Pathogenetic examinations of preeclampsia on an animal model and on humans

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1. Introduction

With an incidence of 1-5% preeclampsia is a major cause of fetal and maternal morbidity (1). Manifested generally in the mid-to-late stages of gestation, preeclampsia is diagnosed on the basis of three clinical signs: hypertension, proteinuria and edema. Severe and acute complications include a convulsive condition called eclampsia and/or a condition with multiorgan dysfunction, most often appearing as Hemolysis, Elevated Liver Enzymes and Low Platelet count (HELLP) syndrome (2). Risk for the development of the disease is increased in the case of primiparity, work-related psychosocial strain during pregnancy, bad social background, the mother's own low birthweight, prematurity and young age (3, 4, 5). Chronic hypertension, diabetes and obesity predispose women to preeclampsia (6, 7). Severe early-onset preeclampsia is often associated with clotting abnormalities, such as the Leiden mutation of factor V, prothrombin 20210A allele, protein C, protein S and antithrombin deficiency, possibly leading to a more accelerated course of the disease by inducing an abnormal interaction between endothelial cells, platelets, leukocytes, and plasmatic factors, as well as causing intervillous or spiral artery thrombosis and consequently inadequate placental perfusion (6, 8, 9).

The onset and progression of the disorder are unpredictable, and the only entirely effective treatment for preeclampsia/eclampsia is termination of the pregnancy. The etiology and pathogenesis of the disease are still unknown. Since preeclampsia may occur in women with abdominal pregnancies, it is not likely or at least not necessary for the uterus to play a part.

2. Etiology and pathomechanism of preeclampsia

2.1. Two-stage disorder theory

According to the widely accepted theory suggested by Redman and Roberts, preeclampsia is a two-stage disorder (10). The illness derives primarily from a placental ischemia (11, 12). Poor placental perfusion, as tested in animals (13), either as a result of immunologically mediated abnormal implantation, excessive placental size or microvascular disease derived from pre-existing hypertension, diabetes or other factors, predispose pregnancies to preeclampsia. Pregnancy induces the uterine spiral arteries to remodel into dilated uteroplacental vessels by an unknown mechanism called "physiological change" (14). During this process extravillous cytotrophoblasts invade the uterine endometrium and myometrium and migrate in a retrograde direction up the spiral arteries, transforming them into large-bore tortuous vessels of low resistance (15). This physiological remodeling process, characterized by a gradual loss of the

normal musculoelastic structure of the arterial wall, is required for a successful pregnancy and spiral artery transformation failure has been well documented in preeclampsia (16, 17).

Consequent (secondary) clinical symptoms reflect a generalized vasoconstrictive disorder as a result of abnormal vascular endothelial function. These changes are similar to the changes observed in disseminated intravascular coagulation and hemolytic uremic syndrome. The serum levels of a certain adhesion molecule, the vascular cell adhesion molecule (VCAM-1), were found to be elevated. Higher VCAM-1 levels may represent a possible mechanism by which endothelial cells attract leukocytes, which cause endothelial cell damage (18). Disturbances in endothelial cell function may lead to elevated shear stress of erythrocytes, resulting in hemolysis, increased red blood cell turnover (19) and release of red blood cell content, all of which can aggravate endothelial damage. Furthermore, there is a breakdown in the balance between vasodilator substances such as prostacyclin, prostaglandin E_2 and nitric oxide and the vasoconstrictors including angiotensin II, thromboxane A_2, serotonin and endothelin (20, 21). In preeclampsia, the placenta produces seven times more thromboxane A_2 than prostacyclin (21). As postulated by Roberts et al. (22), blood-borne products originating from a poorly perfused fetal-placental unit could injure or activate endothelial cells and the resultant changes in the function of these cells could activate intravascular coagulation and increase sensitivity to pressors. During the hypoxia-reperfusion injury of the ischemic placenta the enzyme xanthine oxidase, which has also been detected in the human placenta (23), generates superoxide anions. Xanthine oxidase is one of the main sources of reactive oxygen species in hypoxic tissues, and so it contributes greatly to consequent oxidative tissue injury (24). The transfer of oxidative stress processes from the intervillous space to the systemic circulation may involve activated leukocytes, stable products of lipid peroxidation and even cytokines. Such cytokines, originating from the foci of placental hypoxia/ischemia, are tumor necrosis factor-α and interleukin-1 α and β, the production of which were found to be elevated in hypoxic conditions of villous explants from the human placenta (25). Similarly, the expression of placental cytokines, such as tumor necrosis factor-α, interleukin-1 β and interleukine 10, was increased in the placentae of preeclamptic subjects (26).

Beyond the two-stage theory of the disease, three further hypotheses are currently at the heart of extensive investigation. It must be stressed that all of these theories, which attempt to explain the etiology and pathomechanism of the disease, are not mutually exclusive but probably interactive.

2.2. Very Low-Density Lipoprotein versus toxicity-preventing activity

Pregnancy in general, and preeclamptic pregnancy in particular, are associated with a marked hyperlipidemia. In preeclampsia, circulating free fatty acids are increased as early as 15 to 20 weeks before the onset of the clinical disease (27). In compensation for increased energy demand in women with low albumin concentration, transporting extra non-esterified fatty acids from adipose tissues to the liver is likely to reduce albumin antioxidant activity to a point at which very low-density lipoprotein toxicity is expressed and triglyceride accumulation occurs in endothelial cells (28). As suggested by Lorentzen et al., when placentally derived endothelial disturbing factors, like lipid peroxides and trophoblastic components, are released into the maternal circulation, their effects on the endothelium may be enhanced by the hyperlipidemia-mediated activation or "sensitization" of the endothelial cells (29). As further support for the involvement of the lipid metabolism in the pathomechanism of preeclampsia, common coding sequence variations in the lipoprotein lipase gene have been shown to cause a substantially increased risk of preeclampsia (30). A recurrent theme is that free radical reactions, promoted by a "cross-talk" between the diseased placenta and maternal dyslipidemia, promote a vicious cycle of events that render cause and effect difficult to distinguish but likely contribute to the progression of preeclampsia (31). In preeclampsia increased red blood cell turnover can also be detected, as demonstrated by its indicators (19). Since heme derivatives may mediate lipoprotein oxidation and consequently endothelial damage, hemolysis may be an additional factor in the pathogenesis of the disease (32, 33, 34).

2.3. Immune maladaptation

Epidemiological evidence suggests that immune mechanisms ought to be involved in the etiology of preeclampsia (35, 36, 37). Genuine preeclampsia is primarily a disease of first pregnancies. A previous normal pregnancy is associated with a markedly lowered incidence of preeclampsia. Even a previous abortion provides some protection. The protective effect of multiparity, however, is lost with change of partner: thus, preeclampsia may be a problem of primipaternity rather than primigravidity (36).

The decidua contains an abundance of cells of bone marrow origin as is demonstrable by their cell surface antigen expression (CD45). These antigens are designated by their CD number, an arbitrary number assigned to each Cluster of Differentiation. In the late secretory

phase of the endometrium an unusual population expressing CD56, a marker of peripheral blood large granular lymphocytes, becomes dominant. These uterine large granular leukocytes resemble natural killer (NK) cells but do not express such strong NK cell activity as peripheral blood NK cells. About 75% of decidual cells express CD45, reflecting their bone marrow origin (i.e. 45% uterine large granular leukocytes, 19% macrophages, and 8% T cells). As pregnancy progresses, the number of macrophages and T cells remains constant but the number of uterine large granular leukocytes declines dramatically (38, 39, 40). The immunologically competent cells of the decidua may be important in the regulation of cytotrophoblast invasion (41).

What can be the link between immune maladaptation and endothelial cell activation in preeclampsia? The decidual cells of bone marrow origin can be activated and release mediators that may interact with endothelial cells. Such mediators most often regarded as etiological are cytokines and oxygen free radicals.

2.3.1. Involvement of Human Leukocyte Antigens (HLA) in the development of preeclampsia

In preeclamptic women and in their spouses there is a greater homozygosity at the HLA B locus (42). Also, antigen sharing at the A and B loci and HLA DR4 is greater between affected women and their spouses (43). The inducibility of TNF-α is HLA class II dependent, thus also providing a possible role for the HLA system.

On the other hand, so far the numerous studies on the role of the HLA system in preeclampsia have failed to lead to the detection of any definite influence (1). Wilton et al. (44) were able to rule out the linkage of maternally expressed susceptibility genes to the HLA region, but fetal involvement must still be taken into consideration (45). Preeclampsia is unlikely to be the simple result of excessive HLA antigen sharing between mother and fetus, as was first thought, but a more complex mechanism involving feto-maternal compatibility cannot be excluded. HLA-A, -B and -C genes are blocked in the placental trophoblast cells (46). Instead, HLA-G, a non-specific HLA I group antigen, is expressed by the trophoblast cells (47). We may suppose that HLA-G is involved in the development of immune tolerance (48). Although there was no detectable relationship found between susceptibility to preeclampsia or being born of a preeclamptic pregnancy and the HLA-G genotype (49), a more recent study revealed that an absence/reduced level of HLA-G expression in extravillous

cytotrophoblasts is associated with preeclampsia. According to this study, trophoblasts lacking HLA-G may be vulnerable to attack by the maternal immune system (50).

Many of the findings concerned with the role of HLA in the development of preeclampsia are inconsistent or contradictory. However, further examinations of HLA in preeclampsia are likely to find new links.

2.4. Genetics of Preeclampsia

2.4.1. Role of Genetics: family analysis, genomewide analysis

According to epidemiological studies, preeclampsia/eclampsia has a strong familial component (51, 52, 53). Daughters of preeclamptic women have a higher chance of themselves developing preeclampsia/eclampsia. Phenotyping patients with preeclampsia is vital for any genetic study. In a genomewide screen of Icelandic families representing 343 affected women, a significant locus was found on 2p13 (54), while in another genomewide linkage study of preeclampsia/eclampsia evidence was found for a candidate region on 4q (55). With a mathematical analysis of three or four generations hypothetically either a single dominant gene with incomplete penetrance or a single recessive gene could fit the data (56). Still, it is unlikely that there is a single gene responsible for the pathogenesis of preeclampsia. We may, however, suppose that a cluster of polymorphisms, possibly in conjunction with environmental factors, predisposes one to the development of the disease. It is also likely that a fetal genetic contribution may also be involved in the pathogenesis: there is an increased risk of preeclampsia in women who become pregnant by a man who has already fathered a preeclamptic pregnancy in another woman (3).

