**Treatment with the PAI-1 inhibitor and investigating its effect on kidneys and placenta in a mouse model of eclampsia**

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Preeclampsia in pregnancy is associated with modified coagulation parameters, fibrin deposits and increased plasminogen activator inhibitor (PAI) activity. This study examined the effect of PAI-1–derived peptide (PAI-1-DP), a PAI-1 antagonist, on kidneys and placentas in a mouse model of preeclampsia.The study group was treated with PAI-1-DP (1mg/kg IP BID). Immunohistochemistry was used to examine glomerular podocyte integrity and glomerular endotheliosis. Placental cytokine levels were examined using lysate production. Treated animals presented significantly preserved glomerular integrity, reflected by intensified stains of antibodies. Results indicate that treatment with PAI-1-DP in preeclampsia preserved kidney morphology and may reduce kidney injury by restoring function.

**Keywords:**

**Introduction**

Preeclampsia is a distinct heterogeneous syndrome [1] that occurs specifically during pregnancy and is one of the leading causes of morbidity and mortality of the fetus and the mother. This syndrome occurs in 5% to 10% of pregnant women [2] and is characterized by the development of hypertension and proteinuria after 20 weeks of pregnancy [3]. According to the World Health Organization, 20% of the 15 million preterm births reported each year are associated with preeclampsia [2]. Common clinical manifestations include maternal vascular dysfunction, chronic activity of the immune system, renal dysfunction and intrauterine growth restriction.

Left untreated, preeclampsia can lead to seizures, stroke, multisystem failure and death of the mother [4]. Stage one in the development of preeclampsia occurs as two parallel events that influence each other: partial breakdown of immune tolerance leading to impaired cytotrophoblast invasion and placental malformation leading to vascular pathology [5]. The collapse of maternal-fetal immune tolerance and placental malformation cause the second stage. Maternal syndrome, characterized by generalized impairment of endothelium function including hypercontraction of blood vessels, glomerular endotheliosis and vascular permeability, is the basic cause of the various symptoms of preeclampsia, such as hypertension and proteinuria [6].

Certain factors present in the placenta, such as anti-angiogenic factors (soluble fetal liver tyrosine sintase [sFlt-1] and soluble endoglin [sEng]) and free nucleic acids, are released into the maternal circulation and act on the walls of the blood vessels, altering the secretory ability of the endothelial cells and the responsiveness of vascular smooth muscles to constriction and relaxation stimuli. These factors are part of the placenta, providing a link between the placenta and the maternal vasculature. The maternal response to these factors depends on the health of the maternal vasculature, which could be compromised under conditions such as obesity, diabetes and poor nutrition, all of which are risk factors for the development of preeclampsia [3]. In preeclampsia, exaggerated apoptosis or clearance of apoptotic debris of cytotrophoblast cells in the placenta leads to an increase in the pro-inflammatory cytokine tumor necrosis factor alpha (TNF-α) and his receptor in the blood circulation that secreted by the macrophages [7]. High levels of TNF-α, which have a strong effect on endothelium and blood platelet function, are the cause of increased thrombin production, increase in hypercoagulability, vascular leakage, activation of endothelial cells and production of angiogenic factors, thus causing endothelial damage [8–10].

In addition, high levels of mRNA and the protein interleukin 6 (IL-6) have been seen in white blood cells in women with preeclampsia [11–15]. Maternal endothelial cells have demonstrated the ability to phagocytize dead cytotrophoblast cells and secrete IL-6, the cause of endothelial and vascular permeability [16,17]. In a healthy pregnancy, anti-inflammatory cytokines interleukin 4 (IL-4) and interleukin 10 (IL-10) are produced by the placenta, but in preeclampsia, production of these cytokines by the placenta is reduced [18,19]. In a healthy pregnancy, IL-10 is a potent depressor of pro-inflammatory cytokines such as TNF-α and interferon gamma (INF-γ), and it is produced by leukocytes such as macrophages, natural killer cells and T cells in the decidua [20–23]. In preeclampsia, the placenta responds to hypoxia by reducing the production of IL-10 and IL-4, leading to an increase in the production or uncontrolled production of pro-inflammatory cytokines that participate in the impaired invasion of cytotrophoblast cells into the maternal spiral arteries [24,25]. In addition, IL-10 has anti-apoptotic capabilities, and therefore, its decrease in the preeclamptic placenta is responsible for the increase in apoptosis of the cytotrophoblast cells [26].

Vascular endothelial growth factor (VEGF), placental growth factor (PLGF) and endoglin have angiogenic properties and are active in blood vessels. They play an important role in the formation of the placenta and regulate the activity of the maternal placental vasculature. In preeclampsia, production and signal transmission of these factors are altered because of the production of two anti-angiogenic factors, sFlt-1 and sEng, by the placenta. As a result, there is a disturbance in vascular function and maternal placental development, which causes the hypertensive phenotype [27]. VEGF expresses its biological effect by linking the two high-affinity tyrosine kinase receptors: VEGF receptor 1 (known as fetal liver tyrosine like [Flt-1]) and VEGF receptor 2 (known as kinase domain-related receptor [KDR] or fetal liver kinase 1 [Flk-1]) [28]. Activation of KDR in endothelial cells causes the release of nitric oxide (NO) and prostacyclin and promotes vascular relaxation [29]. sFlt-1 is the soluble form of PLGF/VEGF receptor and is produced by alternative splicing of mRNA Flt-1 [30]. Under normal conditions, it regulates the level of VEGF, but under hypoxic conditions, ischemia and oxidative stress, it is produced at high concentrations during formation of the placenta. sFlt-1 competes with Flt-1 for VEGF or PLGF and causes disruption of the process of angiogenesis by reducing the affinity of VEGF or PLGF for Flt-1 or Flk-1, thus inhibiting vascular expansion [27].

