Short thesis for the degree of doctor of philosophy (PhD)

The role of Plasminogen activator inhibitors in the corneal wound healing processes

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UNIVERSITY OF DEBRECEN

Doctoral School of Clinical Medicine

Debrecen, 2015.

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The Examination takes place at the library of Department of Obstetrics and Gynaecology, Faculty of Medicine, University of Debrecen at 11:30 a.m. on October 21, 2015.

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1. INTRODUCTION AND OBJECTIVES

The need for an ever more perfect correction of both congenital and acquired refractive defects has come more and more to the foreground in the past century. Out of these tendencies, one of the most developing segments is the refractive laser eye surgery method done with an excimer laser. These surgical interventions modify the eye's focusing power and thus the need for correction by changing the refractive power of the cornea. The first excimer lasers, working principle of which is the same as today's lasers', started to appear by the end of the 1980s. However, despite the significant development of technology, even nowadays, after the now routine excimer laser treatments, adverse reactions causing impaired vision or the reduction of visual acuity may appear.

Recently, the most frequent type of refractive excimer laser treatments is photorefractive keratectomy (PRK) the laser-assisted in situ keratomileusis (LASIK), the laser-assisted epithelial keratomileusis (LASEK) and the epithelial laser in-situ keratomileusis (Epi-LASIK).

Based on our current knowledge, corneal wound healing processes are controlled by two big systems with the help of activators and inhibitors. One of the systems is the plasminogen activator-plasmin system, whose significance lies in degradation and the removal of the damaged extracellular matrix. The other system is responsible for the creation of the newly synthesized collagen fibrils in place of the damaged collagen structures with the help of activated keratocytes. The correct operation of the above mentioned systems is inevitable for re-epithelization, i.e. for proper wound healing. The disturbance in the equilibrium of the two systems can result in protracted, pathological wound healing, in serious cases, corneal ulcer.

In 2000, Csutak et al. reported on the activity pattern changes of urokinase plasminogen activators (uPA) in human tears following PRK treatments. They observed a reduction of the uPA activity in case of normal wound healing right after laser treatment, then an increase above the preoperative values on the postoperative third day and their return to the level of preoperative values on the postoperative fifth day. Conversely, in case of the development of corneal stromal haze, the increase of uPA activity did not take place on the postoperative third day.

The connection between the change of plasminogen activators and the development of haze was examined even after the photorefractive laser treatment of pregnant rabbits (Csutak et al., 2004). During their animal experiments, the activity pattern of plasminogen activators in the tear samples treated with serine protease inhibitor (aprotinin, Gordox, Richter Gedeon Rt., Budapest, Hungary), as well as in pregnant animals' tear samples that were not treated with aprotinin matched with the activity pattern measured in the tear samples of patients that had corneal haze and were previously treated with PRK. In these cases, uPA growth in tear was not detectable on the postoperative third day. During the examinations, haze developed in case of seven out of eight pregnant rabbits treated with PRK. When the eyes of pregnant rabbits were treated with uPA, none of them had stromal haze developed. This result that is, most of pregnant rabbits who were cured from corneal haze following the excimer laser treatment indicated the possibility of a relationship between pregnancy and pathological corneal wound healing in case of human pregnancies too. Therefore, we started to examine the change of plasminogen activator inhibitors level in human pregnant tears.

In the human body, the operation of plasminogen activators is blocked by the plasminogen activator inhibitors, which

have two types, namely, PAI-1 and PAI-2. The plasminogen activator cascade plays a role in several physiological and pathological processes, e.g. fibrinolysis, wound healing, inflammatory processes, angiogenesis, ovulation, neuroplasticity as well as the growth, invasion and metastasis of malignant tumours.

The accumulation of inhibitors in blood can lead to thrombosis due to their antifibrinolytic impact. Normal pregnancy is accompanied by the reduction of fibrinolytic activity and the growth of PAIs concentration in blood, thus creating a hypercoagulable state. Both the coagulation and fibrinolysis strengthens over time during pregnancy, especially in the 2^{nd} and 3^{rd} trimester. The PAI-1 and in particular the PAI-2 concentration as an antifibrinolytic factor are continuously growing during pregnancy, already in the early periods and it creates a complex both with the tPA and uPA, thus less plasminogen transforms into active plasminogen and the fibrinolytic activity reduces. However, the full fibrinolytic activity remains constant in pregnant women in spite of the significant change in the tPA, uPA, PAI-1 and PAI-2 levels. In possession of the above mentioned results, we started to examine the changes in level of plasminogen activator inhibitor-2 following the refractive laser surgery operations (PRK, LASIK), and also in pregnant women in order to get closer to a more accurate understanding of the background of biochemical processes during corneal wound healing.

