

Evaluation of spiramycin for topical applications: a cell culture study

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Abstract. – OBJECTIVE: Through a cell culture test, we analyzed the cytotoxic effects of topical spiramycin on NIH/3T3 fibroblast cells.

MATERIALS AND METHODS: Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin was used for the growth of NIH/3T3 fibroblast cells in a 5% CO₂ incubator. Spiramycin's cytotoxicity was measured using the MTT assay. 5,000 NIH/3T3 cells per well of a 96-well plate were seeded in each well, and the cells were treated with spiramycin (3.13-100 µM) for 24, 48 and 72 hours while the plates were incubated at 37°C in a humidified 5% CO₂ atmosphere. First, 10⁵ NIH/3T3 cells were seeded onto coverslips in 6-well plates for morphological analysis of both untreated and spiramycin-treated cells. For 24 hours, NIH/3T3 cells were exposed to a 100 µM dosage of spiramycin. The cells in the control group were grown in complete growth media alone.

RESULTS: Spiramycin was non-toxic to NIH/3T3 fibroblast cells in a MTT test. The concentration of spiramycin used to stimulate cell growth increased as the concentration was increased. After 24 and 48 hours of treatment with 100 µM NIH/3T3, the cells showed the most significant increase in size. Cell viability was shown to be significantly reduced at spiramycin doses of 50 and 100 µM. All MTT findings revealed that spiramycin enhanced cell viability and was not harmful to the fibroblast cells for short-term application of 24 and 48 hours but lowered the viability of fibroblast cells at the doses of 50 and 100 µM for long-term application duration of 72 hours. Confocal micrographs showed that spiramycin treatment did not affect the cytoskeleton or nucleus of fibroblast cells, in contrast to the control NIH/3T3 cells. Both untreated and treated with spiramycin, fibroblast cells were found to be fusiform and compact, with their nuclei remaining unaltered and unreduced in size.

CONCLUSIONS: It was concluded that spiramycin has a beneficial effect on fibroblast cells and is safe for use over short periods. Spiramycin reduced fibroblast cell viability when applied for 72 hours. Confocal micrographs showed that fibroblast cell skeletons and nuclei were unharmed and undamaged, that cell shapes were fusiform and compact, and that nuclei were neither broken nor shrunken. Topical spiramycin could be recommended for septorhinoplasty procedures due to anti-inflammatory effects for short-term usage if clinical trials will confirm experimental data.

Key Words:

Spiramycin, NIH/3T3 fibroblast cells, MTT assay, Confocal micrographs, Viability, Toxicity.

Introduction

Seventy years ago, in 1952, a macrolide antibiotic called spiramycin was developed in a *Streptomyces ambofaciens* growth medium. Spiramycin is widely used to treat toxoplasmosis and others soft-tissue infections in "cattle, pigs, poultry, and sheep¹⁻³" due to its antibiotic action, which inhibits protein synthesis in bacterial cells during translocation. This antibiotic is very effective against "Gram-positive bacteria and mycoplasma species^{4,5}". In certain countries, doctors use spiramycin to treat periodontitis and other illnesses that might cause a foul odor. It is safe for youngsters because it has been shown⁶ to have antiviral action against enterovirus A71 *in vitro* and *in vivo*. Several studies⁷ have shown that spiramycin, by its ability to block adipogenesis,

significantly reduces obesity and hepatic steatosis brought on by a high-fat diet (HFD).

Spiramycin is an alternate drug for gonorrhea in people allergic to penicillins. It is suggested to treat infections of the respiratory system, buccal cavity, skin, and soft tissues caused by sensitive organisms. *C. difficile*-associated diarrhea (CDAD) and pseudomembranous colitis have been linked to antibiotic overuse, and CDAD has been seen⁸ in patients for more than two months after antibiotic discontinuation.

To avoid infecting the fetus, spiramycin is used off-label to treat pregnant women with either acute or reactivated latent toxoplasmosis. Asymptomatic infection in the mother does not rule out the possibility of congenital transmission. Chorioretinitis, congenital disabilities, and developmental delay are all possible consequences of *Toxoplasma gondii* infection during pregnancy. While the likelihood of fetal infection rises as the baby ages, the severity of congenital diseases spikes earlier in pregnancy⁹.

Here, utilizing a cell culture experiment, we looked at the cytotoxic effects of topical spiramycin on NIH/3T3 fibroblast cells. These compound cytotoxic effects were examined using an MTT colorimetric test and a confocal microscopic assessment in a cell culture investigation.

