MICROSCOPIC EVIDENCE OF PLACENTA AS A NATURAL BARRIER FOR A PHOTOSENSITIZER

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Abstract. The aim of this work was to find out if there is selective accumulation of Photofrin II in placenta of a pregnant rat and an embryo at different stages of the embryogenesis and to make preliminary estimations of possible PDT application effect on the embryo.

Fluorescence microscopy methods were used to evaluate accumulation of the photosensitizer in an embryo and placenta at the 7th, 14th and 20th days of rat gestation, 24 hours after intravenous administration of the photosensitizer.

Fluorescence microscopy results revealed that there is no selective accumulation of Photofrin II in embryos during the first stage of gestation (7th day of embryogenesis) as well as after formation of placenta (14th day of embryogenesis) or before the birth (20th day of embryogenesis). However, spectroscopy results show relatively high fluorescence of Photofrin II in embryo at the 7th day of embryogenesis (if to compare with fluorescence in uterus). During the study, it was determined that after formation of placenta (14th day of embryogenesis) it accumulates photosensitizer in an active way. It is clear that placenta serves as an active natural barrier and protects the embryo from phototosensitizer. This proposes that embryo would be safer from the direct PDT effect at later stages of embryogenesis (after formation of placenta). However, even if the embryo does not accumulate photosensitizer, PDT application may have some indirect negative effects – PDT could damage the placenta and lead to abortion, birth defects, premature birth and many other complications.

Spectroscopic and fluorescence microscopy data revealed the presence of high concentrations of endogenous photosensitizers in the uterus before the birth (at the 20th day of gestation), therefore PDT applications at the latest stages of embryogenesis might have stronger side effects.

Further experiments must be performed in order to determine possible direct and indirect effects of Photofrin II administration and PDT on embryo.

Keywords: fluorescence spectroscopy, fluorescence microscopy, photosensitizer, uterus, placenta, embryo.

PLACENTOS APSAUGINIŲ SAVYBIŲ NUO FOTOSENSIBILIZATORIŲ MIKROSKOPINIAI TYRIMAI

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Santrauka. Šio darbo tikslas buvo nustatyti, ar fotosensibilizatorius fotofrinas II priklausomai nuo embriogenezės stadijos selektyviai kaupiasi žiurkingos patelės placentoje, numatyti, kokią įtaką vaisiaus vystymuisi gali daryti fotosensibilizuota navikų terapija (FNT).

Fotosensibilizatoriaus kaupimasis tirtas 7-tą, 14-tą ir 20-tą embriogenezės parą. Taikant fluorescensinės mikroskopijos metodą, prieš 24 valandas suleidus fotosensibilizatorių į veną, nustatyta, kad iki susiformuojant placentai (7-tą embriogenezės parą), placentai susiformavus (14-tą embriogenezės parą) ir prieš atsivedant (20-tą embriogenezės parą) fotosensibilizatorius embrione selektyviai nesikaupia. Tuo tarpu fluorescensinės spektroskopijos eksperimentų rezultatai rodo, kad fotosensibilizatorius 7-tą embriogenezės parą santykinai intensyviau kaupiasi embrione nei gimdoje. 14-tą embriogenezės parą fotosensibilizatorių selektyviai kaupia susiformavusi placenta. Vadinasi, placenta pradeda veikti kaip aktyvus barjeras ir apsaugo embrioną nuo fotosensibilizatoriaus kaupimosi. Taigi tiesioginis FNT poveikis turėtų būti mažesnis, taikant šį metodą vėlesnėmis embriogenezės paromis (placentai susiformavus). Tačiau negalima atmesti tikimybės, kad FNT šioje embriogenezės stadijoje taip pat gali turėti neigiamą netiesioginį poveikį embrionui, mat terapijos metu gali būti pažeista placenta. Tas gali sukelti vaisiaus žūtį, įvairių sklaidos ydų ir kitų

komplikacijų.

Pažymėtina, kad ir fluorescencinės spektroskopijos, ir mikroskopijos tyrimai parodė pastebimą endogeninių fotosensibilizatorių kaupimąsi 20-tą embriogenezės parą. Vadinasi, FNT paskutinėmis embriogenezės stadijomis gali daryti nepageidaujamą poveikį embrionui.

