

## ORIGINAL ARTICLE

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# Microwaves as modulators of membrane stability parameters during hepatic cancer

Guo Cunli, Bi Yumei, Zhang Lei, Xu Yi, Wang Qiucheng

Department of Ultrasound, Harbin Medical University Cancer Hospital, Harbin, 150081, China

### Summary

**Purpose:** The present study explored the potential of microwaves on membrane fluidity changes in diethylnitrosamine (DEN) induced hepatocellular carcinoma (HCC), *in vivo*.

**Methods:** Rats were segregated into four groups: normal control, DEN-treated, microwave-treated, DEN+microwave-treated. Brush border membranes (BBM) were isolated from the rats and, using the membrane extrinsic fluorophore pyrene, we assessed the viscosities as well as fluidity parameters.

**Results:** DEN treatment resulted in a significant rise in lipid peroxidation (LPO). Reduced glutathione levels (GSH) and the activities of glutathione reductase (GR), glutathione transferase (GST), superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) were found to be significantly decreased following DEN treatment. On the other hand, microwave treatment in DEN-treated rats resulted in

a significant decrease in the levels of lipid peroxidation but caused a significant rise in the levels of GSH as well in the activities of GR, GST, SOD, CAT and GPx. The results further demonstrated a marked decrease in membrane microviscosity following DEN treatment. On the other hand, a significant increase was observed in the excimer/monomer ratio and fluidity parameter of DEN-treated rats when compared to normal control rats. However, the alterations in membrane microviscosity and the fluidity parameters were significantly restored after microwave treatment.

**Conclusion:** The study, therefore, concludes that microwave proved quite useful in the modulation of membrane stability parameters following DEN-induced hepatic cancer.

**Key words:** hepatic cancer, membrane dynamics, microwave

### Introduction

Liver is the largest glandular organ in the body and it is responsible for various critical functions to keep the body free of toxins and harmful substances [1]. Liver cancer is classified as primary cancer which begins in the liver cells, and as secondary cancer that develops from cells from other organs that spread to the liver. Unfortunately, it is the second leading cause of cancer related mortality worldwide [2,3]. Membranes are developed to perform a crucial role in the process of carcinogenesis as peculiar characteristics of cancer growth and its progression involves membrane-membrane

interactions [4]. Furthermore, the key attributes of the membranes, which are widely focused in cancer research, are lipid composition, membrane fluidity and their correlation to malignant transformation [5,6]. A recent study observed alterations in the membrane fluidity and its composition at an early stage of malignant transformation process [7]. In cell physiology, membrane fluidity is an important facet that might be manipulated by diet. Improper dietary habits are one of the prime concerns as these exert influence on the key molecular events associated with onset of cancer.

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Correspondence to: Dr. Guo Cunli. Department of Ultrasound, Harbin Medical University Cancer Hospital, Haping Rd no.150, Harbin, 150081, China.  
Tel: +86 18110059182, E-mail: docguocunli@163.com  
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Microwave hyperthermia is an upcoming tool for cancer treatment by sending microwave energy of suitable frequency range to provide localized heating [8]. This strategy heats the tumor more than 42 °C, while keeping the healthy tissues' temperature normal [9]. However, there are still many hurdles in its path to use it as a clinical procedure [10]. In the present study we have tried to explore the potential of microwaves to protect membrane integrity. Besides, there is a paucity of information with regard to the role of microwaves as a membrane stabilizer, especially during hepatic carcinogenesis. Therefore, the present study was conceived to explore the potential of microwaves as a possible intervention on the membrane parameters and in the modulation of membrane fluidity following DEN treatment in rats.

## Methods

### Chemicals

DEN, NADPH, GSH, NBT and DTNB were procured from Sigma Aldrich Co. New York, USA. Sodium selenite was purchased from Merck Co, New Jersey, USA.

