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Spectroscopic evidence of photodynamic reactions in rat embryo and maternal tissues

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Background. One of the problematic points of photodynamic therapy is low selectivity of the accumulated photosensitizer – it accumulates in healthy tissues as well. It is necessary to find out if photosensitizer accumulates in embryo and to see if photodynamic reactions take place in an embryo. This was the aim of our study.

Materials and Methods. *Wistar* line pregnant white rats were used in experimental studies, and PhotofrinII® was chosen as a photosensitizer (i.v. administration). Therapy dose (5 mg/kg) of photosensitizer and light was used. The experiments where performed on three experimental animal groups: the first group – rats on the 7th day of gestation (photosensitizer administrated on 6th day of gestation), the second group – rats on the 14th day of gestation (photosensitizer administrated on the 13th day of gestation) and the third one – control rats group on the 7th day of gestation (no photosensitizer administration). Fluorescence spectroscopy methods to evaluate accumulation of a photosensitizer were used, and photobleaching of PhotofrinII® after irradiation was observed.

Results. During our experiments it was obviously shown that photodynamic reactions appear in embryo on the 7th day of embryogenesis while on the 14th day of embryogenesis photobleaching of photosensitizer was not observed.

Conclusions. This study showed that photodynamic reactions occur in embryo, but the possible effect depends on the stage of embryogenesis.

Key words: photosensitizer, photobleaching, embryo

INTRODUCTION

A new method to treat tumours based on administration of light-sensitive drug (photosensitizer), selective accumulation of it in tumour followed up with irradiation of a certain wavelength light that leads to the generation of singlet oxygen and targeted tumour cell death – a photodynamic therapy – is already approved worldwide (USA, Canada, Japan, EU et al.) for skin, brain cancers etc. One of the problematic points of this treatment is low selectivity of the accumulated photosensitizer – it accumulates in healthy tissues as well (1–4). This means that photo oxidation reactions also occur in healthy tissues and may damage them. Therefore, a lot of experiments were made to determine the effect of PDT on healthy tissues, and there are many results indicating that PDT is safe enough in most cases. PDT might be very useful in treating pregnant women, while the other methods are too toxic and teratogenic for foetus. De Santis et al. showed that after the administration of photosensitizer Verteporfin to pregnant women (3rd week of embryogenesis), there were no side effects on newborns (5). On the other hand, Yang et al. demonstrated that after the administration of 5-aminolevulinic acid into pregnant rats following the irradiation, resorptions were observed (6). Graželienė et al. showed that the accumulation of photosensitizers in rat embryo depends on the stage of embryogenesis (7). However, there is lack of information about the accumulation of photosensitizers in the maternal organs at pregnancy period, and more experiments in this field should be performed.

During the process of embryogenesis, placenta formation begins on the 6th day and completes nearly on the 13th day of embryogenesis. The most important subject for the formation of placenta is angiogenesis when inside the chorion's villi blood vessels form. The formed placenta serves as a natural barrier, which starts preserving the embryo from various exogenous harmful factors, ensures tolerance between the mother and the foetus (8). However, this barrier also allows the passage of many

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chemical agents like some embryotoxic and teratogenic antioxidants that have negative effect on the cell proliferation and embryo development (9, 10).

The aim of this study was to find out if photosensitizer dihaematoporphyrin ether (Photofrin II®), accumulated in the embryo and maternal organs, induces photodynamic reactions. Photobleaching of a photosensitizer in tissues was taken as an evidence of initiated photodynamic reaction. Also it was estimated how photodynamic reactions in embryo and maternal tissues correlate with the stage of the embryogenesis.

MATERIALS AND METHODS

Observation of photosensitizer's photobleaching was chosen as a method to evaluate if photodynamic reactions take place in the tissues under examination. Irradiated photosensitizer in oxygenous environment generates singlet oxygen (Fig. 1.) which destroys photosensitizer and lowers its concentration in tissue and fluorescence as well. This phenomenon is called photobleaching signifying that photodynamic reactions appear where photobleaching of photosensitizer is observed (1, 12, 13).

Wistar line white rats (160–240 g) were used in experimental studies, and 50 embryos were examined. During the oestrous cycle, female rats where kept together with male rats for a night.