Materials and Methods

This study was undertaken at the ENT Department of Eskisehir Osmangazi University, working alongside the Department of Biology within the Faculty of Science at Eskisehir Technical University.

NIH/3T3 Cell Culture

Under 5% CO₂ incubator conditions, fibroblast cells (NIH/3T3) (Commercially available from: <https://www.atcc.org/products/crl-1658>) were grown in Dulbecco's Modified Eagle Medium (DMEM) containing fetal bovine serum (10%) and penicillin/streptomycin (1%) as the complete medium. At 85% confluence, cells were employed for testing after being passaged twice a week without contamination.

Multi-Temperature Toxicity Assay (MTT)

Spiramycin's cytotoxicity was measured using the MTT assay. NIH/3T3 cells in 96-well plates were seeded at a density of 5x10³ per well. The cells were exposed to spiramycin concentrations

ranging between 3.13 and 100 µM for 24, 48, and 72 hours while in a cell culture incubator. MTT (20 µL/well, 5 mg/mL in PBS) was added to each well after incubation, and the plates were kept in the incubator for additional 4 hours. After that, 200 µL of dimethyl sulfoxide (DMSO)/well was used to dissolve the formazan crystals generated by the live cells. The absorbances were measured at 560 nm using an ELISA reader (BioTek HTX Synergy, Winooski, VT, USA). The measured absorbances were then used to determine the percentage viability of NIH/3T3 cells.

Confocal Microscopy-Based Cellular Morphology Analysis

First, 10⁵ NIH/3T3 cells were seeded onto coverslips in 6-well plates for morphological analysis of both untreated and spiramycin-treated cells. Over 24 hours, NIH/3T3 cells were exposed to a 100 µM dosage of spiramycin. The cells in the control group were grown in complete growth medium alone. All cell samples were phalloidin stained for 1 hour at room temperature in the dark after being fixed with glutaraldehyde. Before being stained with acridine orange for 10 minutes at room temperature in the dark, the stained cells were rinsed with an immediate blocking solution. Using a confocal microscope, we imaged samples under cover slips on microscope slides (Leica SP5-II, Wetzlar, Germany).

Statistical Analysis

One-way ANOVA and post-hoc Tukey tests were employed alongside the GraphPad prism (La Jolla, CA, USA) 6 tools for statistical analysis. *p*-values lower than 0.05 were considered statistically significant.

Results

Using the MTT test, spiramycin was found not to be harmful to NIH/3T3 fibroblast cells, suggesting that it is safe. Figures 1 and 2 show that cell proliferation increased as spiramycin concentration increased. After 24 and 48 hours of treatment with 100 µM NIH/3T3, the cells showed the most significant increase in size. Figure 3 displays a statistically significant reduction in cell viability at 50 and 100 µM spiramycin doses. All MTT findings revealed that spiramycin enhanced cell viability and was not harmful to the fibroblast cells for short-term application of 24 and 48 hours

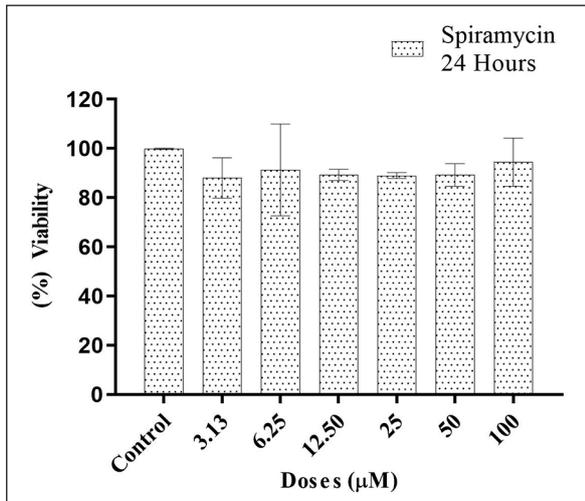


Figure 1. Viability percentages of NIH/3T3 cells treated with spiramycin for 24 hours.

