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Increasing the Efficiency of Photodynamic Therapy by Improved Light Delivery and Oxygen Supply Using an Anticoagulant in a Solid Tumor Model

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Background and Objective: The main factors in photodynamic therapy (PDT) are: photosensitizer retention, photon absorption, and oxygen supply. Each factor has its unique set of problems that poses limitation to the treatment. Both light delivery and oxygen supply are significant bottlenecks in PDT. Vascular closure during PDT reduces oxygen supply to the targeted tissue. On the other hand, with the changes in blood perfusion, the tissue optical properties change, and result in variation in irradiation light transmission. For these reasons, it becomes very important to avoid blood coagulation and vascular closure during PDT.

Study Design/Materials and Methods: The efficiency of PDT combined with the anticoagulant heparin was studied in a BALB/c mouse model with subcutaneous EMT6 mammary carcinomas. Mice were randomized into three groups: control, PDT-only, and PDT with heparin. The photosensitizer Photofrin® was used in our experiments. Light transmission, blood perfusion, and local production of reactive oxygen species (ROS) were monitored during the treatment. The corresponding histological examinations were performed to determine the thrombosis immediately after irradiation and to evaluate tumor necrosis 48 hours after the treatment.

Results: The results clearly demonstrated that PDT combined with pre-administered heparin can significantly reduce thrombosis during light irradiation. The blood perfusion, oxygen supply, and light delivery are all improved. Improved tumor responses in the combined therapy, as shown with the histological examination and tumor growth assay, are clearly demonstrated and related to an increased local ROS production.

Conclusion: Transitory anticoagulation treatment significantly enhances the antitumor effect of PDT. It is mainly due to the improvement of the light delivery and oxygen supply in tumor, and ultimately the amount of ROS produced during PDT. Lasers Surg. Med. 42:671–679, 2010. © 2010 Wiley-Liss, Inc.

Key words: blood coagulation; PDT; Photofrin; ROS; thrombus

INTRODUCTION

Photodynamic therapy (PDT) is used to treat malignant and non-malignant diseases [1]. A photosensitizer is pre-administered, and the target tissue is exposed to light irradiation with a suitable wavelength. Cytotoxic reactive oxygen species (ROS) is generated in the oxygen-mediated photochemical reactions. PDT causes target tissue destruction by either directly killing the cells or by damaging the local vasculature, and may even involve certain immune responses when used to treat tumors [2]. PDT-induced vascular damage can lead to tumor hypoxia and then induce the transcriptional activation of vascular endothelial growth factor (VEGF) via the hypoxia-inducible factor 1α (HIF-1α) pathway causing neovascularization [3–8]. It is one of the reasons for tumor recurrence.

Hence, the time course of vascular closure is crucial [9]. The collapse of the tumor blood vascular system during PDT may increase tissue hypoxia, and thus be a barrier to effective treatment. Otherwise, obstruction of blood vessels after PDT is beneficial for killing tumor cells by anoxia. A solid tumor is often hypoxic as a result of the cells' fast proliferation and imperfect vascular system. In addition to the naturally occurring tumor tissue hypoxia, a PDT treatment consumes local oxygen by itself [10]. Without the presence of molecular oxygen, a typical PDT treatment will have literally no effect on its target. So it is imperative to supply oxygen during PDT. It has been reported that manipulation of tumor oxygenation during PDT can improve tumor response [11]. In addition, carbogen breathing significantly enhances the penetration of red light

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because of improved tumor oxygenation [12]. We proposed that delayed blood coagulation and vascular closure during PDT can enhance the treatment efficiency by improving the blood perfusion and light transmission.

In the present study, in order to temporarily prevent vessels from shutting down to enhance the oxygen supply and light delivery, heparin was combined with PDT. Heparin is a clinically used drug for preventing thrombosis and disseminated intravascular coagulation. Photofrin, a well-established clinical photosensitizer, was employed in the current study due to its known vascular effects and a significant light absorption at its active wavelength. An EMT6 mammary carcinoma model in BALB/c mice was used to investigate tumor response in the combined therapy with heparin. The light transmission and blood perfusion and ultimately the ROS production were measured during the treatment. The enhanced results of the combined therapy were also studied by histological examination and tumor growth assay.

MATERIALS AND METHODS

Reagents

Photofrin® (Porfimer sodium, Axcan Pharma Ltd, Dublin, Ireland) was dissolved in distilled water at a concentration of 500 μg/ml and stored at 4°C in dark until needed. The anticoagulant agent heparin 12,500 U, 2 ml was purchased from The Third Affiliated Hospital of Sun Yat-sen University, China. Fluorescenyl cypridina luciferin analog (FCLA, Tokyo Kasei Kogyo Co., Tokyo, Japan) was dissolved in double-distilled water at a concentration of 100 μM and stored at −80°C until needed. Five percent 2,3,5-triphenyltetrazolium chloride (TTC, Sigma Aldrich, St. Louis, MO) in phosphate-buffered saline (PBS) was stored at 4°C in the dark. Sodium pentobarbital (SERVA Ltd, Heidelberg, Germany) was dissolved in distilled water at 2% (w/v) and stored at 4°C.

Cell Culture and Animal Models

Mouse mammary carcinoma EMT6 cells, purchased from Fourth Military Medical University Experimental Centre, were grown in RPMI-1640 culture media under 5% CO_2 at 37°C. Cells were detached from the substrate using 0.5% trypsin in PBS, centrifuged, and then resuspended in PBS at a concentration of 1×10^7 cells/ml.

Female BALB/c mice (Center of Experimental Animal, Sun Yat-sen University, Guangzhou, China) were housed in an environmentally controlled animal facility with regular 12/12 cycle. Hair on the upper backs of the mice was removed with a chemical depilatory agent (Na_2S 8% aqua-solution), then each mouse was injected with a 200 μl cell suspension solution in the sub-dermal dorsal area.

