Effects of low intensity electromagnetic irradiation of 70.6 and 73 GHz frequencies and antibiotics on energy-dependent proton and potassium ion transport by \textit{E. coli}

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The effects of low intensity (flux capacity 0.06 mW/cm$^2$) coherent electromagnetic irradiation (EMI) of 70.6 and 73 GHz frequencies and their combined effects with antibiotics — ceftriaxone or kanamycin (0.4 or 15 µM, correspondingly) on \textit{E. coli} K12 growth and survival have been reported previously. To further study the effects of EMI and antibiotics and mechanisms, decrease in overall energy (glucose)-dependent H$^+$ and K$^+$ fluxes across the cell membrane was investigated in \textit{E. coli}. The depression of H$^+$ and K$^+$ fluxes rate was maximally achieved with the 73 GHz frequency. The EMI strengthened the effect of N,N’-dicyclohexycarbodiimide (DCCD, an inhibitor of the F$_0$F$_1$-ATPase). The 73 GHz EMI had more influence on H$^+$ efflux inhibition, whereas 70.6 GHz on K$^+$ influx. Also, EMI strengthened the depressive effects of ceftriaxone and kanamycin on the overall and DCCD-inhibited H$^+$ and K$^+$ fluxes. The 73 GHz EMI strengthened the effect of ceftriaxone on both ions fluxes. Kanamycin depressed H$^+$ efflux more as compared to ceftriaxone, which was also strengthened with EMI. The results of \textit{E. coli} H$^+$ and K$^+$ transport systems activities depression by irradiation and the irradiation effect on DCCD and antibiotics action indicated the EMI and antibiotics causing primary changes in the bacterial membrane.

**Keywords:** Extremely high frequency electromagnetic irradiation, \textit{E. coli}, Ion transport, Antibiotics, N,N’-dicyclohexycarbodiimide

Bacteria and other organisms are sensitive to the electromagnetic irradiation (EMI) of extremely high frequencies (ranging from 30 to 300 GHz)\textsuperscript{5,6}. Especially, in \textit{E. coli}, non-thermal intensity of “noise” EMI (broadband frequencies and randomly changing phases) of 45 to 53 GHz\textsuperscript{5,7,22} and coherent EMI (in time) of 42, 54, 66, 78 GHz\textsuperscript{5,7}, 51.8 and 53 GHz\textsuperscript{9,11} and 70.6 and 73 GHz\textsuperscript{12,14} have shown bactericidal effects. The most significant targets responsible for the cellular effects of EMI at resonant frequencies are water\textsuperscript{5,6,12,15,16,22}, bacterial membrane and its surface characteristics, which are responsible for substance transport and energy-conversing processes\textsuperscript{5,14,17-19}, and genome\textsuperscript{5,20,21}. EMI in a wide frequency range has shown effects on ligand binding to hydrophobic receptor proteins as an early event of the interaction with the cell\textsuperscript{5,22}.

The bactericidal effects of 51.8 and 53 GHz EMI are attributed due to complex effects associated with alterations in bacterial membrane structure, conformation and function\textsuperscript{5,9,11}. These frequencies depress the ion (H$^+$, K$^+$) transport and enzymatic activities (F$_0$F$_1$-ATPase and hydrogenase) of bacterial cell membrane. Also, the 51.8 and 53 GHz EMI have shown to strengthen the antibacterial effects of antibiotics\textsuperscript{5,14,23}. Moreover, bacteria and other cells are known to interact with each other through irradiation of sub-extremely high frequency range\textsuperscript{24}. Thus, the external EMI can have effect on bacteria at specific frequencies\textsuperscript{5,24} and can probably alter bacterial reaction to other factors, such as antibiotics and other chemicals\textsuperscript{5,8,9,14,17,23,25}. Especially, the effectiveness of combined action of EMI and antibiotics can be useful in future applications to overcome bacterial resistance to the antibiotics\textsuperscript{11,14}. Also, 51.8 and 53 GHz EMI have shown to alter the processes occurring in bacterial membranes (H$^+$ and K$^+$ transport and F$_0$F$_1$-ATPase activity) and the sensitivity of these processes towards antibiotics, such as ceftriaxone and kanamycin\textsuperscript{11,23}, thus indicating that the chemical and physical factors affect F$_0$F$_1$-ATPase, the main enzyme of bacterial membrane in anaerobic conditions\textsuperscript{5,9,11,23}.

