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Full Length Research Paper

Antimicrobial effect of amphotericin B electronically-activated water against *Candida albicans*

J. Antonio Heredia-Rojas[1], Ricardo Gomez-Flores[2], Abraham O. Rodríguez-de la Fuente[1]*, Enriqueta Monreal-Cuevas[2], Antonio Cayetano Torres-Flores[3], Laura E. Rodríguez-Flores[4], Michaela Beltcheva[5] and Antonio Cayetano Torres-Pantoja[3]

[1]Universidad Autónoma de Nuevo León, UANL. Facultad de Ciencias Biológicas, Av. Universidad S/N. Ciudad Universitaria, San Nicolás de los Garza Nuevo León, C.P. 66451 México.
[2]Universidad Autónoma de Nuevo León,UANL. Facultad de Ciencias Biológicas, Departamento de Ciencias Exactas y Desarrollo Humano, Av. Universidad S/N, Ciudad Universitaria, San Nicolás de los Garza, Nuevo León, C.P. 66451 México.
[3]Universidad Autónoma de Nuevo León,UANL. Facultad de Ciencias Biológicas, Departamento de Microbiología e Inmunología, Av. Universidad S/N, Ciudad Universitaria, San Nicolás de los Garza, Nuevo León, C.P. 66451 México.
[4]Universidad Autónoma de Nuevo León UANL, Facultad de Medicina, Av. Francisco I. Madero pte. S/N, Col. Mitras Centro, Monterrey, Nuevo León, C.P. 66460 México.
[5]Bulgarian Academy of Sciences, Institute of Biodiversity and Ecosystem Research, 1, Tzar Osvoboditel Blvd., 1000 Sofia, Bulgaria.

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The activation of water by physical means stimulates a new scientific approach to microbiology, in particular, antimicrobial methods. However, many of these methods are unproven or have not been properly tested. Since the 1980s, a promising procedure known as biophysical-information therapy or bioresonance therapy (BRT) has emerged as an alternative method against microbial diseases, but it has not yet been properly evaluated. It was demonstrated that by transferring amphotericin B (125 µg·ml^{-1}) information to water samples by an electronic amplifier (BRT device), the growth of cultured *Candida albicans* was significantly (*P<0.05*) inhibited (46% growth inhibition), compared with those cultures treated with sham electro-activated water samples (0% growth inhibition), and a positive control of amphotericin B (125 µg·ml^{-1}; 80% growth inhibition). Evidence for a measurable biological effect by electro-activated water samples that somehow acquires, or at least mimics, the antifungal property of amphotericin B has been demonstrated in the present study. More studies, however, are necessary to elucidate the mechanism by which such electro-activated water resembles the activity of an antimicrobial agent.

Key words: Antimicrobial effect, activated water, bioresonance, amphotericin B, growth inhibition, *Candida albicans*.

INTRODUCTION

There is a number of products to inhibit microbial growth, including the conventional (orthodox medicine) antibiotics or antimicrobial agents (Mc Donnell and Rusell, 1999; Lavin, 2000; Takahashi et al., 2003) or by using unconventional approaches such as electric and magnetic fields; for instance, an early contribution from Rowley et al. (1974), showed an inhibition of infecting microorganisms in human wounds by exposure to alternated electric fields. More recently, Qin et al. (1996) reported that processing liquid foods with high-intensity pulsed electric fields inactivated microorganisms, with the advantage of only a small increase in food temperature. On the other hand, it is known that magnetic fields affect

*Corresponding author. E-mail: abrrodriguez@hotmail.com. Tel: (81)83294000. ext. 6455.

the growth and reproduction of microorganisms and may be used as an antimicrobial procedure and to preserve foods (Pothakamury et al., 1993).

Regarding microbial growth inhibition using unconventional procedures, *Entamoeba invadens* trophozoite growth and encystation inhibition, using 60 Hz sinusoidal magnetic fields at 1.5 and 2.0 mT on cultures was previously observed (Rodríguez de la Fuente et al., 2008). Furthermore, it was recently demonstrated that by transferring metronidazole (a well known cytotoxic drug against parasites) information to water samples via an electronic amplifier (BRT device), the growth of axenically-cultured trophozoites of *Entamoeba histolytica* and *Trichomonas vaginalis* was significantly inhibited, compared with those cultures treated with sham electro-activated water samples (Heredia-Rojas et al., 2011). These results suggest that it is possible to transfer and store biological information to pure water, and that water sample that has undergone such a information transfer, can effectively interact with other biological systems, such as amoeba and yeasts.