PDT Treatment and Transmission Light Measurement

The mice were treated with PDT when the tumor diameter was about 5–9 mm. Photofrin was administered via tail vein at 5 mg/kg, 12 hours prior to the PDT laser irradiation. Mice were randomized into three groups: control, PDT (only), PDT combined with the anticoagulant heparin. The irradiation protocols were 60 J/cm^2 at 20 mW/cm^2, 60 J/cm^2 at 60 mW/cm^2, 100 J/cm^2 at 100 mW/cm^2, and a high dose, 216 J/cm^2 at 60 mW/cm^2. The irradiation light source was a semiconductor laser (635 nm, 2 W, model NL-FBA-2.0-635, nLight Photonics Corporation, Vancouver, WA). The laser output was coupled into an optical fiber (core diameter 400 μm) with a custom distal microlens for an even 3 cm^2 irradiation area that covered the perspective tumors. Heparin (50 U) was administered via tail vein 1 hour before illumination for each mouse in the PDT with heparin group. All the mice were injected with 100 μl 2% sodium pentobarbital 10 minutes before laser irradiation for anesthesia. After the treatment, the tumor dimensions were measured with a caliper two or three times a week. The tumor volume was calculated with a formula (tumor volume = diameter×width^2×3.14/6) [7].

For light transmission measurement, a mouse was positioned on a platform. The light was incident from one side of the tumor via an optical fiber positioned horizontally to illuminate an area of approximately 10 mm diameter on the tumor. The transmitted light was collected with an optical fiber positioned coaxially on the opposite side of the tumor, and detected with a laser power meter (FieldMate, Coherent, Inc., Santa Clara, CA). To minimize animal movement during each measurement, an EMT6 tumor was raised and positioned with a custom-built apparatus on a micromanipulator. By carefully setting up the tumor position and allowing the optical fiber probe to gently touch the surface of the tumor, there was little external interference to the tissue vasculature. The light treatment protocols were 60 J/cm^2 (60 mW/cm^2) and 100 J/cm^2 (100 mW/cm^2). As a control, light transmission was also performed in skin with a dose of 100 J/cm^2 (100 mW/cm^2). The output signal of the power meter was collected by an A/D converting signal acquisition card (NI5102, National Instruments Corporation, Austin, TX) which was controlled with a 10 Hz pulse sequence generated by a function generator (AFG3120, Tektronix, Inc., Beaverton, OR). The schematic is shown in Figure 1A. Each time point was averaged from 100 samplings. For a clearer presentation, only 11 data points, with a 100 seconds interval in between are shown.

Histology

Three mice in each group irradiated with 216 J/cm^2 (60 mW/cm^2), with or without heparin, were sacrificed immediately after PDT. Tumor specimens were harvested and fixed in Bouin's solution for 12 hours. They were dehydrated, cleaned, and then embedded in paraffin, and finally sectioned to 4 μm thick perpendicular to the irradiation light axis. The sections were stained with hematoxylin and eosin (H&E), inspected and photographed under an optical microscope (ECLIPSE 80i, Nikon, Tokyo, Japan). Vessels containing thrombi were identified under high power (20×) magnification. In order to prove the inhibitive effect of heparin, the counting of thrombi in the sections in PDT-only and PDT with heparin groups was performed by direct observation through the microscope

