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Research Article

ANTI-LEISHMANIAL EFFECTIVENESS OF MILTEFOSINE-LOADED MESOPORUS ZINC OXIDE NANOPARTICLES IN MACROPHASES OF RAW 264.7 CELL LINES: AN *IN-VITRO* EVALUATION

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ABSTRACT

Objectives: Visceral leishmaniasis (VL) is a deadly parasitic disease lacking safe and effective treatments. This study investigated the anti-leishmanial efficacy of a novel compound, miltefosine-loaded Mesoporus Zinc Oxide nanoparticles (MF-ZnONPs), *in vitro* using the murine macrophage RAW 264.7 cell line.

Methods: RAW 264.7 cells were cultivated and exposed to *Leishmania donovani* promastigotes with or without MF-ZnONPs treatment, and a battery of assays was performed. Cell viability was assessed by MTT assay, parasite growth by microscopy and trypan blue exclusion, and cellular effects by nuclear staining (DAPI) and ethidium bromide/acridine orange dual staining. A scratch wound assay evaluated the impact on cell migration, and reactive oxygen species (ROS) generation in promastigotes was measured.

Results: MF-ZnONPs were non-toxic to macrophages at concentrations up to 10%, while significantly impairing *L. donovani* promastigote viability. Treated macrophages showed preserved nuclear integrity and reduced apoptosis/necrosis compared to infected cells without treatment. MF-ZnONPs also mitigated parasite-induced inhibition of wound healing in macrophages and induced a marked increase in ROS within promastigotes.

Conclusion: The compound MF-ZnONPs demonstrated potent anti-leishmanial activity *in vitro*, killing parasites and protecting host macrophages. These findings suggest MF-ZnONPs as a promising candidate for VL therapy, warranting further evaluation in animal models to confirm efficacy and safety *in vivo*.

Keywords: Visceral leishmaniasis, Miltefosine-loaded nanoparticles, Mesoporous zinc oxide, RAW 264.7 macrophages, Anti-leishmanial activity.

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INTRODUCTION

Visceral leishmaniasis (VL), also known as kala-azar, is one of the most severe parasitic diseases worldwide and is considered a high-priority neglected tropical disease by the World Health Organization. Globally, VL is responsible for an estimated 200,000–400,000 new infections each year, with over 90% of cases concentrated in parts of South Asia (notably India, Bangladesh, and Nepal), East Africa (Sudan, Ethiopia, Kenya, and Somalia), and South America (particularly Brazil), as shown in Fig. 1 [1,2]. VL is transmitted by the bite of infected female phlebotomine sand flies, which inoculate the flagellated promastigote form of *Leishmania* into the human host. The parasites are phagocytosed by host macrophages and transform into non-flagellated amastigotes that proliferate within cells. Infection typically involves the spleen, liver, and bone marrow, leading to symptoms such as prolonged fever, weight loss, anemia, and massive hepatosplenomegaly. If left untreated, VL is generally fatal, with a nearly 100% mortality rate reported in endemic regions [3-5]. Past epidemics have had devastating impacts; for example, an outbreak in the 1990s in Sudan caused an estimated 100,000 deaths [6]. Despite the gravity of VL, current therapeutic options are limited and fraught with challenges. Pentavalent antimonial drugs were long the first-line treatment, but their widespread use (especially in the Indian subcontinent) has led to extensive drug resistance [7,8]. Moreover, existing medications such as sodium stibogluconate, amphotericin B (including its liposomal form), paromomycin, and the oral drug miltefosine can cause serious adverse effects (e.g., hepatotoxicity, nephrotoxicity, gastrointestinal distress, teratogenicity) and require prolonged treatment courses. These drawbacks, combined with high costs and the emergence of drug-resistant *Leishmania* strains, result in many patients discontinuing therapy prematurely [9,10]. To date, there is no effective vaccine available for human leishmaniasis. This situation underscores an urgent need for new, safer, and more affordable anti-leishmanial agents. Researchers are increasingly turning to novel compounds, including those derived from plants or other natural sources, as potential alternatives. In this context, the present study examines the anti-leishmanial role of a new compound, miltefosine-loaded mesoporus Zinc Oxide nanoparticles (MF-ZnONPs), through *in vitro* experiments on the murine macrophage RAW 264.7 cell line [11,12]. RAW 264.7 macrophages serve as a model host cell to assess both the efficacy of MF-ZnONPs against *Leishmania donovani* promastigotes and the compound's impact on host cell viability and function. We evaluated the cytotoxicity of MF-ZnONPs on macrophages, its ability to kill or inhibit *Leishmania* promastigotes, and its effects on infection-induced cellular damage. A range of cellular assays and microscopy techniques were employed to characterize how MF-ZnONPs influence parasite survival, macrophage nuclear morphology, cell death pathways, oxidative stress, and cell migration. By elucidating these effects, we aim to determine whether MF-ZnONPs show promise as a therapeutic candidate for VL [13,14].

