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Bioassay for assessing cell stress in the vicinity of radio-frequency irradiating antennas†

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The 24 h exposure of water plants (etiolated duckweed) to RF-EMF between 7.8 V m⁻¹ and 1.8 V m⁻¹, generated by AM 1.287 MHz transmitting antennas, resulted in alanine accumulation in the plant cells, a phenomenon we have previously shown to be a universal stress signal. The magnitude of the effect corresponds qualitatively to the level of RF-EMF exposure. In the presence of 10 mM vitamin C, alanine accumulation is completely suppressed, suggesting the involvement of free radicals in the process. A unique biological connection has thus been made between exposure to RF-EMF and cell stress, in the vicinity of RF transmitting antennas. This simple test, which lasts only 24 h, constitutes a useful bioassay for the quick detection of biological cell stress caused in the vicinity of RF irradiating antennas.

Introduction

The occurrence of electromagnetic irradiation in the human environment has risen dramatically over the recent 150 years, to almost a billion (109) times greater than previously. Exposure to electromagnetic fields of various frequencies accompanies technological innovation, but may also be hazardous to humans' health.

The time-varying electromagnetic fields produced by power plants and high-voltage power lines (50–60 Hz) are an example of extremely low frequency (ELF) fields. ELF fields generally have frequencies up to 300 Hz. Other technologies produce intermediate frequency (IF) fields with frequencies between 300 Hz and 100 kHz, and radio frequency (RF) fields with frequencies of 100 kHz to 300 GHz.^{1,2}

While there is no direct evidence that exposure to EMF fields causes damage to biological molecules, including DNA, Wertheimer and Leeper^{1,3} reported that even in ELF an association can be found between childhood leukemia and certain features of the wiring connecting their homes to the electrical distribution lines. Since then, numerous studies have been conducted to follow this important result. Analysis of these publications by the US National Academy of Sciences in 1996 suggested that residence near power lines was associated with an increased risk of childhood leukemia (relative risk RR = 1.5), but not with other cancers. A similar association between cancer in adults and residential exposure to RF fields was not found from these studies.4 Although the precise biological mechanism by which electromagnetic fields are mutagenic and/or co-carcinogenic needs further clarification, it clearly appears that, on the basis of available clinical and experimental evidence, EMFs should be considered cancer-causing agents.5-14

For many decades, radio and TV broadcast stations have been emitting radio frequency electromagnetic fields (RF-EMFs) in the frequency range of 10 kHz to 870 MHz. Amplitude-modulated (AM) transmitters operate at relatively high power levels. ¹⁵ In the vicinity of AM transmitters (100 m radius), high field strengths of more than

Environmental impact

Governmental environmental regulation of the maximum allowed human exposure to RF-EMF is aimed to be below intensities which may evoke bioeffects. And yet, a ten-fold difference is found between two such regulated limitations, issued by different authorities, suggesting that little is known on the real long-term effect of RF-EMF on ecosystems. In this study we demonstrate that etiolated duckweed plants, *Landoltia punctata* (=*Spirodela oligorrhiza*), are suitable for this type of bio-monitoring. This simple test for stress, which lasts only 24 h, constitutes a useful bioassay for the quick detection of biological stress caused in the vicinity of RF irradiating antennas.

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10 V m⁻¹ can occur. Exposure decreases with increasing distance from the transmitter. 16 RF electromagnetic fields generated by radio and television transmitters have been classified as possibly carcinogenic to humans, mainly based on epidemiological studies consistently showing an association between long-term average exposures and cancer. 17-21 However, recent large-scale systematic studies in Korea^{22,23} and Germany¹⁶ show no association between exposure to radio frequency electromagnetic fields emitted from broadcast towers (above 0.917 V m⁻¹) and childhood leukaemia risk.²⁴

Studies on the effect of long-term exposure to low level RF electromagnetic radiation showed increased stress in humans, manifested in elevated excretion rates of stress hormones, and modifications in the human immune system.²⁵⁻³¹

In our previous work^{32,33} we reported that under stress conditions applied to tiny water plants, etiolated duckweed, the initial metabolic response of the plant was production and accumulation of free alanine. A literature survey encompassing a variety of living systems revealed a large number of previous reports of alanine production (among other metabolites) in response to a variety of abiotic stress conditions. Stress conditions included: heat and cold shocks; freezing conditions; exposure to excess ammonia; exposure to UV irradiation; to heavy metal ions, drought, phosphorus and nitrogen deficiencies and osmotic stress. It was then concluded that alanine accumulation was a universal first stress signal in a wide variety of organisms, an observation not previously realized. 32 Hence sudden accumulation of alanine in etiolated duckweed may serve, and is now proposed, as a useful bioassay for the detection of stress conditions.