Objectives:

1. Our first goal was to examine the change in level of plasminogen activator inhibitor in tears after photorefractive eye treatments.

2. The second aim was to study the change of plasminogen activator inhibitor level and make comparisons following photorefractive keratectomy and laser in situ keratomileusis.

3. Our additional objective was to analyse the change of the plasminogen activator inhibitor-2 level in the tear samples of pregnant women.

4. Finally, we examined the change of the plasminogen activator inhibitor-2 level in blood of pregnant women comparing the results to the values taken from the tear samples.

2. LITERATURE REVIEW

2.1. The plasminogen activator-plasmin system

In mammals, the plasminogen activator system is controlled on numerous levels. One of the most important of these is the activity blocking of plasminogen activators by their inhibitors. Plasminogen activators are specific serine proteases that are responsible for the breaking of bond between the plasminogen Arg_{560}-Val_{561} in the human body, thus for transforming inactive plasminogen into active ones, thereby catalysing the extracellular proteolysis. The two types of plasminogen activators are the tissue-type plasminogen activator (tPA) and the urokinase-type plasminogen activator (uPA).

Primarily, tPA plays a role in the fibrinolytic activity of blood, for which fibrin cofactor is necessary, and for its production and secretion into blood vascular endothelial cells are responsible first and foremost. The plasminogen activator inhibitor-1 is the most important participant in its blocking.

The major role of uPA is in extracellular proteolysis, and most probably plays an active part in the creation of metastasis and invasion in tumours. There is no need for fibrin cofactor for its operation. The uPA is synthesized by endothelial cells, macrophages and different types of leukocytes. The uPA is a tear component that is produced by conjunctival and corneal epithelial cells. uPA activity increases with the appearance of several corneal illnesses, e.g. ulcer, and even after the refractive laser treatments accompanying corneal wound, which refers to the fact that the fibrinolytic system

plays a role in the eye's wound healing processes. Several studies dealing with the re-epithelization of corneal wound reported about the existence of uPA and its receptors in keratinocytes. The plasminogen activator inhibitor-2 plays the most significant role in its blocking.

2.2. Plasminogen activators and the change of their inhibitors in corneal wound healing

We can witness the decrease in corneal transparency in case of scarring after injuries and surgeries, in case of corneal degeneration or dystrophy, several inflammatory diseases or as a side effect of certain medications (e.g. epinephrine). Occasionally, as we reported it previously, it can happen that corneal pigmentation develops in the corneal stroma following the phagocytosis of the pigment granules by endothelial cells in the anterior chamber and their transportation through the Descemet's membrane. In these cases, we can see the incorporated pigment granules in the macrophages that migrated from the limbal areas to the corneal stroma and in some keratocytes. The slow clearance of pigment-filled macrophages starts in the limbus and continues its way towards the centre, whereas the keratocytes containing pigments remain in the stroma, thus causing the decrease in corneal transparency, and, ultimately, the deterioration of visual acuity.

From today's perspective, corneal wound healing is controlled by two big systems with the help of activators and inhibitors. One of the systems is the plasminogen activator-plasmin system, whose significance lies in in degradation and in covering the damaged extracellular matrix. The other system is that of activated keratocytes, that is responsible for the creation of the matrix of

newly synthesized collagen fibrils in place of damaged collagen structures and of glycosaminoglycan surrounding collagen fibrils. The proper operation of the above mentioned systems is inevitable for re-epithelization. The imbalance in the two systems can cause protracted, pathological wound healing, in serious cases corneal ulcer creation.

The corneal, conjunctival and lacrimal gland cells are all capable of producing plasminogen activators. The plasminogen activator found in the cornea is of urokinase-type, whereas the lacrimal gland can only produce the tissue-type plasminogen activator. Both types of activators can be detected in the conjunctiva. In these tissues, uPA is probably originating from the epithelial cells of cornea and conjunctiva, while tPA is produced by the vascular endothelial cells of the conjunctiva. We know that normal tear contains protease inhibitors in a very small concentration, however, in tears of people suffering in certain ophthalmological diseases (e.g. corneal ulcer, vernal kerato-conjunctivitis) the inhibitor concentration significantly grows and returns back to the normal level only after the healing process.

In normal tears, uPA activity is very low, while tPA activity is practically non-detectable. PAI-1 has not been detected in normal tear so far, therefore we assume that PAI-2 plays a role in the blocking of uPA produced by the epithelial cells of the anterior segment of the eye.

In case of the damaged epithelial cells in the cornea and conjunctiva (e.g. inflammation processes, certain surgical interventions), larger quantity of uPA is identified in tears. uPA is capable of activating the plasminogen in tears, transforming it into plasmin, which process is set back by inhibitors.

