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Radical Scavengers Suppress Low Frequency EMF Enhanced Proliferation in Cultured Cells and Stress Effects in Higher Plants

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Summary. In previous publications, we reported that sinusoidal varying magnetic fields (SVMF) modify the activity and dynamics of the malignancy marker adenosine deaminase, and enhance the proliferation of chick embryo fibroblasts (CEF). While the SVMF examined by us (50, 60 & 100 Hz / 0.06–0.7 mT) were all below kT, they may have the potential of altering chemical processes in which excited radicals are involved.

We tested this hypothesis in two experimental systems: CEF in culture and *Spirodela oligorrhiza* (*Lemnaceae*) (a small aquatic plant, commonly known as Duckweed). CEF were exposed to SVMF of 100 Hz/0.7 mT for 24 h. The addition of the exogenous radical scavengers catalase, superoxide dismutase or vitamin E to the cells during exposure significantly suppressed enhancement of cell proliferation caused by the field (by 79, 67 and 82%, respectively, as evaluated by the MTT colorimetric assay). ^{15}N NMR analysis of Duckweed plants fed by ^{15}N-labeled ammonium chloride and exposed to SVMF at 60 and 100 Hz/0.7 mT for 24 h, revealed augmented alanine production. Alanine did not accumulate in the absence of SVMF. The addition of vitamin C, a radical scavenger, reduced alanine production by 82%.

Exposure to SVMF resulted in specific metabolic stress effects in Duckweed plants and enhanced proliferation of CEF. In both cases, it is suggested that free radicals are involved.

Keywords: SVMF, CEF, MTT, free radicals, catalase, superoxide dismutase, vitamin E (α-tocopherol), *Spirodela oligorrhiza* (*Lemnaceae*) (Duckweed), ^{15}N NMR, vitamin C (ascorbic acid), alanine production, nitrogen assimilation, etiolated plants

Abbreviations: SVMF = sinusoidal varying magnetic fields; CEF = chick embryo fibroblasts; ADA = adenosine deaminase; SOD = superodxide dismutase.

Introduction

Substantial evidence concerning the effects initiated by static and dynamic magnetic fields, such as those produced by power distribution lines, appliances and electronic devices on many general physiological and biochemical processes have been published. Most studies have concluded that exposure of biological tissues to SVMF causes marked changes in cell proliferation and constitutes physiological stress (Hileman, 1993; Parola and Markel, 1994; Scaiano *et al.*, 1994; Markov, 1994; Lin *et al.*, 1997; Lacy-Hulbert *et al.*, 1998; Katsir and Parola, 1998; Pipkin *et al.*, 1999; Adair, 2000; Liboff and Jenrow, 2000; Gutzeit, 2001; Blank and Goodman, 2002).

We have previously found that SVMF affected cell proliferation as well as the activity and dynamics of the malignancy marker adenosine deaminase, ADA (Parola *et al.*, 2000; Ben-Shooshan *et al.*, 2002): exposure of CEF to a 100 Hz/0.7 mT SVMF resulted in

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reduced cell proliferation, decreased activity of ADA, and a remarkable rise of segmental rotational relaxation rate of ADA bound to adenosine deaminase complexing protein (ADCP = DPPIV = CD26) as determined by multifrequency phase fluorometry. The changes in membrane dynamics were characteristics of those observed in malignant viral cell transformation (Parola and Markel, 1994; Porat *et al*., 1988; Parola *et al*., 1993; Katsir *et al*., 1998).

Our working hypothesis was that while the SVMF examined were all below kT, they might have the potential of altering chemical processes in which excited radicals are involved (Parola *et al*., 1993; Cohen *et al*., 1973; Grissom, 1995; Mohtat *et al*., 1998). In some photochemical reactions performed at 1.0 mT magnetic field, a mechanism of "radical solvent cage dissociation and recombination" was suggested (Fig. 1): the energy gap between the excited triplet vibrational sublevels—triplet-zero and singlet states depends on weak magnetic fields. Since singlet radical pairs will recombine within a solvent cage more readily than triplets, triplet radical pairs stand a better chance of escaping from the solvent cage in which they are initially formed. This will result in a different radical-recombination-product profile (Scaiano *et al*., 1994; Grissom, 1995; Mohtat *et al*., 1998; Turro and Weed, 1983; Scaiano *et al*., 1994; Turro *et al*., 1995; Turro, 1996; Saxena *et al*., 2003). The energy gap between the singlet and triplet excited radicals in the proposed hypothesis of solvent caged radical pair calls for magnetic field intensities similar to those reported here.

