OXIDATIVE STRESS AND DETOXIFICATION IN REPRODUCTION WITH EMPHASIS ON GLUTATHIONE AND PREECLAMPSIA

Maarten T.M. Raijmakers

OXIDATIVE STRESS AND DETOXIFICATION IN REPRODUCTION WITH EMPHASIS ON GLUTATHIONE AND PREECLAMPSIA

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Promotores:	Prof. Dr. J.B.M.J. Jansen
	Prof. Dr. J.M.W.M. Merkus
Co-promotores:	Dr. W.H.M. Peters
	Dr. E.A.P. Steegers
Manuscriptcommissie:	Prof. Dr J.J.H.H.M. De Pont
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	Mw. H.M.J. Roelofs

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Lightning crashes, a new mother cries

her placenta falls to the floor

the angel opens her eyes

the confusion sets in before the doctor can even close the door

(Taken from "Lightning Crashes", by Live)

ALAT	alanine aminotransferase
ASAT	aspartate aminotransferase
BP	blood pressure
BPDS	bathophenanthrolinedisulfonic acid
CAD	coronary artery disease
CI	confidence interval
CDNB	1-chloro-2,4-dinitrobenzene
CVD	cardiovascular disease
CYP	cytochrome P450 monooxygenase
CYS	cysteine
CysGly	cysteinylglycine
DBP	diastolic blood pressure
EDTA	ethylenediaminetetra-acetic acid
ELISA	enzyme linked immunosorbent assay
EPHX	epoxide hydrolase
FRAP	ferric reducing ability of plasma
fThiol	free thiol
GA	gestational age
GPX	glutathione peroxidase
GSH	glutathione
GSSG	glutathione disulphide
GST	glutathione S-transferase
GSTA	glutathione S-transferase Alpha
GSTM	glutathione S-transferase Mu
GSTP	glutathione S-transferase Pi
GSTT	glutathione S-transferase Theta
G6PDH	glucose-6-phosphate dehydrogenase
Hcy	homocysteine
HELLP	haemolysis, elevated liver enzymes and low platelets
Hp	haptoglobin
H_2O_2	hydrogen peroxide
ISSHP	international society for the study of hypertension in pregnancy
IU	international unit
IVS	intervillous space

kD	kiloDalton
LDH	lactic dehydrogenase
LDL	low density lipoprotein
MDA	malondialdehyde
mmHg	millimeter mercury (1 mmHg = 133.322 Pa)
n	number of subjects / observations
NEM	N-ethylmaleimide
NO	nitric oxide
OR	odds ratio
oThiol	oxidised thiol
O_2^-	superoxide radical
PACs	polycyclic aromatic hydrocarbons
PBS	phosphate buffered saline
PBST	phosphate buffered saline supplemented with 0.05% (v/v) Tween20
PCA	perchloric acid
PCR	polymerase chain reaction
PGH	prostaglandin H
PO_2	partial oxygen pressure
PGI_2	prostacycline
RFLP	restriction fragment length polymorphism
ROS	reactive oxygen species
r_s	Spearman's rank correlation coefficient
rThiol	free-to-oxidised ratio of thiol
SeGPX	selenium dependent glutathione peroxidase
SBDF	7-fluorobenzofurazane-4-sulfonic acid
SOD	superoxide dismutase
TBARS	thiobarbituric acid reactive substances
TGPX	total glutathione peroxidase
TXA_2	tromboxane
UGT	UDP-glucuronosyltransferase
v/v	volume per volume
w/v	weigth per volume

Part I

General Introduction.

Chapter 1

Objectives and outline of the thesis.

Despite many years of intensive research, preeclampsia is still a common complication of pregnancy with high maternal as well as foetal morbidity and mortality. The haemolysis, elevated liver enzymes and low platelets (HELLP) syndrome often complicates preeclampsia, although HELLP may also occur solely. Recently, the hypothesis of maladaptation of throphoblast invasion in early pregnancy resulting in poor placental function has been postulated as a key factor in the aetiology of preeclampsia. Placental oxidative stress may lead to oxidative stress in maternal circulation. Part 1 of this thesis provides a general overview of the concept of oxidative stress, biotransformation and the implication of oxidative pressure and antioxidant defence in normal pregnancy and preeclampsia.

In the last decade many studies on the pathophysiology of severe preeclampsia and HELLP syndrome have been performed in a co-operation between the departments of Gastroenterology and Obstetrics & Gynaecology. This has resulted in the insight that oxidative stress and its detoxification by glutathione and related enzymes play an important role in the pathogenesis of these disorders. In the second part of this thesis the role of these enzymes in physiological pregnancy and during embryonic development is described. The main objective, however, is to study the specific role of aminothiols in preeclampsia.

Besides their role in protein synthesis, aminothiols such as cysteine, homocysteine and glutathione are important elements in the defence against oxidative and chemical stress. When oxidative stress occurs, aminothiols are oxidised to scavenge all kinds of free radicals or reactive oxygen species in order to maintain the intracellular redox-balance, which is important for normal functioning of numerous cellular processes. The third part of this thesis describes the role of aminothiols in oxidative stress during preeclampsia.

Maternal oxidative stress is an important feature of preeclampsia. Several proteins are able to "capture" molecules that induce oxidative stress or produce reactive oxygen species. Therefore, we were also interested in the role of polymorphisms in the genes encoding for such proteins as outlined in the fourth part of this thesis.

The main objectives of this study were:

1) To investigate the importance of the glutathione / glutathione biotransformation system in male fertility and physiological pregnancy.

2) To investigate the role of the aminothiol redox-balance in women with severe preeclampsia.

3) To investigate the association between polymorphisms in oxidative stress related genes and the pathogenesis of severe preeclampsia.

Chapter 2

The concept of detoxification and oxidative stress.

2.1. Biotransformation

During life, numerous harmful compounds, including carcinogens and reactive oxygen species (ROS) are introduced to organisms at several different ways, including nutritional intake, inhalation of polluted air or generation by UV-radiation (1). In addition, organisms itself may produce toxic compounds, such as free radicals and ROS, by metabolic processes that may lead to oxidative stress. Since many of these compounds are associated with disease in humans, both non-enzymatic as well as enzymatic defence mechanisms are present (2). The major defence against xenobiotic compounds and their breakdown products is provided by the two-stage mechanism of phase I and phase II biotransformation reactions (Figure 2.1). Phase I reactions involve oxidation, reduction, hydrolysis or dealkylation of many compounds and is mainly catalysed by a variety of cytochromes P450 monooxygenases (CYP) and two epoxide hydrolases (EPHX) (3). The purpose of this initial reaction is to create or liberate free hydroxyl- or amino-moieties, which can readily be conjugated resulting in hydrophilic molecules by phase II reactions. These phase II reactions are largely catalysed by the glutathione S-transferase (GST) and UDP-glucuronosyltransferase (UGT) families, which conjugate glutathione (GSH) and UDP-glucuronic acid, respectively. Many other Phase II enzymes including sulfotransferases and acetyltransferases may play an important role. After phase II reaction, the end products are less biologically active and more water-soluble, thereby facilitating their excretion via urine or bile.

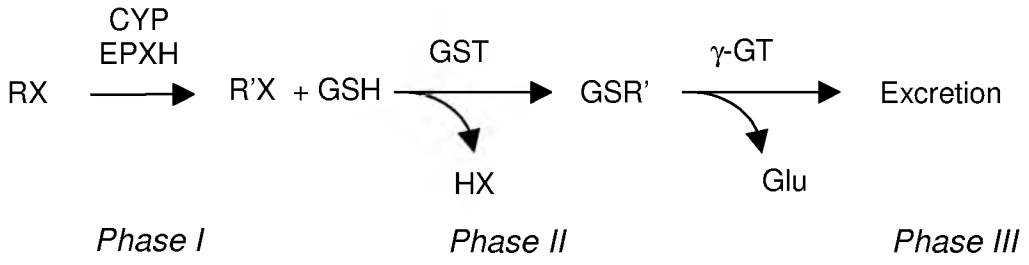

Figure 2.1 Schematic overview of phase I and phase II biotransformation:
In phase I toxic compounds (RX) are activated by e.g. cytochromes (CYP). In phase II GSH is conjugated to the reactive intermediate by GST after which the conjugates can be secreted via bile or urine (phase III).

2.1.1 Glutathione S-transferase

One of the most important protective systems, at least in a quantitative sense, is the glutathione dependent GST enzyme system (1;4). In eukaryotes the family of GSTs compromises two different multigene superfamilies. One family consists of membrane-bound transferases, whereas the other family is soluble, also referred to as cytosolic GSTs. So far, seven classes of the cytosolic GST family have been characterised, which were designated Alpha, Pi, Mu, Theta, Sigma, Zeta and Kappa (4). In humans the first four classes of GSTs are primarily expressed (1). The genetic origins of these four classes are located on chromosomes 6, 11, 1 and 22, respectively. Generally, GSTs that share over 40% homology belong to the same class, whereas those with less than 30% homology are assigned to different classes. For both GSTMu and GSTTheta a null-genotype, corresponding with no detectable expression of GST enzyme activity, can be found in approximately 54% and 18% of the North-West European population, respectively (5). However, the presence of both polymorphisms differs markedly between human races (1).

Each class of GSTs is composed of homo- or heterodimeric enzymes with subunits of approximately 25 kDaltons (kD) molecular mass (ranging from 23 to 28 kD). This different subunit composition results in substrate specificity for each GST isoform, although substrate overlap exists. Additionally, expression of GST isoforms is tissue specific and a diverse expression pattern of GST isoforms is seen, which results in specific protection for each tissue. Generally, GSTAlpha is the main isoform in liver, kidney and adrenal gland (6-8), GSTPi is predominantly expressed in placenta, oesophagus, stomach, lung and heart (7;9;10), whereas in bladder GSTMu is the main isoform (11). During embryonic and foetal development GST isoforms are also expressed. However, amounts of specific isoenzymes may differ between foetal and adult tissues (12;13), indicating that some foetal tissues exert different functions in comparison with those in adults. For instance, foetal liver primarily expresses GSTPi, whereas in adult liver GSTAlpha is the predominant form in even higher amounts than that of GSTPi in foetal liver. Such findings demonstrate the hepatic change of erythrocyte synthesis during antenatal development to its main task as detoxification organ in adult live. Furthermore, expression of GSTPi in foetal lung decreases with gestational age (14). Since, induction of GST expression may be an adaptive response to chemical or oxidative stress (1), presence of GST isoforms may be a reflection of a specific toxic environment.

Besides catalysing the conjugation of GSH to reactive intermediates in phase II reactions, which is the most important function, the family of GSTs has several other functions. By its peroxidase activity GSTs play an important role in the reduction of reactive oxygen species (ROS), as outlined in Figure 2.2. Furthermore, GSTs are capable in the non-catalytic binding and transport of several exogenous and endogenous compounds including bilirubin, genotoxic electrophiles etc. However, this function is relatively unknown as compared to the transferase and peroxidase activities (1).

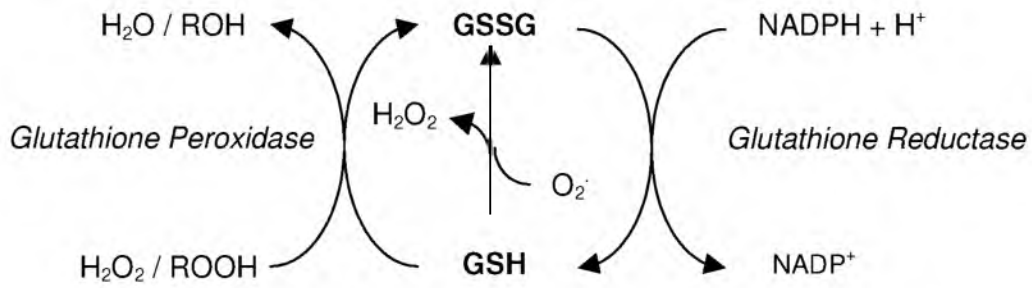

Figure 2.2 Reduction of reactive oxygen species by glutathione and related enzymes: Hydrogen peroxide (H_2O_2) or organic hydroperoxides (ROOH) can be reduced enzymatically by glutathione peroxidase as well as non-enzymatically by direct oxidation of glutathione (GSH), resulting in H_2O or corresponding alcohols (ROH). The inactive, oxidised form of glutathione (GSSG) is reduced by glutathione reductase thereby consuming nicotinamide-adenine-dinucleotide phosphate (NADPH). Superoxide anion may be converted to H_2O_2 by oxidation of GSH.

2.1.2. Glutathione

Glutathione is a water-soluble antioxidant, which has as main functions to serve as co-factor in phase II conjugation reaction by GSTs and to protect cells from oxidative damage induced by ROS. In the latter reaction GSH itself may directly scavenge ROS or indirectly by action of glutathione peroxidase (GPX). Besides these two functions glutathione has a myriad of other functions, of which maintenance of –SH groups in the reduced state, storage and transport of amino acids in particular cysteine, and formation of leukotriene C_4 and derivatives are the most important ones (15;16).

Synthesis of glutathione takes place in two consecutive steps that are catalysed by γ-glutamylcysteine and glutathione synthetase, respectively (Figure 2.3) (15;17). In the first and rate-limiting step cysteine is covalently bound to glutamate and subsequently glutathione is formed by the subsequent addition of glycine. In the first reaction a very stable γ-glutamyl bond, instead of the normal peptide bond, is formed. This γ-glutamyl bond cannot be cleaved

by the abundantly present peptidases, but only by γ-glutamyl transpeptidase. Regulation of glutathione synthesis takes place at the first reaction in two different ways: a) by feedback inhibition of glutathione and b) the availability of free cysteine.

Glutathione is widely distributed in human tissues and is present in high intracellular amounts. Therefore, GSH is the most prevalent cellular thiol and accounts for over 90% of non-protein sulphur (17). In blood, GSH levels in erythrocytes are high (≈1000 µmol/L) (18), whereas in human plasma they are relatively low (<20µmol/L) (19).

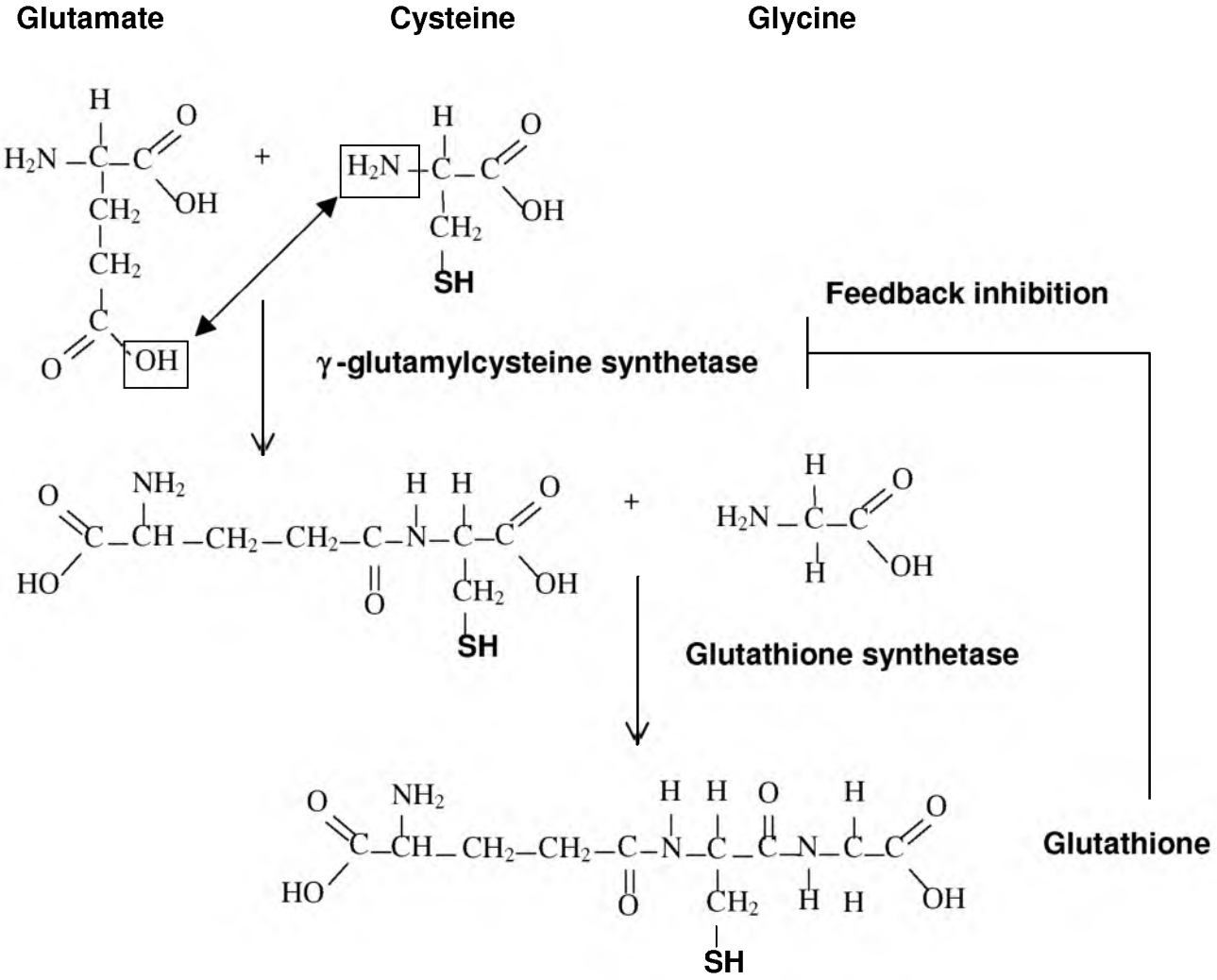

Figure 2.3 Synthesis of glutathione:

The initial rate-limiting step is catalysed by γ-glutamylcysteine synthethase, which couples cysteine and glutamate, subsequently followed by the addition of glycine catalysed by glutathione synthetase, resulting in glutathione. The first reaction is feedback inhibited by glutathione.

GSH can be excreted from tissues as glutathione S-conjugate or as glutathione disulphide (GSSG) (20) by multidrug resistance proteins (21) or by other ATP-dependent transporters (22;23). Excessive amounts of GSSG may be produced during periods of oxidative stress and

in the absence of sufficient reducing equivalents some of the GSSG may be excreted to preserve cellular thiol balance. Therefore, the rate of GSSG transport may represent intracellular oxidative status (23).

2.2 Oxidative stress

Oxidative stress is defined as disturbance in the balance between antioxidants and (pro)oxidants in favour of the latter. Oxidative stress occurs when free radical generation exceeds the capacity of antioxidant defences due to an inadequate dietary intake of antioxidants or by an increase of cellular oxidants, which can be defined as substances with one or more unpaired electrons (24). The amount of oxidants may increase due to exposure to free radical generating toxins such as those from cigarette smoke (2) or radiation (25). A small part (1 – 3%) of the inhaled oxygen is converted into ROS, including the superoxide radical (O_2^-) and hydrogen peroxide (H_2O_2). Some of it is deliberately produced to inactivate viruses or to kill bacteria or fungi (2). However, most intracellularly generated ROS are unavoidable by-products of oxidative metabolic pathways (25). Unless properly scavenged, ROS may lead to lipid peroxidation, which represents an important manifestation of oxidative stress (24). Lipid peroxidation is initiated when a free radical interacts with polyunsaturated fatty acids of cell membranes and may finally result in a chain-reaction forming lipid hydroperoxides (26). Therefore, ROS may induce damage to DNA, proteins, carbohydrates or even disturb cell membrane integrity (2;27;28), which might result in impairment of enzymatic properties as well as cell membrane functioning.

On the other hand, oxidative stress induces the expression of redox-sensitive transcription factors like activating protein-1, hypoxia inducible factor-1α and nuclear factor-κB (29;30), which results in the expression of several genes including those of the GST family (31). Thus oxidative stress indirectly initiates antioxidant defence. Biological antioxidants can be defined as compounds that protect biological systems against the harmful effects of processes or reactions that can cause excessive oxidation (32). This antioxidant defence comprises enzymatic as well as non-enzymatic systems.

2.2.1 Glutathione peroxidase and other enzymatic antioxidants

First line defence against ROS is provided by the glutathione peroxidases (GPXs), which catalyse the reduction of organic hydroperoxides or hydrogen peroxide into their corresponding alcohols or H_2O by the oxidation of glutathione (Figure 2.2). Like the family of GSTs, GPXs consist of a multigene family of six members (4). Most members require selenium for catalysis (SeGPX) and are characterised by a covalently bound selenocysteine in its active centre. At least four members are described: a. the classical cellular/cytosolic GPX (GPX1), b. gastrointestinal GPX (GPX2), c. extracellular/plasma GPX (GPX3) and d. phospholipid hydroperoxide GPX (GPX4). SeGPXs are capable to reduce organic hydroperoxides as well as H_2O_2. The second type of GPXs are selenium independent and probably mainly consist of GSTs (4). This GPX is only reactive towards organic hydroperoxides. GPXs are ubiquitously expressed including placental and decidual tissue (33;34), but most abundantly in erythrocytes, kidney and liver (4).

