

# Enhancing Effect of 100.414-kHz Electromagnetic Field Produced by Defender's Pulse Generator on the ChIFN $\gamma$ -Like Molecule Inducing Capacity of *Lens culinaris* Agglutinin and 10% PBS Washouts of Different Holocene Minerals

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## Abstract

Macrophages play key role in host defense and tissue repair, and thus understanding regulation of their function is important. For instance, our previous results have shown that in chicken macrophage system (CoMA cell line), application of a pulse of electromagnetic fields of frequencies 0.618, 1.054, 5.229, and 100.414 kHz induces production of interferon  $\gamma$ -like molecules. In this study, we have shown that the electromagnetic field of 100.414 kHz is the most effective in inducing synthesis of chicken interferon  $\gamma$  and chicken interferon  $\gamma$ -like molecules in CoMA cells, especially when combined with *Lens culinaris* agglutinin and 10% phosphate-buffered saline washouts of different Holocene minerals. A 2-minute pulse of electromagnetic field was produced by Defender's pulse generator. Both chicken interferon  $\gamma$  and chicken interferon  $\gamma$ -like molecules from the cell supernatant were evaluated by an antiviral assay and were also analyzed with reverse-phase high-performance liquid chromatography on Phenomenex, Aeris peptide columns. Our results show that application of a single inducing factor (*Lens culinaris* agglutinin, 100.414 kHz electromagnetic field, 10% phosphate buffer saline washout) or combined usage of 2 of them moderately stimulated production of chicken interferon  $\gamma$ -like molecules (from 1.550 to 48.028 IU/mL), whereas the combination of 10% phosphate-buffered saline washout of Koprivnica rock + *Lens culinaris* agglutinin + 100.414 kHz/9 V resulted in an output of 162.122 IU/mL. Hence, we may conclude that a combined use of electromagnetic field, Holocene minerals, and *Lens culinaris* agglutinin greatly stimulates synthesis of chicken interferon  $\gamma$ -like molecules in CoMA cells.

## Keywords

CoMA cells, ChIFN  $\gamma$ , LCA, 10% PBS washout, Holocene minerals, induction, antiviral assay, RP-HPLC

## Abbreviations

ChIFN  $\gamma$ , chicken interferon  $\gamma$ ; EMF, electromagnetic field; EMEM, Eagle's minimal essential medium; IFN, interferon; LCA, *Lens culinaris* agglutinin; LCL, *Lens culinaris* lectine; PBS, phosphate buffer saline; rChIFN  $\gamma$ , recombinant ChIFN  $\gamma$ ; RP-HPLC,

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reverse phase high-performance liquid chromatography; SD, standard deviation; TFA, trifluoroacetic acid; VSV, vesicular stomatitis virus

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## Introduction

Macrophages are heterogeneous cells that play key roles in host defense and tissue repair following challenges from injury, infection, or malignancy.<sup>1,2</sup> Their function is determined by integration of signals from the microenvironment that, to date, are predominantly characterized as biologic and chemical stimuli, such as microbial products, cytokines, and metabolic factors. One important overlooked physical clue in regulation of macrophage functions are the endogenous and direct current electromagnetic fields (EMFs). Situations where EMFs arise include wounded tissues, where epithelial barriers were broken. For example, directional ion transport leads to a transepithelial potential difference of 50 to 100 mV across intact skin. This collapses topically to 0 at the breached epithelium giving rise to a steady voltage gradient of 40 to 200 mV/mm directed toward the wound edge and parallel to the epithelial layer, with the wound negative about distal tissue.<sup>3-5</sup> Pretnar and colleagues<sup>6</sup> reported that one or few strong electric impulses could induce interferon (IFN)-like proteins in human leukocytes that are antigenically different from any contemporary IFN molecule. Kovacs and colleagues<sup>7</sup> found that in porcine leukocytes, the addition of Sendai virus and electric impulses produced porcine interferon  $\alpha$ -like molecules. It was found<sup>8-10</sup> that 50 Hz EMFs caused phytohemagglutinin (PHA) induction of IFN  $\gamma$  and interleukin 6 (IL-6). This implies that electric impulses have a threshold, over which the impulses work as a stress, inducing the release of the inflammatory cytokines. The question is whether the same threshold exists in the case of the electromagnetic field, and, if there is, whether the changes are of the gene expression as in the case of direct impulses.<sup>10</sup>

Our previous experiments show that in CoMa cells, the pulses of EMFs of 0.618 kHz/9 V, 1.054 kHz/9 V, 5.229 kHz/9 V, and 100.414 kHz/9 V all for 2 minutes alone induce chicken interferon (ChIFN)  $\gamma$ -like molecules,<sup>11</sup> suggesting that no threshold exists for induction of ChIFN  $\gamma$ -like molecules by EMF pulses.

The aim of the present study is to determine whether induction of the production of ChIFN  $\gamma$  and ChIFN  $\gamma$ -like molecules upon irradiation of CoMA cells with EMF pulses of 0.618 kHz/9 V, 1.054 kHz/9 V, 5.229 kHz/9 V, and 100.414 kHz/9 V can be further facilitated by the addition of *Lens culinaris* agglutinin (LCA) and 10% phosphate buffer saline (PBS) washout of different Holocene minerals.