Earlier, we have reported the effects of 70.6 and 73 GHz frequencies EMI and antibiotics — ceftriaxone and kanamycin from different groups of antibiotics (‘third generation’ semi-synthetic cephalosporins and aminoglycosides) on \textit{E. coli} growth and survival\textsuperscript{14}. These effects could be due to the reorganizations in
membrane and alterations inside the cell\textsuperscript{5,14,26}. Thus, the action of EMI on ions fluxes is important to understand the role of bacterial membrane. \textit{E. coli} has been reported to carry out H\textsuperscript{+} and K\textsuperscript{+} exchange in the presence of energy source by secreting 2 H\textsuperscript{+} with F\textsubscript{0}F\textsubscript{1}-ATPase and uptaking a K\textsuperscript{+} by TrkA system\textsuperscript{5,7,10,11,14,27-29}. This indicates that inhibitor of F\textsubscript{0}F\textsubscript{1}-ATPase, \textit{N,N\textprime}-dicyclohexylcarbodiimide (DCCD) and antibiotics (kanamycin and ceftriaxone) that primary react with cell membrane might be useful in revealing of the mechanism of enhancement of bactericidal action of antibiotics by the EMI. Therefore, in this paper, the effects of coherent 70.6 and 73 GHz frequencies EMI, DCCD and kanamycin and ceftriaxone on H\textsuperscript{+} and K\textsuperscript{+} ions transport across membrane have been investigated in \textit{E. coli} K12.

Materials and Methods

\textbf{Bacteria and preparation to assays}

\textit{E. coli} wild-type strain K-12 (\(\lambda\)) was used for the experiments. Bacteria were grown in a glucose (0.2\%) containing peptone medium (0.2\% peptone, 0.5\% NaCl and 0.2\% K\textsubscript{2}HPO\textsubscript{4}, pH 7.5) under anaerobic conditions till stationary growth phase (18-20 h) as described\textsuperscript{9,14}. Grown cells were concentrated following the 15 min centrifugation, washed and diluted in bi-distilled water. Thereafter, the bacterial suspension (10 ml) was transferred on to the plate (Petri dish) with suspension thickness of \(\sim\)1 mm (cell density was \(\sim\)2 \times 10\textsuperscript{9} cell in 1 ml suspension) for subsequent irradiation\textsuperscript{9,14}.

The 100 mM Tris-phosphate buffer containing 0.4 mM MgSO\textsubscript{4}, 1 mM NaCl and 1 mM KCl (pH 7.5) was used to determine H\textsuperscript{+} and K\textsuperscript{+} transport across bacterial membrane\textsuperscript{10,11,14}. pH of growth and assays mediums was determined by pH-potentiometer with selective electrode (HJ1131B, HANNA Instruments, Portugal)\textsuperscript{12}.

\textbf{Electromagnetic irradiation of bacteria}

Bacterial suspension was irradiated with coherent electromagnetic waves of 70.6 and 73 GHz frequencies using an EMI generator of G4-142 type (State Scientific-Production Enterprise (SSPE) "Istok", Fryazino, Moscow Region, Russia). The frequency stability of generator in continuous wave mode was up to 20 MHz; the amplitude-modulation frequency was 1 Hz. The distance from the radiating end of the conical antenna to the object of irradiation was \(\sim\)20 cm (the far zone). For this distance, the power flux density measured using a M5-49 thermistor and a M3-10A wattmeter (SSPE "Istok", Fryazino, Moscow Region, Russia) was 0.06 mW/cm\textsuperscript{2}. The frequency of output signal was controlled by a CH-25 wavemeter (SSPE "Istok", Fryazino, Moscow Region, Russia). The conical antenna with diagonal of 24 mm and altitude of 34 mm provided equal distribution of power to the exposed sample in mentioned distance. The power reflected to the waveguide system was \(\sim\)30\%; the actual intensity reaching cells on the surface was \(\sim\)70\% and on the bottom \(\sim\)50\%. These provided weak EMI power intensity\textsuperscript{14}.