Levels of VEGF and PLGF in blood are reduced in women with preeclampsia. These data can be attributed to an increase in the level of sFlt-1 in the circulation [31]. The organ that is mainly affected by preeclampsia is the kidney. Proteinuria is one of the hallmarks of preeclampsia. Endothelial injury in the glomerulus is thought to be responsible for the kidney damage in preeclampsia. One of the factors involved in endothelium function at the glomerular barrier is endoglin (CD105), a type 1 glycoprotein located in the membrane of endothelial cells and a coreceptor for transforming growth factor beta (TGF-β) [32]. The link between them causes secretion of vasodilatory factors such as NO. In preeclampsia, as a result of oxidative stress in the placenta, membranal endoglin is split and soluble endoglin is formed that is secreted into the circulation. The soluble endoglin competes with the membranal endoglin in the glomerular endothelium for binding with TGF-β and thus inhibits the pathway [33]. There is a reduction in the secretion of NO followed by reduced vasodilation and hypertension, leading to endothelial dysfunction in the glomerulus and loss of filtration capacity [34].

In contrast, other studies have shown that podocytes, which are major components of the glomerular barrier, are responsible for the loss of renal filtration ability [35]. The glomerular barrier is made up of endothelium, glomerular basal membrane and podocytes. Podocytes are nondividing, large-body epithelial cells containing foot processes connected to each other by a diaphragm between them. Proteins essential for normal glomerular maintenance are located on the foot processes [36]. The function of the podocytes is to respond to high hydrostatic pressure in the kidney and produce proteins of the basal membrane and of the endothelium [37,38]. In preeclampsia, the damage in the podocyte could be related to damage in one or more of the proteins found in the foot processes. One of these proteins could be alpha actin 4 (ACTN4). ACTN4 is expressed in high levels in a number of human tissues, but mutations in this protein have only been detected in the kidneys. Mutations in ACTN4 demonstrate a strong binding to F-actin, which could change the mechanical properties of the podocyte and lead to destruction of the glomerular barrier and to renal disease [39,40].

In recent years, a number of reports have suggested treatment with the plasminogen activator inhibitor 1–derived peptide (PAI-1-DP) in patients with brain injury such as stroke and traumatic brain injury, because treatment with recombinant tissue plasminogen activator (tPA) leads to bleeding [41–43]. PAI-1-DP is a new peptide with 18 amino acids based on the hexapeptide EE11MD derived from PAI-1 [44–46]. The peptide interacts with the regulatory region of PAI-1 in tPA, which is outside its catalytic site and therefore cannot affect the fibrolytic function of tPA, which activates plasminogen that degrades fibrin [47]. In this way, PAI-1-DP inhibits the activity of PAI-1 and allows tPA to act to break down fibrin without interference.

In conclusion, despite studies on preeclampsia, its causes are not completely clear, and there is no effective treatment. In this study, we attempt to harness the inhibitory activity of PAI-1-DP that causes the disassembling of fibrin to prevent placenta and kidney injury in preeclampsia.

***Aims of the research***

This research aimed to examine if the use of the inhibitor PAI-1-DP would reduce kidney damage in a mouse model with preeclampsia symptoms by

(1) reducing the damage to podocytes. We assessed this with a histological test (immunohistochemistry on the kidney tissue) by staining with the antibody to ACTN4, which is expressed in podocytes that form part of the glomerular barrier.

(1) reducing endothelial damage. We assessed this by histological methods (immunohistochemistry on the kidney tissue) by staining with an antibody against endoglin (CD105), the receptor for TGF-β, expressed in the endothelium, which is part of the glomerular barrier.

This research also aimed to examine whether the use of the inhibitor PAI-1-DP would cause the emergence of changes in cytokines that participate in the angiogenic or inflammatory process, expressed in the placenta, in the mouse model with symptoms of preeclampsia. We measured this using a cytokine assay kit.

**Materials and methods**

***Animals***

Mouse model CBA/J\*DBA/2 of recurrent abortions is also a model for preeclampsia. Spontaneous pairing between males DBA/2 and females CBA/J led to a strain that developed many traits of preeclampsia (albuminurea, endotheliosis, increase in sensitivity to angiotensin 2, increase in levels of plasma leptin and antagonism of VEGF by VEGF receptor [sFlt-1]) that are involved in damage to the placenta and fetus [48]. The mice were 6 to 14 weeks old. They were mated to create a model for recurrent abortions and preeclampsia. Mating between female BALB/C and male CBA/J constituted the controls (normal pregnancy). Vaginal plug is evidence of mating; the appearance of the plug is considered day 0.5 of the pregnancy. Sacrifice of the animals was performed on day 14.5 (out of 21 days).

***Injection of PAI-1-DP***

We received the PAI-1-DP from Prof. Abd Al-Roof Higazi, Department of Clinical Biochemistry, Hadassah Hospital, Jerusalem, Israel. Mice in which a vaginal plug was seen were treated twice per day with intraperitoneal injection of PAI-1-DP at a concentration of 1 mg/kg until day 14.5. The control group included two groups: pregnant mice with signs of preeclampsia, with intraperitoneal injections of saline, and pregnant mice without symptoms of preeclampsia (W.T.).

***Histological test (immunohistochemistry)***

This method was performed by an automatic immunohistochemical device (Ventana, Tucson, Arizona, USA) using Chromogen DAB.

Kidneys were removed from the experimental mice and fixed in 4% formalin. Tissue sections were prepared and stained with specific antibodies. Microscopic examination was performed to measure intensity of the stain density in the glomerulus.