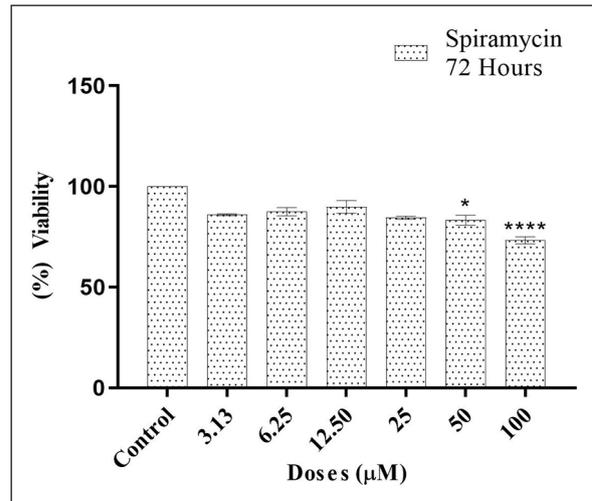


Figure 3. Viability percentages of NIH/3T3 cells treated with spiramycin for 72 hours (* $p \leq 0.0386$, **** $p \leq 0.001$).

but lowered the viability of fibroblast cells at 50 and 100 μM doses at the long-term application period of 72 hours.

Confocal micrographs showed that spiramycin treatment did not affect the cytoskeleton or nucleus of fibroblast cells, when compared to the control NIH/3T3 cells (Figure 4-5). Nuclei were not fractured or shrunken, and the morphology of the cells was fusiform and compact in both untreated and spiramycin-treated fibroblasts.

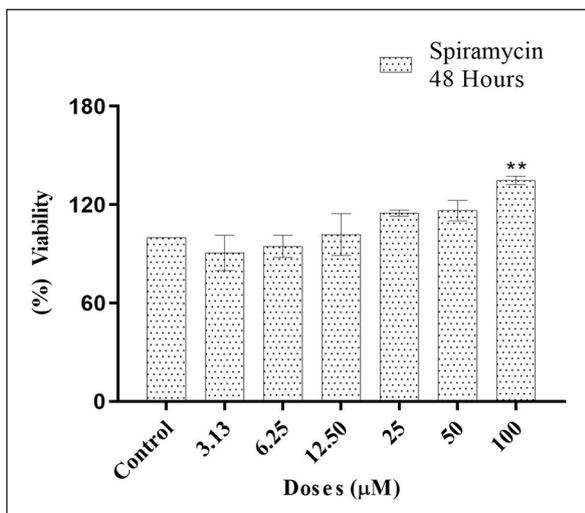


Figure 2. Viability percentages of NIH/3T3 cells treated with spiramycin for 48 hours (** $p \leq 0.0014$).

Discussion

An analysis by Chavanet and Portier¹⁰ found that spiramycin's high concentration in tonsillar and pharyngeal tissues was an excellent treatment for pharyngitis and tonsillitis. Saliva contained spiramycin at amounts 1.3-4.8 times higher than serum. For 4.5 hours after a single 1.5 g dosage of spiramycin was given to a patient for three

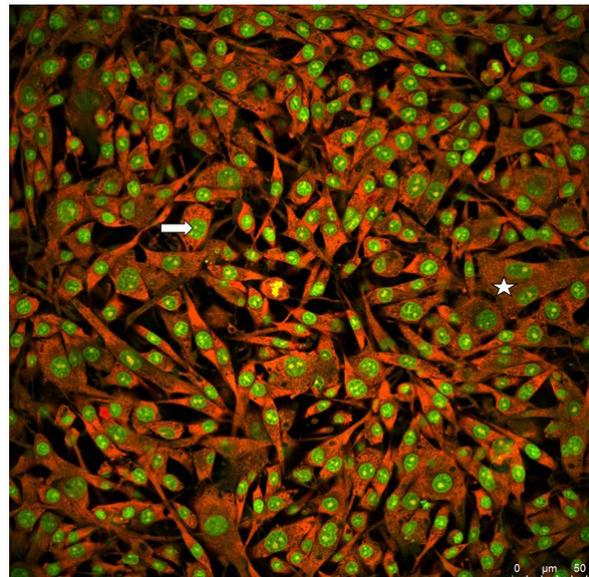


Figure 4. Confocal micrograph of NIH/3T3 cells applied with spiramycin for 24 hours. Arrow-nucleus (Green). Asterisk-cytoskeleton (Red) (Scale bar: 0-50 μm).