Atsižvelgiant į tyrimų duomenis būtina toliau atlikti bandymus ir nustatyti tiesioginį bei netiesioginį FNT poveikį embrionui.

Raktažodžiai: fluorescensinė mikroskopija, fluorescensinė spektroskopija, fotosensibilizatorius, gimda, placenta, embrionas.

Introduction. A relatively new method to treat tumours – the photodynamic therapy – has been already approved worldwide (USA, Canada, Japan, EU, etc.) for skin, brain and other types of cancer. This therapy is based on administration of a light sensitive drug (photosensitizer), its selective accumulation in malignant lesions and subsequent light exposure at certain wavelengths that leads to generation of singlet oxygen and inactivation of targeted tumour cells (John Wiley et al., 1989). However, the efficacy of this method is limited because of the limited selectivity – photosensitizers accumulate in healthy tissues as well. This means that photooxidation reactions could also occur in healthy tissues and may damage them. Therefore, a lot of experiments were made to determine the effect of photodynamic therapy (PDT) on healthy tissues and there are many results indicating that PDT is safe enough in most cases (John Wiley et al., 1989; Jori, 1990; Dolmans et al., 2003; Dougherty et al., 1998). PDT might be very useful for treatment of pregnant women, whereas the other methods are too toxic for the bearing organism and the embryo. It has been shown by de Santis *et al.* that after the administration of the photosensitizer Verteporphin to pregnant women (on 3rd week of embryogenesis) there were no side effects on newborns (De Santis et al., 2004). However, the study performed by Yang *et al.* demonstrated that after the administration of 5-aminolevulinic acid followed by the irradiation, resorptions in pregnant rats were observed (Yang et al., 1994).

During the rat pregnancy, formation of tissues surrounding the embryo begins at the 6th day and completes with the formed placenta at the 12th–14th day of embryogenesis. The most important process for the development of placenta is angiogenesis, when blood-vessels form inside the chorion's labyrinths. The formed placenta serves as a natural barrier, which starts preserving embryo from various exogenous harmful factors, ensures tolerance between the mother and the foetus (Marin et al., 2004) and is now viewed as a metabolic barrier rather than a physical barrier (Gupta, 2009). However, this barrier also allows the passage of many chemical agents, which are embryotoxic, teratogenic and have a negative effect on cell proliferation and embryo development (Miller et al., 1998; Cross, 2005).

The aims of this study were to determine the pattern of photosensitizer's dihaematoporphyrin ether (Photofrin II®) accumulation in embryo and placenta in order to estimate if a natural placenta barrier could prevent an embryo from the accumulation of photosensitizer and to make preliminary estimations of possible PDT application effect on embryo.

Materials and methods. *Wistar* line white rats (160-240 g) were used in the experimental studies. 27 rats (9 in a control group and 18 in a treated group) and 81 embryos (27 control and 54 treated) have been examined. After being acclimated for at least 7 days, female rats were mated overnight with males of the same strain. Vaginal smears from each female rat were collected and subjected to microscopic examination on the following morning in order to determine the oestrous cycle and the presence of sperm. The day of sperm detection in vaginal smears was designated as day 0 of gestation.

Photofrin II ® (Axan Pharma Inc. - Canada) was used as a photosensitizer at a dose of 5 mg/kg.

There are critical periods during the rat embryogenesis, which have been found as being the most sensitive for embryo formation: 6th–9th days – implantation and early organogenesis, as well as 10th–14th days – placenta formation and active organogenesis. Therefore a photosensitizer was intravenously administrated into experimental animals at the 6th, 13th and 19th days of embryogenesis. 24 hours after administration (at the 7th, 14th and 20th days) rats were sacrificed and tissues were examined for the accumulation of the sensitizer.

Spectroscopic measurements for qualitative analysis were performed by using spectrophotometer LS-50B (Perkin Elmer, Inc., USA). Haematoxylin and Eosin (H&E) staining and the fluorescence microscopy (excitation at 360 nm and observation at 420–700 nm) were used for visualization of histological sections. Coloured digital photomicrographs were taken by using a microscope (Olympus BX60) and a photo camera.

The animal husbandry and experiments on animals were carried out according to the European regulations (experiments comply with EU Directive 86/609/EEC and the EC recommendation 2007/526 EC) as well as according to the law No. 8-500 and other legal acts of the Republic of Lithuania and were approved by the Lithuanian Animal Care and Use Committee.