MATERIALS AND METHODS

Materials

All chemicals used here were of analytical grade and used without further purification. All chemicals were purchased from Sigma Aldrich, St. Louis, United States. The active pharmaceutical ingredient of miltefosine was received from Alert David, Kolkata, India, as a gift sample. RAW 264.7 is

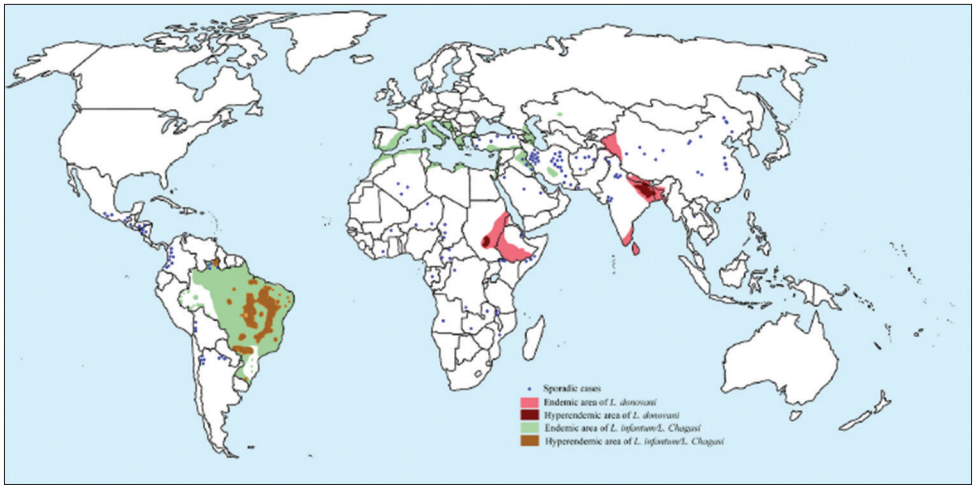

Fig. 1: Map of the global distribution of visceral leishmaniasis

a murine macrophage cell line, procured from the National Centre for Cell Science, Pune, India. Promastigotes of *L. donovani* sensitive strain (MHOM/IN/80/Dd8) were acquired from HiMedia, India. Adenosine, folic acid, biotin, hemin, $NaHCO_3$ for medium, and resazurin dye were purchased from Sigma Aldrich. In addition to this, M199, RPMI-1640 medium, L-glutamine, and fetal bovine serum (FBS) were supplied by invitrogen. The storage temperatures for the reagents were observed to ensure that they were in the correct conditions, and new stocks were prepared whenever changes were observed in the reaction results.

Cell culture (RAW 264.7 macrophages)

RAW 264.7 cells were cultured in medium with 10% FBS and antibiotics in a humidified incubator with 5% CO_2 environment. For experiments, cells were seeded in 96-well plates or 6-well plates after recording cell viability using the trypan blue exclusion method using a hemocytometer [15].

***Leishmania* promastigote culture**

The NNN and milk agar media were prepared after mixing the respective ingredients in an appropriate manner by adding them to the containers for solidification, following autoclaving. Five types of liquid overlays were prepared, namely dextrose, skim milk, full-fat milk, human urine, and saline. *Leishmania* parasites were inoculated into culture vessels containing solid medium overlaid with these liquids and incubated at 26°C. Observations were made every day, and media changes were done every 3 days. Overlays were centrifuged, supernatants discarded, and cultures continued with parasite counts performed by a formalin-based method. Parasites were cultured in NNN medium and RPMI-1640 with 10% FBS at 22±2°C. The *L. donovani* promastigotes were maintained in the stationary phase and infected RAW 264.7 cells with 1×10^6 parasites/ml. They were incubated for a few days. After incubation, the char slides containing promastigotes were cleaned, fixed, and then stained with Giemsa for the determination of the infection index [16].