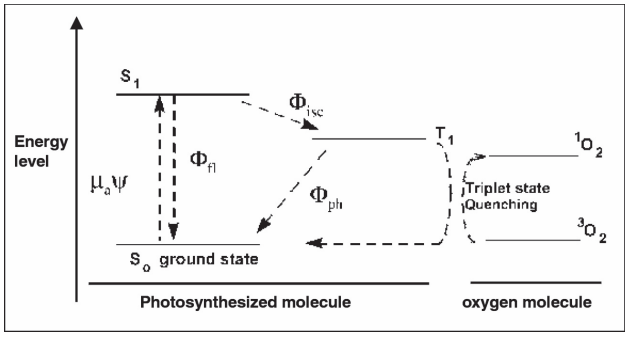

Fig. 1. Singlet oxygen generation scheme. The molecules are excited from the ground state, S_0, to the excited singlet state, S_1. The excited molecule can relax by fluorescent photon emission (with quantum yield Φ_{fl}) or intersystem cross to the first triplet state, T_1 (quantum yield Φ_{isc}). From triplet state, the molecule can either relax by phosphorescent photon emission (quantum yield Φ_{ph}), or be quenched by interaction with a ground state oxygen molecule, $^3O_2$, to produce singlet state oxygen, $^1O_2$ (13)

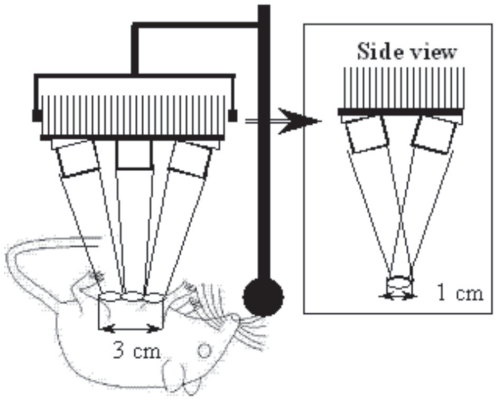

Fig. 3. Scheme of irradiation procedure on rat. The irradiation of 6 light sources (in two rows) is focused on the area of the left horn of the uterus (3 cm² area). The total fluence rate is 120 mW/cm², irradiation time - 28 minutes (energy dose: 201.6 J/cm²)

24 hours later, the presence of spermatozoa in vaginas was checked, and the day when the spermatozoa were found was assumed as zero day of the embryogenesis. The animals where divided into three experimental groups: the first group included rats on the 7th day of gestation (photosensitizer administrated on the 6th day of gestation), the second group consisted of rats on the 14th day of gestation (photosensitizer administrated on the 13th day of gestation) and the third was control rats group on the 7th day of gestation (no photosensitizer administration). We used toxicology experiment methodology to choose the embryogenesis days for our experiments (14).

Photofrin II® (Axan Pharma Inc., Canada) was used as a photosensitizer. It was intravenously administrated to experimental animals by a dose of 5 mg/kg on the 6th and 13th day of embryogenesis. 24 hours after the administration the rats were irradiated, sacrificed, and the tissues were examined.

The uterus of rat consists of two horns (Fig. 2). 1st and 2nd animal groups received the same photosensitizer dose but only the left horn was irradiated. So the embryos and maternal tissues in the left horn were rated as incubated and irradiated while the embryos and maternal tissues in the right horn – as incubated only. This model was chosen, because photosensitizer is not toxic in the dark. Irradiation scheme (Fig. 3) was adjusted to irradiate only the left (3 cm² area) horn with a fluence rate of 120 mW/cm² for 28 minutes (energy dose 201.6 J/cm²). Emission spectrum of light used for irradiation is presented in Fig. 5.