## Materials and Methods

### Materials

Recombinant ChIFN  $\gamma$  (rChIFN  $\gamma$ ; 40.000 IU/mL) was a gift from Prof Dr John Lowenthal (CSIRO Division of Animal

Health, Australian Animal Health Laboratory, Geelong, Victoria, Australia); LCA was from Merck (Micro-Polo, Maribor, Slovenia); and Holocene minerals Eko-Rast was from Multi Natura D.o.o., Peteranec, Croatia. Different samples of alluvial Holocene minerals were from Prof Dr Hrvoje Mazija (CIETO, Zagreb, Croatia). Chicken macrophage cell line (CoMA) was provided by Lidija Gradišnik (Institute of Biomedical Sciences, Faculty of Medicine, University of Maribor, Slovenia). High-performance liquid chromatography (HPLC) column was Phenomenex, Aeris peptide column 3.6 mm XB-C18, 250 mm  $\times$  4.6 mm. Defender's device was from BIOEL D.o.o. (Novi Sad, Republic of Serbia). Eagle's minimal essential medium (EMEM) with L-glutamine and antibiotics and Fetal Calf Serum (FCS) were from Merck (Micro-Polo). 10% PBS washout of Holocene minerals is 10% PBS washout of Holocene minerals. Ten grams of different Holocene minerals were put in 100 mL of PBS and centrifuged the suspension at 2500 rpm. The supernatant is its 10% PBS washout.

## Methods

### Cells

Cultivation of CoMA cells were performed in Sigma 175-cm<sup>2</sup> cell culture flasks from Merck (Micro-Polo) in EMEM with L-glutamine and antibiotics penicillin, streptomycin, gentamicin and 10% FCS for cell growth. (All mentioned ingredients are from Merck, Micro-Polo). After reaching the confluence, the CoMA cells were dispersed with help of Sigma trypsin solution (Merck, Micro-Polo) and placed in glass tubes (5 mL) with rubber stoppers. All cells were cultivated at 37 C with 5% CO<sub>2</sub>.

### Defender's Pulse Generator

#### Defender's Construction

Defender is a Zapper-like (by Dr Hulda Clark)<sup>12</sup> DC (9 V/0.5 mA) symmetric square pulse (positive offset) generator with an output signal amplitude of 5 V and generating frequencies of 0.664, 1.055, 5.229, and 100.414 kHz (6-100.000 pulses/s), with a current limit output using a 9-V battery voltage. Its basic construction is shown in Figure 1. The heart of the Defender is a processor unit generating frequencies among 0.618, 1.055, 5.229, and 100.414 kHz. The timed outputs of the different frequencies are regulated by the Piezo Buzzer and Driver unit. The duty cycle of the square impulses at each frequency is 50%, which means equal active impulse and pause duration.

