# Induction of plasminogen activators in pregnant women with *Toxoplasma gondii* infection

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**Summary**

*Objective:* To determine whether plasminogen activators (PAs) are involved in the pathologic process of toxoplasmosis. *Materials and Methods:* Out of 220 pregnant women the study included 26 with a diagnosis of toxoplasmosis: six based on seropositivity for *Toxoplasma gondii* IgM and 20 based on seropositivity for *T. gondii* IgG. We measured serum activities and protein levels of PAs by casein zymography and Western blotting, respectively. *Results:* Serum PAs were higher in healthy pregnant women than in their healthy nonpregnant counterparts. Furthermore, serum PAs were significantly higher in pregnant women infected with *T. gondii* than in their healthy counterparts. *Conclusion:* PAs participate in the pathogenesis of toxoplasmosis in pregnant women and may be useful markers of *T. gondii* infection.

*Key words:* Plasminogen activators; Pregnancy; *Toxoplasma gondii;* tPA; uPA.

## Introduction

*Toxoplasma gondii* is an intracellular coccidian parasite that infects a wide variety of warm-blooded animals, including humans, and causes toxoplasmosis, a common zoonotic disease worldwide [1, 2]. Systemic infection follows oral ingestion and passage through the intestinal epithelium, basement membrane, and lamina propria. Active traversal of cellular barriers and upregulated migratory capacity are used for tissue dissemination to gain access to biologically restricted organs, e.g., the brain and the placenta [3]. Specifically, *T. gondii* uses proteinases to degrade the extracellular matrix (ECM) and reach immunoprivileged sites [4, 5].

The activation of plasminogen into plasmin is mediated by two types of activators, urokinase-type plasminogen activator (uPA) and tissue-type plasminogen activator (tPA) [6]. Microorganisms including bacteria, fungi and also parasites interact with components of the fibrinolytic pathway [7]. The highly invasive pathogens *Borrelia burgdorferi* [8], streptococci [9], and *Yersinia pestis* [10] express plasminogen activators. These pathogenic pathogens are capable of subverting the function of proteases, activators or inhibitors for their own benefit including dissemination within the host and evasion of host inflammatory immune response. Thus, several pathogenic bacterial species interfere with the mammalian proteolytic plasminogen-plasmin system. This interference promotes ECM damage as well as bacterial spread and organ invasion during infection [11]. PAs are associated with brain barrier disruption and pathogenesis in helminthic infections such as angiostrongyliasis [12, 13]. uPA participates in the release of the malaria parasite *Plasmodium falciparum* from infected erythrocytes [14].

The pathophysiologic role of the plasminogen system was deduced indirectly from correlations between levels of its components and clinical disease states. However, the role of PAs during *T. gondii* infection in pregnant women remains poorly understood. The purpose of this study was to determine the expression of PAs during *T. gondii* infection in pregnant women.

## Material and Methods

### *Patients and serum sampling*

Out of 220 pregnant women during the third trimester of pregnancy (38-40 weeks), the study included 26 with a diagnosis of toxoplasmosis: six based on seropositivity for *T. gondii* IgM and 20 based on seropositivity for *T. gondii* IgG. The serum titer was obtained using a commercially available enzyme-linked immunoassay for *T. gondii* IgM/IgG antibody (Euroimmun, Cardiff, UK). Further, using *T. gondii* IgG avidity enzyme immunoassay (Ani Labsystems, Finland), all six IgM seropositive samples had a low IgG avidity suggesting recent infection. Serum was obtained from fresh blood samples drawn from all 220 women. The Research and Ethical Review Committees and Internal Review Board of Chung Shan Medical University Hospital approved the protocol and procedures for the project.

### *Casein zymography*

The serum samples were centrifuged at 300 *g* for 10 min to remove debris. Activities of PAs were determined using casein zymography according to the method of Hou *et al.* [12] with slight modifications. Briefly, the samples were diluted in a standard loading buffer containing 0.1% sodium dodecyl sulfate (SDS) without β-mercaptoethanol, and the diluted samples were not boiled before loading on 7.5% (mass/volume) SDS-polyacrylamide gels copolymerized with 0.1% casein (Sigma, St. Louis, MO, USA) and plasminogen (13 mg/ml, Sigma, St.