Results and discussion. The accumulation of the intravenously administered photosensitizer was observed in uteri, placenta and embryos of rats by registering fluorescence spectra of specimens. As expected, autofluorescence spectra did not possess distinctive peaks in the red spectral region, while after incubation with Photofrin II® it was possible to distinguish relatively intense fluorescence peaks at 630 nm and 690 nm in the

spectra. This is in agreement with previously reported results of our group (Grazeliene et al., 2006) and is not the subject of this paper except the latest stage of embryogenesis what revealed unexpected results.

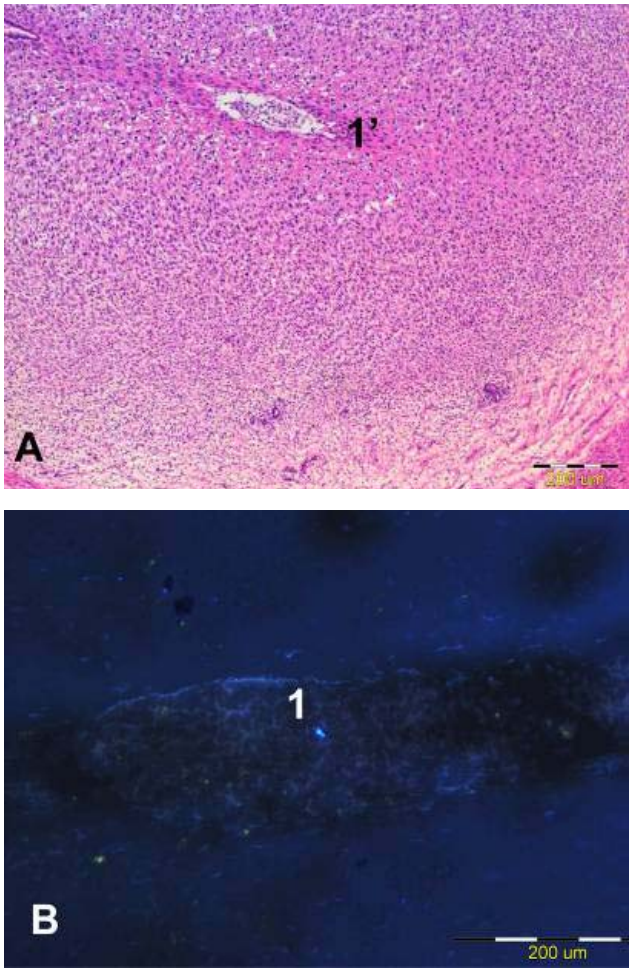

Fig 1. (A) – **Histological visualization of an embryo** (1') **from the control group**; (B) – Fluorescence visualization of an embryo (1) after administration of Photofrin II ®. Pictures were taken *ex vivo* at the 7th day of embryogenesis

The formation of new tissues is in progress during the embryogenesis and depends on its stage, so it is possible to identify different tissues morphologically (corresponding to the stage of the embryogenesis) and to examine them using fluorescence microscopy methods. Because of that, the fluorescence measurements on the selected tissues were performed.

The results reported below are presented following the different stages of the *Wistar* line white rats embryogenesis.

At the 7th day placenta is not formed yet, so photosensitizer molecules from maternal tissues can reach the embryos because of diffusion and according to our previously published data the fluorescence intensity of the photosensitizer in an embryo was almost as high as in uterus (Grazeliene et al., 2006). Fig. 1 shows embryos microphotographs: stained with Haematoxylin and Eosin (A) and obtained in fluorescence registration mode (B). The intensity of the fluorescence signal was very low and no selective accumulation pattern of the fluorescing sensitizer was observed in the embryo tissues. This is because photosensitizer reaches embryo in a passive way.