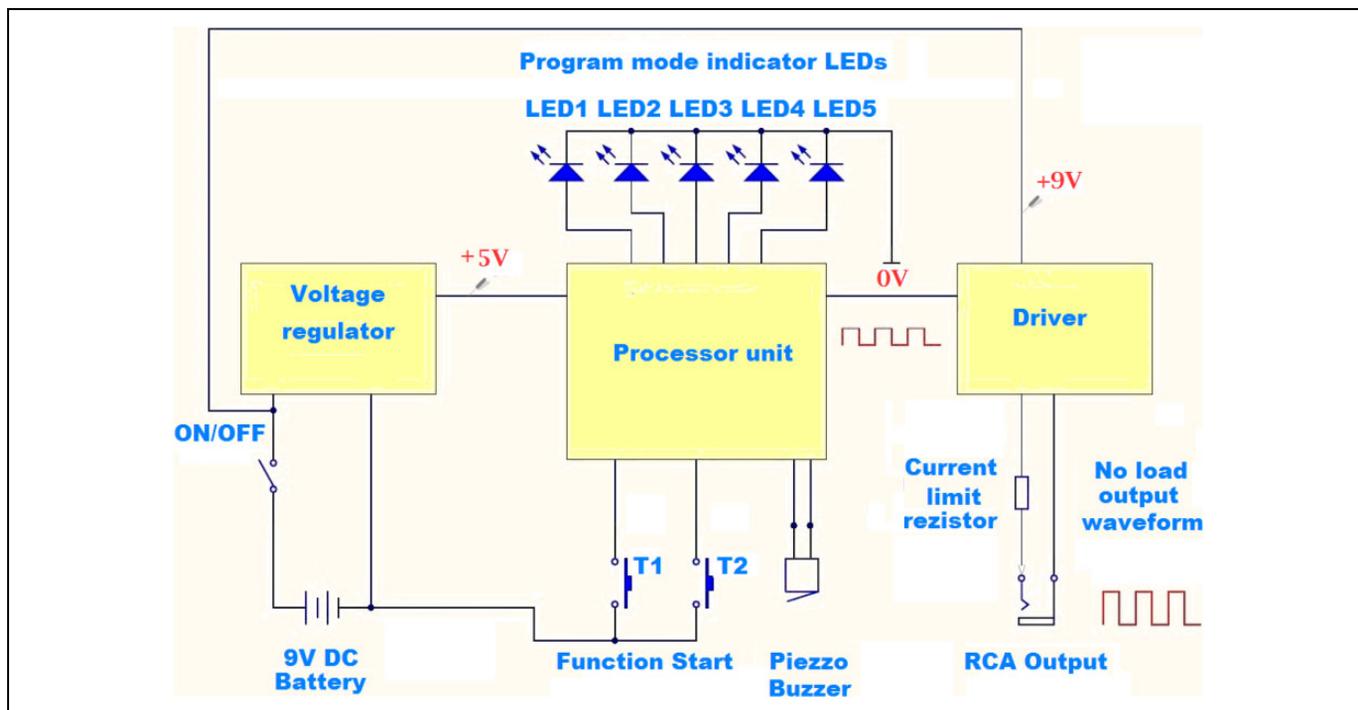


Figure 1. Basic construction of Defender’s pulse generator (Bioel D.o.o., Novi Sad, Republic of Serbia).

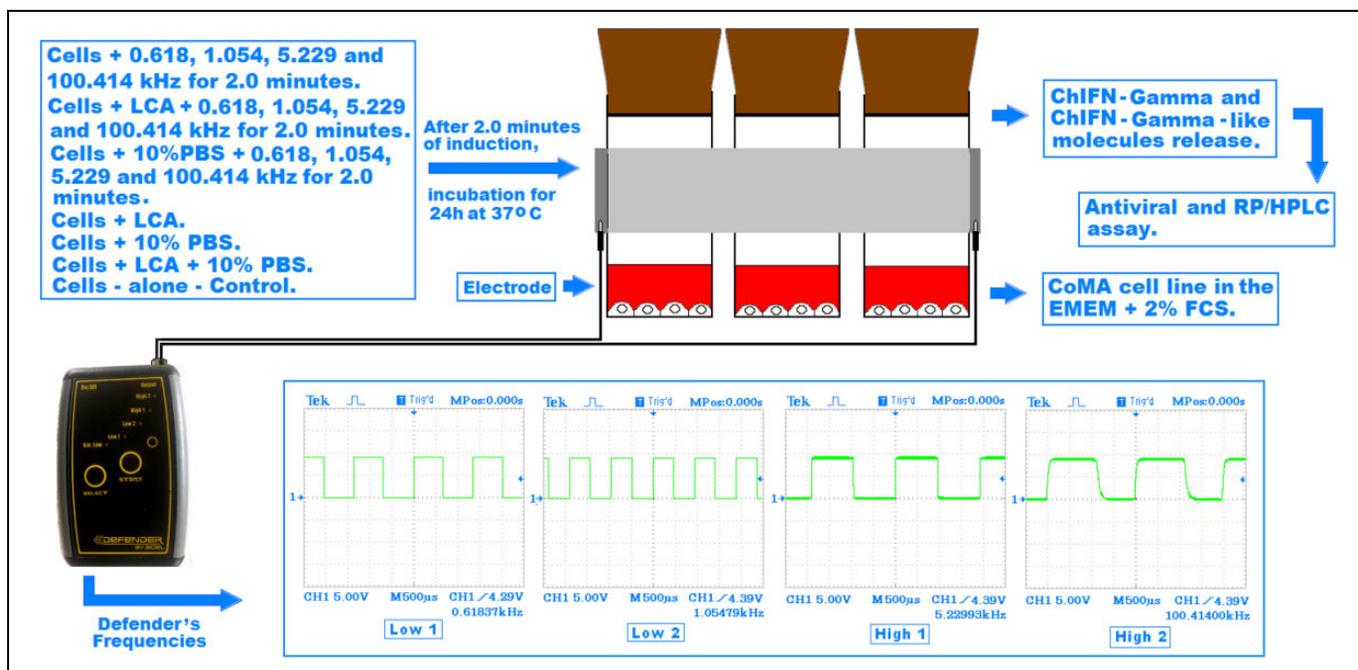


Figure 2. Scheme of the experiments.

### Symmetric Square Pulses

Different symmetric square pulse frequencies were measured on the oscilloscope Tektronix TDS 1001B (40 MHz, 500 ms/s), as shown in Figure 2.

### Generation of Frequencies’ Electric Fields

Different electric fields are calculated according to Yoshie<sup>13</sup>:  $J = \sigma E = \pi R f \sigma B$ , where  $\sigma$  is the electric conductivity. The values of the cell (as the highest value of the radius of the loop

**Table 1.** Generation of Frequencies' Electric Fields.

Frequencies, kHz	Electric Field, N/C
0.618	120.115
1.054	204.888
5.229	1.015.893
100.414	19.505.023