Two other enzymes play a major role in defence against the superoxide anion. Superoxide dismutase (SOD) converts superoxide anion to H_2O_2 and oxygen. Since H_2O_2 is highly reactive and can easily cross the cell membrane, SOD works in parallel with the H_2O_2-removing enzymes GPX and catalase, both quickly reducing H_2O_2 to H_2O and oxygen (2). Both enzymes are expressed at different locations, GPX is mainly present in cytosol, whereas catalase is localised in the matrix of peroxisomes. Therefore, both enzymes have their own specific function and complement each other (24).

2.2.2. Non-enzymatic antioxidants

A relatively large number of compounds are capable to prevent lipid peroxidation or metal-catalysed radical reactions and thus possess antioxidant capacity (32). In general, non-enzymatic antioxidants can be grouped in lipid-soluble and water-soluble antioxidants.

The major lipid-soluble antioxidants are the family of tocopherols including α-tocopherol, or vitamin E. All tocopherols are effective inhibitors of the propagation step of lipid peroxidation by reacting with one ore two peroxyl radicals. Carotenoids, precursors of e.g. vitamin A, show a similar antioxidant capacity as tocopherols, thus quenching at least two peroxyl radicals, by their conjugated double-bond systems. The main function of ubiquinone, one of the quinones, is to reduce the α-tocopherol radical. However, it may also directly act on either peroxyl or alkoxyl radicals. Although bilirubin is a product of heme-metabolism, it

is also an important lipid-soluble antioxidant, which has similar properties as α-tocopherol (32).

Major water-soluble antioxidants include glutathione, ascorbic acid (vitamin C), uric acid, metal-binding and heme-binding proteins. Ascorbic acid acts synergistically with α-tocopherols, since it is capable to quickly regenerate α-tocopherol by reduction of the α-tocopherol radical (2;32). Though uric acid is a waste product like bilirubin, at physiological concentrations it shows a strong antioxidant capacity towards water-soluble oxidants. Furthermore, metal-binding (transferrin) as well as heme-binding proteins (haptoglobin) prevent metal-initiated oxidation by binding to transition metals like iron (32).

2.2.3. Oxidative stress during pregnancy

Increased lipid peroxidation is a normal phenomenon of pregnancy (35). Several studies have reported higher blood levels with an increase throughout pregnancy of lipid peroxidation markers, such as thiobarbituric acid reactive substances (TBARS), lipid hydroperoxides and conjugated dienes in pregnant women as compared with those in non-pregnant controls (26;36;37). However, not all studies report increasing TBARS levels during gestation (38). Elevated levels of oxidative damage products normalise within days after delivery (26).

In order to maintain a physiological metabolism, the progressive increase of oxidants has to be counterbalanced by a parallel increase of total antioxidant capacity (39). Two major enzymatic antioxidant enzymes, SOD and GPX, were shown to be progressively increased during pregnancy (37;38). Also levels of the non-enzymatic antioxidant, α-tocopherol, which is tightly bound to β-lipoproteins, increase during pregnancy (37;40). In contrast with other antioxidants, the level of vitamin A is slightly lower during pregnancy (40). Both lipid peroxidation products and antioxidants systems normalise after delivery (26;37;40).

Thus during uncomplicated pregnancy the transiently increased lipid peroxidation is followed by a parallel increase of antioxidant capacity. However, when not properly counteracted this could lead to several complications of pregnancy, including hypertensive disorders of pregnancy such as preeclampsia (17;18;24;35).

2.3. Preeclampsia

Preeclampsia complicates approximately 5% of all pregnancies (41) with poor maternal as well as foetal outcome (42). It is accompanied by high maternal and foetal morbidity and mortality (41;43). According to the definition of the International Society of the Study of Hypertension in Pregnancy preeclampsia is classically defined as pregnancy-induced hypertension with proteinuria (diastolic blood pressure (DBP) >90mmHg (Korotkoff V), measured on two or more consecutive recordings more than 4 hours apart and an urinary protein excretion of >300 mg/24 hours) (43). Severe preeclampsia is defined as a DBP above 110mmHg or preeclampsia accompanied with complications such as HELLP (haemolysis, elevated liver enzymes, low platelets) syndrome. HELLP is biochemically defined by lactic dehydrogenase > 600 IU/L, both aspartate and alanine aminotransferase > 70 IU/L, and a platelet count < $100x10^9$/L (44). Preeclampsia is a multi-system disorder in which many organs may be affected including the kidney and the liver.

Although the definite aetiology of preeclampsia is not yet elucidated, several underlying pathogenetic mechanisms are clear. A central pathological feature of preeclampsia is dysfunction of the endothelium layer lining the vascular walls, which normally maintains vascular integrity (42). This results in several pathophysiological features including an activated clotting cascade, and high levels of endothelin, von Willebrand factor and fibronectin, (41). Furthermore, related to endothelium dysfunction increased oxidative stress is present, which also shifts the balance between prostacycline (PGI_2) and tromboxane (TXA_2) in favour of increased vasoconstriction and trombocyte aggregation (43;45).

Preeclampsia has a familial occurrence (41;43). Offspring of mothers with preeclampsia, sisters of women who had preeclampsia or being pregnant from a partner who fathered a preeclamptic pregnancy (42) are all risk factors to develop preeclampsia. Therefore it is likely that a genetic predisposition is involved, which could be from both maternal and paternal origin (41-43;45-48). However, instead of originating from a single "preeclampsia-gene" it is rather a complex of several genetic polymorphisms contributing to the development of preeclampsia (42). Furthermore, the fact that a) preeclampsia predominantly affects first pregnancies, b) long-term exposure to seminal fluid diminishes preeclampsia risk, whereas c) change of partner increases the risk to develop preeclampsia, may indicate that an inadequate immunological response plays a role in the pathogenesis of preeclampsia (41;43;45;49). Additionally, preeclampsia can only occur in the presence of placental tissue, which is partly

from paternal origin. Hydatidiform moles, i.e. a pregnancy with only placental tissue and absence of a foetus, are at higher risk (43) and symptoms and lesions of preeclampsia resolve after delivery of the placenta (41;49;50). Therefore, it is proposed that preeclampsia originates from a disturbed placental development and/or function.

2.3.1 Hypothesis of pathogenesis of preeclampsia

Adequate maternal-foetal exchange is a key requirement for a successful pregnancy (51). The current concept of the pathogenesis involves the early development of the placental unit (Figure 2.4). Until approximately the 10th week of gestation maternal blood flow is absent from the precursors of the intervillous space (IVS) due to the presence of aggregates of cytotrophoblastic cells derived from the developing placenta. Therefore, a capillary circulation or simple diffusion performs the exchange of oxygen, nutrients and waste products between the developing embryo and mother (51). Around the 10th week of gestation the arterial plugs loosen and gradually disappear and maternal blood is able to enter the IVS. As a result of this maternal blood flow the oxygen tension (pO_2) rises, with subsequent generation of ROS and oxidative stress (51). This transient burst of oxidant stress may trigger differential pathways that develop a full maternal circulation to the placenta and can be counterbalanced by the induction of several antioxidant systems (51) or exaggerated by chemical stress induced by e.g. medication, environmental factors or metabolic disorders. One of the responses during a physiologically normal pregnancy is the invasion of syncitiotrophoblasts into the spiral arteries, resulting in an increased diameter. During this process the endothelium of the vascular wall and the musculature are destroyed by interstitial trophoblasts, converting them into flaccid sinusoidal sacs lined by endovascular trophoblasts (41;52). These changes are necessary to have a sufficient placental blood flow in the intervillous space to provide the developing foetus with adequate amounts of oxygen and nutrients.

At this regulatory point oxidative stress is controlled by maternal antioxidant capacity, which is determined by genetic predisposition, nutritional intake as well as by the expression of antioxidants by trophoblasts, which is influenced by both maternal and paternal genotype. When antioxidant capacity is not sufficient or when fluctuations of pO_2 or too high levels of pO_2 cause an extensive temporal oxidative stress that may overwhelm the antioxidant capacity, trophoblast degeneration may occur resulting in trophoblast malfunction and decreased invasive capacity. This may initiate diminished remodelling of the spiral arteries and lead to poor placental perfusion, which most probably occurs in women who develop

preeclampsia. Poor placental perfusion may lead to chronic oxidative stress in the placental unit, which has damaging effects on DNA and cell structure integrity.

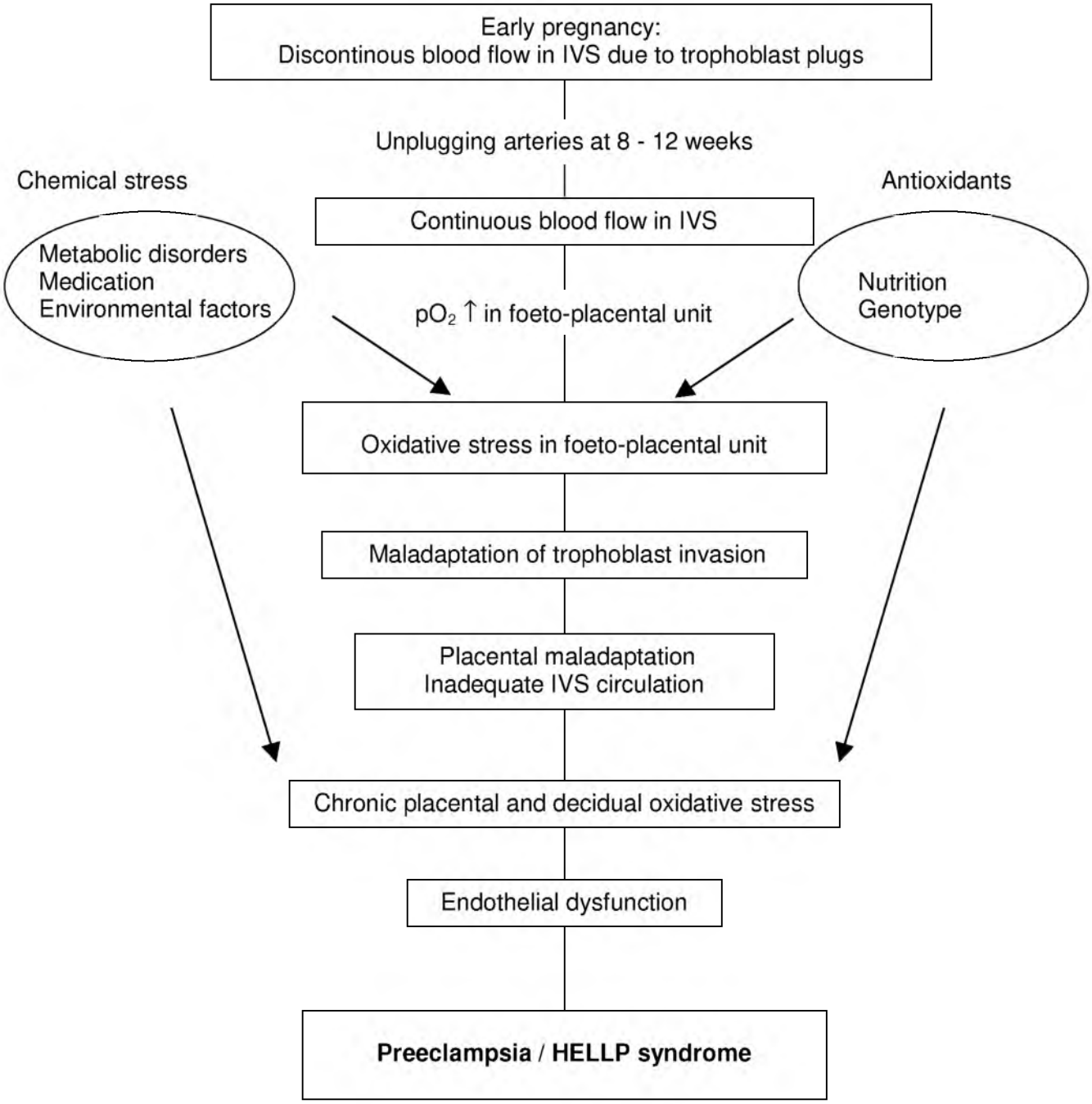

Figure 2.4. Proposed pathogenesis of preeclampsia (adapted from Jauniaux et al. (51):
In short; after unplugging spiral arteries the continuous blood flow in the intervillous space (IVS) results in a rise of oxygen tension and transient oxidative stress. If not properly counterbalanced by antioxidants or when exaggerated by chemical stress, this may lead to trophoblast degeneration, resulting in maladaptation of trophoblast invasion. As a consequence of poor placental circulation, chronic placental stress may evolve, which finally may affect maternal endothelium in the systemic circulation.

Although, increasing enzyme activity of GPX and other antioxidants are found in placental tissue to prevent oxidative damage (33;51), the amount of oxidative products may exceed the placental antioxidant capacity. Eventually, the overproduction of lipid peroxides may also affect maternal circulation due to leakage of ROS to maternal vascular system and may finally result in the clinical symptoms of preeclampsia and HELLP syndrome.

2.3.2. Oxidative stress in preeclampsia

In line with the role of placental oxidative stress in the pathogenesis of preeclampsia, numerous independent markers of oxidative stress indicate the presence of placental oxidative stress in women with preeclampsia. However, the problem with the measurement of oxidative damage is that these products can easily be formed *in vitro* and therefore no golden standard to measure oxidative stress is present, which may explain the contradictory findings as reported in literature (35). Several studies reported higher placental levels of lipid peroxidation (53-55), whereas recently also higher levels of oxidative protein damage (56) or ROS (57) were described in women with preeclampsia as compared with those in uncomplicated pregnancies. However, Poranen et al. (58) described lower levels of conjugated dienes, which are formed in the process of lipid peroxidation, in placental tissue of women with preeclampsia. In the same study elevated levels of thiobarbituric acid reactive substances, which include malondialdehyde (MDA) and a higher peroxidation potential were described in placentas of women with preeclampsia as compared to those in normal pregnant women (58).

In most of these studies it was also shown that placental antioxidant capacity was decreased in placental tissue of women with preeclampsia (53;54;56). In preeclampsia the expression of the important enzymatic antioxidants Cu-Zn SOD and GPX is down regulated in placental tissue as demonstrated by lower mRNA levels (53;59). Furthermore, also protein levels of these specific enzymatic antioxidants as well as those of G6PDH are lower in women with preeclampsia (35;58;59), whereas the level of GSTPi, the major GST isoform in placenta, is lower in placental tissue of women with preeclampsia (9). Additionally, levels of the non-enzymatic antioxidant vitamin E were found to be lower (59).

In contrast with the studies that show decreased placental antioxidant capacity some studies describe that glutathione levels (33;55), GPX enzyme activity (33), and catalase enzyme activity (59) are higher in placentas from women with severe preeclampsia. It is postulated

that the increase of these specific antioxidant systems is necessary to cope with increased oxidative stress.

In conclusion, the elevation of lipid peroxidation markers and the down-regulation of the antioxidant system as often noticed provide a clear indication for the presence of placental oxidative stress in women with preeclampsia.

Reports of oxidative stress in maternal circulation have been variable, however, most of them indicate higher oxidative damage and lower antioxidant capacity (35;54) or an increase of superoxide generation from circulating neutrophils (24;60). Furthermore, some studies showed an elevated oxidant potential of preeclamptic blood by measurement of increased oxidation of vitamin C (61) and the increased generation of superoxyde or peroxynitrite (24;35;62). Increased serum levels of MDA, a major breakdown product of lipid peroxides, was one of the first markers showing elevated lipid peroxidation in women with preeclampsia (50). Numerous other studies have confirmed that MDA levels or the concentrations of TBARS, which mainly consists of MDA, are higher in women with preeclampsia as compared to normotensive pregnant women (29;35;38;63-67). However, one study could not demonstrate a difference in MDA levels, although the ratio of MDA over total antioxidant capacity was much higher in women with preeclampsia versus uncomplicated pregnancies, indicating increased oxidative stress (68). A consequence of lipid peroxidation is the formation of conjugated dienes, which involves bond migration in the unsaturated fatty acid hydrocarbon chain (50), thereby being specific markers of lipid peroxidation. Levels of conjugated dienes are elevated in plasma and platelets of women with preeclampsia (50;63;69). Free radical attack of arachidonic acid results in the generation of stable products the so-called iso-prostanes. Results on measurement of iso-prostanes levels seem to be ambiguous. In plasma of women with preeclampsia higher levels of 8-isoprostane were detected (70), whereas in urine no differences were found (71). This controversy might be explained by an impaired renal clearance in preeclampsia (70). Since extra-cellular low-density lipoproteins (LDL) may be exposed to cell-derived oxidants and may be less protected by antioxidants, they are prone to oxidative attack. In several disorders associated with oxidative stress including preeclampsia, increased levels of antibodies against an epitope on the oxidised form of LDL are found (35;72).

Besides damage to lipids, oxidative stress may have noxious effects to carbohydrates, amino acids, proteins, RNA, DNA and other molecules. Proteins may be modified by direct oxidative attack or by lipid peroxidation products, affecting their amino acid side chains

resulting in the formation of additional carbonyl groups. In plasma of women with preeclampsia higher protein carbonyl levels were found (73). Free radicals may also react with the free sulfhydryl group of aminothiols, such as cysteine, homocysteine, cysteinylglycine and glutathione, resulting in the formation of (mixed) disulphides. A relatively higher increase of the oxidised thiol level as compared with the rise of the corresponding free thiol level in whole blood results in a lower free-to-oxidised ratio and may be a direct measure of oxidative stress. The free-to-oxidised ratios for cysteine, cysteinylglycine, and homocysteine were found to be lower in women with preeclampsia. (74, this thesis).

Besides the elevation of oxidative damage products, the decrease of specific antioxidants or the total antioxidant capacity are other indirect indications for oxidative stress in preeclampsia. However, measurement of total antioxidant capacity may be confusing, since depending on the method used –SH containing antioxidants are not measured (56) or uric acid, a breakdown product of purines that is elevated in women with preeclampsia, is also measured (58;75). The elevation of uric acid levels in preeclampsia may reflect an adaptive mechanism in the defence against oxidative stress, be an indication for the increased destruction of tissue, or point at an altered metabolism or clearance of purines (76). Overall total antioxidant capacities were reported to be lower in women with preeclampsia (39;68).
Results on vitamin E levels in preeclampsia are ambiguous; some papers report lower levels (39;64), whereas others demonstrate increased levels (75) or no differences (35;61) between preeclamptic and uncomplicated pregnancies. Since vitamin E is transported by ß-lipoproteins, the discrepancy is most likely explained by the presence of hyperlipoproteinemia in preeclampsia and absence of correlation of vitamin E with lipoprotein levels (35). The same contradiction is found for vitamin C, which is reported to have a synergistic effect on the activity of vitamin E. Some studies found lower levels in preeclampsia (39;61), whereas in others studies unchanged levels were reported (66;75).
Other important non-enzymatic antioxidants comprise the thiol-containing peptides, especially glutathione. Total thiol content in blood was reported to be lower (66;77). Studies on glutathione show inconsistent results. Levels in plasma are lower in women with preeclampsia (60;78), whereas free levels of glutathione were shown to be lower in women with HELLP (18).
In preeclampsia the presence and activity of enzymatic oxidants have hardly been studied. In studies with a small number of subjects, SOD activity was found to be lower in preeclampsia

(38;62;66), whereas GPX activity was found to be slightly lower (38), or remained unchanged (66). However, in a recent larger study SOD was lower in preeclampsia, whereas GPX activity was higher (67). In the same study catalase enzyme activity was found lower in preeclampsia (67), whereas others have reported unchanged enzyme activity (38).

In addition, the antioxidant therapies applied by Chappell et al. (79) and Gülmezoglu et al. (80) indirectly support the concept of oxidative stress in the pathogenesis of preeclampsia. In both studies supplementation with high doses of vitamin E and vitamin C was given, which reduced the risk of preeclampsia in the study of Chappell et al, whereas no effect was found in that of Gülmezoglu et al. However, treatment protocol was different, Gülmezoglu et al. started the vitamin treatment at onset of severe preeclampsia, whereas Chappell et al. supplemented a high risk population starting in early pregnancy (16 – 22 weeks) until delivery. This indicates that early intervention might be essential. Since placental oxidative stress arises after unplugging of the spiral arteries at approximately the 10th week of gestation (Figure 2.4) and lipid peroxidation progressively increases with gestational age, even in uncomplicated pregnancy, the positive effect of vitamin intervention might indicate that oxidative stress is an early factor in the pathogenesis of preeclampsia.

2.3.3 Pathophysiological consequences of oxidative stress in preeclampsia.

Oxidative stress may alter vascular function by direct action on endothelial cells or through vasoactive pathways including nitric oxide (NO) synthase, prostaglandin H (PGH) synthase and endothelin (24). Lipid peroxides may interact with endothelial cells or alter their function (81) and may have a contractile effect on the vascular system by the formation of increased levels of oxidised LDL, which may inhibit endothelial-dependent relaxation (24;50). The levels of NO synthase and nitric oxide are increased in women with preeclampsia. Although NO is a potent vasorelaxant; it may rapidly react with superoxide yielding peroxynitrite, which a) reduces the availability of NO and thus prevents its action as a vasorelaxant, b) is involved in necrosis and apoptosis, and c) may directly damage endothelial cells due to its pro-oxidant capacity (24;62). Additionally, peroxynitrite along with lipid peroxides may increase phospholipase A_2 activity, which activates prostaglandin synthase resulting in overproduction of prostacyclin (24;62). However, high levels of lipid peroxides inhibit PGH synthase. Normally prostacyclin is a vasorelaxant, however, it also binds to the tromboxane

receptor causing a vasoconstrictive response (24). Furthermore, both cell damage and oxygen radicals stimulate the release of endothelin, a potent vasoconstrictor, into the circulation.
The above-mentioned effects of ROS, lipid peroxides and other radicals may explain the characteristics of preeclampsia, since vasoconstriction results in hypertension, whereas damage of the endothelium, including that lining the renal glomular capillaries, may result in proteinuria (35).

