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Original Article

Nanoemulsion of Spiramycin against Tachyzoites of *Toxoplasma gondii*, RH Strain: Preparation, Toxicology, and Efficacy Studies

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Abstract

Background: *Toxoplasma* infection is caused by *Toxoplasma gondii*, which is an intracellular protozoan parasite. This infection consequently lead various congenital disabilities during pregnancy in patients. Spiramycin (Spi), a macrolide antibiotic, is typically recommended for *T. gondii* infection in pregnant women. We aimed to prepare the nanoemulsion of spiramycin (NE-Spi) and to evaluate the activity of this formulation in tachyzoites of *T. gondii*, RH strain.

Methods: This study was conducted in 2019-2021 at the School of Public Health, Tehran University of Medical Sciences, Tehran, Iran. NE-Spi was prepared by spontaneous emulsification. The effects of this nanoemulsion on the viability of cultured cells were measured using MTT assay. To estimate the effects of NE-Spi on tachyzoites of *T. gondii*, RH strain, different concentrations of NE-Spi, S-Spi (suspension of spiramycin), and NE (nanoemulsion without any spiramycin) were added to tachyzoites and then stored for 30, 60, 90, 120 min and 24 h in 250 µg/ml concentration at room temperature. Finally, Tachyzoites mortality rates were evaluated by trypan blue staining. Of note, flow cytometry was conducted to confirm the obtained results.

Results: The final particle size of NE-Spi was calculated to be 11.3 nm by DLS and TEM. Thereafter, using MTT assay, in 62.5 µg/ml concentration of NE-Spi, the Vero cells viability was obtained as 82%. The highest mortality rates of tachyzoites of *T.gondii*, RH strain were observed at 250 µg/ml concentration and after 120 min of exposure, but it was not significantly different from 24 h of exposure.

Conclusion: NE-Spi has lethal efficacy on *T. gondii* RH strain in-vitro.

Keywords: Nanoemulsion; Spiramycin; *Toxoplasma*; Tachyzoite; RH strain

Introduction

Toxoplasma gondii, the coccidian protozoan parasite that is distributed worldwide, is the agent of toxoplasmosis in human beings and animals (1). Depending on the detection method used and geographical regions, the prevalence rates of *T.*

gondii infection are estimated between 0 and 100 % (2).

The life cycle of *T. gondii* has three infective stages as follows: fast-dividing tachyzoite, slow-growing bradyzoite in tissue cyst, and oocyst containing sporozoite (3). *Toxoplasma* infection is caused by consuming raw or undercooked meat with tissue cysts and food or water contaminated with oocysts (4).

Using host machinery factors, tachyzoites multiply, differentiate, and consequently lead to the development of severe diseases in immunocompromised patients and congenitally infected newborns (5). Infection with *T. gondii* during pregnancy leads to fetal death or various congenital disabilities, mainly when acute congenital disease occurs in the first trimester (6). Serological assays are the most commonly tested for toxoplasmosis diagnosis.

Toxoplasmosis has been diagnosed using various methods, including microbial isolation, protein analysis, immunological (serological) techniques, and molecular techniques. Serological techniques are the most widely employed detecting specific antibody classes of antigens against toxoplasmosis among these methods. Production and Evaluation of *T. gondii* Recombinant Surface Antigen 1 (SAG1) (7), Diagnosis of antigenic markers of acute toxoplasmosis by IgG avidity immunoblotting are examples of serological investigations (8). Teimouri et al. also researched the significance of *T. gondii* IgG avidity testing in differentiating between acute and chronic toxoplasmosis in pregnancy (9).

The treatment of toxoplasmosis in pregnant women is necessary as well as in immunocompromised patients suffering from acquired immune deficiency syndrome (AIDS) or those undergoing chemotherapy, because the parasite can cause extreme morbidity and mortality among them (10). Sulfadiazine and pyrimethamine with some potentially significant side effects such as hematologic toxicity, allergy, folic acid deficiency, and bone marrow suppression are used as the standard treatment for acute toxoplasmosis (1). Spiramycin produced by *Streptomyces ambofaciens* is an anti-parasitic antibiotic, which belongs to macrolide category, known as the first-line treatment for *Toxoplasma* infection among pregnant women with successful results and no teratogenicity (11, 12).