Two factors were measured in this test:

(1) Alpha actin 4(ACTN4): by staining with an antibody to alpha actin 4 (Rabbit anti Alpha actinin 4, 19096-1-AP, Proteintech) at a dilution of 1/500

(2) Endoglin (CD105): by staining with an antibody to endoglin (Rabbit anti Endoglin, 10862-1-AP, Proteintech) at a dilution of 1/100

***Cytokine assessment (mouse cytokine antibody array)***

To determine the cytokine levels in the placenta of the experimental and the W.T. mice, we examined cytokines with a predetermined cytokine kit (Mouse cytokine antibody array C1000, RayBio C-Series). The placentas were harvested on day 14.5, which parallels the second third of the human pregnancy. The tissues were collected into a test tube with lysis buffer containing protease inhibitors (150 µL protease inhibitor per 1 mL lysis buffer) and homogenized in a homogenizer. The liquid was centrifuged at 4 degrees, 30 min, 10,000 g. The liquid layer was transferred to a new test tube, and protein was determined by the BCA protein assay kit. The membranes containing the primary antibodies were inserted into the tray wells. The blocking buffer was added to each well and incubated for 30 min at room temperature. The samples were added at a concentration of 50µg to 500µg protein and incubated 1.5 to 5 h at room temperature on a shaker. The samples were washed with wash buffer, and the secondary antibody cocktail was added into each appropriate well and incubated for 1.5 to 2 h at room temperature. The samples were washed with wash buffer, and the substrate was added to the wells and incubated for 2 h at room temperature. The samples were washed again, and detection buffer was added on each membrane and incubated for 2 min at room temperature. Immediately after the incubation, the samples were exposed and photographed.

**Results**

***The effect of PAI-1-DP on the expression of ACTN4 in podocytes***

*Microscopic observation*

Microscopic observation of the renal glomerular tissue can discern the staining intensity of the antibody that binds to ACTN4. W.T. pregnant mice, without signs of preeclampsia, showed intense staining, reflecting the level of ACTN4 (Figure 1A). In mice with symptoms of preeclampsia without treatment with PAI-1-DP, the staining was weaker (Figure 1B). When the mice were treated with PAI-1-DP, the intensity of the stain increased significantly, and the level of ACTN4 increased (Figure 1C).

*Intensity of stain density*

Intensity of the stain density was measured in the glomerulus of the experimental mice (Figure 1D). In W.T. mice, intensity of the density observed was 0.3596. In mice that developed preeclampsia but were not treated with the inhibitor (N.T.), intensity of the density was 0.1294. In mice that developed preeclampsia and were treated with the inhibitor (PAI-1DP), the stain density observed was 0.3015. [FIGURE 1 NEAR HERE]

***The effect of PAI-1-DP on the expression of endoglin (CD105)***

*Microscopic observation*

By microscopic observation of the glomerulus in kidney tissue, the intensity of the color of the antibody that binds to endoglin can be seen. In W.T. pregnant mice, without signs of preeclampsia, a strong intensity of color can be seen, reflecting the high level of endoglin (Figure 2A). However, in mice with preeclampsia syndrome, without treatment with PAI-1-DP, the color is weak, reflecting the decrease of the level of endoglin (Figure 2B). When these mice were treated with the inhibitor, the intensity of the color increased and the level of endoglin increased (Figure 2C).

*Intensity of stain density*

The intensity of stain density was measured in the glomerulus of the experimental mice (Figure 2D). In W.T. mice, the intensity of the density was 0.3375. In untreated preeclamptic mice (N.T.), intensity of the density was 0.1625. In mice that developed preeclampsia and were treated with PAI-1-DP, stain density was 0.2292. [FIGURE 2 NEAR HERE]

***Cytokine experiment (mouse cytokine antibody array)***

*Expression of the angiogenic cytokine VEGF and its receptors*

***VEGF.*** Experimental mice that developed signs of preeclampsia and were not treated with PAI-1-DP showed a decrease in VEGF compared to W.T. mice that did not develop any signs of preeclampsia. Compared to mice without treatment, the experimental mice that were treated with PAI-1-DP showed a large increase in VEGF. In W.T. mice, the normal level of VEGF was 100%. In experimental mice without treatment, the level of VEGF was 75.6%, and in the experimental mice that received treatment, the level of VEGF was 223.15%, as can be seen in Figure 3.

***VEGF-R1 / VEGF-R2*.** At the same time that levels of VEGF were tested, the levels of its two main receptors were also tested: VEGF-R1 and VEGF-R2. VEGF-R1 was seen to decrease in untreated experimental mice, while with the addition of PAI-1-DP, there was an increase in this receptor but not above normal levels found in the W.T. strain. The receptor VEGF-R2 levels declined in mice without treatment. After addition of PAI-1-DP, the levels showed an additional reduction. In experimental mice without treatment, the level of VEGF-R1 was 16.5%, while the level in the experimental mice with treatment was 58.65%. VEGF-R2 levels in experimental mice without treatment was 79.4%, and after addition of treatment, the level dropped to 56.3%, as seen in Figure 3. [FIGURE 3 NEAR HERE]

*Expression of pro-inflammatory cytokines IL-6 and TNF-α and the receptors sTNFR1 and sTNFR2*

***IL-6.*** Experimental mice that developed signs of preeclampsia and were not treated with PAI-1-DP showed an increase in IL-6 compared to W.T. mice that did not develop any signs of preeclampsia. In comparison to the experimental mice without treatment, experimental mice treated with PAI-1-DP showed an additional increase in the level of IL-6. In W.T. mice, the normal level of IL-6 was 100%. In experimental mice without treatment, the level of IL-6 was 45%, while in the experimental mice with treatment, the level of IL-6 was 163.8%, as seen in Figure 4. [FIGURE 4 NEAR HERE]

***TNF-α.*** Experimental mice that developed signs of preeclampsia and were not treated with PAI-1-DP demonstrated a greater increase in the level of IL-6 compared to W.T. mice that did not develop any signs of preeclampsia. In comparison to untreated experimental mice, experimental mice treated with PAI-1-DP showed an additional increase in the level of TNF-α. In W.T. mice, the normal level of TNF-α was 100%. In untreated experimental mice, the level of TNF-α was 181.9%, and in the treated experimental mice, the level of TNF-α was 239.7%, as seen in Figure 5.

