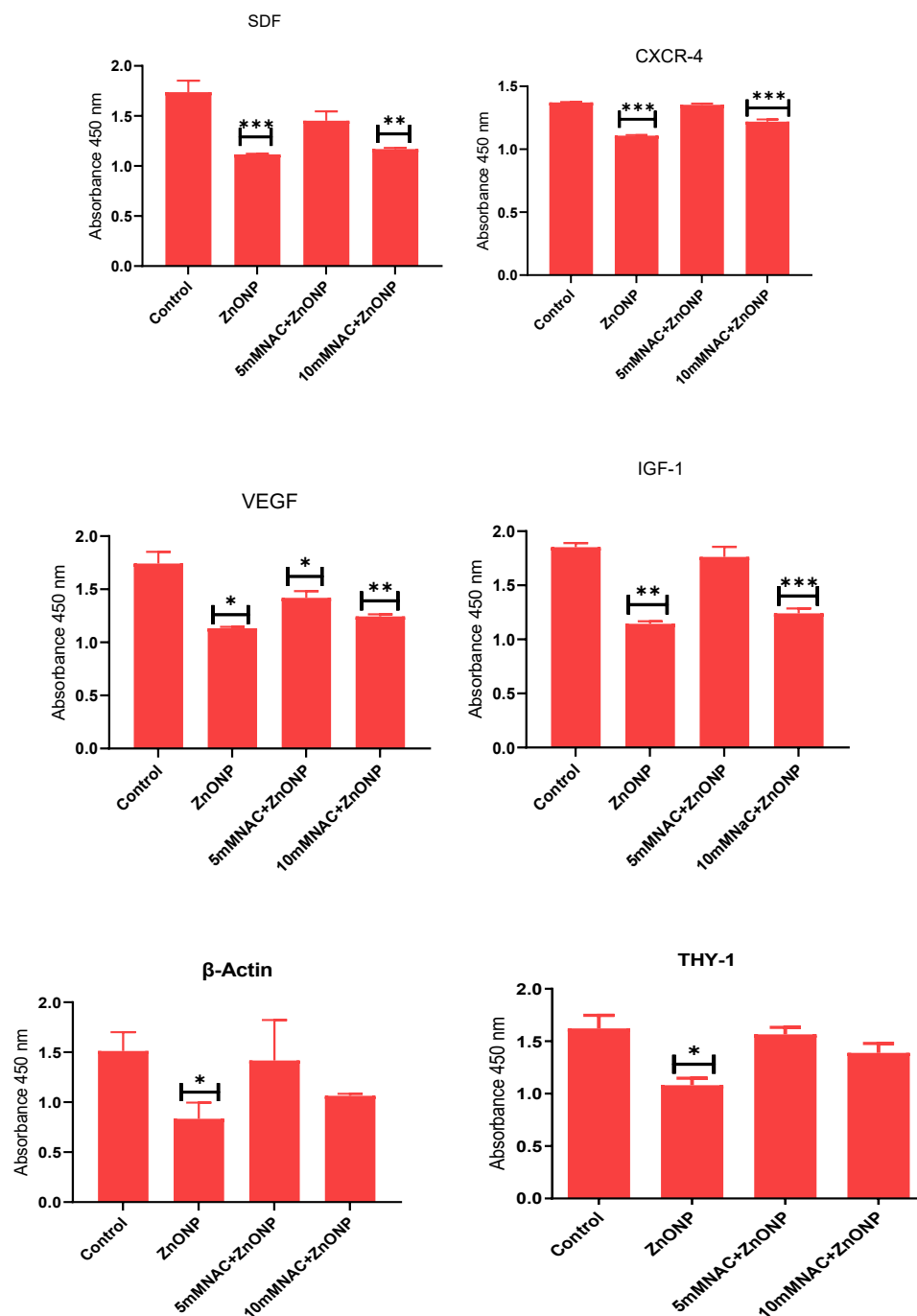


Fig 5- Graphical representation of assessment of ELISA markers on BM-MSCs. All values are expressed as mean $\pm$ SEM. p value significance as ^{ns} $p > 0.05$, * $p < 0.05$, ** $p < 0.001$, *** $p < 0.0001$.