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Part II

Glutathione / glutathione S-transferases in reproduction

Chapter 3

Glutathione and glutathione S-transferases A1-1 and P1-1 in seminal plasma may play a role in the protection against oxidative damage to spermatozoa.

Maarten T.M. Raijmakers, Hennie M.J.Roelofs, Eric A.P. Steegers, Régine P.M. Steegers-Theunissen, Theo P.J. Mulder, Maarten F.C.M. Knapen, Wai Yee Wong, and Wilbert H.M. Peters.

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Abstract

Objective: To study the levels of glutathione, glutathione S-transferase A1-1 and glutathione S-tranferase P1-1 in seminal fluid of fertile and subfertile men.

Design: Retrospective case-control study.

Setting: The Departments of Gastroenterology, Obstetrics / Gynaecology, and Epidemiology / Biostatistics of the University Medical Center Nijmegen, The Netherlands.

Patients: Twenty-five subfertile males visiting the fertility clinic and twenty-five fertile males from midwife practices were recruited.

Main outcome measures: Plasma levels of glutathione, glutathione S-transferase A1-1 and P1-1 in relation to seminal characteristics.

Results: Glutathione, glutathione S-transferase A1-1 as well as glutathione S-transferase P1-1 were found in considerable amounts in seminal fluid of subfertile and fertile men. No differences between groups were found for glutathione S-transferase A1-1 and P1-1. Also no associations with sperm count, motility or morphology could be detected. Fertile men had significantly higher glutathione levels as compared with subfertile males. Associations of glutathione with sperm motility quality (r_s = 0.321) and abnormal sperm morphology (r_s =-0.496) were found.

Conclusions: Presence of glutathione S-transferase A1-1 and P1-1 in seminal fluid suggest a role in the protection against (oxidative) damage of spermatozoa, whereas glutathione may play a role in male fertility.

Introduction

Human spermatozoa are capable of generating reactive oxygen species and this activity is accelerated in cases of defective sperm function (1). Because of the presence of extraordinary high levels of polyunsaturated fatty acids in the plasma membrane of spermatozoa, human spermatozoa are highly susceptible to oxidative stress, which may lead to peroxidative damage of the cell membrane, resulting in more permeable membranes (2). This may have a negative effect on sperm quality, motility, and fertilization and may result in defective sperm function and conception, or even infertility (3). Protection against reactive oxygen species and prevention of other damage is of critical importance and can be provided by both enzymatic or non-enzymatic antioxidants (2;4-7). However, due to the high density of mitochondria, which may leak oxygen radicals in the cytoplasm, the ability of spermatozoa to scavenge oxidants in the small midpiece is limited. Therefore, antioxidant capacity has to be present in the seminal fluid as well. In spermatozoa and in seminal fluid the presence of considerable amounts of the antioxidants α-tocopherol, uric acid, and vitamin C, as well as the enzymes superoxide dismutase, glutathione peroxidase and catalase have been described (3;5).

An important endogenous antioxidant in humans is the tripeptide glutathione, which plays a central role in the defence against oxidative damage and toxins, where it serves as co-factor for glutathione peroxidases and glutathione S-transferases. The family of human cytosolic glutathione S-transferases consists of four main classes, Alpha, Pi, Mu and Theta, each subdivided in one or more different isoforms (8). Due to the existence of many different isoenzymes broad substrate specificity is achieved.

It was already shown that glutathione and glutathione S-transferases play an important role in reproduction (9). However, data on glutathione and glutathione S-transferases in human seminal fluid are limited (5). Therefore, we investigated the amounts of glutathione, glutathione S-transferase P1-1, and glutathione S-transferase A1-1 in seminal fluid of both fertile and subfertile males.

Materials and Methods

Patient material

The local Institutional Review Board approved the experimental protocol. Fertile subjects were recruited from midwife practices. They had no history of fertility problems and their partners had a spontaneously pregnancy at the time of participation. Subfertile males were recruited at the fertility clinic of the University Medical Center Nijmegen, The Netherlands, from couples who failed to conceive after one year of regular unprotected intercourse with the same partner and who had a sperm count of 5 – 20 million per milliliter. After informed consent was given semen samples were collected standardised by masturbation after a sexual abstinence of at least 48 hours. Semen samples were collected for a large study on zinc and folic acid deficiency in male factor subfertility (10). Subgroups of fertile (n=25) and subfertile (n=25) men were randomly selected from this study. Semen samples were characterised by the semen parameters: sperm count, percentage of motile spermatozoa, quality of sperm motility (on a scale of 1-6) and percentage of spermatozoa with abnormal morphology, as described earlier by Menkveld et al. (11). The semen characteristics of both fertile and subfertile males are summarised in Table 3.1. Seminal plasma was prepared by centrifugation of the ejaculate (1,500xg, 10 min and 4°C) and was stored at –30°C until use.

Table 3.1. Age and Semen Characteristics of the Study Groups.

	Fertile males (n=25)	Subfertile males (n=25)	P-Value
Age (years)	34 (27 – 42)	33 (27 – 41)	NS
Sperm count (x10^6/mL)	75 (35 – 175)	5 (0.1 – 13)	0.0001
Quality of sperm motility *	4/5 (3 – 5/6)	3 (2 – 4)	0.0001
Motile spermatozoa (%)	60 (30 – 70)	20 (1 – 60)	0.0001
Abnormal Morphology (%)	63 (35 – 90)	81 (54 – 98)	0.0001

* Quality of sperm motility is given on a scale of 1-6.

Data are presented as median (range). Abbreviation: NS = not significant

Assays

Levels of glutathione S-transferase A1-1 (GSTA1-1) and glutathione S-transferase P1-1 (GSTP1-1) in seminal plasma were determined by ELISA as previously described for blood plasma (12;13). In short; polystyrene microtiter plates (Microlon®-600 High Binding

Capacity, Greiner, Alphen a/d Rijn, The Netherlands) were coated overnight with purified monoclonal antibody against GSTA1-1 (14) or GSTP1-1 (15). Plates were washed 5 times with phosphate-buffered saline supplemented with 0.05% (v/v) Tween 20 detergent (PBST). 100 µL of standard solutions (0.8 – 20 µg/L for GSTA1-1 and 0.4 – 100 µg/L for GSTP1-1) and seminal plasma samples (diluted up to 1:200) were added to the wells and incubated overnight. After washing with PBST, plates were incubated with rabbit anti-glutathione S-transferase A1-1 or anti-glutathione S-transferase P1-1 antiserum. Plates were washed again and incubated with horseradish peroxidase-labeled swine anti-rabbit antiserum (DAKO, Glostrup, Denmark). After a final wash, plates were stained with *o*-phenylenediamine/H_20_2 (Sigma Chemicals, Zwijndrecht, The Netherlands). The detection limits are 0.04 µg/L and 0.4 µg/L for GSTA1-1 and GSTP1-1, respectively.

For determination of glutathione concentrations, 100 µL of 12% perchloric acid (Merck, Darmstadt, Germany) was added to 20 µL seminal plasma to precipitate all proteins in the sample. After centrifugation (13,000xg, 10 min and 4°C) clear supernatants were stored at – 30°C until analysis. Glutathione concentrations were determined after conjugation to 7-fluorobenzofurazane-4-sulfonic acid (SBDF; Fluka Chemie AG, Bornem, The Netherlands) using a high performance liquid chromatography (HPLC) method, essentially as described previously (16). In short; 100 µL sample was reduced for 30 min at room temperature with 10 µL 10% (w/v) tris-2-carboxyethyl-phosphine (Fluka Chemie AG). Subsequently, samples were neutralised with 75 µL 2M NaOH. 100 µL of the neutralised sample was derivatised with SBDF, thereafter, 60 µL derivatization buffer (50 µL borate buffer (125 mmol/L $K_2B_4O_7{\cdot}4H_2O$ (Fluka Chemie AG) with 4 mmol/L EDTA, pH 9.5), 5 µL SBDF (4 mg/mL borate buffer) and 5 µL 1.55 M NaOH) was added. Subsequently, samples were incubated for 1 hour at 60°C. Samples were cooled and 20 µL was injected into the HPLC system.

Statistics

Differences between fertile and subfertile males were analysed by a Wilcoxon-Mann-Whitney test. Data from the fertile and subfertile group were pooled for the calculation of associations between GSTA1-1, GSTP1-1 and glutathione with spermatozoa count, percentage of motile spermatozoa, quality of sperm motility and morphology, which were computed by Spearman's rank correlation test. All statistical tests were performed with the Astute Statistical Add-In for Microsoft Excel 5 version 1.50.

Results

In most seminal plasma samples of both fertile and subfertile males considerable amounts of glutathione, glutathione S-transferase A1-1 and glutathione S-transferase P1-1 could be detected (Table 3.2). Median levels of glutathione were significantly higher in fertile males as compared with subfertile males (1.2 (0 – 3.9) versus 1.8 (0.1 – 7.7) µmol/L, respectively; P=0.02). No statistical differences between subfertile and fertile males were found for GSTA1-1 and GSTP1-1 concentrations. In the pooled data, values of glutathione showed 80-fold variation, whereas those of GSTA1-1 and GSTP1-1 both showed approximately 20-fold variation. Median GSTP1-1 concentration was approximately 6 times higher than that of GSTA1-1.

Table 3.2. Levels of glutathione, glutathione S-transferase A1-1 and P1-1 in Human Seminal Plasma

	Fertile males	Subfertile males	Total study group
Glutathione	1.8 (0.1 – 7.7)	1.2 (0 – 3.9)*	1.5 (0 – 7.7)
GSTA1-1	238 (34 – 660)	143 (34 – 674)	176 (34 – 674)
GSTP1-1	1174 (430 – 2622)	981 (265 – 4837)	1104 (265 – 4837)

Data are presented as median (range) and expressed as µmol/L (glutathione) and ng/ml (GSTA1-1 and GSTP1-1).

Statistics: * P=0.02 fertile versus subfertile males

Neither glutathione S-transferase A1-1 nor glutathione S-transferase P1-1 concentrations were correlated with glutathione levels, sperm count, percentage of motile spermatozoa, motility quality, or abnormal morphology. Glutathione concentrations in seminal plasma were significantly correlated with semen morphology and motility quality (r_s = -0.496, P≤0.001 and r_s = 0.321, P=0.02, respectively).

Discussion

Due to the generation of oxygen free radicals by spermatozoa both seminal plasma as well as spermatozoa itself should be capable of scavenging these reactive oxygen species (ROS) for

protection against oxidative damage. Lipid peroxidation in the cell membranes of spermatozoa, generated by ROS, may result in impairment of function (5). The presence of enzymatic (superoxide dismutase, catalase, glutathione peroxidase) and non-enzymatic antioxidants (vitamin C, vitamin E, urate, albumin, taurine, hypotaurine, and glutathione) (2;4-7;17;18) or total antioxidant capacity (19) in seminal plasma or spermatozoa have been previously studied .

Previous reports on glutathione in seminal plasma are in line with the results of our study. Ochsendorf et al. (5) reported median (range) levels of 0.6 (0.3 – 1.1) μM and 0.7 (0.2 – 2.4) μM in oligozoospermia and normozoospermia, respectively, whereas in the study of Yeung et al. (17) amounts of glutathione were below the limit of detection (<2.5 μM) in seminal plasma samples. However, in contrast with the studies of both Ochsendorf et al. and Yeung et al. we established significantly lower glutathione concentrations in seminal plasma of subfertile males compared with those of fertile males. In addition, we showed that higher glutathione levels in seminal plasma were associated with a higher motility quality index and that lower glutathione levels were associated with a higher degree of spermatozoa with abnormal morphology. This emphasizes the previous findings by Lenzi et al., who showed that glutathione therapy improved semen quality (20). Therefore, our results provide evidence that the levels of glutathione in seminal plasma seems to play a role in fertilty, since glutathione may protect against oxidative damage of the cellular membranes of spermatozoa.

So far, no attention was given in literature to the presence of glutathione S-transferase isoenzymes in seminal plasma. We now demonstrate that, besides their co-factor glutathione, glutathione S-transferase A1-1 and glutathione S-transferase P1-1 are present in considerable amounts in seminal plasma of all subjects investigated. However, a broad inter-individual variation was seen. Exposure to pesticides or the recent use of antibiotics were associated with male factor subfertily (10), whereas the importance of glutathione for semen quality was noticed (20). Therefore, we also expected to find differences in seminal plasma concentrations of GSTA1-1 and GSTP1-1 between fertile and subfertile men, or associations of these GSTs with semen quality factors. Although both GSTA1-1 and GSTP1-1 tended to be lower in subfertile versus fertile men, no statistical significant differences were found, which is most probably due to high inter-individual variation and the multifactorial cause of subfertility (10). In addition, no of the associations between GSTA1-1 or GSTP1-1 and semen quality factors could be found. These results indicate that GSTA1-1 and GSTP1-1 may have no dominant protective function in seminal plasma. The protective effect of glutathione may, therefore, not mainly originate from its co-operation with GSTA-1-1 or GSTP1-1, but may be

explained by its direct scavenging of ROS, by its contribution to glutathione peroxidases, or by a combination of these functions.

In conclusion, glutathione S-transferase A1-1, glutathione S-transferse P1-1 and their co-factor glutathione are present in considerable amounts in most seminal plasmas of both fertile and subfertile men. Glutathione might play a role in fertility, since glutathione concentrations in seminal plasma were higher in fertile than in subfertile males. Furthermore, higher levels of glutathione seem to improve or protect the quality of sperm motility and morphology of spermatozoa, independently from glutathione S-transferase levels.

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Chapter 4

Developmental aspects of glutathione and glutathione S-transferases.

Paragraph 4.1

Glutathione S-transferases and thiol concentrations in embryonic and early foetal tissues.

Maarten T.M. Raijmakers, Eric A.P. Steegers, Wilbert H.M. Peters.

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Abstract

Background: Glutathione S-transferases (GSTs) are important in intracellular binding and transport of numerous compounds and play a central role in human detoxification. Human GSTs mainly consist of class Pi (GSTP), Mu (GSTM), Alpha (GSTA), and Theta (GSTT) enzymes, each subdivided into one or more isoenzymes. They catalyse the conjugation of glutathione to toxic compounds resulting in more water soluble and less biologically active products that can easily be excreted from the body. The reactive –SH group in glutathione (GSH) is provided by cysteine, which is an important amino acid in glutathione synthesis.

Methods: In this study we investigated GST expression, enzyme activity, and levels of cysteine and glutathione in cytosolic fractions of organs from an embryo and a foetus at 8 and 13 weeks of gestational age, respectively.

Results: $GSTP_1$ was predominantly present in all tissue samples of both the embryo and foetus. GSTA ($GSTA_1$ + $GSTA_2$) levels were moderate as compared to $GSTP_1$ levels, whereas $GSTM_1$ was present in only low amounts. $GSTT_1$ could not be detected in any of the tissue samples. GST enzyme activity was highest in organs directly exposed to amniotic fluid. In all embryonic and foetal organs considerable amounts of glutathione and cysteine could be detected, with higher GSH concentration in organs where lower cysteine concentrations were demonstrated.

Conclusions: These results suggest that in embryonic and early foetal development cysteine, glutathione, and glutathione S-transferases are present in high amounts and that $GSTP_1$ is the most important GST isoform at these developmental stages.

Introduction

The family of glutathione S-transferases (GSTs) consists of homo- or heterodimeric cystolic proteins composed of subunits with an estimated molecular mass of approximately 25 kilodaltons (range 23-28 kilodaltons). Today four main subclasses of GST isoforms have been recognised in humans: Alpha, Mu, Pi, and Theta (GSTA, GSTM, GSTP, and GSTT), each divided into one or more distinct isoforms with different reactivity towards specific substrates. Therefore, a diverse expression pattern of GSTs isoforms is seen in the various adult tissues. GSTAlpha is mainly expressed in liver but it is also present in kidney, testis, adrenal gland, and small intestine (1) whereas $GSTP_1$ is mainly expressed in lung, placenta, breast, and urinary bladder. $GSTM_1$ and $GSTT_1$ are found in relatively low levels in many organs. GSTs catalyse the conjugation of glutathione with a wide variety of xenobiotics such as carcinogens and pharmacologically active agents as well as reactive oxygen species. This conjugation may result in the formation of more soluble and less biologically active compounds, which can easily be excreted in urine (1-5) . The characteristic feature of the tripeptide glutathione (γ-glutamyl-cysteinylglycine) is the presence of the reactive sulfhydryl (-SH) group donated by cysteine, which dictates the chemistry of glutathione. Besides its role in detoxification, glutathione is important in storage and transport of amino acids (5).

Embryonic and foetal growth and development depend on a constant flow of nutrients from the mother (6). During the first weeks of development the embryo exchanges nutrients and waste products by diffusion, followed by exchange via the placental intervillous space. Despite the large detoxification capacity of the placenta (7;8), almost every drug present in the maternal circulation is able to pass the placental barrier and may reach the foetal organs (9). Therefore, cysteine and glutathione may have a function in foetal detoxification in combination with GSTs, which may be vital to scavenge toxic compounds passing the placental barrier. Levels of cysteine, glutathione, and distribution of GST isoforms in combination with GST enzyme activity have been extensively studied in adult tissues. However, little is known about embryonic or foetal tissues, which is subject of this descriptive study.

Materials and Methods

Tissue samples

The Institutional Review Board approved the study protocol. After informed consent was obtained stomach, small intestine, liver, kidney, adrenal gland, lung, heart, and testis were excised on ice from a male extra-uterine located embryo with gestational age of 8 weeks and a crown-rump length of 31 mm (10). Another male foetus with a crown-rump length of 85 mm was removed from an uterus and placed on ice, together with samples of decidual and placental tissue, from a patient undergoing hysterectomy for cervical cancer. From this foetus with a gestational age of 13 weeks oesophagus, stomach, small intestine, liver, spleen, kidney, adrenal gland, lung, heart, bladder, testis, brain, and brain stem were excised on ice. Gestational ages of both embryo and foetus had been confirmed by ultrasound examination. All tissue samples were frozen in liquid nitrogen and stored at –80°C until analysis.

Tissue homogenates were prepared on ice by adding 4 volumes of ice-cold homogenisation buffer (0.25 M saccharose, 20 mM Tris/HCl buffer pH 7.4, and 1 mM dithiothreitol). After 5 strokes using a glass-glass potter tube, homogenates were centrifuged for 1 hour at 100,000xg and 4 °C. Immediately after centrifugation cytosols were stored in aliquots at –80°C until analysis. Protein was determined using the method of Lowry *et al.* (11) using bovine serum albumin as a standard.

Quantification of glutathione S-transferase isoforms:

Levels of the GST isoforms were determined as described before (12). Samples of the embryo and foetus were run in parallel on different blots. In short, cytosolic samples were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (12% acryl-/bisacrylamide w/v, 37.5:1, Bio-Rad Laboratories, Veenendaal, The Netherlands) and separated under standardised conditions. Subsequently, proteins were transferred to nitrocellulose membranes (Protran®; Schleicher and Schuell, 's Hertogenbosch, The Netherlands) using a semidry blotting system (Novablot II, Pharmacia, Uppsala, Sweden). After blocking with 1% gelatine (w/v) in PBS-T, western blots were incubated with monoclonal antibodies (ascites diluted 1:5,000) against human GST class Alpha (both A_1 and A_2) (13) $GSTM_1$ (14), $GSTP_1$ (15) and $GSTT_1$ (16) as described in detail before. After three wash cycles with PBS-T, specific binding of monoclonal antibodies to the isoforms was detected by incubation with peroxidase-

conjugated rabbit anti-mouse immunoglobulin (Dakopatts, Glostrup, Denmark) followed by subsequential development of the peroxidase label with 0.1% 3,3' diaminobenzidine in PBS (Sigma Chemicals, Zwijndrecht, The Netherlands) containing 0.01% hydrogen peroxide (Merck, Darmstadt, Germany), 0.34 g/L imidazole (Merck) and 0.26 g/L cobalt-chloride.$6H_20$ (ICN Biomedicals B.V., Zoetermeer, The Netherlands). Staining intensity on the immunoblots was quantified using a laser densitometer (Ultroscan XL, LKB, Bromma, Sweden). Quantification of GST isoforms in the cytosolic fractions was performed with known amounts of purified GSTs, which were run in parallel with the samples. The detection limit of each GST isoform was approximately 50 nmol/mg protein (12), whereas the within-assay and day-to-day variation were 10 and 15%, respectively.

Determination of glutathione S-transferase enzyme activity

Glutathione S-transferase enzyme activity was determined in duplicate according to Habig *et al.* (17). In short, 10 µL of each cytosolic fraction was added to 2.0 mL 0.1 M potassium phosphate buffer pH 6.5 containing 1.0 mM 1-chloro-2,4-dinitrobenzene (CDNB; Sigma Chemicals) and 5.0 mM glutathione (Sigma Chemicals) at 25 °C in a disposable cuvette. The change in absorbance at 340 nm was followed for 3 mins using a Lambda 12 spectrofotometer (Perkin Elmer, Nieuwerkerk a/d IJssel, The Netherlands).