In case the created plasmin does not get activated, it can set in motion several processes on the anterior segment of the eye (e.g. it can lead to ulcer due to the dissimilation of the collagen constituting the basis of the cornea and of the collagenase activated with the help of the plasminogen activator-plasmin system). In pathological conditions, affecting the anterior segment of the eye (e.g. conjunctivitis, keratitis, etc.), the permeability of conjunctival vessels usually intensifies, thereby proenzyme forms of proteinases and proteinase inhibitors get into the tear. If the damage reaches the level when the uPA activity depletes the inhibitors that get into the tears with transudation, tears can become proteolytically active due to the continuous production of plasmin. The great amount of plasmin activates the pro-collagenase into collagenase, as a result of which, corneal ulcer can also develop. However, the not too excessive activation of the plasminogen activator-plasmin system is inevitable during the wound healing processes. Their activation plays a significant role in the disposal of cell and tissue debris, as well as in repairing the damaged collagen and extracellular matrix.

In the light of the above, we can say that the plasminogen activator-plasmin system can play a key role in the degrading dissimilation of protein during the pathological processes in the anterior segment of the eye, in a similar way to other extracellular proteolytic processes such as malignant cell invasion. It seems that the uPA plasminogen-plasmin system plays a crucial role in the wound healing processes, including the pathogenesis of epithelial defects. Although an exact mechanism, which would be the basis of corneal wound complications following surgical interventions, is not known, we can assume that individual variations in corneal wound healing are also key players in the refractive regression following surgical interventions and in the development of corneal stromal haze.

The low activity of the plasminogen activator-plasmin system can lead to protracted wound healing, or even to chronic epithelial erosion.

2.3. The change of the plasminogen activator/activator inhibitor system during pregnancy

The work of plasminogen activators is held back by the plasminogen activator inhibitors, which has two types, PAI-1 and PAI-2. The plasminogen activator inhibitor-1 is produced by the vascular smooth muscle cells, thrombocytes and hepatic cells. The plasminogen activator inhibitor-2 is primarily produced by trophoblasts, and they have an important part in the blocking of uPA during pregnancy. The multiplication of fibrinolytic inhibitors can lead to thrombosis. Normal pregnancy is accompanied by the reduction of fibrinolytic activity and by the increase of PAIs concentration in the blood, thus creating a hypercoagulable state.

Both coagulation and fibrinolysis strengthen with the development of pregnancy, especially in the 2^{nd} and 3^{rd} trimester. This hypercoagulable state protects women from fatal bleeding during child-birth, but also predisposes them to thromboembolic diseases. During pregnancy, the primary fibrinolytic components, the amount of tPA and uPA temporarily decreases in the first and second trimester, whereas, in the third trimester, the concentration of uPA significantly grows compared to the level of concentration before pregnancy. The seriously reduced fibrinolytic activity can cause pre-eclampsia, placental abruption and intrauterine growth restriction in pregnancy. PAI-1 and mainly the concentration of PAI-2, as an antifibrinolytic factor is constantly growing even starting from early pregnancy and creates a complex both with tPA and uPA, and therefore less plasminogen transforms into active plasmin, and the fibrinolytic activity decreases. Up until now, it is still not clarified

whether the plasminogen activator level drop measured in the first two trimesters is the result of the reduced synthesis, the increased "turnover" or the complexes created with inhibitors, but the biologically active tPA and uPA levels measurably decrease. In the 32nd week of pregnancy, increased amount of uPA can be measured, which suggests that in this period of pregnancy the increased synthesis of activator is formed. The full fibrinolytic activity remains persistent in spite of the significant change of the tPA, uPA, PAI-1 and PAI-2 levels.

The coagulation activity, anticoagulation activity, the fibrinolytic and antifibrinolytic activity increase during normal pregnancy, but these activities are balanced. This balance and change is necessary so that the hemodynamics in the umbilical artery and in the uterine artery become baby-friendly (high stream, low resistance) with the development of pregnancy.

In view of the findings described above, we started to analyse the changes of the plasminogen activator-plasmin system in healthy, human pregnancies, of which, as we know, there is no available information in the literature. Furthermore, we studied the PAI level changes during LASIK and PRK to get closer to a more accurate understanding of the background of biochemical processes during corneal wound healing based on the results obtained.

