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MINIREVIEW

Molecular and Genetic Aspects of Preeclampsia: State of the Art

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Preeclampsia is a major cause of fetal and maternal morbidity. It has an incidence of 1–5% (1). Manifested generally in the mid-to-late stages of gestation, preeclampsia is diagnosed by the three clinical signs: hypertension, proteinuria, and edema. Severe and acute complications are 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 case of primiparity, work-related psychosocial strain during pregnancy, poor social background, the mother's own low birthweight, prematurity, and young age (3–5).

The onset and progression of the disorder are unpredictable, and the only 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 that the uterus plays a part.

Among several concepts (Table 1), according to the theory suggested by Redman and Roberts, preeclampsia is a two-stage disorder (6). The illness derives primarily from a placental ischemia (7,8). Poor placental perfusion, as tested in animals (9), as a result of immunologically mediated abnormal implantation, excessive placental size, or microvascular disease derived from preexisting 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" (10). 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 (11). 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 (12,13).

Consequent (secondary) clinical symptoms reflect a generalized vasoconstrictive disorder as a result of abnormal vascular endothelial function. As postulated by Roberts *et al.* (14), 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 intracellular coagulation and increase sensitivity to pressors. Some of these factors, originating from the foci of placental hypoxia/ischemia, are cytokines, such as tumor necrosis factor-α and interleukin-1 α and β, the production of which was found to be elevated in hypoxic conditions of villous explants from the human placenta (15). Similarly, the expression of placental cytokines, such as tumor necrosis factor-α, interleukin-1-β and interleukine

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TABLE 1
Summary of Current Concepts of Preeclampsia

Concept	Basis of theory
Placental ischemia	Hypoxia/reperfusion in the placenta initiates local oxidative processes and leads to release of factors that consequently cause endothelial damage.
Hyper/dyslipidemia	High serum lipid (VLDL) levels with insufficient antioxidant activity may lead to oxidative processes and consequently to endothelial damage.
Immune maladaptation	Immune processes at the placenta due to insufficient immune tolerance of the fetus may lead to immune processes, release of cytokines, and consequently endothelial damage.
Genetic imprinting	The development of preeclampsia may be based on a single recessive gene or a dominant gene with incomplete penetrance.
Mitochondrial defects	Invasion of cytotrophoblasts into the maternal endometrium is a highly energy-consuming process. This process may be incomplete in case of a mitochondrial defect.
Disturbance of the invasion of placental extravillous cytotrophoblasts	Histological observations confirmed incomplete invasion of cytotrophoblasts to the maternal endometrium. This failure may be secondary to any predisposing factor.

10, was increased in the placentas of preeclamptic subjects (16). As a newly described agent of placental origin, neurokinin B, a member of the tachykinin family with vasoactive properties, was found to be secreted excessively by the placenta (17). It is not clear yet, whether it is a marker or an etiologic factor of preeclampsia.

Observations of Possible Predisposing Factors

There are predisposing factors, which are not necessary for the development of preeclampsia (Table 2). Such well-known factors are diabetes mellitus and previous hypertension. Most recently a mineralocorticoid receptor mutation was described, causing early-onset hypertension that is markedly exacerbated in pregnancy (18).

Clotting abnormalities. Severe preeclampsia may be associated with intervillous or spiral-artery thrombosis and consequently inadequate placental perfusion. The increased incidence of mutations predisposing patients to thrombosis (Leiden mutation of factor V, prothrombin 20210A allele, protein C protein S, and antithrombin deficiency) has been reported (19). However, in a study from the Netherlands no difference was found in the prevalence of practically the same genetic risk factors compared to control subjects (20). Recently, methylenetetrahydrofolate (MTHF) reductase polymorphism has been reported as a possible genetic factor; however, the data are inconclusive (19,21,22). MTHF reductase catalyzes the conversion of 5,10-MTHF to 5-MTHF a cosubstrate for homocysteine remethylation to me

TABLE 2
Summary of Factors Possibly Predisposing to Preeclampsia

Factors independent from genetics and molecular mechanisms	Poor socioeconomical conditions
	Primiparity
	Young age of the mother
	Maternal stress
	Low birthweight (prematurity) of the mother
Factors which may have molecular relationship and may be influenced by inheritance	Previously existing hypertension
	Diabetes mellitus
	Clotting abnormalities
	Hyper-/dyslipidemias
Susceptibility factors which are possibly influenced by genetics	Preeclampsia genes?
	Involvement of mitochondrial dysfunction
	Interactions between maternal and fetal HLA genes
	Genetic variability of the renin-angiotensin system
	Genetic variability of endothelial nitric oxide synthas

hionine. Due to a common functional mutation in he MTHF gene (C677T; alanine for valine), reduced nzyme activity and increased plasma homocysteine oncentrations occur. Raised plasma homocysteine oncentration is associated with increased lipid per-xidation, blunted endothelium-dependent vasore-axation and enhanced coagulation. Thrombomodu-in gene polymorphism may also be involved in the athogenesis of severe early-onset preeclampsia 23). Defects of fibrinolysis seem to contribute to the levelopment of the disease, as it was suspected al-eady in the 1980s (24). Recently a deletion/inser-ion polymorphism (4G/5G) in the promoter of the lasminogen activator inhibitor-1 gene was found to e associated with severe preeclampsia: 4G allele requency was significantly higher in patients than n the control group (25).

Hyper-/dyslipidemias. Pregnancy in general, nd preeclamptic pregnancy in particular, is associ-ted with a marked hyperlipidemia. As it is postu-ated in another theory explaining the pathomecha-ism of preeclampsia, a low ratio of toxicity-reventing activity to very low-density lipoproteins VLDL) would result in cytotoxicity and triglyceride ccumulation in endothelial cells (26). In preeclamp-ia, circulating free fatty acids are already increased 5 to 20 weeks before the onset of clinical disease 27). As suggested by Lorentzen *et al.*, when placen-ally derived endothelial disturbing factors, like ipid peroxides and trophoblastic components, are eleased into the maternal circulation, their effects n the endothelium may be enhanced by the hyper-ipidemia-mediated activation or "sensitization" of he endothelial cells (28). Recently, common coding equence variations in the lipoprotein lipase gene ave been shown to cause substantially increased isk of preeclampsia (29). A recurrent theme is that ree radical reactions, promoted by a "cross-talk" etween diseased placenta and maternal dyslipide-nia, promote a vicious cycle of events that make ause and effect difficult to distinguish but likely ontribute to the progression of preeclampsia (30). n preeclampsia increased red blood cell turnover an also be detected, as demonstrated its indicators n an animal model and in humans (31,32). Since eme derivatives may mediate lipoprotein oxidation nd consequently endothelial damage, hemolysis nay be an additional factor in the pathogenesis of he disease (33–35).

Tumor necrosis factor-α. TNF-α has fundamen-al effects on endothelial cells by several means, including altering the balance between oxidant and antioxidant, changing the pattern of prostaglandin production, and affecting expression of several cell surface components. TNF-α mRNA expression is significantly elevated in preeclamptic patients compared with the other two control groups. The high expression of TNF-α may be associated with the TNF1 allele, whose frequency is markedly increased in preeclamptic patients (36). A mutation in the promoter region of the TNF-α gene (TNF T2) has been described which is associated with increased transcription of the gene. However, the frequency of the TNF T2 allele is not increased in patients with preeclampsia or HELLP syndrome. Therefore, this promoter mutation is probably not a major genetic cause of preeclampsia (37).

Role of Genetics: Family Analysis, Genome-wide Analysis

According to epidemiological studies, preeclampsia/eclampsia has a strong familial component (38–40). 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 genome-wide screen of Icelandic families representing 343 affected women, a significant locus was found on 2p13 (41), while in another genome-wide linkage study of preeclampsia/eclampsia evidence was found for a candidate region on 4q (42). 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 (43). 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 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).

Genetic Factors Possibly Involved in the Pathogenesis of Preeclampsia/Eclampsia

Mitochondrial Dysfunction

In the late 1980s Tobergsen and his co-workers observed that a high incidence of preeclampsia was present in a family with mitochondrial dysfunction (44). The same research group found that mutations

in the mitochondrial transfer ribonucleic acid genes in two families were also associated with preeclampsia (45). 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 (3,46): while mitochondrial genes are transmitted through the mothers, paternal genes clearly have contribution to the risk of preeclampsia.

Human Leukocyte Antigens (HLA)

In preeclamptic women and in their spouses there is a greater homozygosity at the HLA B locus (47). Also, antigen sharing at the A and B loci and HLA DR4 is greater between affected women and their spouses (48). The inducibility of TNF-α is HLA class II dependent, which might also provide a role for the HLA system. On the basis of epidemiologic evidence immune mechanisms (immune maladaptation) ought to be involved in the etiology of preeclampsia (46).

On the other hand, studies abound on the role of the HLA system in preeclampsia without any definite influence having been found (1). Wilton *et al.* (49) 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 (50). 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 (51). Instead, HLA-G, a nonspecific HLA I group antigen is expressed by the trophoblast cells (52). We may suppose that HLA-G is involved in the development of immune tolerance (53). Although there was no detectable relationship found between susceptibility to preeclampsia or being born of a preeclamtic pregnancy and the HLA-G genotype (54), 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 (55).

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 relations.

The Role and the Effect of Genetic Variability of 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 (56,57). In this system, AT II can act in an autocrine/paracrine fashion (58): it can stimulate angiogenesis and also is capable of antiproliferative actions by mediating the inhibition of endothelial cell proliferation (59,60). In humans, the actions of AT II are mediated by at least two receptors, AT 1 and 2 receptor subtypes (61). The vasoconstrictive and growth-promoting effects of AT II are mediated by the AT 1 receptor, the presence of which has been proved in the human placenta (62,63). On the other hand, AT 2 receptors exerting an antiproliferative effect which counteracts the growth action of AT 1 receptors, are practically not present. Thus, it is unlikely that AT II mediates any significant action via the AT 2 receptors (57,64). Since renin, ACE, and AT receptor 1 are all expressed in and around remodeling spiral arteries (56), the known actions and presence of 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 members of renin-angiotensin system in the pathogenesis of preeclampsia has been extensively examined.

Angiotensinogen. Shortly after an association of angiotensinogen molecular variants with hypertension had been demonstrated (65), 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 (66). Moreover heterozygous women were shown to exhibit significantly elevated expression of the T235 allele compared to the M235 allele. These observations sug

ested that the elevated expression of the T235 llele in decidual spiral arteries may cause first rimester atherotic changes leading to preeclampsia 67). This proposal was confirmed by the same research team: women homozygous for the angiotensinogen T235 allele had a greater area/diameter atio than women homozygous for the normal angiotensinogen M235 allele (68). On the other hand, ewer studies based on examinations in Chinese, ustralian, and Japanese populations have suggested that the contribution of angiotensinogen gene olymorphism to the occurrence of preeclampsia/ clampsia is small and is not constant across populations (69,70).

