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DIFFERENT EFFECTS OF MICROWAVES AND CONVENTIONAL HEATING ON BACTERIOPHAGE Λ PROLIFERATION IN *E. COLI*

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Abstract – The proliferation of bacteriophage λ in *E. coli* was used as a model to compare the effects of microwaves and conventional heating on cell metabolism. Irradiation was carried out in a single-mode focused reactor at 2.45 GHz, under an absorption rate of 0.8±0.1 W/g and at constant temperature (33°C or 37°C). The kinetic curve of phage release from cells irradiated at 37°C was decreased as compared to the control, but significantly increased at 33°C. This was attributed to local overheating of cells by microwaves. Based on the conventional heating experiments, performed at 33°C, 37°C, 41°C and 45°C, we estimated that microwave-induced overheating inside cells was between 4°C and 8°C. However, this should have a limited effect on virus proliferation at 33°C, contrary to the obtained results. The increased expression of heat shock proteins (HSP) was proposed to be responsible for the observed effect of microwaves on virus proliferation.

Key words: Bacteriophage λ, *E. coli,* microwaves, heating

INTRODUCTION

It is generally recognized that microwaves (MWs) induce thermal effects by inducing frequent rotations of dipolar molecules (mainly water) and oscillations of solvated ions, causing the constant breaking and re-forming of weak intermolecular bonds in the irradiated medium; this allows the efficient transfer of electromagnetic energy of MWs into heat, producing a rapid increase in temperature throughout the irradiated medium (Halliday et al., 1997). If the medium contains phases with different dielectric properties, MWs can also induce specific thermal effects, i.e. differential heating of phases, which is not reproducible by conventional heating. When thermal and specific thermal effects cannot completely explain observed MW-induced phenomena, it is hypothesized that non-thermal effects of MW irradiation are involved (De la Hoz et al., 2005; Kappe and Stadler, 2005; Pavelkić et al., 2009; Pereux and Loupy, 2002; Stanisavljev et al., 2004;). Putative mechanisms of the non-thermal effects in chemical reactions were discussed by Pereux and Loupy (2001).

The increased use of MW appliances in everyday life has encouraged numerous investigations into the biological effects of MWs. The harmful consequences of MWs are often attributed to the thermal effects, although non-thermal mechanisms cannot be excluded. Early studies have indicated that MWs decreased the viability of both bacteria and viruses (Fujikawa et al., 1992; Heddleson and Doores, 1994; Woo et al., 2000) and could be applied for sterilization (Almajhdi et al., 2009; Datta and Davidson, 2000; Hong et al., 2004; Jamshidi et al., 2010; Park et al., 2006; Pucciarelli and Benassi, 2005). Animal

studies indicated that chronic low-intensity MW irradiation decreased the reproductive capacity of males (Akdag et al., 1999; Kesari et al., 2011). The MW emission from mobile phones induced significant cytotoxic effect in rat cortical neurons *in vitro* and increased the level of Bcl-2 and Bax apoptotic proteins in brain neuronal cells of rats with cranial defect (Zhu et al., 2008). In addition, MWs induced oxidative stress and increased lipid peroxidation in the blood, brain cortex and hippocampus of rats (Koylu et al., 2006; Yurekli et al, 2006). Chronic MW irradiation caused DNA damage in rat leukocytes, at least partially by oxidative stress (Garaj-Vrhovac et al., 2009). Moreover, significant DNA damage in peripheral blood leukocytes was induced by occupational exposure to MW irradiation (Garaj-Vrhovac and Oreščanin, 2009). However, Lantow et al. (2006) detected no conclusive effects on the production of reactive oxygen species (ROS); the authors reported that MWs significantly increased ROS production only in human monocytes, but not in human lymphocytes, and did not affect the expression of heat shock protein (HSP) 70 in either cell line. Moreover, several comprehensive analyses of the scientific data concerning the possible health implications of radio frequency and MW radiation have presented conflicting results, and the question of whether exposure contributes to cancer risk has remained unresolved (for review see Breckenkamp et al., 2003; McNamee et al., 2009; Moulder et al., 2003; Valberg et al., 2007).

Bacteriophage proliferation is a simple but very sensitive model for the evaluation of bacterial metabolism since almost all metabolic processes are involved in proliferation (Madigan et al., 2009), so that changes in different cellular processes can accumulate and result in alterations of bacteriophage proliferation curve. In our previous work, we showed that MW irradiation can lead to opposing effects on bacteriophage proliferation under different conditions, which were tentatively ascribed to the local overheating of infected cells, i.e. to specific thermal effects of MWs (Milojević et al., 2010). In the present work, we used the same model system to estimate the magnitude of MW overheating inside the cells by comparing the effects of MW irradiation with the effects of conventional heating.