Production of free radicals and active oxygen species is ubiquitous in all respiring organisms, and is enhanced in many disease states by carcinogen exposure as well as by conditions of stress. A literature survey reveals a vast number of reports on alanine production and accumulation (among other metabolites) in response to stress (Guyton and Kensler, 1993; Ben-Izhak *et al*., 2003; Mayr *et al*., 2004; Koppitz *et al*., 2004). It seems that alanine accumulation is a universal stress signal in a wide variety of organisms. Evidence of formation of "heat shock proteins" and enhanced protein kinase activity under various stress conditions has been reported widely (see literature survey in (Ben-Izhak *et al*., 2003). It has recently been proposed that enhanced production and accumulation of specific amino acids precedes this stress activity (Ben-Izhak *et al*., 2003).

To test the applicability of the radical hypothesis to our system, the effect of SVMF on two experimental systems was studied. One system examined CEF proliferation in the presence of the enzymatic antioxidants, superoxide dismutase (SOD) and catalase, both of which are known to scavenge superoxide anions, as well as vitamin E (α- tocopherol), a lipid peroxidation scavenger (Lacy-Hulbert *et al*., 1998). The second system examined the metabolism of ammonium ions in *Spirodela oligorrhiza* (*Lemnaceae*, a small aquatic plant, commonly known as Duckweed), grown in darkness (Fig. 2), in the presence of vitamin C (ascorbic acid) (Ben-Izhak *et al*., 2003). The accumulation of alanine was specifically followed. The addition of exogenous radical scavengers was expected to reduce the SVMF effects.

Materials and methods

SVMF

SVMF exposure was conducted in a pair (for field- and sham-control exposure) of matched, temperature-controlled Wedco incubators (concurrently run), each fitted with modified Helmholtz coils. Special care was taken to eliminate spurious and ambient fields in the laboratory environment as well as cross talk between the exposure and the control incubators. This was achieved by adequate positioning of the two incubators. In all experiments a randomly chosen incubator was activated as a field incubator while the other identical incubator served as a sham control. Cells were introduced blindly into the incubators. Results were not dependent upon which incubator was chosen for exposure or control. Magnetic field inside incubators was measured with a gaussmeter or a Single Axis magnetometer (Fig. 3), as previously described (Katsir and Parola, 1998).

CEF proliferation

CEF cultures were prepared and maintained as previously described (Katsir and Parola, 1998; Parola *et al*., 1993). In all experiments, 2×10^5 cells per well were seeded in 24-well culture plates and allowed to grow for 24 hr. Prior to field exposure each of the enzymes and radical scavengers was added to the growth media. Each treatment in a single independent experiment was performed at 8 repeats. Number