Nanomedicine is characterized by the application of nanotechnology in the prevention, treatment, monitoring, and control of diseases (13). Drug delivery systems based on nanotechnology will enhance the pharmacokinetic profile of drugs, meaning that by increasing the solubility area, its stability, dissolution rate, surface of drug, modulating therapy, and permeability increase through absorption into membranes, so the bioavailability of drug increases at lower doses (14). Nanoemulsion drug delivery systems, are innovative methods for increasing the bioavailability of hydrophobic medicines and those with a high first-pass metabolism (15). Nanoemulsions or nano-sized oil dispersions have some advantages such as increasing drug absorption and penetration and reducing the effective doses (1). The effects of various formulations of anti-*Toxoplasma* drugs such as nanoemulsions and nanoparticles, on *T. gondii* tachyzoites have been previously studied in several studies with promising results (1, 3, 16-18).

The present study was conducted to prepare NE-Spi and to evaluate its in-vitro effects on tachyzoites of *T. gondii*, RH strain compared to both S-Spi and NE.

Materials and Methods

Materials

Soybean oil was provided from Sigma-Aldrich Co., St Louis, MO, USA and polysorbate 80, polysorbate 85, and ethanol were purchased from Merck KGaA, Darmstadt, Germany. Deionized distilled water (DW) was used in all the procedures in this study. Moreover, Spiramycin tablets (Rovamycin®-Sanofi Aventis Pharmaceutical Company, France) were weighed and then crushed into powder. The calculation of active ingredient was done according to the weight of each tablet (14).

Parasite

This study was carried out in 2019-2021 at the School of Public Health, Tehran University of Medical Sciences, Tehran, Iran.

Tachyzoites of *T. gondii*, RH strain (type I) were inoculated in BALB/c mice using intra-peritoneal (IP) injection. Mice were housed at Animal Center of School of Public Health, Tehran University of Medical Sciences, Tehran, Iran, under standard laboratory conditions (light-dark cycle conditions, at the regulated temperatures of 22 °C) with ad libitum foods and fresh drinking water. Tachyzoites were harvested from peritoneal cavity by passing three days from the infection, washed in phosphate-buffered saline (PBS, pH 7.2), and finally counted with hemocytometer microscopic slide (1).

NE-Spi: Preparation and characterization

For NE preparation, soybean oil was selected as the oily phase, dimethyl sulfoxide (DMSO) as co-solvent, a mixture of polysorbate 80 and polysorbate 85 as surfactants, and ethanol was selected as co-surfactant. The spontaneous emulsification method was used to prepare NE-Spi. Briefly, Spiramycin powder (1%) was dissolved in oil phase containing soybean oil (5%), DMSO (5%), polysorbate 80 (24%), and polysorbate 85 (14%). This mixture was then added to the aqueous phase containing distilled water (41%) and ethanol (10%), and adequately stirred at room temperature.

Characterization of nanoemulsion

Particle size and zeta potential

At this stage, particles' diameter and zeta potential of NE were measured using Nano-ZS90 dynamic light scattering (Malvern Instruments, Malvern, UK) at 90° fixed angle and at room temperature.

Transmission electron microscopy (TEM)

Morphological features of NE-Spi were observed using Zeiss transmission electron microscope (TEM) (EM10C-Germany) operating at 100 kV. Thereafter, the aqueous solution of the sample was sonicated for 15 min. Next, 20µl of NE-Spi was dropped onto formvar carbon film on copper grid 300 mesh (EMS-USA) and then allowed to be dried at room temperature. Finally, the images were prepared.

Stability tests

To determine the stability of NE-Spi solution, freeze and thaw method was conducted for three periodic cycles. NE-Spi was monitored for phase separation, creaming, and discoloration after the storage for one month at room temperature. After three times of repeating the freeze-thaw cycles, particle size and zeta potential were re-calculated.

Cell viability assay

Totally, 5×10^6/ml of Vero cells were cultured in Dulbecco Modified Eagle Medium (DMEM) (Sigma-Aldrich Co., USA) with 10% of fetus bovine serum (FBS, Gibco, Germany) and 1% of penicillin/streptomycin in cell culture flasks (SPL, South Korea) with 5% CO_2 for 24 h at 37 °C.

In order to estimate Vero cells viability rate at different concentrations of each spiramycin formulation (including NE-Spi, S-Spi, and NE), MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assay was performed in terms of the manufacturer's instructions (Kiazist, Hamadan, Iran). Finally, the Vero cells viability was calculated.