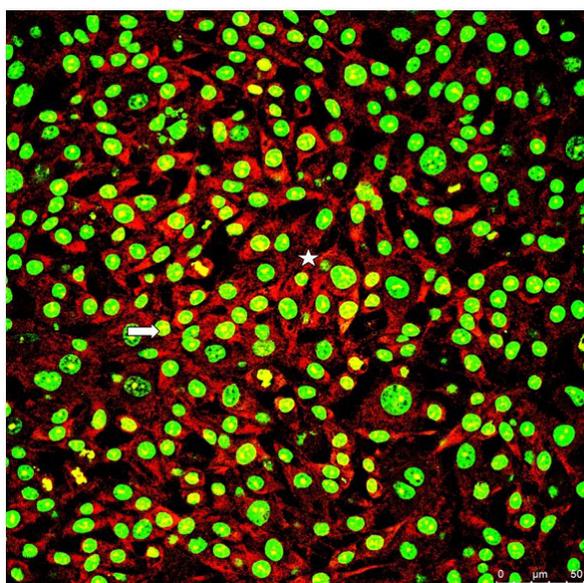


Figure 5. Confocal micrograph of NIH/3T3 fibroblast control cells. Arrow-nucleus (Green). Asterisk-cytoskeleton (Red). (Scale bar: 0-50 μm).

days, the minimum inhibitory concentration values of streptococci, pneumococci, and *Moraxella* (previously *Branhamella*) were attained or exceeded¹¹.

Spiramycin's antibacterial activity against respiratory tract *Haemophilus influenzae* strains¹² and modest action against anaerobic bacteria¹³ have been demonstrated *in vitro*. When *Staphylococcus aureus* was tested for its antibacterial reaction to spiramycin and erythromycin, spiramycin was shown¹⁴ to be more effective, with a more noticeable and longer post-antibiotic effect. Clinical trials^{15,16} have shown that spiramycin is as practical as or more effective than doxycycline in treating acute sinusitis. It is as effective as penicillin in treating acute bacterial tonsillitis in adults. Due to its high intracellular activity, spiramycin has also been advocated for treating acute and chronic bronchitis¹⁷.

Here, utilizing a cell culture experiment, we looked at the cytotoxic effects of topical spiramycin on NIH/3T3 fibroblast cells. Spiramycin was shown to be non-toxic to NIH/3T3 fibroblast cells in an MTT cytotoxicity experiment. The concentration of spiramycin used to stimulate cell growth increased as the concentration was increased. After 24 and 48 hours of treatment with 100 μM NIH/3T3, the cells showed the most significant increase in size. Cell viability was shown to be significantly reduced at spiramycin doses of

50 and 100 μM . All MTT findings revealed that spiramycin enhanced cell viability and was not harmful to the fibroblast cells for short-term application of 24 and 48 hours but lowered viability of fibroblast cells at the doses of 50 and 100 μM at the long-term application period of 72 hours.

Photos taken using confocal microscopes showed that the cytoskeleton and nucleus of spiramycin-treated fibroblast cells were unharmed and unaltered from those of control NIH/3T3 cells. Nuclei were not fractured or shrunken, and the morphology of the cells was fusiform and compact in both untreated and spiramycin-treated fibroblasts.

Using lipopolysaccharide (LPS) as a model, Kang et al¹⁸ showed that spiramycin dramatically reduced nitric oxide (NO), interleukin (IL)-1, and IL-6 levels in RAW 264.7 cells. The spiramycin-induced drop in NO production may be explained by the fact that spiramycin also reduced the expression of NO synthase (iNOS). In addition, spiramycin blocked the activation and "nuclear translocation of nuclear factor B (NF-B)" and "the phosphorylation of mitogen-activated protein kinases (MAPKs) extracellular signal-regulated kinase (ERK)" and "c-Jun N terminal kinase (JNK)". This demonstrated that spiramycin inhibited NF-kappa B and MAPK signaling pathways, resulting in increased iNOS expression and decreased IL-6, IL-1, and NO release from macrophages.

The effectiveness of spiramycin against amoxicillin in treating individuals with acute community-acquired upper respiratory tract infections (URTIs) was studied by Bunnag et al¹⁹. Patients were given either spiramycin (3 MIU; 2 tablets, 500 mg or 1.5 MIU each tablet) twice daily after meals, for a total of 6 MIU/day, or amoxicillin (1 capsule, 500 mg/day, after meals), for a total of 1,500 mg/day. This research¹⁹ involved 99 individuals, 49 of whom were given spiramycin, and 50 were given amoxicillin. Investigators evaluated 40 of the 45 patients treated with spiramycin as a "success" (89%) and five as a "non-success" (11 percent). In contrast, for the 48 patients treated with amoxicillin, 40 were classed as a "success" (83.3 percent), and eight were classified as a "non-success" (16.7%). Concerning treatment efficacy as a whole, no statistically significant differences were shown among the available options. This research¹⁹ showed that spiramycin and amoxicillin regimens equally effectively treated adult acute URTIs. Both medications were equally well-tol-

erated. It was also mentioned that spiramycin's twice-daily dosing schedule might increase patient compliance.