### Experimental design and animal treatment

The Institutional Ethical Committee on Experimental Animals approved the animal care procedures followed in the current study for Biomedical Research. Male Wistar rats weighing 120-150 g were procured from the Central Animal house and were housed in the animal facility of our institute. All the animals were housed in polypropylene cages under hygienic conditions, and were provided with pellet diet and drinking water *ad libitum*. Animals (n=40) were segregated into 4 treatment groups. Animals in Group 1 served as normal controls and were given water and diet *ad libitum*. Rats in this group also received pure olive oil, which was used as the vehicle for treatment in DEN-treated animals. Animals in Group 2 were given weekly intra-peritoneal injections of 50mg/kg DEN, diluted in pure olive oil in order to obtain a fully developed HCC on a cirrhotic liver after 14 weeks [11]. Group 3 animals received microwave treatment by using fabricated quasi-yangi antenna. Animals in Group 4 were given a combined treatment of DEN as well as microwaves in a similar manner as given to Groups 2 and 4 animals.

### Lipid peroxidation and antioxidant defense system enzymes

Lipid peroxidation assay was done by the method of Wills [12].

Reduced Glutathione (GSH): Estimation of GSH was performed in the tissue homogenates of liver by the method of Ellman [13].

Catalase: The method of Luck was used for the estimation of catalase [14].

Superoxide Dismutase (SOD): The activity of SOD was estimated by using the method of Kono [15].

Glutathione Reductase (GR): The enzyme was assayed by the method of Carlberg and Mannervik [16].

Glutathione-S-Transferase (GST): This enzyme was assayed by the method of Habig et al [17].

Glutathione Peroxidase (GPx): This enzyme was assayed by the method of Pagila and Valentine [18].

Protein: Protein assay was done by the method of Lowry et al. [19].

### Isolation of liver brush border membrane

Animals were sacrificed using ether anesthesia, and the livers were excised. Intestinal BBM was isolated by homogenizing the tissue in chilled sodium maleate buffer (10% homogenate) in a waring blender fitted with a Teflon pestle and using a glass homogenizing tube with 0.05-mm clearance. All the procedures were done strictly at 4°C including the excision of the liver, flushing with physiologic saline, and dissecting into several small pieces for homogenization. The homogenate was passed through two layers of surgical bandage, and anhydrous CaCl<sub>2</sub> was added to the filtrate with constant stirring on a magnetic stirrer to a final concentration of 10 mM. The tubes were centrifuged at 2000 g for 10s at 4°C and the supernatant so obtained was further recentrifuged at 42,000 g for 20s. The pellet was suspended in 20 volumes of 50 mM sodium maleate buffer (pH 6.5-6.8) and was recentrifuged at 42,000 g for 20 min. The final pellet obtained was suspended in the 50 mM sodium maleate buffer. The method has been based on the procedure of Schmitz et al. [20].

### Membrane stability studies in BBM

Pyrene fluorescence excimer [dimer] formation was used to study the lateral diffusion in the membrane following the method of Massey et al [21]. The fluorescence intensity was read in a fluorescence spectrophotometer by using the excitation wavelength of 365 nm and emission wavelengths of 515 nm and 475 nm for the excimer and monomer fluorescences, respectively. The viscosity ( $\eta$ ) was calculated from the monomer/excimer fluorescence intensity ratios by using the following relationship:

$$E/M(\text{excimer/monomer}) = (\text{Pyrene}) T/k/n$$

where  $T$  is the absolute temperature,  $k$  is the Boltzmann constant ( $1.38062 \times 10^{-23}$  Joules/Kelvin), and  $n$  is the microviscosity while the pyrene concentration was kept at 5 mM.

### Microwave treatment

The microwave hyperthermia was provided by utilizing a wideband quasi-yagi antenna. The antenna was compact, directional and wideband and generated circular array for a microwave hyperthermia system. It was operated across wideband centered at 7.5 GHz.

### Statistics

The statistical significance of the data was determined by using one-way analysis of variance (ANOVA) followed by a multiple *post-hoc* test least significant difference (LSD) with 5% considered significant. The results are presented as mean  $\pm$  SD.

## Results

The results obtained from various experiments conducted in this study are depicted in Tables 1-3. The data from various treatment groups have been compared with the normal control animals. However, results obtained from microwave+ DEN-treated group were additionally compared with those of DEN group.

