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Photodynamic ablation of a selected rat embryo: a model for the treatment of extrauterine pregnancy

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BACKGROUND: To test the feasibility of photodynamic therapy (PDT)-based ablation of rat embryos as a model for PDT of extrauterine pregnancy (EUP) in humans. METHODS: A controlled pre-clinical study. Selected rat embryos [one per litter, $n = 30$, embryonic day 14 (E14)] were subjected to placental injection of a Palladium-bacteriochlorophyll derivative and illuminated to achieve selective photo-ablation. Histopathology studies were performed 48 h after treatment (E16). Parturition (E21) and breeding ($\sim$12 weeks) after treatment were also evaluated. RESULTS: Using direct placental injection, nearly 80% of the treated rat embryos were selectively photo-ablated, leaving the remaining litter unharmed to achieve normal parturition. Treated animals retained fertility and normally implanted in both treated and untreated uterine horns attesting to the confined toxicity inherent to this approach. CONCLUSIONS: Although requiring respective adaptation to clinical application in terms of treatment protocols and designated hardware, photodynamic interventions using novel bacteriochlorophyll-based photosensitizers may prove applicable to treatment of EUP, as well as other gynecological pathologies and malignancies in a safe, minimally invasive manner.

Keywords: extrauterine pregnancy; photodynamic therapy; fertility preservation; rat

Introduction

Extrauterine pregnancy (EUP) is the abnormal implantation of an embryo outside the uterus. The high prevalence of EUP ($\sim$10–20/1000 pregnancies) (Pisarska *et al*., 1998) makes it the leading cause of pregnancy-related maternal death during the first trimester and the second leading cause of overall pregnancy-related maternal mortality in the USA. Major risk factors include a history of pelvic inflammatory disease and assisted reproductive technology, as well as previous EUP and pelvic surgery (Bouyer *et al*., 2003). Early diagnosis, by transvaginal ultrasonography and serum β-hCG monitoring, usually early in the first trimester (Carson and Buster, 1993), allow for conservative intervention prior to Fallopian tube rupture, enhancing fertility preservation. Treatment options consist of medical (methotrexate) or surgical therapy. The adverse effects of methotrexate include acute abdominal pains, impaired liver functions, stomatitis, cytopenia and, rarely, pneumonitis (Miller and Griffin, 2003). Methotrexate is only applicable in asymptomatic patients presenting with embryonic mass size of <4 cm, absence of fetal heart beat and low blood β-hCG levels (Miller and Griffin, 2003).

Many EUP patients require conservative or radical surgery. The main risk in conservative surgery is incomplete placental removal and persistent disease ($\sim$15% of patients) necessitating further surgery or methotrexate treatment (Hajenius *et al*., 2000). Radical surgery, while effective, usually impairs fertility (Bangsgaard *et al*., 2003, Fujishita *et al*., 2004). All surgery entails risks of anesthesia, hemorrhage as well as pelvic adhesions and mechanical infertility (Bangsgaard *et al*., 2003). Prolonged hospitalization and recovery times make surgery significantly more costly compared with medical treatment (Lecuru *et al*., 1998, Lecuru *et al*., 2000).

These limitations demand search for novel treatment options. One possible approach is photodynamic therapy (PDT), best known for its applications in cancer therapy (Dougherty *et al*., 1998, Dolmans *et al*., 2003) and macular degeneration (Bressler, 2002, Dougherty, 2002).

In the USA, there are several Federal Drugs Agency (FDA) approved PDT drugs with others still in development (Bressler, 2002, Oseroff *et al*., 2006). In PDT, a non-toxic photosensitizer drug locally combines in the treatment site with focused light of a matched wavelength to induce cellular/tissue damage in an oxygen-dependent manner. Light can be delivered interstitially via optic fibers in a highly controlled and minimally-invasive manner (Dolmans *et al*., 2003). Photosensitization elicits transfer of energy or an electron to molecular

oxygen, generating cytotoxic reactive oxygen species (ROS). The short half-life of ROS in the biological milieu (<0.04 μs) restricts diffusion distance to <20 nm, confining the damage to the illuminated area (Dolmans *et al*., 2003). Upon i.v. or local administration of the photosensitizer depending on the drug and the treatment protocol, phototoxicity can be directed toward targeted tumor cells or the tumor vasculature (Abels, 2004).