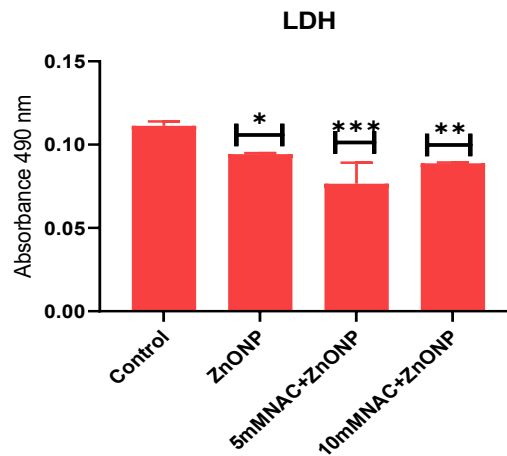


Fig 6- Graphical representation of LDH assay. All values are expressed as mean $\pm$ SEM. p value significance as ^{ns} $p > 0.05$, * $p < 0.05$, ** $p < 0.001$, *** $p < 0.0001$.

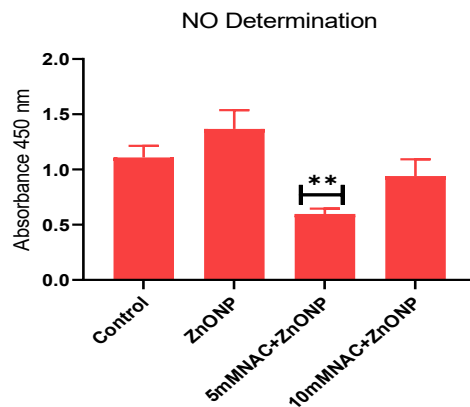


Fig 7- Graphical representation of nitrite determination assay. NO value found to be lower in NAC+ZnONP groups. All values are expressed as mean $\pm$ SEM. p value significance as ^{ns} $p > 0.05$, * $p < 0.05$, ** $p < 0.001$, *** $p < 0.0001$.

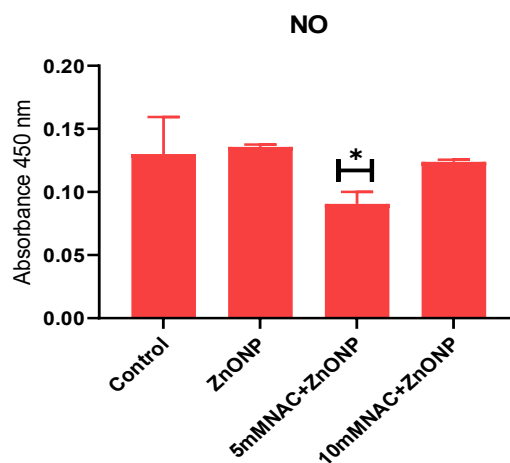


Fig 8- Graphical representations of *in vivo* nitrite determination assay. NO value found to be lower in NAC+ZnONP groups. All values are expressed as mean $\pm$ SEM. p value significance as ^{ns} $p > 0.05$, * $p < 0.05$, ** $p < 0.001$, *** $p < 0.0001$.