ACE gene polymorphism. In two studies evaluting the effect of ACE insertion-deletion polymorhism on the incidence of preeclampsia/eclampsia, o evidence was found that the polymorphism of the CE gene was associated with preeclampsia/ clampsia (71,72).

Renin. In an investigation on two or three genrations of affected females, although only in a reltively smaller number of families, gene restriction ragment length polymorphism did not exhibit linkge with preeclampsia/eclampsia (73).

AT receptor type 1. In a recent study, Morgan *et l.* found that allele and genotype frequencies in four olymorphic regions did not differ between normoensive and preeclamptic groups. However, two varints (an 573T variant in the coding exon of the eceptor gene and a dinucleotide repeat polymorhism in its 3′ flanking region) showed a similar istortion of maternal-fetal transmission (74). Acording to this finding, the AT1 receptor transmision in the fetus may contribute to the etiology of reeclampsia.

Endothelium-Derived Nitric Oxide Synthase (eNOS)

Of the three isoforms of NOS, eNOS is widely istributed in the placental tissue (75), producing itric oxide (NO), a potent vasodilator and inhibitor f platelet aggregation (76). Although eNOS is exressed by syncytiotrophoblasts, no proof of expresion was found in the case of extravillous cytotrohoblasts at any time during invasion (77). This fact uggests that NO is unlikely to contribute to spiral rtery dilatation. On the other hand, reduction of NOS activity was detected in preeclamptic placenta 78,79), implicating the eNOS gene in the pathohysiology of preeclampsia/eclampsia. A linkage study using preeclamptic families reported evidence for a preeclampsia/eclampsia susceptibility locus in the eNOS region on chromosome 7q36 (80). This, however, was not confirmed in a recent repetition study using the same markers (81), while in another recent linkage study using 25 microsatellite markers from chromosome 7, a strong suggestion of linkage was found for one marker, D7S1805 (82). The results of these examinations raise the possibility that a putative preeclampsia/eclampsia susceptibility locus may be located on 7q36.

Conclusion

In summary, preeclampsia/eclampsia seems to be a multifactorial disease. The search for genetic factors is complicated by environmental/risk factors that may interact with one or more causative genes. The pathogenesis of preeclampsia/eclampsia is altered by numerous genetically influenced predisposing factors, affecting either the first or the second stage of the disorder. Although genetic aspects modify the development of the disease, they seem not to be the major answer to our questions concerned with the pathomechanism of preeclampsia. As it is suspected, the disease may be induced by a multifactorial disturbance of trophoblast invasiveness. In this process, the placental renin-angiotensin system and the expression of HLA antigens may play a significant role.

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The Pathogenetic Role of Heme in Pregnancy-Induced Hypertension-like Disease in Ewes

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Toxicosis syndrome of fasting pregnant ewes has a close similarity to human preeclampsia (hypertension, albuminuria). The common etiological factor might be oxidative hemolysis and heme-induced endothelial damage. Ewes (5 starving, 5 control) at 130–135 gestational days with a 96-h fasting period followed by refeeding were used. Blood pressure, platelet count, electrolytes, kidney and liver function parameters, as well as plasma glucose, hemoglobin/heme, free thiol groups and Trolox equivalent antioxidant capacity, and plasma iron and ferritin levels were measured. Statistical significance was assessed using Student's *t* test ($P < 0.05$). Besides hypertension and renal disturbances, hemolysis, elevated liver enzymes and low platelet count, characteristic of human HELLP syndrome, were also present. In the first 24 h of glucose deprivation there was a significant rise in both the plasma hemoglobin/heme and indirect bilirubin concentrations. The antioxidant free thiol levels decreased significantly the next day, without any change in the total antioxidant capacity of the plasma. While the loss of calcium and magnesium levels related to the similarity to preeclampsia, reduced plasma iron concentrations referred to species differences in iron homeostasis. An oxidative stress causing hemolysis in a glucose-6-phosphate dehydrogenase-deficient animal model was proven by the loss of free thiols after glucose deprivation. The activation of the oxidative stress protein heme oxygenase was a signal of endothelial cell injury, the primary cause of pregnancy-induced hypertension. © 1997 Academic Press

Pregnancy-induced hypertension (PIH), usually manifested in late gestation, is a major cause of fetal and maternal morbidity and mortality. If associated with albuminuria, edema, or both, the disease is termed preeclampsia (1). A severe and acute complication of preeclampsia is the Hemolysis, Elevated Liver enzymes, and Low Platelet count syndrome (HELLP syndrome) (2).

Although the etiology of preeclampsia is unknown and the pathogenesis is poorly understood, the clinical symptoms reflect a generalized vasoconstrictive disorder as a result of abnormal vascular endothelial function (3). Hypertension is explained by increased vascular reactivity (4). This increased reactivity is due to endothelial dysfunction, with the consequence of an imbalance between prostacyclin and thromboxane A2 and possibly dysfunction of endothelial relaxig factor (EDRF) and endothelin synthesis (5–7). There is evidence of an increased demand for antioxidant protection during pregnancy and the presence of oxidative stress, i.e., increased free radical activity and reduced antioxidant protective systems in the pathogenesis of human PIH (8,9). The imbalance between vasoconstrictor thromboxane A2 and vasodilator prostacyclin in preeclampsia was associated with an imbalance between lipid peroxides and vitamin E in maternal blood (10). Recent studies on the mechanism of cultured endothelial cell damage revealed that heme, especially when free, is dangerous to the endothelium. It is an oxidative hazard because iron released from hemoglobin-derived

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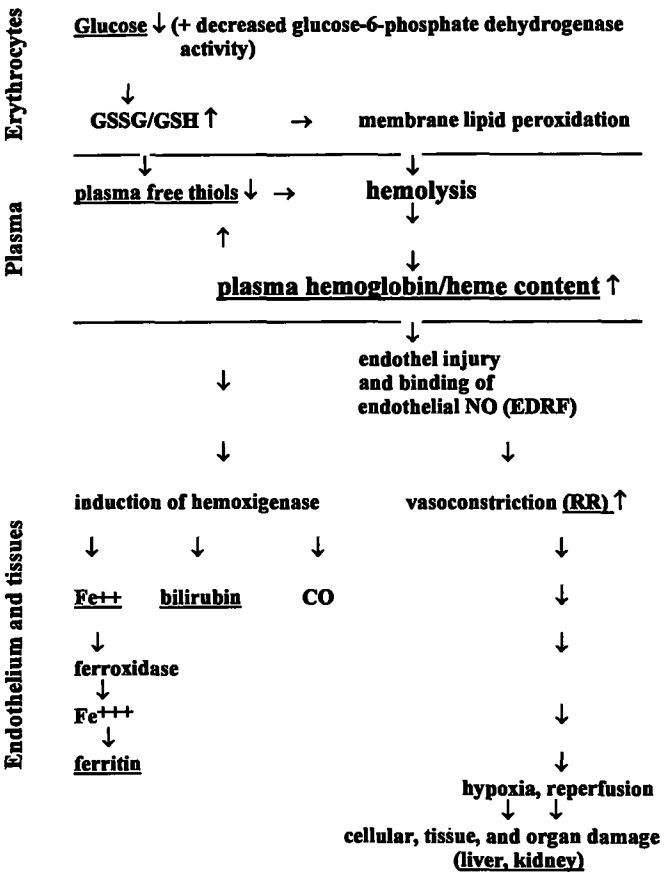

FIG. 1. Hypothesis of the pathogenesis of pregnancy-induced hypertension and HELLP syndrome. Parameters examined are underlined.

heme both catalizes the oxidation of low-density lipoproteins and sensitizes the vascular endothelial cells to other oxidants (11–13). Endothelium defends itself from heme by induction of the heme degrading enzyme heme oxygenase (14).

On the basis of these data we offer the hypothesis that the endothelial dysfunction resulting in toxicosis-like syndrome in pregnant fasting ewes—very similar to human PIH (15)—may be derived from the deleterious effect of free hemoglobin/heme on the vascular endothelium (Fig. 1). All the pathophysiological symptoms, such as vasoconstriction, hypertension, and kidney and liver damage may be explained by the oxidative injury of endothelial cells caused by heme from hemolysis of red blood cells. Sheep red blood cells are extremly sensitive to oxidants because of their low antioxidant protective glucose-6-phosphate dehydrogenase (G6PD) activity (16). As a consequence of insufficient capacity of the hexose monophosphate shunt, erythrocytes fail to regenerate oxidized glutathione (GSSG) to its reduced form (GSH). The decline of G6PD activity during human pregnancy has already been reported (17).

The aim of this study was to follow the measurable biochemical consequences of glucose deprivation in this animal model with known metabolic disorder so as to draw some conclusions on their possible participation in the pathogenesis of human PIH.

MATERIALS AND METHODS

Prior to the experiment a license was issued by the University Committee for Ethics in Animal Experiments (permit No. ÁTB 83/1993). The experiment was performed at the animal stables of the Veterinary College of the Debrecen Agricultural University, located in Hódmezővásárhely. Ten pregnant merino ewes at the gestational age of 130–135 days (terminus: 142–145 days) were randomly divided into control and fasting groups (5 animals each). During their pregnancy animals were fed hay and straw. Prior to the experiment twin pregnancies were documented by ultrasound examination, although one singleton pregnancy in the fasting group was revealed only at birth. Development of the syndrome was examined during a 96-h withdrawal of food, followed by refeeding (for 3 days). Water was given ad libitum. The diagnosis was established when symptoms of the syndrome occurred (muscular tremors, increased proteinuria, hypertension, low platelet count, and elevated liver enzymes).

Measurements

Blood pressure readings were taken every day throughout the experiment. Blood pressure cuffs of a digital sphygmomanometer were placed on the left thoracic limb over the anterior cephalic artery, and pressure was recorded three times over a 30-min period.

Ten milliliters of heparinized blood was collected by the direct puncture of the external jugular vein eight times: at the very beginning of food withdrawal and subsequently at 24-h intervals until the third day of refeeding (Days 0–7). Blood was centrifuged at once (10 min at 1500 rpm).

Because sheep platelets are smaller than those of humans, they would not have been measured effectively using hematological automates; thus, the platelet count was determined using a Buerker's chamber and a polarization microscope.

Plasma and Urine Biochemistry Studies

Plasma electrolytes, as well as plasma creatinine, urea nitrogen, albumin, bilirubin, glutamate oxalate transpeptidase (GOT) and glutamate pyruvate transpeptidase (GPT) levels were measured by conventional laboratory techniques each day. Iron and iron binding capacity were measured on Days 0 and 5. Plasma ferritin levels were determined by chemiluminescence immunoassay (Sangtec Diagnostica GmbH BYK LIA-mat Ferritin) on Days 0 and 5.