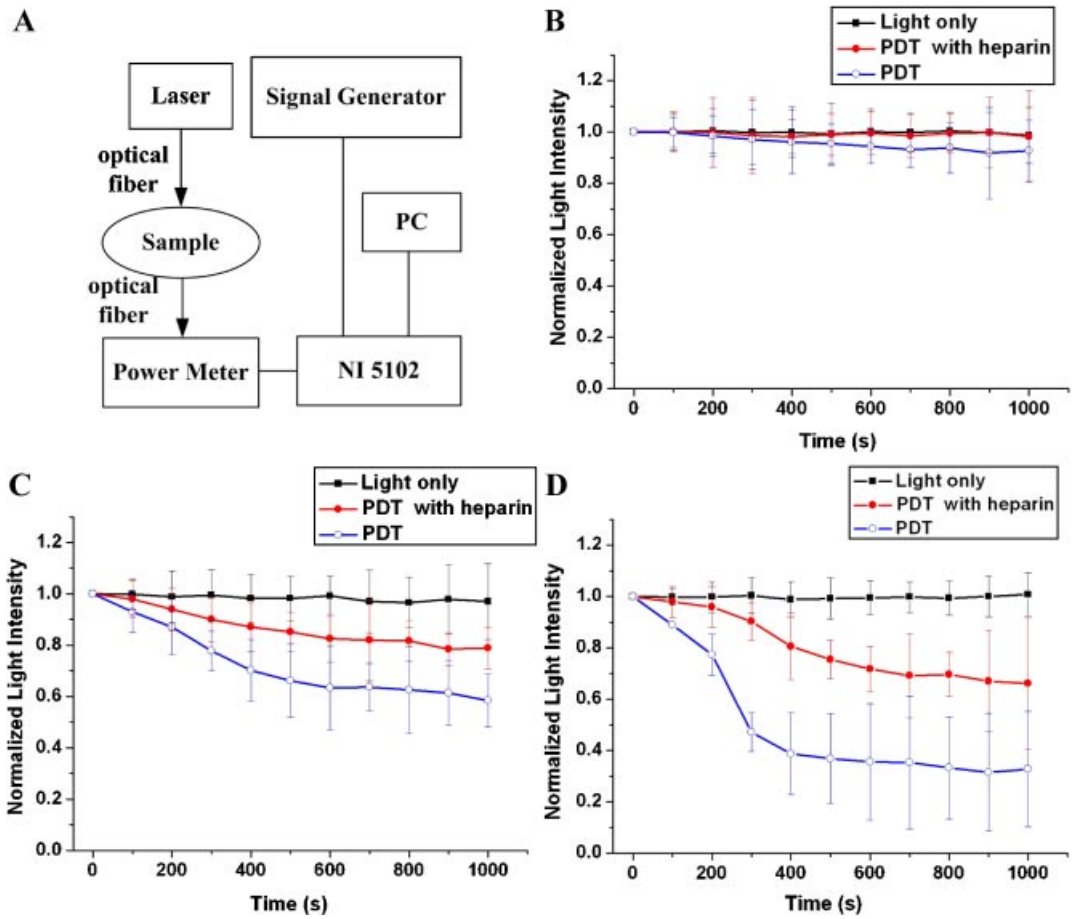

Fig. 1. Light transmission intensity in the EMT6 tumor during PDT and the effect of heparin. **A**: The schematic of the experimental system for light transmission measurements. **B**: Normalized light transmission intensity of skin was measured in control group (■, $n = 5$, light only), PDT group (○, $n = 5$), PDT with heparin group (●, $n = 5$), respectively. The irradiation protocol was 100 J/cm^2 at 100 mW/cm^2. **C**,**D**: Normalized light transmission intensity of tumor was measured in control group (■, $n = 5$, light only), PDT-only group (○, $n = 5$), and PDT with heparin group (●, $n = 5$), respectively. The irradiation protocols were 60 J/cm^2 at 60 mW/cm^2 (C) and 100 J/cm^2 at 100 mW/cm^2 (D). Statistical analysis reveals significant differences among the PDT-only, PDT with heparin and the control groups (C and D, $P<0.05$, repeated measures ANOVA tests).

(10×, DM5000B, Leica, Wetzlar, Germany). The quantitative analysis was performed by counting the total number of thrombi from five random visual fields of the microscope on each tumor section.

Additional mice were treated with the same irradiation dosage (216 J/cm^2, 60 mW/cm^2), and sacrificed 48 hours after PDT, with or without heparin treatment ($n = 4$/group). The tumor tissue was harvested for measuring the area of tissue necrosis. Specimens were immediately incubated in 5% TTC in PBS at 37°C for 30 minutes in the dark. The samples were then washed with PBS, frozen at −20°C for 10 minutes, and embedded in tissue freezing medium for frozen section (CM1850, Leica). The sections were cut at about 150 μm thick perpendicular to the irradiation light axis, and inspected and photographed under a microscope (Stereo Lumar.V.12, Carl Zeiss, Oberkochen, Germany). For a reasonable comparison of the tissue necrosis, the tenth sections from the irradiated surface were displayed and the corresponding necrotic area was calculated with computerized planimetry [13,14]. The results are shown as both the whole tumor area and the necrotic tumor area in each group.

Singlet Oxygen Monitoring

The ROS production during PDT was monitored with a well-established chemiluminescence (CL) technique [15,16]. The study was performed for animals that received 216 J/cm^2 (60 mW/cm^2) PDT laser irradiation, with or without prior heparin treatment. Each mouse was pre-administered with 100 μM FCLA in situ, 1 hour before PDT. During each PDT treatment, the delayed CL signal was measured during brief interruption of the irradiation. The time-sequenced irradiation and CL collection (5/0.5 seconds, respectively, sampling rate: 20 Hz) was achieved with a custom apparatus controlled with a computer [16]. In a brief summary of the setup, the CL

was measured using a photomultiplier tube (PMT, Model MP 952, PerkinElmer Optoelectronics, Wiesbaden, Germany) with a counter (PCL-836, Advantech Co., Ltd, Taibei, Taiwan). The laser output and CL measurement system were controlled with LabVIEW (LabVIEW version 8.0, National Instruments Corporation). The time sequence and length of the irradiation and data acquisition were also controlled by the same program.

Perfusion Measurement

For the blood perfusion measurements, a mouse was positioned on a platform. The probe (Probe 418-1, Perimed, Stockholm, Sweden) of the laser Doppler flow meter (PeriFlux System 5000, Perimed) was inserted into the tumor at a depth of about 2–3 mm below the light irradiated tumor surface, perpendicular to the light irradiation axis, with the guidance of a dermal needle mounted on a 3-D micromanipulator stage. To evaluate the effect of blood perfusion by the anticoagulant heparin, light treatment protocols were 60 J/cm^2 at 20 mW/cm^2, 60 J/cm^2 at 60 mW/cm^2, and 100 J/cm^2 at 100 mW/cm^2, respectively. Blood perfusion in tumors was also monitored with and without heparin in light-only controls (100 J/cm^2, 100 mW/cm^2). The PDT light irradiation and the Doppler measurement were time sequenced with a LabVIEW-controlled computer interface (PCI 1751, Advantech Co., Ltd). This allowed the perfusion data to be collected in a 10-second "dark period" following every 100 seconds of PDT laser irradiation, so as to eliminate the interference from the treatment light. The blood perfusion was measured 110 seconds before the light irradiation started. Except for the irradiation light and the light for the Doppler measurement, the experiments were performed in a completely dark environment.

Statistical Analysis

Data from the light transmission, blood perfusion, and CL measurements were normalized to that at the beginning of the PDT light irradiation, and were processed with Origin 6.0 (OriginLab, Hampton, MA). Data were presented as mean ± SD. Repeated measures ANOVA tests were performed to determine any statistical difference in the blood perfusion and light transmission measurements between the combined therapy and PDT treatment, as well as the control. The counts of thrombi, relative cumulative CL, necrotic tumor area and tumor volumes (volumes were measured at the fifteenth day after treatment), were all tested with the Student's t-test for any statistical difference between the combined therapy and PDT treatment. The statistical analysis was performed with SPSS 13.0 program (SPSS, Chicago, IL). A value of $P<0.05$ was considered statistically significant.