MTT cytotoxicity assay (macrophage viability)

RAW 264.7 cells were seeded in 96-well plates and allowed to adhere for 24 h. Cytotoxicity of MF-ZnONPs through MTT assay was done at variable concentrations from 2% to 14% for dose standardization and up to 120 min for time standardization. MTT working solution (5 mg/mL) was added post-treatment, incubated, followed by the addition of DMSO to dissolve formazan crystals. Cell viability in percentage was assayed by measuring optical density at 570 nm and calculating percent viability relative to untreated cells. Triplicate testing for each concentration was necessary to ensure reproducibility; these were independently repeated three times. In addition, a positive control (cells exposed to substances with known cytotoxic agents) was set in place as a validation of the sensitivity of the assay [17,18].

Cell viability as a percentage was computed using the following formula:

$$\% \text{ Cell Viability} = \frac{\text{Mean absorbance in Test cells}}{\text{Mean absorbance in Control cells}} \times 100$$

DAPI nuclear staining (host cell nuclear integrity)

Cells cultured to ~40% confluence on coverslips were exposed to MF-ZnONPs, followed by staining with DAPI at room temperature, washing, and observation with a fluorescence microscope at 20×. Nuclear fragmentation, shrinkage, or condensation was considered a sign of cellular damage due to the treatment. Control groups included untreated cells and cells treated with known apoptosis-inducing agents to confirm the specificity of nuclear alterations. Images were captured and analyzed using dedicated imaging software [19].

Ethidium bromide/acridine orange (EtBr-AO) dual staining (apoptosis/necrosis assay)

EtBr-AO staining was based on discrimination of cell death with fluorescent dyes AO and EtBr, depending on a varying degree of chromatin condensation. Viable cells will display a bright green fluorescence with a completely illuminated, evenly colored nucleus. The apoptotic cells show normal bright green entering the nucleus; the necrotic cells exhibited bright orange nuclei. RAW264.7 cells were stained with 100 µg/mL of EtBr-AO dye and followed by incubation at room temperature for 30 min in the dark and analyzed under the radical fluorescence at ×20 magnification. Three independent replicates were performed for reliability, and 100 cells per field were randomly selected for quantification of the viable, apoptotic, and necrotic cell proportions. Images were analyzed with software to ensure accuracy in cell classification [20].

Proliferation assay

RAW 264.7 cells were plated into 6-well plates for expansion in the proliferation assay. After 2 days, the medium was replaced by FBS-free media DMEM before the assay. A scratch across the cell monolayer was made using a sterile pipette tip. After gentle washing with phosphate-buffered saline (PBS), cells were further incubated in a medium containing drug MF-ZnONPs for 36 h. The scratches were imaged at ×10 magnification using an inverted microscope (Olympus Inc.) with a digital camera for analysis. For quantification, scratch width was measured at different positions using ImageJ software and expressed as a percentage of scratch closure to its initial width. Control treatments consisted of non-treated cells and a standard active proliferation-inhibiting agent that confirmed the validity of the assay [18,22].

Reactive oxygen species (ROS) assay in parasites

Intracellular ROS generation in *Leishmania* promastigotes was measured to explore a possible mechanism of MF-ZnONPs's parasiticidal

effect. Promastigotes (1×10^6 cells/mL) were incubated with MF-ZnONPs (at an effective lethal concentration, e.g., 50 µg/mL) or with medium alone (untreated control) for 72 h at 22°C. After treatment, the cell-permeable fluorogenic probe 2′,7′-dichlorodihydrofluorescein diacetate (H2DCFDA) was used to detect ROS. H2DCFDA (10 µM final concentration) was added to each sample and incubated in the dark at room temperature for 20 min. Within cells, H2DCFDA is deacetylated by esterases to a non-fluorescent compound, which in the presence of ROS is oxidized to form the highly fluorescent 2',7'-dichlorofluorescein (DCF). After incubation, promastigotes were washed twice with PBS to remove excess dye. Fluorescence intensity of DCF was measured in a microplate reader (excitation ~504 nm, emission ~529 nm). Mean fluorescence units from three independent experiments were recorded for each condition. Elevated DCF fluorescence indicates higher ROS levels [18,22,23].

Statistical analysis

The results were expressed as mean±standard deviation. All experiments above were performed at least in triplicate. Statistical analysis was carried out (where needed) using Student's t-test or one-way analysis of variance with *post hoc* tests, considering $p<0.05$ as statistically significant. Data are presented in figures with appropriate error bars representing the standard error of the mean.

