中国激光医学杂志 2013 年 2 月第 22 卷第 1 期 Chin J Laser Med Surg，February 2013，Vol 22，No. 1

Photodynamic Therapy(PDT) is a minimally invasive cancer treatment that has been approved in many countries. It's indications include skin cancer, precancerous skin lesions, esophagus cancer, stomach cancer, lung cancer, head and neck cancer, bladder cancer and gynecological cancer, etc. It can even be used to treat several types of non-cancerous ophthalmic diseases[1,2]. There are two steps in PDT: firstly patients are given photosensitizers locally or systematically, after a period of time the photosensitizers will selectively stay in the tumor cells, while those in the normal cells will be completely metabolized; then light in wavelengths that are consistent with the photosensitizers' absorption spectrum will be shined locally on the tumor sites. With the light being absorbed, the molecules of the photosensitizers will then be transformed into ROS(reactive oxygen species), of which the main component is singlet oxygen. ROS can destroy tumor tissues in many ways, the mechanisms of which include the following: (1) Damaging and killing tumor cells directly and selectively, causing necrosis and apoptosis of the tumor cells; (2) Damaging tumor's blood vessels, leading to insufficient oxygen and nutrition supply to the tumor; (3) Reacting with patients' immune systems, it can either stimulate or suppress immune response[2,3].

光动力疗法(photodynamic therapy，PDT) 是一种微创治疗肿瘤的方法，目前已在世界多个国家获得批准应用，其适应证包括皮肤癌和皮肤癌前病变、食管癌、胃癌、肺癌、头颈部肿瘤、膀胱癌、妇科肿瘤等，还可以用于眼科多种非肿瘤疾病的治疗[1, 2]。PDT 包括两个步骤: 首先全身或局部给予光敏剂，一段时间后光敏剂选择性滞留于肿瘤组织中，而正常组织中的光敏剂代谢殆尽，然后用波长与光敏剂吸收光谱相符的光照射肿瘤局部，滞留在肿瘤组织内的光敏剂吸收光后使分子氧转化为以单态氧为主的活性氧物质(reactive oxygen species，ROS) ，ROS 可以通过多种途径破坏肿瘤组织。PDT 杀伤肿瘤的作用机制包括: (1) 直接选择性杀伤肿瘤细胞，使肿瘤细胞坏死或凋亡; (2) 损伤肿瘤血管，从而引起肿瘤缺乏氧和营养物质供应; (3) 作用于免疫系统，既可以激发免疫反应，也可以抑制免疫反应[2, 3]

Light source, photosensitizers and molecular oxygen are the three key elements of PDT, among which photosensitizers are the core. Hence it's essential to explore and find out safe and effective photosensitizers for PDT to be better applied for clinical use. Through observing the differences in curative effects of PDT on S180 sarcoma in mice with Duteroporphyrin, Photosoft and 5-ALA, the objective of this research is to study the similarities and differences in the anti-tumor mechanisms of the three types of photosensitizers mentioned above, providing experimental proof for PDT clinical application.

光源、光敏剂和分子氧是 PDT 的三大要素，其中光敏剂是核心。探索安全有效的光敏剂是光动力疗法更好应用于临床的关键。本研究通过观察多替泊芬、Photosoft 和 5-氨基酮戊酸(5-ALA) 对小鼠 S180 肿瘤的光动力治疗疗效，初步探讨它们抗肿瘤机制的异同，为临床应用提供

实验依据。

Materials and methods

1. Materials

1) Experimental animals and tumor inoculation

40 Kunming mice, of which half were male and the other half were female, all 4~6 weeks old, weighing 18~22g. The mice were purchased from the Experimental Animal Center of Southern Medical University and the male and the female were kept in separate cages. The subcutaneous S180 tumour cells for mice were provided by Key Laboratory of Clinical Biotechnology of Southern Medical University.

材料与方法

一、材料

1. 实验动物和瘤株

昆明小鼠 40 只，雌雄各半，4～ 6 周龄，体重 18 ～ 22 g。雌雄小鼠分笼饲养，购自南方医科大学实验动物中心。S180 小鼠纤维肉瘤细胞株，由南方医科大学临床生物技术重点实验室提供。

2) Reagents

Duteroporphyrin, batch number 071129, provided by Shanghai Fudan-Zhangjiang Bio-Pharmaceutical Co., Ltd.; 5-ALA, batch number 101001, provided by Shanghai Fudan-zhangjiang Bio-Pharmaceutical Company; Photosoft (NGPDT), batch number 2010316, provided by THE CHO GROUP. FITC Annexin V/PI Apoptosis Detection Kit, production of BD company in America. 1640 culture media and fetal bovine serum, both were productions of Hyclone Company. Ethanol, xylene, eosin, haematoxylin and sodium sulfide, productions of Guangdong Guanghua Chemical Co., Ltd.