2.4.2 Genetic factors possibly involved in the pathogenesis of preeclampsia/eclampsia

2.4.2.1.The role and the effect of genetic variability in the renin-angiotensin system

All major components of the renin-angiotensin system (renin, prorenin, angiotensinogen, angiotensin (AT) I, angiotensin II, angiotensin converting enzyme (ACE) and angiotensin receptors) are present in the human placenta and related tissues, forming one of the examples of the recently accepted local renin-angiotensin system (57, 58). In this system, AT II can act in an autocrine/paracrine fashion (59): it can stimulate angiogenesis and also is capable of antiproliferative actions by mediating the inhibition of endothelial cell proliferation (60, 61). Since renin, ACE and AT receptor 1 are all expressed in and around remodeling spiral arteries (57), the known actions and presence of the renin-angiotensin system suggest that the local

spiral artery renin-angiotensin system may play a role in the pregnancy-induced remodeling of these vessels. During the last decade, the possible role of genetic variability in the constituents of the renin-angiotensin system in the pathogenesis of preeclampsia has been extensively examined:

Angiotensinogen: A significant association of preeclampsia with a molecular variant of angiotensinogen, M235T (threonine for methionine), was observed in a series of Caucasian women with pregnancy-induced hypertension (62). Moreover, heterozygous women were shown to exhibit a significantly elevated expression of the T235 allele compared to the M235 allele. Further observations suggested that the elevated expression of the T235 allele in decidual spiral arteries may cause first-trimester atherotic changes leading to preeclampsia (63). On the other hand, the contribution of angiotensinogen gene polymorphism to the occurrence of preeclampsia/eclampsia seems not to be constant across populations (64).

ACE gene polymorphism: In two studies evaluating the effect of ACE insertion-deletion polymorphism on the incidence of preeclampsia/eclampsia, no evidence was found that the polymorphism of the ACE gene was associated with preeclampsia/eclampsia (65, 66).

Renin In an investigation on two or three generations of affected females, although only in a relatively smaller number of families, gene restriction fragment length polymorphism did not exhibit linkage with preeclampsia/eclampsia (67).

AT receptor type 1: In a recent study, Morgan et al. found that allele and genotype frequencies in four polymorphic regions did not differ between normotensive and preeclamptic groups. However, two variants (a 573T variant in the coding exon of the receptor gene and a dinucleotide repeat polymorphism in its 3' flanking region) showed a similar distortion of maternal-fetal transmission (68). According to this finding, the AT1 receptor transmission in the fetus may contribute to the etiology of preeclampsia.

2.4.2.2. Endothelium-derived nitric oxide synthase (eNOS)

Of the three isoforms of NOS, eNOS is widely distributed in the placental tissue (69), producing nitric oxide (NO), a potent vasodilator and inhibitor of platelet aggregation (70). Although eNOS is expressed by syncytiotrophoblasts, no proof of expression was found in the case of extravillous cytotrophoblasts at any time during invasion (71). This fact suggests that NO is unlikely to contribute to spiral artery dilatation. On the other hand, reduction of eNOS activity was detected in preeclamptic placentae (72, 73), implicating the eNOS gene in the pathophysiology of preeclampsia/eclampsia. A linkage study using preeclamptic families

reported evidence for a preeclampsia/eclampsia susceptibility locus in the eNOS region on chromosome 7q36 (74). This, however was not confirmed in a recent repetition study using the same markers (75), while in another recent linkage study using 25 microsatellite markers from chromosome 7, a strong suggestion of linkage was found for one marker, D7S1805 (76). The results of these examinations raise the strong possibility that a putative preeclampsia/eclampsia susceptibility locus may be located on 7q36.

2.4.2.3. Is preeclampsia a mitochondrial disease?

In the late 1980's Tobergsen and his co-workers observed that a high incidence of preeclampsia was present in a family with mitochondrial dysfunction (77). The same research group found that mutations in the mitochondrial transfer ribonucleic acid genes in two families were also associated with preeclampsia (78). Differentiation of the early embryonic trophoblast (which forms the placenta) and invasion of the trophoblast into the maternal endometrium are highly energy-consuming processes, requiring cell migration and synthesis of a large variety of molecules. It is therefore a reasonable speculation that a defect in the energy-producing system of the trophoblasts may impair normal placentation. Although the involvement of mitochondria in the pathogenesis of preeclampsia is possible, population-based studies do not support the hypothesis (3, 37): while mitochondrial genes are transmitted through the mothers, paternal genes clearly contribute to the risk of preeclampsia.

2.5. Unifying hypotheses (Figure 1)

Endothelial cell dysfunction appears to be the final common pathway of preeclampsia, yet the etiology of the disease remains obscure. Genetic factors are involved; however, it is likely that there is not one major preeclampsia gene but several genetic factors associated with maternal susceptibility. Placental ischemia and increased syncyciotrophoblast deportation probably represent an end-stage disease phenomenon. Immune maladaptation and the genetic conflict hypothesis are hypotheses that are congruous with data on the effects of changing partners and the protective effect of sperm exposure. Presence of an abundance of decidual leukocytes and the complex cytokine network underlines the pivotal impact of a correct fetal-maternal (or paternal-maternal) interaction. Leukocyte-, lipoprotein- and cytokine-mediated oxidative stress, caused by immune maladaptation or a genetic conflict, fits with data on cytokines, oxidative stress, lipid changes, and the type and chronologic sequence of endothelial cell dysfunction in preeclampsia.

3. Animal models developed and used in the examination of preeclampsia

Although animal models for preeclampsia are no alternative to clinical studies, they are furthering our understanding of pathophysiology in numerous areas. There are several models, which mimic the human disease. In these models most characteristic symptoms of the disease, such as proteinuria, hypertension and fetal growth retardation, are present. However, since the etiology of preeclampsia remains unclear, it is doubtful that the artificial induction of the disease resembles the etiologic factors of human preeclampsia to any extent.

3.1. Preeclampsia-like conditions produced by NOS inhibition

NO, synthesized by endothelial cells from the amino acid arginine, plays a pivotal role in the maintenance of normal vascular tone (79). In the pathomechanism of preeclampsia, reduced eNOS activity plays an important role; however, its primary role is debated (2.4.2.2.). Acute blockade of NO synthesis by arginine analogues, such as L-nitro-arginine methylester (L-NAME), leads to transient but marked hypertension in both gravid and non-pregnant rats (80). After chronic administration of L-NAME either enterally or parenterally, rats demonstrated hypertension, proteinuria, glomerular injury and decreased platelet count (81, 82). Intrauterine growth retardation was also present in the pups of the treated animals (81, 82). The model can be easily reproduced, making it appropriate to test various therapeutic strategies. On the other hand, since the exact role of reduced eNOS activity in preeclampsia remains unclear, the pathomechanism of the experimental illness may differ greatly from the human disease.

3.2. Induction of a preeclampsia-like disease by ultra-low dose endotoxin infusion.

In the mid 1990s, rats infused with an ultra-low dose of endotoxin were shown to express relevant features of preeclampsia (83, 84). Endotoxin infusion resulted in a decrease of pup weight and number, as well as causing an increase in mean arterial pressure when compared to control animals. The model is based on the observation that preeclampsia shares a number of pathophysiologic phenomena with the so-called generalized Shwartzman reaction, which can be induced by endotoxin (85). Although endotoxin is unlikely to play a pathogenetic role in the development of preeclampsia, it has been suggested that the two diseases may share a final common pathway.

3.3. Experimental preeclampsia produced by chronic constriction of the lower aorta.

This model is based on the theory that preeclampsia is a disease characterized by an increase in vascular tone associated with reduced uteroplacental flow leading to a breakdown in the balance of vasodilators and vasoconstrictors. Reduction of the lower aorta pressure/uterine blood flow resulted in hypertension, proteinuria and glomerular endotheliosis similar to human preeclampsia in several species, e.g. in rabbits, dogs and primates (13, 86, 87). The syndrome evoked in experimental conditions resembles preeclampsia. However, the primary mechanisms (placentation errors and causes of placentation errors) resulting in uterine artery constrictions differ. Thus, the model mimics the second phase of the illness.

3.4. Studies on the sheep model of preeclampsia

Pregnant ruminants, especially sheep, after 2-3 days of fasting and a consequent decrease of blood glucose levels, a significant elevation in blood pressure as well as increased proteinuria, may exhibit decreased uteroplacental blood flow and decreased glomerular filtration rate (88, 89, 90). Histological alterations of the kidneys and liver are similar to that of human preeclampsia. Unlike in human preeclampsia, disturbed carbohydrate metabolism (hypoglycemia and ketonemia) is always present in this model. The symptoms in fasted ewes are not entirely identical to the human disease and may have different physio-pathological points. However, the illness in sheep mimics the human disease, and it can be induced through simple food withdrawal, without causing any artificial change in the animals' organism, thus facilitating an investigation of pathomechanical alterations from the onset of the disease.

4. Aims of our studies

— We wanted to reproduce the sheep model of preeclampsia, so as to examine the pathomechanism of the disease.

— Since increased erythrocyte turnover is present and may be involved in the pathomechanism of endothelial damage in human preeclampsia, we examined the occurrence of hemolysis and the onset of protective biochemical processes in the sheep model, whether they precede or follow the appearance of the first symptoms of the illness.

— We planned to examine the effects of two methylxanthines, allopurinol and pentoxifylline on the occurrence of biochemical and clinical signs of preeclampsia in the sheep model. Since allopurinol inhibits the enzyme xanthine oxidase and pentoxifylline improves microcirculation, consequently decreasing red blood cell damage, we hypothesized that both compounds may have inhibitory effects on the development of symptoms.