Plasma hemoglobin/heme concentration was measured spectrophotometrically at 415 nm (Soret band) expressed in μmol/liter using $\epsilon_{mM}^{415\,nm} = 125$ (18). The antioxidant activity of the plasma (Trolox Equivalent Antioxidant Capacity = TEAC) was compared with that of 1 mmol/liter Trolox, a water-soluble vitamin E derivative (19). Plasma concentration of free thiols was estimated spectrophotometrically (reduction of 5,5'-dithiobis-2-nitrobenzoic acid) (20).

Urine samples were taken every day, after introduction of a ballooned catheter. Total protein was measured every day.

Statistical analysis was calculated using Student's one-tailed t test within the groups and two-tailed t test between groups. If P values were less than 0.05, alterations were considered statistically significant. Performability of t test was checked by F test.

RESULTS

There was no significant difference between the two groups at the onset of the experiment. Control animals demonstrated no significant changes during the 8-day experiment period, either in blood and urine chemistry or in physiologic state (see Figs. 2A and 3A).

The serum glucose values of the fasting animals decreased, while hemoglobin/heme and indirect bilirubin increased significantly after a 24-h period of food withdrawal (see Fig. 2B).

Plasma hemoglobin/heme exhibited a quick rise after 24 h of starvation, and it stayed discretely elevated during the whole period of experiment (Fig. 2B).

Plasma free thiol levels in the fasting group declined by the second day of food withdrawal. An increasing trend of free thiol level occurred during refeeding (Fig. 2B).

The serum Ca^{2+} level already decreased after 24 h (from 2.45 ± 0.08 to 2.22 ± 0.16; $\bar{x}$ ± SD, mmol/liter), and this change became significant after 48 h (2.02 ± 0.19). The lowest level of serum Ca^{2+} was reached by the last day of fasting (1.73 ± 0.23). Serum Mg^{2+} levels exhibited the same trend; however, significant alteration from baseline value (0.92 ± 0.06; $\bar{x}$ ± SD, mmol/liter) occurred only on the final day of fasting (0.68 ± 0.08, $P < 0.05$).

A significant rise in blood pressure occurred after a 72-h withdrawal of food. Arterial mean pressure rose from 94 ± 4.2 to 114 ± 3.8 mm Hg and by Day 6 it was as high as 118 ± 3.7 mm Hg ($\bar{x}$ ± SD).

By Day 3 the platelet count in the fasting animals decreased to 127,500 ± 32,000/μl, from the baseline value of 292,000 ± 32,400/μl ($\bar{x}$ ± SD).

Blood urea nitrogen increased significantly on Day 2; however, from Day 4 on no significance could be detected (Fig. 3B). Serum creatinine levels showed a similar tendency, with a significant difference between the two groups on Days 1 and 2. There was a significant rise also in proteinuria. The amount of protein in the urine elevated by Day 4 from 485.3 ± 83.6 to 2150.8 ± 385.7 mg/liter ($\bar{x}$ ± SD). Plasma albumin concentrations decreased by Day 4 from 36.2 ± 0.9 to 32.6 ± 1.9 g/liter ($\bar{x}$ ± SD). Hemoglobinuria could not be proven.

The plasma GOT increased significantly by the first day of refeeding, while a significant rise in GPT levels could be detected on the last day of the experiment (Fig. 3B).

The plasma iron level showed a significant decrease (from 24.6 ± 3.7 to 16.9 ± 2.8 μmol/liter) by Day 7, though not accompanied by an alteration of iron binding capacity (53.8 ± 3.9 and 51.1 ± 10 μmol/liter) ($\bar{x}$ ± SD). The ferritin level remained under the detectable range in both groups.

The concentrations of TEAC were in the range of 1.0 and 1.35, without any significant change between the groups.

Outcome of Pregnancy

In the control group all the animals delivered healthy lambs. In the fasting group one animal expired after convulsions, two animals aborted, and two ewes delivered viable lambs.

DISCUSSION

Previous studies have revealed that pregnancy provoked unique physiological situation in pregnant fasting ewes, a 72-h-long withdrawal of food caused toxicosis-like syndrome, while nonpregnant animals showed neither biochemical nor hemodynamic changes (21). In our experiment some other pathophysiological fea-

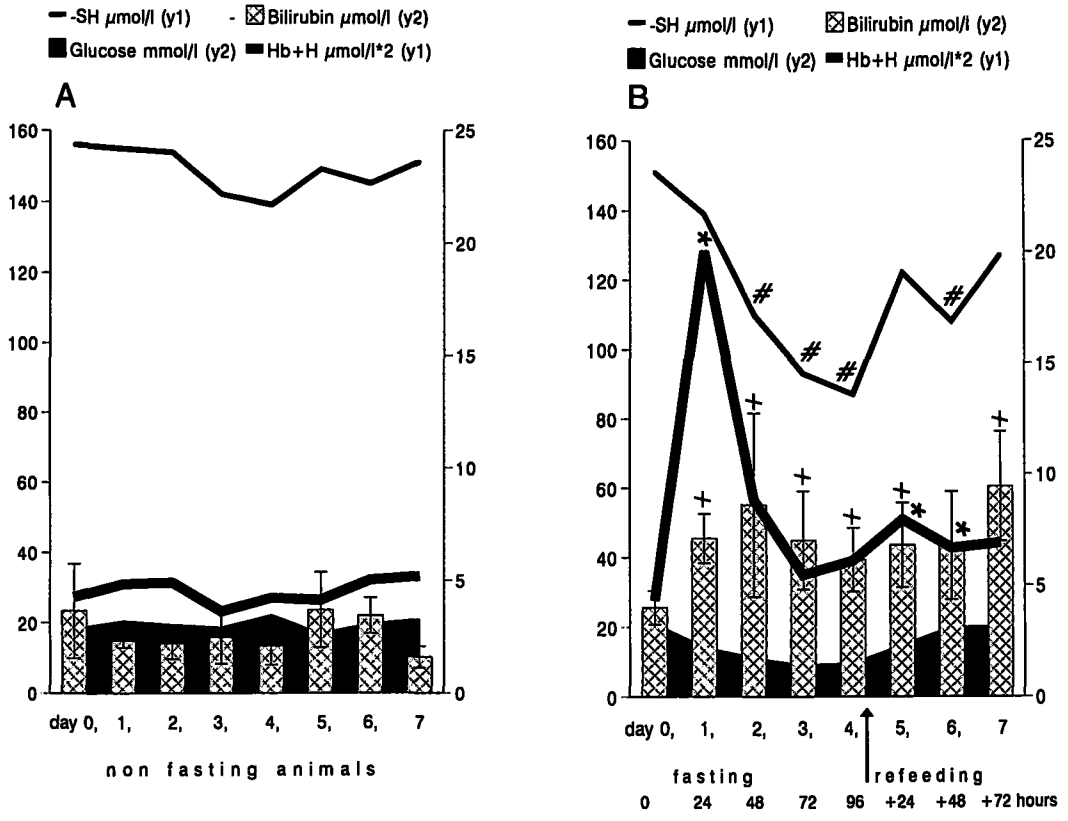

FIG. 2. Change of plasma glucose, hemoglobin/heme, indirect bilirubin, and free thiols in the toxicosis of ewes. (A) Control group ($n = 5$). (B) A 96-h-long fasting group. Changes significant ($P < 0.05$) with both one-tailed and two-tailed Student's t tests are indicated by X (glucose), * (Hb + H = hemoglobin/heme), + (bilirubin = indirect bilirubin), and # (-SH = free thiols).

tures characteristic of human HELLP syndrome were also shown. Besides hypertension and renal disturbances (Fig. 3), changes in calcium and magnesium metabolism, a fall in platelet count, and hepatic insufficiency were also measured. A significant increase in plasma hemoglobin/heme concentration with simultaneous reduction in free thiols (Fig. 2) proved that the oxidative stress as the common result of increased oxidant activity and insufficient antioxidant capacity of the red blood cells resulted in hemolysis. An increased heme catabolism, estimated as a concomitant rise in plasma bilirubin levels, was also seen in fasting pregnant animals during the whole experiment (Fig. 2) as a signal of heme oxygenase enzyme activation (14). All the above-mentioned changes were in concordance with biochemical signs documented in human pathology. The measured decline in plasma free thiol groups, which offer a nonspecific buffer to oxidative stress, is also one of the most sensitive antioxidant markers in human PIH (8). Induction of heme oxygenase, a 32-kDa 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, the antioxidant biliverdin is produced, which is quickly reduced to bilirubin, also antioxidant (22). Since carbon monoxide and iron are the two other products of the heme oxygenase enzyme reaction, increased levels of carboxyhemoglobin, serum iron, and bilirubin in severe cases of preeclampsia (23) were not only the indicators of increased red cell turnover but also the signals of induced oxidative stress protein activation.

Plasma iron and ferritin levels are increased in human PIH (24). It was an unexpected result of our study to measure the loss of plasma iron concentrations in fasting and refed pregnant ewes on the sixth day of the experiment together with undetectable plasma ferritin. There might be some species differences in iron homeostasis in favor of fast iron elimination instead of storage in ferritin molecules, a process proven to be the ultimate antioxidant defence in other animals and human during an oxidative

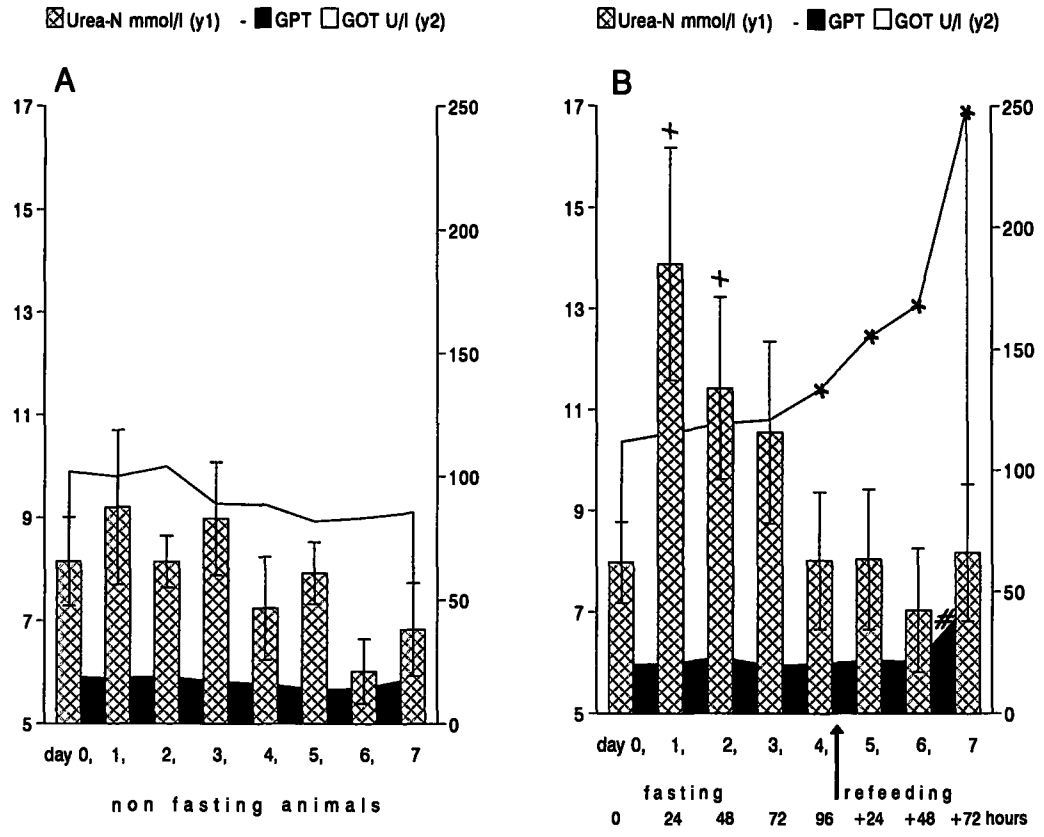

FIG. 3. Change of plasma urea nitrogen, glutamate oxalate transpeptidase, and glutamate pyruvate transpeptidase in the toxicosis of ewes. (A) Control group ($n = 5$). (B) A 96-h-long fasting group. Changes significant ($P < 0.05$) with both one-tailed and two-tailed Student's t tests are indicated by + (Urea-N = urea-nitrogen), * (GOT = glutamate oxalate transpeptidase), and # (GPT = glutamate pyruvate transpeptidase).

