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The investigation of the effect of NAD, H_2O_2 and weak magnetic field on the antibacterial mechanism of isoniazid (INH) that first line antibiotic against *M. tuberculosis* agent

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Abstract

Tuberculosis is an infectious disease, which is caused by the *Mycobacterium tuberculosis* complex. This disease leads to up to 1.3 million deaths out of more than eight million cases every year. A prodrug called isoniazid has been proven to be effective and widely used in the treatment of infections caused by tuberculosis. Despite its use for more than six decades clinically, the action mechanism of this prodrug is yet to be elucidated. INH action agains mycobacteria requires catalase−peroxidase (KatG) function, and IN-NAD adduct formation is catalyzed in vitro by *M. tuberculosis* KatG under a variety of conditions. Low-intensity EMF (Electromagnetic Field) has been used in therapeutic practices in addition to its use in telecommunication systems and food protection. EMF is used in medicine and food industries especially for its bactericidal effects. In this study, we aimed to investigate the effects of weak magnetic field application and the addition of NAD and H_2O_2 on the action mechanism of isoniazid. We added H_2O_2 and NAD individually and together, to the different groups at varying concentrations. Also, one experimental group was exposed to a 5mT, 50Hz magnetic field for 4 to 5 hours per day (total of 45 hours in 10 days). The agar proportion method was used to evaluate the results. It was determined that the addition of 100 µM NAD and H_2O_2 together increased the effectiveness of isoniazid to some extent. However, the application of a weak magnetic field did not change the effectiveness of the drug.

Keywords: *Mycobacterium tuberculosis*, Isoniazid, Magnetic Field, NAD, H_2O_2

Introduction

According to the WHO 2018 Tuberculosis Report, millions of people continue to get sick every year with tuberculosis. It is estimated that fatalities related to tuberculosis have reached 1.3 million among HIV negative people and 300000 among HIV positive people in 2017. It is evaluated that approximately 1.7 billion people, 23 percent of the world's population, carry tuberculosis [1].

Tuberculosis is an infectious disease caused by bacilli called *Mycobacterium tuberculosis* complex. In addition to factors such as increasing and spreading of multi-drug resistant strains and co-infection with HIV, the long-term, combined, complex, expensive treatment regimen of tuberculosis and the inability to provide adequate patient compliance makes the control of the disease increasingly difficult [2].

Isoniazid, which is a prodrug, is an isonicotinic acid hydrazide and is a widely used and effective drug in the treatment of tuberculosis. The isonicotinic acid radical formed by KatG dependent isoniazid activation is oxidized by NADH to form the covalent bond compound of INH-NADH. The NADH/NAD ratio is extremely important for maintaining the viability of the bacterium and it also affects the susceptibility of mycobacteria to isoniazid. The reduction of NAD in the environment causes isoniazid resistance. In oxidative phosphorylation, NADH dehydrogenase enzyme forms NAD by NADH oxidation [3,4].

WHO accepts ICNIRP (International Commission on Non-Ionizing Radiation Protection) when it comes to the effects of non-ionizing radiation and IARC (International Agency for Research on Cancer) when it comes to cancer. In June 2001, IARC announced that electromagnetic fields (ELF) formed near energy transmission (high voltage) lines could be carcinogenic. ELF magnetic fields have been accepted as "Possibly Carcinogenic" (Group-2B) in the WHO classification of cancer [5].

The electromagnetic field affects the ion transport systems, membrane potential and ions related cellular response, especially in the cell membrane. Thus, it affects many cell functions such as cell growth and differentiation, apoptosis, DNA synthesis, RNA transcription, protein expression and phosphorylation, lipid reoxidation, ATP synthesis, hormone production, antioxidant

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enzyme activities, and metabolic activity [6-9].

This study aims to demonstrate the role of NAD, and H_2O_2 added to the environment in varying concentrations in isoniazid susceptible and resistant strains of M.tuberculosis, and at the same time, to determine the possible effects of 5mT, 50Hz weak magnetic field application on the efficacy of the drug.

Materials and Methods

In the first stage of the experiment, five groups (40 samples in total) were studied with four susceptible (H37Rv) and four resistant (H37Ra) strains total of 8 samples in each group for ten days. Cultivation was done in MD7H9 liquid media. Mc Farland turbidity was set to 0.5 ($1x10^6$ CFU/mL) and 0.2µg/ml of INH was added to each tube.

1. Group (Control 1 group): It was incubated in the absence of any treatment.

2. Group: 8 samples with four susceptible and four resistant strains were exposed to a magnetic field of BDC = 4.95 (± 0.02) mT, for 45 minutes every day for 4.5 hours in total. The room temperature was kept at 23 ± 2^0C.