— In the human study our aim was to measure the activity of xanthine oxidase, one of the main sources of reactive oxygen specimens, in patients with mild pregnancy hypertension, so as to assess if there was any measurable elevation in the enzyme activity *in vivo.*

5. Examinations concerning the pathogenesis of preeclampsia

5.1. Studies on the sheep model of preeclampsia

Subjects and Methods

Animals

The experiment was performed at the animal stables of the Debrecen Agricultural University Veterinary College, located in Hódmezővásárhely, Hungary. Before starting the study, a permit was obtained from the Albert Szent-Györgyi University Committee for Ethics in Animal Experiments (permit no. ÁTB 83/1993).

Twenty pregnant merino ewes at the gestational age of 130-135 days (terminus: 142-145 days) were randomly divided into the following groups of 5 animals each: control; fasting; fasting, pentoxifylline (PTX)-treated (15 mg/kg/die, per os, divided into two doses, administered from day 0 to delivery); and fasting, allopurinol (AP)-treated (20 mg/kg/die, per os, divided into two doses, administered from day 0 to delivery). Prior to the experiment, during their pregnancy, the animals were fed hay. Water was supplied *ad libitum*. Before starting the dietary intervention animals were habituated to the stables. The pathomechanism of the disease was examined during a food withdrawal period (for 4 days), followed by a period of refeeding (for 3 days). The diagnosis was established according to the occurrence of symptoms: muscular tremors, increased proteinuria, hypertension, low platelet count and elevated liver enzymes.

Measurements

Ten ml of heparinized blood were collected by the direct puncture of the external jugular vein 8 times: at the very beginning of fasting and at 24-hour intervals until the third day of refeeding (days 0-7). Blood was centrifuged immediately (10 minutes at 4°C, 1500 rpm).

Since sheep platelets are smaller than human thrombocytes, they could not have been measured effectively using hematological automates; thus, the platelet count was determined using a Buerker's chamber and a polarization microscope.

Plasma biochemistry studies

Plasma glucose (glucose-oxidase/peroxidase enzymatic test), calcium (flame photometry), as well as creatinine (Jaffe's method), urea-nitrogen (UV-test), albumin (bromcresol-green method), bilirubin (Jendrassik-Gróf method), glutaminate-oxalate-transferase (GOT) and glutaminate-pyruvate-transferase (GPT) levels (enzymatic UV-test), were measured each day.

Iron and total iron-binding capacity were assessed on days 0 and 5 (with batho-phenantroline-disulfonic acid).

Plasma hemoglobin/heme concentration was measured spectrophotometrically at 415 nm (Soret band) expressed in μmol/l, using $\varepsilon_{mM}^{415nm} = 125$ (91). 25 μl of plasma was diluted in 1 ml of phosphate buffer saline (pH 7.4, 5 mM) and the extinction was measured at 415 and 700 nm. The 700-nm value (at which wavelength hemoglobin/heme does not give extinction) was considered the blind control of the sample and was extracted from the 415-nm value. Plasma concentration of free thiols was estimated spectrophotometrically (reduction of 5,5'-dithiobis-2-nitrobenzoic acid, Sigma) (92).

Hypoxanthine, xanthine, uric acid and alloxanthine levels were determined using the high-performance liquid chromatography method (93).

After the introduction of a ballooned catheter, urine samples were taken every day. Total protein and hemoglobin contents were measured.

Daily blood pressure readings were taken every day throughout the experiment. Blood pressure cuffs connected to a digital sphygmomanometer were placed on the left thoracic limb over the anterior cephalic artery, and pressure was recorded 3 times over a 30-minute period.

Statistical analysis

Multiple-choice analysis of variance was assessed. Factors for the analysis were: days (0-7), groups (1-4) and animals (1-5, within the groups). Pairwise comparisons were performed using Tukey's test. If p-values were less than 0.05, alterations were considered to be statistically significant.

5.2. Human examinations

Subjects and methods.

Subjects

Pregnant women with mild hypertension but without superimposed preeclampsia were detected at routine obstetric visits after 24 weeks of gestation. Sixteen subjects met the criterion of gestational hypertension, defined as an increase of 30 mmHg in the systolic or 15 mmHg in the diastolic blood pressure as compared with the values obtained before 20 weeks of gestation, or an absolute blood pressure of >140/90 mmHg if the earlier blood pressure was not known (12). Neither proteinuria nor any impairment in renal function was observed (defined as >500 mg per 24-h urine collection, and physiological creatinine clearance values).

Most patients (n=12) had concomitant hyperuricemia, defined as >330 μM uric acid (UA) in plasma. There was a >15 kg rise in body weight during pregnancy in 14 patients, and there was transient diabetes in 4 of them. Six patients were regularly treated with antihypertensive drugs (methyldopa or nifedipine) during the study period. Thrombocytopenia, increased packed cell volume and abnormal liver enzyme activity were not seen.

Fourteen women with a clinically normal pregnancy, matched for maternal age, parity and gestational age, but with a significantly lower body weight acted as pregnant controls. The control women were age-matched members of the laboratory staff.

This study was approved by the Committee for Ethics of the University.

Blood and urine samples

Blood was drawn into ice-preheated tubes and separated within half an hour, and the sera were stored frozen at -70 °C until assayed (within 6 days). Patients were asked to refrain from methylxanthine containing food and beverages before 48 hours of investigation. Blank urine was collected before the patients received a dose of caffeine (10 mg/kg). Urine was collected over a 6-h period after caffeine consumption.

Determination of concentrations and ratio of caffeine metabolites

Urine concentrations of 1-methyl-xanthine (1MX) and 1-methyl-urate (1MU) were determined by the high-pressure liquid chromatographic (HPLC) method described by Grant et al. (94) with some modifications by Kilbane et al. (95). After adjustment of each urine sample to pH = 3.5 with 6 N hydrochloric acid, a 2 ml aliquot was stored at -20 °C until assay. A 0.2 ml aliquot of each sample was saturated with 120 mg of ammonium sulfate. Six ml of chloroform:isopropanol (85:15, v/v) was added, followed by 0.1 ml of internal standard (5-fluorouracil, 40 mg/l), and the tube was vortexed vigorously for 30 sec. After centrifugation at 2,500 g for 5 min at room temperature, the organic phase was evaporated to dryness under a gentle stream of nitrogen at 45 °C. The residue was redissolved in 0.4 ml of the mobile phase. A 20 μl volume of the sample was injected onto a Waters μBondapak^{TM} phenyl 125 A 10 μm column (3.9x150 mm; Waters) in tandem with a reverse-phase octadecylsilane column (Super Pac Spherisorb ODS2; 5 μm, 4.6 x 250 mm; Pharmacia, LKB), eluted with 0.05% acetic acid:methanol (88:12, v/v) at a flow rate of 0.8 ml/min, and the eluate was monitored by the absorbance at both 272 and 280 nm. The HPLC system consisted of the following components: a Pharmacia LKB Pump 2248, an Autosampler 2157 and a Variable Wavelength Monitor 2141.

Standard plots were constructed in which the integrated signal values (relative to the internal standard 5-fluorouracil) were related to known amounts of 1 MX and 1 MU in a urine sample processed as above. Because of the wide range of the urine concentrations of the metabolites, two calibration plots were used for each compound of interest. The plots were linear in the concentration ranges 1-10 μM and 10-100 μM. Subsequent urinary 1MX and 1MU concentrations were calculated by interpolation from the linear standard plots. The minimum quantifiable concentration for the analytes in 0.2 ml of urine was 0.5 μM. The intra-assay and interassay coefficients of variation at a concentration of 25 μM were in the ranges 2.5-4.2% and 4.8-7.8%, respectively, and the accuracy was within ± 7%.

Xanthine oxidase (XO) activity index was used to characterize the functional XO activity, it was calculated as the molar ratios of 1MU/(1MX+1MU) (96).

Other biochemical methods

The carbon monoxide-hemoglobin (COHb), and total hemoglobin levels were estimated by Hemoximeter (Radiometer Copenhagen) within 15 min after venapuncture. Concentrations of COHb were given as a measure of increased red blood cell turnover (19).

Plasma concentrations of hypoxanthine (HX), xanthine (X) and UA were determined by a reverse-phase HPLC method and UV detector (Pharmacia LKB) (93). The plasma contents of the lipid peroxidation products, fluorescent lipids and conjugated dienes were also estimated (97). The level of free thiols (SH groups) in the plasma was measured with Ellman reagent at 412 nm (92).

Statistical analysis

Normally distributed demographic data were compared between groups using the *t*-test for continuous variables and the χ^2 test for categorical data. Because the distribution of both metabolites and calculated ratios were highly skewed, logarithmic transformation preceded the statistical analyses. Analysis of variance and Scheffe's *post hoc* test were used. Correlations between parameters were characterized by calculation of the linear regression and correlation coefficients. The significance level for all tests was taken as $p < 0.05$.

5.3. Results of the animal experiments

No significant difference was noted among the various groups at the onset of the experiment. Apart from transient decreases of plasma indirect bilirubin and GOT levels, control animals showed no significant change from their initial values (Table 1, Figures 2A, 2B, 2C, and 3A). In all the fasting animals, blood glucose levels decreased significantly, as early as 24 hours after the onset of food withdrawal, from 2.88-3.1 mmol/l concentration and reached its bottom line level of 1.42-1.54 mmol/l concentration ($p<0.01$) by the end of the 96-hour fasting period. After refeeding, glucose levels quickly rose and reached the 2.9-3.2 mmol/l concentration (the same as that of the control animals) 48 hours after refeeding. Blood calcium concentration also decreased from 2.28-2.4 mmol/l and reached the minimum level by the end of the 4-day fasting period (1.88-1.61 mmol/l, $p<0.01$) without a significant difference between the fasting groups.