Our experiments were carried out in Kibbutz Na'an (Fig. 1), located in the center of Israel, between the years 2005 and 2009. There are about 800 inhabitants. In 1960, 32 radio broadcasting antennas were placed near the Kibbutz. The radiation levels produced by the antennas were 87 V m⁻¹, within limits set by the Israeli law at that time. In the early 90s it was realized that too many Kibbutz members suffered from cancer. Between the years 1993 and 2004, 60 members of the Kibbutz, suffered from cancer. All patients under the age of 55 (including children) suffered from various forms of cancer: leukaemia (a 15-fold greater proportion than elsewhere in Israel (SIR = 15.15; p < 0.001), lymphoma and brain tumor (a 7.69-fold greater proportion than elsewhere in Israel (SIR = 7.69; p = 0.03)) according to Richter.³⁴ These results provided a statistically significant association between cancer and residential exposure to RF antenna radiation.

In the present work the proposed bioassay is applied to examine stress conditions in etiolated duckweed plants, possibly resulting from exposure to RF irradiation, generated by radiotransmitter antennas in a residential area.

Materials and methods

Plant culture

Axenic cultures of duckweed, Landoltia punctata (G. Mey.) Les & D. J. Crawford Synonym of Spirodela oligorrhiza (Kurz) Hegelm. Clone Kefar Hayaaroq,35 were used. Etiolated plants

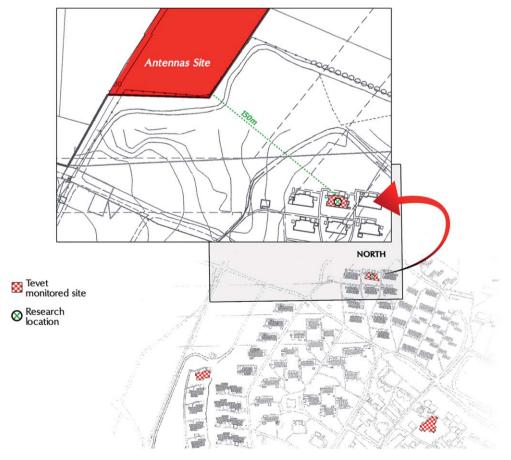


Fig. 1 The antennas site, Tevet monitored sites and the research location in Kibbutz Na'an.

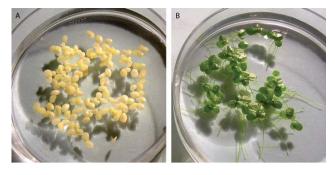


Fig. 2 (A) Etiolated (grown in continuous darkness for 5 months) and (B) light-exposed duckweed, *Landoltia punctata* (G. Mey.) Les & D. J. Crawford Synonym of *Spirodela oligorrhiza* (Kurz) Hegelm. Clone Kefar Hayaaroq. ³⁵

(grown in continuous darkness for 5 months) (Fig. 2) were grown under sterile conditions in 250 ml Erlenmeyer flasks (Kimax, Lawrence, Kansas, USA) containing 100 ml Hutner's growth medium³⁶ containing 0.5% sucrose at 24 ± 2 °C.³⁷ Ammonium ion assimilation was studied by introducing 30 ml of sterilized Hutner's growth medium into a 100 ml Erlenmeyer flask, followed by filter-sterilized (Sartorius, Goettingen, FRG; membrane filter 0.45 μm) ¹⁵N-labelled NH₄⁺ solution (30 mM ¹⁵NH₄C1, 99 atom%; Sigma Chemical, St Louis, MO, USA). Where mentioned, vitamin C (10 mM Sigma Chemical, St Louis, MO, USA) was added. Approximately 1 g (fresh weight) of axenic cultures of etiolated duckweed was added to each flask and incubated for 24 h at room temperature 24 ± 2 °C under continuous darkness exposed to RF-EMF. Etiolated plants were