First generation photosensitizers [such as Photofrin© (Dolmans *et al*., 2003)] posses several drawbacks including: limited tissue penetration (due to absorption in the visible spectrum) limiting treatable tumors volumes, slow accumulation in the tumor (1–3 days prior to treatment) and very slow clearance from the circulation (weeks) inducing prolonged skin phototoxicity, a known side effect of PDT (Moan and Peng, 2003). These drawbacks prompted the development of new photosensitizers, including the bacteriochlorophyll-based family, developed by us (Rosenbach-Belkin *et al*., 1996; Scherz and Salomon, 1997, 1998; Zilberstein *et al*., 1997, 2001; Schreiber *et al*., 2002; Koudinova *et al*., 2003; Preise *et al*., 2003; Scherz *et al*., 2003; Plaks *et al*., 2004; Mazor *et al*., 2005; Vakrat-Haglili *et al*., 2005). These drugs target the tumor vasculature resulting in rapid vascular stasis (minutes) and hypoxia, culminating with necrosis and tumor eradication (weeks). Bacteriochlorophyll-based photosensitizers have high quantum yields (Vakrat-Haglili *et al*., 2005) and absorb in the near infra-red (750–765 nm) enabling deep light penetration ($\leq$20 mm) with large volume tumor ablation (Dougherty *et al*., 1998; Zilberstein *et al*., 2001; Chen *et al*., 2002). These photosensitizers act immediately upon injection in the target vasculature, not requiring time for tissue accumulation (Photofrin©) or time to metabolize into an active form [5-aminolevulinic acid (5-ALA) (Morton, 2002, Lopez *et al*., 2004)]. Moreover, these photosensitizers clear rapidly from the circulation (minutes to hours), practically eliminating skin photosensitivity (Mazor *et al*., 2005). Vascular-based PDT with Palladium (Pd)-bacteriochlorophyll- based Tookad (WST09), developed in collaboration with Steba-Biotech, France, is presently in phase II clinical trials for treatment of prostate cancer (Gertner *et al*., 2003; Weersink *et al*., 2005a, b; Trachtenberg *et al*., 2007) in the UK (Pendse *et al*., 2007) and recently also approved by the FDA for a multi-center study in the USA.

The analogy between tumors and newly implanted embryos is striking: both are masses of rapidly dividing cells, invading surrounding tissues, with a developing neo-vascular system (Lala *et al*., 2002). It was therefore logical to examine PDT for EUP termination. The only previous report of photoablating rat pregnancies applied systemic administration of 5-ALA resulting in indiscriminate removal of all pregnancies with high infertility rates (only 66.2% of animals implanting in the treated horn, with ~28% fewer embryos per litter), indicative of lasting endometrial damage (Yang *et al*., 1994). The use of 5-ALA-PDT as a potential treatment for endometriosis was later reported (Yang *et al*., 1996; Reid *et al*., 2000; Krzemien *et al*., 2002).

Lacking a suitable laboratory animal model for EUP (Wang *et al*., 2004), we decided to attempt the ablation of a single selected embryo in the pregnant rat model by local placental PDT. Although not functionally homologous, an analogy can be drawn between human Fallopian EUP and rat embryos, being of similar size [~2 cm diameter at embryonic day 14–15 (E14–15) in the rat, and <4 cm diameter surgically treatable EUP] and implanted in tubular organs.

The goal of this study was to achieve successful feto-placental ablation in the rat uterus without adversely affecting the rest of the litter and consequent fertility, using Pd-bacteriochlorophyll-based photosensitizers. This would suggest the appropriateness of applying PDT to the treatment of EUP.

Materials and Methods

Animals

Pregnant 10-week old female Wistar rats (litter sizes of 10 ± 2) (Harlan Laboratories, Rehovot, Israel) were delivered between E7–E12 and allowed acclimatization of at least 48 h. All animal protocols were approved by the Weizmann Institutional Animal Care and Use Committee (IACUC).

Photosensitizers

Positively charged Pd-bacteriochlorophyll derivative (PMRDA), a novel experimental PDT agent, was synthesized in the laboratory of A.S. This photosensitizer, previously found effective in the treatment of rat mammary carcinoma tumors *in vivo* (E. Neumark, 2004, unpublished work), was received as powder from Steba Laboratories, Rehovot, Israel and kept under argon (Ar) at -20°C in the dark. The photosensitizer was dissolved in methanol and concentration was determined spectrophotometrically (molar extinction coefficient $= 1.2 \times 10^5$ at 747 nm) and further stored as dried aliquots under Ar at -20°C in the dark until use.

Light source

A 1 W 755 nm laser (Ceramoptec, Germany) was used. Light was delivered *trans*-uterine as a beam (6 mm ϕ) by optic fiber illumination equipped with an FD1 frontal light distributor (Medlight S.A., Ecublens, Switzerland) for even light distribution on the target.