stress caused by heme (25–27). Nevertheless, the species difference of sheep ferritin, which may be undetectable with an immunoassay developed for human ferritin, cannot be excluded. There was also another discrepancy between our results and the findings of human preeclampsia. In spite of the significant decrease in the plasma thiol concentrations in fasting animals parallel to the rise of hemoglobin/heme, there was no deficit in the total antioxidant capacities, i.e., TEAC values of plasma compared to those of controls. In human studies measured with the same method plasma total antioxidant capacities of women with preeclampsia were regularly reduced by approximately half (28). However, that difference resulted from the progressive increase in antioxidant activity of normal pregnancy, while no rise occurred in human PIH (28). During the short time period there was no measurable rise in the total plasma antioxidant capacity of control animals.

Recent studies showed that even small amounts of the highly reactive hemoglobin in plasma suffice to trigger oxidation of low-density lipoproteins (along with its lipid oxidation), which can damage vascular endothelial cells (29). In addition, heme has a direct injurious effect on the endothelial cells (11). Heme is a physiologic iron chelate that could facilitate endothelial iron uptake in cultured endothelial cells (12). Iron-loaded cells become extremly 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 lipide peroxides (11–13). Furthermore, other heme-containing proteins, such as myoglobin and cytochromes, might be released subsequent to ischemia or reperfusion. In this instance, heme released from necrotic cells could enter endothelium from the abluminal side, without being intercepted by the heme and iron-binding proteins normally present in plasma (11). Induction of heme oxygenase has been

proven to be a common, protective antioxidant response to heme burden in different animal models of tissue injury in which enhanced oxidative stress in implicated (25–27) and at the same time a signal of endothelial cell oxidative stress. Additional evidence for our hypothesis concerning hemoglobin as the direct cause of endothellial cell injury with consequent heme oxygenase activation was given in the endothelial cell cultures exposed to different acellular hemoglobin solutions (30). 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 (7), in response to erythrocyte lysates (31).

Endothelial injury caused by heme/hemoglobin resulting in hypersensitivity of endothelial cells to exogenous and endogenous oxidant may be sufficient to explain the increased vascular reactivity and consequent endothelial dysfunction with imbalances among vasodilator prostacyclin, EDRF, which has been identified as nitric oxide (NO), as well as the vasoconstrictors prostanoid thromboxan A2 and endothelin-1. A decrease of plasma calcium level may also contribute to increased vascular reactivity (32).

The hypothesis that increased free hemoglobin concentration was the direct cause of vasoconstriction underlying preeclampsia has previously been proposed on the basis of its effects on EDRF (33). Hemoglobin and the hemoglobin/haptoglobin complex bind to and inhibit the effects of EDRF (34). The theory was criticized on the grounds that while having a morphologically intact endothelium, free hemoglobin can only inhibit luminally released, locally acting EDRF. The more substantial abluminal component thus remains protected (35).

Glucose deprivation in pregnant ewes, with low G6PD activity, induced a toxicosis-like syndrome very similar to human PIH. 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 (36). Hematologic data in severe deficient women suggested slight hemolysis in the first trimester of pregnancy (37). The percentage of unsuccessful pregnancies with spontaneous abortion occurring in the first trimester was higher (22%) in the heterozygous carrier state than in control pregnancies (9.3%) (38). A significant decline in G6PD activity was reported in late gestosis compared to healthy pregnant women (39). 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 (17). Genetic predisposition to PIH is very likely because of the high recurrency risk of 4 to 27%.

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 PIH.

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Inhibitory effects of methylxanthines on the pre-eclamptic-like symptoms in ewes

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Abstract

Objective: Our objective was to determine whether two methylxanthines, pentoxifylline (PTX) and allopurinol, would have beneficial effects on experimental pregnancy-induced pre-eclampsia- like disease in ewes.

Study design: About 20 animals at the gestational age of 130–135 days were divided into four groups (control; fasting; fasting, PTX-treated; and fasting, allopurinol-treated). The illness was provoked with a 4-day fasting period. Electrolytes, glucose, conventional parameters, plasma haem content, indirect bilirubin concentration and free thiol levels were measured.

Results: Unlike in the fasting group, conventional signs of the disease, such as hypertension, kidney and liver injury and platelet count decrease, were all mitigated in the fasting, drug-treated animals. In the treated animals plasma haem content increased by a less significant level, while indirect bilirubin concentration showed a more rapid rise.

Conclusions: Both methylxanthines partly inhibited the pre-eclamptic-like symptoms in ewes. We speculate that the better induction of haem oxygenase might play an important role in this inhibitory effect on this particular animal model. © 2001 Published by Elsevier Science Ireland Ltd.

Keywords: Ewe; Pre-eclampsia; Haem oxygenase; Methylxanthines

1. Introduction

Pregnancy-induced hypertension (PIH), one of the major complications in pregnancy, develops in more than 10% of pregnant women. More severe forms of the disease are pre-eclampsia (albuminuria and oedema also arise) and HELLP syndrome (pre-eclampsia complicated by haemolysis, elevated liver enzymes and low platelet count) [1]. Although, there are several theories which attempt to explain the aetiology of the disease, the pathogenesis is still doubtful.

Poor placental perfusion, which is the result of immunologically mediated abnormal implantation, microvascular disease, or excessive placental size, is a unique feature of pregnancies predisposed to pre-eclampsia [2]. It has also been postulated that inadequate placentation in pre-eclampsia creates foci of placental ischaemia/hypoxia leading to the elaboration of factors that compromise systematic endothelial function to produce disease sequelae [2]. During hypoxia-reperfusion injury the enzyme xanthine oxidase, which has also been detected in the human placenta [3], generates superoxide anions [4].

According, to the observations mentioned, we speculated that two methylxanthines, pentoxifylline (PTX) and allopurinol (AP), might beneficially modify the alteration of biochemical parameters and symptoms of the disease in an animal model during the development of the syndrome. AP is a potent inhibitor of xanthine oxidase, while PTX has beneficial effects on microcirculation [5,6].

In pregnant sheep fasting 3 days, a significant elevation in blood pressure as well as increased proteinuria, decreased uteroplacental blood flow and decreased glomerular filtration rate occur [7]. Unlike in human PIH, disturbed carbohydrate metabolism (hypoglycemia and ketonaemia) 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 in some way the human disease, making the animal model appropriate for further examination.

As we observed previously, in the development of PIH-like disease in ewes the change in haem metabolism (accelerated haemolysis, increased plasma haem content) might have a triggering effect [8]. Since both AP and PTX may inhibit the development of the disease via modification of the placental circulation and the oxidative processes, we hypothesised that a treatment with these methylxanthines

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could postponed the occurrance of symptoms and might also alter the onset and degree of haemolysis.

2. Subjects and methods

2.1. 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).

About 20 pregnant merino ewes at the gestational age of 130–135 days (terminus: 142–145 days) were randomly divided into the following groups of five animals each: control; fasting; fasting, PTX-treated (15 mg/kg per die, per os, divided into two doses, administered from day 0 to delivery); and a fasting, AP-treated (20 mg/kg per 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.

2.2. Measurements

10 ml of heparinised blood were collected by the direct puncture of the external jugular vein eight times: at the very beginning of fasting and at 24 h intervals until the third day of refeeding (days 0–7). Blood was centrifuged immediately (10 min at 4°C, 1500 rpm).

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

2.3. 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 (brom-cresol-green method), bilirubin (Jendrassik-Gróf method), glutaminate-oxalate-transferase (GOT) and glutaminate-pyruvate-transferase (GPT) levels (enzymatic UV-test), were measured each day.

Plasma haemoglobin/haem concentration was measured spectrophotometrically at 415 nm (Soret band) expressed in μmol/l, using $\varepsilon_{mM}^{415\,nm}=125$ [9]. About 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 haemoglobin/haem 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) [10].

Hypoxanthine, xanthine, uric acid and alloxanthine levels were determined using the high-performance liquid chromatography method [11].

After the introduction of a ballooned catheter, urine samples were taken every day. Total protein and haemoglobin 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 three times over a 30 min period.

2.4. 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 <0.05, alterations were considered to be statistically significant.

3. Results

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, Figs. 1A–C and 2A).

As early as 24 h after the onset of food withdrawal, blood glucose levels decreased significantly from 2.88–3.1 mmol/l concentration in all the fasting animals and reached its bottom line level of 1.42–1.54 mmol/l concentration ($P < 0.01$) by the end of the 96 h fasting period. After refeeding, glucose levels quickly rose and reached 2.9–3.2 mmol/l concentration (the same as that of the control animals) 48 h 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 pre-eclampsia (hypertension, proteinuria, tremors, elevation of serum creatinine and urea-nitrogen), the disease was present in all the fasting, non-treated animals. 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

Table 1
Effects of pentoxifylline and allopurinol treatment on changes in plasma albumin, urea nitrogen and creatinine as well as total urine protein in pre-eclampsia-like disease in ewes[a]

	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		Fasting		Fasting, pentoxifylline-treated		Fasting, allopurinol-treated	
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[b] (4)	35.9 ± 0.8	34.5 ± 1.7[b] (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[c,**] (6)	39.8 ± 6.5	165.8 ± 19.4[c,*] (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[d] (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[b] (1)	99.6 ± 4.4	182.5 ± 45.4[b,*] (2)

[a] Each group consisted of five animals. Fasting: 96 h from day 0 to day 4. In the fasting, pentoxifylline-treated group sheep were treated with 15 mg/kg per die pentoxifylline, while in the fasting, allopurinol-treated group 20 mg/kg per die allopurinol was administered.

