Original Article

Photodynamic Therapy: A New Approach to Remove Embryos of the Wistar Rat

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Abstract

Background: Photodynamic therapy (PDT) is a promising new cancer treatment strategy which inactivates tumor cells by simultaneoulsy using light and a photosensitizer. The similarity between tumors and newly implanted embryos is notable. Extrauterine pregnancy (EUP) does not have a definite treatment and previous therapeutic options (medical and surgical) have not been effective or suitable. Therefore, PDT is suggested as a possible treatment for EUP.

Materials and Methods: The photosensitizer, hematoporphyrin, was injected locally into the placenta of one selected embryo from a pregnant Wistar rat (E15). Then, a laser beam was illuminated at the same point and 48 hours later, changes in the embryo and placenta were investigated. Furthermore, the integrity of the uterus was examined by macroscopic evaluation and sonographic images.

Results: Sections obtained from treated and control groups demonstrated that the embryo and placenta were damaged in the PDT group, whereas the control ones were intact. Furthermore, macroscopic observations and sonographic images during the second parturition after treatment showed that the uterus was intact and fertility was preserved.

Conclusion: Successful ablation of the treated embryo with no clear damage to the uterus attests to the success of this approach. The successful use of hematoporphyrin, as a first generation photosensitizer, should be further investigated for its possible clinical applications.

Keywords: Photodynamic Therapy (PDT), Ectopic Pregnancy, Embryo, Placenta, Fertility

Introduction

In recent years, photodynamic therapy (PDT) has been a promising new modality for cancer treatment. PDT is based on the combination of a photosensitizing agent (photosensitizer) that is preferentially taken up by tumor cells and the visible light of a wavelength matched with the absorption spectrum of the drug. In fact, individually these two factors are harmless; whereas, when combined in the presence of oxygen, they produce cytotoxic products. These products cause permanent cellular damage and tumor destruction (1).

PDT is suitable as a practical treatment for different types of cancers. PDT destroys tumor cells by forming cytotoxic reactive oxygen species, which are caused by the effect of the photosensitizer that is uptaken and retained by tumor cells, followed by light irradiation of an appropriate wavelength (2).

The photosensitizer, activated by light, interacts with molecular oxygen to produce a cytotoxic, shortlived species named singlet oxygen. PDT may cause both apoptotic and necrotic responses within treated tumors and produces microvascular injury resulting in inflammation and hypoxia (3).

Photodynamic therapy is actually a modality that consumes oxygen, which causes local hypoxia. The features of photosensitizer localization in tumor tissue and photochemical formation of reactive oxygen species combining with close delivery of laser-generated light can produce a treatment offering local tumoricidal activity (4, 5).

Extrauterine pregnancy (EUP) is still a common cause of morbidity and mortality. Between the years 2003 and 2005, early pregnancy bleeding was the one of the commonest cause of maternal death in developed countries, of which over 60% of these cases were due to ruptured tubal ectopic pregnancies, and is the most common cause of

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pregnancy-related first trimester death (6).
Ectopic pregnancy (EP) is a common event which threatens fertility and can be fatal. EP occurs in approximately 1.3-2% of all reported pregnancies in the developed world. EPs have increased over the past three decades from 4.5 per 1000 pregnancies in 1970 to 16.8 per 1000 in 1989. There are at least two causes for this increase: i) there have been an increase in the prevalence of different risk factors that cause EP and ii) an improvement in diagnostic methods such as sensitive pregnancy tests and transvaginal ultrasound which result in earlier detection (7, 8).
Some of the important risk factors include a history of pelvic inflammatory disease, assisted reproductive technology (ART), previous EUP and pelvic surgery. Early diagnosis, by transvaginal ultrasonography and serum β-hCG monitoring, can enhance fertility preservation through more conservative performances prior to fallopian tube rupture. Therapeutic options for EP are medical (methotrexate) or surgical therapy (9).
Adverse effects associated with methotrexate include acute abdominal pain and impaired liver functions, to name a few (9).
Many EUP patients need conservative or radical surgery. One major risk of conservative surgery is the incomplete placental removal which therefore, necessitates additional procedures. Radical surgery, while effective, usually impairs fertility. All surgeries involve the risks of anesthesia, hemorrhage, pelvic adhesions and mechanical infertility, in addition to prolonged hospitalization and increased cost which make surgery significantly more difficult when compared with other medical treatments. These limitations require a search for novel treatment options. One possible approach is PDT, which is primarily known for its applications in cancer therapy and age-related macular degeneration (AMD) (9).
However there are similarities between tumors and newly implanted embryos. Both are cell masses which divide quickly, invade surrounding tissues and have newly developing vascular systems. It was therefore logical to examine PDT for EUP termination (9).
Thus, our object is to ablate the selected embryo in a pregnant rat, without damage or harm to the uterine integrity and animal's fertility, with the intent to create a model to treat the EP in humans.

