Acute toxic effects of sonodynamic therapy on hypertrophic scar fibroblasts of rabbit ears

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ABSTRACT. The objective of this study was to observe the acute cytotoxic effects of hematoporphyrin monomethyl ether sonodynamic therapy (HMME-SDT) on hypertrophic scar fibroblasts of rabbit ears. We first assessed the effects of different irradiation times and HMME concentrations on the survival of hypertrophic scar fibroblasts using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay to determine the optimum irradiation time and HMME concentration. The hypertrophic scar fibroblast cell suspensions of the rabbit ears were divided into four groups, the survival rates were detected using the MTT assay, and the type of cell death was detected by Annexin V/propidium iodide (PI) double staining flow cytometry. Our results showed that HMME-SDT significantly reduced the viability of hypertrophic scar fibroblasts of rabbit ears at ultrasonic irradiation times of 30, 60, and 90 s, but not 10 s ($P < 0.05$). HMME alone had no significant effect on the cell survival rate at any irradiation time ($P > 0.05$). In contrast, the cell survival rate was significantly decreased at an irradiation time of 10 s and HMME concentrations of 20 and 50 μg/mL ($P < 0.05$). Furthermore, Annexin V/PI double staining showed both

necrosis and apoptosis of the hypertrophic scar fibroblasts. Given our results, HMME might be an effective sound-sensitive agent for SDT as it has a significant lethal effect on hypertrophic scar fibroblasts of rabbit ear cultured *in vitro*. HMME-SDT may therefore provide a new method for the treatment of hypertrophic scar formation.

Key words: Sonodynamic therapy; Hypertrophic scar; Necrosis; Hematoporphyrin monomethyl ether; Apoptosis

INTRODUCTION

Sonodynamic therapy (SDT), also called sonodynamic chemistry therapy, consists of the intravenous injection of a specific dose of a sound-sensitive agent, or sonosensitizer, in patients followed by ultrasonic irradiation at a specific frequency and duration. This results in the accumulation of the sonosensitizer at the lesion site, which produces reactive oxygen molecules that attack and inhibit the growth of the diseased cells (Lv et al., 2012). The sonosensitizer and ultrasound are two important components of SDT. The discovery of novel sonosensitizers is an important focus of SDT research. Several sonosensitizer s are derived from photosensitizers, such as porphyrin complexes and porphyrin sources (e.g., hematoporphyrin, Photofrin, ATX-70, 5-ALA, quinolone, and HPD). Hematoporphyrin monomethyl ether (HMME) is a second-generation photosensitizer indigenously developed in China, which has the advantages of being a single component with stable performance, lower light-sensitivity, and an improved safety profile. However, so far no correlation studies have been conducted to determine its suitability as a sonosensitizer and its behavior in SDT (Dai et al., 2011). The current study investigated the sound sensitization and kinetic effects of HMME on hypertrophic scar fibroblasts of rabbit ear, which may provide a research model for the testing the feasibility of hypertrophic scar treatments.

MATERIAL AND METHODS

Experimental animals

Thirty-six rabbits with body weights ranging from 1.8 to 2.4 kg were purchased from the experimental animal center of Harbin Medical University, Harbin, China. The experiments were performed under license No. 2011-7812 and under clean conditions. HMME was developed by the Second Military Medical University, Harbin, China, with a specific concentration of 10 mg/mL and batch number 111209. The rabbits were randomly divided into two groups: a normal control and a scar model group. The animals were acclimatized one week before the experiment at a temperature of $23 \pm 2°C$ and a relative humidity of $40 \pm 10\%$. Water and solid feed was available *ad libitum*. Both groups were fed under the same conditions and environment throughout the experiment.

Establishment of the rabbit ear hypertrophic scar model

We established a rabbit ear hypertrophic scar model according to a modified version of a previously established method (Morris et al., 1997). The rabbits received anti-infective

treatment after surgery and had access to food and water *ad libitum*. At day 21 post-surgery, the wound was found to be healing and was clearly protruding from the surface of the skin, which is indicative of hypertrophic scar formation.

Figure 1. Molding of the hypertrophic scar

Figure 2. Hypertrophic scar model

Cell recovery and cell growth

After dissecting the hypertrophic scar tissue of the rabbit ears and recovering normal skin tissue cultured *in vitro* for 4 h the specimens were rinsed several times with NS. The epithelial tissue sections were subsequently removed, cut into 2 mm x 2 mm pieces, placed in a sterile flask, and spread onto the flask's surface at a certain density by adding a small amount of medium to the flask. The tissue was cultured at the bottom of the flask and the cells were in

the logarithmic growth phase after treatment and 3-4 subculture cycles. The fifth generation of cells was used in this study at which point the cell fusion was 70 to 80%.