[b] The occurrence of pathologic changes was noted in the case of either the pentoxifylline- or allopurinol-treated, fasting groups: when they arose later. Values are given as mean ± standard deviation.

[c] The occurrence of pathologic changes was noted in the case of either the pentoxifylline- or allopurinol-treated, fasting groups: when the amount of change was less. Values are given as mean ± standard deviation.

[d] The occurrence of pathologic changes was noted in the case of either the pentoxifylline- or allopurinol-treated, fasting groups: when the alteration was less and appeared later, when compared to the non-treated, fasting group. Values are given as mean ± standard deviation.

* Significance was calculated by multiple-choice ANOVA followed by pairwise comparison using Tukey's test $P < 0.05$.

** Significance was calculated by multiple-choice ANOVA followed by pairwise comparison using Tukey's test $P < 0.01$.

simultaneous increase in plasma GOT level by the end of the fasting period also occurred (Table 1, Fig. 1A–C), 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 remained in 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, Fig. 1A-C). The increase of GPT concentration was not significant in the treated groups. Plasma enzyme activity rose by day seven 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 $\pm$ standard deviation) to 31.4 ± 9.12 IU/l

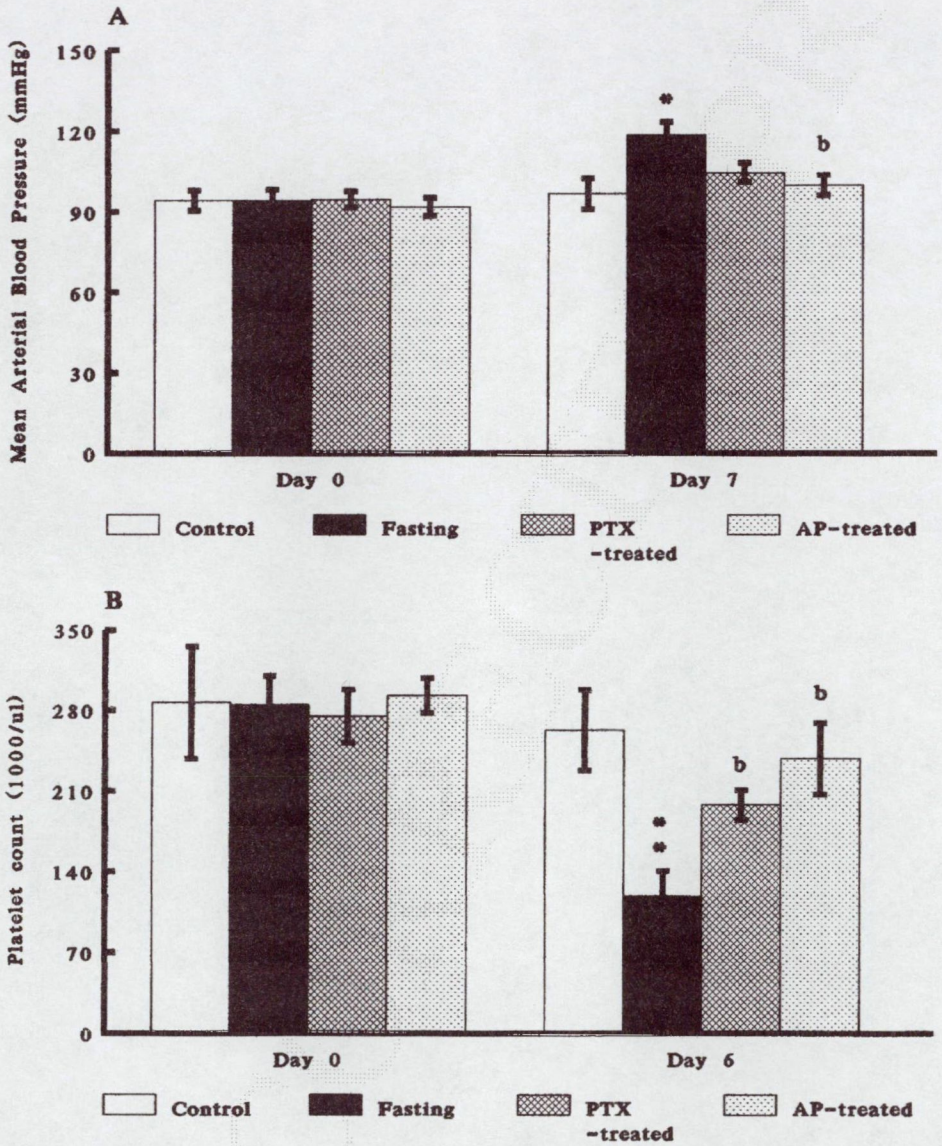

Fig. 1. Initial values and values at maximal change in mean arterial blood pressure, platelet count and GOT activity: in pregnant, non-fasting (control); fasting; and fasting, treated ewes. Groups: control group; fasting group: 96 h-long fasting period; PTX-treated group: animals fasting 96 h, medicated with PTX (15 mg/kg per die from day 0 to delivery); AP-treated group: allopurinol-treated group fasting 96 h (20 mg/kg per die AP from day 0 to delivery). Number of animals in each group: 5. (A) Initial values and values at maximal change in mean arterial blood pressure (7 days after the beginning of the 96 h fasting period in fasting and fasting, treated groups). (B) Initial values and values at maximal change in whole blood platelet count (6 days after the beginning of the 96 h fasting period). (C) Initial values and values at maximal change in plasma GOT activity (7 days after the beginning of the 96 h fasting period). Values are depicted as mean $\pm$ standard deviation. Significant alterations were calculated by multiple-choice ANOVA (Factors for the analysis were days (0–7), groups (1–4) and animals (1–5, within the groups)) followed by pairwise comparison using Tukey's test (*): $P < 0.05$, and (* *): $P < 0.01$). Significant differences ($P < 0.05$) between the treated, fasting groups and the fasting, non-treated group were indicated by b.

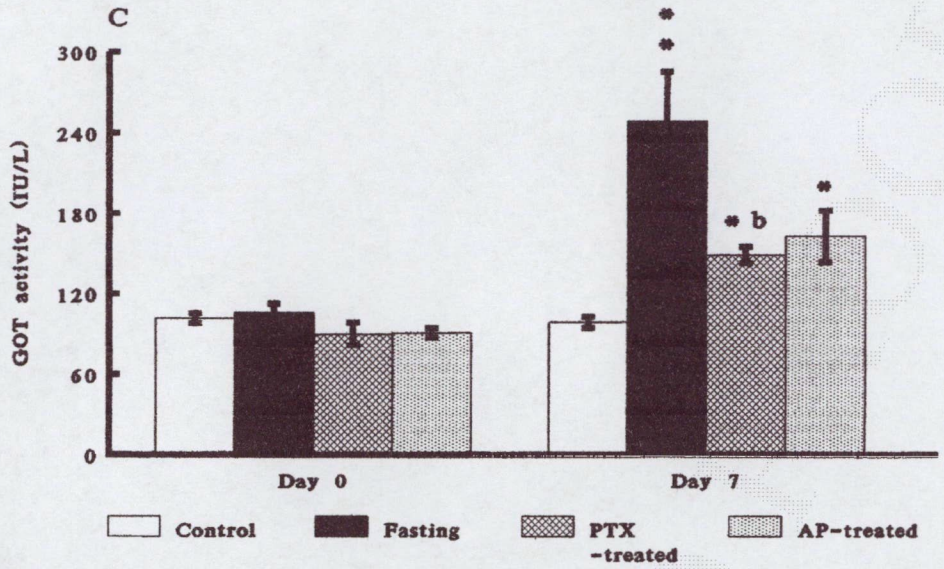

Fig. 1. (*Continued*).

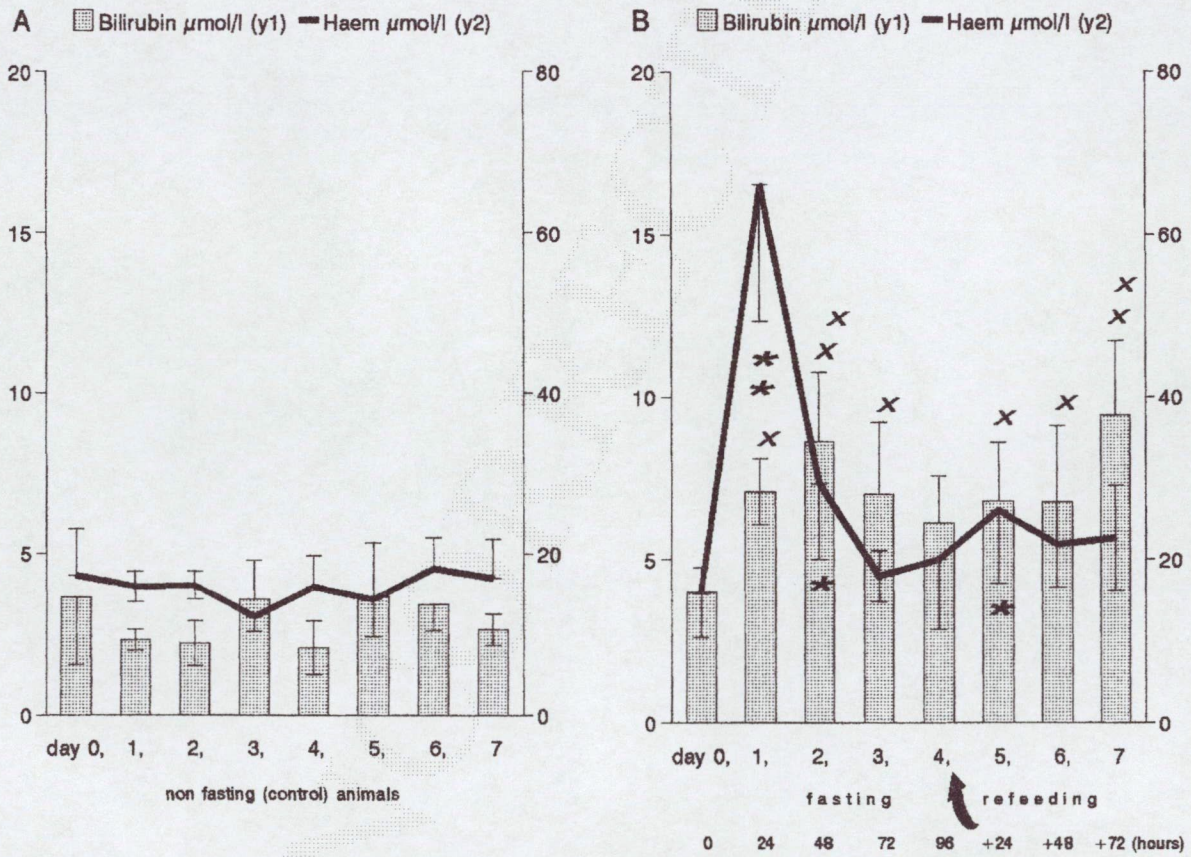

Fig. 2. Changes in plasma indirect bilirubin and total haem in pregnant, non-fasting (control) and fasting, non-treated ewes. Values are depicted as mean ± standard deviation. (A) Control group ($n = 5$). (B) Group fasting 96 h. Significant alterations ($P < 0.05$ and $P < 0.01$) were calculated by multiple-choice ANOVA (factors for the analysis were: days (0–7), groups (1–4) and animals (1–5, within the groups)) followed by pairwise comparison using Tukey's test, and indicated by × and ×× (indirect bilirubin) and * and * * (free haem). Standard deviation is indicated.