Materials and Methods

All animal care and surgical interventions were undertaken with the approval of the TMU Institutional Review Board and Institutional Ethics Committee. A pregnant rat (E15) was anesthetized by injecting ketamine and xylazine and placed in the supine position. The lower abdomen was shaved and washed with 70% ethanol and iodine. A midline incision about 2 cm in length, in the lower abdomen was performed. The uterus was gently drawn out from the abdominal cavity and placed on the abdomen.
The photosensitizer, hematoporphyrin (Sigma-Aldrich, USA) an iron-free derivative of heme, a product of the decomposition of hemoglobin (mol. mass: 598.69 g/mol), which was preferentially prepared and used freshly. Hematoporphyrin (1 g), was dissolved in 0.1 N sodium hydroxide and the solution was neutralized to pH 7.1 with 0.1 N hydrochloric acid. Sodium chloride was added to make the final solution isotonic. Hematoporphyrin was administered in an approximate dose of 10 µg/g body weight (10).
An embryo was selected, whose placenta was identified visually. The photosensitizer (30 µl prepared solution) was injected trans-uterine into the placenta using an insulin syringe. Then, 5 minutes later, a laser beam [Nd: YAG (GP532.50.CW, Germany), 532 nm, 50 mW] was delivered locally to the same point for 15 minutes.
At least four embryos were selected from each rat and subjected to the following treatments: light control (just taken laser light), drug control (just taken drug: hematoporphyrin), PDT (drug and light; D/L), sham (just taken the solution made for solving the photosensitizer: the solvent) and the rest of the embryos were considered as control group.
After treating the groups, the uterus was eased back into the abdominal cavity. The abdominal muscle facia and skin were sutured [sterile 4-0 braided silk thread (Al Ashaer, UAE)]. The rat was placed in a cage, being kept in the dark and monitored closely for 48 hours.
After 48 hours, the animal was sacrificed and the abdomen was surgically opened. The treated embryos were removed and placed in bouin's fixative for 20-24 hours. Fixed tissue samples were placed in ascending levels of alcohol, paraffin embedded and 4 mm thick slides were prepared. Slides were stained with hematoxylin and eosin (H&E), and then monitored and evaluated with a light microscope (Zeiss, Germany).
A group of 20 rats experienced these steps, whereas, we considered 10 post-PDT rats not to be sacrificed, to pass following events.
These treated rats were actually allowed to reach parturition (~E21.5) and pup weaning (P21).
Fertile males were then introduced into the

cages and rats were allowed to mate until pregnancy could be verified by observing vaginal plugs. Pregnancy in both uterine horns was verified by sonography images (Vouloson 730 Pro, USA) and/or by animal sacrifice and macroscopic examination.
To fully prove fertility of rats post-PDT, those examined via sonography were allowed to bear the second litter to parturition and pup-weaning.

Results

The operated animal were sacrificed 48 hours after treatment and the treated and control units were excised. Obvious differences in size and color were observed in the treated embryos. In the control and sham units, the placenta and the embryo were intact. Light and drug controls were intact either. While in treated groups (PDT; D/L group), the placenta and the embryo were both damaged in appearence. Post-PDT embryos were discolored, extremely loose and fragile which was in contrast with the control intact units (Fig 1).
These macroscopic observations were verified by sections made through histological analyses.
Upon histological evaluation of the sections it was clear that organs and structures in the control units, in contrast with the post-PDT embryos, were normal and distinguishable. As shown in fig 2, see intact organs such as the heart, vertebra, liver and lung in the control sections are seen, whereas in the post-PDT units, only a shadow of the vertebra can be visualized.
Additionally, sections from the placenta showed observabel changes between the groups. The two main layers of placenta, including labyrinth and junctional zones, were damaged and changed in the PDT groups when compared with the control groups (Fig 3).

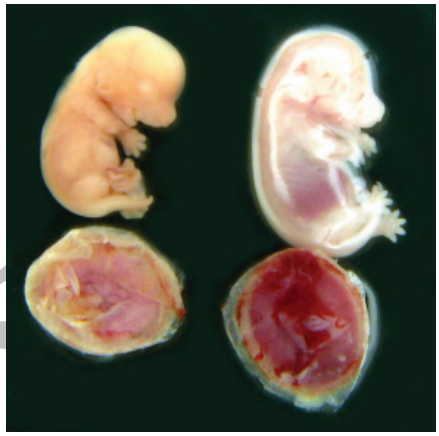

Fig 1: Comparison of control and post-PDT units at the 17th day: The left embryo and placenta were treated. They are discolored and fragile. No clear organ is distinguishable through the opaque embryo. The right are control and normal. The embryo is transparent and internal organs such as the liver are distinguishable.