According to the conventional signs of pregnancy-induced preeclampsia (hypertension, proteinuria, tremors, elevation of serum creatinine and urea-nitrogen), the disease was present in all the fasting, non-treated animals (Table 1, Figure 2A). Plasma albumin concentration showed a slight decline, which became significant by day 4 only in the non-treated group. A significant drop in platelet count and a simultaneous increase in plasma GOT level by the end of the fasting period also occurred (Figures 2B, and 2C), while a significant increase of GPT activity could be observed by day 7: the plasma enzyme activity increased from the initial value of 20.02 ± 6.04 to 44.80 ± 14.21 IU/l (mean ± standard deviation), while in the control group it stayed within the 15-25 IU/l range.

In the case of PTX- and AP-treated, fasting animals pathological alterations of kidney and liver function parameters, blood pressure and platelet count were also present; however, these changes occurred later than in the fasting, non-treated group, and the changes were smaller, especially in the case of plasma GOT activity (Table 1, Figures 2A, 2B, and 2C). The increase of GPT concentration was not significant in the treated groups. Plasma enzyme activity rose by day 7 from the initial values of 20.75 ± 5.89 IU/l (PTX-treated, fasting) and 16.76 ± 5.72 IU/l (AP-treated, fasting) (mean ± standard deviation) to 31.4 ± 9.12 IU/l (PTX-treated, fasting) and 31.9 ± 9.08 IU/l (AP-treated, fasting). Although significant proteinuria could be detected, plasma albumin levels did not decrease significantly (Table 1).

Plasma free thiols declined from the initial value of 145-160 μmol/l in all the fasting groups, and a significant ($p<0.05$) decrease occurred after 48 hours of food withdrawal. By

the end of the fasting period, plasma free thiols reached the bottom line level of 80-90 μmol/l ($p<0.01$) in the case of the fasting and fasting, PTX-treated groups, while in the case of the fasting, AP-treated animals the concentration of thiols decreased to a less significant level (104.5 ± 9.8 μmol/l, $p<0.05$, mean ± standard deviation). After refeeding, elevation occurred in all three fasting groups; however, thiol levels did not reach that of the control animals.

Plasma free heme level, as the first predictive sign of the illness, showed a sharp elevation simultaneously with the decrease of glucose levels in the fasting group, and subsequently it remained slightly elevated (Figure 3B). The elevation of plasma heme content was smaller than in both of the treated groups, and a significant increase was postponed to day 2 (AP-treated group) and day 5 (PTX-treated group). In addition, there were somewhat higher levels and an earlier increase of plasma indirect bilirubin (by day 1) in the treated groups than in the fasting, non-treated group (day 2) (Figures 3C and 3D). The summarized molar plasma indirect bilirubin/(indirect bilirubin + plasma free heme) ratios as product/(product + substrate) ratios, which provided indirect evidence of heme oxygenase activity (the enzyme which degrades heme), were more favorable in the drug-treated, fasting groups, compared to the fasting, non-treated group. In the pentoxifylline-treated group this ratio was 0.296 ± 0.059, significantly higher ($p < 0.05$) than in the fasting, non-treated group (0.222 ± 0.060), while in the allopurinol-treated group it was non-significantly higher (0.259 ± 0.050) (mean ± standard deviation).

While plasma iron level practically did not change in the control group, in the case of the fasting groups, it showed a significant decrease, which was slightly larger in the methylxanthine-treated groups. Iron concentrations decreased from 24.6 ± 3.7 to 16.9 ± 2.8 μmol/l in the fasting, non-treated group while from 24.9 ± 1.1 and 24.4 ± 5.2 to 14.4 ± 2.0 and 14.3 ± 3.8 in the PTX- and AP-treated groups by day 5 (mean ± SD). Although this decrease was not accompanied by the significant alteration of iron-binding capacity, a slight decrease could be observed toward the end of pregnancy in each group of animals. This decline was larger in the treated groups (from 54.5 ± 4.2 and 57.1 ± 8.0 to 45.7 ± 5.3 and 48.2 ± 2.3 in the PTX- and AP- treated animals) versus the control and fasting group (from 53.1 ± 2.0 to 50.8 ± 6.8 and from 53.8±3.9 to 51.1 ± 2.1 μmol/l) (mean ± SD).

Plasma hypoxanthine, xanthine and uric acid levels were similar in the control, fasting and fasting, PTX-treated groups: hypoxanthine and uric acid concentrations ranged between 4 and 6 μmol/l, while xanthine levels remained between 1 and 2 μmol/l (sheep purine metabolism is

different from that of humans: the enzyme uricase degrades uric acid, and consequently uric acid concentrations are low). In contrast, in the case of AP-treated, fasting animals, an inhibition of xanthine oxidase occurred after 24 hours. Uric acid level was under 2 μmol/l throughout the treatment period, while hypoxanthine and xanthine concentrations ranged between 7 and 12 μmol/l. Plasma concentration of alloxanthine, an active metabolite of AP, ranged between 6 and 12 μmol/l.

Outcome of Pregnancy: In the non-fasting group all the animals delivered healthy lambs (three twins and two singleton lambs) at 10.6 ± 2.3 days after the beginning of the experiment (mean ± standard deviation). In the fasting, non-treated group, one ewe (carrying twin pregnancy) died in convulsions. Lambs were born earlier than among the control animals (7.2 ± 2.5 days). Two ewes aborted (twin lambs) and two gave birth to viable lambs (one singleton lamb and one twin pregnancy). In the PTX-treated, fasting group two singletons and one from each twin pregnancy survived (time of birth 8.9 ± 3.1 days after the beginning of the experiment). In the AP-treated, fasting group two singletons and two pairs of twin lambs survived, while one pair of twins died (birth at 8.6 ± 2.9 days).

5.4. Results of the human examinations

The demographic data of the study groups are shown in table 2. The concentration of free thiols showed a decrease even in the normotensive pregnant women. Elevated contents of fluorescent lipids and conjugated dienes were measured in the hypertensive patients as compared with either the normotensive ones or the non-pregnant controls (Table 3).

There was no difference in the whole blood hemoglobin or COHb concentrations as potential markers of increased red blood cell catabolism (Table 3).

The sum of the plasma concentrations of physiological purine metabolites (HX+X+UA) was increased in the pregnant women *versus* the non-pregnant controls ($p<0.05$). An increased level of UA, the end product of XO, was demonstrated in the hypertensive individuals, while the amounts of substrate for XO (HX) were also significantly higher in the normotensive pregnant subjects than in the non-pregnant controls (Figure 4).

A significant decrease in the urinary concentration of 1MX, the caffeine metabolite serving as a substrate for XO, was measured in the hypertensive subjects without any decrease in the urinary concentration of 1MU, the product of XO (Figure 5). As a result the XO activity index, the molar ratio 1MU/(1MX+1MU), displayed a significant rise in this group (Figure 6).

5.5. Discussion of the animal experiments

Previous studies have already revealed that fasting provoked a unique physiological situation in pregnant ewes: a 72-hour-long withdrawal of food caused a human preeclampsia-like syndrome, while non-pregnant animals showed neither biochemical nor hemodynamic change (89).

In our experiment some other pathophysiological features characteristic of human HELLP syndrome were also shown. Besides hypertension and renal disturbances (Table 1), a fall in platelet count and hepatic insufficiency were also measured (Figures 2A, 2B and 2C). Pentoxifylline (PTX) and allopurinol (AP) had beneficial effects on the development of experimental pregnancy-induced preeclampsia-like disease in ewes. While in the fasting, non-treated animals, all the mentioned characteristics of the disease (hypertension, renal and liver function disturbances and low platelet count) were detected, and pathological changes were smaller and occurred later in the treated animals. A significant decrease in blood calcium levels was also present in this model, similar to previous reports (90); however the three fasting groups showed a similar decline, which was not accompanied with the parallel onset of symptoms.

The decrease of plasma free thiols – the first line of antioxidant activity, offering a non-specific buffer to oxidative stress – was similar in all the fasting groups. Presumably, the plasma level of free thiols was affected rather by alimentation than by oxidative processes. The less significant decline in the AP-treated group may be a result of the inhibition of xanthine oxidase activity.

A significant increase in plasma hemoglobin/heme, prior to the development of the disease, proved that oxidative stress was the common result of increased oxidant activity and insufficient antioxidant capacity of the red blood cells resulting in hemolysis. Sheep red blood cells are extremely sensitive to oxidants because of their low antioxidant protective glucose-6-phosphate dehydrogenase (G6PD) activity (98). In fasting pregnant ewes a decline in the plasma glucose level occurred even after a short period because of the accelerated glucose metabolism. As a consequence of insufficient capacity of the hexose-monophosphate shunt, erythrocytes fail to regenerate oxidized glutathione (GSSG) to its reduced form (GSH).

An increased heme catabolism, estimated as a concomitant rise in plasma bilirubin levels, were also seen in fasting pregnant animals during the whole experiment (Figures 3B, 3C and 3D) as a signal of heme oxygenase enzyme activation (99). All the above-mentioned changes were in concordance with biochemical signs documented in human pathology.

Plasma hemoglobin/heme levels increased later and less in both of the treated, fasting groups than in the non-treated, fasting animals, and higher concentrations of plasma indirect bilirubin levels were measured as early as day 1. Since PTX improves microcirculation oxygenation by improving the flexibility of the erythrocytes and decreasing blood viscosity (100), these effects of the drug may also improve placental microcirculation. Consequently, local hypoxia and hypoxic-reperfusion injury may be postponed. AP could also defend against local oxidative stress by specifically inhibiting the enzyme xanthine oxidase (101), the presence of which in the placenta has been proved (22, 102).

Induction of heme oxygenase, a 32 kD oxidative stress protein, the rate-limiting enzyme in the catabolism of cellular heme indicated the protective processes of endothelial cells against heme toxicity. As it breaks down heme, it produces the antioxidant biliverdin, which is quickly reduced to bilirubin, which is also antioxidant (103). Iron and carbon monoxide are the two other products of the heme oxygenase enzyme reaction, the latter being a potent relaxant of vascular smooth muscle. Increased levels of carboxyhemoglobin, serum iron and bilirubin in severe cases of preeclampsia (19) were not only indicators of increased red cell turnover but also signals of induced oxidative stress protein activation.