3. Group: For each of the eight samples, 100µL of H_2O_2 prepared as 1mM per day, was added for ten days. Hence, H_2O_2 was obtained in the 10µM final concentration in the test tube.

4. Group: NAD was added to achieve a 10µM final concentration.

5. Group: H_2O_2 and NAD were added together for ten days to ensure that both H_2O_2 and NAD were present at the 10µM final concentration.

The agar proportion method was used. At the end of 10 days, 10µL was taken from each tube (from a total of 40 tubes) and were cultured by being taken with sterile loops into the Middlebrook 7H10 solid media, in which antibiotics were added and was previously prepared. The experiment was terminated to count the colonies formed in the plates that were kept in the incubator for 14 days.

In the second stage of the experiment, four groups were prepared which included five liquid media (MD7H9) each. ATCC H37Rv susceptible strains were cultured on these. They were exposed to H_2O_2 and NAD for 12 days such that their final concentrations were 50, 100, 150 and 200µM. To evaluate again by agar proportion method, the experiment was terminated to count the colonies by culturing on solid media (MD7H10).

Result

As the results of the normal distribution test, which is the first statistical evaluation, did not conform to the normal distribution, Kruskal Wallis Variance Analysis and Mann Whitney U tests, which are non-parametric statistics tests, were used in the analysis. When all the groups were evaluated by performing Kruskal Wallis variance analysis in more than two-group comparisons and a statistically significant difference was found between the groups ($p < 0.05$), the Mann Whitney U test was performed to determine which groups caused the difference.

Table 1. Effects of every 10 µM H_2O_2 and NAD on susceptible and resistant strains

GROUPS	SUSCEPTIBLE STRAIN H37Rv ATCC 27294 (colony numbers)	RESISTANT STRAIN H37Ra ATCC 25177 (colony numbers)
Control 2	a 17	a 83
	b 16	b 82
	c 17	c 84
	d 18	d 83
10 µM H_2O_2	a 18	a 82
	b 16	b 84
	c 15	c 83
	d 16	d 83
10 µM NAD	a 15	a 84
	b 15	b 81
	c 14	c 82
	d 16	d 81
10 µM H_2O_2 +10 µM NAD	a 7	a 83
	b 8	b 82
	c 7	c 82
	d 8	d 83

Table 2. The effects of susceptible and resistant strains of 45 hours 5mT magnetic field application on colony numbers

GROUPS	SUSCEPTIBLE STRAIN H37Rv ATCC 27294 (colony numbers)	RESISTANT STRAIN H37Ra ATCC 25177 (colony numbers)
Control 1	a 14	a 83
	b 16	b 81
	c 15	c 84
	d 15	d 80
Magnetic Field	a 16	a 80
	b 15	b 81
	c 15	c 82
	d 15	d 81

While the addition of H_2O_2 and NAD individually or combined in resistant strains did not affect colony numbers ($p > 0.05$), the addition of 10µM H_2O_2 and NAD, when added together, inhibited bacterial growth significantly by enhancing the efficiency of isoniazid dramatically in susceptible strains ($p < 0.05$). We then tested the dose-dependent effect by adding different concentrations of H_2O_2 and NAD only to susceptible strains.

When we compared the four experimental groups, in which H_2O_2 and NAD were added, such that their final concentrations were 50, 100, 150, and 200 µM, we found that the most effective concentration was achieved by adding 100µM H_2O_2 and NAD together. In this concentration, the growth of isoniazid-susceptible bacilli was more strongly inhibited as compared to others ($p < 0.05$)

Table 3. Colony numbers obtained by the addition of H_2O_2 and NAD

GROUPS	SUSCEPTIBLE STRAIN H37Rv ATCC 27294 (colony numbers)
Control Group 2	a 17 b 16 c 17 d 17 e 18
50 µM H_2O_2 +50 µM NAD	a 14 b 16 c 14 d 14 e 14
100 µM H_2O_2 +100 µM NAD	a 7 b 7 c 7 d 8 e 7
150 µM H_2O_2 +150 µM NAD	a 10 b 9 c 9 d 10 e 10
200 µM H_2O_2 +200 µM NAD	a 12 b 13 c 15 d 14

