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ORIGINAL ARTICLE

Z-scan method to measure the nonlinear optical behavior of cells for evaluating the cytotoxic effects of chemotherapy and hyperthermia treatments

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Abstract

The effects of new treatments must be investigated in vitro before using clinically or in vivo. The aim of this study was to introduce the Z-scan technique as a fast, accurate, inexpensive, and safe in vitro method to distinguish the cytotoxic effects of various treatments. C6 and OLN-93 cell lines were prepared and treated with Temozolomide (TMZ), radiofrequency hyperthermia (HT), and chemo-hyperthermia (HT+TMZ). The cytotoxic effects of different treatments on both cell lines were evaluated using colony formation assay and Z-scan method. The results of colony assay showed that the surviving fraction (SF) of C6 cells treated with TMZ, HT, and HT + TMZ were significantly decreased compared to the control group. Whereas, hyperthermia treatment had no significant effect on the SF of OLN-93 cells. The results of Z-scan technique indicated that the control group of C6 cells had the negative nonlinear refractive index (n_2). Whereas, the C6 cells treated with HT, TMZ, and HT + TMZ had the positive n_2 index. The sign of n_2 index in the control and HT groups of OLN-93 cells was positive but treatment of cells with TMZ and HT + TMZ changed the sign of it. Moreover, with increasing the cytotoxic effects of different treatments, the SF value of both cell lines decreased and the magnitude of n_2 index increased. The results of Z-scan technique were completely in line with the results of colony assay. Therefore, Z-scan method could distinguish the cytotoxic effects of various treatments by examining the nonlinear optical properties of the samples.

Keywords Z-scan technique · Nonlinear optical behavior · Nonlinear refractive index · Temozolomide · Radiofrequency hyperthermia

Introduction

Chemotherapy treatment includes the use of low-molecular-weight anticancer drugs to selectively destroy cancer cells. Temozolomide (TMZ) is a chemotherapeutic drug that is currently used in the treatment of glioblastoma multiforme (GBM) cancer cells [1]. TMZ is a methylating agent of the imidazotetrazine class and spontaneously decomposes to the cytotoxic alkylating agent 5-(3-methyltriazen-1-yl) imidazole-4-carboximide under physiologic conditions [2, 3] that results in cell death by methylating O6 position of guanine in DNA [4, 5]. Growing evidence shows that combination of chemotherapy and hyperthermia (chemo-hyperthermia) leads to highly toxicity in cancer cells [6, 7]. Nowadays, new treatments are under development to enhance the efficacy of cancer therapy [8–10].

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The effects of all new treatment methods must be investigated in vitro before using clinically or in vivo. In vitro assays must have capability to identify the cytotoxic or genotoxic effects of different treatments in cells. Moreover, these techniques should be precise, repeatable, and fast.

Nowadays, there are many different methods for the evaluation of various treatment effects such as clonogenic assay, flow cytometry techniques, quantitative real-time PCR, Western blot analysis, colorimetric assays, cytogenetic methods, etc. [11–15]. These methods are expensive, laborious, and sometimes require very specific training.

Clonogenic assay is an in vitro cell survival method, which has been used broadly to determine the long-term cytotoxicity of various treatments [16]. Colony formation assay is based on the capability of a single cell to reproduce a colony. The colony is defined as a collection of at least 50 cells [17]. This method has been used in the decades for a large variety of research, but it is time-consuming that limits the widespread applicability of it.

In the present study, it was desirable to introduce closed aperture Z-scan technique as a short-term, precise, and inexpensive method to identify the effects of cell treatments using laser and nonlinear optical properties of cells. The Z-scan method was developed by Mansoor Sheik Bahae et al. [18, 19], which can rapidly measure the nonlinear optical properties of cells, cell components, materials, and biomaterials [20–23].

Chemotherapeutic agents, hyperthermia, or chemo-hyperthermia treatments lead to changes in cells (such as structural, morphological, biochemistry, molecular, and genomic changes) [24–26], that result in the variation of nonlinear optical properties of them [27, 28]. Z-scan analysis can detect the alteration of the light that is scattered by cells or materials; therefore, it can be used to identify the changes of cells associated with various treatments. Previously, in many studies, Z-scan technique was used to determine the concentration of biomaterials in the body, such as proteins [29], cholesterol, triglycerides [30], and glucose [31] in the blood of patients. Additionally, the authors of the present study have previously demonstrated that Z-scan technique is a useful method for distinguishing MCF-7 cell line from SK-BR-3 breast cancer cell line [21].