Effects of spiramycin formulation on T. gondii tachyzoites

Tachyzoites of *T. gondii*, RH strain were exposed to four concentrations (including 250, 125, 62.5, and 31.25 µg/ml) of each spiramycin formulation (including NE-Spi, and S-Spi) for 30, 60, 90, and 120 min and for 24 h at the concentration of 250 µg/ml at room temperature. Additionally, one well was treated with nanoemulsion without any Spi (NE). Each experiment was conducted in duplicate. 1.5×10^6/ml fresh tachyzoites were added to sterile test tubes with 50µl of each concentration of NE-Spi, S-Spi, and NE for performing in-vitro experiments and with distilled water as the control group. Afterward, each tube was mixed

properly, and then stored at room temperature. The number of dead parasites was estimated using trypan blue dye (Sigma-Aldrich Co., USA) and counting was done with light microscopy, then the mortality rates were determined.

Flow cytometry

At this stage, 2×10^5 tachyzoites were exposed to the highest concentration (250 µg/ml) of NE-Spi, and then stored for 24 h at room temperature. Subsequently, propidium iodide (Sigma-Aldrich Co., USA) was added to it and the staining procedure was done in dark for 30 min at 4 °C and the mortality rate was assessed using flow cytometry (FACSCalibur B, BD, and America). The blank tube contained tachyzoites and distilled water.

Results

Characterization of NE-Spi

The particle size of NE-Spi was estimated as 10.4 nm using DLS (Dynamic light scattering), and zeta potential was measured to be -13.7 mV.

Stability of NE-Spi

No changes were observed in color, cloudiness, or phase separation on NE-Spi in one month. After performing stability tests, the particles size reached 11.3 nm. No considerable changes were found in particles size after the freeze and thaw cycles. Of note, no phase separation or appearance changes were seen in the prepared nanoemulsion after the freeze and thaw cycles.

Morphology of NE-Spi

To confirm the spherical shape and size of nanoemulsion particles, TEM was used at this stage. (Fig.1).

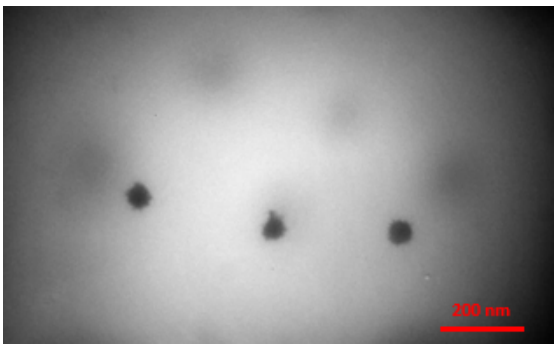

Fig.1: Transmission electron micrograph (TEM) of NE-Spi

Cell viability assay

By conducting MTT assay, it was shown that more than 82% of Vero cells remained viable at the maximum concentration of NE-Spi (62.5µg/ml). By decreasing the NE-Spi concentration, the viability of Vero cells increased to 94%. Furthermore, Vero cells viability for S-Spi at the concentration of 62.5µg/ml was obtained as 92%, which increased to 96.6% at 7.81µg/ml. The effects of NE-Spi, S-Spi, and NE on Vero cells at four different concentrations in this study are shown in Fig. 2.

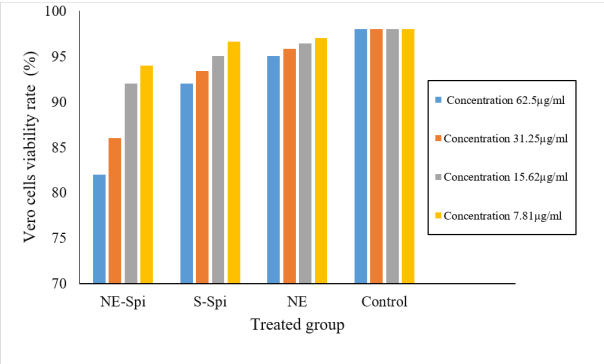

Fig. 2: Vero cells viability rate (%) after the exposure with NE-Spi, S-Spi, and NE compared to the control group at four concentrations (including 7.81, 15.62, 31.25, and 62.5 µg/ml) by MTT assay

The effects of spiramycin formulation on T. gondii tachyzoites

NE-Spi, S-Spi, and NE at the concentrations of 31.25, 62.5, 125, and 250µg/ml were found to be effective on tachyzoites of *T.gondii*, RH strain after exposure for 30, 60, 90, 120 min, and 24 h (for 250µg/ml). The highest mortality rate was seen at the concentration of 250µg/ml and after 120 min of exposure. No significant differences were observed in mortality rates for NE-Spi at the concentration of 250µg/ml between 120 min and 24 h of exposure. The results of the tachyzoites of *T. gondii*, RH strain exposed to NE-Spi, S-Spi, NE, and control group are shown in Fig. 3.