RESULTS

Improvement of Light Distribution

Light is one of the important factors in PDT. It is shown that the relative transmitted light intensity was gradually reduced in the PDT treatment at all dose levels in tumor (Fig. 1). As a control in skin, due to a lower injury caused by photochemical reaction, the light transmission remained stable (Fig. 1B). In the tumors, with the dose of 60 J/cm^2 (60 mW/cm^2), transmission was 58% in the PDT group and 79% in the PDT with heparin group, respectively (Fig. 1C), relative to light-only controls. With the dose of 100 J/cm^2 (100 mW/cm^2), in the PDT group, light fluence rate at the end of treatment was 33% of that at the beginning of treatment, while it was 65% in the PDT with heparin group (Fig. 1D). The results clearly show a significant improvement of light transmission, more than 30% of light fluence rate in the deep tissue of tumor, when heparin was administered.

Blood Perfusion Changes During PDT

To examine the oxygen supply, the blood perfusion was monitored during PDT. The perfusion changed little in the light only but no photosensitizer group (Fig. 2A). With 60 J/cm^2 (20 mW/cm^2), the blood perfusion decreased slowly in both PDT-only and the combined therapy groups for the first 30 minutes. This was followed by a sharper decline in the PDT-only group (solid arrow, Fig. 2B), compared to that observed in the heparin-PDT group. Comparing to that at the beginning of treatment, with the dose of 60 J/cm^2 (60 mW/cm^2), the blood perfusion dropped to 47% and 72% in the PDT and PDT with heparin groups, respectively (Fig. 2C). The results also show that with the dose of 100 J/cm^2 (100 mW/cm^2) blood perfusion did not significantly decrease (Fig. 2D) suggesting that the photo-thermal effect may stimulate the blood vessels.

Thrombus Formation and Inhibition During PDT

In order to detect blood vessel closure and thrombus formation, the tumor was harvested immediately after PDT. The area near and within the irradiated volume was examined with H&E staining. The targeted tissue was examined for thrombosis (Fig. 3). Thrombosis was seen in the PDT-only group, but was significantly inhibited in the PDT with heparin group as shown in Figure 3A,B. Erythrocyte aggregation can also be found in both the PDT-only and PDT with heparin groups (data not shown) [17,18]. These show that in the PDT group, the blood vessels could be closed during the treatment in the superficial tumors which were near light irradiation.

Enhancement of ROS Production

As singlet oxygen is the main ROS induced by PDT and is a critical factor for PDT therapeutic efficacy, the generation of singlet oxygen was monitored with the delayed CL method [15,16]. The CL was positively correlated with the ROS. The results show that the CL decayed exponentially during the treatment, likely due to both the FCLA and oxygen molecules consumption. The decay of the CL in the PDT with heparin group was slower than that in the PDT group (Fig. 4A). Overall, the cumulative CL in the PDT with heparin group was significantly higher ($>50\%$) compared to that in PDT-only group (Fig. 4B).

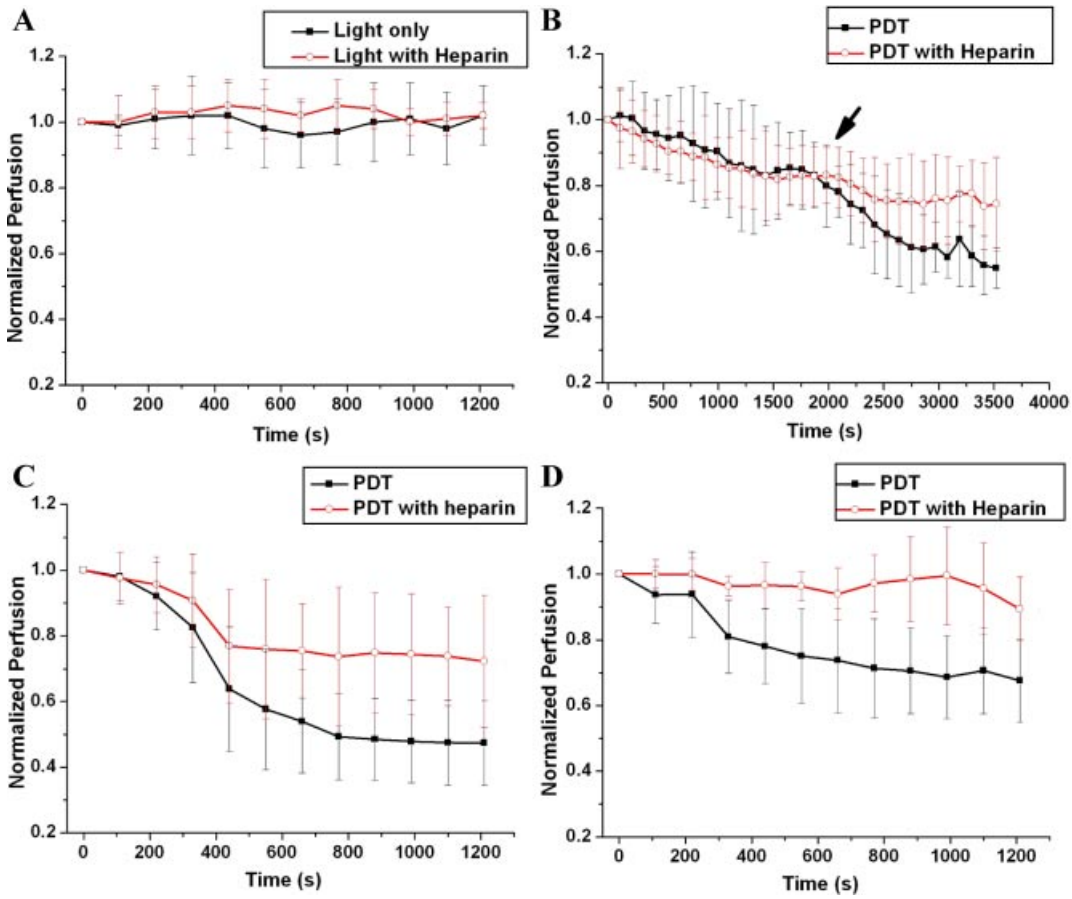

Fig. 2. Blood perfusion in the superficial tumor during PDT and effect of heparin. **A**: Normalized perfusion changes while light exposed at 100 J/cm^2 (100 mW/cm^2) without Photofrin and with (○, $n=5$) or without (■, $n=5$) heparin as a control. The normalized perfusion changes during PDT with different irradiation protocols: **B**, 60 J/cm^2 (20 mW/cm^2, $n=10$); **C**, 60 J/cm^2 (60 mW/cm^2, $n=5$); and **D**, 100 J/cm^2 (100 mW/cm^2, $n=5$) in PDT group (■) and PDT with heparin group (○). Statistical analysis reveals significant difference between the PDT with heparin and PDT-only groups (C and D, $P<0.05$, repeated measures ANOVA tests).