The changes in temperature of exposed sample were below 0.1\ºC (non-thermal effects), and measured by thermometer; the sample kept at room temperature \((\sim\)25\ºC) during the exposure\textsuperscript{11-14}. Moreover, EMI with the flux capacity of 0.06 mW.cm\textsuperscript{2} was found to have no effect on the temperature of bacterial suspension during irradiation (non-thermal effect). The overall procedure was the same as that described elsewhere\textsuperscript{11-14}. The EMI effects were not dependent from \textit{E. coli} cells concentrations in suspension\textsuperscript{9,14}.

After 1 h irradiation (the optimal period for irradiation established earlier\textsuperscript{12,13}) of bacterial suspension, cells were washed following 15 min centrifugation (at room temperature) and immediately transferred into the assay buffer (Tris-phosphate buffer). In the control (without irradiation), bacteria were held in the same condition as exposed to irradiation.

\textbf{Ion transport assays}

The H\textsuperscript{+} and K\textsuperscript{+} fluxes through the non-damaged bacterial membrane were measured using appropriate selective electrodes (Hanna Instruments, Portugal; Cole Parmer Instruments Co., USA) as described elsewhere\textsuperscript{10,11,14}; correction for energy (glucose)-dependent H\textsuperscript{+} and K\textsuperscript{+} flux was made for the cells without and with glucose. Electrode readings data in mVs were outputted automatically by LabView computer program (National Instruments Co., USA). Electrode readings were calibrated by titration with 0.01 N HCl and 0.02 mM KCl. Ions fluxes were expressed as the change in external activity of the ion in mM/min per number of cells in a unit of medium volume (ml).

\textbf{Reagents and antibiotics used}

Agar, peptone, \textit{N,N\textprime}-dicyclohexycarbodiimide (DCCD) (Sigma, USA), glucose (Borisov Medical Preparations Plant, Borisov, Belarus) and other
reagents of analytical grade (Carl Roth GmbH, Germany) were used.

DCCD, a non-specific inhibitor of the $F_{0}F_{1}$-ATPase at 0.2 and 0.4 mM concentrations was used in the treatment of bacterial cells. The effects of DCCD (DCCD-sensitive values) were determined as a difference between the values in the presence and absence of DCCD in parallel measurements.

Bacteria were treated with antibiotics ceftriaxone (Rusan Pharma Ltd., USA) and kanamycin (Sinthez Ltd., Kurgan, Russia) for 10 min prior to the addition of glucose and subsequent assays. The minimal effective concentrations of ceftriaxone and kanamycin (4 and 15 µM correspondingly) were determined experimentally.

**Data processing**

All assays were carried out under anaerobic conditions at 37°C. The average data from three replicates with determination of the standard error (3%) and of the Student’s validity criteria (p) for the difference between experimental and appropriate non-irradiated control measurements were presented. These calculations were done using the SigmaPlot software. The statistically valid data above the columns in all figures were marked with *(asterisk); when not mentioned, p<0.01.