### Lipid peroxidation and reduced glutathione (GSH) levels

The present study showed a significant increase in the levels of lipid peroxidation (Table 1) in the livers of rats following 14 weeks of DEN treatment. However, microwave treatment in DEN-treated rats significantly moderated the lipid peroxidation levels when compared to rats treated with DEN alone. On the other hand, significant decline in the GSH

levels were observed in the DEN-treated rats that showed a significant elevation upon treatment with microwave.

### Antioxidant enzymes

DEN treatment resulted in a significant increase in the activities of glutathione reductase (GR) (Table 2) but caused a significant decrease in the activities of glutathione-S-transferase (GST), superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx). Microwave treatment in DEN-treated rats resulted in significant decrease in the activities of GR. Furthermore, microwave resulted in a significant increase in the activities of GST, SOD, CAT and GPx.

### Membrane dynamics studies

The lateral diffusion has been measured by the excimer formation of the pyrene. In the present

**Table 1.** Effect of microwave treatment on the lipid peroxidation and GSH levels

| Groups                  | Lipid peroxidation<br>(nmoles of MDA formed min <sup>-1</sup> 100mg protein <sup>-1</sup> ) | GSH<br>(μmoles GSH gm tissue <sup>-1</sup> ) |
|-------------------------|---|--|
| Normal control          | 3.30 ± 0.20   | 0.61 ± 0.01                                  |
| DEN treated             | 4.29 ± 0.20 <sup>c</sup>  | 0.47 ± 0.01 <sup>c</sup>                     |
| Microwave               | 3.11 ± 0.24   | 0.69 ± 0.01                                  |
| Microwave + DEN treated | 3.50 ± 0.15 <sup>y</sup>  | 0.57 ± 0.02 <sup>c,x</sup>                   |

Values are expressed as mean ± SD. <sup>c</sup>P ≤ 0.001 by least significant t-test (LSD) when values are compared with control group. <sup>x</sup>P ≤ 0.05 and <sup>y</sup>P < 0.01 by least significant t-test (LSD) when values of Group 4 are compared with Group 2

**Table 2.** Effect of microwave treatment on the antioxidant enzymes

| Groups                  | CAT<br>(mmoles of H <sub>2</sub> O <sub>2</sub><br>decomposed min <sup>-1</sup><br>1mg protein <sup>-1</sup> ) | SOD<br>(I.U)               | GST<br>(nmoles of CDNB<br>conjugates/min/mg<br>protein) | GR<br>(nmoles /min/mg<br>protein) | GPx<br>(μmoles NADPH<br>oxidized min <sup>-1</sup> mg<br>protein <sup>-1</sup> ) |
|-------------------------|--|----------------------------|---|-----------------------------------|--|
| Normal control          | 3.81 ± 0.17  | 4.2 ± 0.34                 | 0.29 ± 0.12   | 2.90 ± 0.58                       | 0.71 ± 1.31  |
| DEN-treated             | 2.30 ± 0.13 <sup>c</sup>   | 2.05 ± 0.51 <sup>c</sup>   | 0.06 ± 0.04 <sup>c</sup>                                | 1.87 ± 0.39 <sup>b</sup>          | 0.54 ± 0.39  |
| Microwave               | 2.79 ± 0.32 <sup>a</sup>   | 3.01 ± 0.54 <sup>a,x</sup> | 0.29 ± 0.06 <sup>b</sup>                                | 1.90 ± 0.34 <sup>b</sup>          | 0.78 ± 16.06   |
| Microwave + DEN treated | 2.41 ± 0.36 <sup>c</sup>   | 4.29 ± 0.78 <sup>b</sup>   | 0.20 ± 0.21 <sup>a</sup>                                | 2.03 ± 0.34 <sup>b</sup>          | 0.65 ± 0.48  |

Values are expressed as mean ± SD. <sup>a</sup>P ≤ 0.05, <sup>b</sup>P ≤ 0.01 and <sup>c</sup>P ≤ 0.001 by least significant t-test (LSD) when values are compared with control group. <sup>x</sup>P ≤ 0.05 by least significant t-test (LSD) when values of group 4 are compared with Group 2.