***sTNF-R1 and sTNF-R2*.**In parallel with the assay for the level of TNF-α, the levels of its two soluble receptors were examined: soluble tumor necrosis factor receptors 1 and 2 (sTNF-R1 and sTNF-R2). The level of sTNF-R1 showed a slight decrease compared to the normal level found in the W.T. strain. In treated experimental mice, after the addition of PAI-1-DP, the level remained the same compared to untreated experimental mice. The level of sTNF-R2 in the untreated experimental mice was similar to the normal levels in the W.T. strain. In treated experimental mice after the addition of PAI-1-DP, there was no change in this receptor compared to the untreated experimental mice and the W.T. mice. The level of sTNF-R1 in untreated experimental mice was 89.5%, and the level in the treated experimental mice was 91.73%. The level of sTNF-R2 in untreated experimental mice was 103.65%, and the level in the treated experimental mice was 109.2%, as seen in Figure 5. [FIGURE 5 NEAR HERE]

*Expression of the anti-inflammatory cytokines IL-4 and IL-10*

***IL-4.*** Experimental mice that developed signs of preeclampsia but were not treated with PAI-1-DP did not show changes in the level of IL-4 compared to W.T. mice that had not developed any signs of preeclampsia. Comparing experimental mice without treatment to the W.T. mice, the experimental mice that were treated with PAI-1-DP showed an increase in the level of IL-4. The normal level of IL-4 in W.T. mice was 100%. In untreated experimental mice, the level of IL-4 was 97.45%, while in the treated experimental mice, the level of IL-4 was 146.13%, as seen in Figure 6.

***IL-10*.** Experimental mice that developed signs of preeclampsia but were not treated with PAI-1-DP did not show changes in the level of IL-10 compared to W.T. mice that had not developed any signs of preeclampsia. Comparing experimental mice without treatment to the W.T. mice, the experimental mice that were treated with PAI-1-DP showed a slight increase in the level of IL-10. The level of IL-10 in W.T. mice was 100%. In untreated experimental mice, the level of IL-10 was 102.7%, while in the treated experimental mice, the level of IL-10 was 123%, as seen in Figure 6. [FIGURE 6 NEAR HERE]

**Discussion**

The first aim of the study was to investigate whether the use of PAI-1-DP would reduce kidney damage in a mouse model with preeclampsia, by way of reducing damage to the podocytes. Measurements of ACTN4 reflect damage to podocytes in the glomerulus of the kidneys.Following reports in the literature of the involvement of ACTN4 in the injury to podocyte cells in certain nephrotic syndromes [40], and because there are also reports of injuries in these cells in preeclampsia [35], we decided to investigate the effect of PAI-1-DP on levels of ACTN4 in glomerular podocytes in kidneys of experimental mice. Levels of ACTN4 would reflect kidney damage and injury to the podocytes in particular. Relating to the results that we found microscopically and the intensity of the stain density in the glomerulus of the W.T. mice, levels of ACTN4 appeared elevated, as we expected (Figure 1A). In the glomeruli of mice with signs of preeclampsia but without treatment, the levels of ACTN4 were reduced as we expected (Figure 1B). We expected to see very low levels of the protein, what would indicate a significant injury to the podocytes. When we added the PAI-1-DP to the experimental mice, a significant increase was seen in the levels of ACTN4, as we had anticipated (Figure 1C). The conclusion from these data is that the inhibitor is indeed raising the levels of ACTN4 (Figure 1D). The mechanism is not known, but we hypothesize that the disruption of the fibrin deposits in the kidney and reduction of coagulability via the inhibitor lead to an increase of perfusion to the kidney tissue and prevention of injury to the podocyte cells by preserving the structure of this protein.

The second aim of the study was to investigate whether the use of PAI-1-DP would reduce kidney damage in mice with preeclampsia via reduction of the injury to the endothelium. Measurements of endoglin (CD105) and the TGF-β receptor reflect endothelial damage in the glomerulus in the kidneys. The reason to investigate the levels of endoglin in the kidneys is because under normal conditions, there is normal biological activity of it with its ligand TGF-β on the glomerular endothelium. Reduction of endoglin levels will impede the TGF-β pathway and will cause swelling of the endothelial cells and loss of glomerular filtration [34]. In addition, a decrease in the level of endoglin can provide a general view of its biological activity and endothelial damage as a result of the development of preeclampsia. It is known from the literature that the kidneys are the major organs injured as a result of hypertension, mainly the endothelium of the kidneys, which functions as part of the glomerular barrier [35]. Therefore, endoglin is a good marker for the measurement of endothelial damage.

Regarding the results, when we received W.T. pregnant mice without signs of preeclampsia, endoglin levels seemed to be high, as seen in Figure 2A. When the intensity of stain density was measured, the result was high, as we expected (Figure 2D). In mice with signs of preeclampsia without treatment, we hypothesized that we would see a low level of endoglin, as reported in the literature [49,50]. The results showed a low level of endoglin, microscopically (Figure 2B) as well as with measurements of intensity of stain density (Figure 2D). However, in preeclamptic mice that received treatment with PAI-1-DP, there was a substantial increase in the endoglin level (Figure 2C). The conclusion reached from these results is that the inhibitor activates the expression of membrane endoglin, which causes angiogenesis and proliferation of endothelial cells and secretion of NO and prostacyclin, causing vasodilation.