**Results**

**EMI effects on ion transport across E. coli membrane**

The EMI lowered the glucose-dependent $H^{+}$ and $K^{+}$ transport processes across bacterial membrane (Fig. 1A, B). Non-damaged E. coli K-12 cells carried out $H^{+}$ and $K^{+}$ exchange at the expense of energy from glucose fermentation by secreting 2 $H^{+}$ with $F_{0}F_{1}$-ATPase and uptaking a $K^{+}$ by TrkA system. The EMI, however, showed no effect on the $H^{+}/K^{+}$ exchange direction and ratio (2/1). The overall $H^{+}$ secretion from 70.6 and 73 GHz irradiated cells decreased ~1.2-fold (p<0.035) and ~1.3-fold (p<0.05), respectively (Fig. 1A). Addition of DCCD (0.2 and 0.4 mM) inhibited glucose-dependent $H^{+}$ efflux ~1.6-fold (p<0.02) and ~1.9-fold (p<0.035), respectively. DCCD addition also had depressive effect on irradiated bacteria. The $H^{+}$ efflux in presence of 0.2 mM DCCD from 70.6 GHz and 73 GHz irradiated bacteria was depressed ~1.6-fold (p<0.035) and ~1.8-fold (p<0.015), respectively, but in presence of 0.4 mM DCCD depressed ~2-fold and ~2.7-fold (p<0.05), respectively (Fig. 1A).

The $K^{+}$ influx was only slightly depressed (~1.1-fold; p<0.025) after the exposure to 73 GHz compared with the control flux (Fig. 1B). Furthermore, $K^{+}$ flux in control (non-irradiated) after DCCD addition (0.2 and 0.4 mM) was inhibited ~2.63-fold (p<0.05) and ~11.5-fold (p<0.025),...

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**Fig. 1**—Glucose-dependent total $H^{+}$ (A) and $K^{+}$ (B) fluxes in % through the whole E. coli K12 cell membrane after EMI of 70.6 and 73 GHz frequencies [DCCD was added into assay medium with final concentrations of 0.2 mM and 0.4 mM. The fluxes were determined as described in ‘Materials and Methods’ and presented the average data from three replicates. *(asterisk) mark above the columns indicated the statistically valid difference between of non-irradiated and irradiated data. Also, comparisons were done between data non-irradiated without DCCD and with DCCD of different concentrations. For irradiated samples with DCCD, comparisons were done with their appropriate DCCD concentrations of non-irradiated samples. The p values were presented in the text]
respectively. Such depressive effects for two DCCD concentrations on irradiated bacteria were ~2.72-fold (p<0.01) and ~22-fold (p<0.03) with irradiation of 70.6 GHz and ~2.52-fold (p<0.01) and ~19-fold (p<0.035) with irradiation of 73 GHz, respectively (Fig. 1B).

The change in ions fluxes, especially by 73 GHz EMI could be due to the alterations in the activities of F0F1 and TrkA and interaction between these proteins. This correlated well with the changed number of accessible SH-groups in membrane vesicles of irradiated bacteria (higher alterations were found with 73 GHz), as a consequence of rearrangement in intra- and inter-molecular disulfide bonds in membrane proteins\(^{13}\). Also, decrease of organic acids secretions, the end products of fermentation processes might have effect on the H\(^+\) flux rate\(^{5,14,28,30}\). Addition of 0.4 mM DCCD in the case of irradiated bacteria exhibited higher inhibiting effect on both ion fluxes than in the case of control. Thus, DCCD addition showed that 73 GHz had higher influence on the H\(^+\) efflux and 70.6 GHz on the K\(^+\) influx.

**EMI and antibiotics effects on overall and DCCD-sensitive ion transport**

The effects of EMI and antibiotics on energy-dependent H\(^+\) and K\(^+\) transport across the membrane of whole cells was also studied. The results for overall and DCCD-sensitive fluxes are shown in Figs 2 and 3. Ceftriaxone (Fig. 2) and kanamycin (Fig. 3) depressed

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**Fig. 2**—Glucose-dependent total H\(^+\) (A) and K\(^+\) (B) fluxes through the whole *E. coli* cell membrane after 70.6 and 73 GHz frequencies EMI [Irradiated bacteria were treated with ceftriaxone (4 µM) for 10 min prior to the addition of glucose and subsequent assays. The “non-irradiat” column presented the data of ion flux for ceftriaxone and without irradiation in % (compared with non-irradiated and without antibiotics control; the difference was statistically significant - p ≤ 0.01). For others, see legends to Fig. 1]