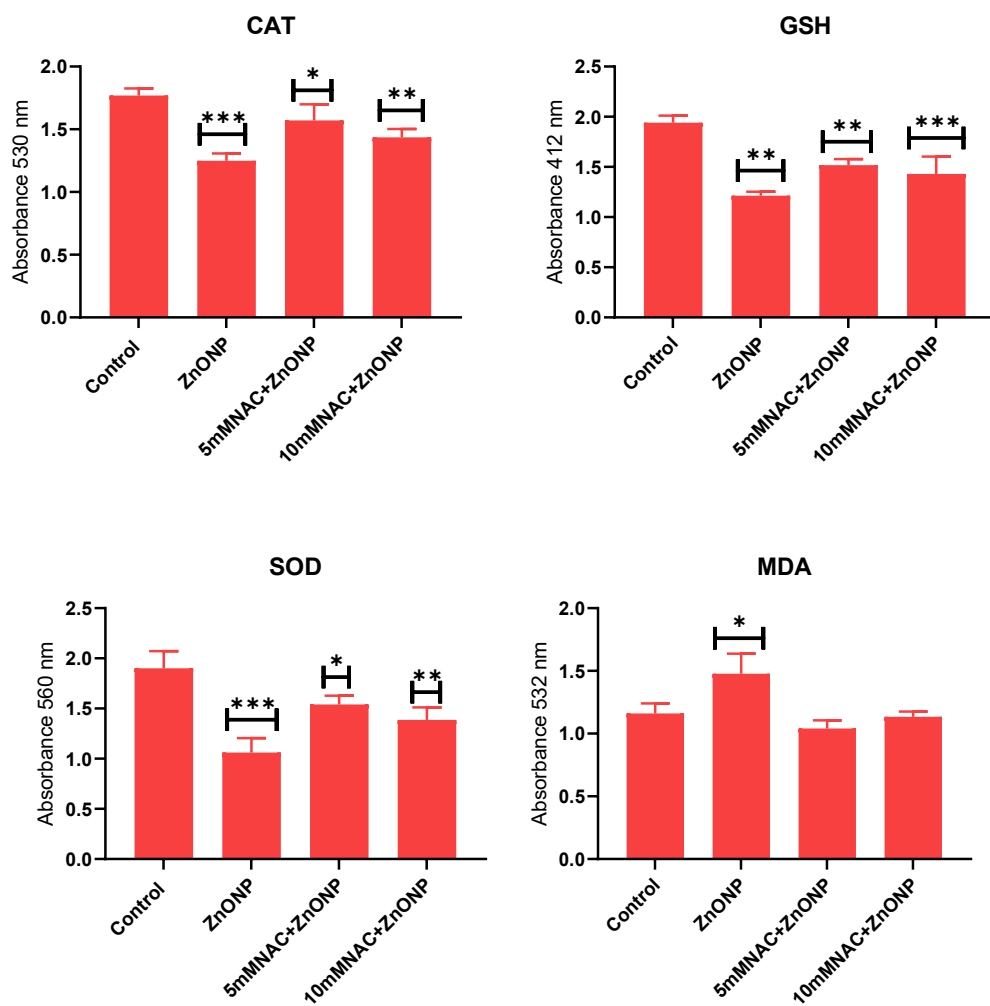


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