RESULTS

Macrophage viability (MTT assay)

MF-ZnONPs exhibited low cytotoxicity toward RAW 264.7 macrophages at the concentrations and exposure times tested. The MTT assay revealed that cell viability remained above 70% even at concentrations up to 10% (v/v) of MF-ZnONPs and exposure durations up to 120 min. In 15, 4 dose–response tests (40 min treatment), concentrations of 2%, 4%, 6%, 8%, 10%, 12%, and 14% of MF-ZnONPs showed minimal reduction in cell viability compared to untreated controls, whereas higher doses (12% and 14%) led to more pronounced viability loss. Similarly, in time-course tests using 10% MF-ZnONPs, cell viability was largely maintained for treatment times up to 60 min, with a slight decline observed at 120 min. These results indicate that MF-ZnONPs are relatively safe for macrophages at ≤10% concentration for at least 1 h of exposure (Fig. 2a, 2b). Consequently, subsequent experiments in infected cultures were performed using ≤10% MF-ZnONPs and short incubation periods (typically 40 min) to ensure host cell safety.

Effect on *Leishmania* promastigote growth

The compound MF-ZnONPs demonstrated a clear inhibitory effect on the growth and survival of *L. donovani* promastigotes. In drug-treated parasite cultures (10–100 µg/mL range), promastigote counts were significantly reduced in a dose-dependent manner relative to untreated controls. Notably, at the highest concentration tested (100 µg/mL), the parasite population showed near complete growth arrest over 72 h, with a majority of promastigotes losing motility and membrane integrity (as evidenced by trypan blue uptake). Lower concentrations (e.g., 10–25 µg/mL) caused a slower proliferation rate compared to control cultures. Microscopic examination of Giemsa-stained promastigotes confirmed the presence of typical elongated, flagellated promastigote forms in control samples. In contrast, promastigotes exposed to MF-ZnONPs exhibited abnormal morphology, including rounding of cells and shortening or loss of the flagellum, suggestive of stress or damage. These findings indicate that MF-ZnONPs have direct anti-parasitic activity against *L. donovani*, substantially impairing promastigote viability and multiplication *in vitro*.

ROS induction in parasites

One mechanism by which MF-ZnONPs may exert anti-leishmanial effects is through the induction of oxidative stress in the parasite. Measurement of ROS levels using the H2DCFDA assay showed a marked increase in fluorescence in drug-treated promastigotes compared to untreated parasites. As shown in Fig. 3, *L. donovani* promastigotes incubated with MF-ZnONPs had significantly higher DCF fluorescence, indicating elevated intracellular ROS production, whereas control promastigotes maintained low baseline ROS levels. The ROS increase in the presence of MF-ZnONPs was statistically significant ($p<0.05$) and may contribute to parasite killing, since excessive ROS can damage cellular components and induce death in *Leishmania*. These results support the idea that oxidative stress is involved in the antiparasitic action of MF-ZnONPs. Parasites experiencing high ROS likely suffered oxidative damage, consistent with their inhibited growth and abnormal morphology noted above. ROS are crucial for cellular functions but can cause oxidative stress and cell death if in excess or out of balance. ROS have been known to fight against a wide range of diseases due to their ability to cause damage either to the cells or the promastigotes. Intracellular levels of ROS post-drug treatment were determined using the fluorogenic dye H2DCFDA. This dye, once inside the cells, is converted by ROS into the highly fluorescent DCF. Results have shown that cells treated with leishmanial promastigotes exhibited much higher ROS production compared to control cells, where the cells were not treated with promastigotes. Drug-loaded MZONPs lowered the ROS production, hence contributing to lowering the oxidative stress as depicted in Fig. 4.

Nuclear integrity in infected macrophages (DAPI staining)

Infection of RAW 264.7 macrophages with *L. donovani* promastigotes led to pronounced nuclear changes characteristic of early apoptosis, whereas treatment with MF-ZnONPs protected the cells from these changes. DAPI-stained fluorescence microscopy images (Fig. 4) illustrate the nuclear morphology under different conditions. Uninfected control cells (Fig. 4a) displayed round, homogeneously stained nuclei with no signs of chromatin condensation or fragmentation. In sharp contrast, macrophages exposed to *Leishmania* promastigotes for 40 min without treatment (Fig. 4b) showed irregular, condensed, and fragmented nuclei. The nuclear membrane appeared shrunken or distorted in many infected cells, indicating the induction of apoptosis by the parasite. Notably, cells that were infected and then treated with MF-ZnONPs (Fig. 4c) retained near-normal nuclear morphology, similar to that of control cells. The chromatin in these drug-treated cells remained mostly intact and evenly distributed. This suggests that MF-ZnONPs effectively mitigated the parasite-induced nuclear damage. By preventing chromatin condensation and nuclear fragmentation, MF-ZnONPs treatment preserved the structural integrity of the host cell nuclei, implying a cytoprotective effect on infected macrophages.