The aim of the present study was to introduce the closed aperture Z-scan technique as a fast, accurate, inexpensive, and safe in vitro method to distinguish the cytotoxic effects of various treatments on cells. In order to justify this hypothesis, the C6 (glioblastoma multiforme) and OLN-93 (normal glial) cell lines were treated with Temozolomide, radiofrequency hyperthermia, or combined chemo-hyperthermia, then the nonlinear properties of them were evaluated using a single beam Z-scan method. Finally, the results of Z-scan method were compared with the results of colony formation assay.

Materials and methods

Materials

Formaldehyde solution, Temozolomide (TMZ), and Crystal violet 0.5% were obtained from Sigma Chemical Company (St Louis, MO, USA). Penicillin-streptomycin, Trypsin-EDTA (0.25%), fetal bovine serum (FBS), Dulbecco's modified Eagle's medium (DMEM) and Ham's F12 were purchased from Gibco (Invitrogen, USA). Also, the C6 and OLN-93 cell lines were provided from Pasteur Institute of Iran.

Cell culture

Glioblastoma multiforme (C6) and glial (OLN-93) cell lines were employed in this study. C6 cell line was cultured in Ham's F12 and OLN-93 cultured in DMEM medium containing 10% FBS, 100 U/mL penicillin, and 100 mg/mL streptomycin and maintained at 37 °C under 5% CO_2 and 95% air humidified environment incubator at a density of 10^4 cells/cm^2.

Cultured cells were detached from the T-25 cell culture flasks with trypsin-EDTA 0.25% and centrifuged at 1200 rpm for 5 min. After cell counting, a density of 2×10^5 cells were seeded in the 60-mm cell culture Petri dish with one sterilized glass slide at the bottom of it and incubated for 24 h to adhere on the Petri dish.

Treatment of cells with Temozolomide, hyperthermia, or chemo-hyperthermia

In this study, C6 and OLN-93 cell lines were evaluated in four groups: (1) control, (2) TMZ (30 μg/mL), (3) radiofrequency hyperthermia (HT) (13.56 MHz, 80 W, 30 min), and (4) HT + TMZ.

After 24-h incubation, both cell lines were treated with TMZ (30 μg/mL) for 24 h. Treated cells, as well as cells that had not been exposed to TMZ, were placed in the center of the RF coil and irradiated with it (13.56 MHz, 80 W, 30 min) to reach the average temperature of 43 °C. The temperature variations of cells were monitored in real time using a digital infrared (IR) thermal camera (Testo 875-1i, Germany) (Fig. 1). In both cell lines, untreated cells were considered as the negative controls.

Evaluation of the cytotoxic effects of various treatments

Colony formation assay

After treatment, the C6 and OLN-93 cell lines were washed with phosphate buffer saline (PBS), detached with trypsin-EDTA 0.25%, and centrifuged. Single cell suspensions were