Doxycycline and spiramycin were used to treat patients with acute maxillary sinusitis with secretion in research by Axelsson and Brorson²⁰. Doxycycline was administered for nine days, initially at 200 mg, then at 100 mg once a day. For ten days, 1.5 g of spiramycin was administered twice daily. For this study, we used the antibiotics' ability to suppress bacterial growth to analyze the antibiotic concentration in 149 samples of sinus discharge, either aspirated or irrigated, from 58 individuals. Antibiotic concentrations were determined physiologically by plating suitable bacterial strains over thick blood agar plates. The concentration exceeded the bacteria's minimal inhibitory concentration (MIC) by a wide margin. A statistically significant rise in spiramycin concentration was seen as treatment time progressed.

Two sets of 4- to 10-week-old calves were used in Friis et al²¹ pharmacokinetic determinants of spiramycin and its distribution into the respiratory tract study. Measurements of spiramycin distribution into nasal and bronchial secretions, as well as pharmacokinetic characteristics, were obtained in Group A calves (n = 4), following intravenous (IV) and oral administrations of the medication at doses of 15 and 30 mg/kg of body weight, respectively. The spiramycin distribution in lung tissue and bronchial mucosa was analyzed using Group B calves (n = 4). Spiramycin accumulates in high amounts in the fluids and tissues of the respiratory system. Three hours after spiramycin injection, the tissue-to-plasma concentration ratio was 58 for lung tissue and 18 for bronchial mucosa, and after 24 hours, these ratios increased to 137 and 49, respectively. The ratio of secretion concentration to plasma concentration was relatively stable throughout time (4 for nasal secretions and 7 for bronchial secretions). This demonstrates that spiramycin is able to go further into the respiratory system, but its effectiveness in bronchial secretions is lower than in lung tissues and bronchial mucosa. The calculated maintenance dosage of spiramycin against bovine pathogens in bronchial secretions is 20 mg/kg IV once a day following an initial loading dose of 45 mg/kg IV.

After being treated with spiramycin, IL-1, and IL-6 secretion were decreased. The innate immune response relies heavily on many pro-inflammatory cytokines, including tumor necro-

sis factor-alpha (TNF- α), interleukin-6 (IL-6), and interleukin-1 beta. Macrophage-produced pro-inflammatory cytokines have a crucial role in the onset and maintenance of chronic inflammation²². Spiramycin showed substantial inhibitory activity to counteract LPS's stimulation of the mitogen-activated protein kinase/nuclear factor B (MAPK/NF-B) signaling pathway. Many inflammatory mediators and cytokines can be controlled by the intracellular signaling pathways nuclear factor B and mitogen-activated protein kinases (MAPKs)²²⁻²⁵. Spiramycin's antiviral properties and ability to reduce IL-6, IL-1, NO, and iNOS suggest that it may help treat cytokine storms. In this regard, spiramycin has been proposed¹⁸ as a topical anti-inflammatory treatment option.

Conclusions

Spiramycin has a beneficial effect on fibroblast²⁶ cells and is safe to be used over short periods. Spiramycin reduced fibroblast cell viability when applied for 72 hours. Confocal micrographs showed that fibroblast cell cytoskeletons and nuclei were unharmed and undamaged, that cell shapes were fusiform and compact, and that nuclei were neither broken nor shrunken. Topical spiramycin could be recommended for septorhinoplasty procedures due to anti-inflammatory effects for short-term usage if clinical trials will confirm experimental data.

Conflict of Interest

The Authors declare that they have no conflict of interests.

Ethics Approval

This is a cell-culture study conducted by commercially available NIH/3T3 cells. Therefore, Ethics Committee approval was not needed.

Informed Consent

This is a cell-culture study conducted by commercially available NIH/3T3 cells, and there is no need to take informed consent.

Funding

There are no funds for this study.

Authors' Contribution

Ozlem Yagiz Aghayarov: Planning, designing, literature survey, active intellectual support. Nuray Bayar Muluk: Planning, designing, literature survey, interpretation of the results, active intellectual support, writing, submission. Canan Vejselova Sezer: Planning, designing, data collection, literature survey, statistical analysis, interpretation of the results, active intellectual support. Hatice Mehtap Kutlu: Planning, designing, data collection, literature survey, interpretation of the results, active intellectual support. Cemal Cingi: Planning, designing, literature survey, data collection, interpretation of the results, active intellectual support, English editing.

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