2. 试剂

多替泊芬，批号 071129，由上海复旦张江生物医药股份有限公司提供; 5-ALA，批号 101001，由上海复旦张江生物医药股份有限公司提供; Photosoft (NGPDT) ，批号 2010316 由 THE CHOGROUP 提供。FITC Annexin V/PI 凋亡试剂盒，美国 BD 公司产。1640 培养基、胎牛血清，Hyclone 公司产。乙醇、二甲苯、伊红、苏木素、硫化钠，广东光华化学有限公司产。

3) Instruments

Photodynamic therapy apparatus in the wavelength of 630nm, production of Guangxi Guilin Xingda Co., Ltd. THE CHO GROUP dual-frequency photodynamic therapy apparatus in the wavelengths of 660~795 nm. FACS Calibur flow cytometry, production of BD Company in America. Optical

Microscope, production of a Japanese Company OLYMPUS.

3. 仪器

光动力治疗仪，波长 630 nm，广西桂林兴达有限公司产。THE CHO GROUP 双频光动力治疗仪，波长 660 ～ 795 nm。FACS Calibur 流式细胞仪，美国 BD 公司产。光学显微镜，日本 OLYMPUS 公司产。

2. Methods

1) Tumor cell culture and establishment of ascitic tumor model

S180 fibrosarcoma cells for mice were resurrected and cultured in vitro. S180 Cells that were in good state were tested and trypan blue staining showed that viable cells were >95%, the cell count was $1x10^7$ cells/ml. Kunming mice that were 4~6 weeks old were injected with 0.2 ml of S180 cell suspension intraperitoneally($2x10^6$ cells), the growth of ascites was observed.

二、实验方法

1. 细胞培养与腹水瘤模型的建立

复苏、体外培养 S180 小鼠纤维肉瘤细胞，取生长状态良好的 S180 细胞，经台盼蓝染色测定活力在 95% 以上，计数 $1 \times 10_7$ 个/ml。取 4 ～ 6 周龄昆明小鼠，腹腔注射 S180 细胞悬液 0. 2 ml(即 $2 \times 10_6$ 个) ，观察小鼠腹水生长情况。

2) Establishment of subcutaneous solid tumor model

Ascites was drawn from ascitic type tumor-bearing mice that had been implanted with S180 tumor cells for 7~10 days. The ascites was then diluted with stroke-physiological saline solution into cell suspension in concentration of $1x10^7$ cells/ml and put on ice for later use. The hair of the Kunming mice's rumps was removed with 8% concentrated sodium sulfide solution. The Kunming mice were anesthetized by intraperitoneal injection of 1% concentrated pentobarbital sodium solution(60mg/kg), then 0.2ml ($2x10^6$ cells) of mixed S180 cell suspension were injected subcutaneously to each of the mice's bilateral ramps. Observation on the growth of the mice's subcutaneous tumors was made, after 5~7d the tumors grew to 5~7cm by diameter, and the mice were then given PDT.

2. 皮下实体瘤模型的建立

取腹腔种植 S180 肿瘤细胞 7 ～ 10 d 的腹水型荷瘤小鼠，抽取腹水，无菌生理盐水稀释成浓度为 $1 \times 10_7$ 个/ml 的细胞悬液，放于冰上备用。用 8% 硫化钠脱祛昆明小鼠臀部的毛。腹腔注射 1%戊巴比妥钠 60mg /kg，麻醉小鼠后抽取混匀的 S180 细胞悬液 0. 2 ml(即 $2 \times 10_6$ 个/ml) ，

于双侧臀部皮下注射。观察小鼠皮下肿瘤的生长，约 5 ～ 7 d 后肿瘤直径长至 5 ～ 7 mm，开始行光动力治疗。

3) Grouping and treatment application

40 Kunming mice with well grown tumors were randomly divided into four groups: (A) the Duteroporphyrin group (10mg/kg), (B) Photosoft group (20mg/kg), (C) 5-ALA group (100mg/kg) and (D) blank control group. The mice in Group A and Group B were given PDT 8~10h after tail vein injection of photosensitizers, while the mice in Group C were given PDT 3h after tail vein injection of photosensitizers. Lasers in the wavelength of 630nm were applied to Group A and C while Group B was applied with THE CHO GROUP duel-frequency PDT apparatus. All mice had were anesthetized by injection of 1% concentrated pentobarbital sodium solution(60mg/kg) before PDT. The local tumor sites were irradiated vertically, with the diameter of the laser spots being 1.2~1.5cm, it was made sure that the whole tumor and 3~5mm of normal tissues around the tumors were covered by the lasers. With the power density of 150mW/cm^2 and the energy density being 180J/cm^2, the lasers were applied to Group A-C for 20min. Mice in Group D were not given any treatment.