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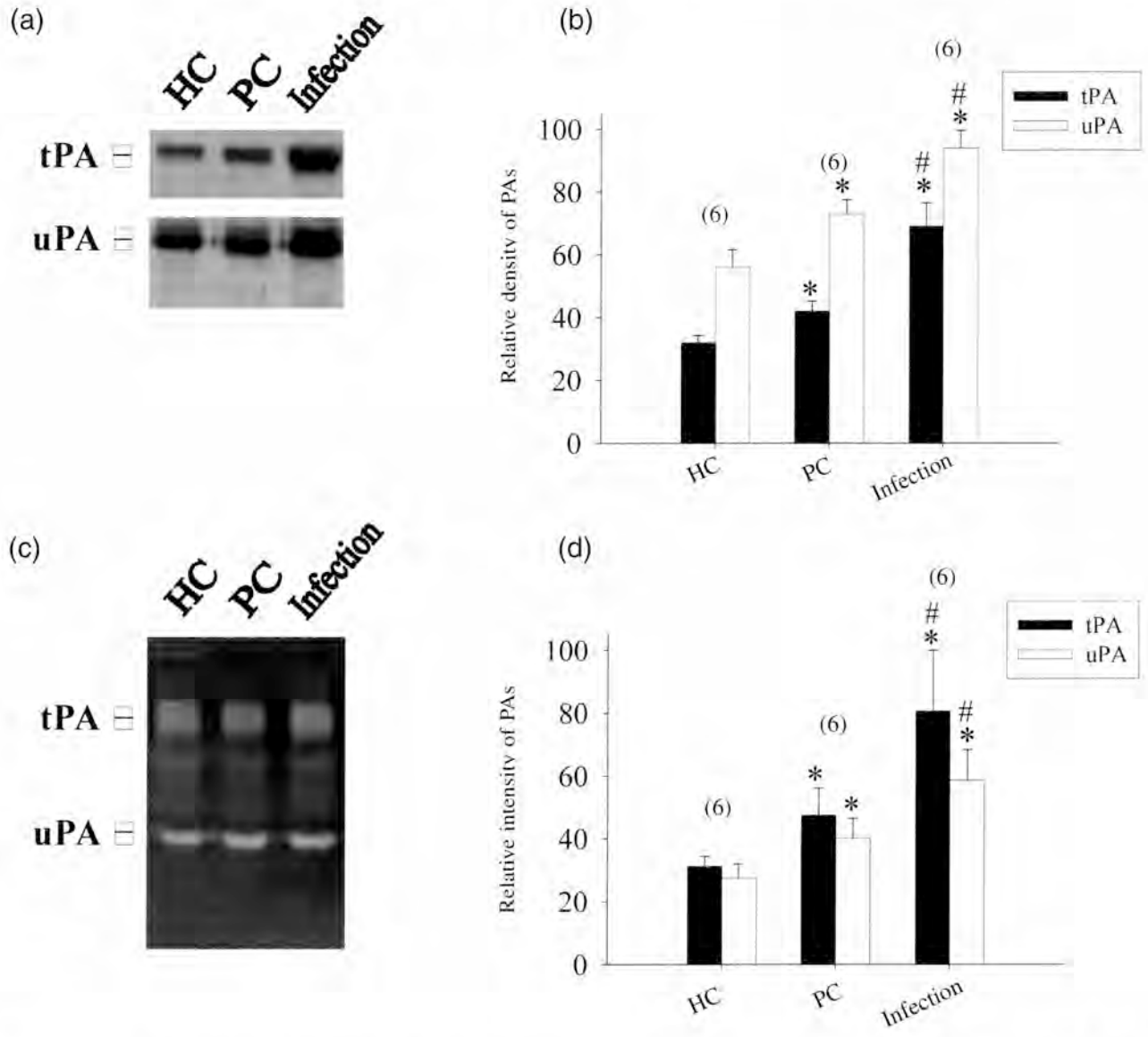