(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 haem level, as the first predictive sign of the illness, showed a sharp elevation simultaneously with a decrease of glucose levels in the fasting group and subsequently remained slightly elevated (Fig. 2A and B). The elevation of plasma haem 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) (Fig. 3A and B). The summarised plasma indirect bilirubin/(indirect bilirubin + plasma free haem) ratios as product/(product + substrate) ratios, which provided an indirect evidence of haem oxygenase activity (the enzyme which degrades haem), were more favourable in the drug-treated, fasting groups, compared to the fasting, non-treated group. In the PTX-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).

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 h 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 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

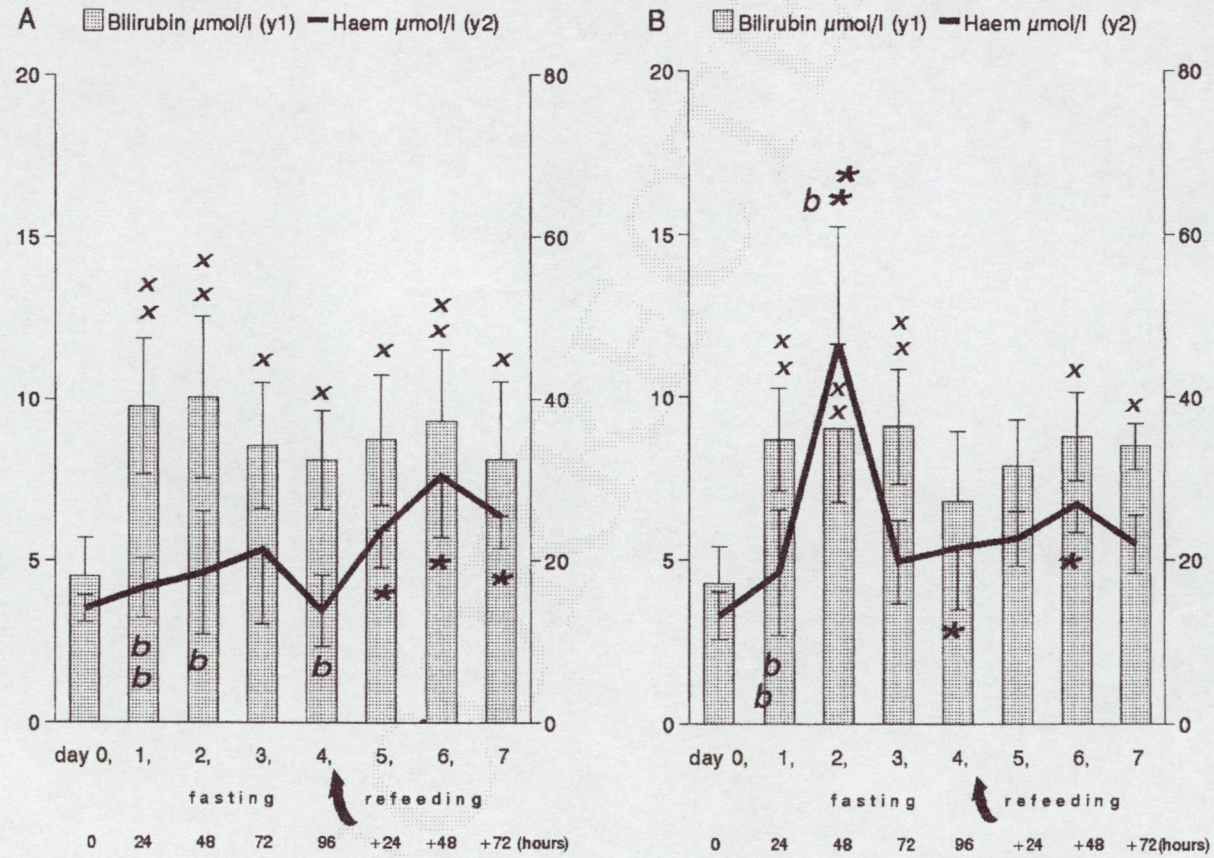

Fig. 3. Changes in plasma indirect bilirubin and total haem in pregnant, fasting, treated ewes. Values are depicted as mean ± standard deviation. (A) Animals fasting 96 h ($n = 5$), medicated with PTX (15 mg/kg per die from day 0 to delivery). 3B: allopurinol-treated group (20 mg/kg per die allopurinol from day 0 to delivery) fasting 96 h ($n = 5$). Significant alterations ($P < 0.05$ and $P < 0.01$) were calculated by multiple-choice ANOVA (factors for the analysis were: days (0–7), groups (1–4) and animals (1–5, within the groups)) followed by pairwise comparison using Tukey's test, and indicated by × and ×× (indirect bilirubin) and * * (free haem). Significant differences ($P < 0.05$) between the treated, fasting groups and the fasting, non-treated group (these values shown in Fig. 2B) were indicated by b.

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 h. The 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.

3.1. 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 lambs) died in convulsions. Lambs were born earlier than in the case of 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).

4. Discussion

In the work presented here, we have demonstrated that PTX and AP have beneficial effects on the development of experimental pregnancy-induced pre-eclampsia-like disease in ewes. While in the fasting, non-treated animals, all characteristics of the disease (hypertension, renal and liver function disturbances and low platelet count) were detected, pathological changes were smaller and occurred later in the treated animals. We also observed a significant decrease of blood calcium levels also in this model, similar to previous reports [12,13]; however the three fasting groups showed a similar decline, which was not accompanied with the parallel development of symptoms.

In this model, haemolysis and haem toxicity seemed to be (according to the time-dependent appearance of changes in plasma biochemistry) the triggering mechanism of endothelial dysfunction and a primary cause of vasoconstriction and placental hypoperfusion [8]. Sheep erythrocytes are very sensitive to blood sugar levels, since sheep glucose-6-phosphate dehydrogenase activity, and consequently the red blood cell antioxidant defence, are very low and the erythrocyte glutathione level is also lower than that of humans [14]. While fasting is tolerated well in the case of non-pregnant ewes, [7], a decline in the plasma glucose level occurred even after a short period of fasting in pregnant ewes because of the accelerated glucose metabolism. In the present experiment, simultaneous, significant haemolysis also occurred, indicated by an increase of plasma haemoglobin/haem content. Haem, as a lipophyl, physiologic iron chelate, facilitated endothelial iron uptake in cultured endothelial cells [15]. Since iron-loaded cells become extremely sensitive to either exogenous or endogenous oxidants, endothelial damage and malfunction may occur, increasing the risk of hypertension.

Plasma haemoglobin/haem 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 [5], 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 the local oxidative stress by specifically inhibiting the enzyme xanthine oxidase, the presence of which in the placenta has been proved [3,16].

Furthermore, the enhanced formation of bilirubin in the treated animals refers to the activation of haem oxygenase, a 32 kDa oxidative stress protein, which is the rate-limiting enzyme in the catabolism of haem. During the haem oxygenase enzyme reaction, the concentration of toxic haem is reduced and equimolar concentrations of the antioxidant bilirubin as well as carbon monoxide are produced, the latter being a potent relaxant of vascular smooth muscle. Induced haem oxygenase activity, as an additional beneficial effect of PTX and AP, indicates more effective protective processes of endothelial cells against haem and also non-haem (iron) toxicity [17,18].

Although, we found no reference to AP or PTX inducing haem 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 haem oxygenase gene expression was increased due to elevation of the intracellular cAMP level [19,20].

The decline of plasma free thiols — the first line of antioxidant activity — 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 decrease in the AP-treated group may be a result of the inhibition of xanthine oxidase activity.

In the animal model of ewes PTX and AP proved to be effective in the prevention of more severe pre-eclamptic-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 haem oxygenase may be important points in

maintaining normal endothelial function and vascular tone regulation during the pregnancy of sheep.

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XANTHINE OXIDASE ACTIVATION IN MILD GESTATIONAL HYPERTENSION

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Running title: **XO activation in gestational hypertension**

Abstract

Objective: We hypothesized that activation of the xanthine oxidase enzyme system is a potential source of free radicals in pregnancy-induced hypertension.

Methods: A prospective observational study was carried out on 16 pregnant women who met the criteria of gestational hypertension (rise in blood pressure of 30 mm Hg systolic or 15 mm Hg diastolic after 20 weeks or blood pressure >140/90 if earlier blood pressure unknown) without proteinuria or any signs of renal impairment. Fourteen women with a clinically normal pregnancy matched for maternal age, parity and gestational age acted as pregnant controls. Non-pregnant control women were members of the laboratory staff (n=15).

Main Outcome Measures: Concentrations of free sulfhydril groups, purine catabolites, lipid peroxidation products in plasma, and blood carboxyhemoglobin levels were used to follow oxidative stress and potential hemolysis. A non-invasive measurement of functional xanthine oxidase activity was carried out; i.e. the urinary ratio of the two metabolites of caffeine was estimated.

Results: A pronounced oxidative stress was demonstrated in plasma samples of patients with hypertension by the elevated concentrations of uric acid and lipid peroxidation products. A reduced level of free sulfhydryl groups and an increased concentration of hypoxanthine were shown in normotensive pregnant individuals. The xanthine oxidase activity index was substantially higher in overweight pregnant subjects with mild hypertension [0.849 ± 0.096; ($p<0.01$)] than in normotensive pregnant women or in age-matched non-pregnant subjects [0.596 ± 0.105; 0.542 ± 0.049 (means $\pm$ SD), *respectively*].

Conclusions: Our study of mildly hypertensive pregnant subject provides addional evidence of the putative role of xanthine oxidase activation as a source of free radicals in the early stage of endothelial dysfunction.

Keywords: 1-methyluric acid, 1-methylxanthine, xanthine oxidase.

INTRODUCTION

Hypertension complicates 6-20% of all pregnancies and ranks among the four most common causes of maternal/perinatal morbidity. Although pre-eclampsia, a severe multisystem disorder characterized by increased blood pressure, proteinuria and oedema, is more than simply hypertension (1), epidemiologic and pathological data support the hypothesis that the pre-eclampsia syndrome stems from different causes of either placental or maternal origin (2). The primary events leading to pre-eclampsia are still unclear; a failure of trophoblast invasion into the myometrium, with a reduced placental perfusion and consequent mitochondrial dysfunction, are triggers for further events (1, 2). We hypothesize that gestational hypertension is due to pre-existing maternal disorders (sometimes not evident outside pregnancy), such as overweight, insulin resistance, hypertension or renal disease (2) and that the different causes converge to cause the pre-eclampsia syndrome by the common mechanisms of endothelial cell activation and injury (3).