3. 分组与治疗

取肿瘤生长良好的昆明小鼠 40 只随机分为以下 4 组: A 组为多替泊芬-PDT 组，多替泊芬药物浓度为 10 mg /kg; B 组为 Photosoft -PDT 组，Photosoft 药物浓度为 20 mg /kg; C 组为 5-ALA-PDT 组，5-ALA 药物浓度为 100 mg /kg; D 组为空白对照组。A、B 组小鼠尾静脉注射光敏剂 8 ～ 10h 后行激光照射，C 组小鼠尾静脉注射光敏剂 3 h 后行激光照射; A、C 组使用波长为 630 nm 激光照射，B 组使用 THE CHO GROUP 双频光动力治疗仪激光照射。照光前，腹腔注射 1% 戊巴比妥钠 60 mg /kg 麻醉小鼠。激光垂直照射肿瘤局部，光斑直径 1. 2～ 1. 5 cm，保证光斑覆盖整个肿瘤，并覆盖肿瘤四周边缘约 3 ～ 5 mm 的正常组织，功率密度 150 mW/cm$_2$，能量密度 180 J /cm$_2$，照光时间各 20 min。D 组不做任何处理。

4) The observation of tumor changes in appearance and size

Changes in tumor appearance were observed and recorded with photograph every three days both before and after PDT. The tumors' long diameters(a) and short diameters(b) were also measured with a vernier scale for volume calculations and the drawing of tumor growth curves. With the 21st day after PDT as the end of observation, the inhibition rates were calculated.

Tumor Volume ab^2 x1/2

肿瘤大体形态及体积变化的观察

治疗前及治疗后每隔 3 d 观察肿瘤外观的变化，拍照记录，并用游标卡尺量度肿瘤长径(a) 与短径(b) ，计算肿瘤体积，绘制肿瘤生长曲线，以治疗后第 21 天为观察终点，计算抑瘤率。

肿瘤体积= $ab^2 \times 1/2$

抑瘤率= 对照组平均体积－ 治疗组平均体积 × 100%

对照组平均体积

5) Analysis of cell necrosis and apoptosis with FITC Annexin V/PI double staining method and observation on pathological changes of tumor tissues via HE staining.

5 mice of each treatment group were intentionally killed 24h after treatment, unilateral subcutaneous tumor tissues were removed and cut into pieces with ophthalmic scissors, the tissues were then filtered with a 200-mesh cell strainer and made into cell suspension. With the instructions on the staining kit followed, tumor cell necrosis and apoptosis were tested with flow cytometry technology. The subcutaneous tumors on the other side of the rumps were cut off, routinely fixed, dehydrated, immersed with wax, embedded, sectioned, stained with HE and observed with optical microscope.

5. FITC Annexin V/PI 双染色分析细胞凋亡、坏死情况，HE 染色观察病理组织学变化

各组治疗后 24 h 处死 5 只小鼠，取一侧皮下肿瘤，用眼科剪剪碎， 200 目细胞筛过滤，制成细胞悬液，按照试剂盒说明书进行操作，上流式细胞仪检测肿瘤细胞凋亡、坏死情况。取另一侧皮下肿瘤，常规固定，脱水，浸蜡，包埋，切片，HE 染色，光学显微镜观察。

3. Statistical processing

The statistics were expressed as mean± and standard deviations(x ± s) and were analyzed with the software SPSS13.0. Multiple mean comparison was made by analysis of variance and LSD-t test, ($P<0.05$), the difference was statistically significant.