The third aim of the study was to investigate whether the use of PAI-1-DP causes changes in the expression of cytokines that participate in angiogenesis or in the process of inflammation in the placenta in mouse models of preeclampsia. It is important to note that the aim of the cytokine investigation was to learn the extent to which a number of cytokines that participate in pregnancy and that are known in the literature may be associated with the development of preeclampsia. We did not invest in a large number of mice in each group but made due with three, simply to view the tendencies of each cytokine in order to continue our work on this subject. Therefore, statistical tests will not be available except for the mean.

***Results of the angiogenic cytokine VEGF and its receptors VEGF-R1 and VEGF-R2***

The level of placental VEGF decreased in mice that were not treated with PAI-1-DP as compared to W.T. mice, as reported in the literature [51], whereas in the experimental mice that were treated with PAI-1-DP, the level of VEGF increased threefold compared to the untreated mice. According to these results, we agree that PAI-1-DP causes an increase in the anticoagulant factor tPA, which activates the production of VEGF and increased angiogenesis of the blood vessels. When we investigated the level of VEGF-R1 receptor, known also as Flt-1, a promotor of vasoconstriction, we saw a sharp decline in the level of the receptor in mice without treatment with PAI-1-DP; when the inhibitor was added, there was an increase in the level of the receptor but not above the level in the W.T. mice.

The VEGF-R2 receptor, known as KDR or Flk-1, causes the secretion of NO and thereby induces vasodilation. This receptor was seen to decrease in untreated mice compared to the W.T. mice. Experimental mice that were treated with PAI-1-DP showed an additional reduction in the level of this receptor. The results obtained with the receptors Flk-1 and Flt-1 in preeclamptic mice without treatment are in agreement with reports from the literature; it is known that the level of the receptors for VEGF on the surface of the cells is altered in preeclampsia. For example, mRNA of Flt-1 is spliced to form sFlt-1. As a result of inadequate invasion of the cytotrophoblast cells into the maternal spiral arteries, an ischemic situation is created, leading to hypoxia and oxidative stress in the placenta. As a result, there is increased production of anti-angiogenic factor sFlt-1 that acts as a soluble receptor of VEGF or PLGF and functions as a regulator of VEGF and PLGF levels. sFlt-1 binds to VEGF or PLGF and competes for their binding to their membrane receptors. As a result, VEGF or PLGF binds to the soluble receptor that promotes vasoconstriction; at the same time, their level decreases in the placenta, and they cannot carry out their biological functions. Because VEGF binds to a receptor that promotes vasoconstriction and cannot bind to the KDR receptor that promotes vasodilation, the maternal blood vessels are in a state of hypercontraction leading to hypertension. As a result of the binding of PLGF or VEGF to sFlt-1, the angiogenic ability decreases in the placental vasculature or blood vessels in other organs such as the kidneys and brain; vascular dysfunction occurs, leading to hypertension and causing damage to the organs themselves [28–31,51].

When we investigated the mice that developed preeclampsia and were treated with PAI-1-DP, the results obtained were contrary to our hypothesis that PAI-1-DP would cause a decrease in the VEGF-R1 receptor that promotes vasoconstriction. Instead, the inhibitor caused an increase in its level. As for the receptor VEGF-R2 that promotes vasodilation, we hypothesized that its level would increase after treatment with inhibitor, but contrary to this, it decreased.

***Investigation of inflammatory cytokines***

There was an increase in the levels of pro-inflammatory cytokines TNF-α and IL-6 in experimental untreated mice that developed preeclampsia. These results are compatible with reports published in the past [52–57]. When we examined the experimental mice that developed preeclampsia and received treatment with PAI-1-DP, the results demonstrated an additional increase in pro-inflammatory cytokines TNF-α and IL-6. TNF-α plays a role in the pathology of preeclampsia via its cytotoxic effect on cytotrophoblast cells, causing death and inhibition of their infiltration into the spiral arteries. In addition, its effect on the endothelium and blood platelet activity cause hypercoagulability, vascular leakage and the production of anti-angiogenic factors [9,10,58]. IL-6 also plays a role in the pathology of preeclampsia by increasing vascular permeability and hyperactivation of the renin-angiotensin system, resulting in increased vasoconstriction and hypertension [16,17,59,60]. With regard to the levels of TNF-α, there was no decrease in the levels of the cytokine after treatment with the inhibitor, as we expected; instead, there was a further increase in its levels. It is known from the literature that part of the pathological activity of TNF-α is to increase coagulation via stimulation of PAI-1 production that inhibits tPA [61]. In contrast, treatment with the inhibitor PAI-1-DP should inhibit the activity of PAI-1 and cause hyperactivity of tPA that would break down fibrin fibers in blood clots and decrease coagulation [44–47,51,61].

The conclusions that can be reached from these data are that it is possible that an inconsistency in the activity of the inhibitor PAI-1-DP exists, because it is known to inhibit PAI-1 and activate tPA, a direct function. On the other hand, it causes an increase in the level and activation of TNF-α in the placenta that is known to promote the production of PAI-1. It is possible that although TNF-α stimulates the production of PAI-1, the inhibitor does the opposite and has a direct effect on PAI-1, leading to a reduction in clotting. There is a competition between production of PAI-1 by TNF-α and inhibition by PAI-1-DP, as can be seen in Figure 7. [FIGURE 7 NEAR HERE]

The model contains two parts:

(1) PAI-1-DP binds to tPA and inhibits PAI-1, resulting in stimulation of tPA and reduction in coagulation.

(2) PAI-1-DP causes an increase in the level of TNA-α that promotes production of PAI-1, resulting in the inhibition of tPA and an increase in coagulability.