MATERIALS AND METHODS

Chemicals and media

Luria-Bertani medium (LB) contained 1% Bacto-tryptone, 0.5% yeast extract, 0.5% NaCl, and was adjusted to pH 7.5 with 1 M NaOH. Tryptone agar semi-solid medium (TA_7) contained 1% Bacto-tryptone, 0.5% NaCl, 0.7% agar (pH 7.5). Tryptone agar (TA_{15}) was the same as TA_7 but contained 1.5% agar. All media were manufactured by Difco & co, Corpus Christi, TX, USA.

Biological materials

Fresh cultures of *E. coli* SY252 (Nikolić et al., 2004) and *E. coli* C600 (Appleyard, 1954), grown overnight at 37°C in LB medium, were used. The wild type bacteriophage λ, previously induced from lysogenic *E. coli* K12, was obtained from the Faculty of Biology laboratory collection.

Microwave irradiation

MW irradiation was performed in a commercial single-mode focused CEM reactor (Model Discover, CEM Co., Matthew, NC, USA) working at 2.45 GHz, with the ability to control output power. Temperature in the reaction vessel was measured directly by a fiber optic temperature sensor specially designed to prevent its interaction with the MWs. The cultures were irradiated with MWs in a glass cylinder (1.8 x 6.5 cm). In all experiments, the same volume of the cultures (6 ml) and a constant irradiation power (100 W), were used. External cooling was applied to keep the temperature of the mixture approximately constant, after a short initial period of heating. In order to keep the temperature within the vessel uniform (uniform bulk temperature) the sample was mixed during irradiation with a built-in magnetic stirrer at 400 rpm. A detailed scheme of the experimental setup has been published elsewhere (Stanisavljev et al., 2004).

The desired final temperature of the irradiated mixture was achieved by finely balancing MW emission and the external cooling (by circulating thermostat). At the beginning of the experiment, the thermostatic fluid and reaction mixture were at the same temperature. Since a constant MW irradiation power was applied, the temperature of the irradiated mixture rose in comparison with the surrounding thermostatic fluid, which facilitated the removal of heat by ordinary convection and conduction mechanisms. After some time, the supply of energy from the MWs was balanced by heat removal, resulting in the steady temperature of the reaction mixture (± 1°C). Only at the very initial stage of the experiment, when the removal of heat was minimal, the rate of temperature increase $(dT/dt)_i$ in the mixture was maximal and gave information on the absorbed power supplied to the reaction mixture by MWs (P_{abs}):

$$P_{abs} = mC\left(dT \div dt\right)_i \qquad (1),$$

where m is the mass of the reaction mixture was approximated with the mass of pure water, i.e. 6g; C is the specific heat capacity of the mixture was approximated with the specific heat capacity of pure water at 25°C, i.e. 4.184 J g^{-1} $°C^{-1}$; $(dT/dt)_i$ is the initial increase in temperature of the mixture. The absorbed power was calculated to be (5±0.5) W in the case of both investigated temperatures for the constant emitted power by the instrument of 100 W. With the applied experimental design, the SAR (specific absorbed rate) was (0.8±0.1) W g^{-1}.

The influence of MW irradiation on bacteriophage λ proliferation in E. coli

The exponential culture of SY252 (OD_{600}~0.5) was centrifuged at 900 *g* for 10 min at 4°C and the cells were resuspended in the same volume of 10 mM $MgSO_4$, used as an isotonic solution. The bacterial suspension was mixed with 0.1 ml of appropriate phage stock dilution, adjusted to obtain the MOI (multiplicity of infection, i.e. the ratio of viable virions and cells) 0.01 and incubated without stirring for 20 min at 37°C in order to enable virus adsorption. After adsorption, cells were separated from unadsorbed virions by centrifugation (1 400*g* for 15 min at 4°C) and resuspended in the same volume of LB medium containing 1% glucose. The infected cell suspension was divided into two vessels, one for MW treatment and the control. One vessel was irradiated with MWs for 100 min at final bulk temperatures of either 37°C or 33°C. Unirradiated control mixtures were incubated under the same conditions. At 25 min intervals the aliquots of 10 μl were taken from irradiated and control mixtures and kept on ice. For plaque forming unit (PFU) measurement, performed in triplicate, the appropriately diluted cell suspensions, used as centers of viral infection, were mixed with indicator *E. coli* C600 cells in melted semi-solid TA7 medium and immediately poured onto TA15 plates. The plates were incubated overnight at 37°C and the number of PFU per ml was calculated:

$$PFU = N \div \left(DV\right) \qquad (2),$$

where N is the average number of plaques per plate; D is the dilution of infected cells; V is the plated volume of infected cells dilution, i.e. 0.1 ml.