三、统计学处理

采用 SPSS13. 0 软件进行统计分析，所用数据以均数± 标准差(x ± s) 表示。采用方差分析，多个样本均数间比较采用 LSD-t 检验。P ＜ 0. 05 为差异有显著意义。

Results

1. Tumor changes in appearance

No obvious difference in tumor appearance of the mice between each group was noted before treatment. Edema and small skin lesions at the irradiation sites were noticed in all treatment groups 1d after PDT. Three days after PDT, skin blackening at the irradiation sites of the mice in Group A and B was noted and some of the mice had eschars, also some red round skin lesions that were consistent with the shape of the lasers were seen in some mice in these two groups. As for the mice in Group C, brownish red eschars were seen where the lasers were targeted at. Six days after PDT, black eschars were noted on the mice in Group A and B, no further growth of tumor was seen in most mice in the two

groups, whereas in Group C, most eschars fell off from the mice and new skin was seen, tumor growth could still be seen in some mice. Most of the mice's eschars in Group A and B fell off 15 days after treatment, skins were fully recovered and fur started to grow back, only a small proportion of the mice's eschars haven't felt off. At the same time most of the mice's skins in Group C have recovered and fur started to grow back, yet tumor growth was also noted. 21 days after treatment, most of the mice's skins and fur in Group A and B were fully recovered while a small amount of the mice's eschars haven't fallen off. No subcutaneous tumor growth was seen in most mice and only in a small amount of mice was residual tumor growth noted after the mice had been intentionally killed and skin sliced through. As for the mice in Group C, after 21 days of treatment, tumor growth could still be seen at and near the laser irradiation sites in most mice, subcutaneous solid tumors were noted after the mice were intentionally killed and skins sliced through. As for the mice in the control group, the tumors in which were growling fast and gradually grew bigger. The mice were intentionally killed on the 21st day of the experiment, the skins of which were sliced through and the subcutaneous solid tumors were relatively bigger than those in Group C. Some of the tumors' middle parts were liquified and necrotized. (Image 1 on Page 64)

结果

一、肿瘤大体形态的变化

治疗前各组小鼠的肿瘤大体形态无明显差异。PDT 治疗后 1 d 各治疗组的照光部位出现水肿和少量皮损。治疗后 3 d，A 与 B 组照光部位的皮肤发黑，部分焦痂形成，部分小鼠的皮肤可见发红圆形皮损，与照光光斑形状相吻合; 而 C 组照光部位形成棕红色焦痂。治疗后 6 d，A 与 B 组黑色焦痂形成，大多数小鼠未见肿瘤继续生长; C 组大多数小鼠的焦痂脱落，露出新鲜皮肤，部分可见肿瘤继续生长。治疗后 15 d，A 与 B 组大多数的小鼠焦痂脱落，皮肤修复完整，毛发开始生长，小部分焦痂未完全脱落;C 组大多数的小鼠皮肤修复，毛发生长，但可见肿瘤继续生长。治疗后 21 d，A 与 B 组大多数的小鼠皮肤修复完好，毛发生长如前，基本覆盖照光部位，少数焦痂未完全脱落，处死小鼠后切开皮肤可见大多数小鼠无皮下肿瘤生长，少数仍有残留肿瘤继续生长; C 组大多数的小鼠肿瘤在照光部位基底及周围继续生长，处死小鼠切开皮肤可见皮下实体肿瘤。空白对照组的小鼠同步观察，其肿瘤逐渐增大，生长速度较快，第 21 天处死小鼠，切开皮肤可见皮下实体肿瘤较大，部分中间液化坏死(图 1 见第 64 页) 。

2. Tumor size changes and tumor inhibition rates

The mean volume differences between each group were not statistically significant before the treatments (P>0.05). Whereas 21 days after the treatments, the mean volume differences between all treatment groups and the control group were statistically significant (P<0.05), so was the difference between Group B and Group C (P<0.05). All the treatment groups showed tumor inhibition compared to the blank control group, among which Group B, which shared similar curative effect as Group A (p>0.05), showed a better therapeutic effect than Group C (P<0.05). The tumor inhibition rates of Group A, B and C were respectively 62.5%, 75.4% and 37.2% (Image 2 on Page 65).

二、肿瘤体积变化及抑瘤率

治疗前各组肿瘤体积均数间差异无显著意义(P ＞ 0. 05) 。治疗后 21 d，各治疗组与空白对照组间差异有显著意义(P ＜ 0. 05) ，治疗组间 B 组与 C 组差异有显著意义(P ＜ 0. 05) 。各治疗组与空白对照组相比都能抑制肿瘤的生长，其中 B 组的疗效好于 C 组(P ＜ 0. 05) ，而与 A 组疗效相当(P ＞ 0. 05) 。A、B 和 C 组的抑瘤率分别为 62. 5%、75. 4%、