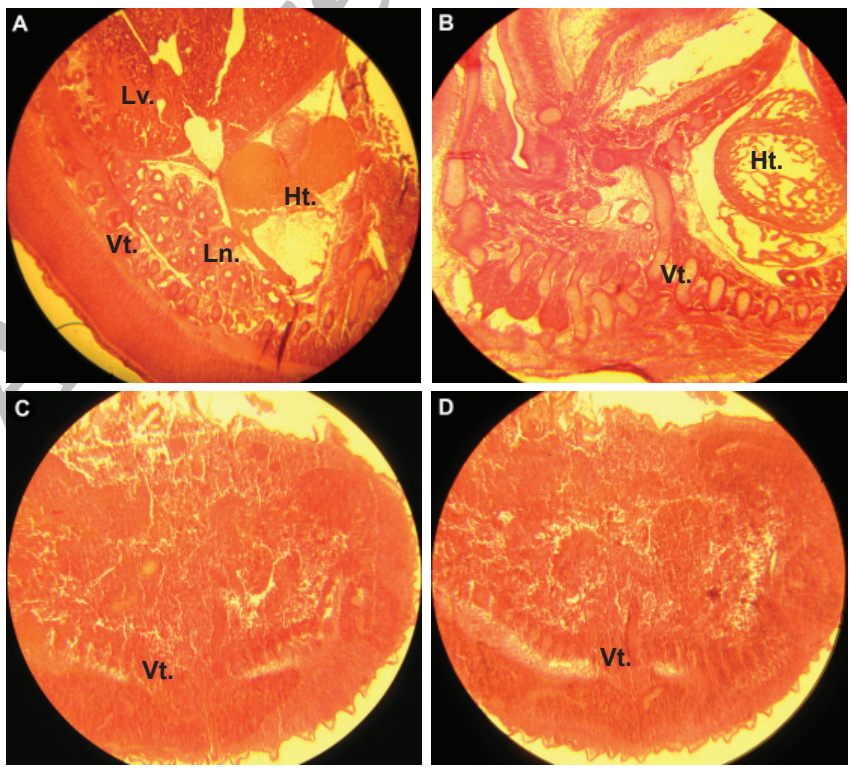

Fig 2: Photomicrograph of 17 day-old embryos. (A,B) Intact organs and structures from the control group. (C,D) damaged structure from L/D (PDT) group. Vt., vertebra; Ln., lung; Ht., heart; Lv., liver.

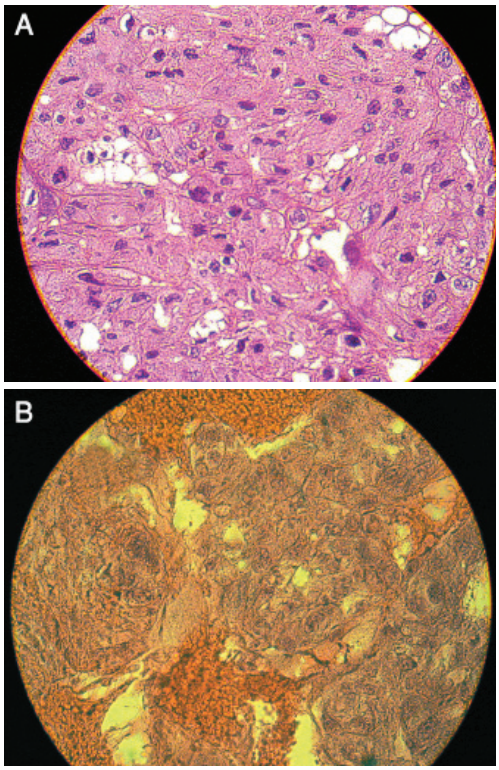

Fig 3: Photomicrograph of junctional layer of (A) an untreated placenta. (B) PDT-treated placenta.