It is not possible to draw a definite conclusion as to the effect of the inhibitor on decrease or increase in inflammation and coagulability. Therefore, additional parameters participating in the coagulation and inflammation processes need to be assessed, and this subject should be explored in depth in the future.

With regard to IL-6, an inflammatory cytokine that promotes vascular permeability and hypertension, we expected to see a decrease in the levels of the cytokine in mice that were treated with PAI-1-DP. Instead, the results we obtained showed an increase in its levels. The conclusion is that the inhibitor activates the cytokine activity and consequently increases the vascular permeability and raises blood pressure, which are not advantageous in preeclampsia and can even exacerbate its symptoms.

In this experiment, we measured the levels of the soluble receptors of TNA-α (sTNF-R1 and sTNF-R2 [sTNFp75, sTNFp55]) and not the membrane-bound receptors of TNA-α that are also present on the surface of placental cells. It was found that the levels of soluble receptors of TNF-α did not increase in the placentas of the experimental mice, compared to the W.T. mice. The soluble receptors of TNA-α are structures that have been split off and formed by shedding of the intracellular regions of the TNA-α receptor that is located on the cell surface. These receptors behave as antagonists and are able to bind to TNA-α and inhibit its biological activities. The aim of this receptor shedding is to regulate the amount of TNA-α and its cellular effects [52,55]. Our results were obtained from placental lysates and demonstrated a level similar to those of untreated experimental mice and experimental mice treated with PAI-1-DP, similar to W.T. mice. In most of the reports in the literature on preeclampsia, the level of the soluble receptors of TNA-α increased in the circulation and in the amniotic fluid, and their elevated levels in the placenta are not mentioned [52,55,62,63].

***Levels of anti-inflammatory cytokines***

Referring to the levels we obtained of anti-inflammatory cytokinesIL-4 and IL-10, there was an increase in mice treated with the inhibitor PAI-1-DP, as opposed to the untreated mice and compared to the W.T. mice. According to the literature, in a healthy pregnancy, the levels of anti-inflammatory cytokines are secreted from the placenta and are increased, while in preeclampsia, these cytokines are reduced [18,19,64,65]. Our results lead us to the conclusion that the inhibitor PAI-1-DP causes activation of cytokines and an increase in their levels. In the case of activating pro-inflammatory cytokines such as TNF-α and IL-6, the results are unsatisfactory, because instead of preventing the development of the syndrome, high levels of pro-inflammatory cytokines can accelerate its development. In contrast, activation of anti-inflammatory cytokines can lead to reduction of preeclamptic symptoms—for example, by preventing apoptosis of the cytotrophoblast cells that could penetrate the spiral arteries of the maternal vasculature in a normal fashion, causing maximum dilation in order to supply the blood to the fetus.

In conclusion, we observed that the inhibitor PAI-1-DP increases the level of expression of ACTN4, which is part of the membrane of the podocyte foot process. Injury to this protein in preeclampsia can be a sign of injury in the podocyte, and therefore, of kidney damage. Increase in the expression of this protein can prevent injury in the podocyte and renal damage in general.

We also observed that the inhibitor PAI-1-DP activates expression of VEGF and endoglin, two factors that participate in angiogenesis and proliferation of the endothelial cell and in the secretion of the vasodilators NO and prostacyclin. When referring to preeclampsia, it is known that these factors decrease as a result of the secretion of soluble receptors that inhibit their activity, and therefore, the action of the inhibitor on VEGF and endoglin is positive. At the same time, the inhibitor PAI-1-DP activates pro-inflammatory cytokines that are not beneficial for preeclampsia, but it also activates anti-inflammatory cytokines that regulate the activities of the pro-inflammatory cytokines. Most of the parameters that were investigated in this study, affected by the inhibitor PAI-1-DP, showed that this inhibitor has the ability to affect the symptoms of preeclampsia. Investigation of the mechanisms that promote the expression of parameters affected by the inhibitor should be continued.