In the case of the treated animals, the enhanced formation of bilirubin, compared to the fasting, non-treated sheep, refers to the better activation of heme oxygenase. Induced heme oxygenase activity, as an additional beneficial effect of PTX and AP, indicates more effective protective processes of endothelial cells against heme and also non-heme (iron) toxicity (99, 103). Although we found no reference to AP or PTX inducing heme oxygenase synthesis in human or sheep endothelial cells, as xanthine derivatives, both compounds elevate the intracellular level of 3',5'-cyclic-AMP (cAMP) via inhibition of phosphodiesterase. Recent articles have revealed that in both rat muscular smooth muscle cells and hepatocytes heme oxygenase gene expression was increased due to elevation of the intracellular cAMP level (104, 105).

Studies in the mid 1990s showed that even small amounts of the highly reactive hemoglobin in plasma suffice to trigger oxidation of low-density lipoproteins, thus possibly damaging vascular endothelial cells (106). In addition, heme has a direct injurious effect on the endothelial cells (107). Heme is a physiologic iron chelate that could facilitate endothelial iron uptake in cultured endothelial cells (108). Iron-loaded cells become extremely sensitive to exogenous or endogenous oxidants. Vascular endothelium in contact with hemolyzing red

cells, might be rendered hypersusceptible to damage by adherent stimulated polymorphonuclear leukocytes or lipid peroxides (32, 107, 108).

Induction of heme oxygenase has been proved to be a rapid, protective antioxidant response to heme burden in different animal models of tissue injury in which enhanced oxidative stress is implicated (109, 110, 111), and at the same time a signal of endothelial cell oxidative stress. Additional evidence for our hypothesis concerning hemoglobin as the direct cause of endothelial cell injury with consequent heme oxygenase activation was found in the endothelial cell cultures exposed to different acellular hemoglobin solutions (112). Furthermore, in cultured endothelial cells there was a dose- and time-dependent enhancement, after a certain lag time (24-72 h), in secretion of endothelin-1, a peptide with a potent vasoconstrictive effect and with higher concentration in preeclampsia (113), in response to erythrocyte lysates (114).

Endothelial injury caused by heme/hemoglobin resulting in hypersensitivity of endothelial cells to exogenous and endogenous oxidants may be sufficient to explain the increased vascular reactivity observed in this animal model of preeclampsia. Consequently, endothelial dysfunction with imbalance between the vasodilator prostacyclin and Endothelium-Derived Relaxing Factor, which later has been identified as nitric oxide (NO), and vasoconstrictors thromboxane A_2 and endothelin-1 might occur. A hypothesis that an increased free hemoglobin concentration was the direct cause of vasoconstriction underlying preeclampsia has previously been proposed on the basis of its effects on NO (33). Hemoglobin and the hemoglobin/haptoglobin complex bind to and inhibit the effects of NO (115).

Plasma iron and ferritin levels are increased in human preeclampsia (116). This was an unexpected result of our study to measure the loss of plasma iron concentrations in fasting and refed pregnant ewes on the 6^{th} day of experiment. There might be some species differences in iron homeostasis in favor of fast iron elimination instead of storage in ferritin molecule which process was proved as the ultimate antioxidant defense in other animals and humans during oxidative stress caused by heme (109, 110, 111).

Glucose deprivation in pregnant ewes, with low G6PD activity induced a toxicosis-like syndrome very similar to human preeclampsia. The significance of this enzyme deficiency, one of the most common genetic enzymatic defects known, in the context of pregnancy was assessed previously. Increased rates of abortions, low-birth-weight infants, and puerperal drops in red cell volumes were noted in the deficient state (117). Hematologic data in severe deficient women suggested slight hemolysis in the first trimester of pregnancy (118). A

significant decline in G6PD activity was reported in late gestosis compared to healthy pregnant women (119). A decrease in enzymatic activity by half during pregnancy was also reported in 65% of pregnant women, while 25% had low activity even at the beginning of gestation (120). According to these data, G6PD enzyme activity, as one of the most important antioxidant protective systems of red blood cells during oxidative stress, might have a role in the pathogenesis of human preeclampsia.

5.6. Discussion of human examinations

Oxidative stress has been suggested as a link between the two-stage model of the preeclampsia syndrome: maternal factors cause reduced placental perfusion as stage 1, and stage 2 involves an activated maternal endothelium with multisystem disorders (10, 121). The enzyme xanthine oxidoreductase (XO/XD) is a key source of oxidants in many pathological processes. This enzyme system catalyses the oxidation of hypoxanthine (HX) to xanthine (X) and to urate (UA), and exists in an innocuous form (xanthine dehydrogenase, XD) in non-ischemic tissues. However, if the tissues are exposed to metabolic stress, such as hypoxia or ischemia, the enzyme is transformed to xanthine oxidase (XO), which can generate the oxidants O_2^-, H_2O_2 and $HO^{\cdot}$ during reperfusion (122). The enzyme system is present in the endothelial and vascular smooth muscle cells (123, 124), and also in the human placenta, localized to trophoblast cells (23). An increase in XO activity has been proposed as a possible cause of oxidative stress in preeclampsia (102), and such an increase has in fact been measured in the placentae of women with preeclampsia (125). Besides direct inactivation of NO as a vasodilator, XO-derived O_2^- reacts with NO to form a potent oxidant peroxynitrite ($ONOO^-$), which has been detected both within the vasculature and in the vessel walls of the placenta after preeclampia (126, 127). The conversion of XD to XO is also induced by $ONOO^-$ via thiol group (SH) oxidation (128).

XO is additionally involved in the metabolism of methylxanthines and, after administration of caffeine as a 'prodrug', the conversion of 1-methylxanthine (1MX) to 1-methyluric acid (1MU) depends exclusively on the XO activity, independently of XD (94). The caffeine metabolic ratio has proved to be a specific indicator of the *in vivo* XO activity (96, 129-131). The clinical status of our overweight subjects met the criteria of preeclampsia syndrome stage 1 with the risk of preeclampsia developing.

The reduced plasma level of free SH groups in pregnancy, even without a hypertensive disorder, are in agreement with the conclusion of a previous study that a pro-oxidant state is present in healthy pregnancy, with reduced plasma thiols (132).

A significant enhancement of the levels of lipid peroxidation products was also measured analogously in the pregnancy-induced hypertensive *versus* the normotensive subjects, as in other studies, in both plasma and placenta samples (133). Placental mitochondria enriched with polyunsaturated fatty acids may be the sources of the abnormally increased lipid peroxidation in the maternal circulation, while increases in both the amount and the oxidative potential of the mitochondria have been demonstrated in the preeclamptic *versus* the normal placenta (134).

The higher sum of the all purine metabolites (HX+X+UA) measured in the plasma samples of the pregnant group as compared with the non-pregnant women points to an increased purine catabolism even during healthy pregnancy. Recent experimental studies have proved that metabolic stress can stimulate the purine catabolism (135). This activation of the purine catabolism was established to be a component of the homeostatic protective response of the mitochondria to oxidant stress by slowing the progressive mitochondrial dysfunction (135).

While in pregnant subjects the plasma concentration of HX, a substrate for XO was increased, in hypertensive patients the levels of UA, the product of XO exhibited a significant increase. The increased level of UA in mild hypertensive subjects with normal renal function, who are under the more pronounced metabolic stress of pregnancy due to their constitution, i.e. overweight or/and with transient diabetes, suggests the concomitant increase in XO enzyme activity.

Previous studies have revealed that certain unique pharmacological and biochemical features of caffeine make it a useful model substrate probe for XO (94, 96, 129-131). Allopurinol treatment caused a specific, dose-dependent inhibition of the conversion of 1MX to 1MU with a high correlation to the urinary ratio UA/X+HX (96, 131). The XO activity index involves only end-products of the caffeine metabolism, and the exact amount and the time of collection of the urine are therefore both relatively unimportant as long as the caffeine intake is large enough for reliable measurement of the metabolites (130). Studies on the caffeine metabolism in pregnancy revealed that smaller amounts of 1MX and 1MU were excreted compared to those of non-pregnant controls whereas their molar ratios were not different (136).

In a population study on 178 young adults (25.5 ± 7.8 years), the XO activity index proved to have a rather consistent value of 0.57 ± 0.13 (mean ± S.D.) (137). Our results in non-pregnant women (0.542 ± 0.049; n = 15) or in normotensive pregnant subjects (0.596 ± 0.105; n = 14) are in good agreement with these values. However, the index was highly increased in pregnant women with mild hypertension (0.849 ± 0.096; n = 16). While 1MU could be produced only from 1MX, a shift in favor of 1MU in our present study should have been a result of XO activation. Thus, the increased purine catabolism during pregnancy was accompanied by a XO activation exclusively in hypertensive pregnant subjects.

In the present clinical study, it was impossible to measure either O_2^- or $ONOO^-$ directly, as has been done in previous studies by histochemical methods in the placenta or in the plasma and the placenta in normotensive and hypertensive pregnancies (125, 126). As XO seems to be a major O_2^- producing enzyme in the vascular system, several experiments were done to clarify the mechanism of XO regulation of NO. The potentiation of NO-mediated vasorelaxation was achieved by a XO inhibitor compound (4-amino-6-hydroxypyrazolo[3,4-d]pyrimidine) on aortic rings from both rabbits and spontaneously hypertensive rats in dose-dependent manner (138). Intravenous injection of the same compound or pretreatment with tungsten diet normalized the characteristic elevation of mean arterial pressure in spontaneously hypertensive rats by the elimination of the increased oxyradical production and detectable XO activity (138, 139).

The role of xanthine oxidase in human plasma H_2O_2 production was also reported (140), and a close correlation between H_2O_2 concentrations and the mean arterial pressure was proven both in normotensive and hypertensive subjects (141).