Figure 1. — Protein levels and activities of serum plasminogen activators in pregnant women seropositive for *Toxoplasma gondii* IgM. (a) The protein levels of PAs were detected by Western blot assay using anti-tPA and anti-uPA, respectively. (c) The activities of tPA and uPA were detected by casein zymography. (b, d) Quantitative analysis of the tPA and uPA bands was performed using a computer-assisted imaging densitometer system. Sample size is shown in parentheses and all data are presented as mean ± standard deviation. HC, healthy non-pregnant women; PC, healthy pregnant women; Infection, women seropositive for *T. gondii* IgM. $^{*}p < 0.05$ indicates a significant difference compared with healthy nonpregnant women. $^{\#}p < 0.05$ indicates a significant difference compared with healthy pregnant

Louis, MO). The stacking gels were 4% (mass/volume) polyacrylamide and did not contain casein and plasminogen substrate. Electrophoresis was performed in running buffer (25 mM Tris, 250 mM glycine, 1% SDS) at room temperature at 120 V for 1 h. After electrophoresis, the gel was washed in renaturing buffer (2.5% Triton X-100) in a shaker for 30 min, with one change after 30 min to remove SDS, washed two times with double-distilled water (10 min each), incubated in reaction buffer (50 mM Tris-HCl, pH 7.5, containing 10 mM $CaCl_2$, 0.02% Brij-35, 0.01% $NaN_3$) at 37°C for 18 h, stained with 0.25% Coomassie Brilliant Blue R-250 (Bio-Rad, Hercules, CA) for 1 h, and destained in 15% methanol/ 7.5% acetic acid. The final gel appeared uniform in all regions except those to which tPA (70 kDa), and uPA (55kDa) had migrated and cleaved their respective substrates. Quantitative analysis of the caseinolytic enzyme was performed with a computer-assisted imaging densitometer system, UN-SCAN-IT$_{TM}$ gel Version 5.1 (Silk Scientific, Orem, UT).