**List of abbreviations**

ACTN4-Alpha actinin 4

Eng- Endoglin

eNOS- Endothelial nitric oxide synthase

Flt-1- Fetal liver tyrosine sintase

GBM- Glomerular basement membrane

IL-4- Interleukin 4

IL-6- Interleukin 6

IL-10- Interleukin 10

INF-γ- Interferon gamma

IUGR- Intra uterine growth restriction

KDR- Kinase domain related receptor

NK- Natural killer

NO- Nitric oxide

PAI-1- Plasminogen activator inhibitor 1

PAI-1-DP- Plasminogen activator inhibitor 1 derived peptide

PGI2-Prostacyclin

PLGF- Placental growth factor

sEng- Soluble endoglin

TGF-β- Transforming growth factor beta

TNF-α- Tumor necrosis factor alpha

TNF-R1- Tumor necrosis factor receptor 1

TNF-R2- Tumor necrosis factor receptor 2

tPA – Tissue plasminogen activator

VEGF- Vascular endothelial growth factor

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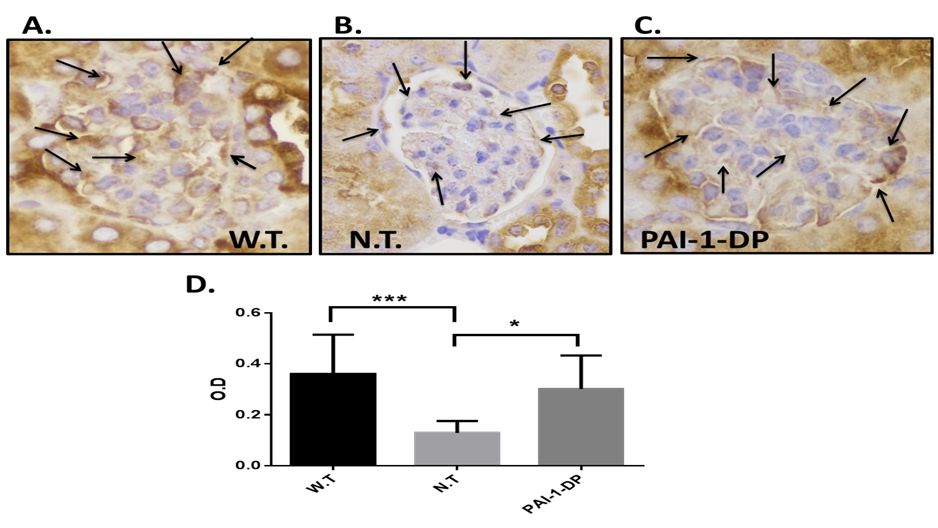
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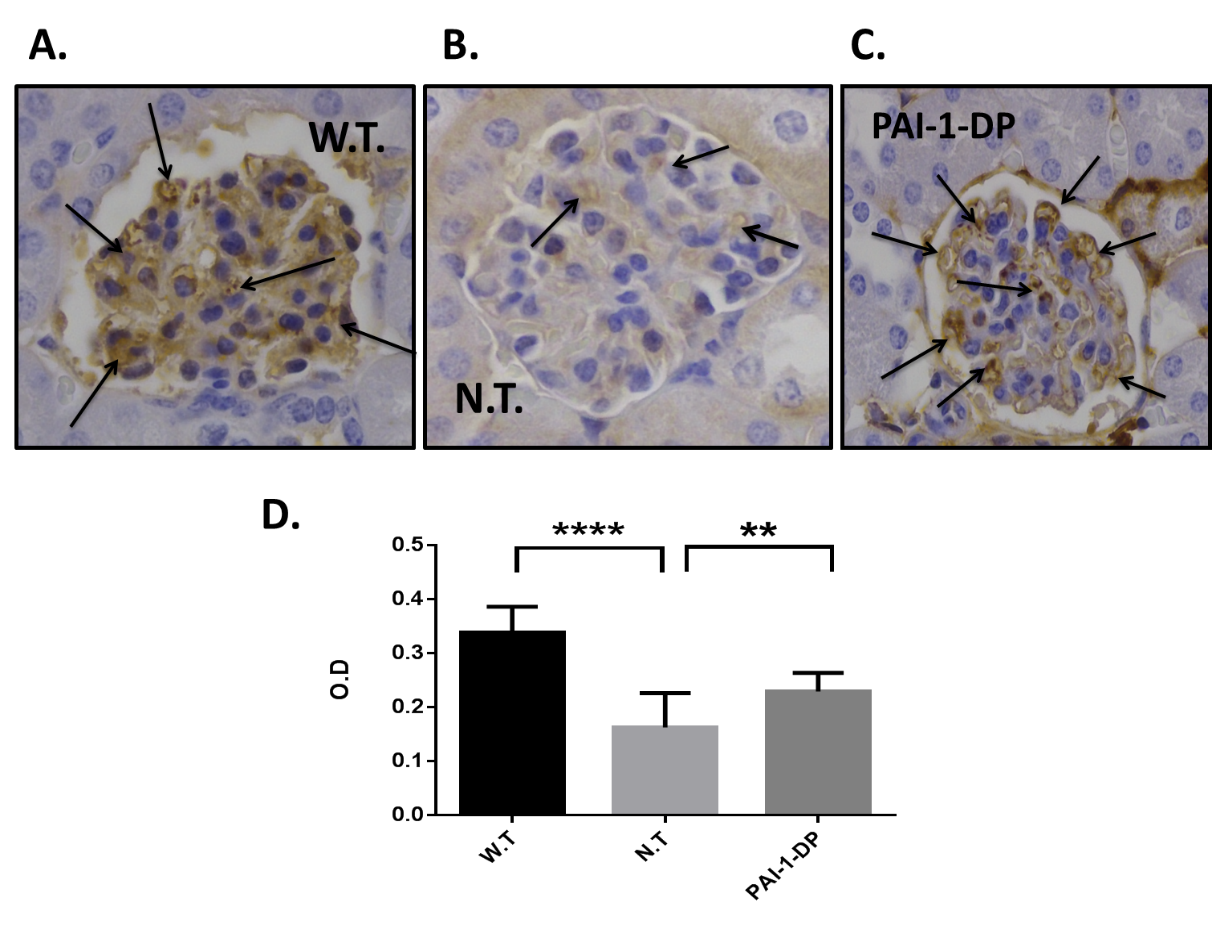
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**Figure 1. The effect of PAI-1-DP on the level of ACTN4 in kidneys of mouse models of preeclampsia.** Levels of alpha actinin 4 (ACTN4) in the glomerulus, light microscope x400. (A) Staining of ACTN4 in the glomerulus of pregnant W.T. mice without signs of preeclampsia. (B) Staining of ACTN4 in the glomerulus mice with symptoms of preeclampsia, not treated with plasminogen activator inhibitor 1–derived peptide (PAI-1-DP). (C) Staining of ACTN4 in the glomerulus mice with symptoms of preeclampsia, treated with PAI-1-DP. (D) Measurement of the effect of the inhibitor on ACTN4 levels, by intensity of stain density, in the kidney glomerulus of mice that participated in the experiments. W.T. = mice that did not develop preeclampsia; N.T. = mice that developed preeclampsia but were not treated; PAI-1-DP = mice that developed preeclampsia and were treated with the inhibitor (PAI-1-DP). \**P* < .05, \*\*\**P* < .001 (one-way ANOVA).