Effects of HMME sonodynamic therapy on the survival of hypertrophic scar fibroblasts of rabbit ears

Cells that were in the logarithmic growth phase were digested and centrifuged, and a single cell suspension was prepared, adjusting the cell concentration to 5×10^5/mL. All experiments were performed using 3 mL of this cell suspension. According to the results of previous experiments, the following experimental groups (N = 3 mL cell suspensions) were used: a control, HMME, ultrasound alone, and acoustic power group. The ultrasonic irradiation conditions were 0.50 W/cm^2 for 10 s with 20 or 50μg/mL HMME. The appropriate ultrasonic irradiation time (10 s) and HMME concentration were selected from the results of these experiments. The experiment was divided into two groups that received either 20 or 50 μg/mL HMME and each group was further divided into four groups: a control, ultrasound alone, HMME alone, and SDT-HMME group. After ultrasound irradiation, the cell suspensions were transferred to 96-wells plates (200 μL cell suspension/well), followed by the immediate addition of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; 5 mg/mL, 20 μL/well). The cells were cultured for 4 h and the supernatant was aspirated after centrifugation. Following the addition of dimethyl sulfoxide (DMSO; 200 μL/well) and an oscillation time of 10 min, the optical density (OD) value of each well was measured with an automatic microplate reader set at 490 nm. The experiment was repeated 3 times.

Detection of the efficacy of HMME against hypertrophic scar fibroblasts of rabbit ear using the MTT assay

Cells cultured as monolayer cultures and that were in the logarithmic growth phase were digested with enzymes and prepared as a single cell suspension. The cell suspensions were transferred to 96-wells plates (200 μL cell suspension/well) and cultured for 24 h, after which HMME was added to the wells at a final concentration of 0, 10, 20, 40, and 50 μg/mL. Three wells were allocated to each treatment group and the cells where cultured for 48 h. MTT (5 mg/mL; 20 μL/well) was added before the end of the culture period. Cells were kept in culture while shaking and the OD value of each well was measured with an automatic microplate reader set at 490 nm. The OD value of each well/group was compared with that of the control wells and calculated as the percentage of viable cells. The experiment was repeated 3 times.

Assessment of the cell death model of hypertrophic scar fibroblasts of rabbit ears using Annexin-V/propidium iodide flow cytometry

Hypertrophic scar fibroblasts of rabbit ear that were in the logarithmic growth phase were digested with commonly used enzymes, centrifuged, and subsequently prepared as a single cell suspension at 1×10^6 cells/mL. The experiments were divided into four groups that were treated with 20 μg/mL HMME and underwent ultrasonic irradiation of 0.50 W x cm^2 for 10 s. After ultrasound irradiation, the cells of all four groups were transferred to 50 mL culture flasks for culturing. Annexin-V/propidium iodide (PI) flow cytometry was used as detection method.

Statistical analysis

All data are reported as means ± SD and analyzed using ANOVA followed by the Student-Newman-Keuls post-hoc test (SPSS software, version 14.0, SPSS Inc., Chicago, IL, USA). P values <0.05 were considered to be statistically significant.

RESULTS

General observations

After wound formation on the ventral surface of the rabbit ear, the wound was crusted and crusted off gradually. Wound healing was concluded at day 12-14 at which point the scleroma could be observed. After a tissue remodeling period of 20-22 days, the induration was more obvious, the thickness of the scar (measured with a vernier caliper) was about 2 times that of the ventral skin side of the rabbit ear, and the observed color and quality of the scar was out of the face and pale red. The extent of hyperplasia was not more than that prior to wound surgery. The scars in the model group reached their peak thickness six weeks after epithelialization of the hypertrophic scar with a thickness of about 3 times more than that of the ventral skin side of the rabbit ear (Figures 1-3).

Figure 3. Scar model 6 weeks after epithelialization.