**Table 2.** CoMA Cells Treatment and Release of the ChIFN  $\gamma$  and ChIFN  $\gamma$ -Like Molecules.

CoMA cells treated with	ChIFN $\gamma$ and ChIFN $\gamma$ -like molecules, <sup>a</sup> IU/mL
0.618 kHz/9 V for 2 minutes <sup>b</sup>	139 (62) <sup>a</sup>
1.1054 kHz/9 V for 2 minutes <sup>b</sup>	457 (94) <sup>a</sup>
5.229 kHz/9 V for 2 minutes <sup>b</sup>	512 (39) <sup>a</sup>
100.414 kHz/9 V for 2 minutes <sup>b</sup>	1.550 (12) <sup>a</sup>
LCA <sup>c</sup>	6.372 (91)
LCA + 0.618 kHz/9 V for 2 minutes <sup>b</sup>	2.441 (79) <sup>a</sup>
LCA + 1.105 kHz/9 V for 2 minutes <sup>b</sup>	2.575 (87) <sup>a</sup>
LCA + 5.229 kHz/9 V for 2 minutes <sup>b</sup>	5.226 (75) <sup>a</sup>
LCA + 100.414 kHz/9 V for 2 minutes <sup>b</sup>	22.191 (180) <sup>a,d</sup>
10% PBS + 100.414 kHz/9 V for 2 minutes <sup>b</sup>	28.077 (105) <sup>a</sup>
LCA + 10% PBS <sup>c</sup>	48.028 (97) <sup>c</sup>
LCA + 10% PBS <sup>c</sup> + 100.414 kHz/9 V for 2 minutes <sup>b</sup>	105.476 (620) <sup>a,d</sup>

Abbreviations: ChIFN  $\gamma$ , chicken interferon  $\gamma$ ; LAC, *Lens culinaris* agglutinin; PBS, phosphate buffer saline.

<sup>a</sup>ChIFN  $\gamma$ -like molecule.

<sup>b</sup>Released from Defender's pulse generator (BIOEL D.o.o., Novi Sad, Republic of Serbia).

<sup>c</sup>LCA denotes *Lens culinaris* agglutinin 25  $\mu$ g/mL.

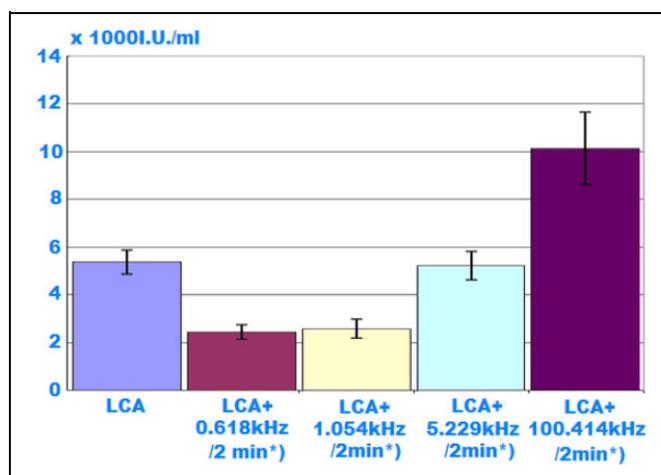
<sup>d</sup> $P = .05$ .

<sup>e</sup>10% PBS denotes 10% PBS washout of Holocene minerals.

for induced current) are as follows:  $R = 7.7$  mm, frequencies = 0.618, 1.054, 5.229, and 100.41 kHz, and magnetic flux density  $B = 3.9$  mT. In this study, the electric conductivity of culture medium was calculated from the measured impedance values of a medium. From the calculation based on the result of the impedance measurement, the electric conductivity was given as  $\sigma = 2.06$  S/m. For different frequencies, the electric fields are presented in Table 1.

### EMF Exposure Arrangement and Conditions

The glass tubes (5 mL) with confluent CoMA cells were covered with a wet bandage and on 2 separate sides connected with the electrodes of Defenders' device. According to the Defenders' protocol and experimental scheme (Figure 2 and Table 2) of CoMA cells treatment with *Lens culinaris* agglutinin (LCA), 10% PBS and combinations among them were exposed to 0.618 kHz, 1.054 kHz, 5.229 kHz, and 100.414 kHz/9 V for 2 minutes as determined by the oscilloscope Tektronix TDS 1001B (40 MHz, 500 ms/s). After EMF treatment for 2 minutes, tubes were incubated (5 mL) for 24 hours at 37 C. The



**Figure 3.** Effect of different strength of EMFs on LCA induction of ChIFN  $\gamma$ -like molecules. \*Released by Defender's pulse generator from BIOEL D.o.o., Novi Sad, Republic of Serbia. EMFs indicates electromagnetic fields; LCA, *Lens culinaris* agglutinin; ChIFN  $\gamma$ , chicken interferon  $\gamma$ .