At the 12th–14th day of the embryogenesis, the placenta is completely formed (Fig. 2A) and as reported in our previous paper (Grazeliene et al., 2006) fluorescence spectra evidenced different fluorescence intensity of photosensitizer accumulated in the embryo, uterus and tissues surrounding the embryo. The chorioallantoic placenta of the rat has two regions – the jungtional zone and the labyrinths zone which consists of giant cells, fetal vessels and mother blood, and is thus the major site of feto-maternal exchange (Davies et al., 1968). The data of the fluorescence microscopy collected on the 14th day of the embryogenesis (Fig. 2, B – E) clearly indicated a selective pattern of Photofrin II® fluorescence in the yolk-sac placenta and in the labyrinths of placenta and no selective accumulation of Photofrin II® was observed in the embryo. However, even if embryo does not accumulate photosensitizer, PDT application at this stage of embryogenesis may damage the placenta. Adequate placental growth and function are fundamental to the well-being, growth, and development of the embryo throughout gestation. At the 18th–20th day of gestation, placenta starts to reorganize before the birth. The images of histological sections of uterus stained with Haematoxylin and Eosin (H&E) are shown in Fig. 3, A.

Fluorescence spectroscopy measurements on the embryonic tissue of the control group at the 20th day of gestation brought unexpected results (Fig. 4, B). The fluorescence spectra registered in the placenta, uterus and an yolk-sac placenta possessed the main peak at 635 nm, which indicated the presence of protoporphyrin IX (PPIX). Another fluorescence peak observed at about 616–620 nm in the spectra of embryos and especially in the spectra of yolk-sac placenta could be attributed to other endogenous porphyrins, such as uro- or copro-porphyrins.

After administration of the photodrug at the 20th day of gestation, no fluorescence peaks of Photofrin II® were detected in embryos, however, fluorescence spectra of uteri and tissues surrounding the embryos indicated the presence of endogenous porphyrins (Fig. 4, A) similarly as corresponding spectra from a control group. Thus, the spectra measured in sensitized tissues reflected the total fluorescence intensity of both endogenous and exogenous porphyrins, the former prevailing over the latter.

Fluorescence microscopy pictures of the embryo and the tissues surrounding the embryo taken 24 hours after administration of Photofrin II® revealed areas of high fluorescence in the labyrinths and uterus (Fig. 3, D, E) but not in the embryo (Fig. 3, E). The spectroscopic data imply that this red glow might be a combined result of fluorescence of endogenous porphyrins and that of administered drug. It is presumable that administered photosensitizer accumulated in the labyrinths and amniochorionic membrane like at the 14th day of embryogenesis, but its fluorescence was overlapped by fluorescence of endogenously produced porphyrins.