Antitumor Effect In Vivo

In order to assess damage, tumors were harvested 48 hours after PDT and stained with TTC (Fig. 5). The results show that the necrotic tumor area in the combined therapy group is larger than that in the PDT-only group in the corresponding histology section (Fig. 5B). Tumor necrosis is also shown in the control group.

The antitumor effect of the combination treatment was investigated in an EMT6 model. The tumor regrowth is shown in Figure 6. It is confirmed that pre-administration of heparin augments tumor destruction in Photofrin-mediated PDT. With a same total dosage (60 J/cm^2), PDT at the low fluence rate (20 mW/cm^2) induced more efficient damage than the medium fluence rate (60 mW/cm^2) in vivo. With the high fluence rate (100 mW/cm^2), due to the fast oxygen consumption, even with a dosage of 100 J/cm^2, the injury to the tumor was diminished. From the data, a high dosage with a medium fluence rate (216 J/cm^2, 60 mW/cm^2) is suitable for a solid tumor treatment. The combined effect with heparin is remarkable (Fig. 6B). It demonstrated that anticoagulation significantly enhanced the antitumor effects of PDT, resulting in retardation of tumor growth, especially with the high dose irradiated at medium fluence rate.

DISCUSSION

Light is a crucial factor in PDT. The application of PDT is often restricted by a limited penetration depth of the irradiating light. With PDT-induced changes of oxygen concentration [19], more oxyhemoglobin changes into deoxyhemoglobin [20]. As the red light absorbance of oxyhemoglobin is lower than that of deoxyhemoglobin [12], the tissue optical property changes accordingly [21]. So, it is important to optimize the light distribution. It has been reported that increasing the tissue oxygen concentration can enhance the penetration of red light [12]. High doses of light irradiation, and photochemical injury during PDT can cause platelet and coagulation-dependent thrombi

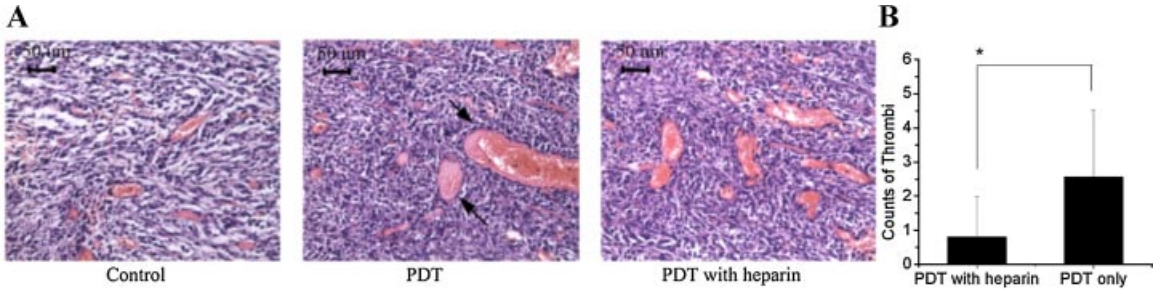

Fig. 3. Thrombus formation by PDT and inhibition of thrombus with heparin during PDT (H&E, 20×). **A**: H&E. Control, $n = 3$; thrombus formation (solid arrow), $n = 3$; inhibition with heparin, $n = 3$. The irradiation protocol was 216 J/cm^2 at 60 mW/cm^2. Bars: 50 μm. **B**: The counts of thrombi in PDT and PDT with heparin groups. The data are presented as mean ± SD per field and analyzed with Student's t-test. *$P<0.05$ compared with PDT-only group.

in the superficial vessels [22]. Our results clearly show thrombus formation with histological evaluation (Fig. 3). With a high irradiation fluence rate, therapeutic effect was reduced due to fast oxygen depletion. With identical irradiation fluence, the lower illumination fluence rate typically results in an improved therapeutic effect. Also the fractionated light delivery can improve the oxygen supplement to enhance the therapeutic effect [23].

PDT can cause changes in target tissue blood flow and perfusion. Although the blood flow rate may be accelerated [10], the diameter of blood vessels became narrower [24]. The combined effect resulted in an overall reduction of blood perfusion, evidenced in Figure 2. The same phenomenon was also found in ALA-PDT [25]. With 60 J/cm^2 (20 mW/cm^2), the blood perfusion decreased slowly in both PDT-only and the combined therapy groups for the first 30 minutes. This was followed by a sharper decline in the PDT-only group (solid arrow, Fig. 2B), compared to that observed in the heparin-PDT group. A possible explanation is that, with the low fluence rate, the treatment was less intensive, and longer irradiation time would be necessary to induce blood coagulation. As the irradiation went on, thrombosis induced a sharp decline of the blood perfusion in the PDT-only group. This was inhibited in the heparin treated group. This observation is similar to that reported by Rosen et al. [22], only pulsed laser illumination at higher laser intensity induces more severe injuries, and more easily induces occlusive thrombi than that with a low light fluence rate.