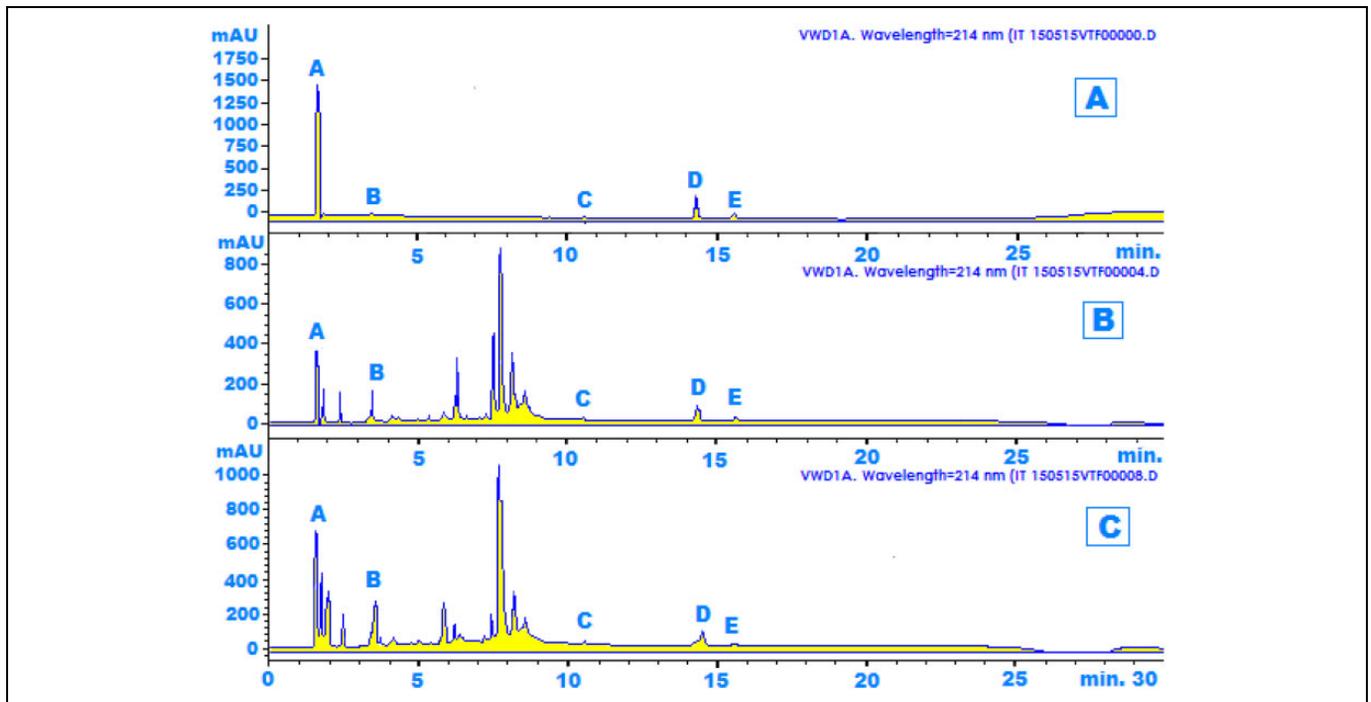
supernatants of the cells were then centrifuged at 1200 rpm for 15 minutes at +4 C (Centric 400R, Domel, Slovenija), collected, and filtered through 0.2- $\mu$ m filters (Corning Syringe filters from Merck, Micro-Polo) and stored at -20 C before analyses.

### Antiviral Assay

Titration of the supernatants for antiviral activity took place with a micro-plaque reduction assay<sup>11</sup> using CoMA cells and vesicular stomatitis virus (VSV; Indiana strain) as challenge virus. With 50 plaque-forming units of VSV, we infected CoMa cell's monolayer, and altogether the samples were tested twice in duplicate. Titrations took place with the International Reference Preparations of rChIFN  $\gamma$ . Titres were expressed mean (standard deviation [SD]).

### Reverse-Phase High-Performance Liquid Chromatography

We analyzed the rChIFN  $\gamma$ , ChIFN  $\gamma$ , and ChIFN  $\gamma$ -like molecule's subtype composition by reverse-phase high-performance liquid chromatography (RP-HPLC).<sup>14-16</sup> The HPLC column was Phenomenex, Aeris Peptide (3.6  $\mu$ m XB-C18, 250 mm  $\times$  4.6 mm). Natural and recombinant samples of 20 to 40  $\mu$ L applied to the column were eluted with the linear gradient of solvent A (water + 0.1% of, trifluoroacetic acid [TFA]) and solvent C (acetonitrile + 0.1% TFA) for 20 minutes with a flow rate of 0.8 mL/min and pressure of 139 to 140 bar. The temperature of the column was 40°C. Monitoring of the absorbance took place at 214 nm. According to the relative hydrophobicity of ChIFN  $\gamma$ -like species in different IFN compositions, they separate in the RP-HPLC.



**Figure 4.** Effect of EMF of 100.414 kHz/9 V on the LCA induction of ChIFN  $\gamma$ -like molecules. At 214 nm, rChIFN  $\gamma$ , ChIFN  $\gamma$ , and ChIFN  $\gamma$ -like RP-HPLC profiles are shown. Panel A: RP-HPLC profile of rChIFN  $\gamma$ ; A, B, C, D, and E marks on it denotes different subtypes of rChIFN  $\gamma$ . Panel B: RP-HPLC profile of with LCA-induced ChIFN  $\gamma$ ; A, B, C, D, and E marks on it denotes different subtypes of ChIFN  $\gamma$ . Panel C: RP-HPLC profile of with 100.414 kHz/9 V affected LCA-induced ChIFN  $\gamma$ -like molecules; A, B, C, D, and E marks on it denotes different subtypes of ChIFN  $\gamma$ -like molecule. EMFs indicates electromagnetic fields; LCA, *Lens culinaris* agglutinin; rChIFN  $\gamma$ , recombinant ChIFN  $\gamma$ ; ChIFN  $\gamma$ , chicken interferon  $\gamma$ ; RP-HPLC, reverse phase high-performance liquid chromatography.