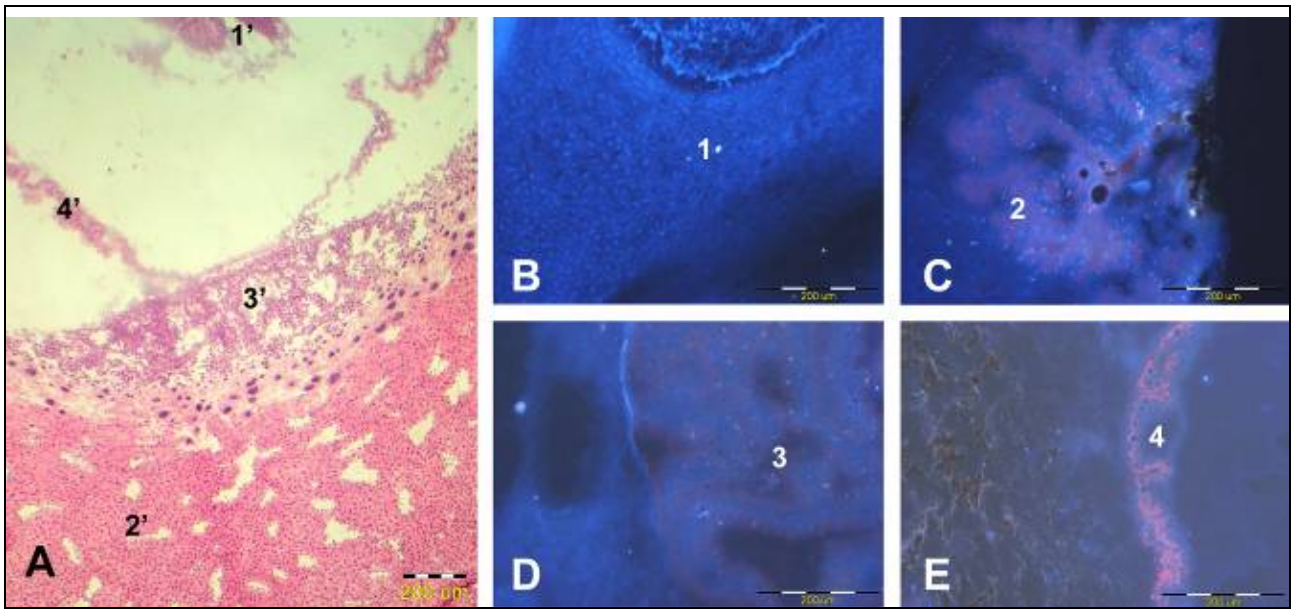

Fig 2. (A) – **Histological visualization of an embryo** (1') **and surrounding tissues – uterus** (2'), **placenta** (3'), **amniochorionic membrane** (4') **from the control group**; (B-E) – fluorescence visualization of embryo (1), uterus (2), placenta (3) and amniochorionic membrane (4) after administration of Photofrin II®. Pictures were taken *ex vivo* at the 14th day of embryogenesis

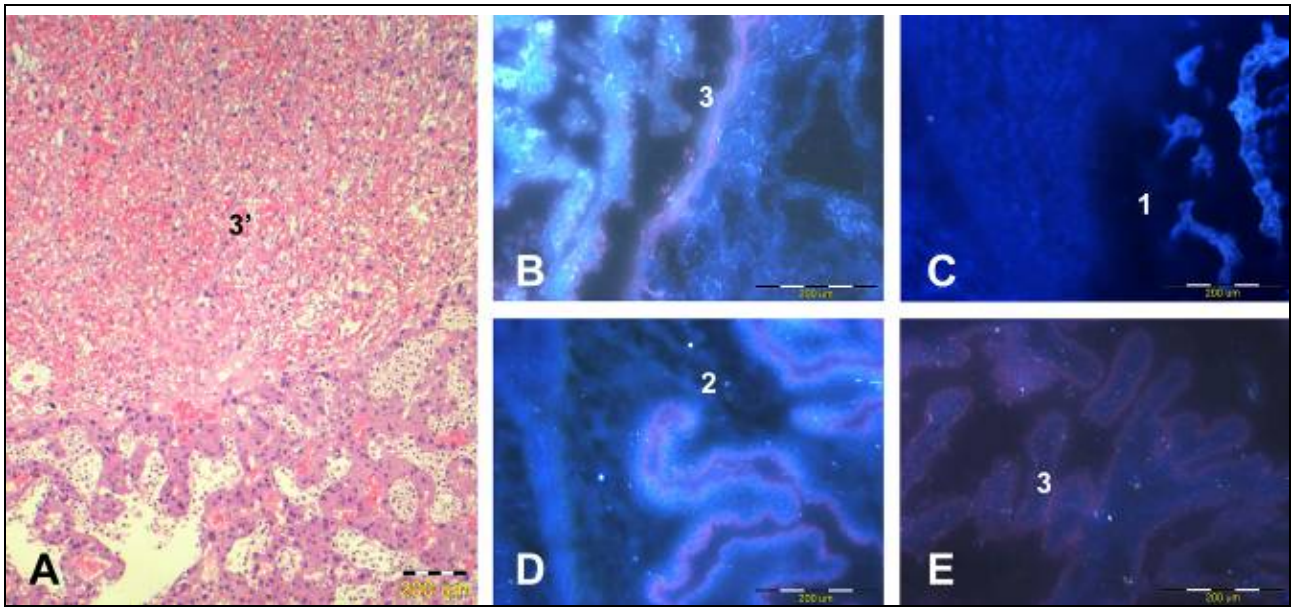

Fig 3. **Histological visualization** (A) **and fluorescence visualization** (B) **of placenta** (chorion labyrinths) (3') **from the control group**; fluorescence visualization (C-E) of an embryo (1), uterus (2) and placenta (chorion labyrinths) (3) after administration of Photofrin II ®. The pictures were taken *ex vivo* at the 20th day of embryogenesis

In order to clarify this situation, fluorescence microscopy experiments were also performed on samples of the control rat group at the stage before the birth (20th day of gestation). Notable is the fact that red fluorescing areas were clearly visible in the chorion labyrinths without administration of any photosensitizer (Fig. 3, B). These findings confirmed that endogenous photosensitizers were responsible for the red fluorescence at the latest gestation stages regardless of whether the Photofrin II® was administrated or not.