PDT protocol

A pregnant rat (E14) was anesthetized by isoflurane inhalation using an IMPAC6 system (VetEquip Inc., Pleasanton, CA, USA) and placed supine; the lower abdomen was shaved, washed with 70% ethanol and draped with sterile gauze. A mid-line incision in the lower abdomen (~2 cm in length) was performed by cutting the skin and abdominal-muscle layers separately. Using round forceps, the uterus was gently drawn out from the abdominal cavity and placed on the gauze. The exposed uterus was continuously irrigated with sterile 0.9% NaCl solution during the procedure (for layout, see Fig. 1A and B) (Geva *et al*., 2005). One embryo was randomly selected for treatment and positioned with the placenta facing up (see picture of the feto-placental close-up, Fig. 1A). As the uterus is bifurcated, the other uterine horn was left in the abdomen for the duration of drug injection and illumination. The placenta was identified visually and the uterus was gently rotated into position as exemplified in Fig. 1A. The laser beam was directed to the exposed placental pole and positioned for therapeutic illumination (Φ 6 mm) using the built-in visible aiming beam (630 nm, 3 mW). No shielding of neighboring embryos was required. The photosensitizer, PMRDA (50 μg dissolved in 30 μl saline) was injected *trans*-uterine into the placenta

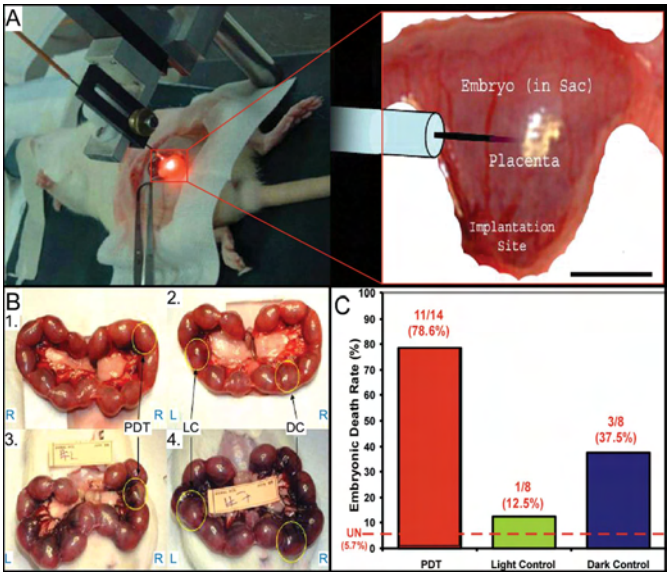

Figure 1: Uterine PMRDA-PDT experimental layout and results. (**A**) The layout of the rat placental PDT procedure is presented during the illumination step (for details see the section Material and Methods). Enlarged picture demonstrating a single feto-placental unit and injection approach (Scale bar 1 cm). (**B**) Exposed rat uteri on embryonic day 14 (E14) with embryos selected for treatment on PDT day (marked by yellow circles, B1) or 48 h after PMRDA-PDT (E16, B3), light/dark controls (LC/DC) before (E14, B2) or 48 h after treatment (E16, B4) are presented. Macroscopic *in utero* analysis of PDT-induced damage to the selected feto-placental unit (note the shrinkage and discoloration, B3) and unharmed embryos following control manipulation (normal size and color, B4) can be observed. (**C**) Uterine PMRDA-PDT summary of results: bars represent embryo-placental unit destruction as embryo death rates, following PMRDA-PDT (red), LC (green) and DC (violet). Dashed line represents background embryonic mortality rate in treated pregnant rats (untreated embryos, UN). PMRDA-PDT, positively charged Pd bacteriochlorophyll derivative-photodynamic therapy

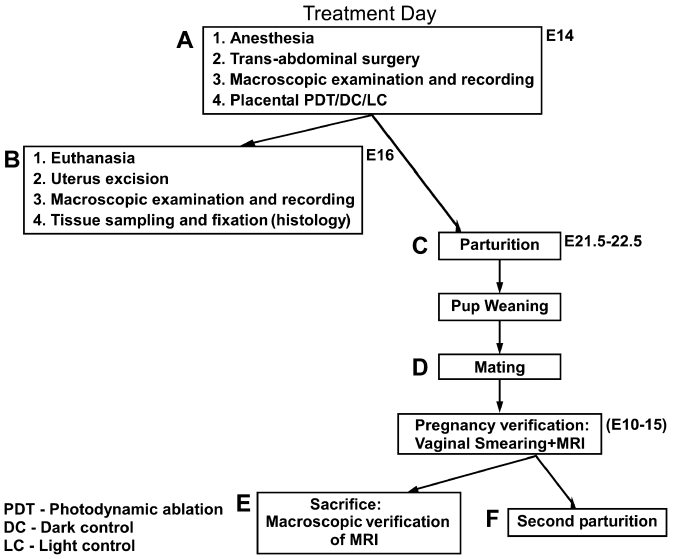

Figure 2: Overview of experimental procedures. (**A**) E14 pregnant rats were anesthetized, subjected to *trans*-abdominal surgery and the uterine horns were exposed, macroscopically examined and photographed. Specific feto-placental units were selected for either PDT (a single embryo per rat), or control manipulations (a single DC or LC embryo per uterine horn, one set per rat) and the rats were allowed to recover for 48 h. (**B**) Rats were either sacrificed (uteri examined, photographed and relevant tissue samples were taken for histological analysis) or (**C**) further followed for ~4 weeks through parturition and pup weaning. (**D**) The same rats were then allowed to mate, conception was verified by vaginal smearing and MRI for identification of bi-uterine implantation. (**E**) Rats were sacrificed to validate the MRI results or (**F**) followed for ~4 weeks through parturition and pup weaning. For further details see Materials and Methods section. MRI, magnetic resonance imaging