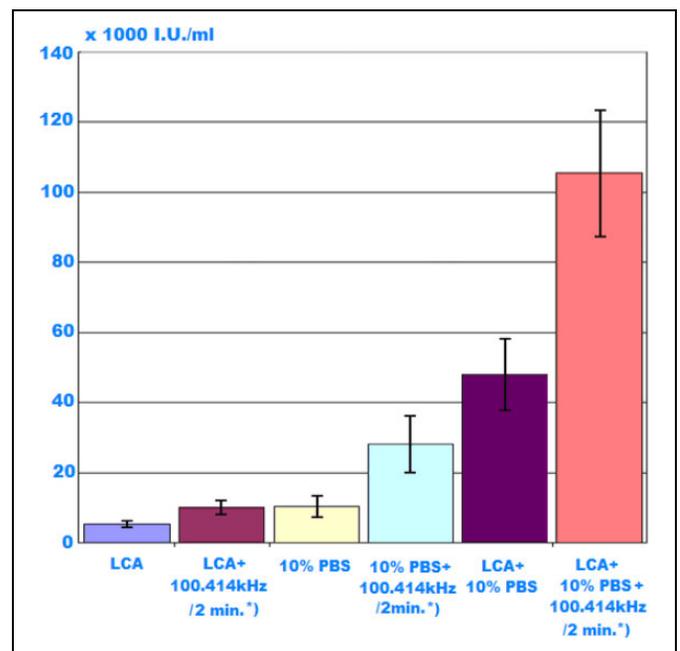
**Statistics**

Results are expressed as IU/mL and reported as mean (SD). Statistical evaluation of the experimental data was performed with a 2-tailed Student *t* test for paired samples with a *P* = .05 as the lowest level of significance.