Cell growth

The hypertrophic scar fibroblast specimens were cultured for 5 days after which cell migration and adherent growth could be observed. The cells were spindle-shaped, transparent, had a full cell body, and had long flat plates, which were arranged in parallel and with a certain radian. Furthermore, round or oval nuclei were found in the center of the cells and two or three swellings were seen around the cells (Figures 4-9).
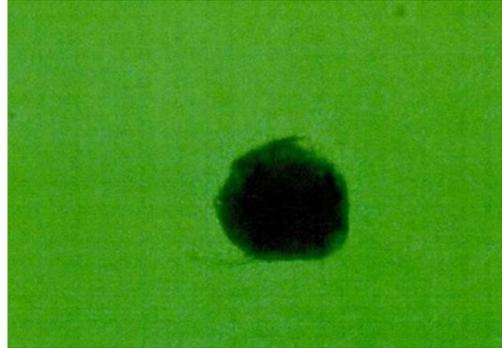
Figure 4. Crawling of the cells out of the tissue after 5 days of primary culture (100X magnification).

Figure 5. Crawling of the cells out of the tissue after 7 days primary culture seven days (100X magnification).
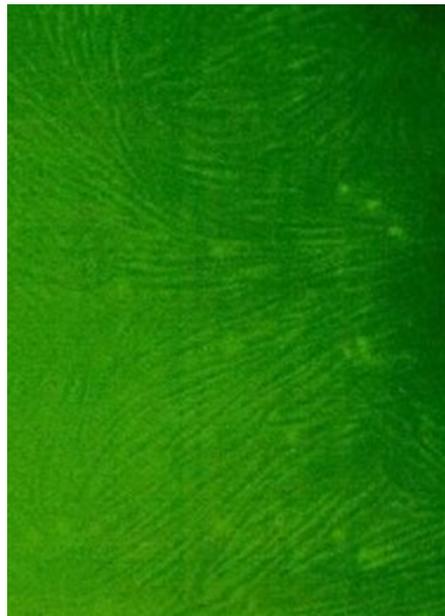

Figure 6. Cells arrayed on the bottom of the culture flask 8-10 days after primary culture (100X magnification).

Figure 7. Cells arrayed in monolayers before generations (100X magnification).

Figure 8. Healthy cell growth on the second day of subculture (100X magnification).

Figure 9. Cells covering the bottom of the culture flask after 7 days of subculture (100X magnification).

Effects of different ultrasonic irradiation times on the survival of hypertrophic scar fibroblasts of rabbit ears

As shown in Table 1, the cell survival rates expressed as a percentage of the control were 95.12, 74.32, 58.37, and 42.86% at an irradiation strength of 0.5 W/cm² and irradiation times of 10, 30, 60, and 90 s, respectively. The cell survival rate was significant with increasing irradiation times, which indicated a certain dose-effect relationship between the cell survival rate and the ultrasound irradiation time under these experimental conditions.

Table 1. Effects of different acoustic power exposure times on the survival of hypertrophic scar fibroblasts of rabbit ears.

Time (s)	Optical density value (OD)	Survival rate (%)
0	1.7123 ± 0.0658	100
10	1.632 ± 0.2147	95.12
30	1.3781 ± 0.2064	74.32
60	0.9634 ± 0.0912	58.37
90	0.793 ± 0.0534	42.86

$P < 0.01$, 0 *vs* 30, 60, and 90 s; $P > 0.05$, 0 *vs* 10 s.

Effects of HMME on the proliferation of hypertrophic scar fibroblasts

No significant difference in OD values were found between the groups treated with different HMME concentrations ($P > 0.05$), indicating that only HMME had no significant effect on the survival rate of hypertrophic scar fibroblasts (data not shown).

Acute lethal effect of HMME in combination with sonodynamic therapy on hypertrophic scar fibroblasts

HMME-SDT had a clear lethal effect on hypertrophic scar fibroblasts; both the OD, which reflects the cell survival rate, was decreased in the HMME-SDT group compared with those of the 3 other groups. (Tables 2 and 3). The highest effect of acoustic power was observed in combination with 20 µg/mL HMME ($P < 0.05$), which suggested that HMME had a strong acoustic sensitization effect.

Table 2. Acute lethal effect of HMME-SDT (20 µg/mL) on hypertrophic scar fibroblasts.

Groups	Optical density value (OD)	Survival rate(%)
Control	3.2371 ± 0.2531	100
Ultrasound (10 s)	2.815 ± 0.164	96.1
HMME (20 µg/mL)	2.642 ± 0.287	94.8
HMME-SDT (20 µg/mL)	1.363 ± 0.298	40.1

$P < 0.01$, the HMME-SDT *vs* the control, ultrasound, and HMME groups.