3. PATIENTS AND METHODS

3.1.1. Patients undergoing excimer laser treatment

In accordance with the content of the Helsinki Declaration, following the authorized signature of ethical permit by the local Ethical Committee, the study of *corneal wound healing* was performed on 46 eyes of 38 patients with PRK (8 patients with both eyes), and 13 eyes of 8 patients (5 patients with both eyes) with LASIK treatment. In the case of PRK, the average value of the pre-surgical refractive errors was -4,11 (±2,22) D, while during LASIK, this value was -5,13 (±1,89) D. The preoperative refractive error in case of patients undergoing PRK and LASIK treatments, examining them with the two-sample t-test there was no significant difference (p=0,152). For those, who were taken tear samples from both eyes, the difference of the refractive error between the eyes was 1,2 (±1,4) D. Using F-test, we compared the refractive error of those patients, whose both eyes underwent laser treatment, with those, whom only had one eye treated, but significant difference was not detectable in this case either (p=0,17). The average age of patients undergoing PRK was 25 (±5), whereas that of patients treated with LASIK was 28 (±9). We have not found any significant difference (p=0,535) among the average ages using t-test.

3.2. Laser treatments

The correction of refractive errors was done with excimer laser treatment together with local anaesthesia (193 nm InproArF - excimer laser, Intraocular Prosthetic GmbH, Norderstedt, Germany),

which was performed by the same ophthalmologist in each and every case.

In the case of PRK, we used keratome blade to remove the epithelial cell layer of the cornea, we applied the Hoffer trephine as a marker, the diameter of which in case of spherical correction was 6,0-6,5 mm, while in case of astigmatic correction it was 7,5- 8,0 mm. We carefully scratched off the epithelium progressing from the periphery of the cornea towards the centre, so that we preferably could avoid injuring the Bowman's membrane. We used sterile tampon gauze to remove the epithelial rests.

In the case of LASIK, we used the Hansatome Model HT 230 microkeratome (Chiron) to create the corneal flap. The flap was 180 µm thick in every eye, when the corneal thickness after the refractive correction (following the deduction of the flap thickness) remained at least 250 µm thick.

If the corneal thickness was thinner, we reduced the flap thickness to 160 µm. The diameter of the ablative zone was 6,5 mm. Comparing the depth of the ablative zones, we did not find any significant difference between the two surgery types (In the case of PRK 48±20 µm; LASIK 59±13 µm, p=0,074).

3.2.1. Pre–and postoperative treatment, control examinations, evaluation of the corneal stromal haze in the case of patients undergoing laser treatment

Before laser surgeries, we gave the patients local anaesthetic drops (Humacain Oxybuprocaine hydrochloride, TEVA Pharmaceuticals). After the surgery, we applied Ciloxan antibiotic eye drops (Ciprofloxacin HCL 0.3%, Alcon) in every hour on the first

day following the surgery, and we continued it for five more days five times a day. Five days later, we instilled steroid filled Flucon (Fluorometholone 0.1%, Alcon) and Tears Naturale (Dextran/Hydroxypropyl Methylcellulose, Alcon) artificial tears. This treatment was repeated five times a day in the first month, 4 times a day in the second month, and three times/day in the third month. Every patient went through control examinations one and three months after the laser surgery.

We performed the tear sampling based on the protocol in the case of preoperative and immediate postoperative state, following both types of laser treatment, as well as in the case of PRK on the 3rd and 5th day, while in case of LASIK it was performed on the first day following the surgery in accordance with the conventional control dates. The evaluation of the corneal stromal haze in this study was performed based on the Hanna's stadium classification.

3.3.1. Pregnant patients

In our further analysis, we collected tear and blood samples from 32 pregnant patients four times in the 8th and 36th week of their pregnancy, and within one week after them giving birth according to the content of the Helsinki Declaration, following the authorized signature of ethical permit by the local Ethical Committee. The sampling was done at the ambulance of the Obstetrics and Gynaecology Clinics, Medical and Health Science Centre of the University of Debrecen.

The age of the pregnant patients was between 19-33, the average being 27,42 (±4,13) years old. Each pregnancy went through normally without any pre- or postnatal adverse reaction. Our

pregnant patients' anamnesis, there occurred no hematopoietic system or other kind of disease, medication. Two pregnant women were wearing contact lenses during pregnancy.

3.3.2. Control examination of pregnant patients

We categorized the ages of our pregnant patients as follows: 8-13, 16, 23-26 and 34-36 weeks, as well as the first week after child-birth. The control examination of pregnant patients was performed in accordance with this classification.