Analysis of thiols

For the analysis of cysteine, homocysteine, cysteinylglycine and glutathione 10 µL 10% (w/v) tris (2-carboxyethyl) phosphine (Fluka Chemie AG, Bornem, The Netherlands) was added to 100 µL of each sample. After reduction for 30 min at room temperature, samples were neutralised by adding 75 µL 2 M NaOH. Subsequently, 100 µL of the neutralised sample was derivatised with 7-fluorobenzofurazane-4-sulfonic acid (SBDF; Fluka Chemie AG) for 1 hour at 60°C by adding 60 µL of derivatizaton buffer containing 50 µL borate buffer (125 mmol/L $K_2B_4O_7 \cdot 4H_2O$ and 4 mmol/L EDTA, pH 9.5), 5 µL SBDF (4 mg/mL borate buffer), and 5 µL NaOH (1.55 M)). Thiols were separated using high performance liquid chromatography as described previously (18). Thiol concentrations were determined using a calibration curve for all thiols which was run in parallel with the samples.

Statistics:

To determine associations between glutathione levels, cysteine levels, glutathione S-transferase isoforms, and glutathione S-transferase enzyme activity the Spearman Rank coefficient of correlation was calculated using Astute for Microsoft Excel 5.0. An association was considered significant if a P-value of 0.05 was reached.

Results

Table 4.1 shows the concentrations in embryonic and foetal tissue of glutathione S-transferase Alpha (A_1 + A_2: GSTA), Pi ($GSTP_1$), Mu ($GSTM_1$), and Theta ($GSTT_1$). In addition GST enzyme activity with CDNB is presented. Due to insufficient amount of material from some organs full analyses of all parameters was not possible.

In the embryo of 8 weeks gestation, $GSTP_1$ was the predominantly expressed GST isoform in all but one tissue sample. Only kidney mainly expressed GSTA instead of $GSTP_1$. Overall, GST was composed of 23% GSTA, 73% $GSTP_1$, and 4% $GSTM_1$. Highest $GSTP_1$ levels were seen in lung and stomach, but only in kidney $GSTP_1$ expression was much lower as compared to the other organs. The highest expression of GSTA was found in liver, intermediate levels were detected in testis, kidney, small intestine and stomach, whereas low levels were found in lung and adrenal gland. In heart no GSTA could be demonstrated. The level of $GSTM_1$ expression was comparable in all tissues studied. In none of the organs $GSTT_1$ could be detected, which was supported by the $GSTT_1$ null genotype found by PCR analysis (19). All organs showed considerable GST activities towards CDNB, with highest enzyme activity in stomach and small intestine.

In the foetus of 13 weeks gestation, $GSTP_1$ was also predominantly expressed in all organs, with the highest levels in small intestine, kidney and lung. Most other tissues had high $GSTP_1$ expression levels, except for spleen, which showed a relatively low level. Overall, GST was composed of 26% GSTA, 69% $GSTP_1$, and 5% $GSTM_1$. Highest expression of GSTA was seen in liver, small intestine and adrenal gland. Oesophagus showed a moderate expression of GSTA, whereas low expression was seen in spleen and lung. No GSTA could be detected in

brain. $GSTM_1$ was mainly present in kidney and in tissues exposed to the amniotic fluid being the oesophagus, small intestine and lung.

Table 4.1. Glutathione S-transferase isoform levels and enzyme activity in embryonic and foetal organs.

	GSTAlpha	$GSTP_1$	$GSTM_1$	$GSTT_1$	GST- Activity
Embryo (8 wks)					
Stomach	1072	8622	325	--	723
Small intestine	2946	7253	353	--	635
Liver	3717	6874	674	--	386
Kidney	2085	531	340	--	511
Adrenal gland	114	4946	117	--	389
Lung	165	8812	264	--	547
Heart	--	6617	341	--	334
Testis	2961	4210	324	--	432
Mean	*1868*	*5983*	*342*	--	*495*
Foetus (13wks)					
Oesophagus	853	6735	764	--	314
Stomach	2036	4233	433	--	NA
Small intestine	4813	9688	728	--	882
Liver	4916	7596	398	--	545
Spleen	263	1159	192	--	61
Kidney	2144	9670	743	--	496
Adrenal gland	4519	6159	155	--	515
Lung	209	9562	907	--	472
Heart	1897	5420	83	--	289
Bladder	1265	5622	551	--	323
Testis	NA	NA	234	--	NA
Brain	--	4075	412	--	228
Brainstem	3080	5705	276	--	144
Mean	*2363*	*6302*	*444*	--	*388*
Placenta	--	8059	98	--	212
Decidua	896	8121	280	1793	268

GST protein levels and enzyme activity are expressed as ng/mg cytosolic protein and nmol/min.mg cytosolic protein, respectively.

Abbrev iations -- = Not Detectable, NA = Not Analysed.

In all other tissues a moderate expression was found with exception of heart, where only faint expression of $GSTM_1$ was seen. $GSTT_1$ was not detectable in all foetal organs examined, but was found in considerable amounts in decidua. PCR analysis revealed that this foetus also bore the $GSTT_1$ null genotype. GST enzyme activity was highest in small intestine, and lower levels were seen in liver, adrenal gland, kidney and lung. In the other tissues moderate enzyme activities were found, except for spleen where enzyme activity was just measurable.

Both the embryo and foetus showed considerable and similar amounts of acid soluble glutathione and cysteine in all organs examined, whereas the levels of cysteinylglycine and homocysteine were much lower (Table 4.2). Overall, thiols were composed of 14% cysteine, 85% glutathione, <1% cysteinylglycine, and <1% homocysteine. Surprisingly, glutathione levels in liver tissues are very low, whereas the amount of cysteine is high compared to the other organs. In the embryo highest cysteine levels were found in liver and testis, while low amounts were shown in kidney and lung compared with the other organs. Glutathione concentrations were highest in stomach, lung, heart and kidney compared to the other organs, whereas in liver almost no glutathione could be detected. Foetal liver and small intestine showed the highest amount of cysteine in comparison with the other foetal tissues. In oesophagus and spleen almost no cysteine was detectable. High glutathione was found in adrenal gland, heart, brain, and brainstem. Liver, testis and spleen showed the lowest glutathione levels.

Expression of GST isoforms in placental and decidual tissue, which was obtained in parallel with the foetus, was comparable to that in the foetal organs. Both placenta and decidua mainly expressed $GSTP_1$. A relatively high level of $GSTT_1$ was found in decidua, whereas GSTA in decidua and $GSTM_1$ in placenta and in decidua were only expressed at relatively low levels. GST enzyme activity was similar in placenta and decidua, however activities were lower in comparison with that of most foetal organs. In both placenta and decidua thiol levels were similar to the levels in most foetal tissues, except for cysteine in decidua where a high level was found.

Significant correlations between GST enzyme activity and expression of GSTA (r_s=0.61, $P<0.05$), $GSTP_1$ (r_s=0.48, P=0.03), and the sum of all GST isoforms (r_s=0.59, P=0.005) were found in the foetus, whereas in the embryo no such correlations could be found. A significant

inversed-correlation between cysteine and glutathione was found in the embryo (r_s=-0.74, P=0.04).

Table 4.2. Cystolic thiol concentrations in embryonic and foetal organs.

	Cysteine	Homocysteine	Cysteinylglycine	Glutathione
Embryo (8wks)				
Stomach	3.6	0.1	0.1	89.7
Small intestine	4.4	<0.1	1.0	54.3
Liver	30.7	<0.1	0.1	2.0
Kidney	2.5	0.1	0.4	64.5
Adrenal gland	5.9	0.4	1.3	38.7
Lung	2.4	<0.1	0.8	68.0
Heart	5.0	0.2	0.9	77.8
Testis	16.2	0.1	0.1	36.7
Mean	*8.8*	*0.2*	*0.6*	*54.0*
Foetus (13wks)				
Oesophagus	0.8	0.2	0.2	62.8
Stomach	6.7	<0.1	0.1	35.5
Small intestine	15.8	0.3	0.8	50.1
Liver	40.1	0.1	0.1	26.2
Spleen	1.4	<0.1	0.1	30.8
Kidney	8.4	0.1	0.9	57.7
Adrenal gland	5.5	0.3	0.6	105.2
Bladder	5.8	<0.1	0.3	46.2
Lung	6.6	<0.1	0.4	66.9
Heart	10.5	0.2	0.8	81.2
Testis	5.3	0.1	0.4	24.0
Brain	7.2	0.2	0.8	80.1
Brainstem	4.9	0.1	0.3	81.3
Mean	*9.2*	*0.2*	*0.4*	*57.5*
Placenta	5.8	<0.1	0.1	32.8
Decidua	19.5	0.4	0.2	33.0

Data are expressed in nmol/mg cytosolic protein.

Discussion

So far, thiol levels and the expression and enzyme activity of glutathione S-transferases in foetuses with comparable gestational ages have been described in several studies (Table 4.3). Although only data of one embryo and one early foetus are presented here, in contrast to earlier studies on development of GSTs, we now present quantitative data on a wide variety of tissues, which allows a direct comparison between organs. All tissues examined express considerable amounts of cysteine, glutathione, glutathione S-transferases P_1, $A_1 + A_2$ and M_1, and show a substantial GST enzyme activity, though in variable range. It was striking, therefore, that $GSTT_1$ was not detectable at all in the embryonic and foetal tissues examined, but was only demonstrated in decidual tissue of the 13 weeks' pregnancy. Of the $GSTT_1$ locus a null genotype exists, which has a frequency of 10 to 30% in a normal West-European population (20). Both embryo and foetus were of the $GSTT_1$ null genotype and are therefore unable to express $GSTT_1$.

Table 4.3. Overview of foetal Glutathione S-transferase isoforms and enzyme activity in literature.

	GSTAlpha	$GSTP_1$	$GSTM_1$	GST-activity	Reference
Liver	+	++	--	260	*22*
	+	++	-- /+-		*23*
Spleen	+	++	--/+-		*23*
Kidney	+	++	+-		*21*
	--	++	-- / +-		*23*
Lung	+-	++	+		*21*
				180	*25*
Brain	--	+			*43*

GST enzyme activity is expressed in nmol/min.mg cytosolic protein.

++: Strong expression, + = normal expression, +- = faint expression, -- = no expression.

Generally, $GSTP_1$ is the predominantly expressed GST isoenzyme in embryonic and early foetal organs, whereas expression of GSTA and $GSTM_1$ is moderately and low, respectively. These results are in line with those previously reported (21) also indicating that $GSTP_1$ is the most important glutathione S-transferase during foetal development.

In liver a relatively high expression of $GSTM_1$ was found, in contrast to results by others (22;23), where no or only faint $GSTM_1$ expression could be detected in foetuses of 10 – 20 weeks of gestation using starch gel electrophoresis. In accordance with these studies we also found high levels of GSTA and $GSTP_1$. Also a higher GST enzyme activity was found in both the embryo and foetus compared to others (22), which might be explained by the high expression levels of $GSTM_1$. In adult liver GSTA is predominantly expressed (Table 4.4), whereas $GSTP_1$ is only seen in bile duct epithelium and blood vessels (24). However, in embryonic liver $GSTP_1$ is the most expressed GST isoenzyme. This indicates that expression of $GSTM_1$ and GSTA increase during human development, whereas the expression of $GSTP_1$ decreases, as reported by others (21). These changes in expression levels of GST isoforms might also explain the higher GST enzyme activity in adult liver as reported previously (24). The different GST isoenzymes expressed in the developing liver and the lower GST enzyme activity as compared to adult liver are in agreement with the different functions of embryonic, foetal and adult liver. In the developing liver synthesis of erythrocytes is the main function, whereas biotransformation of toxic compounds is one of the primary functions of the adult liver.

Table 4.4. Glutathione S-transferase isoforms and enzyme activity in adult tissue in literature.

	GSTA	$GSTP_1$	$GSTM_1$	GST-activity	Reference
Oesophagus	456	8511	518	482	*32*
Stomach	2480	3384	263	331	*32*
Liver	21840	420	+/-	900	*24*
Kidney	5750	610	60	610	*29*
	2000	2500	1000		*26*
				347	*31*
Adrenal gland	3700	2400	1400		*26*
Lung	300	4500	30		*26*
				53	*28*
Heart	--	4700	300		*26*
Bladder	--	1324	2404	176	*33*
Testis	++	--/+-	++	2050	*44*
Brain	200	9800	2100		*26*

GST isoforms and enzyme activity are expressed in ng/mg cytosolic protein and nmol/min.mg cytosolic protein, respectively.

++: Strong expression, + = moderate expression, +- = faint expression, -- = no expression.

In lung a similar expression of GST isoforms as found by us is described earlier (21); levels of $GSTP_1$ were high in early gestation, but decreased during gestation, whereas $GSTM_1$ and GSTA were expressed moderate and weak, respectively. During gestation, GST enzyme activity decreases in parallel with $GSTP_1$ expression (25). The GST enzyme activity remained low in samples obtained more than a year after birth. In adult tissue $GSTP_1$ still is by far the most predominant GST isoform (26;27), however the levels are approximately two times lower than in foetal tissue. GST enzyme activities were found to be much lower in adult than in foetal lung tissue (28;3). When fully functional, the placenta, which has a large detoxification capacity (8;7), removes toxic metabolites from foetal circulation. Amniotic fluid may also contain toxic metabolites, therefore, tissues exposed to the amniotic fluid like those of lung and the gastrointestinal tract might need adaptation to such environment. The high level of $GSTP_1$ and high enzyme activity of lung tissue in early gestation both as compared with other tissues as well as adult levels, may be such an adaptation.
With exception of the low $GSTP_1$ expression in the embryo, the expression pattern of GST isoforms in kidney as reported here were in line with those reported earlier (21) being high, moderate, and faint expression of $GSTP_1$, GSTA and $GSTM_1$, respectively. In contrast to the results of others (23), who found that GSTA was absent in foetal kidney, we measured a moderate expression of this GST subclass. In foetal kidney, levels of GST isoforms and enzyme activity are similar to those found in adult tissue, where the predominantly expressed GST isoform is GSTA (26;29-31). This similarity might indicate that GST subclass development is an early event of pregnancy.

Very similar results were found in literature for the expression and activity of GST isoforms in adult tissue of brain (26), oesophagus and stomach (32) compared with embryonic and foetal tissues as reported here. In contrast, values in adult bladder do differ considerably than compared to corresponding foetal values (33). These discrepancies and similarities between adult and foetal GST capacity may indicate the different development patterns for the various foetal organs.

Mean concentrations of cysteine and glutathione are comparable between the embryo and the foetus studied. The inverse association between glutathione and cysteine levels in the embryo may point to the importance of availability of free cysteine in the synthesis of glutathione or the storage function of glutathione (5). Nutritional cysteine is the only –SH containing amino acid available for glutathione synthesis in the development due to absence of the

cystathionine pathway in early gestation (34), which transsulfurates methionine through homocysteine into cysteine in adults. Cysteine levels in embryonic and foetal liver were higher than those of glutathione, which is in contrast with the levels in most other organs and with the situation in adult liver, where glutathione levels are much higher. This may be explained by functional differences. One of the main functions of adult liver is its role in detoxification, whereas in early gestation liver has mainly a hemopoetic function. It was previously reported (35) that only a small fraction of the detected -SH residues in foetal liver corresponded to glutathione. Most -SH residues originated from cysteine, which was present in higher amounts than glutathione. However, the authors suggested artefacts during sample preparation and handling explaining these results, since glutathione can easily be metabolised to cysteine after foetal death, due to the presence of high foetal γ-glutamyltranspeptidase enzyme activity, but they do not rule out the possibility of high cysteine in foetal liver.

As compared to adults, glutathione levels in embryonic and foetal lung and bladder was approximately six (36) and two times (37-39) higher, respectively. The results in lung can be explained, since glutathione levels might be coupled to the expression of GST isoenzymes and enzyme activity, which are very high during early gestation and decrease to a level which is two times lower in adults (25;26). This change in expression of glutathione, glutathione S-transferase P_1 expression, and glutathione S-transferase enzyme activity during gestation might be explained by the development of the placenta. In early gestation the placenta is not fully developed and subsequently adaptations must be made to prevent damage by toxic products, which are preferably excreted to the amniotic fluid. Therefore, tissues exposed to the amniotic fluid probably have higher concentrations of glutathione, GSTs and a higher enzyme activity. When the placental detoxification starts to function and is able to remove waste products from the foetal circulation, this adaptation is not necessary anymore and consequently levels of glutathione S-transferase and the enzyme activity may be reduced.

Although placenta and decidua are partly and totally, respectively, from maternal origin both tissues probably play an important role in foetal detoxification and protection during growth and development. GST enzyme activity in placenta and decidua is comparable to previous published values in early pregnancy (40) and term placenta and decidua (7;41;42). However, the expression of all GST isoforms found here was much higher compared with the levels reported in term placenta and decidua (8), which may point to another function of the placenta in early gestation as compared with third trimester placenta.

In placental and decidual tissue similar levels of glutathione were found. Although the placental glutathione levels and GST enzyme activity are similar in our study as compared to third trimester values, the glutathione level in term decidua seems to be much higher as reported earlier (7). Knapen et al. reported a level in decidua, which is approximately 5 times higher than in placenta. These results might indicate that decidual GSTs found in early gestation have other functions or is not as important as in third trimester pregnancy.

In conclusion, we have shown that early in embryonic and foetal development cysteine, glutathione, GST isoforms, and GST enzyme activity are expressed in considerable amounts in most tissues examined. In contrast to adult tissue, $GSTP_1$ is the predominant GST isoform in embryonic and foetal organs, whereas moderate levels of GSTA and $GSTM_1$ were found in comparison with adults, which might point to different functions of GSTs as compared to adult tissues.

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Paragraph 4.2

Distribution of components of the glutathione detoxification system across the human placenta after uncomplicated vaginal deliveries.

Maarten T.M. Raijmakers, Sophia W.M. Bruggeman, Eric A.P. Steegers, Wilbert H.M. Peters.

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Abstract

The function of the glutathione-related detoxification system plays an important role to ensure an uncomplicated pregnancy outcome. This study was performed to investigate whether the components of the glutathione-related detoxification system are equally distributed among the different cotelydons in the human placenta. We measured glutathione, cysteine, glutathione S-transferase (GST) isoenzyme levels (GSTA1+A2, GSTP1, GSTM1 and GSTT1), enzyme activities of glutathione S-transferase and glutathione peroxidases, protein carbonyl levels, and antioxidant capacities at twelve different standardised positions in six placentas from healthy women after uncomplicated pregnancy and vaginal delivery. Data were statistically evaluated with a Friedman two-way ANOVA with Bonferroni correction. "Foetal"-side values were not significantly different from those at the "maternal"-side. Except for GSTA1+A2, no significant differences were found between different sampling sites indicating that the distribution of all parameters measured was homogenous throughout the placenta. Since levels of GSTA1+A2 were minor compared to those of GSTP1 and GSTT1, the clinical relevance of this heterogeneity may be limited. These results implicate that the location of sampling is not important as long as biopsies are taken from physiological cotelydons.

Introduction

An optimal maternal-foetal exchange is necessary for a successful pregnancy. Knowledge of placental function and anatomy provides insight into normal as well as pathologic pregnancy. Several complications of pregnancy including intra uterine growth restriction and preeclampsia (1) seem to be related to poor trophoblast invasion and/or a placental insufficiency, which may result in placental ischaemia and oxidative stress (2,3). However, it is not yet understood how a reduction in placental perfusion triggers the characteristic widespread maternal oxidative stress and endothelial dysfunction responsible for the development of severe preeclampsia.

In the defence against oxidative stress, glutathione peroxidases (GPXs) play an important role (4,5). They catalyse the reduction of hydrogen peroxide (H_2O_2) and organic hydroperoxides, thereby simultaneously oxidising glutathione (GSH) (6). Two major types of GPX have been described: a selenium dependent GPX (SeGPX), which is reactive to both organic hydroperoxides and H_2O_2, whereas the other group is selenium independent and probably mainly consists of glutathione S-transferases (GSTs). This second group of GPXs shows only reactivity towards organic hydroperoxides (4,5).

However, the main function of GSTs is the conjugation of GSH to a wide variety of substrates making these compounds less biologically active and more water soluble, thus facilitating their excretion via urine or bile (7). Human cytosolic GSTs are encoded by at least four related gene families constituting of class Alpha, Pi, Mu and Theta. These classes comprehend several hetero- or homodimeric isoforms (7). They display strong substrate specificity though some substrate overlap may occur. Expression of particular isoenzymes is tissue specific and might reflect the presence of certain substrates, representing an adaptive mechanism.

In addition to its role in GST and GPX enzyme activity GSH, a tripeptide possessing a reactive sulfhydryl group provided by cysteine, has several other functions such as storage and transport of cysteine, maintenance of proteins and thiols in a reduced form, and direct protection against reactive oxygen species (ROS) (8). An overview of glutathione and related enzyme system in reproduction is given by Knapen et al. (5).