There are several strategies that use diverse water treatments in an attempt to develop antimicrobial alternatives. Recently, Cloete et al. (2009) observed an antimicrobial mechanism of anolyte-electrochemically activated water against *Pseudomonas aeruginosa* and *Escherichia coli*; anolyte caused bacterial death by complete destruction of proteins or by causing oxidative stress, which resulted in protein fragmentation. Electrolysed water has been also suggested as a disinfectant for fresh-cut vegetables (Izumi, 1999). Furthermore, Bari et al. (2003) have demonstrated the effectiveness of electrolysed acidic water in killing *Escherichia coli*, *Salmonella enteritidis* and *Listeria monocytogenes* on the surface of tomatoes. In fact, most researchers agree that chemically-modified water shows microbial inhibition effect against various microorganisms (Leonov, 1997; Kim et al., 2000). Recently, Vysotskii et al. (2009) found a highly bacteriostatic effect of 93% in cultures of *Staphylococcus aureus* Wood-46 that were previously treated with molecular resonant effect technology (MRET-activated water).

On the other hand, results from Thomas et al. (2000), showed the possibility of transference of biological information by physical means; they observed the activetion of human neutrophils by electronically transmitted phorbol-myristate acetate (PMA), suggesting that PMA molecules emit signals that can be transferred to neutrophils by artificial physical means, in a manner that seems specific to the source molecules. Earlier, Kreisl (1998) observed that by transferring acetic acid information to inorganic salt solutions by means of an electronic amplifier, the pH of the inorganic solutions decreases slightly but significantly. These findings demonstrated that chemical signals can be transferred by an electronic amplifier device as it is used in the present study.

In view of this interesting issue involving unconventional antimicrobial procedures and the possibility to transferring drug information to water molecules by means of resonant circuits, we have undertaken this research to further evaluate the cytotoxic effect of water samples transferred with amphotericin B electronic information, a drug that is used to treat *Candida albicans* infections (Gomez-Flores et al., 1995). Amphotericin B is a polyene antifungal drug originally extracted from *Streptomyces nodosus*, whose mode of action is to bind with ergosterol, the main component of fungal cell membranes, forming a transmembrane channel that leads to monovalent ion (K^+, Na^+, H^+ and Cl^-) leakage, which is the primary effect leading to fungal cell death (Baginski and Czub, 2009).

MATERIALS AND METHODS

Reagents, culture media, and microbial strain

Sodium dodecyl sulfate (SDS), N,N-dimethylformamide (DMF), and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich (St. Louis, MO). Amphotericin B (fungizone) was obtained from Gibco (Invitrogen Corporation, Carlsbad, CA). *Candida albicans* (ATCC 32354) was obtained from the American Type Culture Collection (Rockville, MD). Yeast mold (YM) broth was purchased from Remel (Lenexa, KS). Extraction buffer was prepared by dissolving 20% (wt/vol) SDS at 37°C in a solution of 50% each DMF and demineralized water, and the pH was adjusted to 4.7.

Transmission apparatus

The equipment used for electronic transmission comprised a bioresonance therapy device, Bicom version 4.4 by Regumed (Regulative Medizine Technik GmbH, Germany) serial number 202057299.

Amphotericin B transmission to water

The source flask containing125 $\mu g \cdot ml^{-1}$ of amphotericin B, in a total volume of 1.0 ml of bidistilled water was placed inside the input coil coupled to a bioresonance amplifier, whereas in the output coil, a flask containing 1.0 ml of pure bi-distilled and sterile water was allocated at room temperature. Amphotericin B solutions and bidistilled water were sterilized by filtration. The oscillator was then turned on for the 15 min transmission period; during this procedure, parameters such as power, voltage, capacitance and impedance remained constant. Thus, the nature of the source tube (amphotericin B *versus* vehicle) was the only variable. According to the bioresonance device manufacturer, a specific program labelled as #196 was used for electronic transmission of substance to substance. At the end of the transmission period, the flasks were kept away from light and stored at room temperature for 1 h before being used in bioassays. Fifty microliters of transferred-water or non-treated water were added to each culture tube for treatment and controls.