Since the initial PFU numbers were different in different experiments, for each time point the proliferation factor (PF) was calculated from:

$$PF = \left(PFU\right)_t \div \left(PFU\right)_0 \qquad (3),$$

where $(PFU)_t$ is the PFU per ml at each time point; $(PFU)_0$ is the PFU per ml at initial time point (0 min).

The influence of conventional heating on bacteriophage λ proliferation in E. coli

The effect of incubation temperature produced by conventional heating was investigated as described in the previous subsection, but without MW irradiation. The suspensions of infected cells were incubated for 100 min at the following temperatures: 33°C, 37°C, 41°C or 45°C; PFU measurements were performed at 25-min intervals. The numbers of PFU (Eqn. 2) and PF (Eqn. 3) were calculated as described above.

To compare the effects of MW irradiation and conventional heating on phage proliferation, for each experimental condition (N), we calculated the ratio of PFs (R), taking as a reference the kinetics of phage release during conventional heating at 33°C:

$$R_t = PF_{N(t)} \div PF_{C33(t)} \qquad (4),$$

where $PF_{N(t)}$ is the proliferation factor at each experimental condition, i.e. conventional or MW heating at each time point (t); $PF_{C33(t)}$ is the proliferation factor during conventional heating at 33°C at the corresponding time point.

Statistical analyses

The Student *t*-test (Samuels and Witmer, 2003) was employed for the statistical analysis. All calculated errors represent 95% confidence limits of the mean.

RESULTS

We have previously shown that MW irradiation, under the same conditions as described here, influence the process of intracellular phage proliferation, with no effect on either *E. coli* and phage λ viability, or on the adsorption of phage particles to the bacterial cell wall (Milojević et al., 2010). In Fig.1 we confirm our previous observation that MWs differently influence phage proliferation depending on the experimental temperature: for the same absorbed MW energy, phage release in time was moderately decreased with irradiation at 37°C (optimal temperature for the host cell), while it was significantly increased at 33°C.

Since the desired temperatures were achieved after about 5 min and were constant within ±1°C throughout the experiments (Fig. 2), the obtained results could be attributed to a possible overheating of the cells' interior by the specific thermal effects of MWs.

In order to estimate possible overheating, we tested the influence of conventional heating on phage proliferation and compared it with the effect of MWs. The experiment with conventional heating indicated

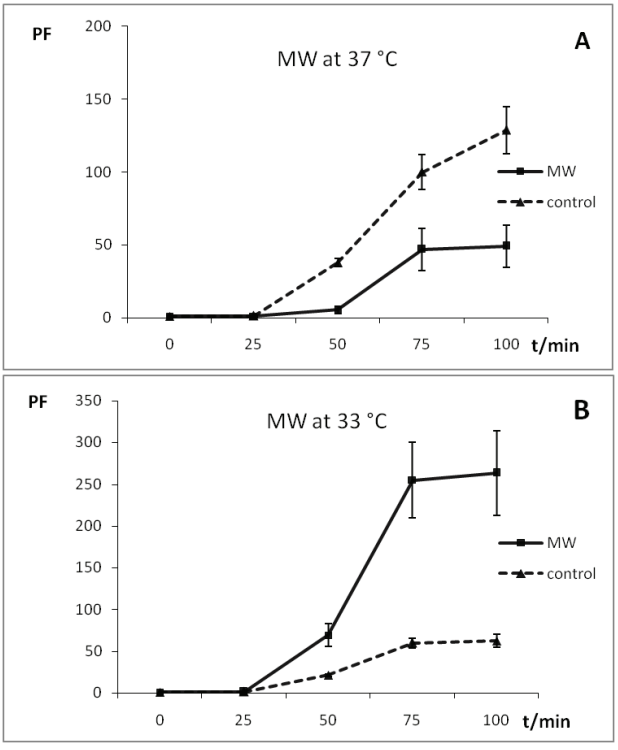

Fig. 1. Proliferation factors (described in Paragraph 2.4) for phage λ proliferation in *E. coli* as a function of time t/min for microwave experiments (A) at 37°C and (B) at 33°C. PF is presented as an average with standard deviation. The initial PFUs/mL were: A – $(1.73\pm0.47)10^7$ for MW irradiation, and $(2.02\pm0.12)10^7$ for control; B – $(0.60\pm0.06)10^7$ for MW irradiation, and $(0.46\pm0.02)10^7$ for the control.