6. Conclusions

— Similarly to previous studies, a sheep disease resembling human preeclampsia and HELLP syndrome was induced through simple food withdrawal.

— In the animal model of ewes, hemolysis, secondary to hypoglycemia and low glucose-6-phosphate dehydrogenase activity, precedes the symptoms of the preeclampsia-like illness in ewes and may trigger the development of the animal disease, which is similar to human preeclampsia.

— Pentoxifylline and allopurinol proved to be effective in the prevention of more severe preeclampsia-like complications. Although the drugs were not completely effective in protecting the animals against certain signs of the illness, organ manifestations (liver and kidney involvement, platelet consumption and hypertension) were reduced and fatal consequences prevented by simple oral medication. The well-known effect of PTX on microcirculation and the inhibition of xanthine oxidase by AP as well as the enhanced induction of heme oxygenase may be important points in maintaining normal endothelial function and vascular tone regulation during sheep pregnancy.

— Our study showed that elevated *in vivo* enzyme activity of xanthine oxidase can be measured in mild hypertensive pregnant subjects, thus providing additional evidence for the putative role of XO as a source of free radicals in human preeclampsia.

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Table 1. Effects of pentoxifylline and allopurinol treatment on changes in plasma albumin, urea nitrogen and creatinine as well as total urine protein in preeclampsia-like disease in ewes.

	Initial value	Value at maximal change (day)	Initial value	Value at maximal change (day)	Initial value	Value at maximal change (day)	Initial value	Value at maximal change (day)
Group	Control n = 5		Fasting n = 5		Fasting, pentoxifylline-treated n = 5		Fasting, allopurinol-treated n = 5	
Albumin (g/l)	36.8 ± 0.7	36.1 ± 0.8 (4)	36.5 ± 0.7	32.3 ± 1.8* (5)	37.1 ± 1.3	34.6 ± 1.2 (–) (4)	35.9 ± 0.8	34.5 ± 1.7 (–) (4)
Urine protein (mg%)	51.5 ± 8.5	63.2 ± 9.4 (6)	48.5 ± 8.3	215.1 ± 35.6** (5)	54.0 ± 6.5	153.0 ± 12.9** (+) (6)	39.8 ± 6.5	165.8 ± 19.4** (+) (6)
Urea nitrogen (mmol/l)	8.15 ± 0.35	10.0 ± 1.1 (1)	7.98 ± 0.18	13.87 ± 2.20 (1)	8.28 ± 0.18	15.92 ± 3.6 (1)	8.25 ± 0.31	15.80 ± 4.5 (†) (2)
Creatinine (µmol/l)	95.5 ± 2.8	115.8 ± 19.7 (2)	90.7 ± 2.6	217.0 ± 55.4* (2)	93.3 ± 3.2	153.6 ± 25.4 (–) (1)	99.6 ± 4.4	182.5 ± 45.4* (–) (2)

Fasting: 96 hours from day 0 to day 4. In the fasting, pentoxifylline-treated group sheep were treated with 15 mg/kg/die pentoxifylline, while in the fasting, allopurinol-treated group 20 mg/kg/die allopurinol was administered. Significance (* and **) was calculated by multiple-choice ANOVA followed by pairwise comparison using Tukey's test ($p < 0.05$ and $p < 0.01$). The occurrence of pathologic changes was noted in the case of either the pentoxifylline- or allopurinol-treated, fasting groups: either they arose later (†); or the amount of change was less (–); or the alteration was less and appeared later (+), when compared to the non-treated, fasting group. Values are given as mean ± standard deviation.

Table 2. Demographic characteristics of the study population (mean ± SD)

	Non-pregnant controls	Normotensive pregnant patients	Hypertensive pregnant patients
	n = 15	n = 14	n = 16
Maternal age (yr)	25.2 ± 1.8	23.2 ± 2.2	24 ± 3.2
Gestational age (wk)	-	35.2 ± 1.3	35.5 ± 2.3
Parity	-	1.2 ± 0.2	1.5 ± 0.3
Body weight (kg)	61.5 ± 1.9	69.5 ± 1.2	82.7 ± 1.5*
Prepregnant body weight (kg)	-	60.3 ± 1.4	67.3 ± 1.8*
Blood pressure (mm Hg)	109/70±9/7	113/73±8/8	154/94±17/7*
Prepregnant blood pressure (mm Hg)	-	110/70±9/6	114/74±13/6

* $p < 0.05$ *versus* normotensive pregnant patients.

Table 3. Concentrations of some biochemical parameters in the plasma and in the whole blood of the study population (mean ± SD)

	Non-pregnant controls n = 15	Normotensive pregnant patients n = 14	Hypertensive pregnant patients n = 16
Plasma values			
Creatinine (μM)	58 ± 12	47 ± 13	63 ± 8
Urea N (mM)	4.3 ± 1.1	3.8 ± 0.9	4.5 ± 1.2
Free sulfhydryl groups (U/L)	388 ± 48	262 ± 42*	220 ± 58*
Conjugated dienes (OD, 233 nm)	0.52 ± 0.26	0.82 ± 0.31	1.70 ± 0.32*#
Fluorescent lipids (OD, 430 nm)	49.4 ± 19.2	68.67 ± 28.2	136.8 ± 57.4*#
Whole blood values			
Total haemoglobin (mM)	8.2 ± 0.4	7.8 ± 0.6	7.5 ± 1.0
Carboxyhemoglobin (μM)	68.5 ± 25	63.7 ± 32	69.2 ± 16

* $p < 0.05$ *versus* non-pregnant controls; # $p < 0.05$ *versus* normotensive pregnants.

Figure legends.

Figure 1. **The pathogenesis of preeclampsia. Unification of theories.**
Abbreviations: RBC: red blood cell, XO: xanthine oxidase, EC: endothelial cell, CK: cytokine

Figure 2. **Initial values and values at maximal change in mean arterial blood pressure, platelet count and GOT activity: in pregnant, non-fasting (control); fasting; and fasting, treated ewes (mean ± standard deviation).** Groups: control group; fasting group: 96-hour-long fasting period; PTX-treated group: animals fasting 96 hours, medicated with pentoxifylline (15 mg/kg/die from day 0 to delivery); AP-treated group: allopurinol-treated group fasting 96-hours (20 mg/kg/die allopurinol from day 0 to delivery). Number of animals in each group: 5.
2A: Initial values and values at maximal change in mean arterial blood pressure (7 days after the beginning of the 96-hour fasting period in fasting and fasting, treated groups). **2B:** Initial values and values at maximal change in whole blood platelet count (6 days after the beginning of the 96-hour fasting period). **2C:** Initial values and values at maximal change in plasma GOT activity (7 days after the beginning of the 96-hour fasting period).
Values are depicted as mean ± standard deviation. Significance was calculated by multiple-choice ANOVA followed by pairwise comparison using Tukey's test (* $p < 0.05$, and ** $p < 0.01$, *versus* control group; ^{b} $p < 0.05$, fasting group *versus* fasting, drug-treated groups).

Figure 3. **Changes in plasma indirect bilirubin and total heme in pregnant, non-fasting (control) fasting, non-treated ewes, and in fasting, treated ewes (mean ± standard deviation).** Values are depicted as. **3A:** Control group (n = 5). **3B:** group fasting 96 hours. **3C:** animals fasting 96 hours (n = 5), medicated with pentoxifylline (15 mg/kg/die from day 0 to delivery). **3D:** allopurinol-treated group (20 mg/kg/die allopurinol from day 0 to delivery) fasting 96 hours (n = 5). Significant alterations were calculated by multiple-choice ANOVA followed by pairwise comparison using Tukey's test.
(^{x} $p < 0.05$ and ^{xx} $p < 0.01$ in the case of indirect bilirubin while * $p < 0.01$ and ** $p < 0.05$ in the case of total heme, *versus* control group; ^{b} $p < 0.05$, fasting group *versus* fasting, drug-treated groups).

Figure 4. Plasma levels of purine metabolites, hypoxanthine (HX), xanthine (X) (A), and uric acid (UA) (B) in controls and pregnant subjects without/with hypertension (mean ± SEM).

Significance was calculated by ANOVA followed by Scheffe's post hoc test.

(** $p < 0.01$; *** $p < 0.001$ *versus* non-pregnant controls; ## $p < 0.01$ *versus* normotensive pregnant subjects.)

Figure 5. Urinary concentrations of two caffeine metabolites, 1-methyl uric acid (1MU) and 1-methylxanthine (1MX) after caffeine intake (mean ± SEM).

Significance was calculated by ANOVA followed by Scheffe's post hoc test.

*** $p < 0.001$ *versus* non-pregnant controls; ### $p < 0.001$ *versus* normotensive pregnant subjects.

Figure 6. Xanthine oxidase activity index (1-methyl uric acid/1-methylxanthine+1-methyl uric acid) in controls and pregnant subjects without/with hypertension

Abbreviations: 1MU: 1-methyl uric acid, 1MX: 1-methylxanthine. Mean values are indicated. Significance was calculated by ANOVA followed by Scheffe's post hoc test.

(*** $p < 0.001$ *versus* non pregnant controls; ### $p < 0.001$ *versus* normotensive pregnant subjects.

Figure 1.