Table 1 The equipment used for electromagnetic field measurements by TeVet Environmental & Occupational Health (Carmey-Yosef, Israel)

Device	Detector type	Threshold sensitivity	Frequency range
Raham 4c	Electric-Model 95	$5~\mathrm{V~m^{-1}}$ $0.2~\mathrm{V~m^{-1}}$ $0.2~\mathrm{V~m^{-1}}$ $16~\mathrm{mA~m^{-1}}$	0.2 MHz to 40 GHz
Narda 8719	Electric-Model 8760		0.2 MHz to 3 GHz
EMC-RFP 40	Electric Model PI-01		0.3 MHz to 1 GHz
Narda 8718	Magnetic Model 8731		300 KHz to 10 MHz

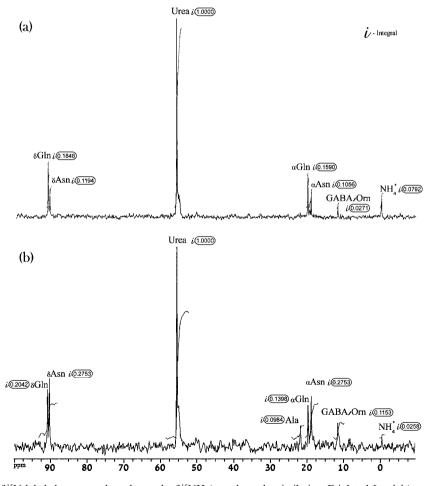


Fig. 3 ¹⁵N NMR analysis of ¹⁵N-labeled compounds as the result of ¹⁵NH₄⁺ uptake and assimilation. Etiolated *Landoltia punctata* (G. Mey.) Les & D. J. Crawford Synonym of *Spirodela oligorrhiza*, (Kurz) Hegelm. Clone Kefar Hayaaroq³⁵ during 24 h exposure to: (a) no RF EMF and (b) RF-EMF of 7.8 V m⁻¹. The spectra reveal ¹⁵N incorporation mainly into glutamine and asparagine (amide-δ-N and amino-α-N), as well as to GABA and/or ornithine, to a lesser extent. Alanine signal appears under RF-EMF of 7.8 V m⁻¹. Measurements are ¹⁵N NMR signal intensities (area under the signal) relative to the corresponding urea reference signal intensity, arbitrarily assigned 1.00. See Methods for details.

Table 2 An independent-sample *t*-test to compare the mean amount of alanine accumulation under different electric field strengths. There is a significant difference in the scores for each of the compared groups (a significance level of $\alpha = 0.05$) 95 percent confidence

Compared electric field strength/V m ⁻¹	Integral ^{a} alanine mean (M)	Standard deviation	Number of repetitions (N)	Degrees of freedom	Tabulated t value ($p = 0.05$)	Calculated <i>t</i> value	Evaluation of the differences between the two groups
0	0.0000	0.0000	6	10	2.228	7.662	Significantly different
1.8	0.0316	0.0101	6				
1.8	0.0316	0.0101	6	10	2.228	3.088	Significantly different
3.3	0.0561	0.0166	6				
3.3	0.0561	0.0166	6	10	2.228	4.053	Significantly different
7.8	0.0938	0.0156	6				
1.8	0.0316	0.0101	6	10	2.228	8.196	Significantly different
7.8	0.0938	0.0156	6				

^a ¹⁵N-Alanine signal intensities (area under the signal) relative to the corresponding urea reference signal intensity, arbitrarily assigned 1.00. See Methods for details.

used as they were found to be EMF-sensitive, while green plants did not show this phenomenon. Control experiments were carried out under similar conditions but without exposure to RF EMF. Exact weighing was not possible at this stage because of the sterile conditions. The plants (1.0 g) were carefully washed five times with distilled water and sonicated (pulsed) for 5 min with 3 ml of distilled water, in order to crush the cells, and the resulting suspension was transferred to a 10 mm NMR tube.