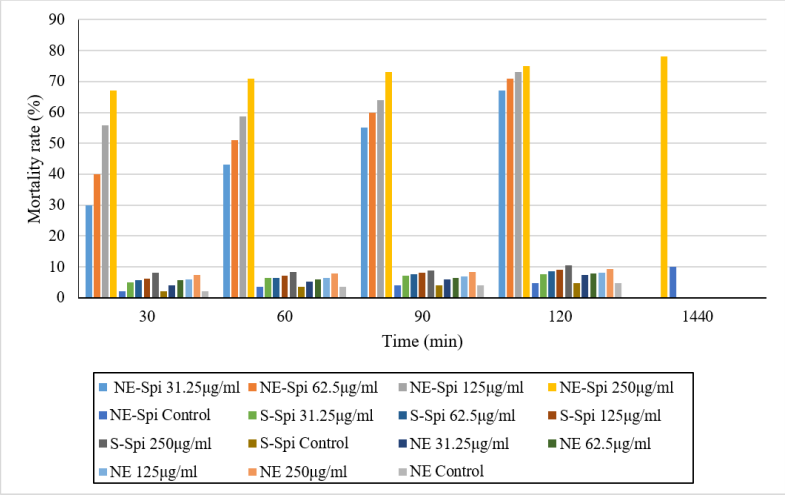

Fig. 3: *In-vitro* assessment of 31.25, 62.5, 125, and 250µg/ml concentrations of NE-Spi, S-Spi, and NE on tachyzoites of *T. gondii*, RH strain compared to the control group. Error bars represent mean ± SD and significant differences are shown as well (P≤0.05)

Flow cytometry

Flow cytometry was used to evaluate the mortality rates of tachyzoites of *T.gondii*, RH strain at the concentration of 250µg/ml and after 24 hours of exposure compared to the control group. Accordingly, the results are shown in Fig. 4. The maximum mortality rate (93.6%) was seen at the concentration of 250µg/ml of NE-Spi.

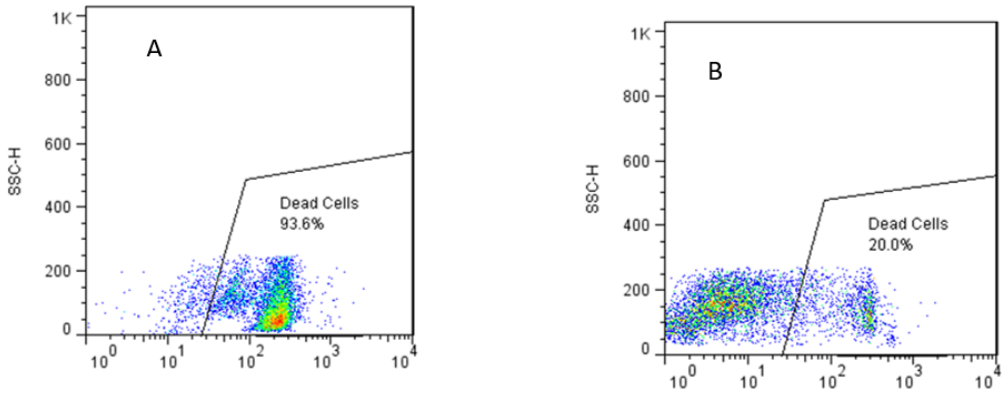

Fig. 4: Flow cytometry results at 250µg/ml concentration of NE-Spi on tachyzoites of *T. gondii*, RH strain after exposure for 24 hours (A) compared to the control group (B)

Discussion

Toxoplasmosis is caused by *T. gondii*, which is an obligate intracellular protozoan parasite infecting many hosts, including human beings (19). Toxoplasmosis is currently treated with the combination of sulfadiazine and pyrimethamine (20). However, due to teratogenic effects of these drugs on fetus, spiramycin was approved for the treatment of *Toxoplasma* infection, in order to reduce the risk of mother-to-child transmission during pregnancy (21, 22). Spiramycin, commonly used for more than 30 years, is the macrolide antibiotic made by *S. ambofaciens* (23).

Membrane permeability, solubility, and dissolution rates of the drug are all aspects affecting the bioavailability of spiramycin. However, the bioavailability of oral spiramycin is variable and incomplete due to its low solubility and poor dissolving rate in water (24). Nanoemulsion, as the drug delivery system, is one of the promising technologies used to improve the oral bioavailability of poorly soluble drugs and reproducibility of drug plasma concentration profiles. Accordingly, it can be designed to deliver drugs via the small size, give a wide surface area, and enable solubilization and penetration (25, 26).