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**Fig. 3**—Glucose-dependent total H\(^+\) (A) and K\(^+\) (B) fluxes through the whole *E. coli* cell membrane after EMI of 70.6 and 73 GHz frequencies [The “non-irradiat” column presented the data of ion flux for kanamycin and without irradiation in % (compared with non-irradiated and without antibiotics control; the difference was statistically significant - p ≤ 0.015). For others, see legends to Figs 1 and 2]
H⁺ flux ~1.25-fold (p<0.025) and ~1.62-fold (p<0.01) (Figs 2A, 3A) and K⁺ flux ~1.13-fold and ~1.5-fold (p<0.02) (Figs 2B, 3B), respectively. However, they did not change the H⁺/K⁺ exchange direction and the ratio. Addition of DCCD showed higher depressive effects on overall H⁺ and K⁺ fluxes; the depression was ~1.53-fold and 2.65-fold (p<0.025) for ceftriaxone (Figs 2A, B) and ~1.4-fold (p<0.01) and 2-fold (p<0.015) for kanamycin (Figs 3A, B) with 0.2 mM DCCD, while with 0.4 mM DCCD, the depression was ~1.94-fold (p<0.03) and 11.8-fold (p<0.035) for ceftriaxone (Fig. 2) and ~1.6-fold (p<0.05) and 8.7-fold (p<0.035) for kanamycin (Fig. 3).

The effects of antibiotics on irradiated bacteria, especially on H⁺ flux were stronger compared with antibiotics alone. Ceftriaxone and kanamycin depressed H⁺ flux of 70.6 GHz irradiated bacteria 1.44-fold (p<0.025), but of 73 GHz irradiated bacteria 1.9-fold (p<0.035) and 2.12-fold (p<0.02), respectively (Figs 2A, 3A). Ceftriaxone also depressed 70.6 and 73 GHz irradiated bacterial K⁺ flux 1.2-fold and 1.33-fold (p<0.015), respectively (Fig. 2B).

Addition of DCCD, especially in higher concentration (0.4 mM) inhibited glucose-dependent ion fluxes, especially H⁺ efflux. The H⁺ flux of 70.6 and 73 GHz irradiated bacteria with ceftriaxone was depressed 1.74-fold and 2-fold, respectively (Fig. 2A) and with kanamycin 3.6-fold (p<0.025) and 4.7-fold (p<0.035), respectively (Fig. 3A). Interestingly, 70.6 GHz EMI and kanamycin also depressed K⁺ flux (11.5-fold; p<0.015), which was a little higher compared with kanamycin used alone (Fig. 3B).

The DCCD-sensitive ions fluxes (the difference between overall and DCCD-treated fluxes), which primarily indicate the F₁/F₀ ATPase operation was calculated as well. The DCCD-sensitive fluxes for both ions were more visible with 0.4 mM DCCD concentration (Figs 4A, B). The decrease of DCCD-sensitive H⁺ flux was insignificant after EMI, but K⁺ flux was decreased ~1.2-fold (p<0.025) after exposure to 73 GHz irradiation Ceftriaxone decreased these ions fluxes 1.2-fold (p<0.015) and kanamycin 1.9-fold (p<0.035) compared with the control (non-irradiated; without antibiotics). On 70.6 GHz irradiated bacterial H⁺ and K⁺ fluxes, only ceftriaxone had higher decreasing effect 1.7-fold and 1.3-fold (p<0.02), respectively. In case of 73 GHz irradiated bacteria, two antibiotics depressed DCCD-sensitive H⁺ flux and decrease with ceftriaxone and kanamycin was 2-fold and 2.6-fold (p<0.05) (Figs 4A, B), respectively. Ceftriaxone had higher decreasing effect on 73 GHz irradiated bacterial DCCD-sensitive K⁺ flux; the flux was decreased 1.5-fold (p<0.025) (Fig. 4B). However, kanamycin showed increasing effect on DCCD-sensitive K⁺ flux of 70.6 GHz irradiated bacteria, but had no effect on 73 GHz irradiated bacteria.