Placental detoxification seems to play an important role during pregnancy, which was shown by the presence of large amounts of GSH, GST isoenzymes and GST and GPX enzyme

activity (5,9-11). Furthermore, recent data suggest that increased placental oxidative stress, measured by elevated levels of protein carbonyls and a lower antioxidant capacity might play a role in the development of pregnancy complications like preeclampsia (12). In many studies on placental tissue, including those on placental detoxification systems, no details are given on the exact site of sampling and whether the parameters under study are equally distributed throughout the placenta. The transverse flow of foetal blood flow in a cotyledon towards maternal flow in the intervillous space could lead to oxygen tension differences and local oxidative stress. Therefore, the expression and function of the above mentioned enzyme system could be different in "maternal"- versus "foetal"-side of the placenta. The objective of our study was to investigate the distribution of several components of the glutathione related detoxification system as well as of markers for oxidative damage and non-enzymatic antioxidant capacity across the human placenta after uncomplicated vaginal delivery.

Materials and methods

Tissue samples

The Institutional Review Board of the University Medical Centre Nijmegen approved the study protocol. Immediately after delivery, six placentas were collected from healthy women with uncomplicated pregnancy outcome. From each placenta six standardised biopsies throughout the whole thickness of the placenta were taken and each divided into two samples originating from "maternal"- and "foetal"-side of the placenta, as indicated in Figure 4.1. Therefore, in total 12 samples of each placenta were analysed. Biopsies were directly frozen in liquid nitrogen and samples were stored at –80°C until analysis.

Before analysis, homogenates (20% w/v) were prepared by adding 4 volumes of homogenising buffer (0.25 M saccharose, 20 mM Tris/HCl buffer pH 7.4, and 1 mM dithiothreitol) to the samples and tissue was homogenised by 5 to 10 strokes at 2500 rpm with a motor-driven glass/teflon homogenizer (B. Braun Melsungen AG, Germany). Subsequently, homogenates were centrifuged for 75 min at 150,000x*g* and 4°C (Optima L-70K, Beckmann Instruments, Palo Alto, CA, USA) after which supernatant (cytosolic fraction) was stored in small aliquots at -30°C or -80°C for the appropriate analysis. Total protein content was

determined according to the method of Lowry et al. (13) using bovine serum albumin as a standard.

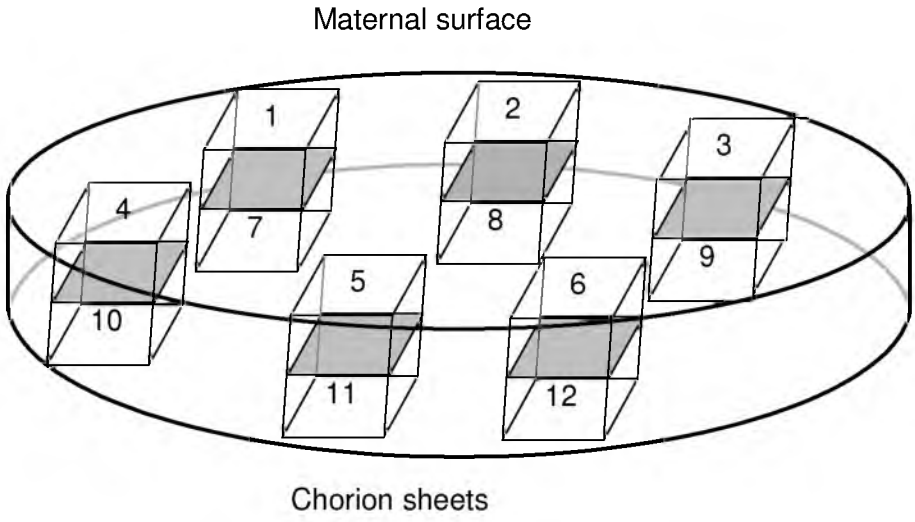

Figure 4.1. A schematic illustration of the human placenta and the regions of which samples were obtained. Each placenta was placed on a clean towel in a similar way; with the umbilical cord placed downwards and located on upper part of the placenta. Subsequently, six transversal biopsies were taken at the indicated places and each was sliced into two pieces of equal size as indicated, resulting in twelve biopsies.

Measurement of non-protein bound glutathione and cysteine

For the measurement of non-protein bound levels of cysteine (CYS) and GSH, proteins were precipitated immediately after ultracentrifugation by adding 100µL 12% perchloric acid to 10µL cytosol, followed by subsequent mixing and centrifugation for 15 min at 16,000x*g* and 4°C. Clear supernatants were stored at –80°C. Concentrations of CYS and GSH were determined using the method described earlier (14) using calibration curves for each thiol, which were run in parallel with the samples. Cysteine and glutathione concentrations were expressed in nmol/mg cytosolic protein.

Quantification of glutathione S-transferase isoenzymes

Levels of the GST isoenzymes were determined as described before (14) using specific antibodies against GST Alpha (GSTA1+A2), Pi (GSTP1), Mu (GSTM1) and Theta (GSTT1). For each isoenzyme, the 12 samples from each placenta were quantificated in the same run. Amounts of each GST isoenzyme were expressed in ng/mg cytosolic protein. The limit of detection of the immunoblot method is approximately 10 ng/mg protein.

Determination of glutathione S-transferase enzyme activity

Total cytosolic glutathione S-transferase (GST) activity was measured by a spectrophotometric assay according to Habig et al. (15), using 1-chloro-2,4-dinitrobenzene (CDNB) as a substrate. GST activity was expressed in nmol/min.mg cytosolic protein.

Analysis of glutathione peroxidase enzyme activity

To measure both selenium dependent (SeGPX) and total glutathione peroxidase (TGPX) enzyme activity, the method according to Howie et al. (16) was followed. Cumene hydroperoxide and H_2O_2 were used as substrates for determination of TGPX and SeGPX activities, respectively. Enzyme activities were expressed in nmol/min.mg cytosolic protein.

Placental antioxidant capacity / oxidative stress

Antioxidant capacity of the cytosolic fractions was determined using the "Ferric Reducing Ability of Plasma" (FRAP) assay, essentially described by Benzie et al. (17) and adapted for tissue cytosol by Zusterzeel et al. (12). Antioxidant capacity was expressed in nmol ferrous ion equivalent per mg cytosolic protein.

To determine the amount oxidative protein damage, as marker for oxidative stress, we analysed the amount of protein carbonyls using an Enzyme Linked Immunosorbent Assay (ELISA) as described by Zusterzeel et al. (12). Carbonyl levels were expressed in nmol/mg cytosolic protein.

Statistical analysis

Since almost all parameters examined showed a large intra-individual variation, we tested if the between-placenta was larger than the within-placenta variation using the Wilcoxon matched-pairs signed rank test. Median between-placenta variation (51%) was larger than the within-placenta variation (33%), p=0.003. To correct for this intra-individual variation we transformed our data to relative values by calculating the mean of all twelve sites sampled per placenta. Individual sites were expressed as a percentage towards this mean. Hereafter, relative values of the 6 placentas were grouped per localization. To test for differences between "maternal"- and "foetal"-side biopsies the Wilcoxon matched-pairs signed rank test was performed, whereas in order to test the homogeneity of each parameter across the placenta a Friedman two-way ANOVA was performed to test for differences between the 12

sites of sampling. A Bonferroni correction was used to correct for multiple testing and significance was reached when $p<0.01$.

When appropriate, differences between individual locations were analysed using the Wilcoxon matched-pairs signed rank test with Bonferroni correction for multiple testing. When $p<0.001$ differences were considered significant.

Correlations between different parameters were calculated using the Spearman-Rank Correlation-Coefficient test. Significance was reached when $p<0.05$.

Results

Table 4.5 presents the median (range) levels per placenta as measured for glutathione, cysteine, glutathione S-transferase isoenzymes, glutathione S-transferase activity, glutathione peroxidase activity, antioxidant capacity and protein carbonyls. In Table 4.6 the median (range) of the relative values of the above mentioned parameters grouped per sample position are given.

No significant difference in any of the parameters investigated were found between "maternal"- and "foetal"-side biopsies or any of the sites sampled, except for GSTA1+A2, which showed a heterogeneous expression between the 12 sampled sites ($P<0.001$). However, when sample positions were mutually tested to further specify where the difference was located, no statistical difference could be found. This indicates that all parameters, except GSTA1+A2, were homogeneously distributed along the cotelydons.

Non-protein bound glutathione and cysteine

Median values of glutathione and cysteine between the six placentas varied from 7.8-15.1 and 0.06-1.48 nmol/mg protein, respectively. A significant correlation was found between the amounts of glutathione and cysteine ($r_s=0.74$, $p<0.0001$).

Glutathione S-transferase isoenzymes

All biopsies contained variable amounts of the four classes of the GST family, except for GSTM1 of which no expression was found in placenta B. The median values in the six placentas of GSTA1+A2, GSTP1, GSTM1 and GSTT1 varied from 107-276, 354-7113, 0-131 and 407-1288 ng/mg protein, respectively. GSTP1 was the predominantly expressed

isoenzyme, followed by GSTT1. Overall, the total GST isoenzyme pool was composed of approximately 68% GSTP1, 25% GSTT1, 5% GSTA1+A2 and 2% GSTM1.

Glutathione S-transferase enzyme activity

Median GST enzyme activities in the six placentas varied between 102-289 nmol/min.mg protein. GST enzyme activity significantly correlated with the expression of GSTA1+A2 (r_s=0.69, p<0.0001), GSTP1 (r_s=0.85, p<0.0001), GSTT1 levels (r_s=0.44, p=0.0001) and the total sum of the GST isoenzymes (r_s=0.72, p<0.0001), whereas, no significant correlation between expression levels of GSTM1 and the GST enzyme activity could be found (r_s=0.01, p=0.90).

Glutathione peroxidase enzyme activity

Median values of the SeGPX enzyme activity of the six placentas varied from 286-409 nmol/min.mg protein, whereas those of the TGPX enzyme activity ranged from 164-302 nmol/min.mg protein. A significant correlation was found between SeGPX enzyme activity and GSTA1+A2 (r_s=0.34, p=0.003), GSTP1 (r_s=0.40, p=0.0006), all GST (r_s=0.40, p=0.0006) isoenzyme levels or GST enzyme activity (r_s=0.34, p=0.003). However, neither correlations between TGPX enzyme activity and GST isoenzymes nor GST enzyme activity could be found.

Placental antioxidant capacity / oxidative stress

Median levels of the antioxidant capacity were between 8.3-20.2 nmol/mg protein, whereas those of the protein carbonyls varied from 2.0-65.5 nmol/mg protein. No significant correlation could be demonstrated between placental antioxidant capacity and protein carbonyl levels.

Discussion

Non-protein bound glutathione and cysteine

Our results indicate that the metabolic requirement for these thiols is the same in different placental cotyledons. Median (range) glutathione levels in our study were lower as compared to the study by Knapen et al. (9); 12.4 (3.3-38.6) versus 25.6 (12.9-49.7) nmol/mg protein,

respectively, which might be due to the small number of placentas analysed or the fact that only placentas after caesarean were used (9).

Malloy et al. (18) assessed total (protein bound as well as free) cysteine levels, but did not relate them to the protein content of the tissue and reported higher levels than those in our study (190 versus ±50 μmol/L). Since approximately 60 percent of total cysteine is protein bound (19,20) these higher levels can thus be explained.

The correlation between levels of cysteine and glutathione may indicate the importance of cysteine in glutathione synthesis and of glutathione in the storage of cysteine.

Glutathione S-transferase isoenzymes

Since the expression of GSTP1, GSTT1 and GSTM1 was homogeneous, no special requirement for one of these isoenzymes at specific sites in the placenta seems to be needed. The predominant expression of GSTP1 (approximately 65% of the total GST content) is similar to that in embryonic and early foetal tissue (14) and most other adult tissues, except for liver in which GSTAlpha is mainly expressed (7). It was proposed that class Pi isoenzymes act *in vivo* to detoxify α,β-unsaturated aldehydes and organic peroxides, which levels increase during oxidative stress (4,21). It is not known whether these compounds represent a major group of substrates for the GSTs in placenta. However, assuming that a certain extent of GST isoenzyme expression may result from adaptation to toxic compounds, the high GSTP1 levels might indicate presence of oxidative compounds even during uncomplicated pregnancies (22,23).

The high median (range) levels of GSTT1, 902 (242 – 2033 ng/mg protein) are in contrast with those found by Zusterzeel et al. (10), 183 (0 – 363 ng/mg protein). This discrepancy could be due to differences in storage of placental tissue. GSTT1 is a relatively unstable enzyme (7) in contrast to the other GSTs quantified here, which showed to be stable up to at least two years storage at –20°C (24). In our study placental tissue was stored for two months at –80°C, whereas in the study of Zusterzeel et al. it was stored up to three years at –30°C, which may have resulted in some breakdown of GSTT1. Little is known about the precise role of GSTT1 in foetal and maternal detoxification, however, the high levels of GSTT1 (approximately 25% of total GSTs) suggest that GSTT1 is important in placental detoxification.

Table 4.5. Glutathione, cysteine, glutathione S-transferase isoenzymes, glutathione S-transferase enzyme activity, glutathione peroxidase enzyme activity, antioxidant capacity and protein carbonyl levels in the six different placentas (A-F).

	A	B	C	D	E	F
Glutathione (nmol/mg protein)	13.2 (3.5-25.8)	15.1 (3.66-24.7)	14.7 (8.06-24.0)	8.1 (3.3-31.4)	8.8 (3.6-24.9)	7.8 (4.1-38.6)
Cysteine (nmol/mg protein)	0.78 (0.02-2.32)	1.48 (0-14.4)	1.01 (0.08-9.28)	0.16 (0.01-9.52)	0.29 (0.06-0.97)	0.06 (0.01-1.43)
GSTA1/GSTA2 (ng/mg protein)	120 (97-219)	129 (53-255)	117 (41-233)	107 (25-122)	276 (197-366)	236 (163-351)
GSTP1 (ng/mg protein)	1151 (646-1867)	1839 (895-4565)	813 (456-2100)	354 (194-1109)	7113 (3611-12486)	3084 (1842-5276)
GSTM1 (ng/mg protein)	79 (50-143)	ND	131 (90-196)	22 (8-67)	97 (72-122)	12 (7-38)
GSTT1 (ng/mg protein)	407 (275-506)	425 (242-543)	1049 (667-1339)	991 (711-1391)	973 (640-1637)	1288 (861-2033)
GST activity (nmol/min.mg protein)	160 (136-193)	226 (161-304)	143 (107-189)	102 (80-123)	289 (214-334)	197 (130-434)
SeGPX activity (nmol/min.mg protein)	286 (228-341)	319 (289-390)	354 (205-428)	356 (277-518)	383 (333-471)	409 (333-522)
TGPX activity (nmol/min.mg protein)	164 (140-193)	210 (179-275)	282 (235-325)	251 (208-366)	233 (217-296)	302 (225-405)
Antioxidant capacity (nmol/mg protein)	14.2 (12.1-20.2)	15.5 (11.6-17.6)	14.2 (8.3-15.0)	12.8 (11.1-14.2)	12.2 (10.8-13.8)	14.4 (12.9-18.2)
Carbonyls (nmol/mg protein)	14.0 (8.0-30.0)	17.0 6.0-26.0)	20.0 (1.0-67.0)	2.0 (0-21.0)	15.0 (5.0-160)	65.5 (19.0-382)

Data are presented as median (range) of the 12 biopsies per placenta. ND = not detectable

Table 4.6. Relative contributions per placental biopsy site for glutathione, cysteine, glutathione S-transferase isoenzymes, glutathione S-transferase activity, glutathione peroxidase activity, and antioxidant capacity and protein carbonyls.

Position	1	2	3	4	5	6	7	8	9	10	11	12
Glutathione	75 (26-144)	58 (34-186)	95 (44-210)	75 (30-115)	74 (34-201)	53 (27-120)	112 (35-285)	134 (49-832)	95 (0-143)	127 (42-1276)	78 (38-262)	104 (0-234)
Cysteine	8 (0-87)	20 (2-271)	31 (9-327)	32 (5-156)	93 (3-431)	19 (1-35)	97 (4-764)	45 (0-280)	60 (0-386)	31 (3-177)	155 (8-330)	138 (25-348)
GSTA1/GSTA2[a]	101 (39-135)	81 (71-114)	73 (60-83)	63 (29-103)	73 (42-87)	89 (44-141)	148 (104-170)	130 (80-188)	120 (106-151)	105 (93-139)	96 (87-133)	110 (71-146)
GSTP1	64 (52-138)	80 (65-97)	78 (47-149)	77 (45-129)	89 (42-106)	118 (67-156)	100 (81-159)	93 (76-148)	107 (65-194)	67 (42-158)	91 (62-122)	158 (67-260)
GSTM1	83 (68-159)	85 (62-101)	147 (73-169)	72 (28-246)	66 (45-85)	104 (54-138)	113 (77-220)	94 (78-115)	144 (116-182)	88 (50-149)	101 (81-125)	83 (60-104)
GSTT1	73 (60-129)	90 (77-93)	87 (69-101)	87 (64-107)	90 (62-100)	104 (65-130)	97 (85-125)	100 (72-124)	113 (98-133)	116 (80-132)	113 (58-158)	118 (113-142)
GST activity	88 (75-117)	90 (79-130)	94 (75-116)	84 (69-100)	96 (76-112)	112 (84-119)	98 (86-113)	97 (89-128)	111 (101-131)	95 (77-124)	96 (60-111)	111 (102-199)
SeGPX activity	106 (89-123)	92 (76-117)	91 (88-119)	98 (88-115)	106 (93-142)	110 (89-121)	103 (79-116)	96 (86-107)	97 (90-123)	87 (59-111)	101 (80-109)	104 (92-124)
TGPX activity	97 (79-120)	90 (84-105)	97 (82-117)	89 (84-114)	102 (92-142)	110 (98-138)	93 (77-110)	92 (81-116)	105 (92-116)	96 (84-105)	101 (86-113)	110 (102-130)
Antioxidant capacity	97 (88-116)	101 (87-115)	102 (84-110)	105 (87-116)	110 (93-139)	98 (74-103)	97 (88-113)	100 (92-108)	95 (77-109)	102 (91-112)	102 (69-113)	101 (64-126)
Carbonyls	108 (19-209)	56 (19-119)	35 (15-112)	39 (0-56)	67 (44-115)	119 (4-208)	89 (18-141)	60 (40-174)	98 (23-248)	80 (39-226)	98 (40-163)	254 (52-491)

Data are presented as percentages in medians (range) for the relative contribution of each biopsy site.

Statistics: [a] $P<0.001$ (Friedman two-way ANOVA)

Expression of GSTM1 is low or even absent, therefore, no major contribution to detoxification in placental tissue is expected. In addition, lack of GSTM1 expression in placenta B, probably due to a GSTM1 null genotype (25), appeared to be consistent with an uncomplicated pregnancy.

In contrast with the other GST isoenzymes, expression of GSTA1+A2 was not homogeneous throughout the placenta, however, to which extent could not be demonstrated. Presence of either foetal or maternal blood in the biopsies could have only slightly influenced these levels, since blood hardly contains any GSTAlpha (26,27). Blood could have been removed by washing, but this might also influence cytosolic levels of GSTs. Additionally, the amount of GSTA1+A2 is relatively low as compared with those of GSTP1 and GSTT1, which may diminish the clinical relevance of this finding.

Glutathione S-transferase and glutathione peroxidase enzyme activities

Our findings confirm the results by McRobie et al. (28) on placental distribution of GST enzyme activity at four different locations. GST enzyme activity was comparable to results of Polidoro et al. (29) and those reported earlier by our group (9,10). However, Poranen et al. (30) reported a much higher GST enzyme activity (12.6 ± 5.1 μmol/min.mg protein) as compared to our study and those mentioned above, however, an explanation for this discrepancy cannot be given.

The correlation found between GST isoenzyme expression and enzyme activity indicates that higher expression of GSTs isoenzymes results in higher enzyme activity. However, the correlation between GST enzyme activity towards CDNB and GSTT1 levels was unexpected, since CDNB is no substrate for GSTT1 (7). Probably, levels of GSTT1 might be coupled to that of other GSTs. Although, GSTM1 has the highest specific activity with respect to CDNB (Hayes and Pulford, 1995; Satoh et al., 1995) no correlation between GSTM1 levels and GST enzyme activity was found. High levels of GSTP1 and GSTA1+A2, being responsible for the majority of the GST enzyme activity, might explain this because low GSTM1 levels (approximately 2% of total GSTs) may only slightly contribute.

In line with the other parameters of the GSH related detoxification system both SeGPX and TGPX enzyme activities were uniformly distributed throughout the placentas. The TGPX enzyme activity was in agreement with that found previously (30), however, both enzyme activities of SeGPX and TGPX were higher than found earlier by us (9). The low number of

placentas investigated or storage conditions, as described above for GSTT1, might be reasons for this discrepancy. Unfortunately, no data on the stability of GPX during storage is available. Furthermore, in the study by Knapen et al. (9) placentas were obtained after caesarean section, whereas here only placentas after vaginal delivery were used. Different circumstances, including repeated contractions during an extended period at vaginal birth or anaesthesia during caesarean section, might have variable effects on placental oxidative or chemical stress. It is likely that during periods of contraction maternal blood flow is discontinuous resulting in variable oxygen tension, which may generate placental oxidative stress (11). Since GPX provides first line defence against ROS (4), the induction of GPX enzymes are an adaptive mechanism to prevent oxidative damage, which was also described in placentas from women with preeclampsia (9).