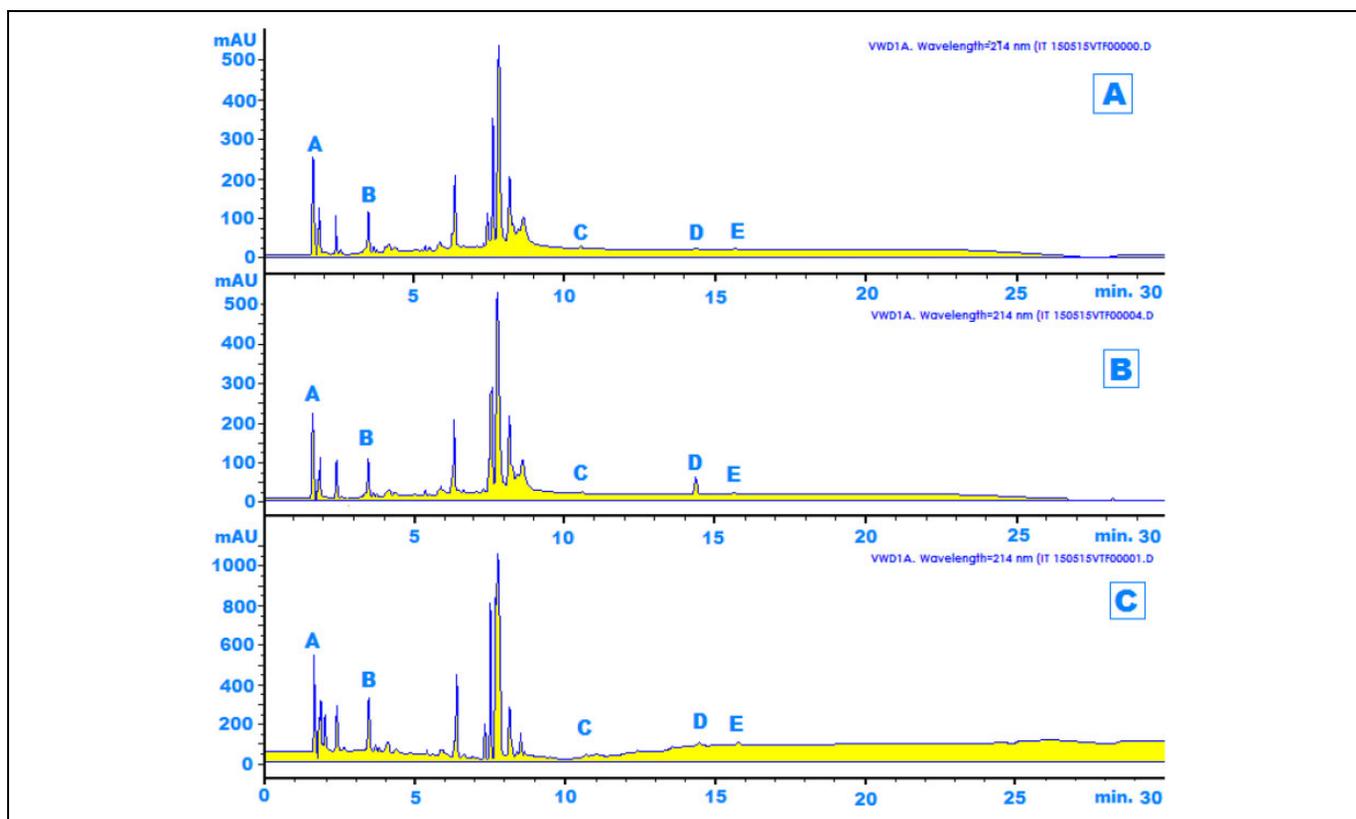
**Results**

*Effect of Different Strengths of EMFs on LCA Induction of ChIFN  $\gamma$  and ChIFN  $\gamma$ -Like Molecules*

During the experiments, a separate induction of ChIFN  $\gamma$  without EMF with 25 $\mu$ g/mL of LCA was attempted, resulting in 6.372 (91) IU/mL. Separate applications of 0.618 kHz/9 V, 1.054 kHz/9 V, 5.229 kHz/9 V, and 100.414 kHz/9 V for 2 minutes on the LCA only produced a statistically significant increase in the titer of ChIFN  $\gamma$ -like molecules with 100.414 kHz/9 V/2 minutes (Figure 5 and Table 2). Therefore, this indicates that EMFs of 0.618 kHz/9 V, 1.054 kHz/9 V, and 5.229 kHz/9 V for 2 minutes have the opposite effect, provoking inhibition of LCA induction of ChIFN  $\gamma$ -like molecules. Figure 4 shows the RP-HPLC profiles, showing an increase in mAU levels after LCA induction + 100 kHz/9 V/2 minutes (Figure 3).



**Figure 5.** Influence of EMF of 100.414 kHz/9 V for 2 minutes on the (1) LCA induction of ChIFN  $\gamma$ , (2) 10% PBS washout of Holocene minerals induction of ChIFN  $\gamma$ -like molecule, (3) LCA + 10% PBS washout induction of the ChIFN  $\gamma$ -like molecule. \*Released by Defender’s pulse generator from Bioel D.o.o., Novi Sad, Republic of Serbia. EMFs indicates electromagnetic fields; LCA, *Lens culinaris* agglutinin; ChIFN  $\gamma$ , chicken interferon  $\gamma$ ; PBS, phosphate buffer saline.



**Figure 6.** Effect of EMF of 100.414 kHz/9 V for 2 minutes on the 10% PBS induction of ChIFN  $\gamma$ -like molecules. At 214 nm, ChIFN  $\gamma$  and ChIFN  $\gamma$ -like RP-HPLC profiles are shown. Panel A: RP-HPLC profile of with 10% PBS washout-induced ChIFN  $\gamma$ -like molecules. A, B, C, D, and E marks on it denotes different subtypes of ChIFN  $\gamma$ -like molecules. Panel B: RP-HPLC profile of with 10% PBS washout + LCA-induced ChIFN  $\gamma$ -like molecules. A, B, C, D and E marks on it denotes different subtypes of ChIFN  $\gamma$ -like molecules. Panel C: RP-HPLC profile of 100.414 kHz affected 10% PBS washout + LCA-induced  $\gamma$ -like molecules. A, B, C, D, and E marks on it denotes different subtypes of ChIFN  $\gamma$ -like molecules. EMFs indicates electromagnetic fields; PBS, phosphate buffer saline; LCA, *Lens culinaris* agglutinin; rChIFN  $\gamma$ , recombinant ChIFN  $\gamma$ ; ChIFN  $\gamma$ , chicken interferon  $\gamma$ ; RP-HPLC, reverse phase high-performance liquid chromatography; PBS, phosphate buffer saline.

### Effect of EMF Field of 100.414 kHz/9 V for 2 Minutes on the Induction of ChIFN $\gamma$ and ChIFN $\gamma$ -Like Molecules

The ChIFN  $\gamma$ -like molecules induced with 10% PBS washout of Holocene minerals alone and in parallel with the 100.414 kHz/9 V applied for 2 minutes to the 10% PBS washouts of Holocene minerals were measured. Furthermore, a combination of LCA + 10% PBS washout of Holocene minerals was exposed to EMF of 100 kHz/9 V for 2 minutes. Figure 5 and Table 2 show that a statistically significant increase in antiviral activity (IU/mL) was found after addition of the combination of LCA + 10% PBS washout of Holocene minerals + 100.414 kHz/9 V for 2 minutes. The RP-HPLC profiles in Figure 6 confirm the increased antiviral activity (IU/mL) with increased mili Arbitrary Units (mAU) values.

Effect of 10% PBS washout of different Holocene minerals was added to LCA and EMF of 100.414 kHz/9 V for 2 minutes on the 10% PBS washout of different Holocene minerals and induction of the ChIFN  $\gamma$ -like molecules.