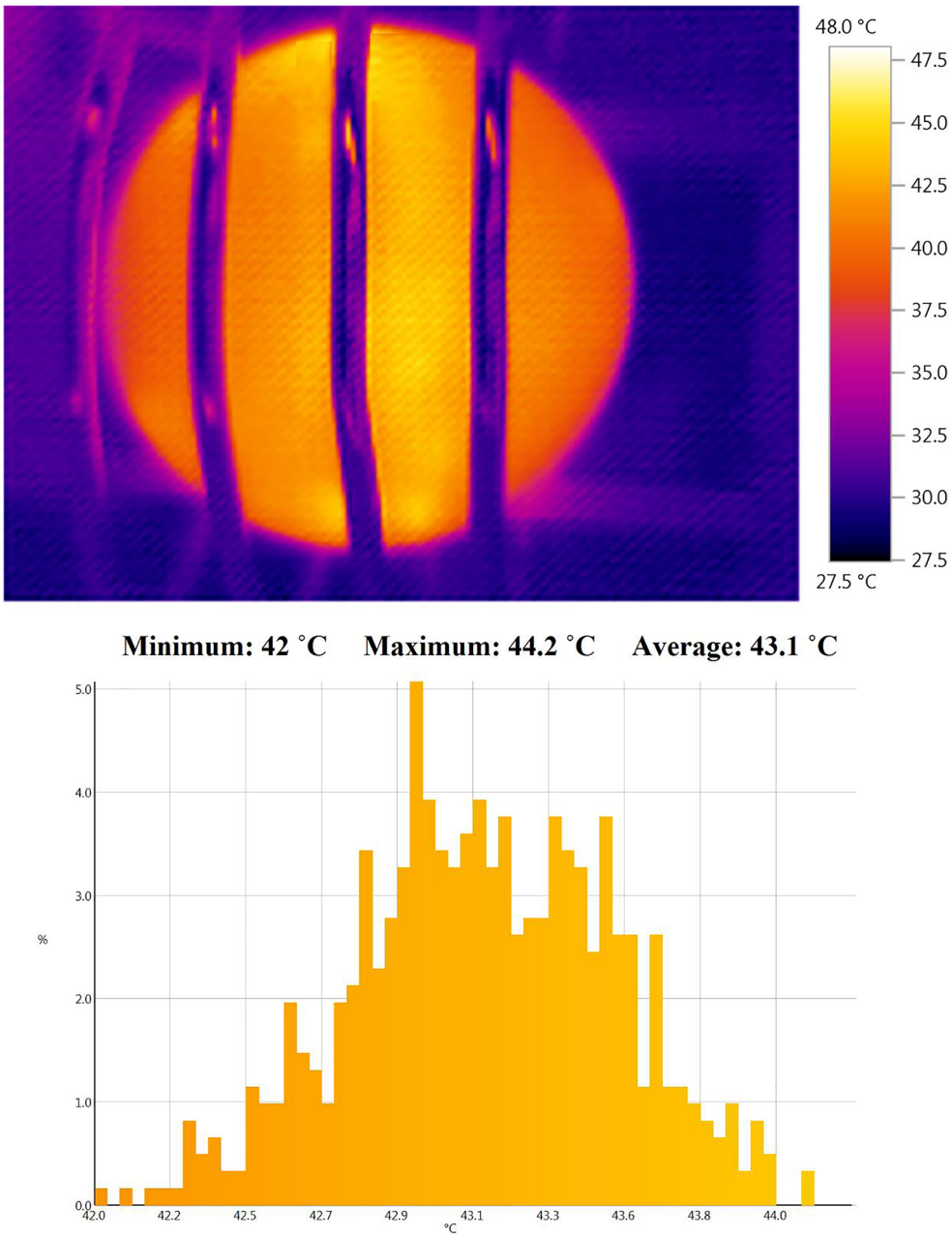

Fig. 1 NIR images of C6 cells irradiated with radiofrequency hyperthermia (13.56 MHz, 80 W, 30 min) to reach the average temperature of 43 °C

seeded in 60 mm cell culture Petri dishes and incubated in the presence of complete medium (DMEM for OLN-93 and Ham's F12 for C6) for a period of 7 days. After that, the colonies were washed with PBS, fixed using 4% formaldehyde solution, and stained with 0.5% crystal violet. The colonies (containing 50 or more cells) were counted using an optical microscope (Zeiss, Germany) and the plating efficiency (PE) and surviving fraction (SF) were determined by Eqs. 1 and 2.

$$\mathrm{PE}\ (\%) = \frac{\text{The number of colonies}}{\text{The number of cell seeded}} \times 100 \tag{1}$$

$$\mathrm{SF} = \frac{\mathrm{PE}_{\mathrm{treated}}}{\mathrm{PE}_{\mathrm{control}}} \tag{2}$$

Closed aperture Z-scan technique

After treatment, the C6 and OLN-93 cell lines were washed with PBS, fixed using 4% formaldehyde solution, and dried at the room temperature. Finally, cells on the glass slides were evaluated using the Z-scan technique. Moreover, optical microscopic images of all samples were taken by an inverted microscope with the magnification of × 400 (BEL, Monza, Italy).