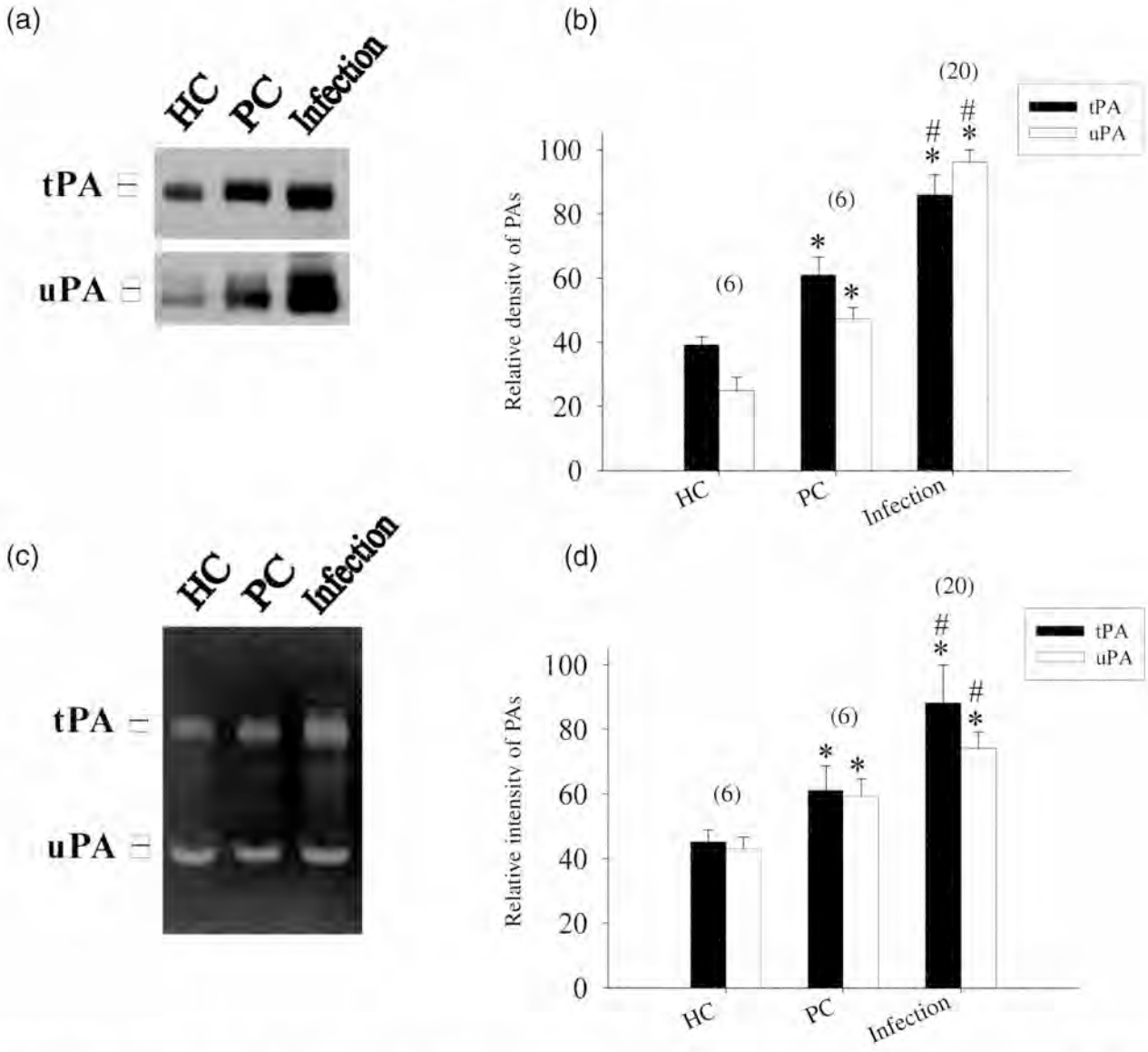


Figure 2. — Protein levels and activities of serum plasminogen activators in pregnant women seropositive for *Toxoplasma gondii* IgG. (a) The protein levels of PAs were detected by Western blot assay using anti-tPA and anti-uPA, respectively. (c) The activities of tPA and uPA were detected by casein zymography. (b, d) Quantitative analysis of the tPA and uPA bands was performed using a computer-assisted imaging densitometer system. Sample size is shown in parentheses and all data are presented as mean ± standard deviation. $^{*}p < 0.05$. HC, healthy non-pregnant women; PC, healthy pregnant women; Infection, women seropositive for *T. gondii* IgG. $^{*}p < 0.05$ indicates a significant difference compared with healthy nonpregnant women. $^{\#}p < 0.05$ indicates a significant difference compared with healthy pregnant women.

*Western blotting*

The protein contents of the serum samples were determined using a protein assay kit (Bio-Rad, Hercules, CA) and bovine serum albumin as the standard. The samples were mixed with an equal volume of loading buffer (62.5 mM Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, 5% 2-mercaptoethanol, and 0.05% bromophenol blue). The mixture was boiled for 5 min prior to electrophoresis on 10% (mass/volume) SDS polyacrylamide gel and electrotransferred to nitrocellulose membrane overnight at a constant current of 30 mA. Afterward, the membrane was washed three times (30 min each) in PBS containing 0.1% Tween 20 (PBS-T) at room temperature, allowed to react with goat anti-human tPA or anti-human uPA polyclonal antibody (1:1000; American Diagnostica, Stamford, CT) at 37°C for 1 h, washed three times with PBS-T, incubated with HRP-conjugated rabbit anti-goat IgG (1: 5000; Jackson ImmunoResearch Laboratories, West Grove, PA) at 37°C for 1 h to detect the bound primary antibody, and then developed using enhanced

chemiluminescence (Amersham Biosciences, Amersham, UK) to visualize the reactive protein. Quantitative analysis of the caseinolytic enzyme was performed with a computer-assisted imaging densitometer system, UN-SCAN-IT$_{TM}$ gel version 5.1 (Silk Scientific, Orem, UT).

*Statistical analysis*

Results in the different groups were compared using the nonparametric Kruskal-Wallis test followed by post-testing using Dunn's multiple comparison of means. All results are presented as mean ± standard deviation. $P$ values of $< 0.05$ were considered statistically significant.

## Results

*Protein levels and activities of PAs in serum containing T. gondii specific IgM antibodies*

Two bands (70-kDa and 55-kDa corresponding to tPA and uPA) were detected in all samples by Western blot analysis. Both tPA and uPA are trypsin-like serine proteinases that activate plasminogen directly. The activity of PAs in serum with *T. gondii* IgM antibody was obtained by casein zymography. The protein levels (Figure 1a, 1b) and activities (Figure 1c, 1d) of PAs were significantly higher ($p < 0.05$) in pregnant women seropositive for *T. gondii* IgM than in seronegative (pregnant or nonpregnant) women.