Pregnancy-induced hypertension has been explained as an endothelial dysfunction with an impaired effect of nitric oxide (NO), the main regulator of the fetoplacental blood flow, which causes a maternal systemic vasodilation during normal pregnancy (4). Free oxygen radicals, i.e. superoxide (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radicals (·HO), mediate endothelial injury through oxidative stress and the activation of neutrophils (5).

The enzyme xanthine oxidoreductase (XO/XD) is a key source of oxidants in many pathological processes (Fig. 1). 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; EC1.1.1.204) in non-ischaemic tissues. However, if the tissues are exposed to metabolic stress, such as hypoxia or ischaemia, the enzyme is transformed to xanthine oxidase (XO; EC1.1.3.22), which can generate the oxidants O_2^-, H_2O_2 and HO·

during reperfusion (6). The enzyme system is present in the endothelial and vascular smooth muscle cells (7,8), and also in the human placenta, localized to trophoblast cells (9). An increase in XO activity has been proposed as a possible cause of oxidative stress in pre-eclampsia (10), and such an increase has in fact been measured in the placentae of women with pre-eclampsia (11).

Besides causing 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 pre-eclampia (12,13). The conversion of XD to XO is also induced by $ONOO^-$ via sulfhydryl group (SH) oxidation (14).

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 (15). The caffeine metabolic ratio has proved to be a specific indicator of *in vivo* XO activity (15-19).

The aim of the present study was to answer the question: Is there any measurable elevation in the *in vivo* XO activity index during normal pregnancy or in cases complicated by mild hypertension?

MATERIALS AND METHODS

Subjects

Pregnant women with gestational hypertension but without superimposed pre-eclampsia were detected at routine obstetric visits after 24 weeks of gestation (Table 1). Sixteen subjects met the criteria 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 (1). Neither proteinuria nor any impairment in renal function was observed (defined as >500 mg per 24-h urine collection, and creatinine clearance values >120 ml/min per 1.73 m^2). Most patients (n=12) had concomitant hyperuricaemia, defined as >330 μM 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, an 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 (Table 1). The control women were age-matched members of the laboratory staff.

This study was approved by the Ethical Committee of the University.

Blood and urine samples

Blood was drawn into ice-prechilled tubes and was separated within half an hour; the sera were stored frozen at -70 °C until assayed (within 6 days). Patients were asked to refrain from methylxanthine containing food and beverages for 48 hours before the 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 1MX and 1MU were determined by the high-pressure liquid chromatographic (HPLC) method described by Grant et al. (15) with some modifications by Kilbane et al. (20). 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%.

XO activity index was used to characterize the functional XO activity, it was calculated as the molar ratios of 1MU/(1MX+1MU)(18).

Other biochemical methods

The carbon monoxide-Hb (COHb), and total Hb levels were estimated by Hemoximeter (Radiometer Copenhagen) within 15 min after venapuncture. Concentrations of COHb were given as a measure of increased RBC turnover (21).

Plasma concentrations of HX, X and UA were determined by a reverse-phase HPLC method and UV detector (Pharmacia LKB)(22). The plasma contents of the lipid peroxidation products, fluorescent lipids and conjugated dienes, were also estimated (23). The level of free SH groups in the plasma was measured with Ellman reagent at 412 nm (24).

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 α=0.05.

RESULTS

The concentration of free SH groups showed a decrease even in the normotensive pregnant women. Elevated contents of fluorescent lipids and conjugated dienes were shown in hypertensive patients compared to both the normotensive ones and the non-pregnant controls (Table 2).

There were no differences in the whole blood Hb or COHb concentrations as potential markers of increased RBC catabolism (Table 2).

The sum of the plasma concentrations of physiological purine metabolites (HX+X+UA) was increased in the pregnant women *vs* the non-pregnant controls [374 ± 27 *vs* 251 ± 18 (μM; means ± SD); $p<.05$]. Increased levels of UA, the end-product of XO, were shown 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 (Fig. 2).

A significant decrease in the urinary concentration of 1MX, the caffeine metabolite serving as a substrate for XO, was shown in the hypertensive subjects without any decrease in the urinary concentration of 1MU, the product of XO (Fig. 3). As a result the XO activity index, the molar ratio 1MU/(1MX+1MU), rose significantly in this group (Fig. 4).

DISCUSSION

Oxidative stress has been suggested as a link between the two-stage model of the pre-eclampsia syndrome: maternal factors cause reduced placental perfusion (stage 1), and stage 2 involves activation of the maternal endothelium with multisystem disorders (25). The clinical status of our overwight subjects met the criteria of pre-eclampsia syndrome stage 1 with the risk of development of pre-eclampsia.

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 (26).

A significant enhancement of the levels of lipid peroxidation products was also measured in the pregnancy-induced hypertensive *vs* the normotensive subjects, as in other studies where this has also been shown in the placenta (27). Placental mitochondria enriched with polyunsaturated fatty acids may be the source of the abnormally increased lipid peroxidation in the maternal circulation, since increases in both the amount and the oxidative potential of the mitochondria have been demonstrated in the pre-eclamptic *vs* the normal placenta (28).

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 purine catabolism (29). This activation of purine catabolism has been shown to be a component of the homeostatic protective response of the mitochondria to oxidant stress (29).

In pregnant subjects the plasma concentration of HX, a substrate for XO was increased, and in hypertensive patients the levels of UA, the product of XO exhibited a significant increase compared to normotensive pregnant or non-pregnant subjects. 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 a concommitant 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 (15-19, 30). 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 (16,19). 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 (18). Studies on 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 diffent (31).

In a population study on 178 young adults, the XO activity index proved to have a rather constant value of 0.57 ± 0.13 (means ± SD)(32). Our results in non-pregnant women (0.542 ± 0.049) and in normotensive pregnant subjects (0.596±0.105) are in good agreement with these values. However, the index was highly increased in pregnant women with mild hypertension (0.849 ± 0.096). While 1MU could be produced only from 1MX, a shift in favour of 1MU in our present study shold have been a result of XO activation. Thus, the increased purine catabolism of 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 (11,12). As XO seems to be a major O_2^- producing enzyme in the vascular system, several experiments have been 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 a dose-dependent manner (33). 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 (33,34).

The role of xanthine oxidase in human plasma H_2O_2 production has also been reported (35), and there is a close correlation between H_2O_2 concentrations and the mean arterial pressure in both normotensive and hypertensive subjects (36). Our study of mild hypertensive pregnant subjects provides some addional evidence for the putative role of XO as a source of free radicals.

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Table 1

Demographic characteristics of the study population (means ± SD)

	Non-pregnant controls n = 15	Normotensive pregnant n = 14	Hypertensive pregnant 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

* $p < 0.05$ vs normotensive pregnant patients.

Table 2

Concentrations of some biochemical parameters in the plasma and in the whole blood of the study population (means ± SD)

	Non-pregnant controls n = 15	Normotensive pregnant n = 14	Hypertensive pregnant 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

* $p < .05$ *vs* non-pregnant controls; # $p < .05$ *vs* normotensive pregnants.

LEGENDS TO FIGURES

Fig. 1

Xanthine oxidoreductase enzyme system in hypoxic tissues.

ATP: adenosine triphosphate; O_2: oxygen; O_2^-: superoxide anion; H_2O_2: hydrogenperoxide; $O^\bullet$: hydroxil radical.

Fig. 2

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

** $p < .01$; *** $p < .001$ *vs* non-pregnant controls;

$p < .01$ *vs* normotensive pregnant subjects.

Fig. 3.

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

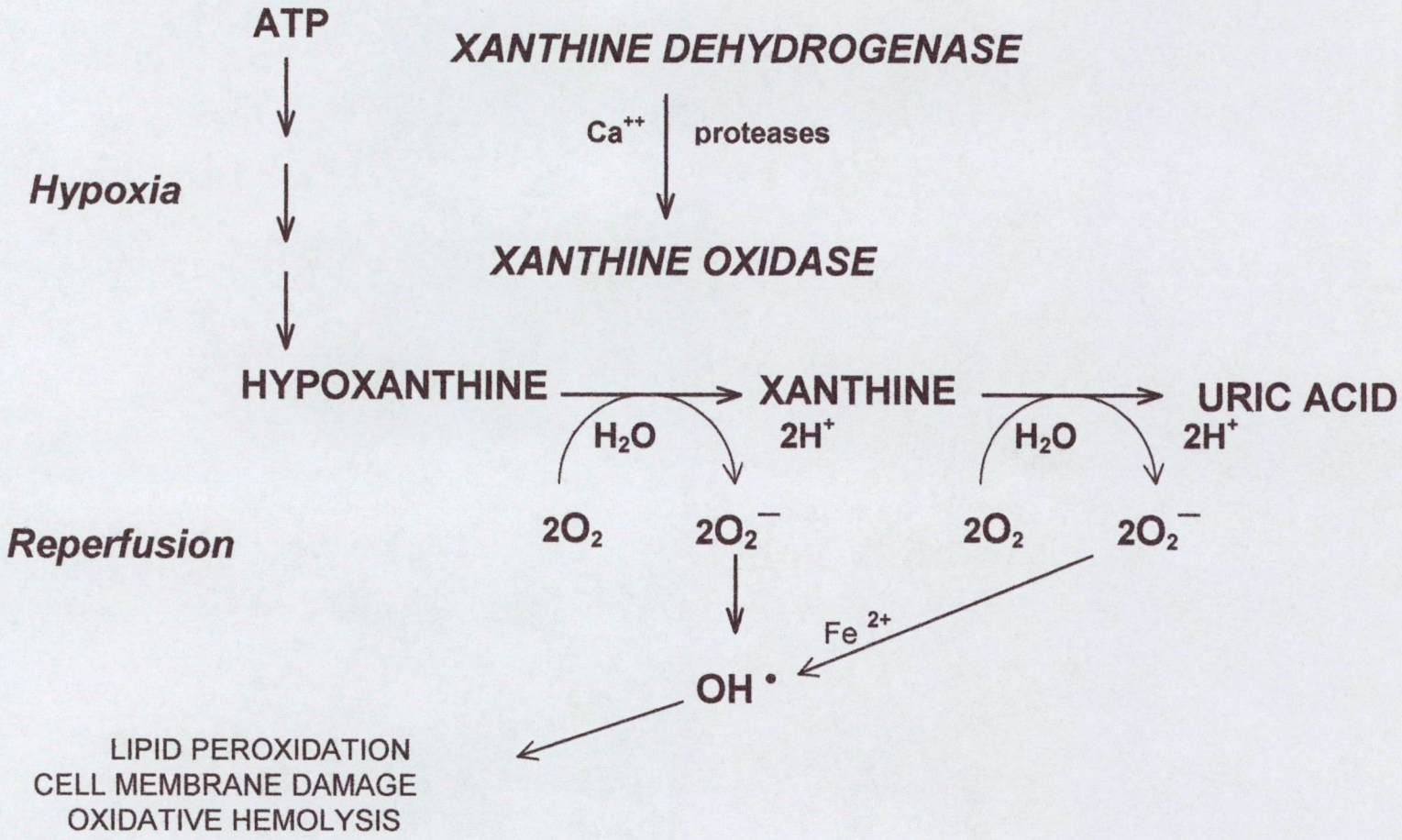
*** $p < .001$ *vs* non-pregnant controls;

$p < .001$ *vs* normotensive pregnant subjects.