Apoptosis and necrosis assessment (EtBr-AO staining)

Dual staining with acridine orange and ethidium bromide further confirmed that MF-ZnONPs rescues macrophages from parasite-induced cell death. In untreated control macrophages, almost all cells fluoresced bright green, indicating viable cells with intact nuclei (no uptake of EtBr). Upon *Leishmania* infection, however, nearly 100% of the cells exhibited a shift to yellow-orange nuclear staining (AO/EtBr overlap) or even red areas, signifying progression from early apoptosis to late apoptosis/secondary necrosis (due to loss of membrane integrity). This dramatic increase in apoptotic/necrotic cells in infected samples (Fig. 5b) correlates with the nuclear abnormalities seen under DAPI. By contrast, infected macrophages that received MF-ZnONPs treatment showed a striking reduction in apoptotic staining. The majority of these cells had nuclei staining green, much like the uninfected controls, with only a minority showing any orange/red fluorescence (Fig. 5c). As the EtBr-AO images demonstrate, MF-ZnONPs treatment substantially decreased the extent of apoptosis and prevented the progression to necrosis in *Leishmania*-exposed macrophages. Quantitatively, the drug-treated group had a lower fraction of apoptotic/necrotic cells compared to the parasite-only group. These results indicate that MF-ZnONPs confer a protective effect on host cells, likely by interrupting the parasite-triggered cell death pathways shown in Fig 5.

Proliferation assay (scratch assay)

Leishmania infection adversely affected the ability of macrophages to migrate and close a wound gap, whereas MF-ZnONPs helped preserve this function. In the scratch wound assay, confluent RAW 264.7

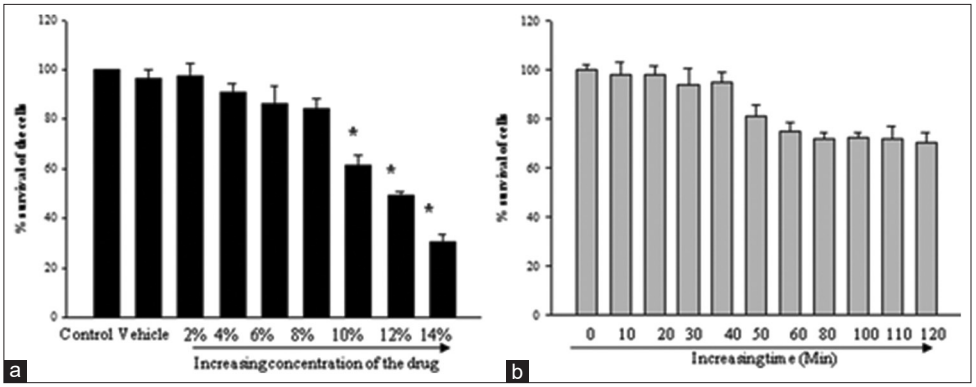

Fig. 2: The effect of drug, miltefosine-loaded mesoporus Zinc Oxide nanoparticles (MF-ZnONPs) on the proliferation of RAW 264.7 cells. (a) Different concentrations (2–14%) of the drug MF-ZnONPs were added for a fixed time span for standardization of the dose. *denotes significantly different from control at p<0.05. (b) 10% of the drug MF-ZnONPs was added for a varied time span (0–120 min) for standardization of time. The viability was measured by the MTT assay. The results were expressed as absorbance at 595 nm. The data were shown as means±standard error, and vertical bars represent standard errors of mean values