In the current study, the formulation of NE-Spi was synthesized to improve both the bioavailability and efficacy of spiramycin on tachyzoites of the *T. gondii*, RH strain. The final particle size of the synthesized NE-Spi was measured to be 11.3 nm by DLS and TEM. Using MTT assay, the Vero cells viability was 82% in cell culture at the highest concentration of NE-Spi (62.5 µg/ml). The results of exposing tachyzoites of the *T. gondii*, RH strain to various concentrations of NE-Spi, S-Spi, and NE revealed that as NE-Spi concentrations and exposure time increased, the mortality rates increased as well, since the better results were obtained after 120 min of exposure to the concentration of 250µg/ml. The most effective concentration was 250 µg/ml after 24 h of exposure according to flow cytometry results. Correspondingly, the results are parallel with in-vitro study, using trypan blue dye.

Several studies have previously investigated the effects of nanostructures such as nanoemulsions, solid lipid nanoparticles, polymer nanoparticles, metal nanoparticles, and nanosuspensions on different stages of *T. gondii* under in-vivo and in-vitro condition (27).

Among the in-vitro studies, the efficacy of 250 µg/ml concentration of nanosilver in both tachyzoites and bradyzoites of *T. gondii*, RH, and Tehran (type II) strains has been reported in a study (3). Moreover, in an in-vitro study, mannosylated paromomycin-loaded solid lipid nanoparticles (PM-SLN-M) showed a substantial antiparasitic activity on tachyzoites of *T. gondii*, RH strain without any significant host cell toxicity (2). Various molecular weights and concentrations of chitosan nanoparticles (CS NPs) showed a significant anti-*Toxoplasma* efficacy on tachyzoites of *T. gondii*, RH strain (28). Nontoxic nature and antiparasitic effects of both chitosan (CS) nanoparticles (NPs) and spiramycin and the use of spiramycin-loaded CS NPs as a potential treatment for human toxoplasmosis (14).

In mice treated with selenium nanoparticles (SeNPs), the mean number of brain tissue cysts decreased compared to the control group. As a result, SeNPs have preventive effects against latent toxoplasmosis with no significant toxicity (29). In addition, curcumin nanoemulsion (CR-NE) was found to be more efficient on *T. gondii*, RH and Tehran strains in mice model compared to curcumin suspension (CR-S). Their results showed the potential ability of CR-NE in the treatment of acute and chronic toxoplasmosis, especially in those with latent tissue cysts in brain (17).

As well, spiramycin loaded poly lactic-co-glycolic acid (PLGA) implants were observed to be highly effective on intracellular parasites in ocular toxoplasmosis, without causing any human retinal pigment and epithelial cell death. Spiramycin-loaded PLGA implants could be considered as promising therapeutic option for the treatment of local ocular toxoplasmosis (11).

The effects of nitazoxanide (NTZ) and combination of spiramycin (SP) and metronidazole on acute experimental toxoplasmosis was investigated and showed that SP-metronidazole had better results in terms of both mice survival rate and parasite load in brain and liver (30). Moreover, zinc nanoparticles (ZnNPs) using *Lavandula angustifolia* Vera., was synthesized and reported that ZnNPs have significant prophylactic effects on chronic toxoplasmosis in mice (31). Anti-*Toxoplasma* activity of biogenically synthetized nanosilver by *Fusarium oxysporum* with the size of 69 nm on Hela cells infected with *T. gondii*, RH strain was reported in another study. According that, inflammatory cytokines were not affected by silver nanoparticles, while interleukin 8 decreased (32). High-pressure homogenization was used to synthesize atovaquone nanosuspensions for in-vitro anti-parasitic activity on *T. gondii*, BK strain, and reported excellent efficacy and low cytotoxicity (33). The synergistic effects of some nanoparticles and spiramycin on immune responses against toxoplasmosis was evaluated, the combined treatment was more effective than single therapy. The best results were observed in mice receiving a combination of chitosan nanoparticles and silver nanoparticles loaded with spiramycin (34).

In this study, spiramycin nanoemulsion was synthesized for the first time that showed a high lethal effects on tachyzoites of the *T. gondii*, RH strain. The in-vivo effectiveness of this combination or in synergism with other medications are suggested for future studies.

Conclusion

NE-Spi is more effective on tachyzoites of *T. gondii*, RH strain at the concentration of 250 µg/ml and after 120 min of exposure compared to the other dilutions (including 31.25, 62.5, 125, and 250 µg/ml) and in different time intervals (including 30, 60, 90, 120 min, and 24 h for 250 µg/ml).

Journalism Ethics considerations

Ethical issues (Including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc.) have been completely observed by the authors.

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Conflict of interest

The authors declare that there is no conflict of interest.

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