37. 2%(图 2 见第 65 页) 。

3. Tumor cell necrosis rates and apoptosis rates

Based on the analysis of the mean value of the test results, it's evident that the differences of proportion of apoptotic cells and necrotic cells (including early apoptotic, late apoptotic and necrotic cells) between all treatment groups and the control group were statistically significant (P<0.01). The difference of early apoptosis rates between all treatment groups and the control group was significant (P<0.01), so was the difference of early apoptosis rates between Group B and Group C (P<0.05). The tumor cell apoptosis rates and necrosis rates were significantly higher in all treatment groups than in the blank control group, whereas normal living cells in the treatment groups were significantly fewer than those in the control group, indicating all treatment groups had obvious cancer-killing effect. (Image 3 on Page 65)

三、肿瘤细胞凋亡率和坏死率

从检测结果的平均值分析，各治疗组的凋亡及坏死的细胞(包括早期凋亡、晚期凋亡和坏死细胞)所占比例与空白对照组比较差异有显著意义(P ＜0. 01) ，其中各治疗组细胞早期凋亡率与空白对照组比较差异有显著意义(P ＜ 0. 01) ，B 组细胞早期凋亡率与 C 组比较差异有显著意义(P ＜ 0. 05) 。各治疗组细胞凋亡率和坏死率比空白对照组明显增多，而正常活细胞明显减少，说明各治疗组杀伤肿瘤细胞效果明显(图 3 见第 65 页) 。

4. Pathological changes of tumor tissues

Under observation with light microscope, small-scale necroses could be seen in the central part of tumor tissues in the blank control group, the marginal tumor cells were highly proliferative and were coated with envelope. The tumor cells were big with dark stained nuclei, high nucleus cytoplasm ratio. Marked nucleus atypia and nuclear fission were noted. Similar results were obtained from tissue observation between all treatment groups. Tumor capsule, tumor cell living zone, necrosis zone and homogeneous red stained zone with no cellular structure were noted from the outside in of the tumor cells. Deposits of erythrocytes in tumor vessels was seen in all treatment groups while angiorrhexis, extravasation of erythrocytes and thrombosis were noted in some vessels. Abundant neutrophil infiltration was observed near some necrotic regions. Of all the groups the most obvious tumorous angiolysis was found in the Duteroporphyrin-PDT group (Image 4 on Page 65).

四、肿瘤组织病理学变化

光镜下观察，空白对照组的肿瘤组织中央部位有小范围的坏死，边缘瘤组织细胞增殖旺盛，外被有包膜; 肿瘤细胞大而核深染，核浆比例大，核异型性明显，可见核分裂。各治疗组所得结果相似，从外到内可见肿瘤包膜，肿瘤细胞存活带，肿瘤细胞坏死带，失去细胞结构的均质红染区。各治疗组可见肿瘤血管内红细胞淤积，部分血管破裂，红细胞外溢，部分血管血栓形成。并可见部分坏死灶周围有大量中性粒细胞浸润。其中肿瘤血管的破坏以多替泊芬-PDT 组最明显(图 4 见第 65 页) 。

Discussion

There are three major types of photosensitizers in clinical use at present:(1) Porphyrins (such as Hematoporphyrin Derivative and 5-ALA, the precursor compound of Protoporphyrin IX); (2) Chlorophyll Derivative (such as mTHPC, NPe6); (3) Dye photosensitizers (such as phthalocyanine) [4] . Among all the most commonly used ones are Porphyrins, as one of their types Photofrin has already been approved for treating various types of solid tumors. When activated by lasers in the wavelength of 630nm, the effective depth of Photofrin can be as deep as 5mm[5] while the effective depth of 5-ALA is 2mm[6] when applied topically, which is mainly for treating superficial diseases such as pre-cancerous skin lesions and skin cancer. The absorption peaks of chlorophyll derivatives are normally higher than 630nm, mTHPC (with the trade name Foscan), for example, which is activated by lasers in the wavelength of 652nm, has a deeper effective depth than traditional porphyrin photosensitizers. However dye photosensitizers can be activated by lasers in wavelengths of 650~850nm, which makes them very promising[4] despite the lack of present clinical use.