The method to register spectroscopic data was developed by our group. Perkin Elmer spectrophotometer was adjusted to register fluorescence of tissues (Fig. 4). The tissues were frozen,

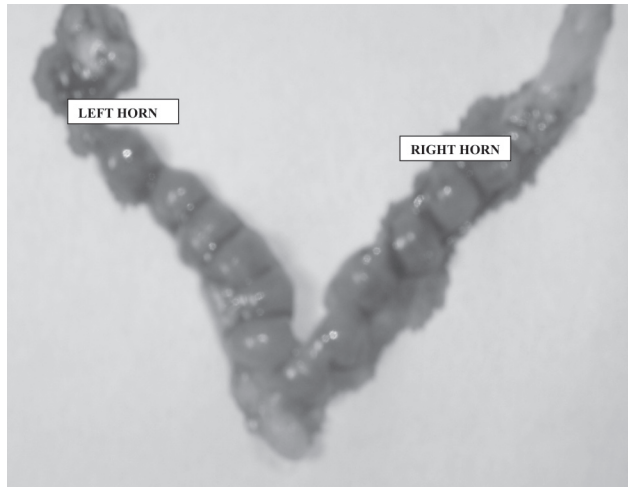

Fig. 2. *Wistar* line white rat uterus

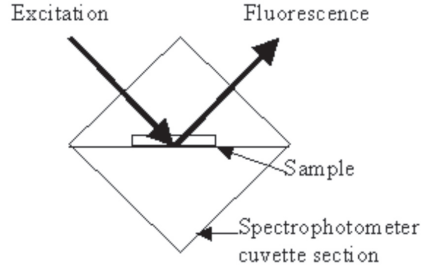

Fig. 4. Fluorescence spectroscopy registration scheme – cuvette section

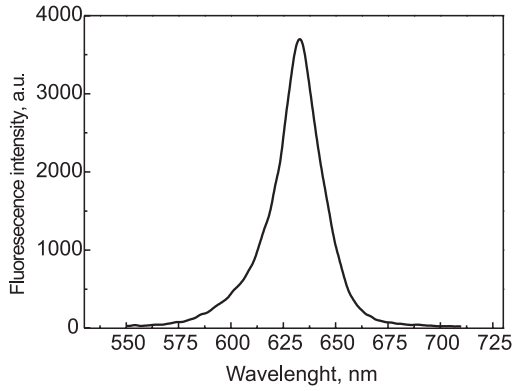

Fig. 5. Emission spectrum of light used for irradiation

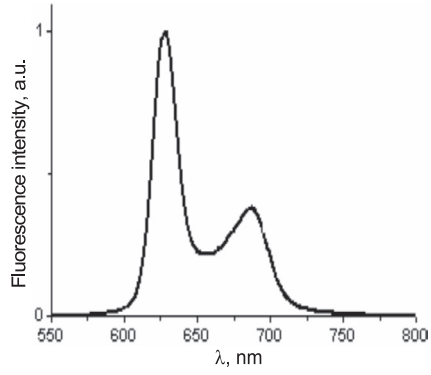

Fig. 7. Photofrin II® fluorescence spectrum in aqueous solution at 10^{-5} M concentration. Excitation wavelength 400 nm

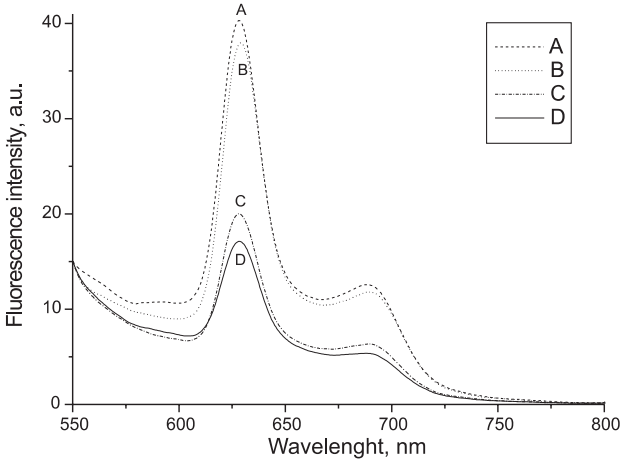

Fig. 9. The fluorescence spectra in rat organs (24 hours after administration of Photofrin II on the 6th day of embryogenesis): A – uterus incubated, B – embryo incubated, C – uterus incubated and irradiated, D – embryo incubated and irradiated

cut into 1 mm slices, placed into Perkin Elmer spectrophotometer cuvette section, and spectra were measured. The autofluorescence intensity differences appeared because of shifting of samples, they were removed by a big number of measurements, averaging spectra and normalizing them to 550 nm. The excitation wavelength of 400 nm was chosen according to absorption maximum of Photofrin II® (Fig. 6). The accumulation of the sensitizer was evaluated according to the fluorescence intensity of the main sensitizer's fluorescence peak at 628 nm (Fig. 7).