Thromboxane and von Willebrand factor (vWF) are released by Photofrin-PDT [26,27]. Thromboxane is a stress molecule, which can cause vasoconstriction and platelet aggregation. Platelet aggregation and thrombin activation are essential parts of blood coagulation and thrombosis. Heparin can be combined with antithrombin, to enhance antithrombin activity and indirectly inhibit the activation of thrombin, which subsequently inhibits blood clotting. Therefore, PDT combined with heparin can improve oxygen supply, maintaining the tissue oxygenation levels better throughout a treatment. Also the improved oxygen supply,

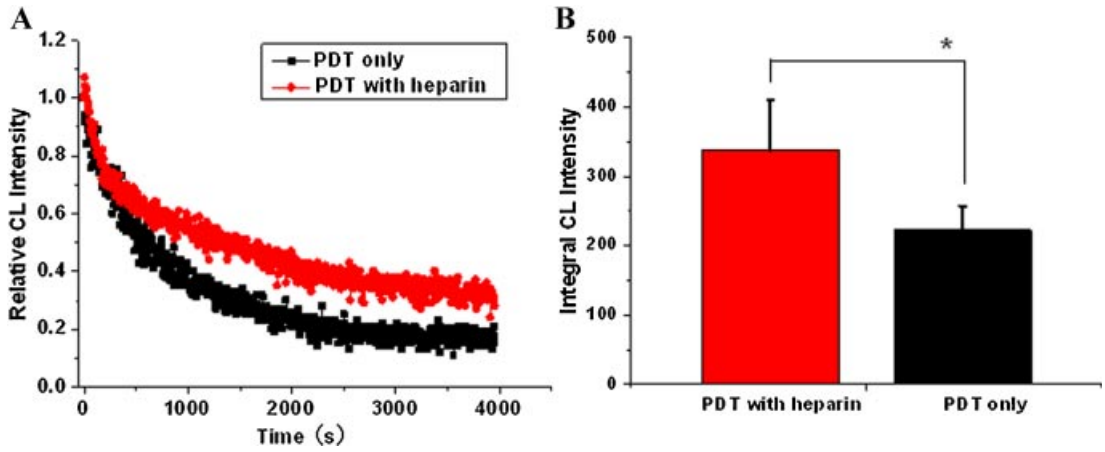

Fig. 4. The generation of singlet oxygen monitored with the CL method during PDT. **A**: Real-time production of singlet oxygen was detected with CL method at the high dose (216 J/cm^2 at 60 mW/cm^2) in PDT group (■, $n = 10$) and PDT with heparin group (●, $n = 10$), respectively. **B**: The cumulative production of singlet oxygen in the entire treatment procedure. The data were presented as mean ± SD. *$P<0.05$ compared with PDT-only group (t-test).

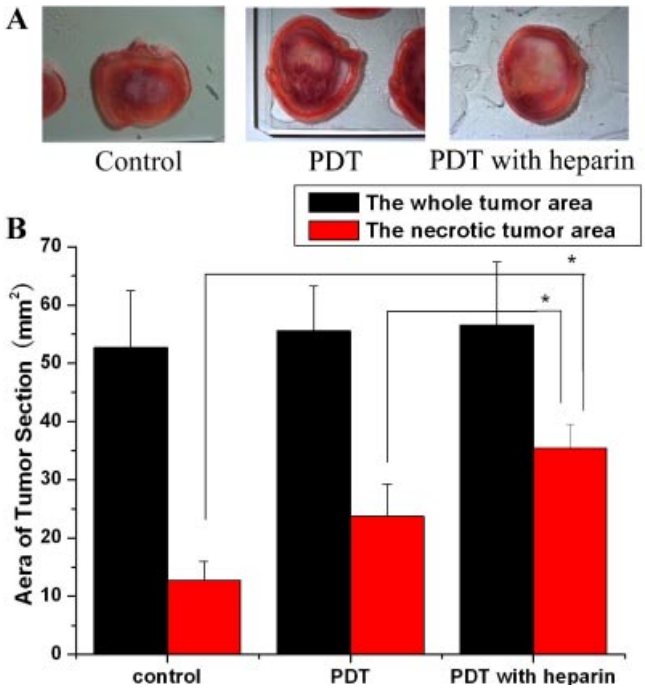

Fig. 5. The necrosis detection of tumor tissues 48 hours after PDT with TTC stain. **A**: TTC stained sections in control ($n = 4$, light only), PDT only ($n = 4$), and PDT with heparin ($n = 4$). Light treatment protocol was 216 J/cm^2 at 60 mW/cm^2. The tenth section was displayed. **B**: The area of the whole tumor and necrotic tumor sections in each group. The tenth sections were analyzed. The data were presented as mean ± SD. *$P<0.05$ compared with the PDT-only group and the control group (t-test).

at the same time, improves the light distribution. Therefore, the combined effects of both oxygen supply and light distribution by the pre-administered heparin enhance the therapeutic outcome.

Vascular injury induced hypoxia leading to cell death is one mechanism of PDT [2,28]. However, tumor vasculature close to the irradiating light source is likely to shut down during treatment, especially with a short drug-light-interval. It is a barrier to the light delivery and oxygen supply. The half-life of heparin in vivo is relatively short, and is about 1 hour for an intravenously administered bolus. So heparin-PDT improves the efficacy of PDT by delaying the time of thrombus formation [17], but has minimal effect on the critically important PDT-induced vasculature damages. Post-PDT ischemia-reperfusion injury has been reported [29,30], due to a recovering tissue oxygen level [20,31]. The administration of heparin may also contribute to an improved post-PDT ischemia-reperfusion and further enhance the therapeutic outcome. It is also reported that the formation of metastatic tumors could be inhibited with heparin by interfering with platelet aggregation or inhibiting tumor cell motility [32]. Other anticoagulants, such as low molecular weight heparin (LMWH), warfarin, and aspirin, have a relatively long half-life, which may diminish the efficacy of PDT by inhibiting the blood vessel closure post-PDT [33,34].