The absence of a correlation between TGPX enzyme activity and GST isoenzyme expression is in agreement with the suggestion of Hayes and McLellan (4) that the contribution of selenium independent GPX to TGPX enzyme activity is low. In this respect, correlations between SeGPX enzyme activity and GSTA1+A2, GSTP1 and total GST isoenzyme levels are remarkable. However, since GSTs are able to scavenge ROS and free radicals, GPX enzyme activity could be induced in parallel with GST expression.

Placental antioxidant capacity / oxidative stress

Protein modifications, either caused by direct oxidative attack or by lipid peroxidation products, can lead to the formation of protein carbonyls (12), which can be counteracted by antioxidants. Therefore, higher protein carbonyl levels or lower antioxidant capacity may serve as markers of oxidative stress. Our results indicate that oxidative damage and antioxidant capacity are equally distributed throughout the placenta. Protein carbonyl levels were higher and antioxidant capacities were lower than those found by Zusterzeel et al. (12) in placentas obtained after caesarean section. These data may provide additional evidence for the hypothesis that a vaginal delivery results in more placental oxidative stress.

In line with the finding of Zusterzeel et al (12) we could not demonstrate a correlation between protein carbonyl levels and antioxidant capacity. This can partially be explained by the fact that the FRAP assay does not measure SH-containing antioxidants such as GSH (17), which greatly contribute to the antioxidant capacity in placental tissue (9).

Conclusion

In placentas from uncomplicated pregnancies after vaginal delivery, components of the glutathione related detoxification system as well as oxidative stress markers were homogeneously distributed at the twelve positions investigated, whereas only GSTA1+A2 levels seemed to vary. Assuming that in complications of pregnancy only placental function is reduced and the physiological structure remains intact, our results implicate that the location of sampling placental tissue is not important as long as biopsies are taken from a physiological normal cotelydon without infarctions. However, since differences were found in placentas obtained after vaginal deliveries and those after caesarean section of previous studies for most of the parameters mentioned above, this might indicate higher placental oxidative stress during vaginal delivery, therefore, it is important to match for type of delivery.

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Chapter 5

Thiols in umbilical cord and maternal plasma in normal pregnancy.

Paragraph 5.1

Thiols in umbilical cord and maternal plasma in normal pregnancy.

Maarten T.M. Raijmakers, Eva Maria Roes, Eric A.P. Steegers, Bas van der Wildt, and Wilbert H.M. Peters.

Clinical Chemistry 2001; 47:749-51

Abstract

Objective: Data on foetal concentrations of the important thiols cysteine, homocysteine, and cysteinylglycine are scarce. We determined reference levels in arterial and venous umbilical cord plasma and investigated maternal-foetal interactions of these thiols.

Methodology: Reference levels of cysteine, homocysteine, and cysteinylglycine were determined by high performance liquid chromatography in 195 arterial and venous umbilical cord plasma samples as well as in 35 corresponding maternal plasma samples.

Results: Median reference levels in µmol/L (central 0.95 interval) in venous and arterial umbilical cord plasma for cysteine, homocysteine and cysteinylglycine are 207 (146 - 299) and 203 (134 - 303), 9.6 (4.8 - 17.4) and 8.8 (4.9 - 20.4), and 33 (20 - 50) and 35 (20 - 51), respectively. Cysteine and homocysteine levels are significantly lower in arterial versus venous samples ($p=0.0002$ and $p=0.009$, respectively), whereas levels of cysteinylglycine ($p=0.005$) are higher in arterial umbilical cord. Arterial umbilical cord levels of homocysteine ($p<0.0001$) are lower, whereas venous umbilical cord levels of cysteine ($p=0.04$) were higher and levels of homocysteine ($p<0.0001$) were lower as compared to maternal concentrations.

Conclusion: Lower homocysteine and cysteine concentrations in arterial umbilical cord as compared to venous levels could implicate that these thiols are used in foetal metabolism. Cysteine may be actively transported from maternal to foetal circulation indicating that cysteine is an essential amino acid for the foetus.

Introduction

Aminothiols such as cysteine and homocysteine have important functions in the human body, where the presence of the reactive sulfhydryl (-SH) group dictates its chemistry (1). Several studies suggested that thiol metabolism is altered during pregnancy. In normal pregnant women plasma levels of cysteine and homocysteine are lowered, whereas elevated homocysteine and cysteine levels are seen in pathologic conditions such as preeclampsia, in which oxidative stress (disturbance of the redox-balance) seems to play an important role (2-4).

Thiols may have important physiological functions in foetal metabolism as well. Although protein and amino acid turnover in the human placenta have been studied extensively (5-7), currently only few data concerning foetal levels of thiols and placental maternal-foetal thiol interactions are available (8). During normal pregnancy foetal growth depends on a steady state supply of nutrients from the mother through the placenta and a clear correlation between maternal and foetal amino acid and homocysteine levels was shown (6-8). Decreased levels of amino acids in the umbilical artery as compared to umbilical vein have been interpreted as uptake of amino acids into foetal tissues where they may be used in protein biosynthesis or as source of energy (5).

We studied foetal and maternal thiol plasma concentrations in normal pregnancies in order to achieve two aims: i.) to determine reference levels of cysteine, homocysteine, and cysteinylglycine in arterial and venous umbilical cord plasma, and ii.) to get more insight into maternal-foetal thiol interactions by correlating umbilical cord thiol levels with those of the mother.

Materials and Methods

Arterial and venous umbilical cord plasma samples from 320 consecutive neonates were drawn immediately after birth in preheparinised 2 mL tubes (no 260545, Kemper Medical BV, Uden, The Netherlands) from March 1997 to January 1998 at the Department of Obstetrics and Gynecology of the 'Nij Smellinghe' Hospital, Drachten, The Netherlands, of

which the Institutional Review Board approved the study protocol. A small volume was used for the assessment of blood gas values on an ABL-330 analyzer (Radiometer Nederland BV, Zoetermeer, The Netherlands). Samples with a difference between arterial and venous pH smaller than 0.02 pH units or neonates born from women with a diastolic blood pressure during gestation above 90 mmHg, an umbilical artery pH below 7.20, a birth weight below the 10th percentile according to Kloosterman (9), or a gestational age of less than 37 weeks were excluded from the study. In parallel with the umbilical cord samples antecubital maternal venous blood samples were collected after informed consent was given. Samples were taken in sitting position after 5 min of rest in 5 mL heparinised tubes (no 367684, Becton and Dickinson, Leiden, The Netherlands), either less than 4 hours before elective caesarean delivery or less than 15 minutes after vaginal birth. Umbilical cord and maternal blood was centrifuged within 10 min at 1,200xg for 10 min at room temperature. Both umbilical and maternal plasma samples were stored at -30°C until analysis. Plasma levels of cysteine, homocysteine, and cysteinylglycine in 195 umbilical cord (102 males / 92 females; no gender was recorded from 1 neonate) and 35 maternal samples were determined by high performance liquid chromatography as described previously (2).

After log transformation to approach normalization, data were analysed by the paired t-test in order to assess statistical differences between maternal, venous and arterial umbilical cord values. The Spearman rank coefficient of correlation was calculated when appropriate using Astute for Microsoft Excel 5.0. A p-value less than 0.05 was considered significant.

Results

Both maternal characteristics studied (age, gestational age, blood pressure, birth weight, and placental weight) and blood gas values (pO_2, $pHCO_3^-$, pH, base deficit, and pCO_2) are representative of the population as admitted for term deliveries in the 'Nij Smellinghe' Hospital. The characteristics of the subgroup (n=35) were not statistically different from the total study group.

Levels of cysteine and homocysteine are significantly lower in arterial as compared to venous umbilical cord plasma (p=0.0002 and p=0.009, respectively), whereas levels of cysteinylglycine are significantly higher (p=0.005), Table 5.1.

Table 5.1. Thiol levels in umbilical cord plasma (n=195).

	Venous		Arterial		P-level
Cysteine	207	(146 - 299)	203	(134 - 303)	0.0002
Homocysteine	9.6	(4.8 - 17.4)	8.8	(4.9 - 20.4)	0.009
Cysteinylglycine	33	(20 - 50)	35	(20 - 51)	0.005

Notes: Data are presented as medians (central 0.95 interval) in µmol/L.

In the sub-population of 35 cases, venous maternal cysteine levels are lower than those in venous umbilical cord (p=0.04), whereas there was a tendency for higher cysteine levels in venous umbilical cord compared with arterial levels (p=0.06), Figure 5.1. Also arterial umbilical cord cysteine tended to be higher than maternal concentrations, however, no significant difference was found (p=0.1). A positive correlation was found between levels of cysteine in maternal and venous umbilical cord plasma (r=0.84, p<0.0001), venous and arterial umbilical cord (r=0.82, p<0.0001), and arterial umbilical cord and maternal plasma (r=0.81, p<0.0001).

Homocysteine showed a decreasing concentration gradient from maternal to venous and arterial umbilical cord plasma (p=0.001 and p=0.04, respectively). This gradient resulted in a significant lowered level of homocysteine in arterial umbilical cord plasma as compared with maternal plasma (p<0.0001). Levels of homocysteine were positively correlated between maternal and venous umbilical cord, venous and arterial umbilical cord, and arterial umbilical cord and maternal plasma (r=0.83, p<0.0001; r=0.82; p<0.0001; r=0.79, p<0.0001; respectively). No correlations were found between maternal, arterial and venous umbilical cord homocysteine levels and neonatal weight (r=0.07, p=0.9; r=0.12, p=0.1; and r=0.0076, p=0.9; respectively).

No significant differences were found for cysteinylglycine levels between maternal and umbilical venous or arterial samples. However, a positive correlation was found between levels of cysteinylglycine in the umbilical artery and the umbilical vein (r=0.59, p<0.001).

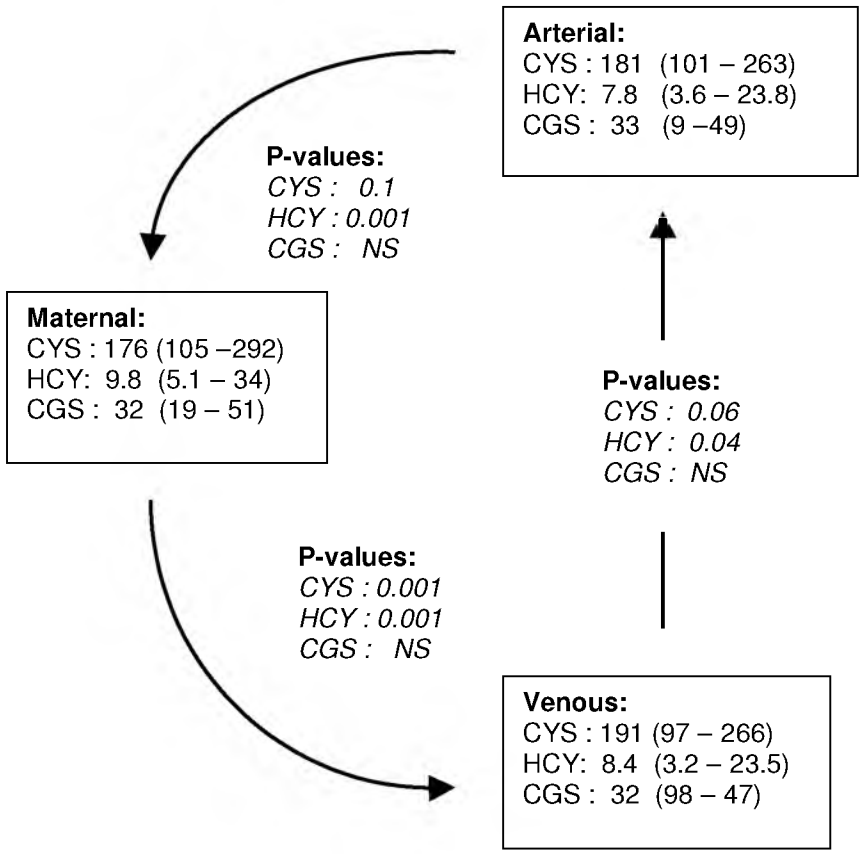

Figure 5.1. Maternal and corresponding venous and arterial umbilical cord plasma thiol levels (n=35). Data are presented as median (central 0.95 interval) in µmol/L. Abbreviations: CYS = cysteine, HCY = homocysteine, CGS = cysteinylglycine, and NS = non-significant.

Discussion

Plasma samples analysed here are from pregnancies with uncomplicated outcome and consequently the values for cysteine, homocysteine, and cysteinylglycine in neonates can be used as reference levels.

Levels of cysteine and homocysteine are lower in arterial umbilical cord plasma as compared to venous levels, indicating the uptake of both thiols into foetal circulation, where they may be used in the biosynthesis of glutathione and protein. Homocysteine passes the maternal-foetal barrier through a decreasing concentration gradient. In contrast, cysteine is transported from maternal to foetal side against a concentration gradient, probably by an active transport

mechanism. Cysteine needs to be taken up in this way by the foetus, because homocysteine cannot be converted to cysteine in the foetus due to the absence of cystathionine-ß-synthase, the enzyme that converts homocysteine to cysteine in adults (10).

Homocysteine values are higher as compared with those in Northern American population (8,11), which might be explained by a higher intake of folate and B vitamins in the American populations as discussed earlier by den Heyer et al. (12). However, maternal levels in this study were comparable (p=0.45) to those previously reported in another study group (2), where we also presented that non-pregnant values of plasma thiols were higher as compared to corresponding values in normal pregnancy, most probably due to an increased plasma volume in pregnancy (2).

In summary; cysteine, which is an essential amino acid in the human foetus may be actively transported in the placenta from the maternal to the foetal circulation, where it is used in biosynthesis processes. Similarly, the foetus may extract homocysteine from the maternal circulation.

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Paragraph 5.2

Umbilical glutathione levels are higher after vaginal birth than after cesarean section.

Maarten T.M. Raijmakers, Eva Maria Roes, Eric A.P. Steegers, Bas van der Wildt, and Wilbert H.M. Peters.

Abstract

Objective(s): The primary goals of our study were to establish reference levels for glutathione in arterial and venous umbilical plasma and to investigate possible differences between vaginal delivery and delivery by cesarean section.

Study design: Glutathione levels were assessed in both arterial and venous umbilical plasma samples of neonates after vaginal delivery (n=140) or after delivery by cesarean section (n=38). From a subset of women who delivered vaginally, also maternal plasma glutathione levels (n=14) were measured.

Results: Both median (5^{th}-95^{th} percentile) glutathione levels in venous as well as arterial umbilical samples were higher after vaginal delivery compared with cesarean section, 2.7 (0.9-7.3) versus 2.0 (0.6-11.5) µmol/L ($P<0.03$) and 3.5 (0.6-22.7) versus 2.3 (0.7-24.3) µmol/L ($P<0.02$), respectively. Maternal glutathione levels were higher, 7.8 (4.3-10.6) µmol/L, as compared to both venous ($P<0.001$) and arterial ($P<0.02$) umbilical levels of their offspring.

Conclusion: Since during oxidative stress high amounts of oxidised glutathione may be excreted from the erythrocyte, the higher umbilical glutathione levels after vaginal delivery suggests that vaginal delivery is associated with higher oxidative stress than delivery by cesarean section.

Introduction

The presence of the reactive sulfhydryl (-SH) group dictates the chemistry of aminothiols such as cysteine, homocysteine and glutathione (1). In humans, glutathione has several important functions. Besides storage and transport of amino acids, glutathione plays an important role in detoxification of toxic compounds by action of glutathione S-transferases and in scavenging of free radicals and reactive oxygen species by action of glutathione peroxidases. By the latter process glutathione is important in the maintenance of the cellular redox balance (1,2). Several studies suggested that glutathione metabolism is altered during pregnancy. In normal pregnancy plasma levels of glutathione are lower as compared to those in the non-pregnant state, whereas even lower levels are seen in gestational complications including preeclampsia, in which oxidative stress (disturbance of the redox-balance) seems to play an important role (3-6).

Foetal growth depends on a continuous supply of nutrients from the mother through the placenta and a clear correlation between levels of maternal and foetal amino acids was demonstrated (7-9). Lower levels of amino acids in the umbilical artery as compared to the umbilical vein have been interpreted as uptake of amino acids into foetal tissues, where they may be used in protein biosynthesis or as source of energy (10). Drugs and other toxic compounds are able to pass the placental barrier. Therefore, besides the storage and transport of amino acids, glutathione may have important physiological functions in foetal detoxification (11). Although protein- and amino acid turnover in the human placenta has been studied extensively (7-10), no data on glutathione concentrations and placental-maternal glutathione interactions are available yet. In a previous study we hypothesised that repeated contractions for a prolonged period may lead to more placental oxidative stress during vaginal delivery as compared with delivery by cesarean section (12). Therefore, we studied foetal and maternal glutathione plasma concentrations in normal pregnancies after vaginal and cesarean deliveries in order to a) define reference levels of glutathione in arterial and venous umbilical cord plasma, b) to investigate possible differences between vaginal and cesarean deliveries, and c) to study maternal-foetal thiol interactions by correlating umbilical cord glutathione levels with those of the mother.

Materials and Methods

Arterial and venous umbilical cord plasma samples from 320 consecutive neonates were drawn immediately after birth in preheparinised 2 mL tubes (Kemper Medical BV, Uden, The Netherlands) from March 1997 to January 1998 at the Department of Obstetrics and Gynecology of the 'Nij Smellinghe' Hospital, Drachten, The Netherlands (13). The Institutional Review Board approved the study protocol. Samples of neonates born from women with gestational diabetes or other complications of pregnancy, a diastolic blood pressure during gestation above 90 mmHg, a gestational age less than 37 or more than 42 weeks, an umbilical artery pH below 7.20, or a birth weight below the 10th or above the 90th percentile according to Kloosterman (14), were excluded from the study. A small volume was used for the assessment of blood gas values on an ABL-330 analyzer (Radiometer Nederland BV, Zoetermeer, The Netherlands). When the difference between arterial and venous pH was smaller than 0.02 pH units, samples were excluded from further analysis. In parallel with the umbilical cord samples, antecubital maternal venous blood samples of 14 women, who delivered vaginally, were collected after informed consent was given (15). Maternal samples were taken in sitting position after 5 min of rest in 5 mL heparinised tubes (Becton and Dickinson, Leiden, The Netherlands) less than 15 minutes after vaginal birth. Umbilical and maternal blood was centrifuged within 10 min at 1,200xg for 10 min. Plasma was stored at -30°C until analysis.

Table 5.2. Population characteristics.

	Cesarean Section (n=38)	Vaginal delivery (n=140)
Maternal age (years)	29 (20 – 40)	29 (21 – 37)
Gestational age (weeks^{+days})	40^{+2} (38^{+3} – 42^{+0})	40^{+1} (37^{+5} – 41^{+6})
Parity	1 (0 – 2)	1 (0 – 3)
Diastolic blood pressure (mmHg)	80 (60 – 90)	78 (67 – 85)
Male offspring (number)	25 (63%)	76 (54%)
Birth weight (gram)	3490 (3094 – 3950)	3244 (2850 – 4055)
Placental weight (gram)	665 (525 – 810)	673 (500 – 875)

Data are presented as medians (5^{th} – 95^{th} percentiles)

Umbilical and maternal total glutathione levels in plasma (i.e. protein bound, reduced and oxidised non-protein bound glutathione) of 180 and 14 subjects respectively were determined by high performance liquid chromatography as described previously (3). Umbilical cord samples were divided into two groups, samples obtained after cesarean section (n=38) and samples collected after vaginal delivery (n=140) of which the characteristics are depicted in Table 5.2 and Table 5.3.

Table 5.3. Blood gas parameters in umbilical cord after cesarean section and vaginal delivery.

	Cesarean Section (n=38)	Vaginal delivery (n=140)
Venous:		
pO_2 (kPa)	28 (16-40)	27 (16 – 39)
pCO_2 (kPa)	43 (35 – 58)	39 (32 – 50)
HCO_3^- (mmol/L)	20 (16 – 23)	20 (16 – 23)
pH	7.33 (7.23 – 7.38)	7.34 (7.22 – 7.42)
BD	-4.0 (-8.1 – -0.9)	-4.4 (-9.0 – -1.1)
Arterial:		
pO_2 (kPa)	15 (9 – 31) [1]	18 (10 – 32) [1]
pCO_2 (kPa)	53 (41 – 64) [1]	53 (38 – 68) [1]
HCO_3^- (mmol/L)	20 (15 – 23) [2]	19 (15 – 23) [1]
pH	7.27 (7.15 – 7.33) [1]	7.26 (7.11 – 7.36) [1]
BD	-4.4 (-11.8 – -0.5)	-4.9 (-12.0 – -1.6) [1]

Data are presented as medians (5^{th} – 95^{th} percentiles). Abbreviations used: BD, base deficit

Statistics:

[1] $P<0.0001$ and [2] $P<0.001$ venous versus arterial umbilical cord

To assess differences between vaginal birth versus cesarean section, different type of vaginal delivery, elective cesarean section versus cesarean section during labor, or anesthetics used during cesarean section the Wilcoxon-Mann-Whitney test was performed. Statistical differences in glutathione levels and blood gas parameters between arterial and venous umbilical samples as well as differences in maternal, venous and arterial umbilical cord values of glutathione in the subgroup with vaginal deliveries were assessed with the Wilcoxon matched-pairs signed-ranks test. The Spearman rank coefficient of correlation was calculated when appropriate. All statistical tests were performed with Astute for Microsoft Excel 5.0. A P-value less than 0.05 was considered statistically significant.