Table 3. Acute lethal effect of HMME-SDT (50 µg/mL) on hypertrophic scar fibroblasts.

Groups	Optical density value (OD)	Survival rate (%)
Control	3.1943 ± 0.2647	100
Ultrasound (10 s)	2.997 ± 0.164	95.1
HMME (50 µg/mL)	2.875 ± 0.113	93.2
HMME-SDT (50 µg/mL)	1.687 ± 0.2076	48.4

$P < 0.01$, the HMME-SDT *vs* the control, ultrasound, and HMME groups.

Determination of the type of cell death using Annexin V/PI flow cytometry

The percentage of necrotic cells, early apoptotic cells, cell debris, and survival of the cells in the HMME-SDT group were 21.21 ± 1.32, 20.53 ± 0.65, 6.19 ± 2.56, and 50.98 ± 2.34%, respectively, at an HMME concentration of 20 µg/mL as determined by flow cytometry (Table 4). These results indicated the presence of two types of cell death induced by HMME-SDT, i.e., apoptosis and necrosis (Figure 10-13).

Table 4. Effect of HMME-SDT on the type of cell death in hypertrophic scar fibroblasts.

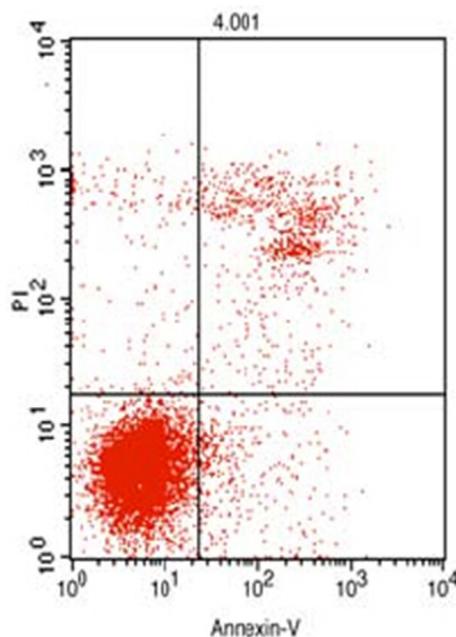| SDT group	Percentage of cells (%)
Cell debris	6.19 ± 2.698
Early apoptosis	23.12 ± 1.79
Late apoptotic and necrotic cells	22.09 ± 2.78
Cell survival	48.96 ± 6.98

Figure 10. Type of cell death of hypertrophic scar fibroblasts treated with HMME-SDT as determined by flow cytometry after double staining with Annexin V/PI (control group).
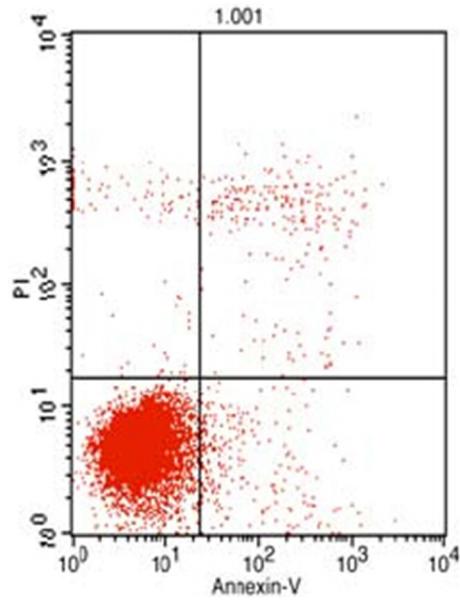
Figure 11. Type of cell death of hypertrophic scar fibroblasts treated with HMME-SDT as determined by flow cytometry after double staining with Annexin V/PI (HMME alone group).
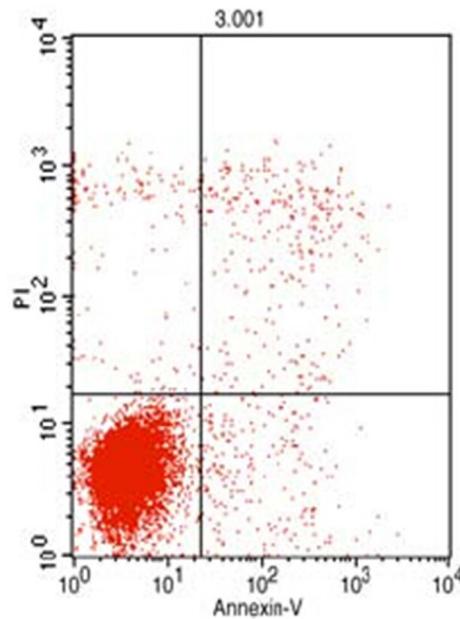