*Protein levels and activities of PAs in serum containing T. gondii specific IgG antibody*

The protein levels (Figure 2a, 2b) and activities (Figure 2c, 2d) of tPA and uPA in serum were significantly higher ($p < 0.05$) in pregnant women seropositive for *T. gondii* IgG than in seronegative (pregnant or nonpregnant) women.

## Discussion

The plasminogen activation system is involved in the regulation of inflammation and is used by *Borrelia* species to enhance its invasiveness [8, 15]. Higher plasma tPA levels have been reported in women infected with human immunodeficiency virus than in normal postpartum women [16]. However, PAs represented in inflammation during pregnancy were scarcity. Here we report that the serum levels of PAs are increased in pregnant women seropositive for *T. gondii* IgM and IgG. These data suggest that PAs participate in the pathogenesis of *T. gondii* infection.

Cytokines regulate the PAs expression/secretion [17]. IL-6 regulates dengue virus-induced tPA production by endothelial cells and may have an important role in the development of dengue hemorrhagic fever and dengue shock syndrome [18]. Cytokines (such as IL-1β, IL-12, IL-15, TNF-α, and IFN-γ) have been shown to be involved in the pathogenesis of toxoplasmosis [19, 20]. Furthermore, *T. gondii* infection upregulates PA activity, and INF-γ upregulates hepatic u-PA activity during infection [21]. Thus, inflammatory cytokines are increased during toxoplasmosis. Therefore, we proposed that the increased expression of PAs in pregnant women with toxoplasmosis might be mediated via inflammatory cytokines.

*T. gondii* migrates across biological barriers to reach immunologically privileged sites where the most severe pathology is observed [3]. Dissemination is via two pathways: 1) active penetration of the epithelium barrier and the ECM and 2) use of nucleated cells (e.g., macrophages) as "Trojan horses" [3, 4]. Furthermore, *T. gondii* takes advantage of the motility of host cells (e.g., dendritic cells) to infiltrate and pass through biological barriers (e.g., endothelial cells and ECM) *in vivo* [3, 22]. The crossing of biological barriers and infiltration into the ECM by *T. gondii* may involve matrix metalloproteinases (MMPs) [5]. The passage of *T. gondii* through vascular basement membranes or through tissue ECM may be facilitated by proteolytic degradation of matrix proteins. The combined effects of PAs, plasmin, and MMPs generate a proteolytic cascade directed at ECM degradation. Therefore, we suggest that PAs participate in ECM degradation to allow immune cell migration through the barrier and *T. gondii* dissemination into adjacent tissues.

Human parturition is a multi-step process involving myometrial contraction, cervical ripening, fetal membrane rupture, and detachment of the placenta and fetal membranes from the maternal uterus [23]. Some of these steps require degradation or remodeling of the ECM by proteolytic enzymes [24]. The PA cascade is thought to play a critical role in labor-associated remodeling events, such as fetal membrane rupture and placental separation [25]. Increased levels of tPA and uPA antigens are found after the third trimester of pregnancy, and levels of PAs remain high through the first stage of labor. However, tPA antigen levels continue to rise during the first few postpartum hours, while uPA antigen levels normalize immediately following childbirth [26]. Although PA activity remains essentially constant during pregnancy, the fibrinolytic system changes in a complex manner [27]. In our study, serum PA activity was higher in healthy pregnant women than in their healthy nonpregnant counterparts. Furthermore, serum PA activity was significantly higher in pregnant women infected with *T. gondii* than in their healthy counterparts. These results suggest that PAs are participants in the physiologic processes required for labor, but overexpression of PAs may cause ECM degradation in pregnant women with *T. gondii* infection.

Although our experiments were not designed to evaluate serum activity in pregnant women infected with *T. gondii* after premature delivery or abortion, we suppose that the toxoplasmosis-induced proteolytic cascade of PAs is involved in premature or abortive pathophysiology. Therefore, we suggest that serum PA levels in *T. gondii* infected mothers might be used to track the risk of infection to fetal health.

In conclusion, serum PA levels are higher in pregnant women with toxoplasmosis than in their noninfected

counterparts during labor. We suppose that PAs participate in the pathogenesis of toxoplasmosis in pregnant women and may be useful markers of *T. gondii* infection.

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