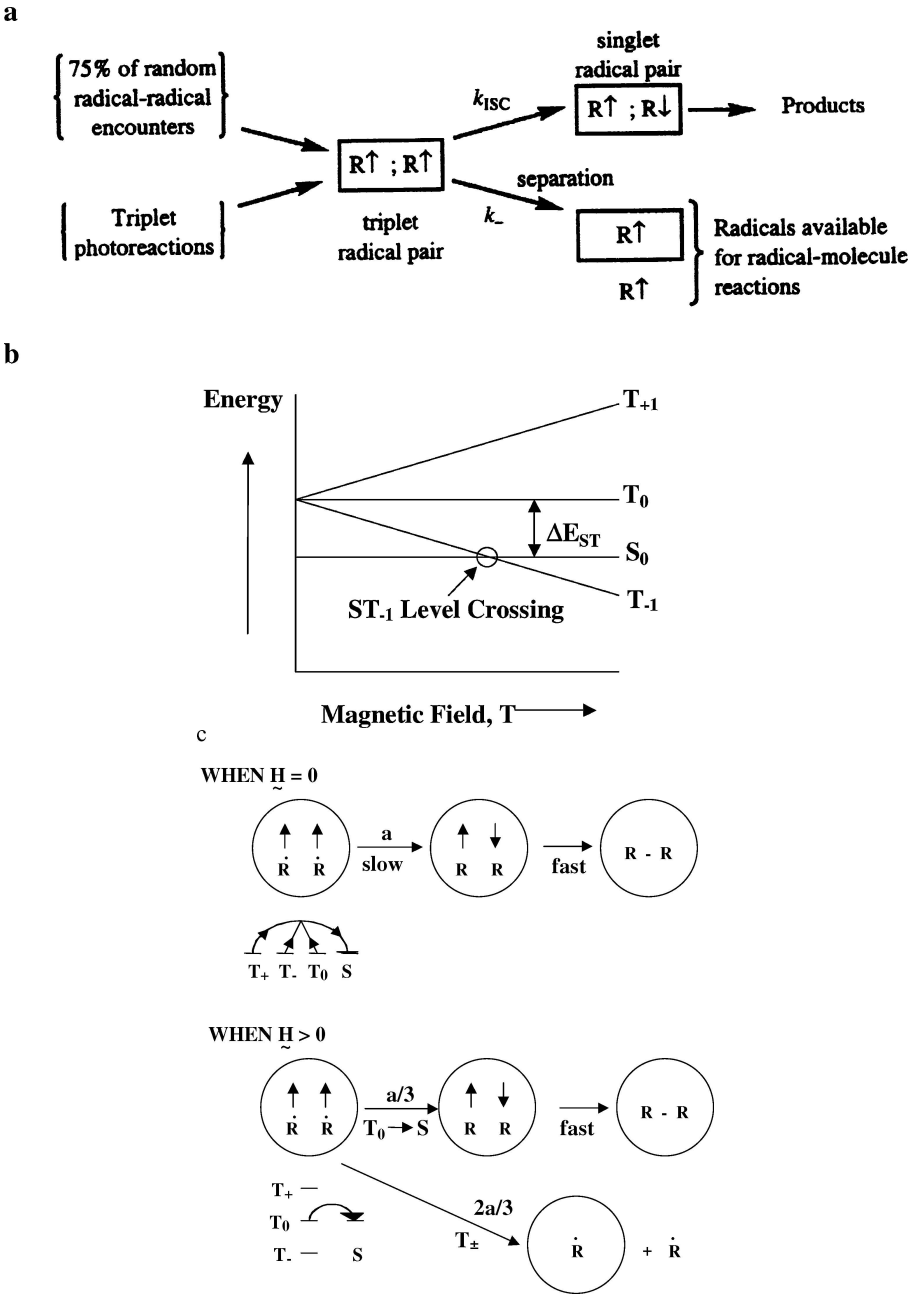

Figure 1. Proposed mechanism of radical solvent cage dissociation and recombination (taken from (Turro and Weed, 1983; Grissom, 1995)). a. Magnetic field effects on radical recombination; b. Application of magnetic field leads to Zeeman splitting of the energies of the triplet sublevels, T_-, T_0 and T_+; c. Exposure to EMF may lead to higher average radical concentration and an increased probability of radical—molecule reactions.

In the absence of an external magnetic field, the slow intersystem crossing of caged triplet radical pairs is followed by fast radical recombination. In the presence of external magnetic fields, the degenerate triplet state of triplet radical pairs splits into three sub-states: T_-, T_0 and T_+. This will result in only a 1/3 probability of intersystem crossing between the energetically adjacent T_0 sublevel and S, leading to fast recombination of these caged radical pairs. The remaining 2/3 of the population, associated with a larger energy gap between the T_+ or T_- and S_o, will have a longer lifetime within the solvent cage. The probability that these radicals will "escape" a radical—radical encounter will increase. Thus, radical dissociation and interaction with neighboring molecules (leading to biochemical alterations) will be enhanced.

Figure 2. Light-exposed (left) and etiolated (grown in darkness, right) *Spirodela oligorrthiza.*