Thus, ceftriaxone and kanamycin had effects on both ions fluxes with irradiation, especially 73 GHz strengthened the effect of ceftriaxone. Kanamycin showed higher effect on H⁺ flux as compared with ceftriaxone, but ceftriaxone exhibited higher effect on DCCD depressed H⁺ and K⁺ fluxes than kanamycin. Interestingly, EMI and antibiotics alone had no effect on these ions ratios, which was 2 and 1 for H⁺ and K⁺.
fluxes, respectively. However, the combined action of EMI of 73 GHz and antibiotics changed the ratio to ~1.5-fold (p<0.01), but in case of 70.6 GHz, such change was only with ceftriaxone.

**Discussion**

Earlier studies have shown that EMI of 70.6 and 73 GHz frequencies enhance the depressive effects of ceftriaxone and kanamycin on *E. coli* growth and survival\(^5,14\), which might be due to the reorganizations within the membrane and inside of cell\(^5,8,14,17,26\). EMI causes alterations in membrane and membrane-associated processes possibly directly\(^5,12\) or such changes follow the alterations in structure and properties of water molecules\(^5,10,14\). Similarly, antibiotics cause modifications in bacterial membrane and change cell membrane properties, cell morphology and biochemical processes\(^14,25,31,33\). Therefore, to understand the action mechanism of EMI and antibiotics on bacteria, especially on membrane properties the ion (H\(^+\) and K\(^+\)) transport in *E. coli* was investigated in this study. The study showed that 70.6 and 73 GHz EMI with the main probability had impact on bacterial membrane. That is why the H\(^+\) and K\(^+\) transport systems activities in *E. coli* were depressed after irradiation, which was strengthened with DCCD and ceftriaxone and kanamycin; the two frequencies had almost similar effects.

*E. coli* in anaerobic conditions at pH 7.5 ferments glucose and yields organic acids and H\(_2\) production. This process is complex and many membrane-bound multi-enzyme complexes are involved, such as formate hydrogen lyase (FHL, responsible for H\(_2\) production, TrkA, K\(^+\) transport system and F\(_0\)F\(_1\)-ATPase\(^5,27-30\)). The latter supplies reducing equivalents from FHL to TrkA. This might specify the relation between H\(_2\) production by FHL, the F\(_0\)F\(_1\)-ATPase activity and H\(^+\)/K\(^+\) transport processes which occur by dithiol-disulfide transitions\(^5,27-29\). The modification of this interaction might initiate the disturbance of all of these processes, mainly the H\(^+\)/K\(^+\) transport process.

This study showed that the number of accessible SH-groups in membrane vesicles of 70.6 and 73 GHz irradiated bacteria were changed (the change was higher with 73 GHz). Thus, it is possible to suggest destruction of intra- or inter-molecular dithiol-disulfide bridges between membrane proteins\(^5,27-29\) having consequent effects on the structure and activity of F\(_0\)F\(_1\) and TrkA. Interestingly, the overall and DCCD-sensitive ATPase activity did not change with 70.6 and 73 GHz irradiation\(^12\), suggesting that the conformational or other changes in F\(_0\)F\(_1\) did not occur with these frequencies. But, the decrease in H\(^+\) flux rate might be due to decrease in organic acids secretions as a result of changed interactions between membrane proteins. However, 70.6 and 73 GHz EMI and antibiotics combined effect on *E. coli* H\(^+\)/K\(^+\) transport was less compared to the 51.8 and 53 GHz\(^3,11,14\), which might be due to the direct depressive effect of 51.8 and 53 GHz on F\(_0\)F\(_1\)-ATPase activity\(^5,9\).

In conclusion, EMI changes bacterial sensitivity towards antibiotics with a specific mechanism related to the alterations in the membrane. Further studies are required about action mechanisms and for application in antibacterial therapy (with EMI and antibiotics)\(^5\).

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