using an insulin syringe (30G/0.3 ml). Following a drug/light time interval of 120 s, light (100 mW/cm^2) was delivered (300 s) to the placenta in a *trans*-uterine manner from above, perpendicular to the uterus (Fig. 1A). Upon protocol completion, the uterus was eased back into the abdominal cavity. The abdominal muscle facia was sutured (sterile 4-0 braided silk thread) and the skin was clipped using 9 mm surgical clips. Anesthesia was discontinued and the rat was placed in a cage and closely monitored for at least 48 h. The animals received analgesia (Oxycod©, 6 ml/l) in the drinking water for at least 48 h. Controls: Dark control (DC): placental administration of photosensitizer without illumination. Light control (LC): placental sham injection of sterile vehicle (saline), followed by illumination with the respective light dose. Untreated control (UN): all untreated feto-placental units in a treated rat (Fig. 2A).

Euthanasia

Animals were sacrificed by CO_2 inhalation, in accordance to IACUC guidelines (Fig. 2B).

Post-PDT fertility assessment

Treated rats were 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 daily vaginal smears and palpation. Pregnancy in both uterine horns was verified by magnetic resonance imaging (MRI) and/or by animal sacrifice and macroscopic examination. To fully prove fertility of rats post-PDT, the MRI examined rats were allowed to bear the second litter to parturition and pup-weaning (Fig. 2C–F).

MRI of pregnant rats

Pregnant female rats (E12–E16) were anaesthetized by i.p. injection of ketamine/diazepam (1:1 ratio by vol). MR images were acquired on a horizontal 4.7T Bruker-Biospec spectrometer using a whole-body birdcage RF coil with a diameter of 7.5 cm. Three-dimensional gradient echo T_2 weighted sequence was acquired with 10 ms repetition time, 3.5 ms echo time, 5° flip angle, 2 scans, 128 × 128 × 128 matrix size, 8 × 4 × 4 cm^3 field of view (Fig. 2D).

Histology

Following animal sacrifice, the abdomen was surgically opened, the uterus was tied off at the cervix, removed and placed in carnoy's fixative (6:3:1 ethanol, chloroform and acetic acid, by vol), or Tris-buffered Zn fixative [for the smooth muscle actin (SMA) stain only, 2.8 mM calcium acetate, 22.8 mM zinc acetate and 36.7 mM zinc chloride in 0.1 M Tris buffer, pH 7.4] for 48–72 h. Fixed tissue samples were placed in 70% ethanol, paraffin embedded and 4 μm thick slides were prepared. Slides were stained with hematoxylin and eosin (H&E). Immunohistochemical staining of specific cell markers was done using sheep anti-von Willebrand factor (vWF) factor (Serotec, Oxford, UK), mouse anti-pan-Cytokeratin (clone

AE1/AE3, Zymed, San-Francisco, USA) and mouse anti-human SMA [a smooth muscle cell (SMC) marker] (clone 1A4, Serotec). Secondary antibodies were the goat anti-mouse Histofine® Universal Immunoperoxidase Polymer (Nichirei corp., Tokyo, Japan) for SMA and anti-pan-Cytokeratin staining) or goat anti-mouse-Alexa-fluor® 488 (Molecular Probes, Oregon, USA) for vWF factor staining. All pathological examinations and reports were conducted by Dr O. Brenner, Weizmann Institute Pathology Service Unit.

Light microscopy

Microscopic images were taken using a Nikon ECLIPSE E600 microscope equipped with a Nikon DXM1200F digital camera (Nikon Instech Co., Kanagawa, Japan).

Photography

Macroscopic images were taken using a Fujifilm MX-2900 ZOOM digital camera (Fuji Photo Film Co., Tokyo, Japan).

Results

The response of in utero fetoplacental units in the pregnant rat to local PMRDA-PDT

The feasibility of employing PMRDA-PDT for the ablation of a single embryo per litter in an E14 pregnant rat was first tested (Figs 1A, 2A and B). PDT-ablated feto-placental units and occasional dead control embryos appeared discolored and shrunken, undergoing uterine absorption (at E16). Otherwise UN, LC and DC control embryos appeared unaffected. Uterine tissue in all PDT and control rats appeared undamaged, including the site of treatment (Fig. 1B).