Figure 12. Type of cell death of hypertrophic scar fibroblasts treated with HMME-SDT as determined by flow cytometry after double staining with Annexin V/PI (ultrasound group).
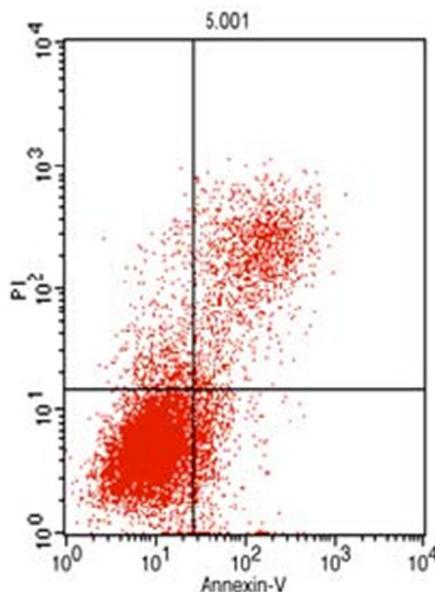
Figure 13. Type of cell death of hypertrophic scar fibroblasts treated with HMME-SDT as determined by flow cytometry after double staining with Annexin V/PI (acoustic power group).

DISCUSSION

Hypertrophic scar formation is a type of fibrous tissue hyperplasia caused by excessive wound healing after skin and tissue injury. Currently, hypertrophic scar formation and clinical research are focusing on this pathology, and clinical treatment result is not satisfactory (Liu et al., 2011). Researchers worldwide have found that SDT can induce apoptosis and inhibit cell proliferation; the underlying mechanisms and whether it is effective in the prevention of hypertrophic scar formation are, however, still unknown (Ma et al., 2011). In our previous study (Li et al., 2014) we that fibroblast proliferation may play an important role in the pathogenesis of hypertrophic scar formation. SDT is a novel therapeutic method that is increasingly used in recent years. Because it focuses the ultrasonic energy mainly on specific parts of a tumor, which activates the accumulation of hematoporphyrin in the tumor with a long retention time, thereby decreasing damage to the surrounding healthy tissue, it has broad application prospects. However, to the best of our knowledge, no research to the effects of SDT on hypertrophic scar formation has been published yet. Similarly, no reports have been published on the use of HMME as a sonosensitizer. Therefore, we applied HMME as a sonosensitizer to investigate whether its combination with SDT could have significant lethal effects on hypertrophic scar fibroblasts of the rabbit ear *in vitro* and whether it may provide a new approach for the treatment of hypertrophic scar formation.

We applied different concentrations of HMME on hypertrophic scar fibroblasts of rabbit ear and found that HMME did not inhibit the fibroblasts' survival. Taking into account that ultrasound treatment of tumor cells could inevitably damage the surrounding healthy cells, we performed the acoustic power experiments with an intensity of $0.5 W/cm^2$ for 10 s based on our initial trial results. Similarly, according to the research using HMME photodynamic therapy,

we used two HMME concentrations (20 and 50 μg/mL) to investigate potential differences in the effect of SDT on hypertrophic scar fibroblasts and found that the survival rate of hypertrophic scar fibroblasts was significantly decreased in the HMME acoustic power group. In contrast, that of the hypertrophic scar fibroblasts in the HMME alone group was not changed. The fibroblasts' survival rate in the HMME alone group was, however, lower than that of those in the ultrasound alone group, which indicated that a certain level of ultrasound could activate the effects of HMME on the hypertrophic scar fibroblasts. Therefore, it may be suggested that HMME might be an effective sonosensitizer .

Our study found that the lethal effect of 20 μg/mL HMME-SDT on hypertrophic scar fibroblasts was higher than that of 50 μg/mL HMME-SDT (cell survival rates of 40.1 and 48.4%, respectively), which may be related to a concentration saturation effect of HMME at these concentrations. Flow cytometry analysis showed that cell death due to both apoptosis and necrosis was present in the HMME acoustic power group.

In summary, our results showed that HMME could be an effective sonosensitizer as HMME-SDT had an acute toxic effect on hypertrophic scar fibroblasts, causing necrosis, apoptosis, and a decreased cell survival rate.

ACKNOWLEDGMENTS

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