3.4. Tear sampling of patients undergoing laser treatment and pregnant women

We collected the tear samples into glass capillary tubes immediately before and after the laser surgery and also on the conventional control days without excitation. Tear sampling of pregnant women during the above mentioned controls was done in the same way as that of patients undergoing laser treatment. In every case, the sampling was performed before applying eye drops (a minimum of two hours after using preventing drops), using glass capillaries (length: 10 mm, diameter: 1 mm), and it was taken from the pre-corneal tear film close to the lower eyelid margin line taking care to avoid touching the conjunctiva. We recorded the duration of sampling and also the amount of tear sample. The secretion rate in the case of laser treated patients was 5-15 µl/min, in the case of pregnant women it was 5-18 µl/min. Every sample was centrifuged (at 1800 rpm) right after sampling, and the supernatant was stored at -80 °C until use. They were defrosted only once directly before measurements. Regarding our control samples, the collection of

samples was performed at the same time and in the same method as that of the sampling of the eye undergoing operation.

3.4. Blood collection from pregnant women

During our analysis, we collected 4 ml blood from the ulnar veins from 25 pregnant women (87 samples), according to the above mentioned control dates, and also within a week after child-birth to diagnose PAI-2 and from 10 pregnant women (20 samples) to detect PAI-1. In addition, we took blood sample during controls from 17 pregnant women (51 samples) to identify the levels of progesterone and estradiol. The collecting was done in anticoagulant (0,5 ml, 0,105 M Trisodium Citrate) tubes that were put on ice after taking the samples. After centrifugation (1800 rpm, 10 min), following the separation of plasma, we froze it to -80°C and only defrosted it at the time of measuring.

3.5. The identification of plasminogen activator inhibitor

With the help of enzyme-linked immunosorbent assay (Imubind Elisa, American Diagnostica GmbH, Pfungstadt, Germany), we determined the PAI-2 level of both tear and blood in accordance with the manufacturer's instructions. We used an enzyme-linked immunosorbent assay (Imubind ELISA) to measure the PAI-1 and PAI-2, with the help of which we can define the human PAI-1 and PAI-2 in human biological liquids.

In the case of PAI-2, the lower limit of detection was 1 ng/ml in case of patients undergoing excimer laser. The free PAI-2

and PAI-2/upA complex can be detected with the same sensitivity. The Imubind PAI-2 ELISA uses polyclonal antibody against the human PAI-2 antibody. We incubated the samples in precoated-microtest cell and adding the antibodies to them, we detected bound PAI-2 molecules. The added Streptavidin-Horseradish Peroxidase covers entirely the production of antibody-enzyme complexes in this case too. The addition of perborate/3,3',5,5'-Tetramethylbenzidine substrate, and its final reaction with the Horseradish Peroxidase results in a blue solution. The sensitivity can be increased with sulphuric acid solution that stops the process and converts the solution into yellow colour. We can measure the PAI-2 level in a quantitative way on 450 nm, and we can also compare the results obtained with the results of a standard diagram. In the case of patients undergoing laser treatment, we identified PAI-2 using 146 tear samples from the PRK treatment of 46 eyes, and 35 tear samples from the LASIK treatment of 13 eyes. Furthermore, 56 tear samples of 19 pregnant women and 87 blood samples of 25 pregnant women were used to define the PAI-2 levels (the lower limit of detection was 100 pg/ml).

The identification of PAI-1 was also done with the help of enzyme immunoassay (Imubind Elisa, American Diagnostica GmbH, Pfungstadt, Germany) in accordance with the manufacturer's instructions. During Imubind PAI-1 ELISA, we used a murine anti-human PAI-1 antibody. Afterwards, we made detection of PAI-1 like we did it in case of PAI-2. The PAI-1 level of tear remained under the assay detection level among patients undergoing excimer laser (1 ng/ml) and among pregnant women as well. The free and complex PAI can be identified with the same sensitivity; the assay is insensitive to PAI-2.

We measured the PAI-1 levels in the tear samples of 61 patients undergoing PRK treatment, and also in 45 tear samples of

13 pregnant women and in 20 blood samples of 10 pregnant women. The free and complex inhibitors were identified with the same sensitivity by ELISA. During our measurements, not any sample PAI value was over the lower level of detectability, therefore we did not perform the measuring of PAI-1 in the tear samples of patients treated with LASIK.

During the analysis of pregnant women's tear samples, as a comparison, we used PAI-1 (in 64 tear samples) and PAI-2 (in 37 tear samples) levels in tears of equal age for negative control, which we extracted from the analysis of tear samples gathered from pregnant women undergoing corneal laser treatment.

3.6. The identification of Estradiol and Progesterone

Out of the total number of our pregnant patients, we identified the estradiol and progesterone levels in 51 blood samples of 17 women. The identification was done with the help of "electro-chemiluminescence immunoassay" (ECLIA, Elecsys Estradiol CalSet II. and Elecsys Progesterone II. CalSet, Roche Diagnostics GmbH, Mannheim, Germany) in accordance with the manufacturer's instructions.