Antimicrobial activity of electronically-transmitted amphotericin B to water

Candida albicans was selected because it is of clinical relevant, particularly in immuno compromised individuals. *C. albicans* was maintained and activated in specific culture medium. The

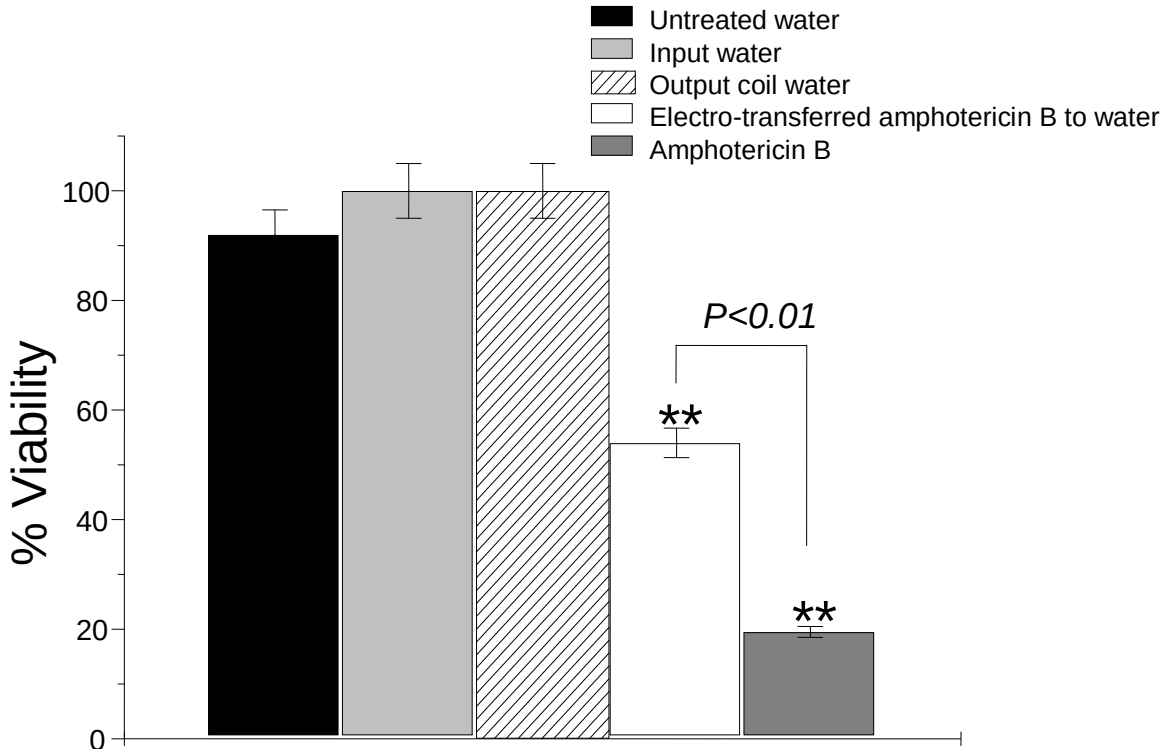

Figure 1. Effect of electronically-activated water on cultured *Candida albicans* growth. Viability of *C. albicans* after exposure to electronically-transmitted amphotericin B to water in liquid medium was determined by a colorimetric technique as explained in the text. Controls used in the present study included amphotericin B (125 µg·ml^{-1}), as a positive control of the antifungal effect, sterile non-activated distilled water (as a negative control or untreated water), sham electro-activated water, this is , water to water electro-transferred water samples, used as the control for a possible artifact effect induced by the bioresonance device on water samples, these controls included water samples that were previously placed in the input coil of the bioresonance apparatus, and water samples that were allocated in the output coil of the bioresonance device. Data represent mean ± SD of 8 replicates per treatment group from 3 independent experiments.