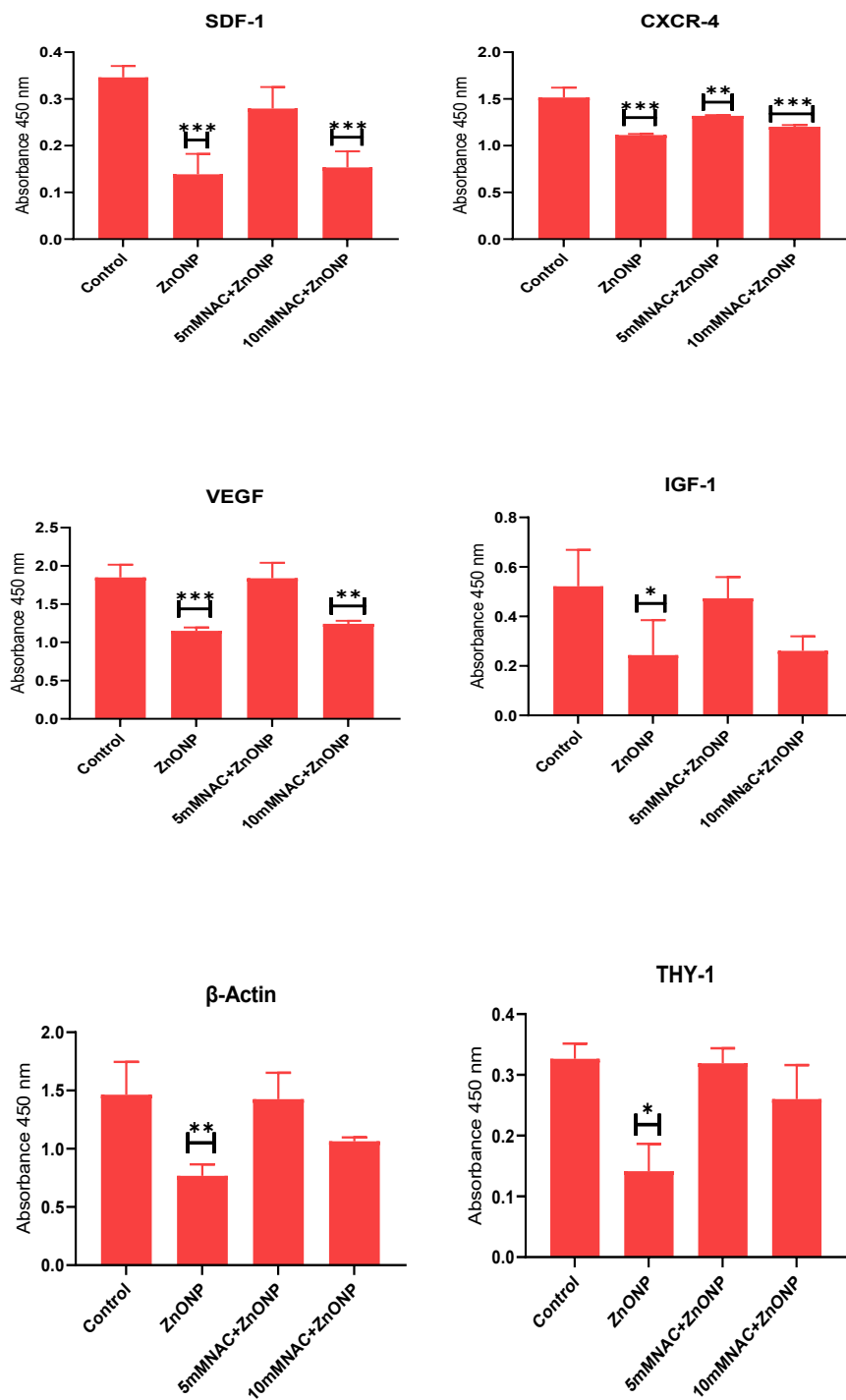