¹⁵N NMR spectrometric measurements

The ¹⁵N NMR method is particularly useful as it shows only metabolites derived from the ¹⁵N-enriched precursor, while the multitude of other (unlabeled) nitrogen compounds remain transparent.

¹⁵N NMR measurements were preformed on a Bruker Avance DMX-500 NMR spectrometer (Karlsruhe, FRG), operating at 500.13 MHz for ¹H and 50.68 MHz for ¹⁵N nuclei. ¹⁵N NMR spectra were measured in H₂O solution, with a concentric sealed 5 mm NMR sample tube containing DMSO-d6 providing the deuterium lock signal and 1.0 mg of 99 atom% 15N-enriched urea as a chemical-shift and relative intensity scaling reference. A 30 s delay between pulses was applied. Usually about 2000 pulses were acquired in overnight experiments. Because the response of ¹⁵N NMR signals arising from different nitrogen environments may differ substantially in intensity, a direct signal intensity comparison between different nitrogen signals may be invalid. We therefore compare signal intensities of the *same* nitrogen type against an external standard, 15N enriched urea. The change in the alanine/urea signal intensity ratio between different experiments reflects the real change in alanine accumulation.

Alanine accumulation was shown previously to be a unique universal stress signal.³² ¹⁵N NMR chemical shift assignments were based on published data and were compared with those of authentic samples of amino acids. Chemical shifts are reported relative to ¹⁵NH₄⁺ at 0 ppm.

RF-EMF conditions

Axenic cultures of etiolated *Landoltia punctata* (*Spirodela oligorrhiza*) were exposed to RF-EMF between 7.8 V m⁻¹ and

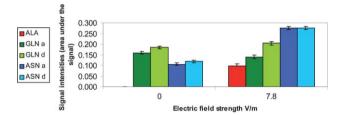


Fig. 4 Column diagram showing quantitatively \pm S.E. changes in alanine and Gln/Asn under irradiation stress. Alanine production under RF EMF exposure was accompanied by a change in the Gln/Asn ratio, from Gln > Asn in the absence of RF irradiation to Gln < Asn under irradiation stress.

1.8 V m⁻¹, generated mainly by AM 1.287 MHz transmitting antennas for 24 h (in the dark) as follows: in experiments carried out during 2005, the electric field strength was 7.8 \pm 0.2 V m⁻¹;‡ during 2006–2007, the electric field strength was 1.8 \pm 0.3 V m⁻¹; and in experiments carried out during 2009, the field was 3.3 \pm 0.2 V m⁻¹.

EMF measurements had been ordered by the Israel Ministry of Environment and the Gezer Regional Council. The measurements have been monitored continuously since 1994 by TeVet Environmental & Occupational Health (Carmey-Yosef, Israel) and were displayed weekly on their web site (http://www.tevetenv.co.il/naan.htm) till the end of December 2009. The instruments used for electromagnetic field measurements are described in Table 1. They were placed in three residential locations in Kibbutz Na'an, including Mr Shaul Avramson's home, where our experiments were carried out. The experiments were conducted inside a residential home located *ca.* 150 m from the antennas (Fig. 1).

The electric field strengths are values which were measured at Mr Shaul Avramson's home during the weeks when our experiments were carried out, using instruments located inside the house.

[‡] Throughout this article electric field strengths are expressed in V m⁻¹. Conversion to power flux density is achieved by: power flux density [W m⁻²] = electric field strength [V m⁻¹]²/377 [Ohm].

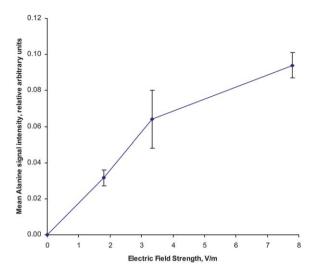


Fig. 5 Intracellular alanine accumulation as a function of RF-EMF intensity. Alanine intensities expressed relative to urea signal intensities (\pm S.E.), arbitrarily assigned value 1.00.