讨论

目前临床应用的光敏剂可以分为三大类: (1)卟啉类(如血卟啉衍生物 HpD 和原卟啉Ⅸ的前体化合物 5-ALA) ; (2) 叶绿素衍生物(如 mTHPC，NPe6) ; (3) 染料类(如酞菁染料) [4]。世界上应用最广泛的是卟啉类光敏剂，如 Photofrin 已被批准用于多种实体肿瘤的治疗，其在波长 630 nm 激光激发下有效杀伤深度可以达到约 5 mm[5]。而 5-ALA 外涂应用的杀伤深度为 2 mm[6]，目前主要应用于皮肤癌前病变与皮肤癌等浅表病变的光动力治疗。叶绿素衍生物类光敏剂的吸收峰普遍大于 630 nm，如 mTHPC(商品名 Foscan) 使用波长 652 nm 的激光激发[4]，比传统的卟啉类光敏剂有更深的杀伤深度。染料类光敏剂能被波长 650 ～ 850 nm 的激光激发，虽然目前临床应用经验较少，但是一种很有前景的光敏剂[4]。

The Duteroporphyrin and 5-ALA used in this experiment are products of Shanghai Fudan-Zhangjiang Bio-Pharmaceutical Co., Ltd.. Duteroporphyrin is a new type of porphyrin photosensitizer, compared to traditional porphyrins such as Photofrin, it’s composition is more stable, and chemical structure better defined. And 5-ALA is a type of externally used photosensitizer that’s approved for treating condyloma. Photosoft is a type of Chlorophyll A Derivative Photosensitizer that was developed by THE CHO GROUP. It has absorption peaks in blue, red and infrared and the PDT apparatus to it uses

double frequency (660~795nm), increasing the effective depth.

本实验所用的光敏剂多替泊芬、5-ALA 都是上海复旦张江生物医药股份有限公司的产品。其中多替泊芬是一种新的卟啉类光敏剂，相对于传统的卟啉类光敏剂如 Photofrin 而言，具有组成成分稳定、化学结构明确的特点。5-ALA 是该公司获准生产上市用于尖锐湿疣光动力治疗的外用光敏剂。Photosoft 是 THE CHO GROUP 研发的一种叶绿素 A 衍生物类光敏剂，在蓝光、红光、红外光都有吸收峰，其光动力治疗仪采用双频(波长 660 ～ 795 nm) ，以加深杀伤肿瘤的深度。

From the observation of tumor appearance changes in the experiment, black eschars were seen in Duteroporphyrin-PDT group and Photosoft-PDT group after treatments while the eschars observed in 5-ALA-PDT group were thinner and in brownish red color. Skins and fur were fully recovered in all three treatment groups after the eschars had fallen off, among those Group C was the fastest in skin repairing. From the tumor growth curves we could see that there was no obvious difference in size of tumors between the treatment groups and the control group 6 days after the treatments. This was possibly caused by the edema and eschars caused by PDT, which occurred at the irradiation sites and thus would increase the measure values of long diameters and short diameters of tumors, from which it could be deduced that the actual sizes of the tumors were smaller than the measured sizes. Yet at later period of observation when edema subsided and eschars fell out, the measured values of long diameters and short diameters of the tumors were closer to truth values. Observation of the tumors' sizes 21 days after the treatments indicated that all treatment groups could inhibit tumor growth, of which Group B, which had similar effect as Group A ($P>0.0.5$), was obviously more effective than Group C ($P<0.05$).

本实验治疗后肿瘤外观形态观察结果提示，多替泊芬-PDT 与 Photosoft-PDT 组光照后都会形成黑色焦痂，而 5-ALA-PDT 组形成的是较薄的一层棕红色焦痂。三组的焦痂脱落后皮肤能修复完整，毛发重新生长，其中 C 组皮肤修复明显比 A、B 组快。从各组肿瘤生长曲线中可以看到，治疗后 6 d 各组较为接近，治疗组与空白对照组暂未出现明显的差异，这可能是因为治疗组照光部位出现水肿、焦痂，一定程度上增大了长短径的测量值，可以推断治疗组的肿瘤体积实际上比测量值要小。而治疗后观察后期，照光部位水肿消退，焦痂脱落，测量出的肿瘤长短径更接近客观值。治疗后 21 d 肿瘤体积观察结果提示各治疗组都能抑制肿瘤的生长，其中 B 组的效果明显好于 C 组($P < 0.05$) ，而与 A 组相当($P > 0.05$) 。