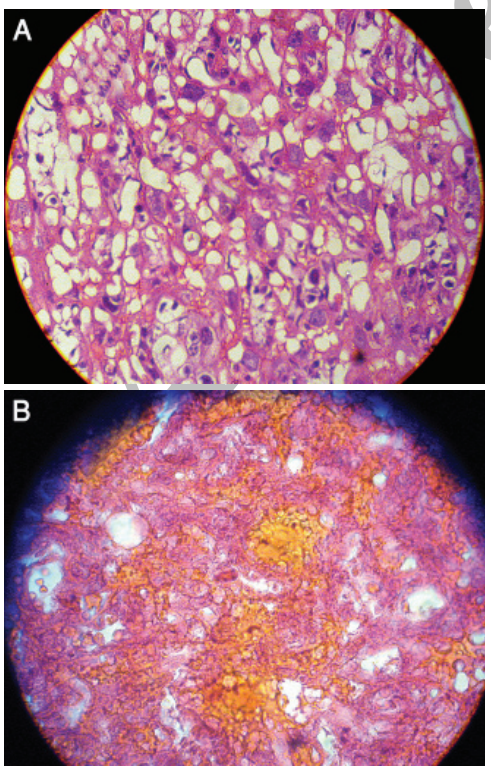

Fig 4: Photomicrograph of labyrinth layer of the (A) untreated placenta. (B) PDT-treated placenta.

In fact, there are two morphologically and functionally distinct regions of the rat placenta: the junctional and labyrinth zones. The junctional zone is the major site of placental hormone production over late pregnancy, whereas the labyrinth zone is the major site of feto-maternal exchange.
The junctional zone, with a vacuolized appearance, and the web-like labyrinth zone were both disarranged and deformed in the PDT group.
Furthermore, the embryos were measured and compared in the case of length and weight rate. The mean length was significantly lower in PDT embryos compared with all four other groups, particularly versus the control group, as shown in figures 5 and 6.

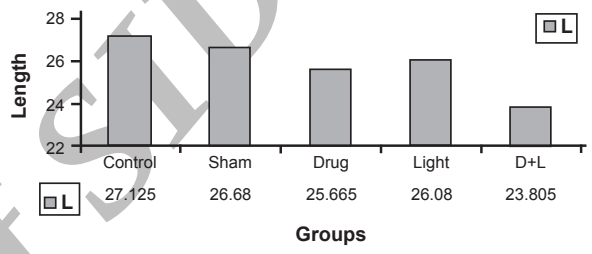

Fig 5: Mean embryo lengths (CR; Crown-Rump) in five groups, 48 hours after surgery

Similarly the mean weight is significantly different and lower in post-PDT units when compared with all other groups (Fig 7, 8).
A total of ten post-PDT rats were not sacrificed and kept alive in a cage with a male adult rat and allowed to mate again.
Pregnancy was verified in all rats by observing vaginal plugs. They were subsequently divided into three separate groups. Sonography was performed for three pregnant rats and the images demonstrated that the embryos were formed in both horns of the uterus which were under treatment in the previous pregnancy (Fig 9). Whereas, three post-PDT animals were sacrificed and checked macroscopically, which similarly verified the existence of embryos in both uterus horns.

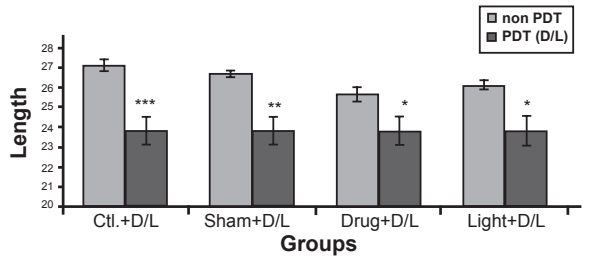

*Fig 6: Comparison of embryo length (CR) in four groups [control, drug, light (non-PDT)] individually with the PDT group (L/D). There is a significant difference in each graph. *** p<0.001, ** p<0.01, * p<0.05.*

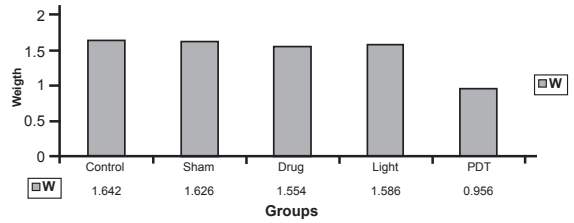

Fig 7: Mean of embryos' weight (CR) in five groups, 48 hours after surgery.