percentage of microbial growth inhibition by electronically-transmitted amphotericin B to water in liquid medium by a colorimetric technique was determined as follows (Gomez-Flores et al., 1995): *C. albicans* was activated by plating an aliquot in YM agar (Difco Laboratories, Detroit, MI) for 24 h at 37°C. Next, a loop of the culture was taken and suspended in YM broth (Difco, Becton Dickinson Microbiology Systems, Sparks, MD), and adjusted to 1 X 10^3 yeasts·ml^{-1}. In this process, we prepared a stock suspension of 1x10^3 yeasts·ml^{-1} which was maintained frozen in a solution of culture media with 5% of glycerol. Fifty micro liters of the microbial suspensions were plated in YM in flat-bottomed 96-well plates (Corning Incorporated, Corning, NY) (8 replicates per treatment group) in the presence of 50 µl of electro-activated amphotericin B (125 µg·ml^{-1}) water, or the controls; amphotericin B (125 µg·ml^{-1}), sterile distilled water, sham electro-activated water, this is, transferring the information from pure water to water, this included water samples that were previously placed in the input coil of the bioresonance apparatus, and water samples that were allocated in the output coil of the bioresonance device. Plates were then incubated for 16 h at 37°C, after which15 µl of the tetrazolium salt MTT was added to all wells at a final concentration of 0.5 mg·ml^{-1}, and plates were incubated for 3 additional hours. At the end of the incubation period, 50 µl of extraction buffer were added to all wells and plates were incubated overnight at 37°C. Optical densities resulting from dissolved formazan crystals were then read in a micro plate reader (DTX 880, Beckman Coulter, Inc., Fullerton, CA) at 570 nm.

Statistical analysis

Data represent mean ± SD of 8 replicates per treatment group from 3 independent experiments. The statistical differences were calculated among groups for the percentage of *C. albicans* viability. The data obtained were first transformed by using the arcsine function, then an analysis of variance for normal distributions and the correspondent parametric Tukey test for establishing individual differences were performed; the normality of the data was determined by the Kolmogorov-Smirnov test (P<0.05). All analyses were done using the SPSS package version 15.0. Differences were considered to be significant when the probability values were lower than 0.05.

RESULTS

The present study evaluated the effect of amphotericin B electronically-activated water samples on *in vitro C. albicans* growth. As observed in Figure 1, electro-activated water caused a significant (P<0.01) reduction of 46% in *C. albicans* viability, compared with amphotericin

B alone (80% growth inhibition) and no effect of the other control groups.

DISCUSSION

The antifungal effect of amphotericin B electro-activated water samples on cultured *C. albicans* was evaluated. There is a trend towards the use of alternative or complementary techniques, mainly in controlling microbial growth. Considering this trend and the lack of consensus on the effectiveness of these procedures, it is of considerable interest to examine if there is a significant and measurable biological effect of such unconventional treatments. The question has been raised as to whether these unorthodox biomedical techniques can really modify the microbial growth.

In this study, a statistically significant reduction of *C. albicans* viability (46% growth inhibition; Figure 1) were observed after pure cultures were treated with water samples activated in a bioresonance device by using a specific program called "substance to substance transference".

These results agreed with previous reports indicating that molecular information can be "scanned" and transferred by a bioresonance instrument. Endler et al. (1995) demonstrated that the metamorphosis of tadpoles could be greatly slowed down by transferring information from a toxic solution of the hormone thyroxin to the aquarium water in a number or parallel blind trials. Thomas et al. (2000) reported the transfer of the activity of 4-phorbol-12-B-myristate acetate, by electronic means, on the activation of human neutrophils. On the contrary, Jonas et al. (2006) found no effects from digital signals on the inhibition of thrombin/ fibrinogen coagulation by a digital signal, used instead of the original molecule.

We have previously reported *E. histolytica* and *T. vaginalis* trophozoites growth inhibition after treatment with metronidazole electro-activated water samples, using a BRT device of identical characteristics and conditions of those used in the present study (Heredia-Rojas et al*.,* 2011). Moreover, we demonstrated that it is possible to transfer drug information to water molecules following the same protocol of transference "substance-to substance" and using the same BRT device in a bacterial model. A significant antimicrobial effect was observed in cultured *Staphylococcus aureus*, methicillin resistant (MRSA), previously treated with vancomycin-electronically activated water samples (unpublished data).

A complete explanation about how it is possible to transfer biological information to pure water and to store it, though not yet clear, however it is accepted that electromagnetic waves interact with water. One mechanism that can explain the effect of electromagnetic fields on water is related to the existence of "defects" in its molecular structure. These stable structural changes were detected in experiments by the UV luminescence spectrophotometer and they have been related to different water structural defects that include specific centers of luminescence; the nuclear proton spins were considered to be a primary target of external magnetic fields, since proton lattice of water molecules is unstable and asymmetric (Binhi, 1998). In this regard, Wong and Lo (1998) have suggested that the "anomalous" states of water are peculiar and unexpected. Their existence may be connected to the occurrence of meta-stable polymorphic states of water at room temperature; it is therefore important to confirm or to refute the observations of these anomalous states by independent experimental investigations. Furthermore, Del Giudice et al*.* (2002) have shown the complex interactions, between water and solute ions, that occur after water solutions are exposed to extremely low frequency-magnetic fields. In the framework of coherent quantum electrodynamics, they suggested that water molecules in the liquid and solute ions are involved in their ground state in coherent ordered configurations and that ions are able to move without collisions among themselves in the interstices between water coherence domains. These interactions could explain some pathways for further interactions of electro-activated water with biological structures.