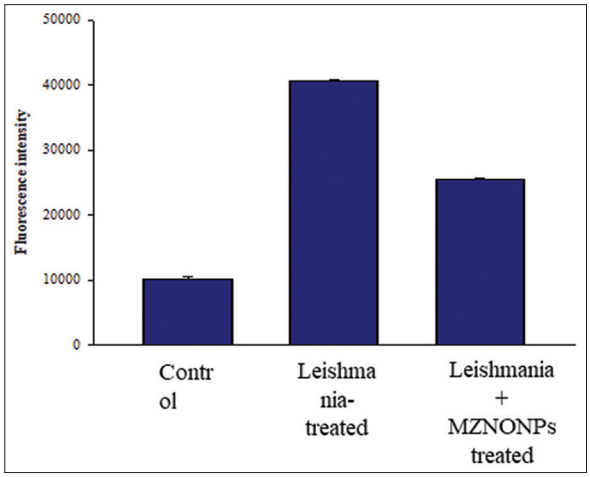

Fig. 3: Reactive oxygen species (ROS) generation in cultured cells after infection with *Leishmania donovani* promastigotes and therapeutic administration with drug-loaded mesoporus Zinc Oxide nanoparticles after infection with *L. donovani* promastigotes. Untreated cells served as a control, and ROS generation in the other two groups was compared with the untreated cells. Results are expressed as the mean±standard deviation

monolayers that were left untreated (control) exhibited significant cell migration into the scratch area after 24 h, leading to partial closure of the wound (Fig. 6a). However, when promastigotes were added to the wounded monolayer, macrophage migration was dramatically inhibited after 24 h, the scratch remained largely open, with few cells moving into the gap (Fig. 6b). In addition, the presence of promastigotes caused notable cell damage along the wound edge, compounding the lack of wound closure. This suggests that *L. donovani* impairs the motility or viability of macrophages required for wound healing. Strikingly, the wells that had been infected and then treated with MF-ZnONPs showed a much improved closure of the scratch after 24 h (Fig. 6c). Macrophages in the MF-ZnONPs-treated group migrated into the wound area more effectively, nearly bridging the gap, in stark contrast to the infected-only group. The cell layer integrity was also better preserved, with fewer signs of cell lysis or detachment. These observations demonstrate that MF-ZnONPs can counteract the deleterious effects of *Leishmania* on macrophage migration and wound healing, likely by both protecting the cells and possibly inhibiting the parasite load. The restoration of near-normal migratory behavior in infected macrophages further highlights the compound's protective benefits to host cell function.

DISCUSSION

This study shows that MF-ZnONPs have significant anti-leishmanial efficacy *in vitro* while preserving acceptable safety profiles for host macrophages. Multiple critical observations substantiate this conclusion. Initially, MF-ZnONPs at concentrations up to 10% did not markedly impair the viability of RAW 264.7 macrophages, corroborating previous research indicating that zinc oxide nanoparticles at regulated dosages demonstrate minimal cytotoxicity toward mammalian cells [24,25]. Second, MF-ZnONPs significantly impeded the proliferation of *L. donovani* promastigotes and generated morphological changes such as rounding and flagella loss, consistent with previous studies indicating that nanoparticle-based delivery systems efficiently restrict parasite growth [26,27].

A significant characteristic of MF-ZnONPs is their ability to elevate ROS in promastigotes, a recognized mechanism of antiparasitic activity. ROS induce damage that compromises the cellular integrity of parasites, resulting in mortality, as evidenced by research assessing metal oxide nanoparticles against *Leishmania* spp. [28]. The observed rise in ROS after treatment corresponds to analogous increases documented when zinc oxide nanoparticles were utilized against *Leishmania* major [29]. This indicates that MF-ZnONPs may induce oxidative stress in parasites beyond their physiological tolerance, resulting in detrimental damage to lipids, proteins, and nucleic acids.

Significantly, MF-ZnONPs provided protective benefits to host macrophages. Infection with *L. donovani* is recognized to provoke apoptosis and necrosis in macrophages as a pathogenic mechanism to circumvent immune responses [30]. Our observations indicated that untreated infected macrophages displayed chromatin condensation, nuclear fragmentation, and ethidium bromide uptake – all characteristics of apoptosis and necrosis. Conversely, infected cells subjected to MF-ZnONPs exhibited nearly normal nucleus morphology and low apoptotic indices, a behavior consistent with observations from other nanoparticle-based formulations, including amphotericin B-loaded PLGA nanoparticles and silver nanoparticles [31,32]. The cytoprotective impact may result from the reduction of parasite load and may be the alteration of macrophage redox equilibrium.

In addition, the scratch wound experiment demonstrated that *L. donovani* infection compromised the migratory capacity of macrophages, an essential role for tissue healing and host defense. MF-MZONP therapy reinstated cell motility, indicating the functional preservation of macrophage physiology. This aligns with prior studies suggesting that nanoparticulate administration of anti-leishmanial drugs can safeguard or rejuvenate macrophage activity *in vitro* [32].