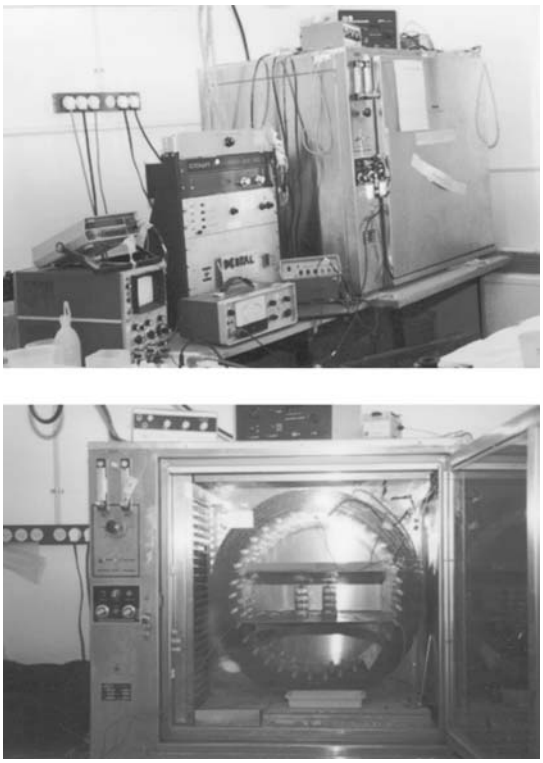

Figure 3. Temperature-controlled Wedco incubator (upper Figure), fitted with modified Helmholtz coils (bottom Figure). Magnetic field inside incubator is measured with a gaussmeter or a Single Axis magnetometer (upper Figure), as described (Katsir and Parola, 1998; Katsir *et al*., 1998).

of independent experiment (n) is indicated. Following 24 h of exposure (100 Hz/0.7 mT) cell proliferation was determined by the MTT colorimetric assay of Mosmann (Mosmann, 1983; Katsir and Parola, 1998). Experiments were repeated at least five times.

Amino acid metabolism in Duckweed

Etiolated *Spirodela oligorrhiza* (grown in darkness for 5 months) (Fig. 2) were fed by ^{15}N- labeled ammonium chloride (30 mM) for 24 h. The plants were exposed (in the dark) to SVMF, 60 and 100 Hz/0.7 mT magnetic field, in the absence or the presence of vitamin C for 24 h, and their $^{15}NH_4^+$ assimilation was studied using ^{15}N-NMR spectroscopy of sonicated plants, as detailed before (Ben-Izhak *et al*., 2003). ^{15}N-NMR chemical shift assignments were based on published data and were compared with reference samples of amino acids. Representative NMR spectra are shown. The same trend was observed in at least six experiments.

Results

The rate of proliferation of sham-exposed cells treated with SOD, catalase or vitamin E did not differ significantly from their controls (SOD, $100 \pm 25\%$ (mean $\pm$ SD, Student's t test), vs. $91 \pm 11\%$ ($p = 0.06$, $n = 7$ independent experiments), catalase, $100 \pm 23\%$ vs. $102 \pm 7\%$ ($p = 0.26$, $n = 5$), and vitamin E, $100 \pm 37.5\%$ vs. $145 \pm 50\%$ ($p = 0.15$, $n = 6$), for sham-exposed control cells in the absence of radical scavengers vs. sham-exposed cells in the presence of radical scavengers, respectively). This implies that under natural conditions, the antioxidant defense of CEF was not influenced by the exogenous addition of SOD, catalase or vitamin E. The mean E/C ratios of CEF proliferation, i.e., the values obtained after SVMF exposure (E) divided by the values obtained in sham-exposed cells (C), in the absence (*Control*) or in the presence of radical scavengers, are presented in Fig. 4 and summarized in Table 1. It was found that at a magnetic field of 100 Hz/0.7 mT, in the absence of radical scavengers, cell proliferation was enhanced, on the average, by $33 \pm 4\%$ compared to the control of sham-exposed cells ($p < 0.05$, Fig. 4 & Table 1). This significantly increased cell proliferation following SVMF exposure, measured by the MTT colorimetric assay of