In summary, treating a single embryo with PMRDA-PDT per rat resulted in pregnancy termination in 11/14 cases (78.6%). Embryo mortality rates in LC and DC controls (one embryo per uterine horn, 2 per litter) were 1/8 (12.5%) and 3/8 (37.5%), respectively (Fig. 1C). In a total of 22 litters (14 PDT and 8 control rats), the death rate of UN was 13/230 (5.7%) (Fig. 1C, dashed line).

Histological analysis of rat feto-placental units following local in utero PMRDA-PDT

Post-PDT uteri were fixed and segments containing the respective feto-placental units were subjected to detailed histopathological examination. The normal placenta appears intact, with the following features discernable: (i) a heavily vascularized pregnant uterine wall (Fig. 3A), (ii) labyrinth layer, where fetal blood vessels cross maternal blood pools and exchange takes place, with fetal nucleated red blood cells marking the fetal vasculature (Fig. 3D), (iii) spongiotrophoblast layer, with its characteristic 'hollow-cell' appearance (Fig. 3F) and (iv) the fetus, with normal well-defined anatomy, such as vertebrae (Vt.), heart, lungs and liver [Fig. 3B and H (higher magnification)].

In post-PMRDA-PDT placentas severe damage was observed. A fully necrotic embryo was observed, presenting ill-defined structures, in a state of disintegration and necrosis, undergoing absorption [Fig. 3C and I (higher magnification), uterus and Vt.]. The placenta featured edema, congestion and moderate but extensive necrosis, involving ~70% of its mass (Figs 3E and G). The surviving feto-placental LC and DC