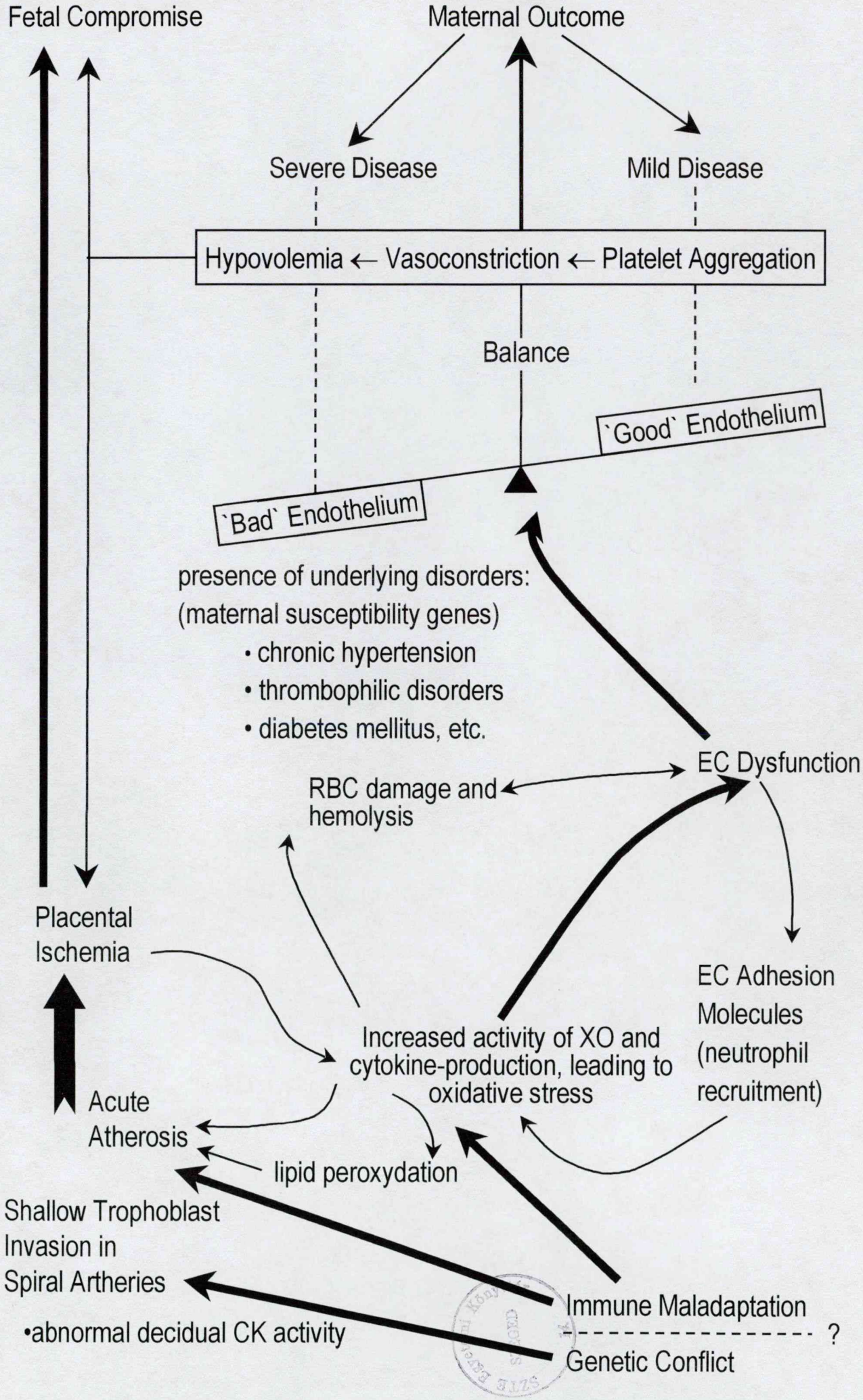

Fetal Compromise
Maternal Outcome
Severe Disease
Mild Disease
Hypovolemia ← Vasoconstriction ← Platelet Aggregation
Balance
`Good` Endothelium
`Bad` Endothelium
presence of underlying disorders:
(maternal susceptibility genes)
• chronic hypertension
• thrombophilic disorders
• diabetes mellitus, etc.
EC Dysfunction
RBC damage and hemolysis
Placental Ischemia
EC Adhesion Molecules (neutrophil recruitment)
Increased activity of XO and cytokine-production, leading to oxidative stress
Acute Atherosis
lipid peroxydation
Shallow Trophoblast Invasion in Spiral Artheries
•abnormal decidual CK activity
Immune Maladaptation
?
Genetic Conflict

Figure 2.

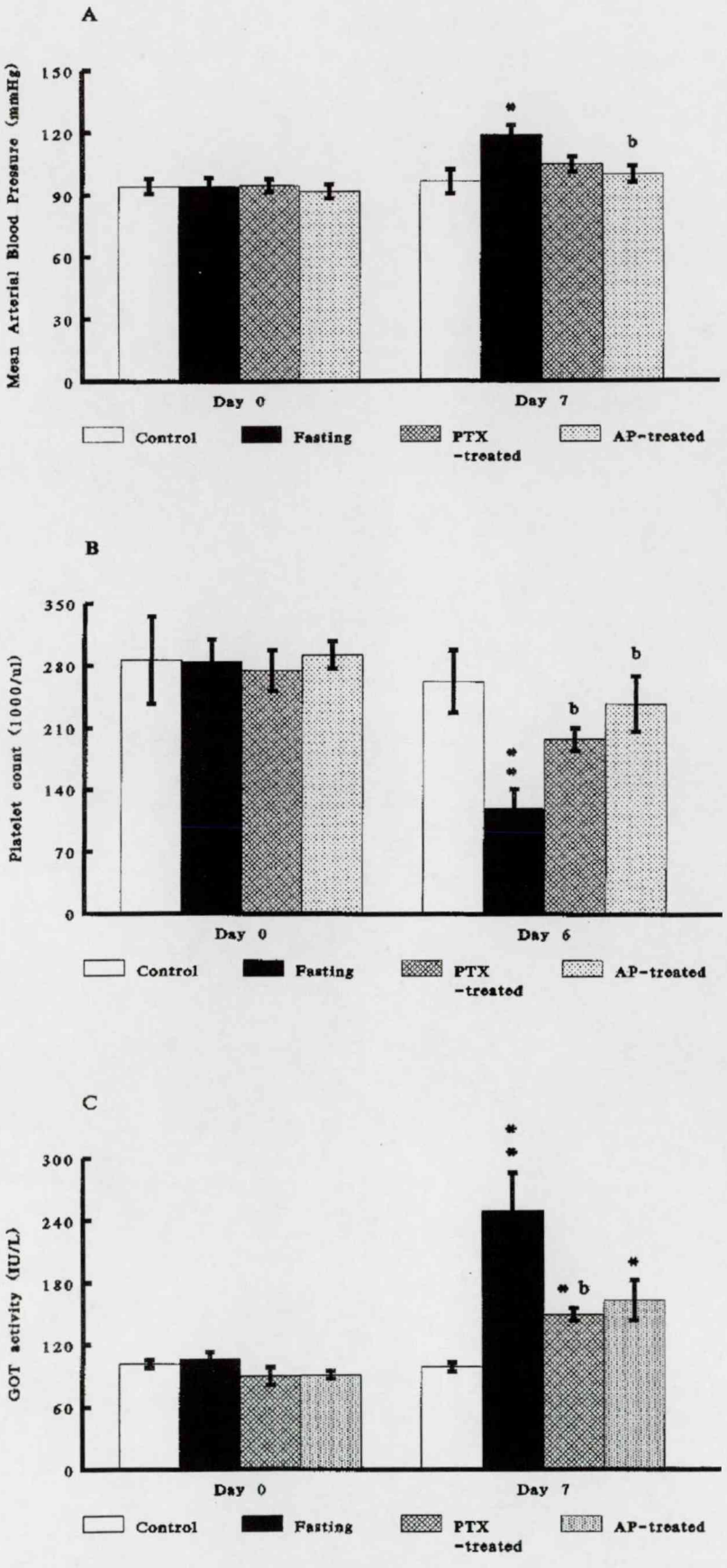

A
Mean Arterial Blood Pressure (mmHg)
150
120
90
60
30
0
*
b
Day 0
Day 7
Control
Fasting
PTX -treated
AP-treated
B
Platelet count (1000/ul)
350
280
210
140
70
0
**
b
b
Day 0
Day 6
Control
Fasting
PTX -treated
AP-treated
C
GOT activity (IU/L)
300
240
180
120
60
0
**
* b
*
Day 0
Day 7
Control
Fasting
PTX -treated
AP-treated

Figure 3.