Testing principle of using FITC Annexin V/PI double staining method to test cellular apoptosis and necrosis: Phosphatidyl serine (PS), for which Annexin V has an affinity, lies within cell membranes in normal cells. Yet once the cells are apoptosed, PS will rapidly be transferred to the outside of the membranes, making it exposed to Annexin V. Annexin V will then quickly unite with PS, of which the reaction is used to test for the exposed PS. However, since cellular necrosis will also cause membrane damage, which will lead to Annexin V uniting with the necrotized cells, PI is used to distinguish living cells, viable apoptotic cells, necrotic cells and non-viable apoptotic cells. Necrotic cells and non-viable apoptotic cells can unite with AnnexinV-FIFC and PI at the same time and show color, while PI can't be united with living cells (negative for FITC) or viable apoptotic cells (positive for FITC). Based on analysis by FCM (flow cytometry), cell histogram consisting of four quadrants was acquired. The left lower quadrant of the histogram represented normal living cells V(-)PI(-), the right lower quadrant

represented viable apoptotic cells V(+)PI(-), while the right upper quadrant represented non-viable apoptotic cells and necrotic cells V(+)PI(+) and the left upper quadrant represented cells that were damaged while being collected V(-)PI(+).

FITC Annexin V/PI 双染色检测细胞凋亡、坏死原理: 在正常细胞中，磷脂酰丝氨酸(PS) 位于细胞膜内侧，一旦发生细胞凋亡则从细胞膜的内侧迅速翻转到细胞膜外侧，使得 PS 暴露在细胞膜表面。Annexin V 与 PS 有很高的亲和力，可以与之迅速结合，所以可以用 PS 与 Annexin V 的相互作用检测外翻的 PS。由于细胞坏死的过程中也会发生细胞膜损伤，坏死的细胞同样也会结合 Annexin V，所以用 PI 区分活细胞、早期凋亡细胞和坏死、晚期凋亡细胞。坏死细胞和晚期凋亡细胞可以同时与 AnnexinV-FITC 和 PI 结合显色，而 PI 则被排除在活细胞(FITC 阴性) 和早期凋亡细胞(FITC 阳性) 之外。经流式细胞仪分析后，获得由四个象限组成的细胞直方图，左下象限代表正常活细胞 V(－) PI(－)，右下象限代表早期凋亡细胞 V(+) PI(－)，右上象限代表晚期凋亡和坏死细胞 V(+) PI(+)，左上象限代表细胞收集过程中出现的损伤细胞 V(－) PI(+)。

Based on the analysis of apoptotic and necrotic tumor cells by FCM 24h after treatments, the proportions of apoptotic and necrotic tumor cells were a lot higher and living cells were obviously fewer in all treatment groups than in the blank control group, indicating all treatment groups showed clear effects in killing cancer cells. However the difference in cell apoptosis rates and cell necrosis rates among the treatment groups were not significant ($P>0.05$), which is inconsistent with the general observation results. The mechanisms of PDT killing tumor cells include directly killing tumor cells, damaging tumor vessels and stimulating patients' immune systems, these factors may benefit each other and they are all long-term tumor-controlling mechanisms[7]. The pathological results from this study showed large-scale tumor cell necroses in all treatment groups 24 hours after PDT treatments. Congestion of blood vessels, angiorrhexis in some blood vessels which caused extravasation of erythrocytes, partial vessel thrombosis and abundant neutrophil infiltration near some necrotic regions were observed. Of all the treatment groups, Duteroporphyrin-PDT group showed the highest damage of tumor vessels, Photosoft-PDT group ranked next while no much tumor vessel damage was seen in 5-ALA-PDT group. Different photosensitizers target at different locations of tumors. Many studies have shown photosensitizers such as Photofrin that were systematically delivered mainly damage tumor vessels. Whereas 5-ALA, which needs to synthetize protoporphyrin IX (the actual photosensitive chemical) within tumor cells, damages mainly the cells[8-12]. Blood vessel damage can cause oxygen and nutrient supply deficiency, which will lead to cell death. To distinguish this and the cell deaths directly caused by PDT, some authors called it delayed death[13]. It's possible that the unsatisfactory anti-tumor response of 5-ALA is relative to it's incapability of tumor vessel damage[12]. Some studies have shown that tumor blood flow was reduced 50%~70% when the 5-ALA inside the tumor cells was being activated by light, yet no continuous blood flow decrease was noted during 3h post-treatment observations.Tumor tissues were obtained for immunohistochemical examination 24 hours after treatment, the result of which showed slightly higher vascular density in 5-ALA-PDT group than in blank control group[11], indicating the possibility of 5-ALA-PDT inducing angiogenesis. This explains why despite the insignificant difference in tumor cell apoptosis and necrosis between 5-ALA-PDT and the other two PDT groups from vitro tests, general observation results revealed 5-ALA-PDT group to

be less effective than the other two treatment groups. All these may be because of tumor recurrence caused by residual tumor vessels or angiogeneses. The tumor inhibition rates are also connected to the objects' immune systems[14]. The influence of immune systems on the therapeutic effect of PDT has received increasing attention[15], the mechanism of which is complicated and unclear at present. Neutrophils are the main effector cells of the inflammatory response caused by PDT, their accumulation is also the starting step of the anti-tumor immune response caused by PDT[16]. The pathological results in this study also showed abundant neutrophil infiltration at the sites of tumor necroses.