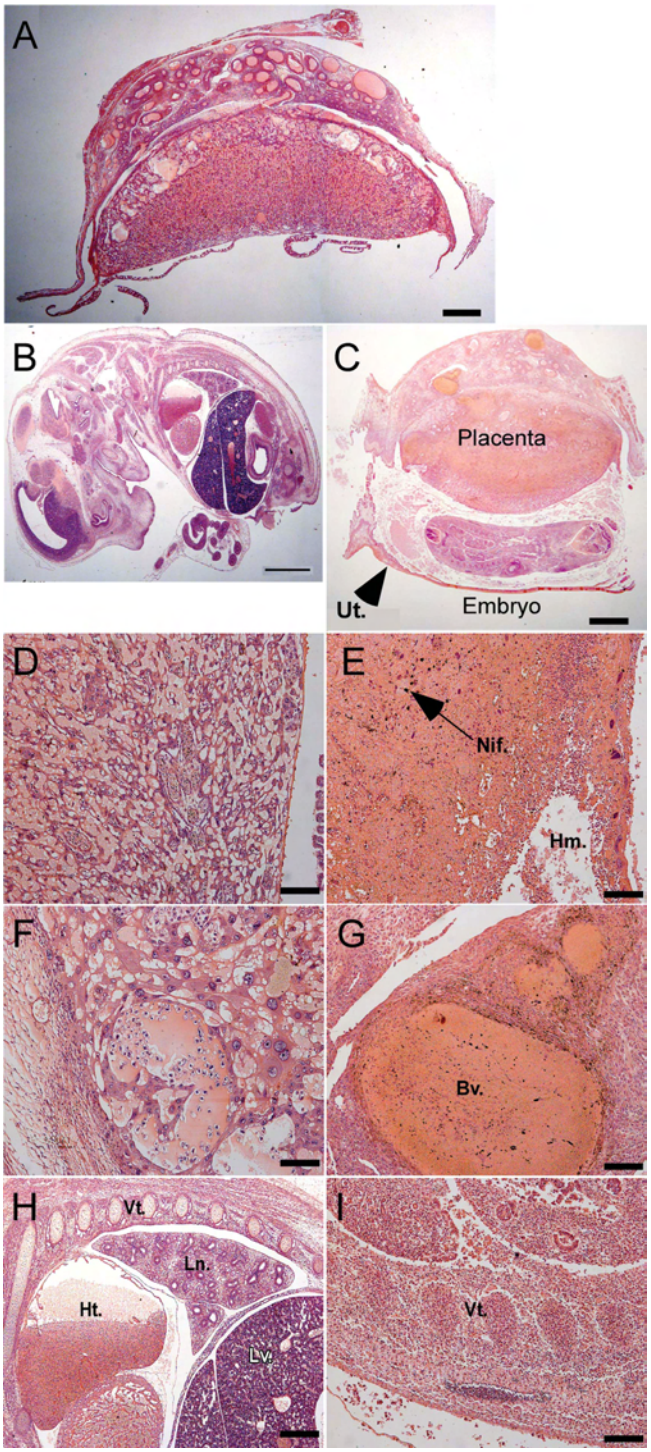

Figure 3: Histopathological analysis of utero-placental tissues following PMRDA-PDT.
(**A**) Overview of intact placenta at E16. (**B**) Overview of intact embryo at E16. (**C**) Overview of PMRDA-PDT-treated placenta and embryo at E16 (Ut., uterus). (**D**) Magnification of labyrinth layer of an untreated placenta. (**E**) Magnification of PMRDA-PDT-treated placenta with immune-cell-infiltrate (Nif.) and visible hemorrhage (Hm.). (**F**) Magnification of spongiotrophoblast layer of an untreated placenta. (**G**) Magnification of PMRDA-PDT-treated placental blood vessel (Bv.) in the spongiotrophoblast layer. (**H**) Magnification of well defined, intact structures (Vt., vertebra; Ln., lung; Ht., heart; Lv., liver) of an untreated fetus. (**I**) Partially dissolved, heavily necrotic PMRDA-PDT-treated embryo, containing ill-defined structures. Scale bars: in A–C 1 mm, in D–I, 0.1 mm

controls presented normal histology, featuring no pathological findings. In cases where feto-placental controls were fatally damaged, histological examination revealed a fully necrotic and/or autolyzed embryo, with small to moderate amounts of necrotic embryonic material at the edges of the placenta, containing a few necrotic cells in the spongiotrophoblast layer. The uterus and placenta remained viable (data not shown).

Importantly, the uterus adjacent to the photoablated placenta appeared intact (Fig. 3C) with only minimal to mild multi-focal neutrophylic infiltration.

It appears that placental PDT induces severe and significant photodamage in placental and embryonic tissues, including hemorrhage (vascular damage), inflammation (immune response) and necrosis (cellular damage) of embryo and placental components, while sparing the uterine tissues. The absence of any apparent injury or damage to the uterus led us to examine the functionality of the reproductive system (parturition and fertility) following PDT.

Focal placental PMRDA-PDT has no deleterious effects on pregnancy outcome and subsequent fertility

To examine the functional impact of PMRDA-PDT on fertility of treated pregnant rats, we examined: (i) the ability of post-PMRDA-PDT rats to achieve successful parturition of the remaining litter (indicative of the functional integrity of the uterine wall in the treated areas) and (ii) the ability of post-PMRDA-PDT rats to conceive again in both uterine horns (indicative of uterine patency). To this end, six pregnant rats (four PDT and two respective control manipulations) were treated and allowed to complete gestation. All six rats achieved normal parturition, delivering 6–11 pups each, without post-parturition maternal or pup mortality. Newborn pups were followed through weaning (P21) with no pathologies or abnormalities found. These results suggested that placental PDT of a selected single feto-placental unit or respective control manipulations induced no adverse effect on the remaining litter or on subsequent parturition. But was fertility conserved?

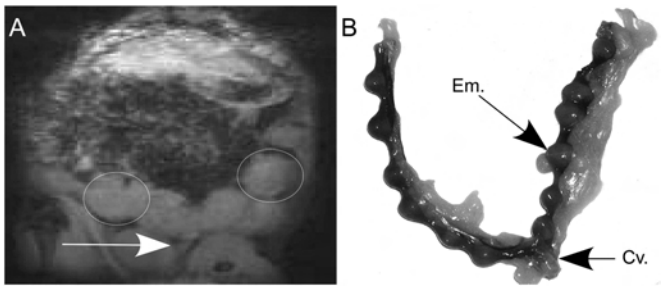

Figure 4: Fertility assessment in post-PDT rats.
(**A**) MRI of uterus in a treated rat in a second pregnancy cycle (following PDT, parturition and subsequent mating, ~E16). Circles mark embryonic sacks *in utero*, arrow marks cervix. Implantation is evident in both uterine horns. (**B**) A uterus from a gestating rat (~E8) was visually examined to verify implantation in both uterine horns. Implanted embryonic sacs are evident in both uterine horns. Em., embryonic sac; Cv., cervix

Following pup weaning (P21), the resumption of the rat's estrus cycle was verified and the rats were allowed to mate, conceive and achieve parturition of an additional litter. All six rats conceived, of which five were subjected to MRI examination to verify pregnancy in both uterine horns (Fig. 4A). Of these rats, two post-PDT and one control were sacrificed for visual inspection (Fig. 4B). The remaining three rats were found to normally complete parturition and maternal care through pup weaning. These experiments demonstrated that focal placental PMRDA-PDT has no deleterious impact on rat fertility, specifically without compromising the ability of the treated uterine horn to re-implant.