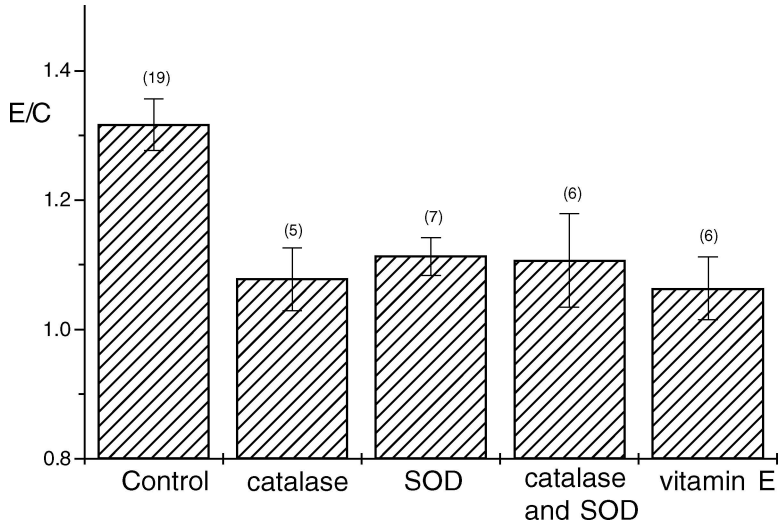

Figure 4. Mean ratio of exposed (E) to sham-exposed (C) CEF. Data presented are the ratio of proliferation of SVMF-exposed to sham-exposed CEF, in the absence (*Control*) or the presence of the indicated radical scavengers, calculated for each experiment, obtained from the indicated numbers of independent experiments (numbers in parentheses). Reprinted from *Biochem. Biophys. Res. Commun.*, 252, G. Katsir and A.H. Parola, Enhanced Proliferation Caused by Low Frequency Weak Magnetic Fields in Chick Embryo Fibroblasts is Suppressed by Radical Scavengers, 753-756 (1998), with permission from Elsevier (J292).

Mosmann (Mosmann, 1983; Katsir and Parola, 1998), was also confirmed by hemocytometer cell count and ^{3}H-thymidine incorporation (Katsir *et al.*, 1998). Treatment with the radical scavengers significantly reduced the SVMF enhancement in cell proliferation, as compared to the control exposed cells i.e., in the absence of radical scavengers ($p < 0.001$, Fig. 4 & Table 1). It is suggested that the addition of exogenous radical scavengers to the cells during exposure to this magnetic field, significantly suppresses the enhancement in cell proliferation caused by the field.

Table 1. Radical scavengers inhibition of the enhanced CEF proliferation induced by SVMF. Reprinted from *Biochem. Biophys. Res. Commun.*, 252, G. Katsir and A.H. Parola, Enhanced Proliferation Caused by Low Frequency Weak Magnetic Fields in Chick Embryo Fibroblasts is Suppressed by Radical Scavengers, 753–756 (1998), with permission from Elsevier (J292).

Radical scavenger	E/C[a]	Extent of reduction of field effect(%)[b]
Control	1.33 ± 0.04	
Catalase	1.07 ± 0.04	79
SOD	1.11 ± 0.03	67
Catalase and SOD	1.10 ± 0.07	70
Vitamin E	1.06 ± 0.04	82

[a]—Data presented are the mean $\pm$ SE of E/C ratio i.e., ratio of proliferation of SVMF-exposed (*E*) to sham-exposed CEF (*C*), in the absence (*Control*) or the presence of the indicated radical scavengers, calculated for each experiment. Number of independent experiments is presented in Fig 4.

[b]—Extent of reduction in the proliferation ratio of SVMF-exposed CEF (*E*) to sham-exposed CEF (C), in the presence of radical scavengers, relative to the 33% increase of *Control* ratio (i.e., in the absence of radical scavengers; column 2, row 1).

Exposure of etiolated Duckweed to SVMF constituted stress, as was evident from the decrease of $16 \pm 3\%$ in the biomass, relative to the control experiments. The ^{15}N NMR analyses revealed ^{15}N incorporation mainly into glutamine and asparagine (amide-δ-N and amino-α-N). However, when plants were exposed to SVMF of 0.7 mT at either 100 or 60 Hz, γ-amino butyric acid (GABA) and/or ornithine and alanine were also produced. Of these metabolites, GABA and ornithine are produced (though to a lesser extent) under normal conditions, while alanine is unique to stress conditions (Mayr *et al.*, 2004; Koppitz *et al.*, 2004; Ben-Izhak and Kost, 1998) & see the comprehensive table with 70 stress cases resulting in alanine accumulation in plants, animals and microorganisms, published in (Ben-Izhak *et al.*, 2003).

Alanine was not produced in the absence of SVMF exposure (Fig. 5). We assumed from the literature and our tests that low intensity and low frequency SVMF