The close-aperture Z-scan technique was used to measure the nonlinear refractive indices (n_2) of samples and its setup has been shown in Fig. 2a. The Z-scan method is based on the movement of samples through the Z-axis and the sign and magnitude of nonlinear refractive index were achieved using it. We applied Nd:YAG laser with a wavelength of 532 nm and a lens with an 80-mm focal length. The direction of the laser beam was fixed and parallel with the Z-axis. The samples were moved along the Z-axis and the laser beam passed through the samples. The transmittance power changes through the aperture (placed at the far field situation) were detected in relation to the sample position. An extreme low-power laser detector (11XLP12-3S-H2, Standa, Lithuania) was used to detect transmittance power changes. Finally, the nonlinear refractive index (n_2) can be computed by Eq. 3:

$$n_2 = \frac{\lambda \Delta T_{P-V}}{2\pi L_{eff}(0.406)(1-S)^{0.25} I_0} \tag{3}$$

where λ is the wavelength of beam source and $\Delta T_{P\text{-}V}$ is the difference between the maximum and minimum values of the normalized transmittance (peak and valley). L_{eff} and S are the effective length and the linear transmittance through the aperture, respectively. I_0 is the maximum intensity at the focal point. The mentioned parameters are given as following:

$$L_{eff} = \frac{1-e^{-\alpha L}}{\alpha} \tag{4}$$

where α and L are linear absorption coefficient and the thickness of the sample, respectively.

$$S = 1-exp\left(-2\left(\frac{r_a}{W_a}\right)^2\right) \tag{5}$$

where r_a is the radius of aperture and W_a is the beam radius at the aperture plane.

$$I_0 = \frac{2P}{\pi w_0^2} \tag{6}$$

where w_0 is the beam waist and P is input power.

The result of scan for the sample with a positive nonlinear refractive index has a valley followed by a peak in the diagram of normalized transmittance (pre-focal and post focal normalized transmittance are minimum and maximum, respectively). When a sample has a negative nonlinear refractive index, the graph is inverted.

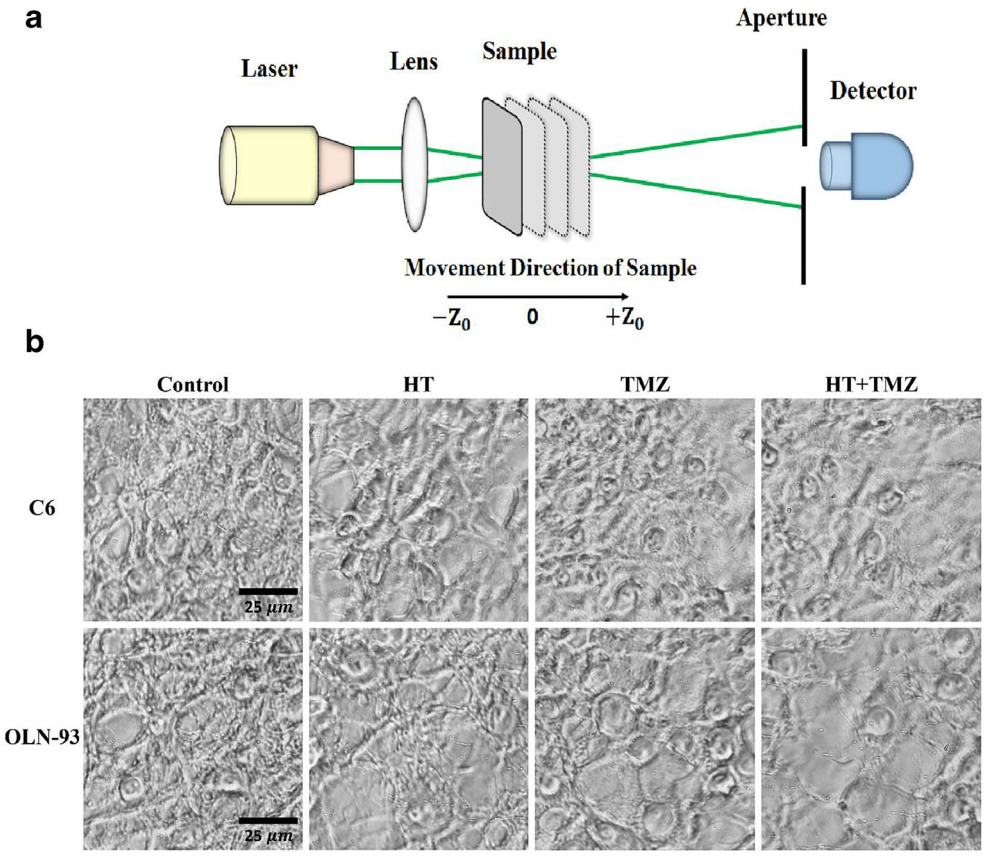

Fig. 2 **a** Experimental set up for the application of closed aperture Z-scan method. **b** The optical microscopic images of C6 and OLN-93 cell lines treated with HT, TMZ, and HT+TMZ with magnification × 400

Statistical analysis

Experimental data were presented as mean ± standard deviation (SD). Statistical comparisons in colony formation assay were performed by one-way ANOVA followed by Tukey's test as the post-hoc analysis using SPSS version 16. $P < 0.05$ was considered significant.