3.7. Statistical methods

For the study of PAI changes during the wound healing process after excimer laser treatment and in the different periods of pregnancy, we performed a standard statistical analysis. For the comparison of the results of PRK and LASIK PAI-2, as well as the

comparison of the PAI-2, estrogen and estradiol levels measured in pregnant women's blood and tear, we used two-sample t-test in the case of samples taken in different periods. We considered the difference as significant when it was $p<0,05$, and we mentioned explicit significance when the value there was a $p<0,01$ value.

4. RESULTS

4.1. The amount of PAI in human tear following refractive laser treatment

We identified the PAI-1 ELISA in 61 PRK tear samples, which affected the samples of all four dates. None of the PAI-1 values reached the lower level of detectability.

We performed the identification of PAI-2 ELISA on 146 PRK and 35 LASIK tear samples. The average values of PAI-2 in the case of tear samples taken four times during PRK: a preoperative 19,8 ng/ml, in the case of the postoperatively taken samples it was 112,7 ng/ml, on the postoperative 3rd day 12,1 ng/ml, and on the postoperative 5thday 15,5 ng/ml. During LASIK, in the case of tear samples taken at three occasions, the PAI-2 average values were measured as follows: in the case of preoperative samples 19,0 ng/ml, directly postoperative 111,5 ng/ml and on the postoperative first day 15,7 ng/ml.

We compared the results of PRK and LASIK with two-sample t-test in the case of preoperative and directly postoperative samples. We did not find any significant difference in any of the cases ($p>0,9$, two-sample t-test). Both in the case of PRK and LASIK, the postoperative PAI-2 level was significantly higher than the PAI-2

level of samples taken at any other time ($p<0,001$ in every case, two-sample t-test). We compared the preoperative tear samples with samples taken on the 1^{st}, 3^{rd} and 5^{th} postoperative days, but did not find any significant difference in any of the cases ($p>0,1$).

In the case of 40 patients undergoing PRK, we found clear cornea during the postoperative three months control period. We detected haze (grade 2) in one of the PRK patients during the three months. Only one eye of this patient was possible to be involved into our study. We detected mild corneal opacification in five more eyes undergoing PRK treatment. Out of these patients, we could identify one with elevated preoperative PAI-2 level (>2 SD), another patient had a high PAI-2 level directly after the laser treatment (>2 SD). On day 3 and day 5, all eyes in the PRK group with opacification had a PAI-2 value within the respective 1 SD of the mean of the PAI-2 values on those days. In the case of clear cornea none of the patients had an elevated preoperative PAI-2 level. However, 3 of the eyes had a PAI-2 value beyond 2 SDs on day 3 and another had a PAI-2 value beyond 2 SDs on day 5. This PAI-2 value distribution beyond the 2 SD level among 146 measurements is to be expected; therefore, no significance can be attached to the individual high PAI-2 values that were associated with eyes with or without corneal opacities. Thus, we could not confirm a significant relationship between the preoperatively elevated PAI-2 level and the development of corneal haze.

We found clear cornea at every patient treated with LASIK during the follow-up period. However, one eye in the LASIK group had a PAI-2 level beyond 2 SDs of the preoperative mean. Another eye had a PAI-2 level beyond 2 SDs of the respective mean at day 1. This distribution is expected and hence there is no statistical significance to this finding.

4.2. The change of PAI-1 and PAI-2 value levels in the tear and blood samples of pregnant women

During the examination of healthy pregnant women, there was no corneal wounding or injury during the investigation period. During pregnancy, the estradiol and progesterone and PAI-2 value highly correlated with the pregnant age, and in accordance with the literary data their level constantly increased. By contrast, the PAI-2 level of tear did not correlate with the pregnancy age. The PAI-1 level in tear samples remained under the detectability limit.

We compared the PAI-2 levels with the results of the non-pregnant patients' tear samples. The average values did not show significant differences in the various periods of pregnancy, except for the non-pregnant patients' results, that was p=0,022 compared to the results of 16 weeks pregnant women.

Between week 8-13 and within the 16th week of pregnancy, regarding the PAI-2 level of blood we could not find significant differences compared to the values measured within one week following child-birth. However, we found a steep increase in the PAI-2 levels of blood analysing the average values of 23-26 and 34-36 weeks pregnant ages. Consequently, the PAI-2 values of blood during pregnancy showed significant connection with the pregnant age. Notwithstanding the above, the PAI-2 levels of tear did not correlate with pregnant age; the values measured in tear samples remained constant during pregnancy.

5. DISCUSSION

As a summary, we can say that the plasminogen-activator/inhibitor system plays an important role in numerous physiological and pathological processes.