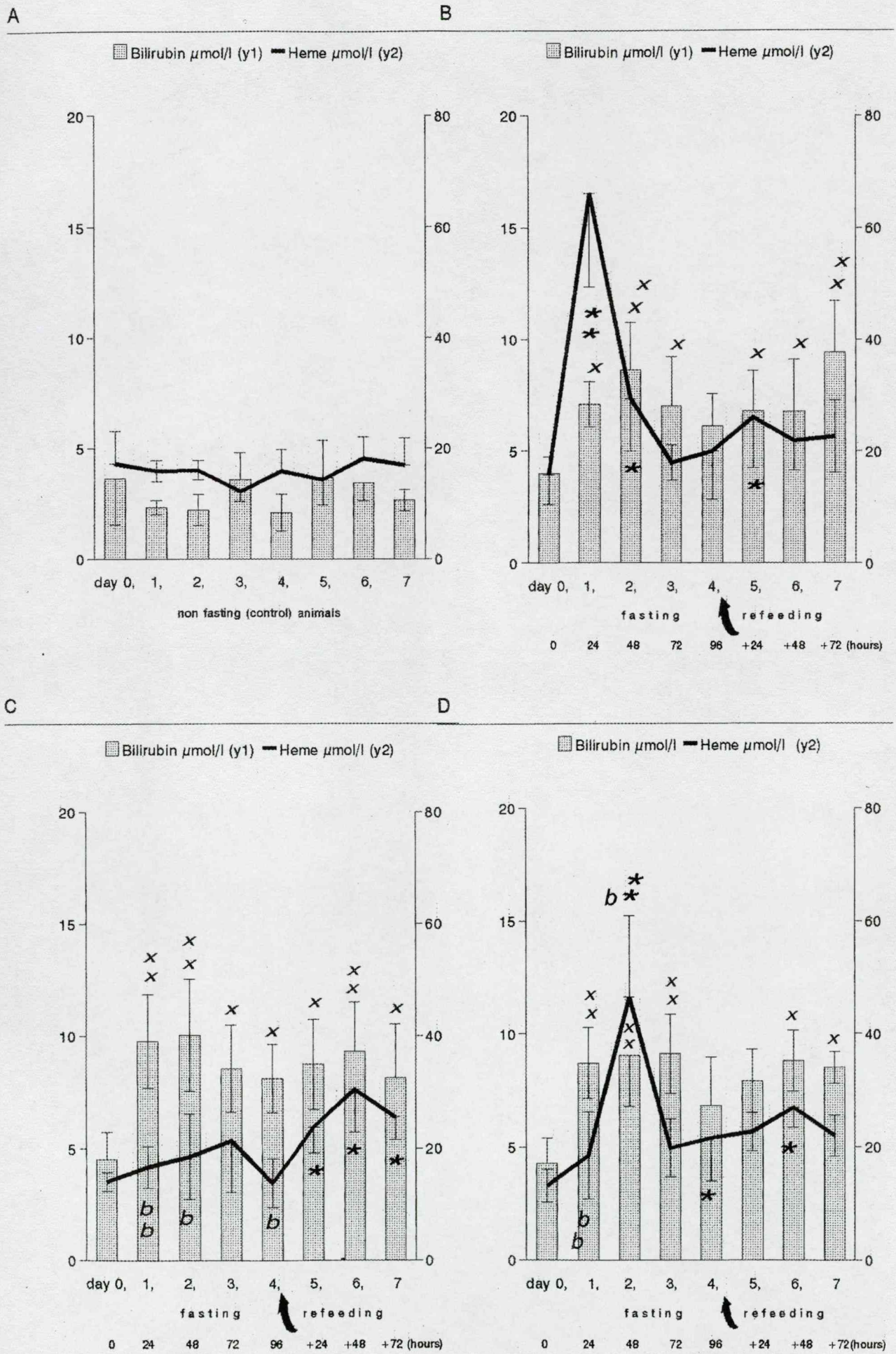

A
B
Bilirubin μmol/l (y1)
Heme μmol/l (y2)
day 0, 1, 2, 3, 4, 5, 6, 7
non fasting (control) animals
fasting
refeeding
0 24 48 72 96 +24 +48 +72 (hours)
C
D

Figure 4.

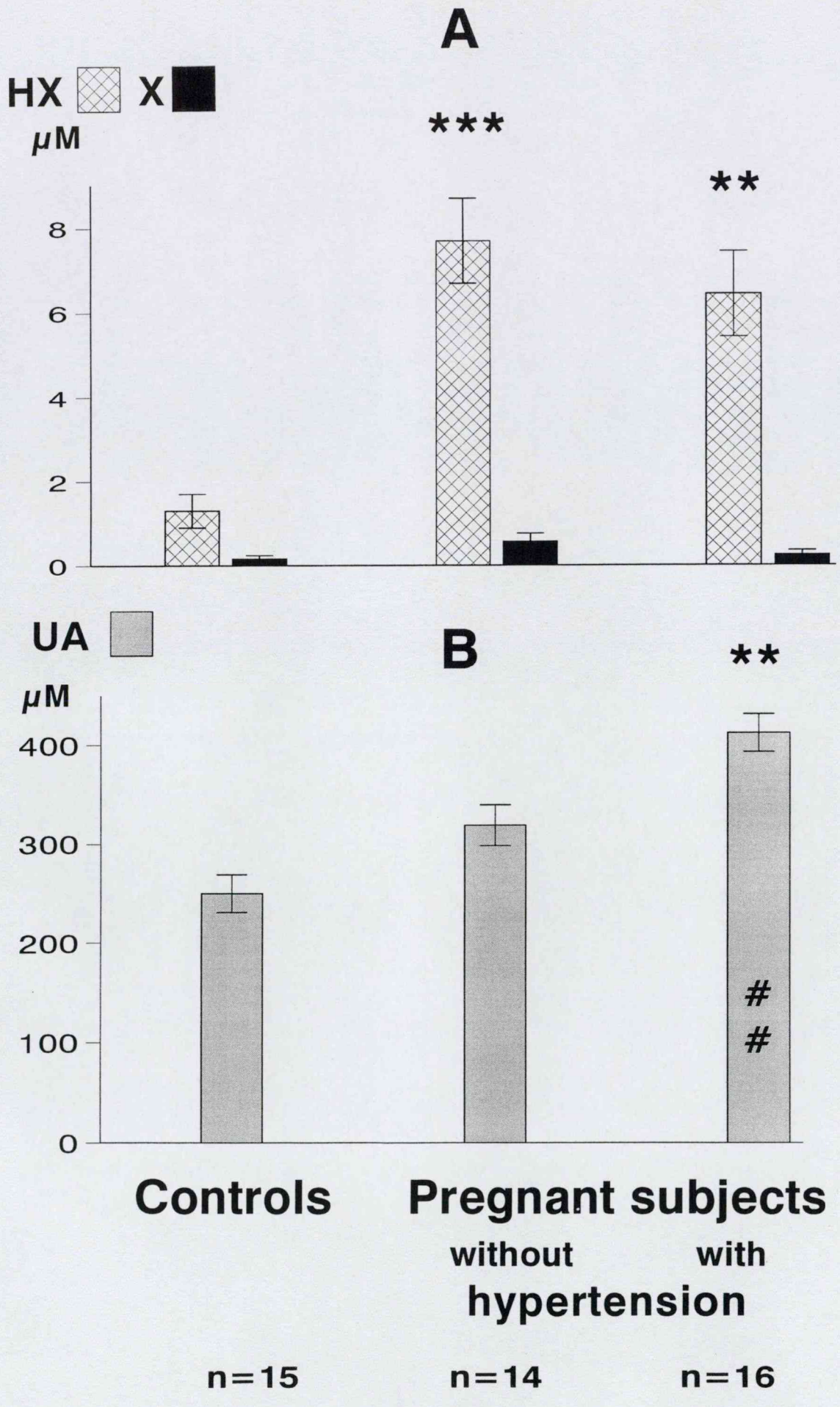
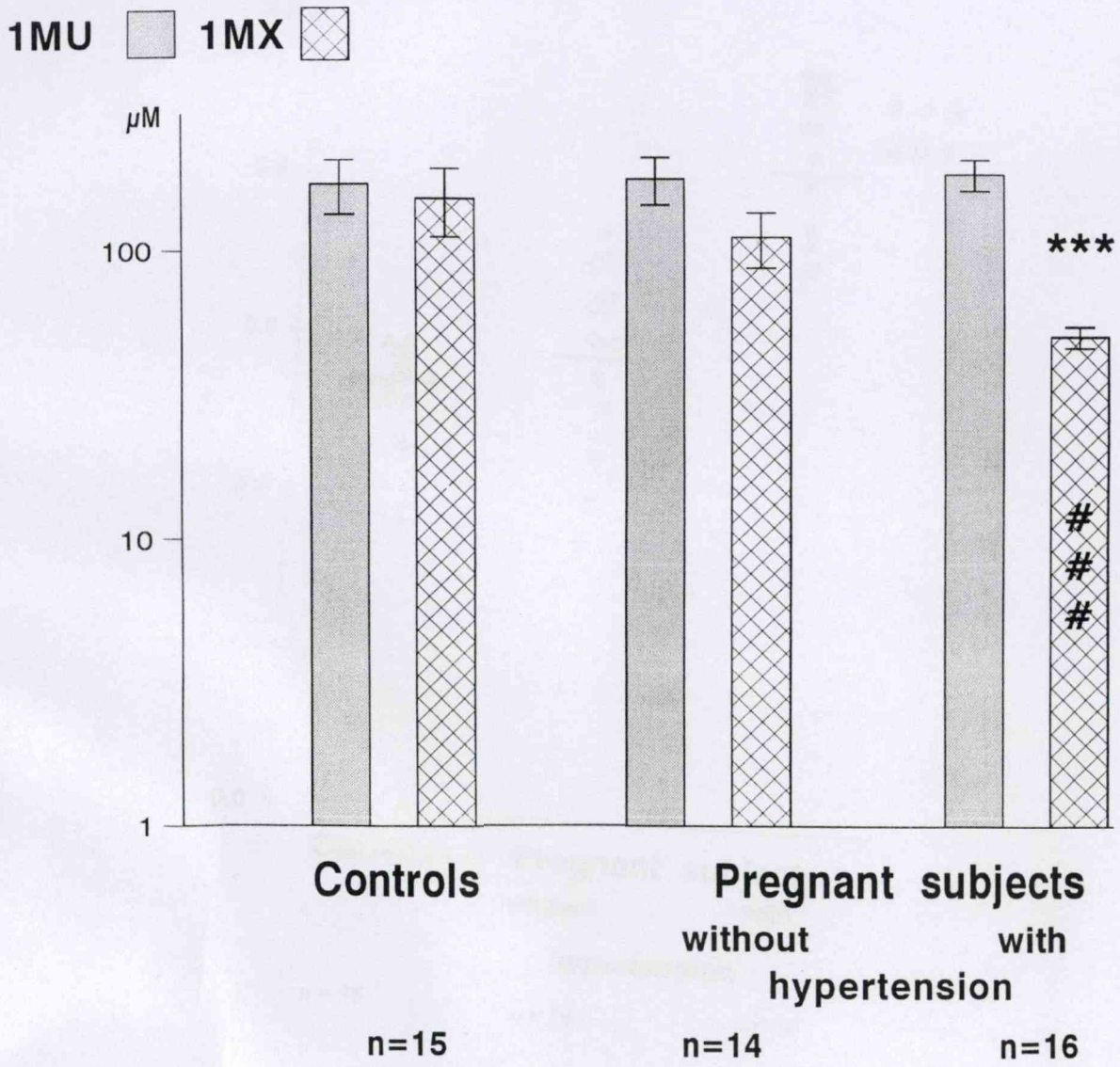

A
HX
X
μM
***
**
8
6
4
2
0
UA
B
μM
**
400
300
200
100
0
#
#
Controls
Pregnant subjects
without
with
hypertension
n=15
n=14
n=16

Figure 5.


1MU
1MX
μM
100
10
1
***
#
#
#
Controls
Pregnant subjects
without
with
hypertension
n=15
n=14
n=16