Results

C6 and OLN-93 cell lines were treated with Temozolomide, RF-hyperthermia, and combined chemo-hyperthermia. Cytotoxic effects of them were evaluated by the colony formation assay and Z-scan method. Moreover, optical microscopic images of them were prepared.

Optical microscopic images

Optical microscopic images of C6 and OLN-93 cell lines after treatment of TMZ, HT, and HT + TMZ have been shown in Fig. 2b. As it can be seen in Fig. 2b, the morphology of both cell lines changed after various treatments and the cytotoxicity of them was confirmed by cellular morphological changes. C6 and OLN-93 cells treated with HT+TMZ exhibited distinctive morphological changes compared to other treatments.

Colony formation assay

The results of colony formation assay have been shown in Fig. 3. As depicted in Fig. 3, RF-hyperthermia (HT) treatment has no significant effect on the surviving fraction of OLN-93 cells compared to the control group ($P > 0.05$). Whereas, TMZ and especially HT + TMZ treatments significantly reduced the SF value of OLN-93 cells ($P < 0.05$).

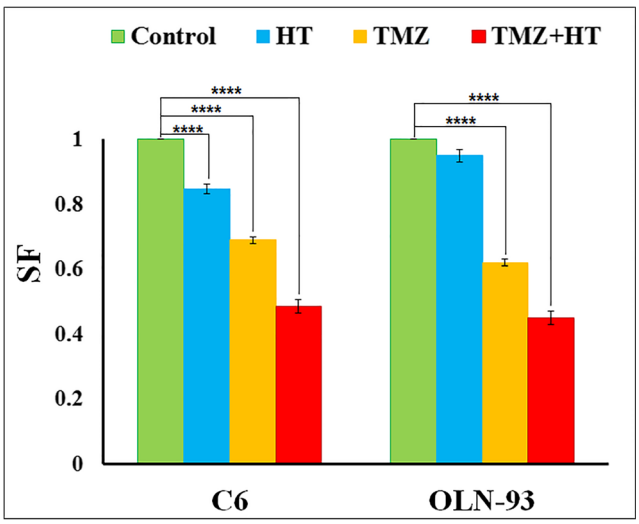

Fig. 3 The SF values of C6 and OLN-93 cell lines treated with HT, TMZ, and HT + TMZ using the colony formation assay (mean ± SD, $n = 3$). Statistical significance was showed with $^{****}P < 0.0001$

In addition, the reduction in SF value of C6 cells caused by TMZ, HT, and HT + TMZ treatments were significant compared to the control cells ($P < 0.05$). Moreover, the results of colony formation assay showed that HT + TMZ treatment group had the highest cytotoxicity on both cell lines.

Closed aperture Z-scan technique

The responses of C6 and OLN-93 cells to various treatments were also evaluated using closed aperture Z-scan method. Diagrams of the normalized transmittance versus *Z*-axis for the C6 and OLN-93 cells after various treatments have been shown in Fig. 4. As shown in Fig. 4a, the control group of C6 cells had the negative nonlinear refractive index. Whereas, the C6 cells treated with HT, TMZ, and HT + TMZ had the positive nonlinear refractive index. In addition, the combined chemo-hyperthermia treatment group (HT + TMZ) had the maximum amount of n_2 index among the samples with positive nonlinear refractive index.

Figure 4b shows the nonlinear optical behavior of OLN-93 cells in different groups. The control and HT groups had the positive nonlinear refractive index and the amount of n_2 in HT group was lower than the control group. The nonlinear refractive index of OLN-93 cells treated with TMZ and HT + TMZ was negative and the magnitude of n_2 index in combined treatment group (HT + TMZ) was higher than that of TMZ alone group.

The nonlinear refractive index of both cell lines after various treatments has been summarized in Table 1. Also, calculated values of n_2 index of all samples were depicted as a chart bar in Fig. 5. As it can be seen in Fig. 5 and Table 1, after changing the sign of n_2 index in both cell lines, the maximum value of n_2 was related to HT + TMZ group, which indicated that combined treatment had the highest cytotoxic effect on both cell lines. The results of Z-scan method were in complete agreement with the results of colony formation assay.