Statistical analysis

Each experiment was repeated six to eight times.

An independent-sample *t*-test (a significance level of $\alpha = 0.05$) was conducted to compare the mean amount of alanine accumulation under different electric field strengths.

Results

Metabolism in untreated plants, fed with ¹⁵N-enriched ammonium chloride for 24 h (see Methods), was analyzed using ¹⁵N NMR spectroscopy of the sonicated plants. The results are presented with the aid of typical ¹⁵N NMR spectra of representative

experiments (Fig. 3), as well as summarized in Fig. 5, which includes the analysis of mean (\pm S.E.) alanine production resulting from all parallel measurements.

An independent-sample *t*-test (a significance level of $\alpha=0.05$) was conducted to compare the mean amount of alanine accumulation under different electric field strengths. Table 2 indicates there was a real significant difference, in the results for each of the compared groups (a significance level of $\alpha=0.05$, 95 percent confidence).

The ¹⁵N NMR analysis revealed ¹⁵N incorporation mainly into glutamine (Gln) and asparagine (Asn, amide- δ -N and amino- α -N), as well as to γ -aminobutyric acid (GABA) and/or ornithine, to a lesser extent (Fig. 3a). However, when etiolated plants were exposed to RF EMF, alanine was also produced in addition to the mentioned metabolites (Fig. 3b). Alanine was not produced in the absence of RF EMF exposure (Fig. 3a). It is thus concluded that alanine is produced in these experiments in response to stress generated by the RF EMF.

Alanine production under RF EMF exposure was accompanied by a change in the Gln/Asn ratio, from Gln > Asn in the absence of RF irradiation to Gln < Asn under irradiation stress (Fig. 4).

The results of the various experiments are summarized in Fig. 5. The intracellular alanine accumulation as a function of RF-EMF intensity is presented. Under lower RF-EMF the level of accumulated alanine decreased substantially, indicating less severe stress conditions.

The correlation between the level of irradiation and the resulting alanine accumulation provides further evidence that the stress experienced by the plants and expressed in alanine production resulted directly from the RF EMF exposure.

The addition of vitamin C to the growth medium during RF-EMF exposure resulted in a dramatic change, namely total

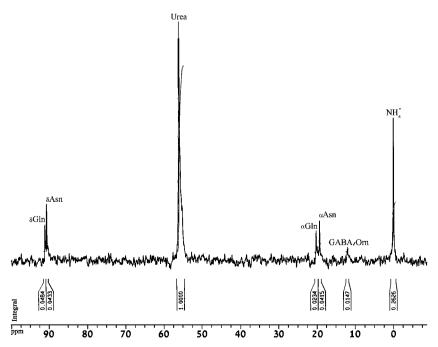


Fig. 6 ¹⁵N NMR analysis of uptake and metabolism of ¹⁵N-labeled ammonium chloride in etiolated plants exposed to RF-EMF of 1.8 V m⁻¹ in the presence of 10 mM of vitamin C (in experiments carried out during 2007). Alanine signal absent.

suppression of alanine production (Fig. 6). The decrease in alanine accumulation in the presence of added vitamin C was accompanied by a decrease in the Gln/Asn ratio and an increase in the residual free ammonium ion, indicating a reduction in the ammonium ion assimilation.

Discussion

Governmental environmental regulation of the maximum allowed human exposure to RF-EMF is aimed to be below intensities which may evoke bioeffects. And yet, a ten-fold difference is found between two such regulated limitations, issued by different authorities: the International Committee for Non Ionizing Radiation Protection (ICNIRP) permits up to 2.8–8.7 V m⁻¹, while the European Union maximum allowed exposure level is 0.3–0.9 V m⁻¹. These large differences suggest that little is known on the real long-term effect of RF-EMF on public health.

The experiments in Na'an were conducted inside a residential home located *ca.* 150 m from the antennas (Fig. 1). At that time (July 2005) five antennas were still broadcasting. During experiments carried out in 2006–2007, only one antenna was still broadcasting, as a result of public protest. This change of situation enabled a comparison of the effects of exposure to different RF EMF levels, under otherwise identical conditions.