Uteri retain structural integrity following PMRDA-PDT and parturition

Having verified fertility preservation, histopathological analysis was conducted in order to identify long-term damage. Uteri in post-PDT and control manipulated rats were examined ~10 days after parturition (17–18 days after treatment). The uteri were stained by standard H&E and markers for SMC (indicative of uterine wall integrity), epithelial cells (indicative of endometrial integrity and absence of adhesions) and endothelial cells (illustrating blood vessel integrity) (Fig. 5). These uteri featured mild to moderate organizing hemorrhages in the endometrium and mesometrium, typical of post-gravid uteri. The only lesion detected histologically was the presence of a low number of hemosiderin-laden macrophages (siderophages) in the endometrium (where such presence is normal) and in the mesometrium. Siderophages were highest in the DC uterus and milder in the post-PDT uterus. No necrotic areas were found in any of the uteri inspected.

Discussion

This study describes the development of a novel accurate local photodynamic application for the ablation of specific feto-placental units in the pregnant rat. The treatment protocol achieved high success rates with no detectable damage or sustained pathological findings in the uterus, neighboring embryos or to the ability of the dams to deliver the untreated pups. Moreover, fertility was fully preserved.

These results therefore suggest the possibility that this procedure may form a reasonable basis for development of a local photodynamic approach for the clinical treatment of EUP. The proposed protocol, once translated to the clinic, would consist of a *trans*-vaginal local administration of photosensitizer directly into the placenta, using a specially designed endoscopic device. Such an endoscope will contain an imaging module for real-time visualization of the treatment target, a syringe with a pre-loaded dose of photosensitizer and an optic fiber-based light delivery system, connected to an external medical laser light source. Simpler and entailing less potential complications, this minimally invasive procedure will also circumvent the need to inject and illuminate via the Fallopian tube wall, as done in the rat uterus in this study.

The proposed procedure is based on localized drug delivery requiring a minute PMRDA dose, all of which remaining confined to the fetal circulation. In the rat model, we found 50 μg

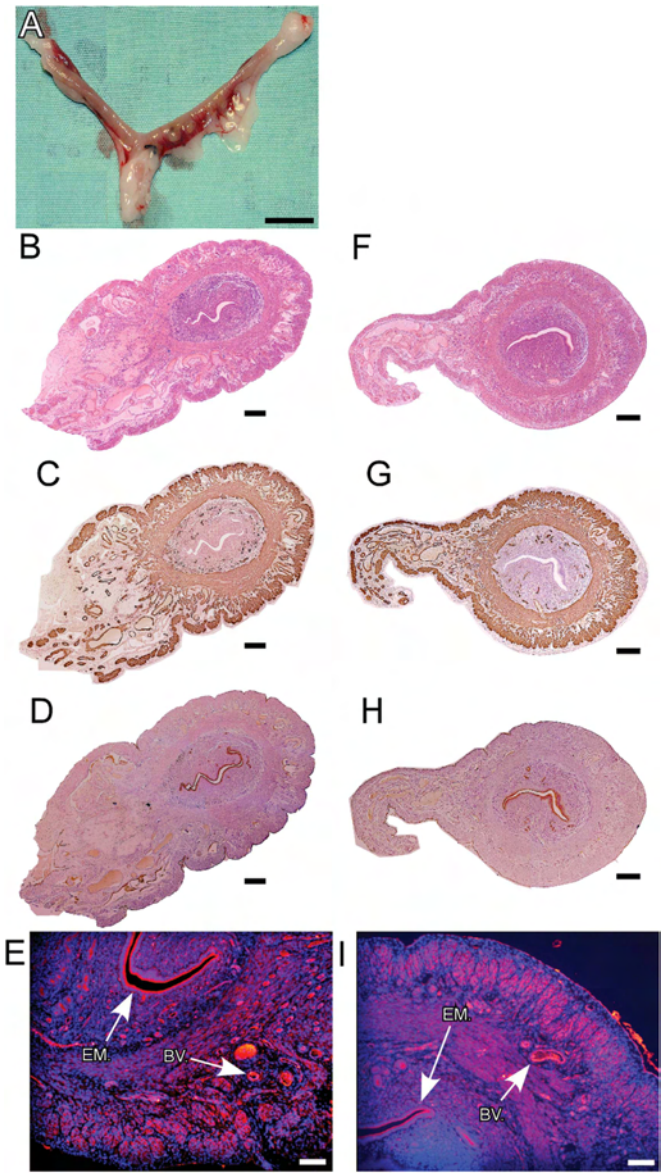

Figure 5: Histopathological analysis of uteri of PMRDA-PDT rats following parturition and pup weaning.
(**A**) Post-PDT uterus sampled ~22 day after parturition (right horn, untreated; left horn, PDT). The uterine horns were separated, fixed in carnoy's fixative, embedded in paraffin and sections prepared from the untreated uterine horn (**B**–**E**) and post-PDT-uterine horn (**F**–**I**) and stained as follows: hematoxylin and eosin (B and F), α-smooth mucle actin, showing smooth muscle layer of uterine wall (C and G), α-pan-cytokeratin, showing uterine epithelial layer (D and H), and α-von Willebrand factor, showing uterine vasculature (E and I). Histological analysis showed no pathological findings in either uterine horn (post-PDT or untreated), both presenting minimal lesions within normal limits, and without any necrotic regions. Scale bars: A, 1 cm; B–D, F–H, 0.2 mm and E and I, 0.1 mm