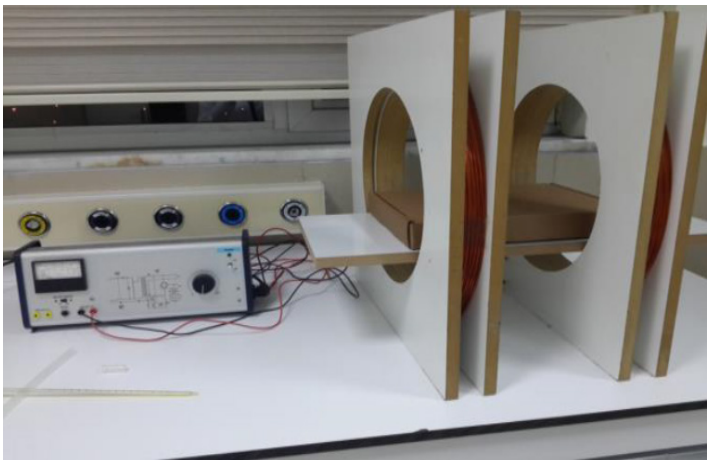

Figure 1. Our experimental setup

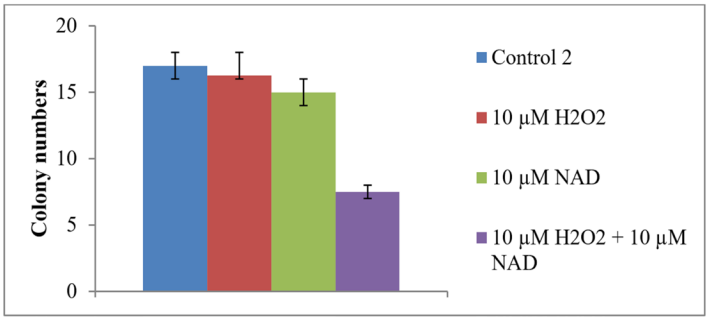

Figure 2. Effects of 10 µM H2O2 and NAD on susceptible strains

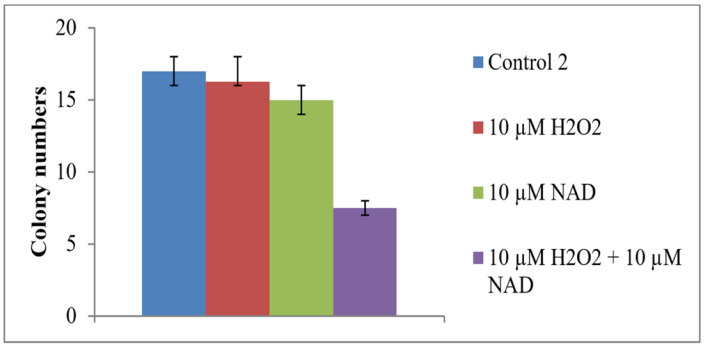

Figure 3. 10 Effects of 10 µM H2O2 and NAD on resistant strains

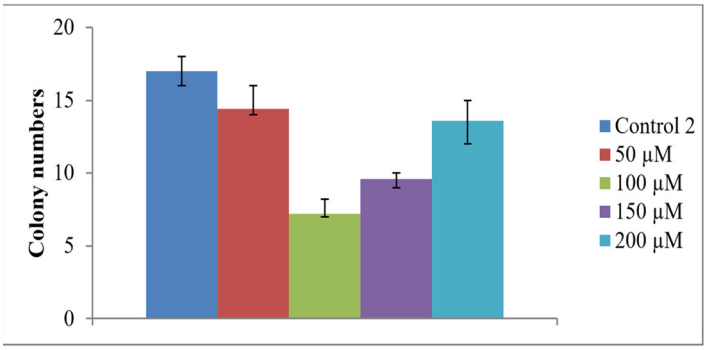

Figure 4. The combined addition of H2O2 and NAD at different concentrations affects the colony numbers of susceptible strains

Discussion

In the literature, studies are showing that the magnetic field increases cell membrane permeability [10]. Also, it is known to alter the activities of enzymes that catalyze the oxy-reduction reactions and contain metal ions in the active center [11,12].

In a study conducted by Fojt et al., they reported that exposure to 10 mT, 50 Hz magnetic field for an hour did not cause any morphological changes on the surface and in the form of both bacillus (*Escherichia coli*) and coccoid (*Paracoccus denitrificans)* shaped bacteria [13]. Another study conducted by the same research team found that there was a decrease in the number of colonies when 50mT, 50Hz electromagnetic field exposure of different bacterial strains (*Escherichia coli, Leclercia adecarboxylata, Staphylococcus aureus*) was compared to the control groups [14]. In another study conducted by Fojt et al., the effects of electromagnetic field exposure of 7.1mT, 50Hz and 24 minutes on the viability of sulfate-reducing bacteria were investigated, and it was shown that the number of colonies decreased by 15% compared to the control group after exposure [15].

Cellini et al. found that the exposure to 50 Hz and 0.1, 0.5, 1.0 mT magnetic fields for up to 2 hours changed the morphology of E.coli and transformed their form from bacilli to cocci. In culture, atypical bacilli were also found along with cocci. Also, the 50Hz electromagnetic field is thought to act as a stress factor in bacteria by inducing phenotypic and transcriptional changes [16].