Hyperoxygenation and carbogen breathing can improve the therapeutic effect of PDT [12,35]. To improve the tissue oxygen supply during irradiation is an important means of enhancing the therapeutic effect [36]. We emphasize that the temporary inhibition of blood clotting to delay the thrombosis can improve the oxygen supply and light delivery, ultimately enhancing the therapeutic effect. It

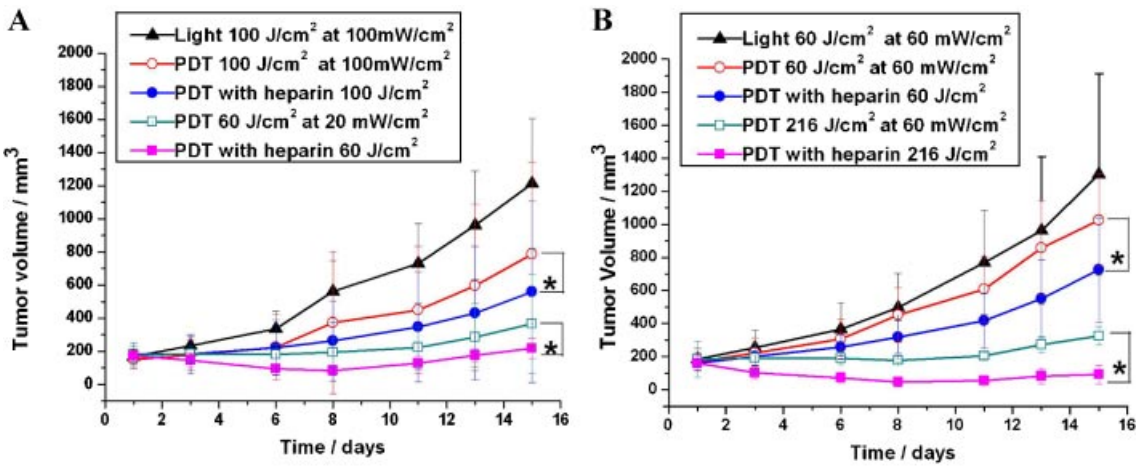

Fig. 6. Antitumor effects of PDT with pre-administered heparin. **A**: Tumor regrowth after treatment, where the irradiation protocol is 100 J/cm^2 at 100 mW/cm^2 (▲, $n = 10$, light only; ○, $n = 10$, PDT only; ●, $n = 10$, PDT with heparin), 60 J/cm^2 at 20 mW/cm^2 (□, $n = 10$, PDT only; ■, $n = 10$, PDT with heparin). **B**: Tumor regrowth after treatment, where the irradiation protocols were 60 J/cm^2 at 60 mW/cm^2 (▲, $n = 10$, light only; ○, $n = 10$, PDT only; ●, $n = 10$, PDT with heparin), 216 J/cm^2 at 60 mW/cm^2 (□, $n = 10$, PDT only; ■, $n = 10$, PDT with heparin). Measurements of tumor diameter were started at the beginning of the experiments and were done every 2 or 3 days. On the fifteenth day, *$P<0.05$ compared with the PDT-only group.

provides a more efficient means for the application of PDT in solid tumors.