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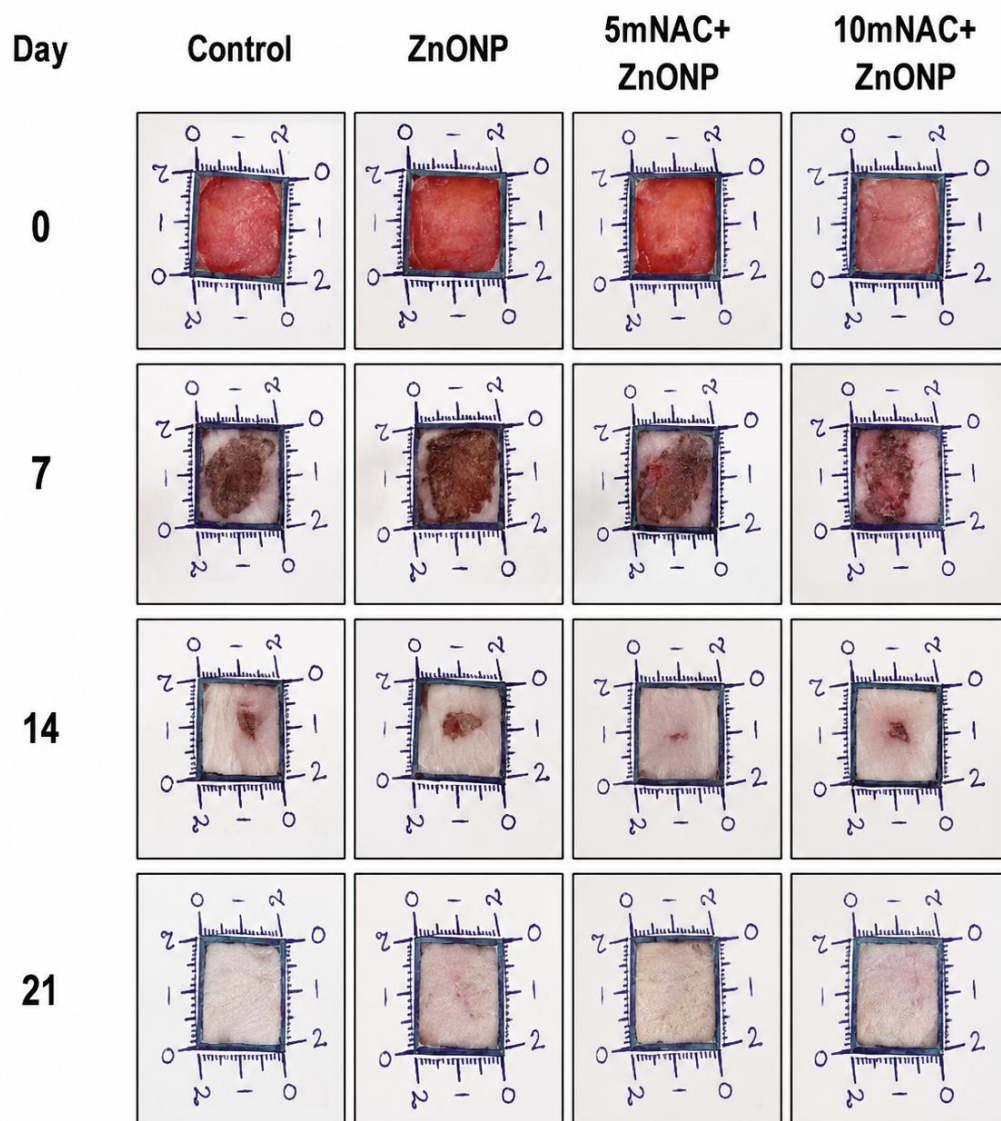


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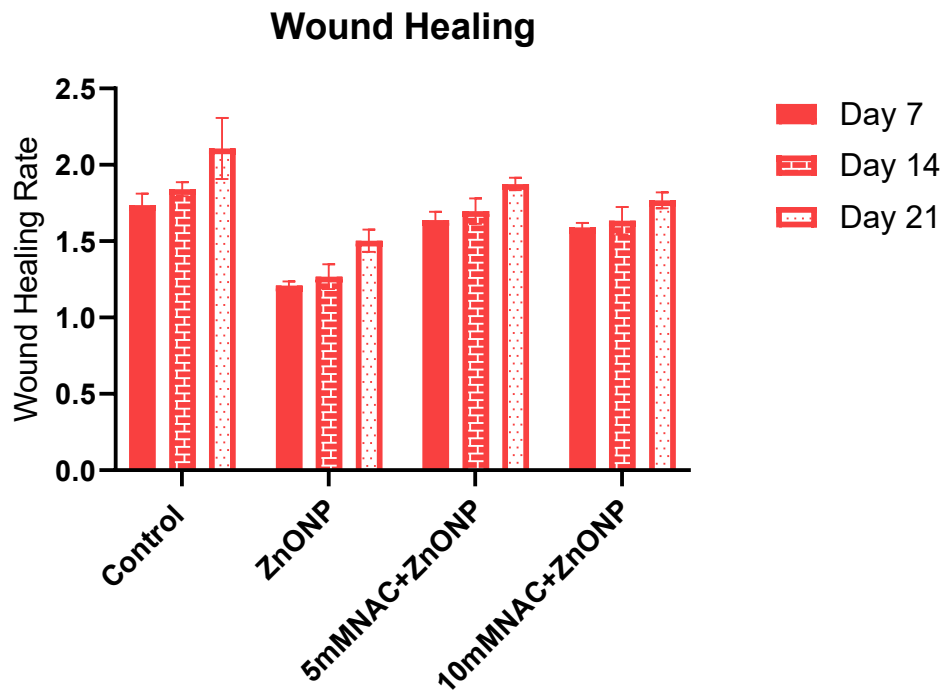