In a study conducted by Strasak et al., it was found that 2.7-10mT, 50 Hz exposure had a negative effect on the proliferation of *E. coli*, and duration and intensity of increased magnetic field caused a bactericidal effect by reducing colony formation ability and oxidoreductive activity of bacteria [17].

In a study conducted by Segatore et al., *Escherichia coli* and *Pseudomonas aeruginosa* bacteria were exposed to 2mT, 50Hz electromagnetic field for 24 hours, and their growth and antibiotic susceptibilities were evaluated. As a result, there were no significant differences in antibiotic susceptibility and growth rates in both bacteria when compared to control groups. In the presence of different class antibiotics (kanamycin, amikacin, ampicillin, cefazolin, ceftazidime, ceftriaxone, mocsalactam and levofloxacin) with different effect mechanisms, the effects of electromagnetic field on the antibiotic susceptibility of both bacteria were tested and no significant change was observed in the minimum inhibition concentrations. According to the study, in the absence of antibiotics, bacteria can recognize the electromagnetic stimulation and regulate their physiological conditions accordingly, and potential cumulative effects may occur when exposed to both sub-inhibitory concentration antibiotics and very-low-frequency electromagnetic fields [19].

According to our comprehensive literature review, there is no such study investigating the possible effects of the magnetic field on the growth potential of *M. tuberculosis*. In this context, our study is the first study investigating the effect of magnetic field on *M. tuberculosis*. The unique cell wall structure of *M. tuberculosis* containing mycolic acid may have made bacilli more resistant to the potential effects of the magnetic field. Or the bacteria may have developed a metabolic adaptation to very long exposure. Even though different microorganisms are exposed to the same magnetic field, it is known that they are affected due to their different biological and molecular structures. The effects of the very low-frequency electromagnetic field are very diverse, depending on the cell type, exposure time, intensity, and frequency of the magnetic field.

These studies show that different magnetic field intensity, frequency and exposure times can have very different effects on different bacilli and strains. Different from these studies, in our study, we investigated *M. tuberculosis*, which has a very different cell wall structure. Low permeability and hydrophobicity of the cell walls of mycobacteria makes bacteria resistant to harmful compounds and makes the usage of some useful metabolites difficult. Also, mycobacteria which have a slow growth rate due to their low elongation rate of the nucleic acid chain, are divided into two in 18-24 hours. Due to the low growth rate of *M. tuberculosis*, we applied a magnetic field to the bacteria for a longer duration as compared to other studies. In conclusion, no significant change was observed when the colonies were counted, compared with the control group ($p > 0.05$).

In their study, Argyrou et al. showed in vivo that the increased NAD addition increased the INH-NAD levels which are effective forms of the drug [20]. Zhao et al., in their study, showed in vivo the effect of NAD and NADH addition, in the presence and absence of additional peroxide, on the rate of formation of INH-NAD compound with isoniazid. In the experiment conducted without the addition of H_2O_2, NADH was more effective in forming compounds than NAD. While NAD was 5 times more effective than NADH when slow-flowing H_2O_2 was added to the medium. InhA enzyme activity was observed with experiments performed with the addition of 200μM H_2O_2 and slow-flowing H_2O_2 with both wild type katG and S315T strains. It was observed that slow-flowing H_2O_2 addition was more effective in lowering the activity of the enzyme. In the study conducted with the wild-type strain, it was determined that the addition of 200μM H_2O_2 significantly decreased the enzyme activity [21]. In another in-vivo study by Cade et al., it was found that isoniazid-resistant strains with different mutations exhibit very different oxidant-specific reactivities. According to the study, the wild-type strain with the addition of 400μM H_2O_2 produces much more INH-NADH products, while the lower peroxide addition causes less product formation. The product formed without the addition of peroxide is extremely low compared to the product formed with the addition of peroxide. These rates were found to be quite different in strains with different mutations [22].

Conclusion

In our study, we determined that the application of a magnetic field to susceptible and resistant strains in the in-vitro environment did not change the efficiency of isoniazid. At the same time, in the experimental groups with different concentrations of H_2O_2 and NAD, the best efficacy was determined where 10μM H_2O_2 and NAD were added together to the susceptible strains. Then, we added H_2O_2 and NAD together into only susceptible strains in different concentrations and we found that the most effective concentrations were obtained by adding 100μM H_2O_2 and NAD together to the susceptible strains. New studies are needed to make isoniazid, which is widely used in the treatment of tuberculosis, more effective.

Competing interests
The authors declare that they have no competing interest.

Financial Disclosure
There are no financial supports.

Ethical approval
We made in-vitro study. We worked only with bacteria. We did not work with the patient sample

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