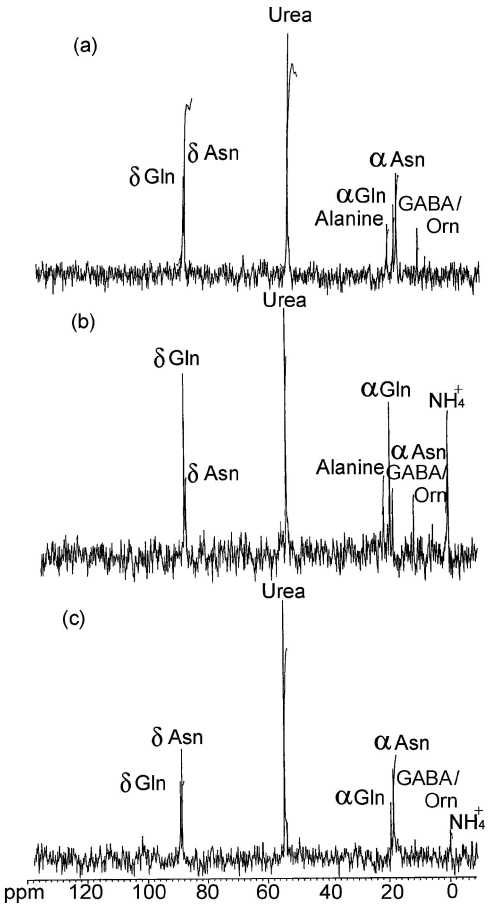

Figure 5. ^{15}N NMR analysis of the uptake and metabolism of ^{15}N-labeled ammonium chloride in etiolated *Spirodela oligorrthiza* during 24 h exposure: (a) exposed to SVMF at 0.7 mT 100 Hz; (b) exposed to SVMF at 0.7 mT 60 Hz; (c) no SVMF exposure. Reprinted from *Biochem. Biophys. Res. Commun.,* 302, E. Ben-Izhak Monselise, A.H. Parola and D. Kost, Low Frequency Electromagnetic Fields Induce a Stress Effect upon Higher Plants, as Evident by the Universal Stress Signal, Alanine, 427–434 (2003), with permission from Elsevier (J292).

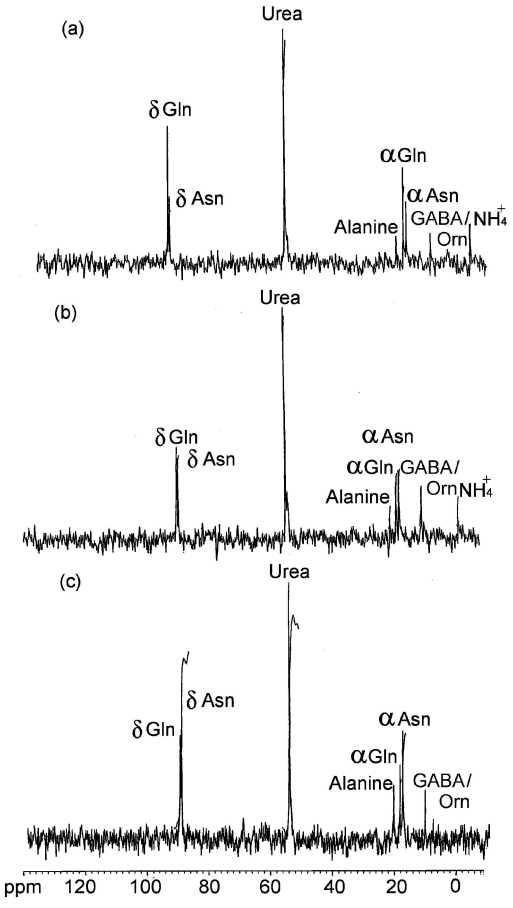

Figure 6. ^{15}N NMR analysis of the uptake and metabolism of ^{15}N-labeled ammonium chloride in etiolated *Spirodela oligorrhiza* during 24 h exposure, with the addition of (a) 10 mM of vitamin C; (b) 2 mM of vitamin C and (c) no added vitamin C. Reprinted from *Biochem. Biophys. Res. Commun.,* 302, E. Ben-Izhak Monselise, A.H. Parola and D. Kost, Low Frequency Electromagnetic Fields Induce a Stress Effect upon Higher Plants, as Evident by the Universal Stress Signal, Alanine, 427–434 (2003), with permission from Elsevier (J292).

can potentially alter chemical processes in which free radicals are involved (Scaiano *et al.*, 1994; Katsir and Parola, 1998; Adair, 2000). It thus follows that (a.) alanine is presumably produced in response to radical reactions, and hence (b.) suppression of the radicals should result in reduced alanine production. Indeed, addition of 2 and 10 mM vitamin C as an exogenous radical scavenger reduced alanine production by 20 ± 10 and $82 \pm 10\%$, respectively, relative to control ($n = 10$, Fig. 6).