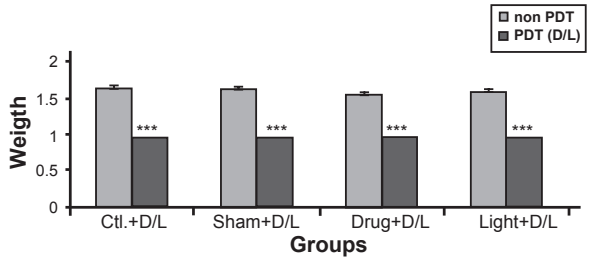

*Fig 8: Comparison of embryos' weight (CR) in four groups [control, drug, light (non-PDT)] individually with PDT group (L/D). There is a significant difference in each graph. *** p<0.001.*

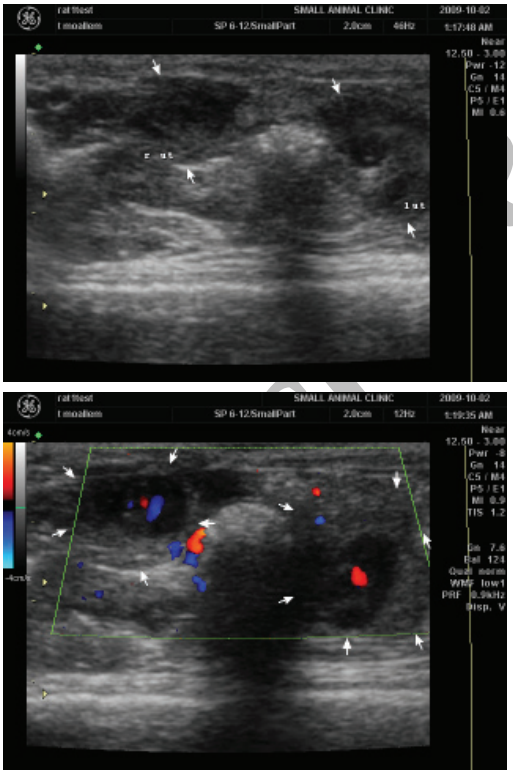

Fig 9: Fertility assessment in post-PDT rats. Sonography of uterus in a treated rat in a second pregnancy cycle (following PDT, parturition and subsequent mating, ~E17. A) The arrows illustrate the boundary of right and left uterus containing embryos. B) Doppler color images; the colors indicate the existanse of blood flow through the embryo's body. The upstream vessels are visible in red, whereas the downstream vessels are blue. These two images attest to the presense of embryos in both uterine horns.

Furthermore, six post-PDT animals were allowed to mate for several successive generations, experienced successful pregnancies, parturition and pup-weaning, all of which showed that fertility was perfectly conserved.

Statistical analysis was performed using INSTAT software and one-way ANOVA in order to determine the possible significant differences in length and weight of the embryos.

Discussion

This study is a novel approach using PDT for ablation of rat embryos as a model for EUP treatment in humans. Additional studies are needed with endoscopic devices in humans, administrating non-invasive and trans-vaginal.

In the just published report combining EUP and PDT, systemic administration of 5-ALA (5-aminolevulinic acid, as a photosensitizer) and illumination of the entire uterine horn in a pregnant rat were applied (11). This technique caused massive endometrial ablation with the loss of all embryos in the treated horn. Additionally, one third of the treated animals in this study did not conceive following PDT treatment. These problems mainly resulted from the protocol which was used with 5-ALA (systemic administration and illumination of the entire uterine horn).

In order to obtain better results, local injection of the photosensitizer into the placenta seems to be a suitable solution.

Glinert et al. who have used PMRDA as the photosensitizer, obtained good results. Their practice was based on localized drug delivery, and injection of the photosensitizer directly into the placenta. The observations around the preservation of fertility provided good evidence for post-PDT uterine integrity (9).

Despite the prolonged skin phototoxicity caused by hematoporphyrin, as the first generation drug, we used it as our photosensitizer, analysing the outputs caused by using it through the same protocol described by Glinert et al. which generally led to identical results.

The prolonged phototoxicity of hematoporphyrin is actually notable when it is administered systemically.

In this study, post-PDT animals were kept in the dark from one week initially and two days at the end. In this study, the injection of a low dose locally into the placenta did not cause damage or harm in the case of phototoxicity.

Histological analysis of treated embryos showed that the post-PDT units of embryos and placentas were successfully ablated, whereas the evalua-

tion of post-parturition treated animals attests to the lack of lasting structural damage to the uteri. Post-parturition treated animals mated again, and underwent successful pregnancy stages and parturition, which were verified by sonographic and macroscopic observations.

Conclusion

This method needs additional research before it can be useful for humans. A minimally invasive clinical approach, through trans-vaginal endoscopic delivery would be useful and acceptable.

Finally, since there is no successful treatment for EP, which has a high prevalence among pregnant women, the use of PDT can be an effective option with which to treat EP.

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There is no conflict of interest in this article.

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