**Table 3.** Effect of microwave treatment on the excimer/monomer ratio, microviscosity and fluidity of brush border membrane

| Groups                  | Excimer/ Monomer Ratio<br>(E/M) | Microviscosity<br>(η) × 10 <sup>-23</sup> | Fluidity<br>(1/η) × 10 <sup>-23</sup> |
|-------------------------|---------------------------------|---|---------------------------------------|
| Normal control          | 0.30 ± 0.04                     | 6.61 ± 0.21                               | 0.17 ± 0.01                           |
| DEN-treated             | 0.49 ± 0.20 <sup>c</sup>        | 4.47 ± 0.19 <sup>c</sup>                  | 0.37 ± 0.19 <sup>c</sup>              |
| Microwave               | 0.35 ± 0.24                     | 5.89 ± 0.01                               | 0.19 ± 0.01                           |
| Microwave + DEN-treated | 0.35 ± 0.15 <sup>y</sup>        | 5.57 ± 0.12 <sup>z</sup>                  | 0.17 ± 0.12 <sup>z</sup>              |

Values are expressed as mean ± SD. <sup>c</sup>P ≤ 0.001 by least significant t-test (LSD) when values are compared with control group. <sup>x</sup>P ≤ 0.05, <sup>y</sup>P ≤ 0.01 and <sup>z</sup>P < 0.001 by least significant t-test (LSD) when values of Group 4 are compared with Group 2

study, DEN treatment for 14 weeks showed a significant increase in Excimer/Monomer (E/M) ratio as compared to normal control group (Table 3). The increased E/M ratio or increased lateral diffusion of the probe (pyrene) was accompanied by a decrease in membrane microviscosity, which further suggests an elevation of membrane fluidity. Simultaneous application of microwave for 14 weeks to DEN-treated rats brought the E/M ratio back to near normal, proving its therapeutic potential.

## Discussion

The present study indicated that co-administration of microwave with DEN to rats modulates appreciably the changes inflicted by DEN on lipid peroxidation, antioxidant defense enzymes and membrane dynamics. So, microwaves hold a promising anticancer potential with respect to liver carcinogenesis.

The relative motional freedom of the lipid molecules in the membrane lipid bilayer is known as membrane fluidity [22]. It basically involves rotational or lateral diffusion of molecules in an array. In the present study, lateral diffusion has been measured by the excimer formation of the pyrene. DEN treatment for 14 weeks showed an increase in E/M ratio as compared to normal control group. The increased E/M ratio or the increased lateral diffusion of the probe (pyrene) indicate a decrease in membrane microviscosity, which further suggests an elevation of membrane fluidity. Increased fluidity entails a less-hindered channel for lipophilic solutes to move across the membrane barrier. The increased lateral diffusion of the probe might be a result of partial lipid removal and more motional freedom of the probe in the hydrocarbon phase.

There could be many possible reasons for the observed increase in membrane fluidity during DEN-induced liver carcinogenesis. However, one of the major factors contributing towards increased membrane fluidity could be the increased lipid peroxidation of membrane phospholipids by

reactive oxygen species (ROS) produced during DEN-induced liver carcinogenesis as observed in the present study. Earlier studies have noticed similar changes in lipid peroxidation levels during cancer [8,28]. Moreover, inhomogeneous state of membrane further hampers pyrene diffusion as well as excimer formation. On the other hand, administration of microwaves in DEN-treated rats significantly decreased the E/M ratio, when compared to DEN-treated group, showing a decline in membrane fluidity. Further, decreased membrane fluidity following microwave treatment to DEN-treated rats could also be indirectly attributed to the regulatory role of microwave in maintaining the ROS levels, as noticed by moderation in lipid peroxidation and antioxidant defense enzymes in DEN-treated rats. Also, the altered surface characteristics of the liver could be due to variability in membrane dynamics, as observed in the present study. Also, changes in mucous production in the membrane could cause surface abnormalities, as mucous production is a natural defense of the system against toxic agents including carcinogens.

So, membrane dynamics modulation by microwaves could be ascribed to the ability of microwaves in maintaining the membrane integrity either by some direct mechanism or indirectly by scavenging the free radicals responsible for increased lipid peroxidation. Secondly, microwaves could have moderated the hyper-proliferation of the hepatic cells that could maintain hepatic surface characters. The study concluded that microwaves act as an effective membrane stabilizer during hepatic carcinogenesis.

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## Conflict of interests

The authors declare no conflict of interests.

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