Discussion

Nowadays, there are several in vitro techniques to identify the effects of different treatments on the cells, but each of them has limitations. In this study, Z-scan technique was introduced as a quick and accurate method for detecting cellular damages. In recent years, Z-scan method was used to determine the concentration of biomaterials in the body [29–32], diagnosis of normal cells from cancer cells [33, 34], and differentiation of different type of cells [21]. In the present study, the proposed method was used to determine cellular damages after chemotherapy, RF-hyperthermia, and chemo-hyperthermia

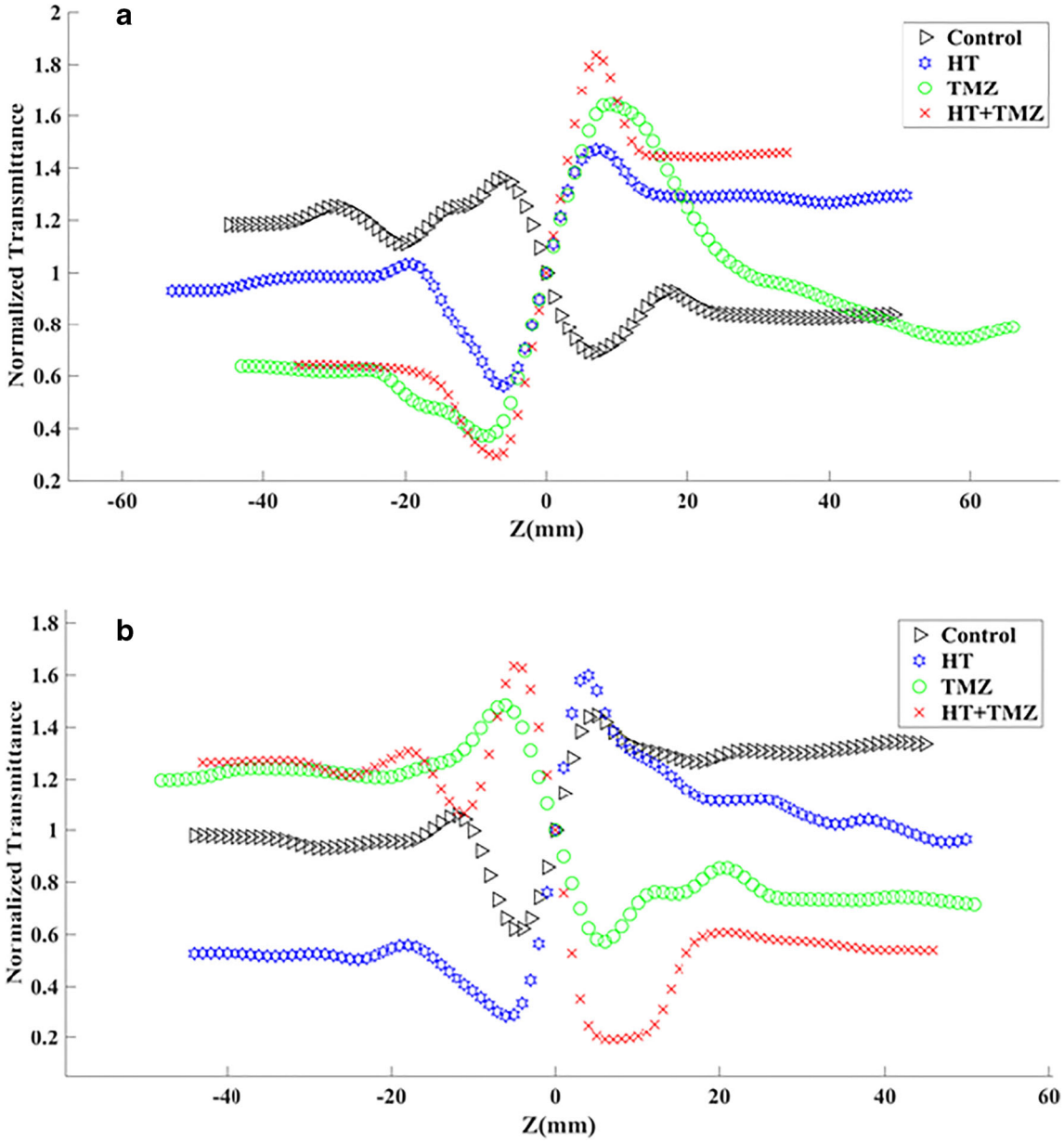

Fig. 4 Closed aperture Z-scan normalized data for **a** C6 glioblastoma and **b** OLN-93 glial cell lines for different treatments. Normalized transmittance represents transmittance power changes through the aperture according to the sample position and was measured by detector