**Figure 2. The effect of PAI-1-DP on levels of endoglin (CD105) in kidneys of mouse models of preeclampsia**. Levels of endoglin (CD105) in the glomerulus, light microscope x400. (A) Endoglin stain in the glomerulus of pregnant W.T. mice, with no sign of preeclampsia. (B) Endoglin stain in the glomerulus of mice with symptoms of preeclampsia, not treated with plasminogen activator inhibitor 1–derived peptide (PAI-1-DP). (C) Endoglin stain in the glomerulus of mice with symptoms of preeclampsia, treated with PAI-1-DP. (D) Measurement of the effect of the inhibitor on endoglin levels, by intensity of stain density, in the glomerulus of mice included in the experiment. W.T. = mice that did not develop preeclampsia; N.T. = mice that developed preeclampsia but were not treated; PAI-1-DP = mice that developed preeclampsia and were treated with inhibitor. \*\**P* < .01, \*\*\*\**P* < .0001 (one-way ANOVA).



**Figure 3. The effect of PAI-1-DP on the expression of VEGF, VEGF-R1 and VEGF-R2 in the placenta of preeclampsia mouse models**. The percentages of protein measured in these experiments are shown as means. The results were normalized to the W.T. mice, where the W.T. was 100. In addition, comparisons were made between mice that developed preeclampsia but were not treated with the inhibitor. (A) The effect of plasminogen activator inhibitor 1–derived peptide (PAI-1-DP) on the expression of VEGF in the mouse model of preeclampsia. (B) The effect of PAI-1-DP on the expression of VEGF-R1 in the mouse model of preeclampsia. (C) The effect of PAI-1-DP on the expression of VEGF-R2 in the mouse model of preeclampsia. VEGF = vascular endothelial growth factor; VEGF-R1 = vascular endothelial growth factor receptor 1; VEGF-R2 = vascular endothelial growth factor receptor 2; W.T. = mice that did not develop preeclampsia; N.T. = mice that developed preeclampsia but were not treated; PAI-1-DP = mice that developed preeclampsia and were treated with inhibitor.



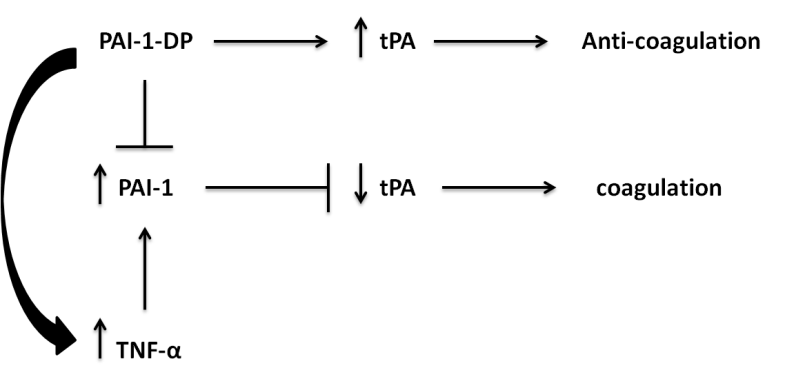
**Figure 4. The effect of PAI-1-DP on the expression of IL-6 in the placenta of mouse models of preeclampsia.** The percentages of protein measured in the experiments are shown as means. The results were normalized to the W.T. mice, where the W.T. is 100. In addition, a comparison was made between mice that developed preeclampsia but did not receive treatment with inhibitor. IL-6 = interleukin 6; W.T. = mice that did not develop preeclampsia; N.T. = mice that developed preeclampsia but were not treated; PAI-1-DP = mice that developed preeclampsia and were treated with inhibitor (plasminogen activator inhibitor 1–derived peptide).



**Figure 5. The effect of PAI-1-DP on the expression of TNF-α, sTNF-R1 and sTNF-R2 in the placenta of the preeclampsia-model mice**. The percentages of protein measured by the experiments are expressed as means. The results were normalized to the W.T. mice, considered to be 100. In addition, comparison was made between the mice that developed preeclampsia but did not receive treatment with the inhibitor. (A) The effect of plasminogen activator inhibitor 1–derived peptide (PAI-1-DP) on the expression of TNF-α in the preeclampsia mouse model. (B) The effect of PAI-1-DP on the expression of sTNF-R1 in the preeclampsia mouse model. (C) The effect of PAI-1-DP on the expression of sTNF-R2 in the preeclampsia mouse model. TNF-α = tumor necrosis factor alpha; sTNF-R1 = soluble tumor necrosis factor receptor 1; sTNF-R2 = soluble tumor necrosis factor receptor 2; W.T. = mice that did not develop preeclampsia; N.T. = mice that developed preeclampsia but were not treated; PAI-1-DP = mice that developed preeclampsia and were treated with inhibitor.



**Figure 6. The effect of PAI-1-DP on the expression of the anti-inflammatory cytokines IL-4 and IL-10 in the placenta of the preeclampsia-model mice.** The percentages of protein measured in the experiments are expressed as means. The results were normalized to the W.T. mice, considered to be 100. In addition, a comparison was made with the mice that developed preeclampsia but did not receive treatment with the inhibitor. (A) The effect of plasminogen activator inhibitor 1–derived peptide (PAI-1-DP) on the expression of IL-4 in the preeclampsia mouse model. (B) The effect of PAI-1-DP on the expression of IL-10 in the preeclampsia mouse model. IL-4 = interleukin 4; IL-10 = interleukin 10; W.T. = mice that did not develop preeclampsia; N.T. = mice that developed preeclampsia but were not treated; PAI-1-DP = mice that developed preeclampsia and were treated with inhibitor.



**Figure 7. Hypothetical model describing the interaction between PAI-1-DP, tPA, PAI-1 and TNF-α.** PAI-1 = plasminogen activator inhibitor 1; PAI-1-DP = plasminogen activator inhibitor 1–derived peptide; tPA = tissue plasminogen activator; TNF-α =tumor necrosis factor alpha.