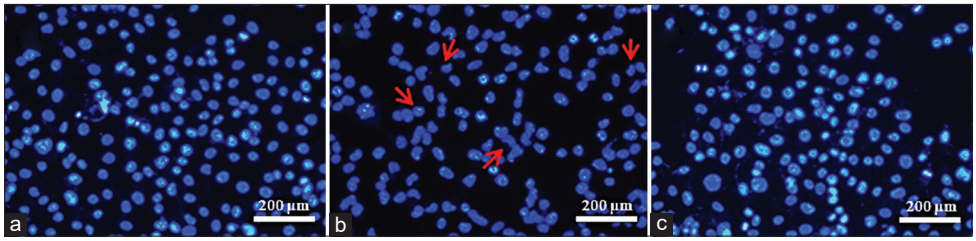

Fig. 4: RAW 264.7 cells were exposed to the leishmanial promastigote for 40 min. Apoptotic nuclear morphological changes, such as chromatin condensation and marginalization (arrows), are evident in cells exposed to leishmanial promastigotes, whereas no marked nuclear alterations were visible in cells that were therapeutically treated with the drug miltefosine-loaded mesoporus Zinc Oxide nanoparticles (MF-ZnONPs). Images are representative of three independent experiments. The pictures were taken in ×20 magnification. RAW 264.7 cells were exposed to the leishmanial promastigotes up to 40 min and therapeutically treated with drug MF-ZnONPs in another set. DAPI-stained micrographs were represented in Fig. 4. DAPI-stained picture of the control set of cells by fluorescence microscopy revealed an intact nucleus without any visible distortion, chromosomal aberration, or shrinkage of nuclear membrane (a). In contrast, leishmanial promastigote treatment revealed a damaged nucleus with gross visible distortion, chromosomal aberration, or shrinkage of the nuclear membrane, which were clear indications of cell damage and apoptosis (b). The therapeutic drug MF-ZnONPs treatment reversed the nuclear damage, and the micrographs (c) of Fig. 4 appeared similar to the control (a)

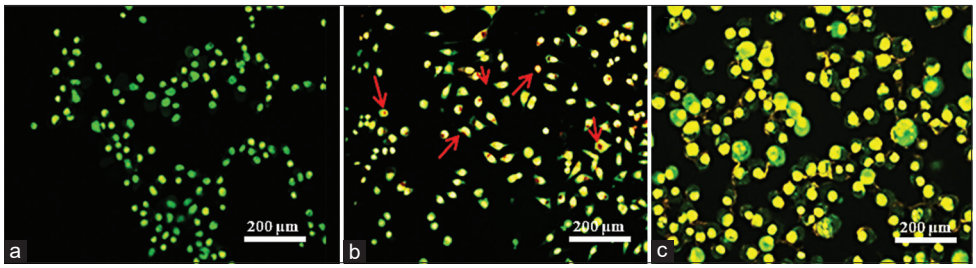

Fig. 5: RAW 264.7 cells stained with ethidium bromide/acridine orange (EtBr-AO) cocktail. The cells revealed the apoptotic states of control sets of cells (a), on exposure to leishmanial promastigote treatment (b), post-treatment with drug miltefosine-loaded mesoporus Zinc Oxide nanoparticles (MF-ZnONPs) followed by leishmanial promastigote treatment (c). The pictures were taken in ×20 magnification. The EtBr-AO stained fluorescence micrographs of RAW 264.7 cells, depicted in Fig. 5, showed that almost 100% of the cells became yellow to orange which signified that cells became pre-apoptotic to apoptotic and also necrotic in some cases (red areas within the cells) on leishmanial promastigote administration, whereas control cell nuclei appeared as uniformly brilliant green. The drug MF-ZnONPs healed the condition significantly by reducing the extent of apoptosis as the colour of nuclei were similar to the control cells