Discussion

We have reported that sinusoidal varying magnetic fields enhance the proliferation of primary chick embryo fibroblasts and induce changes in their membrane lipid-protein interactions (Porat *et al.*, 1988; Parola *et al.*, 1993; Katsir *et al.*, 1998). However, the mechanism through which SVMF affects biological systems is still enigmatic. Our working hypothesis has been the following: assuming that the SVMF tested (60 and 100 Hz, 0.7 mT) have the potential to alter chemical processes in which excited radicals are involved, the

addition of radical scavengers should reduce or eliminate the magnetic field effects.

Free radicals play a vital role in mediating cellular responses to various extracellular ligands. Many extracellular ligands generate and/or require free radicals to transmit their signals to the nucleus. The free radicals' action can be on cytokines, growth factors, hormones, ion transport, transcription and apoptosis (Lander, 1997; Liberto *et al*., 2004; Duchen, 2004; Minotti *et al*., 2004; Haddad, 2004; Ferrari *et al*., 2004; Brandes and Kreuzer, 2005).

The major source of free radicals in cells is electron "leakage" from electron transport chains, such as those found in mitochondria and in endoplasmatic reticulum, to interstitial molecular oxygen, generating superoxide. Damage from such free radicals is kept at minimum in healthy cells by enzymatic and non-enzymatic antioxidant defense (Cheeseman and Slater, 1993). The addition of such natural defense agents is thus expected to reduce effects caused by SVMF-modified radical distribution.

We used the enzymatic antioxidants superoxide dismutase and catalase, which are known to scavenge superoxide anions and H_2O_2, as well as vitamin E, a known lipid peroxidation scavenger, in the CEF proliferation system. In the amino acids metabolism of Duckweed, we used vitamin C as an exogenous radical scavenger.

The addition of radical scavengers to CEF during the exposure to magnetic field (100 Hz, 0.7 mT), significantly suppressed the enhancement in cell proliferation caused by the field. This strongly suggests that radicals are involved in SVMF induced enhancement of CEF proliferation.

Exposure of Duckweed to SVMF resulted in alanine accumulation. The addition of radical scavengers during the exposure reduced alanine accumulation. It may be concluded that alanine is produced in these experiments in response to stress generated by the SVMF, through free radical formation. The decrease in alanine accumulation in the presence of added vitamin C was accompanied by an increase in the Gln/Asn ratio and the appearance of free ammonium ion, indicative of a change in ammonium-assimilation pathway.

Numerous reports have shown that alanine production accompanied exposure to a variety of different stress conditions such as anoxia, osmotic stress, extreme temperature, exposure to heavy-metal ions, water shortage (see Table and Appendix in Ben-Izhak *et al*. (2003)), as well as in response to SVMF, as reported in the present study. The precise function of alanine in the cell in cases of stress is not clear. It has been shown that added alanine (and glycine) stimulates gene encoding for stress-protein synthesis in mammalian kidney (both *in vivo* and *in vitro*), which serves to protect the cells against injury (Nissim *et al*., 1992). It has also been speculated that during anoxia, alanine accumulates as a storage form of pyruvate. Either of these mechanisms may be the reason for alanine accumulation under stress.

One of the most controversial issues regarding the effects of weak magnetic fields at low frequencies on biological systems is the lack of any widely accepted physico-chemical mechanism to account for the observed phenomena: increased cell proliferation, DNA mutation, altered lipid-protein interactions, biochemical reactions involving free radicals, tumor promotion, Ca^{2+} signaling, ornithine decarboxylase activity, gene expression, *src* kinase activity, the involvement of melatonin and Na^+/K^+-ATPase, peroxidase activity, fluid-phase endocytosis, connexin32 hemi channels, effects on the cardiovascular system, as well as interaction at the level of the signaling manifold in general and NO radicals in particular (Antov *et al*., 2004; Portaccio *et al*., 2005; Ramundo-Orlando *et al*., 2005; Mahrour *et al*., 2005; Jeong *et al*., 2005; Kleinerman *et al*., 2005) & see the literature survey in (Katsir and Parola, 1998).

Conclusion

We suggest here a linkage between the application of a weak sinusoidal magnetic field and the well-established chemical phenomenon of free radicals, which can alter a variety of biological processes. Inhibition of the SVMF-enhanced cell proliferation and modified amino acid metabolism by radical scavengers provide experimental observations in line with the radicals hypothesis presented in the introduction, by which excited radical pairs within a solvent cage could be affected by the low magnetic field employed. A Zeeman-like effect (Mohtat *et al*., 1998) may alter the spin state population through interaction with the SVMF employed.

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