Fig. 4.

Xanthine oxidase activity index (1MU/(1MX+1MU) in controls and pregnant subjects without/with hypertension

*** $p < .001$ *vs* non pregnant controls; ### $p < .001$ *vs* normotensive pregnant subjects.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CELL MEMBRANE DAMAGE
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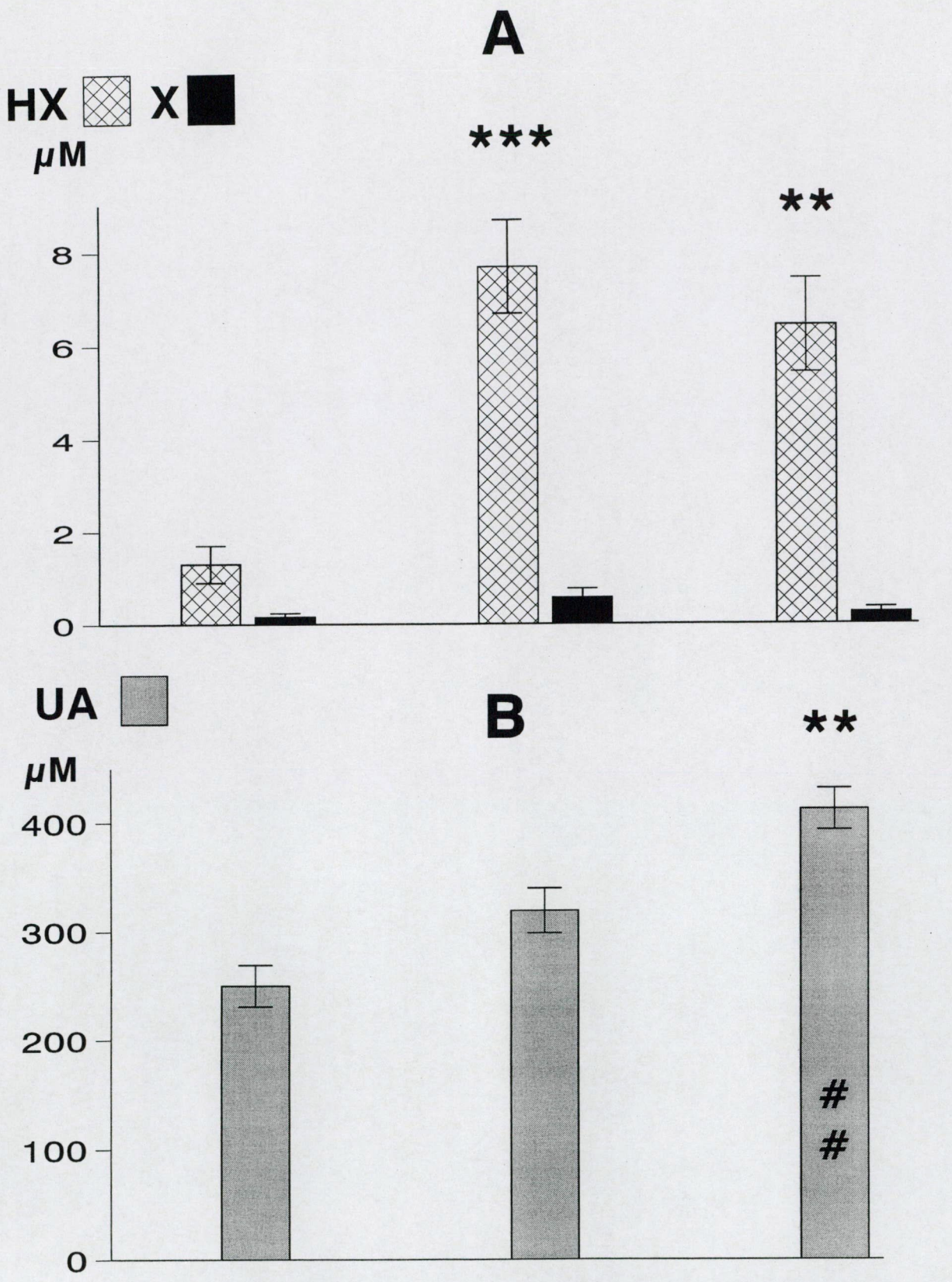
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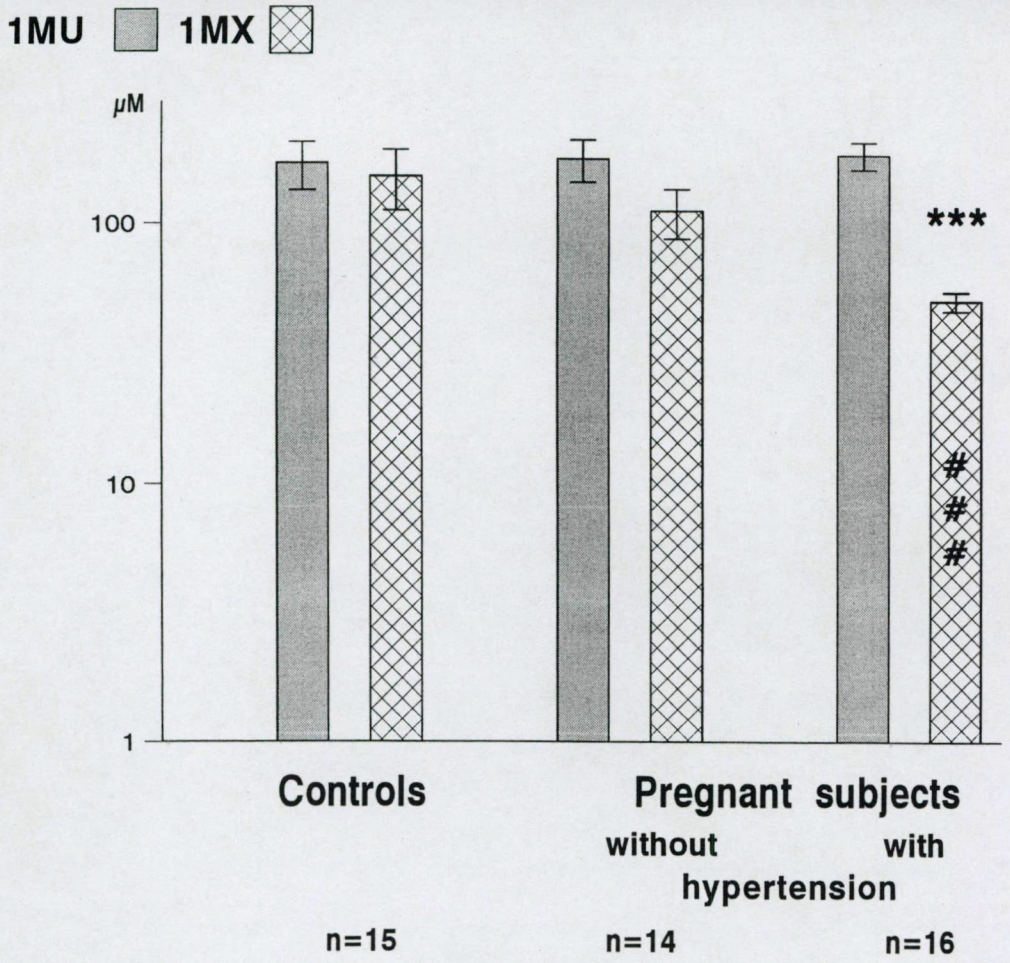
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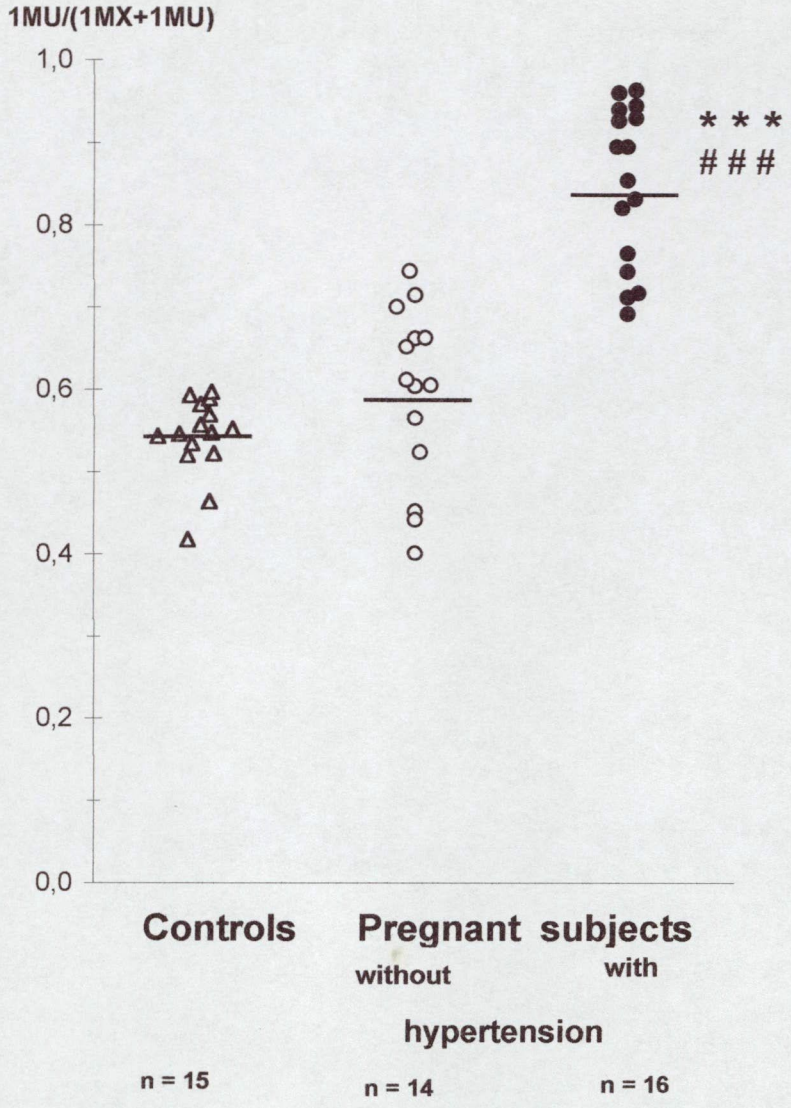
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Controls
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