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REFERENCES

1. Qiang YG, Zhang XP, Li J, Huang Z. Photodynamic therapy for malignant and non-malignant diseases: Clinical investigation and application. Chin Med J (Engl) 2006;119(10): 845–857.
2. Dolmans DE, Fukumura D, Jain RK. Photodynamic therapy for cancer. Nat Rev Cancer 2003;3(5):380–387.
3. Chen B, Roskams T, Xu Y, Agostinis P, de Witte PAM. Photodynamic therapy with hypericin induces vascular damage and apoptosis in the RIF-1 mouse tumor model. Int J Cancer 2002;98(2):284–290.
4. Gomer CJ, Ferrario A, Luna M, Rucker N, Wong S. Photodynamic therapy: Combined modality approaches targeting the tumor microenvironment. Lasers Surg Med 2006;38(5): 516–521.
5. Mitra S, Cassar SE, Niles DJ, Puskas JA, Frelinger JG, Foster TH. Photodynamic therapy mediates the oxygen-independent activation of hypoxia-inducible factor 1α. Mol Cancer Ther 2006;5(12):3268–3274.
6. Solban N, Selbo PK, Sinha AK, Chang SK, Hasan T. Mechanistic investigation and implications of photodynamic therapy induction of vascular endothelial growth factor in prostate cancer. Cancer Res 2006;66(11):5633–5640.
7. Wang HW, Putt ME, Emanuele MJ, Shin DB, Glatstein E, Yodh AG, Busch TM. Treatment-induced changes in tumor oxygenation predict photodynamic therapy outcome. Cancer Res 2004;64(20):7553–7561.
8. Zhang X, Jiang F, Zhang ZG, Kalkanis SN, Hong X, deCarvalho AC, Chen J, Yang H, Robin AM, Chopp M. Low-dose photodynamic therapy increases endothelial cell proliferation and VEGF expression in nude mice brain. Lasers Med Sci 2005;20(2):74–79.
9. Yu G, Durduran T, Zhou C, Wang HW, Putt ME, Saunders HM, Sehgal CM, Glatstein E, Yodh AG, Busch TM. Noninvasive monitoring of murine tumor blood flow during and after photodynamic therapy provides early assessment of therapeutic efficacy. Clin Cancer Res 2005;11(9):3543–3552.
10. Busch TM. Local physiological changes during photodynamic therapy. Lasers Surg Med 2006;38(5):494–499.
11. Chen Q, Huang Z, Chen H, Shapiro H, Beckers J, Hetzel FW. Improvement of tumor response by manipulation of tumor oxygenation during photodynamic therapy. Photochem Photobiol 2002;76(2):197–203.
12. Mitra S, Foster TH. Carbogen breathing significantly enhances the penetration of red light in murine tumours *in vivo*. Phys Med Biol 2004;49(10):1891–1904.
13. Yamamoto J, Yamamoto S, Hirano T, Li SY, Koide M, Kohno E, Okada M, Inenaga C, Tokuyama T, Yokota N, Terakawa S, Namba H. Monitoring of singlet oxygen is useful for predicting the photodynamic effects in the treatment for experimental glioma. Clin Cancer Res 2006;12(23):7132–7139.
14. Kurrelmeyer KM, Michael LH, Baumgarten G, Taffet GE, Peschon JJ, Sivasubramanian N, Entman ML, Mann DL. Endogenous tumor necrosis factor protects the adult cardiac myocyte against ischemic-induced apoptosis in a murine model of acute myocardial infarction. Proc Natl Acad Sci USA 2000;97(10):5456–5461.
15. Wei YC, Xing D, Luo SM, Xu W, Chen Q. Monitoring singlet oxygen *in situ* with delayed chemiluminescence to deduce the effect of photodynamic therapy. J Biomed Opt 2008;13(2): 024023.
16. Wei YC, Zhou J, Xing D, Chen Q. In vivo monitoring of singlet oxygen using delayed chemiluminescence during photodynamic therapy. J Biomed Opt 2007;12(1):014002.
17. Dolmans DE, Kadambi A, Hill JS, Waters CA, Robinson BC, Walker JP, Fukumura D, Jain RK. Vascular accumulation of a novel photosensitizer, MV6401, causes selective thrombosis in tumor vessels after photodynamic therapy. Cancer Res 2002;62(7):2151–2156.
18. Fingar VH, Kik PK, Haydon PS, Cerrito PB, Tseng M, Abang E, Wieman TJ. Analysis of acute vascular damage after photodynamic therapy using benzoporphyrin derivative (BPD). Br J Cancer 1999;79(11–12):1702–1708.
19. Busch TM, Wileyto EP, Emanuele MJ, Del Piero F, Marconato L, Glatstein E, Koch CJ. Photodynamic therapy creates fluence rate-dependent gradients in the intratumoral spatial distribution of oxygen. Cancer Res 2002;62(24):7273–7279.
20. Woodhams JH, Kunz L, Bown SG, MacRobert AJ. Correlation of real-time haemoglobin oxygen saturation monitoring during photodynamic therapy with microvascular effects and tissue necrosis in normal rat liver. Br J Cancer 2004;91(4): 788–794.
21. Chen Q, Wilson BC, Shetty SD, Patterson MS, Cerny JC, Hetzel FW. Changes in *in vivo* optical properties and light distributions in normal canine prostate during photodynamic therapy. Radiat Res 1997;147(1):86–91.
22. Rosen ED, Raymond S, Zollman A, Noria F, Sandoval-Cooper M, Shulman A, Merz JL, Castellino FJ. Laser-induced noninvasive vascular injury models in mice generate platelet- and coagulation-dependent thrombi. Am J Pathol 2001; 158(5):1613–1622.
23. Xiao Z, Halls S, Dickey D, Tulip J, Moore RB. Fractionated versus standard continuous light delivery in interstitial photodynamic therapy of dunning prostate carcinomas. Clin Cancer Res 2007;13(24):7496–7505.
24. Fingar VH, Wieman TJ, Wiehle SA, Cerrito PB. The role of microvascular damage in photodynamic therapy: The effect of treatment on vessel constriction, permeability, and leukocyte adhesion. Cancer Res 1992;52(18):4914–4921.
25. Wang KK, Cottrell WJ, Mitra S, Oseroff AR, Foster TH. Simulations of measured photobleaching kinetics in human basal cell carcinomas suggest blood flow reductions during ALA-PDT. Lasers Surg Med 2009;41(9):686–696.
26. Fingar VH, Wieman TJ, Doak KW. Role of thromboxane and prostacyclin release on photodynamic therapy-induced tumor destruction. Cancer Res 1990;50(9):2599–2603.
27. Foster TH, Primavera MC, Marder VJ, Hilf R, Sporn LA. Photosensitized release of von Willebrand factor from cultured human endothelial cells. Cancer Res 1991;51(12): 3261–3266.
28. Channual JC, Choi B, Osann K, Pattanachinda D, Lotfi J, Kelly KM. Vascular effects of photodynamic and pulsed dye laser therapy protocols. Lasers Surg Med 2008;40(9):644–650.
29. Curnow A, Bown SG. The role of reperfusion injury in photodynamic therapy with 5-aminolaevulinic acid—A study on normal rat colon. Br J Cancer 2002;86(6):989–992.
30. Korbelik M, Sun J, Zeng H. Ischemia-reperfusion injury in photodynamic therapy-treated mouse tumours. Br J Cancer 2003;88(5):760–766.
31. Brown NS, Bicknell R. Hypoxia and oxidative stress in breast cancer. Oxidative stress: Its effects on the growth, metastatic potential and response to therapy of breast cancer. Breast Cancer Res 2001;3(5):323–327.
32. Hejna M, Raderer M, Zielinski CC. Inhibition of metastases by anticoagulants. J Natl Cancer Inst 1999;91(1):22–36.
33. Taber SW, Wieman TJ, Fingar VH. The effects of aspirin on microvasculature after photodynamic therapy. Photochem Photobiol 1993;57(5):856–861.
34. Ranchod TM, Guercio JR, Ping GS, Brucker AJ, Stoltz RA. Effect of aspirin therapy on photodynamic therapy with

verteporfin for choroidal neovascularization. Retina 2008; 28(5):711–716.
35. Huang Z, Chen Q, Shakil A, Chen H, Beckers J, Shapiro H, Hetzel FW. Hyperoxygenation enhances the direct tumor cell killing of photofrin-mediated photodynamic therapy. Optical Methods for Tumor Treatment and Detection: Mechanisms and Techniques in Photodynamic Therapy Xii 2003; 4952: 186–197.
36. Fingar VH, Mang TS, Henderson BW. Modification of photodynamic therapy-induced hypoxia by fluosol-DA (20%) and carbogen breathing in mice. Cancer Res 1988;48(12): 3350–3354.