It seems from previous studies that following the ocular surface corneal wounding (e.g. in case of PRK), the PAA decreases or either because of the not proper production or the significant increase of the PAI-2 level, the cornea heals with opacity, so the development of haze can be observed.

Our study results showed that the measurable amount of PAI-1 antigen is missing from the tear before and after the PRK treatment, but the PAI-2 level has measurable concentration before and after the PRK and LASIK treatments. During LASIK, the PAI-2 level dropped to the preoperative level already by the first postoperative day. We did not have information about the first day following the PRK treatment, but on the third day, when we took the tear samples, we found the PAI-2 level on a preoperative level. We found that the temporal patterns of PRK and LASIK essentially match. In the case of PRK and LASIK treatment, the general parallel in the temporal pattern of PAI-2 suggests that there is a common enzymatic control response in corneal wound healing, even if we would believe that wound healing is different in the two processes.

The uPA activity pattern completes the PAI-2 pattern, which was confirmed by previous studies: usually, in normal cases, PAI-2 is low when the uPA activity is high, and it is true vice versa. They proved that in case the uPA activity is low during the first three days after operation it is an accompanying sign of those PRK

cases where haze developed. The relationship between low uPA activity following PRK and the later developing haze can emerge due to the decreased expression of uPA or the higher PAI concentration. In our recent study, we measured increased PAI-2 concentration on the 3rd and 5th day following PRK and on the 1st day after LASIK in a few cases. However, haze did not develop in any of these cases. Consequently, based on our results we can say that the decreased uPA expression, the non-increasing PAI-2 can be the reason for the later developed opacification and haze.

These observations and the fact that following the photorefractive treatment of pregnant rabbits, corneal haze developed in them brought us into action to study the change of PAI-2 level in blood and tear of human pregnant women as well during the pregnancy periods. The change of the plasminogen activator inhibitor level in tear during pregnancy has never been studied before. As we could observe in non-pregnant women before, the PAI-1 level of tear did not reach the lower level of detectability. This state was present during the whole pregnancy and also within the first week following child-birth. Therefore we believe that the change in PAI-1 level of tear is not significantly affected by pregnancy. It was evident that the PAI-2 level of pregnant women is not higher than the PAI-2 levels of non-pregnant women's tears.

We could see the constant increase of the PAI-2 level of blood during pregnancy, while the PAI-2 levels measured in tear did not correlate with the pregnant age, we could not find any significant differences in their level. Thus, it can be concluded that the increase of the systematic PAI-2 level measured in blood did not lead to a raise in the PAI-2 level of tear.

However, we assume that after the wounding of the ocular surface, an elevated amount of PAI-2 in blood can get through the vessel walls due to the progression of vascular permeability, thus increasing the PAI-2 level of tear. We also assume that some kind of trigger is necessary for the change of level in the PAI-2 in complex in tear, and its release from the complex, e.g. corneal wounding.

A further increase of the PAI-2 level can occur with its release from the complexes found in tears and from the damaged epithelial cells of the ocular surface even with normal PAA, which can trigger imperfect wound healing. This type of abnormal growth of PAIs can lead to corneal wound healing disorders or sometimes to the development of corneal ulcer in case of ocular surface wounding of pregnant women.

Determining the PAI levels of pregnant women's tear samples, we can confirm that the PAI values do not increase during normal pregnancy in the absence of wounding of surgical interventions. In the absence of ocular surface diseases, wounding or surgical intervention, the tear levels are independent of the change in the blood level of enzymes. This suggests that the proteolysis in tears and in the ocular surface might be under local control. On the basis of the above, it can be stated that the proteolytic activity of the human organism – including tears – is under sensitive regulation, a disturbance in which can lead to serious adverse reactions.

A further analysis of this complex enzymatic system is required to perform the incidentally necessary ophthalmological interventions with greater security on pregnant patients.

6. SUMMARY OF NEW RESULTS

1. We examined the typical change of plasminogen activator inhibitors in tear following the photorefractive laser treatment and we found that one eye was detectable with haze by the third month following PRK treatments, and further 5 eyes were affected by corneal opacification. Out of these patients, one patient was measured preoperatively with increased PAI-2 level, and in another case; we detected higher PAI-2 level in the directly postoperative sample. In the case of PRK treated patients, in whose tear samples taken on the postoperative 3^{rd} and 5^{th} days corneal opacification was detectable, the average values of PAI-2 were within 1 SD value. In the case of PRK patients diagnosed with clear cornea, the preoperative PAI-2 average level was not elevated. However, the PAI-2 value exceeded the 2 SDs on the 3^{rd} day in the case of three eyes, and on the 5^{th} day in another case. In the light of the results obtained, we cannot talk about significance in the two cases where we observed corneal opacification, when the PAI-2 level was high. This way we could not prove a significant connection between the preoperative elevated PAI-2 level and the development of corneal haze.