Table 1 Values of non-linear refractive index of C6 and OLN-93 cell lines after various treatments

Samples	Nonlinear refractive index ($n_2 \times 10^{-7}$ cm^2/w ± error)	
	C6	OLN
Control	− 5.44 ± 0.33	+ 6.07 ± 0.36
HT	+ 1.5 ± 0.09	+ 3.24 ± 0.19
TMZ	+ 6.31 ± 0.38	− 8.31 ± 0.5
HT + TMZ	+ 11.41 ± 0.68	− 12.69 ± 0.76

treatments. After that, its results were compared with the results of colony formation assay.

As shown in Fig. 3, the colony formation assay indicated that the treatment of cells with TMZ reduced the proliferation ability of both cell lines compared to untreated cells ($P < 0.0001$), which was in agreement with the results of other study [35]. TMZ is an alkylating chemotherapeutic agent and causes cell death by methylating O6 position of guanine in DNA [36]. The results of Fig. 3 demonstrated that RF-hyperthermia at the average temperature of 43 °C for 30 min inhibited the proliferation rate of C6 glioblastoma cancer cells ($P < 0.0001$). The mechanism of cell death in hyperthermia are denaturation of proteins, alteration in the cellular structure

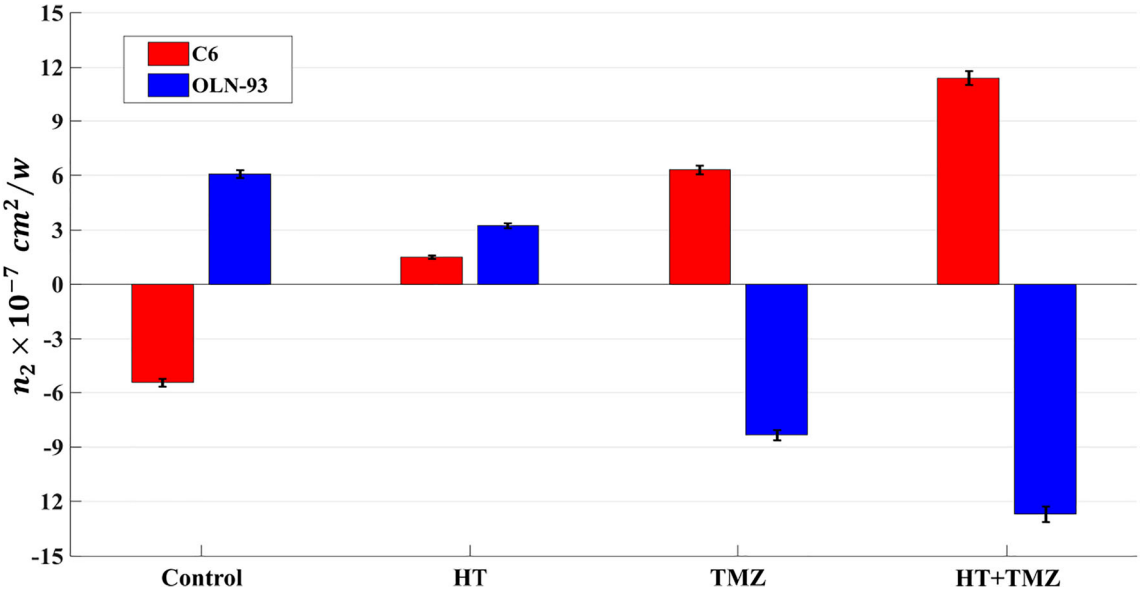

Fig. 5 Values of non-linear refractive index of C6 and OLN-93 cell lines after various treatments (*HT* radiofrequency hyperthermia, *TMZ* temozolomide, *HT + TMZ* radiofrequency hyperthermia + Temozolomide)

such as cytoskeleton and plasma membrane, changes in the function of enzymes and structural proteins, and induction of apoptosis [37, 38].