Results

The characteristics of the population studied are representative for the population as admitted for term deliveries after uncomplicated pregnancies at the 'Nij Smellinghe' Hospital (Table 5.2). In the group with vaginal deliveries 42 neonates were born either by forceps (n=26) or vacuum extraction (n=16). The type of extraction did not significantly influence the results. Cesarean sections (n=38) were performed electively (n=15), without prior contractions, for breech presentation (n=1), cephalo-pelvic disproportion (n=8), repeat cesarean section (n=5) and neonatal auto-immune trombocytopenia (n=1), and during labor (n=23) for foetal distress (n=4), breech presentation (n=2), failure to progress during the first (n=9) or second stage (n=8). No differences in umbilical glutathione concentrations or blood gas values were found between elective cesarean sections compared with those during labor. Type of anesthesia (spinal (n=23), epidural (n=1) or general anesthesia (n=15)) during cesarean section did not influence glutathione levels. No statistical differences in population characteristics were found between women who delivered either vaginally or by cesarean section (Table 5.2). The characteristics of the subset of women who delivered vaginally, of whom also maternal plasma was analysed, were not statistically different from the total group of women with vaginal deliveries.

In neonates born after cesarean section, the following umbilical blood gas values (see Table 5.3) were significantly higher in venous as compared to arterial samples: pO_2 ($P<0.0001$), HCO_3^- ($P<0.001$) and pH ($P<0.0001$), whereas pCO_2 was lower ($P<0.0001$). After vaginal delivery the following parameters were significantly higher in venous than in arterial umbilical samples: pO_2 ($P<0.0001$), HCO_3^- ($P<0.0001$), base deficit ($P<0.0001$), and pH ($P<0.0001$), whereas pCO_2 ($P<0.0001$) was lower.

Levels of glutathione in both venous and arterial umbilical plasma (Table 5.4) were higher after vaginal compared with cesarean delivery, 2.7 (0.9 – 7.3) versus 2.0 (0.6 – 11.5) µmol/L ($P<0.03$) and 3.5 (0.8 – 18.7) versus 2.3 (0.7 – 24.3) µmol/L ($P<0.02$), respectively. After vaginal delivery median arterial umbilical concentrations of glutathione were higher as compared with those in venous umbilical cord ($P<0.0001$), whereas no statistical differences between arterial and venous umbilical samples were found after cesarean delivery. Maternal levels of glutathione, 7.8 (0.6 – 22.7) µmol/L, were higher as compared to both venous, 2.6

(0.9 – 7.3) µmol/L (P<0.001), and arterial, 3.6 (0.6 – 22.7) µmol/L (P<0.02), umbilical samples.

Table 5.4. Glutathione concentrations in umbilical and maternal plasma.

	n	Venous	Arterial	Maternal
Cesarean Delivery	39	2.0 (0.6 - 11.5)	2.3 (0.7 – 24.3)	
Vaginal Delivery	140	2.7 (0.9 – 7.3)[1]	3.5 (0.8 – 18.7)[2,3]	
Vaginal Subgroup	14	2.6 (0.9 - 7.3)[4]	3.6 (0.6 - 22.7)[5]	7.8 (4.3 - 10.6)

Values are given as µmol/L; median (5^{th} – 95^{th} percentile)

[1] P < 0.03 and [2] P < 0.02 cesarean versus vaginal delivery

[3] P < 0.0001 venous umbilical versus arterial umbilical plasma.

[4] P < 0.001 venous umbilical versus maternal plasma

[5] P < 0.02 arterial umbilical versus maternal plasma

Levels in venous and arterial umbilical cord plasma showed a positive correlation after both vaginal and cesarean deliveries (r_s=0.71, P<0.0001 and r_s=0.59, P<0.0001, respectively). However, in the smaller subgroup of vaginal deliveries no significant associations were found between maternal plasma levels and those in both arterial and venous umbilical cord (r_s=0.45, P=0.1 and r_s=0.32, P=0.3, respectively). Gestational age or blood gas parameters did not correlate with glutathione levels in umbilical samples of both cesarean and vaginal deliveries. Furthermore, neither significant associations between the duration of the first stage nor second stage of labor and the levels of glutathione could be found in both arterial (r_s=0.06, P=0.5 and r_s=-0.09, P=0.3, respectively) and venous umbilical samples (r_s=0.15, P=0.1 and r_s=-0.04, P=0.7, respectively) in the group of women with vaginal delivery.

Comment

Foetal glutathione concentrations were studied only in pregnancies with uncomplicated outcome, therefore the values presented here can be used as reference levels in neonates after vaginal or cesarean deliveries.

To our knowledge this is the first study that describes differences in arterial as well as venous umbilical glutathione concentrations between vaginal and cesarean deliveries. Both arterial

and venous levels of glutathione were found to be higher in umbilical samples taken from neonates after vaginal delivery compared with those after cesarean delivery. Furthermore, after vaginal delivery, but not cesarean section, arterial umbilical glutathione concentrations were higher than those in venous umbilical samples. These results might indicate that vaginal delivery is characterised by a higher level of oxidative stress as compared with cesarean delivery. In vaginal deliveries, the repeated contractions or the expulsion period may lead to differences in the oxygen tension of the foetal-placental unit, which probably causes local oxidative stress.

In periods of oxidative stress, characterised by the presence of high levels of reactive oxygen species (ROS), glutathione may by oxidised to glutathione disulfide by glutathione peroxidase in order to scavenge ROS (1,2). Subsequently, oxidised glutathione may be converted into reduced glutathione again by glutathione reductase, thereby consuming NADPH. Both enzymes were shown to be present during foetal development (16-18). However, when high amounts of oxidised glutathione are produced during extended periods of oxidative stress the erythrocyte may excrete oxidised glutathione in order to maintain intracellular redox-balance, which may result in higher glutathione levels in plasma (19). In this way the higher level in umbilical arterial plasma may be explained.

We could not demonstrate significant differences between umbilical glutathione levels in normally delivered neonates and that of neonates delivered by forceps or vacuum extraction. However, the similar glutathione levels in elective cesarean section and those found in cesarean section during labor, suggest that oxidative stress assumable arises during the second stage of vaginal delivery. Furthermore, absence of a correlation between venous and arterial umbilical glutathione levels with duration of first and second stage of labor may suggest that the physiological process of vaginal birth is associated with (local) oxidative stress and not the duration of delivery.

We previously showed that in placental tissue obtained after vaginal delivery the expression levels and enzyme activity of glutathione S-transferases and glutathione peroxidase were higher compared to those after cesarean section (12), which is in accordance with the above presented findings.

In the transport of amino acids across the placental basal and microvillous membranes, several distinctive transport systems have been characterised leading to a number of different metabolic pathways in which amino acids are utilised in the placenta (7). However, a placental transport system for glutathione has not yet been described. Venous umbilical levels

are lower than those in arterial umbilical plasma, whereas maternal concentrations are higher than those in both arterial and venous umbilical cord. Additionally, no association was found between maternal and umbilical glutathione concentrations. Therefore, maternal glutathione probably is not able to pass the placental barrier into the foetal circulation. Besides direct transport of amino acids, protein breakdown and direct synthesis of amino acids play an important role in placental transport processes. Breakdown of glutathione to its amino acids might be important since glutamate is an essential fuel for the placenta (7). On the membranes of most endothelial cells, including the microvillous membrane of throphoblast cells, γ-glutamyl transpeptidase is present (1,20), which initiates the breakdown of glutathione to glutamate and cysteinylglycine, which is further metabolised by the enzymes of the γ-glutamyl cycle (1,21). Cysteine and glycine might be released into the foetal circulation by placental transport and this may result in foetal synthesis of glutathione. Thus, the foetus may be able to synthesize glutathione instead of taking it up from the maternal circulation.

In summary, we presented reference values for glutathione in arterial and venous umbilical cord plasma after vaginal as well as cesarean deliveries. In addition, vaginal delivery may be associated with higher levels of oxidative stress during the second stage of labor as compared with delivery by cesarean section. Furthermore, our data suggest that maternal glutathione is not transported into the foetal circulation, but the foetus itself may be capable to synthesize glutathione.

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Part III

Thiol status in hypertensive disorders of pregnancy

Chapter 6

Plasma thiol status in preeclampsia.

Maarten T.M. Raijmakers, Petra L.M. Zusterzeel, Eric A.P. Steegers, Magda P.C. Hectors, Pierre N.M. Demacker, and Wilbert H.M. Peters.

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Abstract

Objective: To measure plasma thiol levels in control women, uncomplicated pregnant women and women with preeclampsia, in order to define their role in the glutathione homeostasis and in the pathophysiology of preeclampsia.

Methods: Total plasma cysteine, γ-glutamylcysteine, homocysteine, cysteinylglycine, and glutathione levels were measured in ten non-pregnant women, ten women during normotensive pregnancy, and twenty women during preeclampsia at the time of delivery.

Results: Median total plasma levels of all thiols in the normotensive pregnant women were significantly lower than in non-pregnant women. Median total plasma cysteine and homocysteine levels in the women with preeclampsia were significantly higher compared to those in pregnant controls (254 versus 190 μmol/L, $P<0.001$; and 13.3 versus 8.4 μmol/L, $P<0.02$, respectively), whereas glutathione levels were significantly lower in women with preeclampsia as compared to those in pregnant controls (5.1 versus 6.3 μmol/L, $P<0.05$).

Conclusion: In women with preeclampsia homocysteine and cysteine levels, which are lowered in normotensive pregnancy, are comparable to levels in non-pregnant women, whereas glutathione levels are lower. These results suggest that in women with preeclampsia glutathione utilization is higher or its synthesis is disturbed. Therefore, glutathione may play a role in the pathophysiology of preeclampsia.

Introduction

Preeclampsia is a multisystem disorder that affects about 3% of the primigravida mostly after 20 weeks of gestation. It is classically defined by hypertension and proteinuria (1) In severe cases preeclampsia might be complicated by the hemolysis, elevated liver enzymes, and low platelets (HELLP) syndrome (2). Both diseases of pregnancy are associated with an increased maternal and perinatal morbidity (3). Although the etiology of preeclampsia and the HELLP syndrome is unknown, data point to a dysfunction of the endothelial layer lining the blood vessel wall, which is probably caused by the release of a factor from placental origin (4-6). In this respect plasma homocysteine levels may be relevant, because increased concentrations are associated with vascular diseases (7-9). In support of this, elevated homocysteine levels were found in women with preeclampsia (10;11).

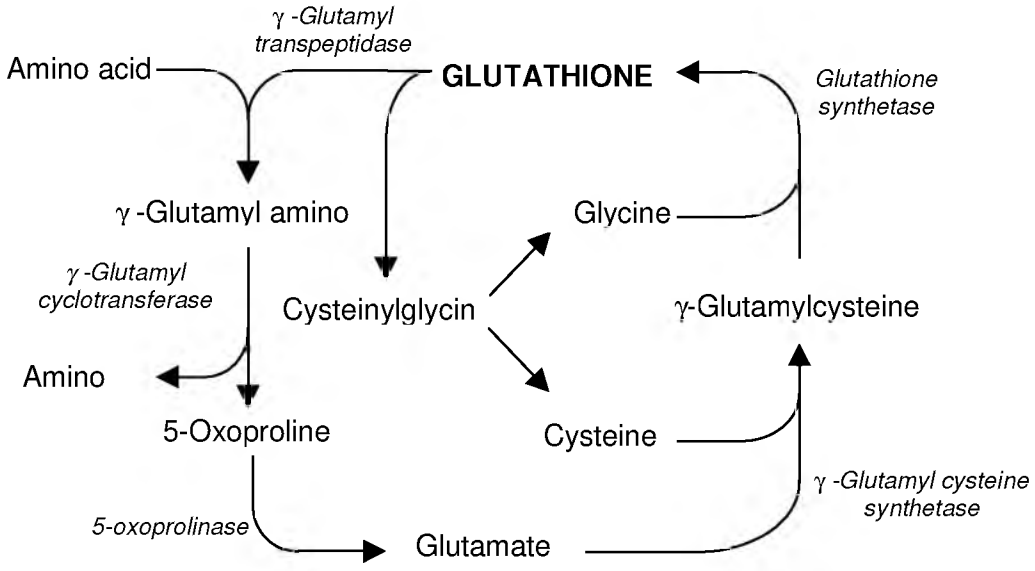

Figure 6.1. Schematic overview of the glutathione metabolism. Glutathione is synthesised in two consecutive steps catalysed by γ-glutamylcysteine synthethase and glutathione synthethase. In breakdown of glutathione γ-glutamyltranspeptidase cleaves the γ-bond resulting in glutatmate and cysteinylglycine.

Homocysteine is situated at a critical regulatory branchpoint in sulfur metabolism. It can be remethylated to methionine, an important amino acid in protein synthesis, or it can be converted to cysteine in the transsulfuration pathway (7;9;12). Cysteine is the only free thiol containing amino acid in proteins. The metabolism of this amino acid is complex and is still

incompletely understood (7). It's degradation proceeds by several pathways leading to formation of taurine or inorganic sulfate (13). In addition, cysteine is needed for glutathione synthesis and provides its thiol residue (7). As shown in Figure 6.1, synthesis of glutathione takes place in two steps. At first, γ-glutamylcysteine synthetase couples glutamate to cysteine forming γ-glutamylcysteine. The availability of cysteine is regulatory in this step. Glutathione is than directly synthesised by coupling γ-glutamylcysteine to glycine catalysed by glutathione synthetase (7;14).

In the human body glutathione has diverse important functions such as storage and transport of cysteine, maintaining the reduced state of proteins and thiols, and protecting cells from toxic compounds; such as reactive oxygen species, drugs, or heavy metal ions (7;14;15). Two different types of detoxification enzymes need glutathione as a substrate. Glutathione peroxidases catalyze the reaction of glutathione with (oxygen) free radicals, whereby glutathione is oxidised. Subsequently, the inactive oxidised form of glutathione can be reduced again by glutathione reductase. Glutathione S-transferases catalyze the conjugation between glutathione and toxic compounds. This glutathione conjugate is then excreted and additional glutathione has to be synthesised.

Earlier we found that whole blood levels of glutathione were lowered in women with preeclampsia (16). We now investigated total (the additional sum of reduced, oxidised and protein-bound) plasma thiol levels in healthy non-pregnant women, women with uncomplicated pregnancies and women with preeclampsia, some of whom developed the HELLP-syndrome as well, in order to better understand the reasons of these lower glutathione levels.

Materials and Methods

DL-homocysteine, DL-cysteine, cysteinylglycine, γ-glutamylcysteine, and glutathione (reduced form) were obtained from SIGMA Chemical (St. Louis, USA). Tris (2-carboxyethyl)-phosphine hydrochloride was obtained from Pierce (Rockford, USA). 7-Fluorobenzofurazane-4-sulfonic acid and $K_2B_40_7{\cdot}4H_20$ were obtained from Fluka Chemie AG (Buchs, Switzerland). All other chemicals were analytical grade and were obtained from Merck (Darmstadt, Germany).

Table 6.1. Characteristics of Women Studied.

	Non Pregnant Controls (n=10)	Pregnant Controls (n=10)	Preeclampsia (n=20)
Age (years)	25 (23 -34)	33 (25-42)	28 (19-33)
Gestational age (weeks^{+days})	---	34^{+1} (29^{+1}-38^{+1})	32^{+2}(26^{+3}-37^{+6})
Diastolic BP (mm Hg; Korotkoff IV)	---	75 (70-90)	115(100-120)
Aspartate aminotransferase (IU/L)	---	---	94(12-155)
Alanine aminotransferase (IU/L)	---	---	82(5-160)
Lactic dehydrogenase (IU/L)	---	---	598(236-1607)
Protein/creatinine ratio (g/10mmol)	---	---	5.45 (1.12-21.54)

Data are given as median (range)

Abbreviations: n = number of subjects; --- = Data not available; BP = blood pressure.

The experimental protocol was approved by the Medical Ethical Review Committee of the University Hospital Nijmegen. Preeclampsia was defined as pregnancy induced hypertension (diastolic blood pressure above 90 mm Hg on two or more consecutive occasions, each more than 4 hours apart) and proteinuria (protein/creatinine ratio above 0.30 g/10 mmol). The HELLP syndrome was defined as a lactic dehydrogenase level above 600 IU/L, both aspartate aminotransferase and alanine aminotransferase above 70 IU/L, and thrombocyte count under 100 x10^9/L. After informed consent was given blood samples were collected of ten non-pregnant healthy women, ten normotensive pregnant and twenty preeclamptic women, of whom ten developed the HELLP syndrome as well. All pregnant women were nulliparous. Patients and controls were randomly selected in the period from July 1995 to May 1998. All women were Caucasians and were matched for age and gestational age on a one-to-one base for non-pregnant versus normotensive pregnant women, and a one-to-two base for normotensive pregnancy versus preeclampsia. Characteristics of subjects studied are described in Table 6.1. Women with diabetes mellitus or essential hypertension were excluded. Seven preeclamptic and all normotensive pregnant women also contributed to studies published previously (16;17).

Directly after admission to the hospital whole blood was collected into sterile vacutainers containing ethylenediaminetetra-acetic acid (EDTA) (Sherwood Medical, Ballymore, Northern Ireland). Whole blood was centrifuged at 1,500xg for 10 min in a Heraeus Christ centrifuge within one hour after blood collection and plasma was stored in small aliquots at -30 °C until analysis.

Plasma was analysed for total cysteine, γ-glutamylcysteine, homocysteine, cysteinylglycine, and glutathione concentrations (the sum of reduced, oxidised and protein-bound). Sample analysis was performed by high performance liquid chromatography with fluorescent detection using an autosampler (Model Marathon, Spark Holland), solvent delivery system (High Precision Pump model 480, Gynkotek), and fluorescent detector (Intelligent Spectrofluorometric Detector model 821-FP, Jasco), operating at an excitation wavelength of 385 nm and an emission wavelength of 515 nm (Separations, H.I. Ambacht, the Netherlands). The column (Inertsil ODS-2, 100 x 3 mm, 5 µm particle size) and the guard column (R2, 10 x 2 mm) were from Chrompack (Middelburg, The Netherlands). Data obtained were analysed with the GynkoSoft chromatography data-system, Gynkotek (München, Germany).

The analysis of thiols is essentially as described by Fortin et al. (9) with some modifications. Shortly: after thawing on ice, 100 µL plasma was reduced by adding 10 µL tris (2-carboxyethyl)-phosphine hydrochloride (10 % in 0.9 % sodium chloride / 4.0 mM EDTA) for 30 min. Proteins were precipitated with 100 µL perchloric acid (0.6 M) / EDTA (1 mM) during 5 min followed by centrifugation for 5 min at 10,000xg in an Eppendorf centrifuge. Subsequently the supernatant (100 µL) was incubated with 20 µL sodium hydroxide (1.55 M), 200 µL borate buffer (125 mM and 4 mM EDTA, pH 9.5) and 20 µL 7-fluorobenzofurazane-4-sulfonic acid (5 mg/mL borate buffer) at 60°C for 1 hour. Of the derivatised sample 20 µL was injected and thiols were eluted with an isocratic eluent (2.0 % methanol in 0.1 M acetic acid pH 5.0) at flow rates of 350 µL/min for 5 min. and 600 µL/min for another 5 min. Concentrations of thiols were determined using calibration curves containing mixtures of all thiols.

Calibration curves for each thiol (100 - 400 µmol/L cysteine, 10 - 40 µmol/L homocysteine and γ-glutamylcysteine, and 5 - 20 µmol/L glutathione) were prepared by diluting stock solutions with 0.9 % sodium chloride / 4 mM EDTA. Stock solutions containing 20 mM of cysteine, γ-glutamylcysteine, homocysteine, cysteinylglycine, or glutathione were prepared in 0.9 % sodium chloride / 4 mM EDTA and stored in small aliquots at -30 °C. In an aqueous matrix calibration curves were linear, correlation coefficients exceeded 0.999 for all analytes in the ranges studied (data not shown). Pastore et al. (18) noticed no substantial matrix effects performing linearity studies on diluted plasma samples.

Sample size of 10 patients was determined based on the homocysteine data reported by Rajkovic et al. (10) assuming $\alpha = 0.05$ and $\beta = 0.95$. The Mann-Whitney U test with Bonferroni correction for multiple comparison was used to assess statistical significance of differences in thiols between groups. Differences were considered significant if $P<0.05$. Correlation between cysteine and homocysteine concentrations was determined with Spearman rank correlation test. Correlation was considered significant if $P<0.05$.

Results

Cysteine, γ-glutamylcysteine, homocysteine, cysteinylglycine, and glutathione eluted in this order with retention times of 3.0, 3.8, 4.3, 5.1, and 6.2 min, respectively. Mean recoveries for cysteine, γ-glutamylcysteine, homocysteine, cysteinylglycine, and glutathione were 99, 101, 102, 101, and 98 %, respectively. In plasma samples all thiols except γ-glutamylcysteine were detectable. Within-run coefficients of variation in plasma were 2.2 % for cysteine, 1.3 % for homocysteine, 3.9 % for cysteinylglycine and 5.9 % for glutathione. Day-to-day coefficients of variation were 1.0 % for cysteine, 7.0 % for homocysteine, 1.0 % for cysteinylglycine and 5.0 % for glutathione.