流式细胞仪检测治疗后 24 h 肿瘤细胞凋亡、坏死的结果提示，各治疗组的凋亡及坏死的细胞所占比例比空白对照组明显增多，而正常活细胞明显减少，说明各治疗组杀伤肿瘤细胞效果明显。但各治疗组细胞的凋亡率和坏死率差异无显著意义(P ＞0. 05) ，与大体观察结果不相符。PDT 杀伤肿瘤的机制包括杀伤肿瘤细胞，损伤血管，以及激发自身免疫功能，这些因素可能互相影响，并且都是肿瘤获得长期控制的机制[7]。本研究病理结果显示，各治疗组在 PDT 治疗后 24 h 出现大范围肿瘤细胞坏死，并可见血管充血，部分血管破裂，红细胞外溢，部分血管血栓形成，部分坏死灶周围有大量中性粒细胞浸润。其中多替泊芬-PDT 组血管损伤最明显，其次是 Photosoft-PDT 组，而 5-ALA-PDT 组血管损伤不明显。不同的光敏剂对肿瘤损伤的主要部位不同，多项研究表明，Photofrin 等通过系统给药后光敏剂损伤的主要部位是肿瘤血管，而 5-ALA 需在细胞内合成真正有光敏作用的原卟啉Ⅸ，损伤的主要部位是肿瘤细胞[8-12]。血管损伤会引起缺氧及缺乏营养物质供应，从而导致细胞死亡，为区别于 PDT 直接引起的细胞死亡，有作者称之为延迟性死亡[13]。5-ALA 未能取得很好的抗肿瘤疗效可能与其不能损伤肿瘤血管有关[12]。有研究显示，5-ALA 在照光过程中肿瘤血流量下降 50% ～ 70%，但照光后持续监测 3 h，血流量没有继续下降。而治疗后 24 h 取肿瘤组织行免疫组化检测显示 5-ALA-PDT 组的血管密度比对照组略高[11]，提示 5-ALA-PDT 可能引起血管再生。为此可以解释，本实验 5-ALA-PDT 组肿瘤体外检测细胞凋亡坏死与其余两治疗组差异无显著意义，而大体观察结果提示 5-ALA-PDT 组疗效较其余两治疗组差，可能是因为体内存在血管残留和再生，引起肿瘤复发。肿瘤最终的抑制率还与免疫系统的作用有关[14]。免疫系统对 PDT 疗效的影响日益受到重视[15]，其作用机制复杂，目前尚未完全阐明。其中，中性粒细胞是 PDT 引起的炎性反应的主要效应细胞，中性粒细胞的聚集也是 PDT 引发的抗肿瘤免疫效应的起始步骤[16]。本研究组织病理学结果也可见肿瘤坏死灶有大量中性粒细胞的浸润。

To sum up, this study verified that PDT with Duteroporphyrin, Photosoft and 5-ALA could all inhibit the growth of S180 sarcoma in mice, and the Photosoft-PDT group, which shared similar therapeutic effect as the Duteroporphyrin-PDT group, showed better effect than the 5-ALA-PDT group. All treatment groups showed significant cancer-killing effects early after the treatments, yet there were some mice that were not cured in all the groups. In order to reach better anti-tumor therapeutic effects, further studies on treatment parameter optimizations (such as methods of administration and dosages of different photosensitizers, light dose, time interval between administration of photosensitizers and irradiation, etc.) and combined applications of different photosensitizers with different mechanisms are

required.

总之，本研究验证了多替泊芬、Photosoft、5-ALA 的光动力治疗都能抑制小鼠 S180 肿瘤的生长，其中 Photosoft-PDT 组比 5-ALA-PDT 组的效果好，与多替泊芬组-PDT 组效果相当。治疗后早期各治疗组杀伤肿瘤细胞效果明显，但各组均有部分小鼠未获得治愈。需要继续深入研究治疗参数的优化，如各光敏剂的给药途径，用药剂量，光照剂量，给药与照光间隔时间等，或不同作用机制的多种光敏剂结合应用以达到更好的抗肿瘤疗效。

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