In these experiments, we tested 10% PBS washouts of different sources: “Koprivnica rock” and “Koprivnica sand.” In both cases, 10% PBS washouts were added to the LCA and

exposed to 100.414 kHz/9 V for 2 minutes, then incubated overnight at 37 C. The highest amounts of ChIFN  $\gamma$ -like molecules (Table 3) were produced after the use of the following combination: LCA + 10% PBS washout of Koprivnica rock + 100.414 kHz/9 V for 2 minutes, with a result of 161.122 (244) IU/mL.

### Discussion

Despite our previous finding that Defender’s pulse generator-released EMF frequencies of 0.618, 1.054, 5.229, and 100.414 kHz/9 V can all induce ChIFN  $\gamma$ -like molecules,<sup>11</sup> our present study demonstrates that only pulse of 100.414 kHz enhanced either LCA induction or LCA+10% PBS washouts of Holocene minerals induction of ChIFN  $\gamma$ -like molecules. This should not be surprising considering that at least 19-fold stronger electric field is produced by a pulse of 100.414 kHz, compared to fields caused by pulses of other used frequencies (Table 1). This result indicates that there is a threshold for induction of ChIFN  $\gamma$ -like molecules by EMF pulse combined with LCA and 10% PBS washouts of Holocene minerals. In contrast to that, our earlier results indicated no apparent

**Table 3.** Effect of EMF of 100.414 kHz on the 10% PBS Washout of Different Holocene Minerals Induction of the ChIFN  $\gamma$ -Like Molecules.

CoMA cells treated with	ChIFN $\gamma$ -like-molecules (IU/mL) <sup>a</sup>
LCA <sup>b</sup> + 10% PBS washout of Holocene minerals	48.028 (177)
LCA <sup>b</sup> + 10% PBS washout of Holocene minerals + 100.414 kHz/9 V for 2 minutes	105.476 (92) <sup>c</sup>
LCA + 10% PBSKR <sup>d</sup>	53.956 (83)
LCA + 10% PBSKR <sup>d</sup> + 100.414 kHz/9 V for 2 minutes	161.122 (244) <sup>c</sup>
LCA + 10% PBSKS <sup>e</sup>	29.640 (98)
LCA + 10% PBSKS <sup>e</sup> + 100.414 kHz/9 V for 2 minutes <sup>e</sup>	78.956 (183) <sup>c</sup>

Abbreviations: ChIFN  $\gamma$ , chicken interferon  $\gamma$ ; EMF, electromagnetic field; LAC, *Lens culinaris* agglutinin; PBS, phosphate buffer saline.

<sup>a</sup>Released from Defender's pulse generator (BIOEL D.o.o., Novi Sad, Republic of Serbia).

<sup>b</sup>LCA denotes *Lens culinaris* agglutinin 25  $\mu$ g/mL.

<sup>c</sup> $P = .05$ .

<sup>d</sup>10% PBSKR denotes 10% phosphate buffer washout of sample of "Koprivnica rock."

<sup>e</sup>10% PBSKS denotes 10% phosphate buffer washout of sample "Koprivnica sand."

threshold in the induction of ChIFN  $\gamma$ -like molecules production by EMF as a sole inducer.<sup>11</sup> Our results show that optimal duration of EMF pulse is 2 minutes; durations higher than 2 minutes caused the development of micro-plaques of dead/destroyed cells in the cells' monolayer.

There has been much research into possible mechanisms through which a pulse of 100.414 kHz/9 V could act. There are two modes of action: genotoxic and nongenotoxic. Of interest for us is nongenotoxic where it affects cell proliferation and cell cycle distribution, apoptosis, gene expression, immune system.<sup>17,18</sup> The radiofrequency EMF of 100.414 kHz affects the calcium-related machinery as a potential mediator of EMF on Ca<sup>2+</sup> ion exchange.<sup>19</sup> Small and somewhat variable "window" of frequencies are sensed when the immune system is exposed to EMFs between 100 kHz and 10 GHz.<sup>20</sup> It was found that the effect of Ca<sup>2+</sup> ions for ChIFN  $\gamma$  induction on the mobilization might be a summation of an influx (Ca<sup>2+</sup>) and release from the intracellular Ca<sup>2+</sup> stores.<sup>21</sup> The EMF of a frequency of 100.414 kHz/9 V for 2 minutes is probably responsible for distinct biological effects, connected with altered specific translation products, such as IL-2, heat shock protein, and ChIFN  $\gamma$ -like proteins.<sup>8,9</sup>

## Conclusion

In conclusion, 10% PBS washouts of different Holocene minerals (Table 3) enhanced the LCA induction of ChIFN  $\gamma$ -like molecules synthesis with the use of EMF of 100.414 kHz/9 V for 2 minutes. Sand from Koprivnica (sample 3) in a combination of 10% PBS washout of sample 3 + LCA + 100.414 kHz/9 V for 2 minutes gave 162.122 (409) IU/mL. Therefore, it is

possible that results depend on the specific chemical composition of various Holocene minerals. It is also important to get the cheap way of production of ChIFN  $\gamma$  and ChIFN  $\gamma$ -like molecules because of their use for poultry infection diseases therapy, as well as for cancer therapy in combination with the NDV virus ZG1999HDS.<sup>22</sup>

## Declaration of Conflicting Interests

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