Interestingly, our results showed that RF-hyperthermia had no significant effect on the OLN-93 normal cells ($P > 0.05$). Zamora-Mora et al. [39] also demonstrated that human glioblastoma cancer cells (A-172) were more sensitive to the magnetic RF-hyperthermia compared to the normal fibroblast cells (FBH). In their study, magnetic RF-HT treatment significantly reduced the viability of A-172 cancer cells, but had no significant effect on the viability of FBH normal cells.

As it can be seen in Fig. 3, the inhibition of cell proliferation could have been further promoted by the combination chemo-hyperthermia treatment. Hyperthermia could cause more cytotoxic effect when combined with TMZ. It can be due to (1) increased cell membrane permeability to the chemotherapy drug, (2) increasing the reaction rate of TMZ, and (3) inhibition of DNA repair system [7, 40, 41]. Minaei et al. [42] showed that the combination of RF-hyperthermia (13.56 MHz, 80 W, 25 min) and TMZ (21.7 μg/mL) significantly reduced the value of plating efficiency and enhanced the apoptosis rate of cells compared to each one treatment alone.

As stated, hyperthermia, TMZ, and HT + TMZ cause cell death and cellular damages through multiple factors such as DNA methylation, denaturation of proteins, changes in cellular structure, alteration in the function of enzymes, induction of apoptosis, etc. These structural, molecular, and biochemical alterations can change the nonlinear optical behavior of cells, pattern of the optical diffraction, the gradient of transmittance curve, and consequently the sign and magnitude of the nonlinear refractive index. Therefore, change in the sign and magnitude of refractive index can be used as an indicator of cell damage.

The results of Figs. 4 and 5 and Table 1 showed that the untreated C6 cell line had a negative nonlinear refractive index with a magnitude equal to − 5.44 ± 0.33. After applying different treatments of hyperthermia, TMZ, and HT + TMZ, the sign of n_2 index changed and its magnitude increased to + 1.5 ± 0.09, + 6.31 ± 0.38, and + 11.41 ± 0.68, respectively. Also, in the clonogenic assay (Fig. 3), the SF value of cells after these treatments decreased to 0.84 ± 0.015, 0.69 ± 0.01, and 0.48 ± 0.02, respectively and were statistically significant compared to the control group ($P < 0.0001$).

Furthermore, the untreated OLN-93 cell line had a positive nonlinear refractive index with a magnitude of + 6.07 ± 0.36. Treatment of cells with TMZ and HT + TMZ changed the sign of it and its magnitude increased to − 8.31 ± 0.5 and − 12.69 ± 0.76, respectively. The SF values also decreased to 0.62 ± 0.01 and 0.45 ± 0.15, respectively, which was significant in comparison to the control group ($P < 0.0001$). The sign of n_2 index did not change in the HT group. Also, in the colony formation assay, there was no significant difference between the hyperthermia and control groups ($P > 0.05$).

Treatments that have caused significant cellular damages based on the results of colony formation assay have also led to a change in the sign of nonlinear refractive index in the Z-scan method. Moreover, with increasing the cytotoxic effects of different treatments, the SF value of cells decreased and the magnitude of nonlinear refractive index increased.

In this regard, the results of Z-scan technique were completely in line with the results of colony assay. Therefore, this study indicated that investigation of nonlinear

optical behavior of cells is a useful method for detecting the amount of cell damage caused by different treatments.

The results of study by Gader et al. [43] also showed that in the CT-26 cell line, by increasing the time of hyperthermia, the magnitude of nonlinear refractive index increases (after changing the sign of n_2 index). Our study was performed on two cell lines (C6 cancer cells and OLN-93 normal glial cells) and demonstrated that Z-scan technique could also detect the cytotoxic effects of various treatments such as radiofrequency hyperthermia and chemotherapy. In addition, it could even detect the increased damage caused by combination therapy (chemo-hyperthermia).

Given that the Z-scan method has several advantages, such as high precision, repeatability, speed of operation, and inexpensive. By conducting further research in the future, this method can be introduced as an appropriate technique for the evaluation of cellular damages.

Conclusion

In conclusion, the closed aperture Z-scan technique could distinguish the cytotoxic effects of various treatments on cells by examining the nonlinear optical properties of the samples and the nonlinear refractive index. Moreover, this method could identify the increased cellular damages caused by combination therapy.

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Compliance with ethical standards

Conflict of interest The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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