PMRDA (~100 μg/g embryo) to be sufficient. Even with the entire dose inadvertently injected into the maternal circulation of the rat, blood concentrations should still be ~50 times lower than those efficient for anti-prostate PDT with Tookad, another drug of this family (Koudinova *et al*., 2003). Considering the embryonic masses of the rat (~0.5 g at E14) and the human (~1–4 g, week 8–10), we predict similar drug doses to be efficient in local application of EUP. Notably, the typical drug doses systemically infused during photodynamic treatment of prostate cancer using Tookad, ~2 mg/Kg (~140 mg for a 70 kg patient) (Weersink *et al*., 2005a, b; Trachtenberg *et al*., 2007), are much higher. These factors therefore contribute to the safety of the suggested intervention, considering the target population of potential EUP patients. While different PDT drugs are discussed here, further optimization of future Pd-bacteriochlorophyll-based sensitizers for PDT of EUP will aim for high efficacy and safety with minimal dark toxicity.

The severe cellular and vascular damage to both placenta and embryo, while the illuminated uterus remained intact (Fig. 3), indicated that the photosensitizer is absent in any significant concentration from illuminated maternal tissues. Injection into the placenta is expected to introduce the photosensitizer into the labyrinth and the feto-placental circulation, with some interstitial drug distribution. This interstitial photosensitizer remains photoactive and contributes to treatment outcome. This protocol assumes that treatment success results from the cumulative damage to the feto-placental unit, independent of accurate targeting of a specific compartment. In addition, the injection injury and the innate dark toxicity of the photosensitizer may also contribute to the final result.

Histological analysis of post-parturition-treated animals (Fig. 5) attests to the lack of lasting structural damage to the uteri: (i) an absence of adhesions or scarring as revealed by pan-cytokeratin staining (reducing the risk for consequent EUP), (ii) structural integrity of the uterine wall as shown by SMA staining (no rupture following treatment and parturition) and (iii) an absence of vascular malformations, thrombosis or other long-term vascu-endothelial effects, shown by vWF staining. These support the localized nature of the damage, limited to the selected feto-placental unit, sparing the uterus and inferred to apply in the case of the Fallopian tube. We take the successful parturition of the PDT-treated litters, subsequent pregnancy and successive normal parturition as quality control measure for this treatment.

In the only published account combining EUP and PDT, systemic administration of 5-ALA and illumination of the entire uterine horn in a pregnant rat were used (Yang *et al*., 1993, 1994). This resulted in massive endometrial ablation and the loss of all embryos in the treated horn. Importantly, a full third of the treated animals did not conceive following PDT. These problems mainly resulted from the protocol used with 5-ALA (systemic administration and illumination of the entire uterine horn), but were probably also related to the limitations of this photosensitizer which can be tested in the future using local illumination. Moreover, the considerable drug–light interval required for conversion of 5-ALA (a pro-drug) into proto-porphyrin-IX (the active photosensitizer), probably negates the suitability of its intra-operative application. Indeed, the limitations of currently used photosensitizers, such as 5-ALA, Foscan® and Photofrin®, probably contributed to the fact that application of PDT in gynecological pathologies and malignancies is rather limited. As recently reviewed by Allison *et al*. (2005), these limitations include insufficient depth of light penetration, problematic light dosimetry over irregular surfaces and prolonged skin phototoxicity. Although

not covering photodynamic-EUP treatment, this review portrays an important clinical role for PDT in gynecology, even with the above limitations. Photodynamic applications in gynecology using bacteriochlorophyll derivatives may exceed EUP treatment to include treatment of pelvic endometriosis, uterine fibroid tumors, vulvar, vaginal and cervical lesions and other pre-cancerous tumors of the reproductive system.

The rat model used here presents encouraging results in terms of efficacy and fertility preservation in spite of the invasive and morbid protocol in the rat compared to the suggested clinical procedure: (i) abdominal surgery with *trans*-uterine PDT in the rat, compared to a minimally invasive clinical *trans*-vaginal approach, (ii) *trans*-uterine injection of photosensitizer and illumination compared to *trans* vaginal endoscopic delivery and (iii) ablation of a single feto-placental unit without detectable damage to neighboring embryos or uterine tissue at the implantation site, unprecedented in current EUP treatment options and their common complications. Successive unimpaired parturition and preservation of fertility provide unequivocal evidence for post-PDT uterine integrity (Fig. 5).

This novel PDT procedure therefore shows promise for possible clinical translation to the treatment of women suffering from EUP, offering a highly controlled, local, short and cost effective minimally-invasive modality.

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