The animal husbandry and experiments on animals were carried out according to the national and European regulations and were approved by the Lithuanian Animal Care and Use Committee.

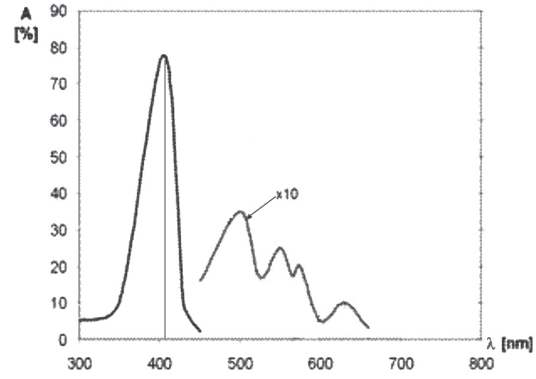

Fig. 6. Photofrin II® absorption spectrum in aqueous solution at $2.6 \cdot 10^{-6}$ M concentration. A – absorption coefficient in arbitrary units (15).

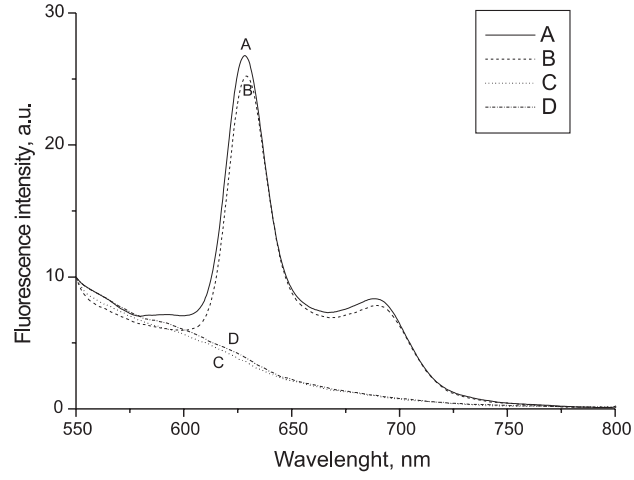

Fig. 8. The fluorescence spectra in rat organs: 24 hours after administration of Photofrin II on the 6th day of embryogenesis in comparison with controls. A – uterus incubated, B – embryo incubated, D – uterus control, C – embryo control

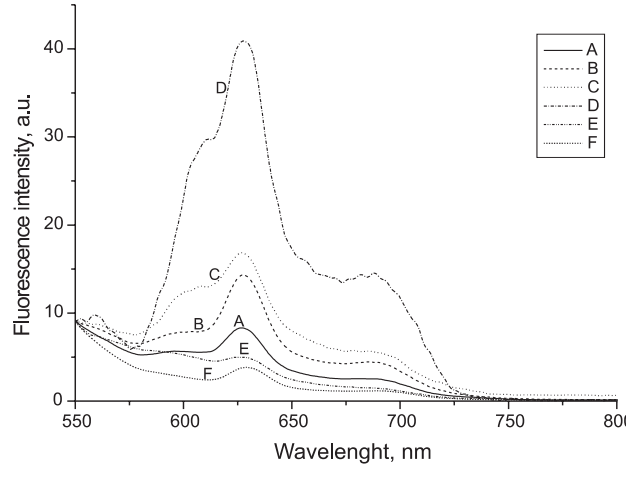

Fig. 10. The fluorescence spectra in rat organs (24 hours after administration of Photofrin II on the 13th day of embryogenesis): A – uterus incubated and irradiated, B – uterus incubated, C – placenta incubated and irradiated, D – placenta incubated, E – embryo incubated and irradiated, F – embryo incubated

RESULTS

The accumulated photosensitizer in rat maternal organs and embryos was observed by registering fluorescence spectra of specimens. Usually autofluorescence spectra do not have very

intensive peaks, while after incubation with Photofrin II rat organs spectra show the presence of Photofrin II fluorescence (Fig. 8). The measurements of tissues were performed at the different stages of embryogenesis. On the 7th day of embryogenesis Photofrin II accumulates in embryo at a very high rate – fluorescence in embryo is as high as in uterus (Fig. 8). This is because placenta is not formed yet, and the embryo is not directly connected to mother's blood system so photosensitizer can easily reach the foetus because of diffusion without any prevention, and because of that fluorescence in embryo is as high as in uterus. After irradiation a significant moderation of fluorescence intensity (it decreases about 2 times) in irradiated horn embryos and uterus was observed (Fig. 9) meaning that photosensitizer was destroyed by generated singlet oxygen.