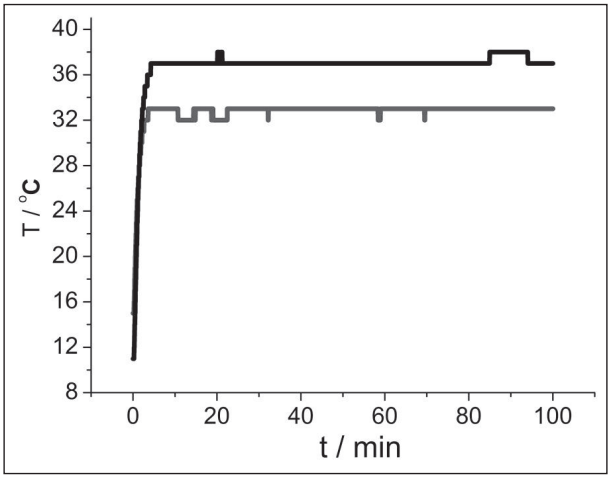

Fig. 2. The temperature profiles during MW heating of the reaction solution to the final temperatures of 33°C and 37°C.

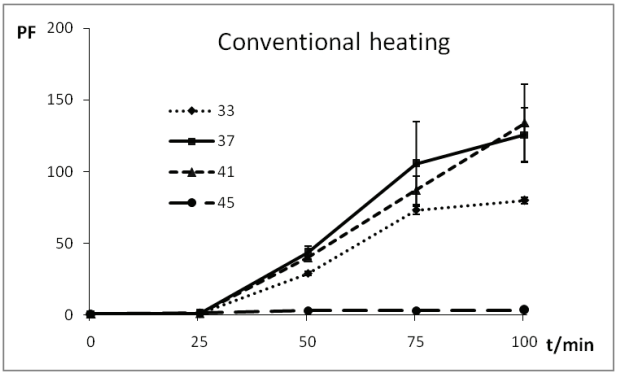

Fig. 3. Proliferation factors for phage λ proliferation in *E. coli* during conventional heating at 33°C, 37°C, 41°C and 45°C as a function of time t/min. PF is presented as average with standard deviation. The initial PFU/mL was 1.06±0.02)10^7.

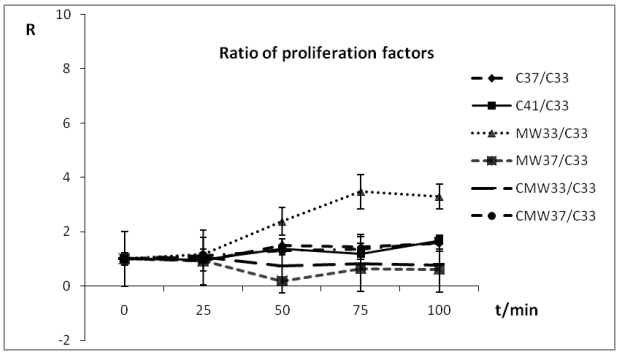

Fig. 4. The R – ratio of proliferation factors for all experiments, taking the conventional heating at 33°C as a reference, during time t/min. R is presented as average with standard deviation.

that in the temperature range 33-41°C (with increments of 4°C) the kinetics of phage release, presented as the function of PFs in time, showed only limited influence on phage proliferation (Fig. 3). On the other hand, the incubation of infected cells at 45°C markedly reduced the release of new phages, and consequently there were virtually no changes in the PFs during the entire experiment.

When comparing the results in Fig. 1 and Fig. 3, one can see that there is a significant difference in the effects of MW irradiation and conventional heating on phage proliferation at 33°C. To further assess this difference, the graph presenting the R values (Eqn. 4) as a function of time is shown in Fig. 4 for all experimental conditions. In order to emphasize the similar behavior of controls in the MW and conventional heating experiments, the C_{MW33}/C_{33} and C_{MW37}/C_{33} R values are included in the graph. As can be seen, the curves presenting the effects of conventional heating at 37°C and 41°C reveal only a minor increase from the reference, and the curve presenting the effect of MWs at 37°C shows only a slight decrease. In contrast, there is a significant difference between the effects of MWs and conventional heating at 33°C, which highly exceeds the statistical deviations. This opens the intriguing question of the non-thermal effects of MWs.