2. We compared the PAI-2 results of PRK and LASIK with the help of t-test in the case of preoperative and directly postoperative sample. We could not find any significant difference between the results PRK and LASIK. During both of these laser treatments there was a significant difference between the results taken directly after operation and in the other periods. We performed every possible combination between the results preoperative and 1^{st}, 3^{rd}, 5^{th} postoperative days in tear samples, but could not find significant difference in any cases during either of the laser treatments. Based on the above results, there can be a common enzymatic control

response during the corneal wound healing process in the studied refractive surgery procedures even if we thought that the wound healing processes differ to a large extent following these two procedures.

3. The PAI-2 levels of pregnant women's tear samples did not correlate with the pregnant age, the values measured in the tear samples remained essentially constant during pregnancy. We compared the PAI-2 values of tear with the results of non-pregnant tear samples. The average values did not differ significantly in the various periods of pregnancy , except for comparing the results of non-pregnant patients to that of the 16 weeks pregnant women, when it was p=0,022. The PAI-1 level in the tear samples remained under the lower level of detectability.

4. Comparing the PAI-2 levels in various pregnant ages, we could observe the followings: the values of the 8-13 weeks and the 16^{th} pregnancy week did not show significant difference compared to the 1^{st} week after child-birth. Conversely, a significant difference was observable between the average values of the 23-26 and 34-36 week pregnant ages. The PAI-2 level of blood constantly increased with the development of pregnancy.

In conclusion: the PAI values of pregnant women did not increase during normal pregnancy in the absence of wounding of surgical interventions. In the absence of ocular surface diseases, wounding or surgical intervention, the tear levels are independent of the change in the blood level of enzyme.

Keywords

plasminogen activator; plasminogen activator inhibitor; excimer laser; pregnancy; corneal wound healing

7. ACKNOWLEDGEMENT

Hereby, I would like to thank my supervisor, Dr. Adrienne Csutak senior lecturer for continuously following my work closely, and offering her utmost support and help during the project. I also thank Prof. Dr. András Berta university professor and my colleagues for assisting me in my work. I would like to express my gratitude to doctor Hassan Ziad for giving me the opportunity to collect tear samples from his patients undergoing surgery. Thanks are also due to Mrs. Bazsa Dr. Kassai Zsuzsa for her irreplaceable assistance in processing and analysing the blood samples taken from pregnant women. Last but not least, I would like to thank my family for supporting me throughout my work and providing the stabile background without which this study could not have been born.

CONTRIBUTION

The following tender made a significant contribution to the successful realization of our work: Vascular and cardiac research network: the pathomechanism, diagnostics and pharmacological controllability of vascular and cardiovascular diseases on basic research level. TAMOP- 4.2.2.A-11/1/KONV-2012-0045.

8. APPENDIX

Registry number: DEENK/164/2015.PL
Subject: Ph.D. List of Publications

Candidate: Zita Steiber
Neptun ID: D06K4O
Doctoral School: Doctoral School of Clinical Medicine
MTMT ID: 10034850

List of publications related to the dissertation

1. **Steiber, Z.**, Tőzsér, J., Silver, D.M., Jakab, A., Németh, G., Berta, A., Csutak, A.: Plasminogen activator inhibitor type 2 in human tears and blood during pregnancy. *Int. J. Ophthalmol. Eye Res.* 3 (7), 121-125, 2015.

2. **Steiber, Z.**, Ehlers, N., Heegaard, S., Hjortdal, J., Berta, A., Prause, J.U.: Brown cornea. *Graefes Arch. Clin. Exp. Ophthalmol. 246* (4), 537-541, 2008. DOI: http://dx.doi.org/10.1007/s00417-007-0736-9 IF:1.77

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4. Treszl, A., **Steiber, Z.**, Schally, A.V., Block, N.L., Dezső, B., Oláh, G., Rózsa, B., Fodor, K., Buglyó, A., Gardi, J., Berta, A., Halmos, G.: Substantial expression of luteinizing hormone-releasing hormone (LHRH) receptor type I in human uveal melanoma. *Oncotarget. 4* (10), 1721-1728, 2013. IF:6.627

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Total IF of journals (all publications): 13,276
Total IF of journals (publications related to the dissertation): 4,278

The Candidate's publication data submitted to the iDEa Tudóstér have been validated by DEENK on the basis of Web of Science, Scopus and Journal Citation Report (Impact Factor) databases.

31 August, 2015

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