Moreover, it has been suggested that the orientation of nuclear proton spins may influence biochemical processes in biological systems, as a result of associations and disintegrations of the above mentioned structural defects of water, since ionic structural defects are chemically active (Smirnov et al., 2005).

There is experimental evidence that demonstrates that, by exposing water to resonant circuits, this may permanently alter some of its physico-chemical properties (Cardella et al., 2001). These researchers concluded that water samples after exposure acquire "biological-like" behavior that lasts for a significant period of time. Based on these findings, it is assumed that such behavior will somehow affect any chemical and/or biochemical reactions in which the exposed water may become involved.

It is possible that despite water exhibits no magnetic properties, the water clusters could be altered in some way due to the action of electromagnetic waves, because electro-activated water samples are under electromagnetic influence of the bioresonance apparatus. Since 1996, it is known that water clusters are extremely sensitive to the influence of physical factors such as magnetic and electric fields, and even low and ultra-low fields (Liu et al., 1996). This water capacity to acquire paradoxical configurations induced by low and extremely low intensity electromagnetic fields should be considered when trying to explain resonant intermolecular transfer of electromagnetic energy in liquid water samples (Woutersen and Bakker, 1999). Recently, it has been reported that electromagnetic transmission of chemical information can be stored in the electric dipole moments of water in close analogy to the manner in which

magnetic moments store information on a computer disk (Widom et al., 2010).

The basis for an understanding of the antimicrobial effects of bioresonance techniques is the assumption that the alternating electromagnetic fields, which are detectably emitted by living organisms and molecules and are characterized by intensity (amplitude) and frequency, contain biologically significant information that is used for transmitting a number of signals between cells, tissues, and even molecules (Likhoded et al., 2007). Recently, a novel property of DNA showing the capacity of some microbial DNA sequences to induce electromagnetic waves at high aqueous dilutions was reported (Montagnier et al., 2009a; 2009b). They found that electromagnetic signals of low frequency can be produced in aqueous dilutions of the human imunodeficiency virus DNA. This also opens the way to the development of highly sensitive detection system for some microbial illnesses in humans and animals.

To our knowledge, this is the first report showing electronically-transferred amphotericin B to water inhibits yeast growth. Research trials conducted primarily in Russia, Germany, and Eastern Europe, indicated that electromagnetic waves can affect biology in single-cell models that can include microorganisms (Islamov et al., 2002).

There is a big challenge in this 21st century; serious infections caused by microorganisms that have become resistant to commonly used antimicrobial drugs have become a major global healthcare problem. By the way, a question of “are we in the post-antibiotic era?” has been raised (Alanis, 2005). Independently of the answer, it is necessary to explore other antimicrobial strategies as we presented in the present study.

In conclusion, our *in vitro* study suggests that water samples that are electronically activated with vibrational information of amphotericin B are capable of inhibiting growth of axenically cultured *C. albicans*. However, with the results presented here, we are not supporting any therapeutical technique nor recommending bioresonance procedures, rather we showed evidence for a significant and measurable biological effect induced by electro-transferred water samples, that is, after the chemical information transferring procedure acquires, or at least mimics, the properties of that specific chemical substance (amphotericin B). Furthermore, an advantage of using a single-cell model for evaluating the effectiveness of bioresonance technique is that, the placebo effect claimed by those who refuse any alternative or complementary therapeutics, is absent.

On the other hand, we consider that today’s biology dominated by the molecular approach developed since about 1940, is suffocated by an immense number of experimental data on molecular aspects of biological functions, which present an extremely fragmented view on the living state. Thus, the holistic approach to biological studies is a complement to the contemporary practice. Field theories, as a central element of holistic models, possibly will be dominant models in the future. In addition, we believe that it is important to draw the scientific community attention on experimental results obtained by an unconventional approach. Every really new approach is labelled as unconventional until we fully disclose its mechanism of action. As a matter of fact, in the biological domain we are more acquainted with chemical than with physical approaches, but the last ones, in a near future, may become the next step in the evolution of pharmacology.

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