DISCUSSION

In the present work, the influence of MW irradiation and conventional heating on bacteriophage λ proliferation in *E. coli* has been compared in order to investigate the effects of MWs on cell metabolism. Contrary to the ordinary multimode reactors (MW ovens) with imprecise measurement and poor controlling of temperature in the investigated system, we used a single-mode focused reactor where MW energy was emitted only in the area of the reaction vessel. This ensured a constant energy density and prevented the existence of hotspots in the reaction medium. In addition, the built-in magnetic stirrer provided efficient stirring of the infected culture, assuring that all cells spent the same average time in every position in the reaction vessel. Moreover, after about 5 min of irradiation, the desired temperature was achieved and it was kept constant within ±1°C till the end of the experiment. Such experimental design eliminated large fluctuations in the bulk temperature due to MWs, but temperature increase inside bacteria, resulting from specific thermal effects, could not be excluded. However, due to the small dimensions of the bacteria compared with the MW wavelength of 12 cm and their simple inner constitution, only a limited increase of temperature inside the cells could be expected. Since MW-induced overheating inside the cells was not feasible to measure, we used the conventional heating experiments for its evaluation. Based on the obtained results, we estimated that MW overheating inside the cells at the bulk temperature

of 37°C should be more than 4°C (since the curves of phage proliferation at 37°C and 41°C are only negligibly different) and less than 8°C (since proliferation was almost completely diminished at 45°C). Consequently, even the maximal overheating due to MW irradiation at 33°C should produce only a limited influence on virus proliferation. Contrary to this, the obtained results clearly show a higher increase of the proliferation factors at 33°C, which cannot be attributed to statistical deviations. Therefore, only part of the MW effects observed in our experiments can be explained as thermal, indicating the possible involvement of non-thermal mechanisms.

During the past decades, a number of papers have claimed that MWs also elicit non-thermal effects on the structure and function of biological molecules (Beneduci et al., 2012; Bohr and Bohr, 2000; Bryant et al., 2007; Copty et al., 2005; George et al., 2008; De Pomerai et al., 2000; 2003). The non-thermal effects could be tentatively related either to the lowering of the activation energy by MWs which accelerates chemical reactions (Perreux and Loupy, 2002), or to their influence on the orientation of water dipoles around organic molecules that slightly influence their conformation (Bohr and Bohr, 2000; De Pomerai et al., 2000; 2003). However, the existence of non-thermal effects of MWs is far from confirmed, mainly because of the imprecision of experimental setups (Gaestel, 2010). Repeated experiments in well-controlled irradiation conditions failed to confirm the non-thermal mechanism in some physical, chemical and biological reactions (Shazman et al., 2007). Moreover, the original authors themselves performed critical analyses of their previous study (De Pomerai et al., 2000) and pointed out that observed effects of MWs were at least partially induced by thermal effects (Dawe et al., 2006).

Currently, the MW-induced biological phenomena that could not be attributed to MW thermal effects are attributed to alterations in biological membranes, influencing their permeability and function (Beneduci et al., 2012; Di Donato et al., 2012), and very often to the heat shock response. Several reports suggest that high frequency electromagnetic radiation, such as radiofrequency and MWs, primarily acts as a stress inducer, causing changes in protein conformation, which in turn induce a synthesis of HSPs in the exposed cells and tissues (French et al., 2001; Fritze et al., 1997; De Pomerai et al., 2000; 2003; Leszczynski et al., 2002; Wang et al., 2006). Moreover, HSPs are induced also by non-thermal stress inducers, which could affect the protein conformation, such as salt stress, acid, and UV-irradiation (Hartke et al., 1997; Kilstrup et al., 1997). Knowing that HSP GroEL and GroES are involved not only in the repair of damaged proteins, but also are required for λ phage maturation and packaging (Murialdo, 1991), we propose that their enhanced expression could be responsible for the increased phage proliferation observed in this study. Since the experiments with conventional heating revealed only a limited effect on phage proliferation in the temperature range 33-41°C, a significant MW-induced effect at 33°C cannot be attributed solely to the heating of the cells. In conclusion, the increased expression of heat shock proteins that were not only induced by overheating could be responsible for the observed effect of microwaves on virus proliferation.

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