Fig 12 - Graphical representation of *in vivo* wound healing measurements. All values were expressed as mean $\pm$ SEM. p value significance as ^{ns} p > 0.05, *p < 0.05, **p < 0.001, ***p < 0.0001.

Table

Table 1: Rats were divided into four experimental groups.

Groups Name	Description of Experimental Groups	Abbreviations of Groups
Control group	Normal BM-MSCs	Control
ZnONP	BM-MSCs treated with ZnONP	ZnONP
5mM NAC and ZnONP	BM-MSCs preconditioned with NAC and treated with ZnONP	5mMNAC+ZnONP
10mM NAC and ZnONP	BM-MSCs preconditioned with NAC and treated with ZnONP	10mMNAC+ZnONP

Table 2: *In vitro* ELISA values of ZnONP and NAC on BM-MSCs.

ELISA Markers	Control	ZnONP	5mM+ZnONP	10mM+ZnONP
SDF-1	1.73 $\pm$ 0.115	1.11 $\pm$ 0.0071	1.45 $\pm$ 0.094	1.17 $\pm$ 0.0118
CXCR-4	1.37 $\pm$ 0.002	1.10 $\pm$ 0.0047	1.35 $\pm$ 0.0109	1.21 $\pm$ 0.0184
VEGF	1.74 $\pm$ 0.108	1.13 $\pm$ 0.013	1.42 $\pm$ 0.063	1.24 $\pm$ 0.019
IGF-1	1.85 $\pm$ 0.039	1.14 $\pm$ 0.022	1.76 $\pm$ 0.093	1.24 $\pm$ 0.446
β -Actin	1.51 $\pm$ 0.105	0.83 $\pm$ 0.092	1.41 $\pm$ 0.234	1.06 $\pm$ 0.012
Thy-1	1.62 $\pm$ 0.096	1.08 $\pm$ 0.064	1.56 $\pm$ 0.066	1.38 $\pm$ 0.089

Table 3: *In vivo* ELISA values of ZnONP and NAC on BM-MSCs.

ELISA Markers	Control	ZnONP	5mM+ZnONP	10mM+ZnONP
SDF-1	0.34 $\pm$ 0.013	0.13 $\pm$ 0.025	0.27 $\pm$ 0.026	0.15 $\pm$ 0.019
CXCR-4	1.51 $\pm$ 0.060	1.11 $\pm$ 0.0047	1.35 $\pm$ 0.001	1.21 $\pm$ 0.018
VEGF	1.84 $\pm$ 0.096	1.15 $\pm$ 0.025	1.84 $\pm$ 0.116	1.24 $\pm$ 0.021
IGF-1	0.52 $\pm$ 0.084	0.24 $\pm$ 0.081	0.47 $\pm$ 0.049	0.26 $\pm$ 0.033
β -Actin	1.46 $\pm$ 0.162	0.76 $\pm$ 0.056	1.42 $\pm$ 0.131	1.06 $\pm$ 0.018
Thy-1	0.32 $\pm$ 0.145	0.14 $\pm$ 0.026	0.31 $\pm$ 0.014	0.26 $\pm$ 0.032