In July 2009 only one antenna was still broadcasting, but this time at higher intensity (3.33 V m $^{-1}$) relative to that of 2006–2007 (1.8 V m $^{-1}$).

A potential link between EMF and its effects on living organisms is the fact that EMF causes an oxidative stress, that is, EMF can alter energy levels and spin orientation of electrons to increase the activity, concentration, and lifetime of free radicals.^{38–40} It also affects enzyme activity, gene expression, iron release from ferritin, the iron cage proteins, and release of calcium from intracellular storage sites.⁴¹ The latter influences the membrane structure, cell growth, and cell death, thereby contributing to cancer and leukaemia.^{29,40,42–45}

Glutamine and asparagine are important products of $\mathrm{NH_4}^+$ utilization initiated by the GS/GOGAT pathway (Fig. 7). Glutamine serves as the primary acceptor of assimilated nitrogen and asparagine serves as a secondary acceptor. The amide nitrogen derived from glutamine is readily transferred to asparagine by asparagines synthetase reaction. Production of alanine under stress conditions may occur either by transamination of pyruvate from glutamate or be derived from aspartate (Fig. 7). The exact mechanism of alanine production under stress remains to be studied.

Numerous previous reports showed that alanine production accompanied exposure to a variety of different stress conditions such as anoxia, osmotic stress, extreme temperatures, exposure to heavy-metal ions, water shortage,³² as well as response to RF-EMF, as reported here for the first time. The precise function of alanine in the cell in cases of stress is unknown. It has been shown that added alanine (but also glycine) stimulated the gene encoding for stress-protein synthesis in mammalian kidney (both *in vivo* and *in vitro*), which serves to protect the cells against injury damage.⁴⁶ It has also been speculated that under stress, alanine is accumulated as a storage form of pyruvate. Either one

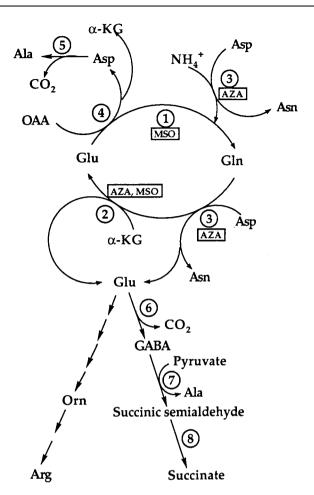


Fig. 7 Schematic representation of the GS/GOGAT and related ammonium ion assimilation cycles. Enzymes: (1) GS + ATP; (2) GOGAT + NAD(P)H or reduced ferredoxin; (3) asparagine synthetase + ATP; (4) aspartate aminotransferase; (5) aspartate decarboxylase; (6) glutamate decarboxylase; (7) GABA:pyruvate transaminase; and (8) succinic semialdehyde dehydrogenase + NAD. Inhibitors are shown in square frames (MSO and AZA).

of these speculations may account for the purpose of alanine production and accumulation under stress.

The mechanism by which RF-EMF affects biological systems is still enigmatic. Our working hypothesis has been the following: assuming that the RF-EMF to which the plants were exposed potentially alters chemical processes in which free radicals are involved^{32,47–49} the addition of radical scavengers should reduce or eliminate the magnetic field effects. Vitamin C is known to function as a highly effective radical scavenger (antioxidant) in living organisms.^{32,50–53} The addition of vitamin C to the growth medium during the RF-EMF exposure caused total suppression of alanine accumulation. It may be concluded that alanine is produced in the described experiments in response to stress generated by RF-EMF through formation of free radicals.

Conclusion

The present work makes a unique *biological* connection between exposure to RF-EMF and real biological stress in living cells. Vitamin C functioned in our experiments as an effective radical

scavenger completely suppressing alanine accumulation. It may be concluded that alanine production resulting from exposure to RF EMF is associated with free radical formation.

Etiolated duckweed plants may serve as a bioassay for the quick detection of biological stress caused in the vicinity of RF irradiating antennas.

Abbreviations

EMFs electromagnetic fields **ELF** extremely low frequency RF radio frequency alanine Ala glutamine Gln Asn asparagine GABA γ-aminobutyric acid

Acknowledgements

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