On the 14th day of embryogenesis placenta is already formed, and it is possible to examine more maternal tissues. Fig. 10 shows that distribution of photosensitizer on the 14th day is different compared with the 7th day of embryogenesis. This is probably the result of natural placenta barrier for photosensitizers (11). On the 14th day of embryogenesis photosensitizer in embryo was almost undetectable using our spectroscopic methods and was detected in uterus in much lower concentration compared to the 7th day of embryogenesis results, but it was detected in placenta in high concentration. Photobleaching experiment showed decrease of photosensitizer fluorescence in all the maternal tissues, and this fact again signifies that singlet oxygen was generated. In embryo photobleaching was not observed because of too low concentration of photosensitizer.

DISSCUSION

In this study we showed than in the primary stages of embryogenesis when embryo is not directly connected to mother and is not protected by placenta the photosensitizer accumulates in foetus and bleaches after irradiation. The authors of other work groups report, that the presence of photosensitizer photobleaching phenomenon is an evidence of photodynamic reactions (12, 13). These reactions may be very harmful for further embryo development. Foetus was shown to be safe enough from the penetration of photosensitizer after the formation of placenta (in agreement with the other papers of our group (7, 11) and from direct photodynamic reactions, however, they occur in the surrounding maternal tissues (placenta and uterus), and it is not clear how damaging the impact of the photodynamic effect could be on the development of embryo after the destruction of placenta or uterus. This study – the evidence of the presence of photodynamic reactions in the tissues examined – gives reason to plan further experiments and observation of resorptions.

CONCLUSIONS

Our study clearly showed that photodynamic reactions occur in embryo and maternal tissues using therapy doses of drug and light. It depends on the stage of embryogenesis, and it is connected with the formation of maternal organs. It is predictable that photodynamic reactions on the 7th or even 14th day of embryogenesis may affect the development of embryo, so experiments on toxicity and teratogenicity must be performed in future.

ACKNOWLEDGEMENTS

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FOTODINAMINĖS REAKCIJOS ŽIURKĖS EMBRIONE IR MOTINOS AUDINIUOSE: SPEKTROSKOPINIAI TYRIMAI

S a n t r a u k a

Viena didžiausių problemų, su kuria susiduriama taikant fotosensibilizuotą navikų terapiją (FNT), yra prastas fotosensibilizatorių selektyvumas – jie kaupiasi ir sveikuose audiniuose. Norint FNT taikyti nėščiosioms moterims, yra būtina ištirti, ar fotodinaminės reakcijos vyksta vaisiuje.

Siekdami tai išsiaiškinti savo eksperimentuose Wistar nėščioms žiurkėms intraveniniu būdu suleidome fotosensibilizatorių Fotofriną II. Terapinė dozė – 5 mg/kg. Tyrimai buvo atlikti su trimis gyvūnų grupėmis: I – žiurkės nėščios 7-ą parą (fotosensibilizatorius suleidžiamas 6-ą neštumo parą), II – žiurkės nėščios 14-ą parą (fotosensibilizatorius suleidžiamas 13-ą neštumo parą) ir kontrolinė grupė – žiurkės nėščios 7-ą parą (fotosensibilizatorius nesuleidžiamas). Apšvitinus tiriamuosius gyvūnus, spektroskopiniais metodais buvo nustatinėjamas fotosensibilizatoriaus fotoblyškimas embrionuose ir motinos organuose.

Tyrimų metu 7-ą parą nėščių žiurkių embrionuose buvo nustatytas fotosensibilizatoriaus fotoblyškimas, tuo tarpu 14-ą parą nėščių žiurkių embrionuose fotosensibilizatorius neblyško.

Išvada: fotodinaminės reakcijos vyksta embrionuose, tačiau tai, kaip ir galimas fotosensibilizuotos terapijos poveikis, priklauso nuo embriogenezės paros.