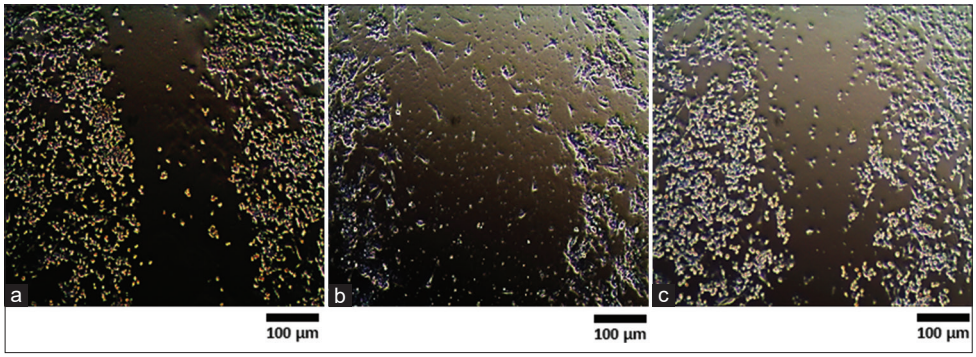

Fig. 6: Scratch assay on RAW 264.7 cells. (a) untreated control, (b) leishmanial promastigote treatment, (c) leishmanial promastigote treatment with MF-ZnONPs. The cell scratch experiment revealed that the leishmanial promastigote treatment dramatically in (b) reduced cell invasion into the wound location in 24 h compared to untreated control (a), while the drug-loaded mesoporus zinc oxide nanoparticles treatment (c) rectified the condition. Representative images from a bright-field microscope (scale bar: 100 µm) are shown

Notwithstanding these encouraging results, many limitations warrant attention. This study predominantly utilized promastigote-stage parasites, which, although suitable for *in vitro* experiments, do not accurately represent the intracellular amastigote stage that causes human disease. Previous research has demonstrated that intracellular amastigotes may display distinct susceptibility patterns in contrast to promastigotes [33]. Consequently, subsequent studies must assess MF-ZnONPs against intracellular amastigotes within macrophages to validate their therapeutic efficacy. Furthermore, whereas *in vitro* cytotoxicity was negligible, zinc oxide nanoparticles have exhibited dose-dependent oxidative stress and genotoxicity in mammalian systems under specific conditions [24,25]. *In vivo* investigations are essential for characterizing pharmacokinetic behavior, tissue distribution, and potential off-target toxicity.

Our findings corroborate the increasing evidence that nanocarrier-based delivery methods can concurrently provide antiparasitic effects and safeguard host cells from dysfunction caused by infection [31]. MF-ZnONPs demonstrate a dual method of direct parasitic eradication, presumably through ROS formation, while maintaining macrophage viability and functionality. This amalgamation of interventions is exceedingly advantageous for VL treatment, considering the limitations

of existing drugs such as sodium stibogluconate and amphotericin B, which are hindered by toxicity and the development of resistance [34]. Consequently, MF-ZnONPs necessitate additional investigation in pre-clinical models to validate their potential as a novel, effective, and safer treatment for leishmaniasis.

CONCLUSION

The *in vitro* studies indicate that MF-ZnONP is an effective anti-leishmanial compound against *L. donovani* promastigotes and is well-tolerated by host macrophages. The substance dramatically inhibited parasite growth, possibly by inducing oxidative stress, and importantly, it safeguarded macrophage cells against the harmful effects of infection. Macrophages exposed to MF-ZnONPs maintained typical nuclear shape and functional capabilities (e.g., migration), underscoring the drug's cytoprotective advantages. These results establish MF-ZnONPs as a promising contender for further advancement in combating VL. Nonetheless, additional study is necessary before clinical applicability, especially *in vivo* investigations. We advocate for the execution of animal model research, namely utilizing *Leishmania*-infected mice, to validate the efficacy of MF-ZnONP in eliminating intracellular amastigotes and to assess pharmacological characteristics and safety within a complete organism. These investigations will ascertain the therapeutic dosage, possible toxicity, and treatment protocol. Upon establishing *in vivo* efficacy and safety, MF-ZnONPs may progress to clinical trials. The objective is to create a novel, efficacious, and safe treatment for VL, fulfilling a vital requirement in world health. MF-ZnONP demonstrates significant potential in this context, and with additional validation, it may enhance outcomes and survival for people afflicted by this overlooked yet lethal condition.

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AUTHOR'S CONTRIBUTION

Parag Ghosh (P.G.) conceived and designed the study, synthesized and characterized the nanoparticles, and prepared the first manuscript draft. Dileep Bharti (D.B.) carried out the in vitro experiments, including macrophage culture, cytotoxicity assays, and microscopy. Dibya Das (D.Da.) contributed to data collection, statistical analysis, and figure preparation. Subas Chandra Dinda (S.C.D.) supervised the research, interpreted results, and provided critical revisions of the manuscript. Anirbandeep Bose (A.B.) coordinated the project administration, provided resources, and approved the final version of the manuscript. All authors reviewed and approved the final manuscript. All authors contributed equally to the drafting, editing, and approval of the final manuscript.

CONFLICT OF INTEREST

The authors hereby declare that they have no conflict of interest either to disclose.

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