The appearance of endogenous photosensitizers can be explained by the principal morphological age changes in the placenta what leads to the accumulation of fibrin and fibrinoid, and the calcification. Significant calcium deposits occurs near the end of pregnancy and these deposits of calcium can obstruct parts of the placenta with clots of maternal blood, block the maternal blood vessels, replaced with fibrous tissue or cause certain small parts of the placenta to die (Akirav et al., 2005). This induces irreversible inflammatory processes and formation of

necrotic areas what leads to appearance of endogenous photosensitizers. These endogenous porphyrins being exposed to illumination during PDT can generate singlet oxygen and thus may increase adverse effects to healthy tissues.

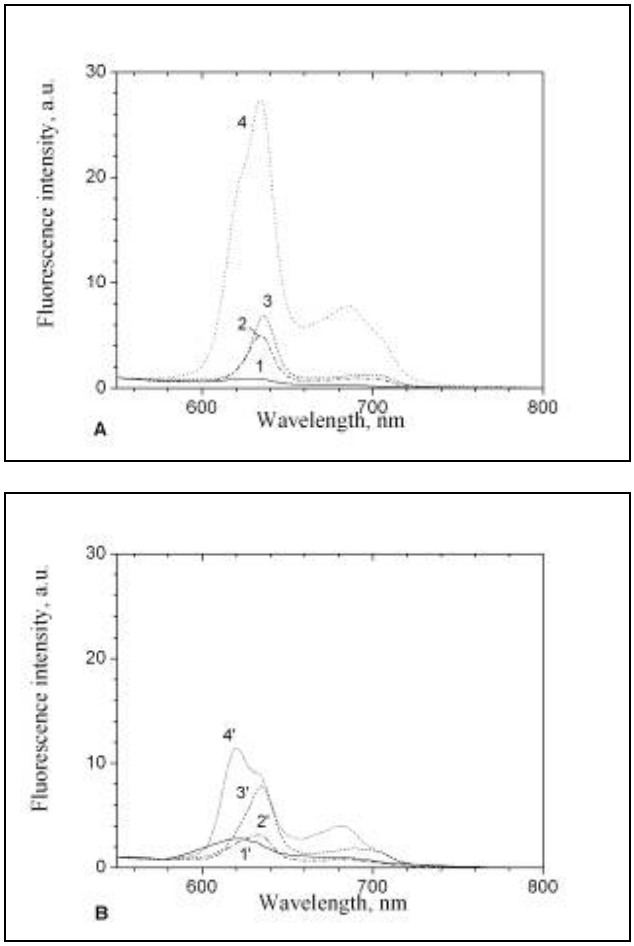

Fig 4. Fluorescence **spectra of a rat embryo and surrounding tissues specimens measured ex vivo at the 20th day of embryogenesis:** (A) – the spectra of an embryo (1), uterus (2), placenta (3) and amniochorionic membrane (4) after administration of Photofrin II ®; (B) – the spectra of corresponding specimens (') from the control group

Conclusions. As fluorescence microscopy results indicate that there is no selective accumulation of photosensitizer in embryo and fluorescence spectroscopy results indicate relatively high accumulation of photosensitizer in embryo at the 7th day of embryogenesis, we can expect that embryo is safe from the direct PDT effects at later stages of embryogenesis. It was shown that the placenta with the active part of it – labyrinths – seem to be a barrier and to protect the embryo from the accumulation of the photosensitizer. However, PDT application at the 14th day of embryogenesis may damage placenta and induce an indirect negative effect. Adequate placental growth and function are fundamental to the well-being, growth, and development of the embryo throughout gestation. Therefore PDT induced damage to the placenta could lead to abortion, birth defects, premature birth and many other complications.

At the 20th day of gestation, the fluorescence spectroscopy and microscopy data revealed the fluorescence of endogenous photosensitizers in uterus. As such photosensitizers may induce PDT effects, it is not possible to state that PDT application at the latest stage of embryogenesis is safe. It is necessary to perform further experiments.

Further experiments must be performed in order to determine possible direct and indirect effects of Photofrin II administration and PDT on embryo.

Acknowledgements

This research was funded by the grant (No. MIP-10440) of the Research Council of Lithuania.

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Received 27 October 2011
Accepted 21 September 2012