Table 6.2. Plasma Thiol Concentrations in Preeclamptics and Controls.

Thiol	Non Pregnant Controls (%)	Pregnant Controls (%)	Preeclampsia (%)
Cysteine	253 (205- 270) (100%)	190 (138 - 232) [a] (75 %)	254 (222 - 533) [c] (100 %)
γ-Glutamylcysteine	nd	nd	nd
Homocysteine	13.7 (9.2 - 17.3) (100%)	8.4(7.0 - 15.6) [a] (61 %)	13.3 (7.5 - 26.4) [d] (97 %)
Cysteinylglycine	28.5 (21.6 - 34.1) (100%)	22.0 (17.7 - 29.8) [b] (77 %)	21.8 (6.0 - 38.6) (76 %)
Glutathione	11.6 (8.6 - 15.5) (100%)	6.3 (4.3 - 9.5) [a] (54 %)	5.1 (1.5 -.10.7) [e] (44 %)

Data are given as median (range) and expressed as µmol/L

nd = not detectable; % = Percentage as compared to non-pregnant controls.

Statistics: a: $p<0.001$, b: $p<0.03$ (Non-pregnant versus pregnant controls)

c: $p<0.001$, d: $p<0.02$, e: $p<0.05$ (Preeclampsia versus pregnant controls)

Median levels of cysteine, homocysteine, cysteinylglycine and glutathione in healthy non-pregnant women were 253, 13.7, 28.5, and 11.6 µmol/L, respectively (Table 6.2). Levels of cysteine, homocysteine, cysteinylglycine and glutathione were significantly lower in women with normotensive pregnancy as compared to healthy non-pregnant women.

No significant differences were found between preeclamptics with or without the HELLP-syndrome. In preeclamptic women median cysteine and homocysteine levels were significantly higher as compared to levels in normotensive pregnant, whereas glutathione concentrations were significantly lower (Table 6.2). Individual data for glutathione levels are shown in Figure 6.2. No significant differences in cysteinylglycine levels were found.

A significant correlation between plasma cysteine and homocysteine levels for all subjects was found ($r=0.74$, $P<0.001$).

Discussion

Thiol levels found in healthy non-pregnant women are consistent with those reported by Pastore et al. (18) and Mansoor et al. (19). However, homocysteine levels were slightly higher than reported by Mansoor et al., but are within the normal range and are comparable to normal levels for the Dutch population (20).

Cysteine, homocysteine, cysteinylglycine, and glutathione levels were significantly lower during normotensive pregnancy as compared to healthy non-pregnant women (75, 61, 77 and 54% of control values, respectively). Changed steroid levels during pregnancy as reported by Kim et al. (21) may contribute to this effect. However, in an overall analysis of 31 studies revealed an increase in plasma volume of approximately 40% during pregnancy (22). It is likely that this also contributes to the decrease of plasma thiol levels in pregnant women. This effect of hemodilution was also shown for homocysteine by Anderson et al. (23). Glutathione levels, however, were even lower than can be explained by hemodilution only. This might point to a higher utilization or lower synthesis of glutathione during pregnancy.

We found higher plasma homocysteine levels in preeclampsia compared to normal pregnancy, which is in agreement with the findings of Rajkovic et al. (10). However, the homocysteine concentrations found in our study were higher than those of Rajkovic et al. (13.3 versus 8.7 µmol/L). The subjects in our study were not uniformly fasting and it is known that this can affect homocysteine levels (24;25), although this effect seems to be minimal. Probably more

important is that different populations were studied, since it is known that homocysteine levels in the Northern American population are lower as compared to the Dutch population, due to a higher dietary intake of folate and vitamin supplements in the United States (26).

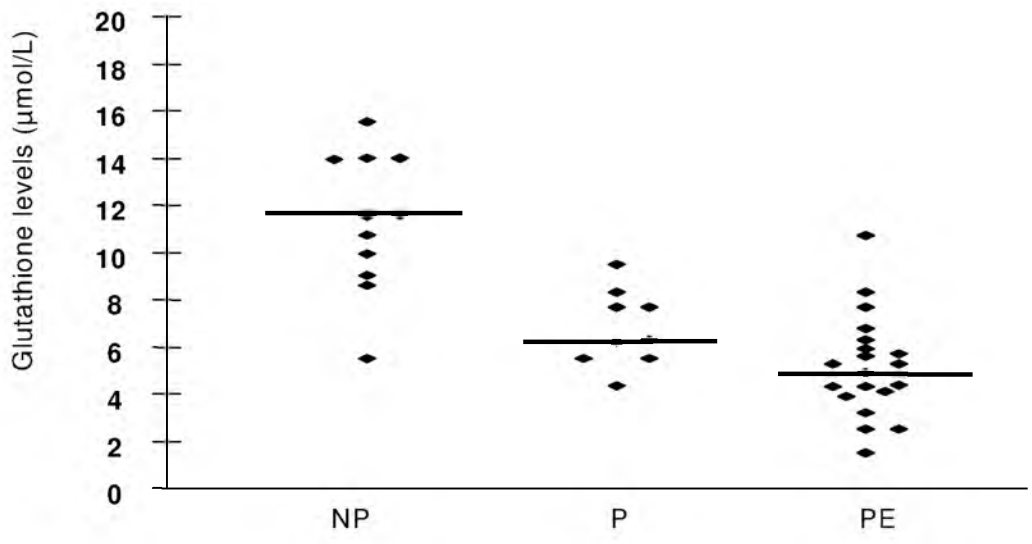

Figure 6.2. Individual glutathione concentrations in plasma of non-pregnant (NP), normotensive pregnant (P), and preeclamptic (PE) women.

Homocysteine is linked to cysteine by the enzyme cystathione-ß-synthase, which converts homocysteine to serine and cystathione, which is further metabolised to cysteine (9;12). Rajkovic et al. (10) suggested that a deficiency of cystathione-ß-synthase may be responsible for the increased homocysteine levels. However, this would result in normal or even lower cysteine levels in normotensive pregnancies, which is not in line with the observed correlation between cysteine and homocysteine. This suggests that a disturbance in the remethylation of homocysteine to methionine has to be considered. Low vitamin B12 or folate levels, both essential for this remethylation, might be contributing factors to the elevated homocysteine levels in preeclampsia. However, Rajkovic et al. (10) found no significant differences in both vitamin B12 and folate levels in women with normotensive pregnancy and preeclampsia.

The levels of homocysteine and cysteine in preeclampsia, which are higher than in normotensive pregnant women, are comparable to the levels in healthy non-pregnant women. These elevated levels during preeclampsia can possibly be explained by the pathophysiological reduction of plasma volume during preeclampsia, which may be as much as 40% in severe cases (5;22). In this line of thinking, higher levels of cysteinylglycine and glutathione were expected than those we actually found in women with preeclampsia. In preeclampsia, plasma glutathione concentrations were even lower than in normotensive

pregnancies. These results are in agreement with our previous findings that whole blood glutathione levels are lower in preeclampsia as compared to normotensive pregnancy (16). Glutathione is essential in the defense against oxidative stress and is important in a wide variety of detoxification reactions in humans (14;27). Therefore, oxidative stress in pregnancy and preeclampsia in particular might cause a higher consumption of glutathione, resulting in low glutathione levels. Another possibility is that the synthesis of glutathione is disturbed in women developing preeclampsia. As a result the amount of glutathione may not be sufficient to fully protect against oxidative stress or toxic factors.

Elevated homocysteine levels may contribute to damage of the vascular endothelium in women with preeclampsia. The question arises whether the slightly elevated homocysteine levels found here, which are comparable to levels in healthy non-pregnant women, may damage the vascular endothelium of preeclamptic women. Vascular endothelium of women with preeclampsia might, however, be more sensitive towards injury. Therefore the moderate elevation in homocysteine levels during preeclampsia may lead to endothelial damage with subsequent activation of several factors such as adhesion molecules or von Willebrand's factor, as shown by several studies (4;5). Damage of the endothelium is more likely caused by an increased oxidative stress due to pregnancy or by the release of a toxic factor from placental origin both resulting in the low glutathione levels observed.

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Chapter 7

Oxidised and free whole blood thiols in women with preeclampsia.

Maarten T.M. Raijmakers, Petra L.M. Zusterzeel, Eva Maria Roes, Eric A.P. Steegers, Theo P.J. Mulder, and Wilbert H.M. Peters.

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Abstract

Objective: Analyses of oxidised and free thiols in whole blood of normotensive pregnant and preeclamptic women, with or without HELLP syndrome, in order to evaluate the role of oxidative stress, which may play an important role in the pathogenesis of preeclampsia.

Methods: We measured whole blood oxidised and free levels of cysteine, homocysteine, cysteinylglycine, and glutathione by high performance liquid chromatography in women with normotensive pregnancies (n=50), preeclampsia (n=29), and preeclampsia complicated by the hemolysis, elevated liver enzymes, low platelets (HELLP) syndrome (n=16).

Results: In preeclampsia both oxidised and free levels (median (range) in µmol/L) of cysteine and homocysteine were higher than in normotensive pregnancy (45 (27 – 81) vs. 29 (9 – 91), P<0.001 and 98 (57 – 193) vs. 69 (33 – 215), P<0.001; 0.8 (0.2 – 4.4) vs. 0.4 (0.01 – 1.6), P<0.001 and 2.1 (0.7 – 9.4) vs. 1.2 (0.2 – 21.2), P=0.01; respectively). The ratios of free to oxidised cysteine, homocysteine and cysteinylglycine were lower in preeclampsia compared with normotensive pregnancy (2.2 (1.3 – 3.0) vs. 2.4 (1.7 – 4.3), P<0.001; 2.3 (0.5 – 5.4) vs. 2.9 (1.1 – 24), P<0.001; 4.1 (2.3 – 11.6) vs. 5.4 (2.6 – 24.3). P=0.02; respectively), indicating a shift in favour of the oxidised form of these thiols. In HELLP syndrome levels of oxidised and free cysteine and levels of oxidised homocysteine were elevated compared with normotensive pregnancy (44 (33 – 63) vs. 29 (9 – 91), P<0.001 and 102 (82 – 133) vs. 69 (33 – 215), P<0.001; 1.0 (0.3 – 2.9) vs. 0.4 (0.01 – 1.6), P<0.001; respectively). No statistical differences were found in oxidised glutathione levels in women with preeclampsia (22 (5 - 49) vs. 17 (2 - 60), P=0.06, or free levels in preeclamptic women with HELLP syndrome (757 (624 - 993) vs. 842 (539 - 1516), p=0.09) as compared with normotensive pregnant women. The ratios of free to oxidised cysteinylglycine and glutathione were higher in women with HELLP syndrome compared with preeclampsia (5.4 (3.3 – 12.7) vs. 4.1 (2.3 – 11.6), P=0.02; 56 (28 – 124) vs. 45 (16 – 166), P=0.02; respectively).

Conclusion: The significantly lower free to oxidised ratios of cysteine, homocysteine, and cysteinylglycine in preeclampsia might indicate oxidative stress.

Introduction

Preeclampsia is one of the most frequent complications of pregnancy (5-7%) leading to foetal growth retardation, premature delivery, and maternal and foetal morbidity and mortality (1). The haemolysis, elevated liver enzymes, and low platelets (HELLP) syndrome may complicate preeclampsia in approximately 10% of the cases. Although the aetiology of these disorders remains obscure, rising evidence indicates that dysfunction of the vascular endothelium may result in increased vasoconstriction leading to maternal hypertension (2) and reduced uteroplacental blood flow (3). Endothelial dysfunction can be mediated by oxidative stress defined as disturbance of the redox-balance, caused either by an increased amounts of oxidants such as cytokines or free radicals, or a deficiency of antioxidants resulting in an overall oxidant insult (3-7). In women with preeclampsia lipid peroxidation products are elevated (3;5;7) and they might contribute to the aetiology of preeclampsia (8). In normal pregnancy lipid peroxidation has been shown to progressively increase with gestational age, whereas the antioxidant capacity, the vitamins A, C, E, and erythrocyte levels of thiols, increased in parallel (3;5;7).

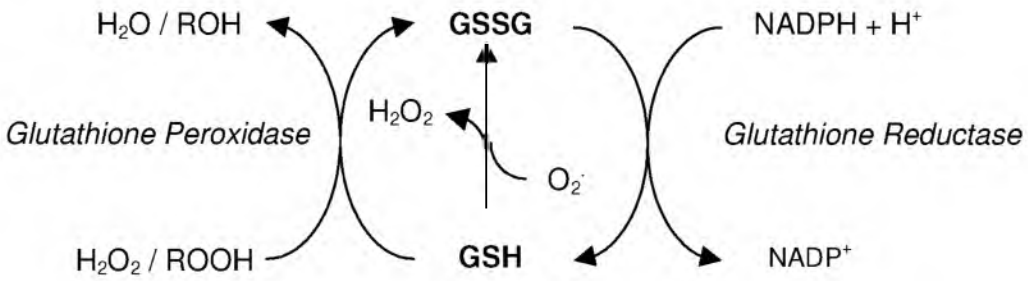

Figure 7.1. Glutathione cycling in detoxification of reactive oxygen species.
Reactive oxygen species ($RO^{\cdot}$), hydrogen peroxide (H_2O_2) and organic peroxides (ROOH) can be detoxificated by action of glutathione peroxidase (GPX) with reduced glutathione (GSH) as a substrate, yielding the oxidised form of glutathione (GSSG) and relative harmless ROH. Oxidised glutathione can be regenerated to GSH by glutathione reductase (GR) thereby consuming nicotinamide adenine dinucleotide phosphate (NADPH).

Red blood cells contain high concentrations (up to 2 mM) of reduced glutathione accounting for almost 98% of the total blood content (7;9). In addition to their detoxification function by conjugation of noxious compounds, glutathione and other thiols, maintain the redox-balance of cells, thereby preventing oxidative damage (7). Glutathione maintains the redox-balance by

action of glutathione peroxidases, which couple the reduction of organic peroxides or (oxygen) free radicals to the oxidation of glutathione (10;11). Subsequently, a glutathione reductase catalysed reaction converts the inactive oxidised glutathione back into the active reduced form (10; Figure 7.1).

Oxidative stress in preeclampsia has already been demonstrated indirectly by high levels of lipid peroxidation products such as malondialdehyde (3;5;6;12;13) and low levels of antioxidants such as the vitamins A, C and E (2;3;5-8;13). We previously noticed lower whole blood (14) and plasma (15) levels of glutathione in patients with preeclampsia, whereas plasma concentrations of cysteine and homocysteine were increased (15). In this present study we report free and oxidised levels of the thiols cysteine, homocysteine, cysteinylglycine, and glutathione in whole blood for the direct assessment of oxidative stress in women with preeclampsia and preeclampsia complicated by HELLP syndrome.

Materials and Methods

The experimental protocol was approved by the Medical Ethical Review Committee of the University Hospital St. Radboud, Nijmegen. Preeclampsia was defined according to the standards of the International Society for the Study of Hypertension in Pregnancy as pregnancy induced hypertension (a diastolic blood pressure above 90 mm Hg on two or more consecutive occasions, each more than 4 hours apart) and proteinuria (protein/creatinine ratio above 0.3 g/10 mmol). HELLP syndrome was defined as hemolysis (lactic dehydrogenase above 600 IU/L), elevated liver enzymes (both aspartate aminotransferase and alanine aminotransferase above 70 IU/L), and low platelets (thrombocyte count under 100 $x10^9$/L). After written informed consent was given, blood samples of normotensive pregnant women (n=50), women with preeclampsia (n=29), and women with preeclampsia complicated by the HELLP syndrome (n=16) were collected before anti-hypertensive treatment was started. Patients and controls were included in the period from July 1998 to November 1999. All women were Caucasian and respectively 40%, 40%, and 70% of women included were nulliparous. Women with diabetes mellitus or essential hypertension were excluded from participation in the study.

N-ethylmaleimide, 3-[N-morpholino]-propanesulfonic acid, and bathophenanthroline-disulfonic acid were obtained from Sigma Chemical (St. Louis, USA). All other chemicals were analytical grade and were obtained from Merck (Darmstadt, Germany).

Whole blood was collected into sterile vacutainers containing ethylenediaminetetra-acetic acid (EDTA) (Sherwood Medical, Ballymore, Northern Ireland) and handled within 1 hour after collection. For assay of the free thiol levels (both reduced and oxidised non-protein bound thiols), proteins were precipitated by adding 500 µL whole blood to 500 µL 12% perchloric acid containing 2.0 mM bathophenanthrolinedisulfonic acid. For assay of oxidised free thiols, another 500 µL whole blood was added to 500 µL 12% perchloric acid containing 2.0 mM bathophenanthrolinedisulfonic acid and 40 mM N-ethylmaleimide. After thorough mixing and centrifugation at 16,000xg for 20 minutes and 4 °C, supernatants were collected and stored at -80 °C until analysis. Samples were analysed within two weeks. Just before analysis of the oxidised and free thiols the excess of N-ethylmaleimide was removed by adding 70 µL KOH (2.0 M) followed by 60 µL HCl (0.01 M in 0.3 M 3-[N-morpholino]-propanesulfonic acid buffer) to 100 µL of sample. Samples were analysed for levels of cysteine, homocysteine, cysteinylglycine, and glutathione by high performance liquid chromatography with fluorescent detection as described previously (15). Data obtained were analysed with the Chromeleon chromatography data-system, Gynkotek (München, Germany). After analysis the ratio of free over oxidised thiol were calculated for each subject.

To be able to detect a difference in whole blood glutathione concentration of 250 µmol/L a sample size of at least 15 women in each group was needed, assuming α=0.05 and ß=0.95. Kruskal-Wallis ANOVA and, when appropriate a Mann-Whitney U-tests were performed to assess statistical significance in thiols between groups; $P < 0.05$ was considered significant.

Results

In Table 7.1 the characteristics of the women studied are summarised. The control women did not differ from the patients in the preeclamptic nor the HELLP group regarding age, gestational age, and hemoglobin and hematocrit levels.

Table 7.1. Characteristics of the Women Studied.

	Controls (n=50)	Preeclampsia (n=29)	Preeclampsia + HELLP (n=16)
Age (years)	33 (22 – 44)	31 (23 - 38)	30 (24 - 35)
Gestational Age (weeks^{+days})	32^{+0} (28^{+0} - 38^{+2})	32^{+3} (22^{+3} - 37^{+2})	29^{+1} (24^{+4} - 40^{+4})
Diastolic BP (mmHg; Korotkoff V)	70 (55 – 90)	110 (95 - 135) †	105 (90 - 120) *
Hemoglobin (mmol/L)	7.3 (6.1 - 8.3)	7.6 (6.6 - 9.1)	7.5 (6.5 - 8.2)
Hematocrit (L/L)	0.35 (0.30 - 0.40)	0.36 (0.30 - 0.93)	0.35 (0.31 - 0.37)
Protein / Creatinine ratio (g/10 mmol)	---	1.7 (0.3 - 21.1)	6.6 (0.3 - 33.8)
Uric acid (mmol/L)	---	0.43 (0.18 - 0.58)	0.44 (0.32 - 0.69)
Lactic Dehydrogenase (IU/L)	---	434 (182 - 1250)	1305 (706 - 4380) ‡
Aspartate Aminotransferase (IU/L)	---	32 (14 - 312)	222 (100 - 1690) ‡
Alanine Aminotransferase (IU/L)	---	21 (9 - 276)	179 (40 - 1760) ‡
Thrombocytes (x10^9/L)	---	200 (62 - 391)	55 (28 - 110) ‡

Data are given as median (range)

Abbreviations: --- = data not available, n = number of subjects, BP = blood pressure

Statistics:

*: P<0.001 controls versus preeclampsia + HELLP

†: P<0.001 controls versus preeclampsia

‡: P<0.001 preeclampsia versus preeclampsia + HELLP

Median values of whole blood free levels of cysteine, homocysteine, cysteinylglycine and glutathione are shown in Table 7.2. Levels of cysteine and homocysteine were significantly higher in the preeclamptic group, whereas cysteine alone was significantly higher in the HELLP group both compared with corresponding values in controls. No significant differences were found for cysteinylglycine and glutathione between controls and either preeclampsia with or without HELLP syndrome.

Oxidised levels of cysteine and homocysteine in both groups of complicated pregnancies were significantly higher compared with those in controls. No significant differences were found for oxidised cysteinylglycine and glutathione levels in preeclamptic women with or without HELLP syndrome compared with control women, however in women with preeclampsia glutathione levels nearly reached significance (P=0.06).

Table 7.2. Free, Oxidised, and Ratios of Free to Oxidised Thiols in Pregnancy

	Normtensive (n=50)			Preeclampsia (n=29)			Preeclampsia + HELLP (n=16)		
	Free	Oxidised	Ratio	Free	Oxidised	Ratio	Free	Oxidised	Ratio
Cysteine	69	29	2.4	98*	45*	2.2*	102*	44*	2.3
	(33 - 215)	(9 - 91)	(1.7 - 4.3)	(57- 193)	(27 - 81)	(1.3 - 3.0)	(82 - 133)	(33 - 63)	(2.0 - 3.7)
Homocysteine	1.2	0.4	2.9	2.1*	0.8*	2.3*	1.5	1.0*	2.6
	(0.2 - 21.2)	(0.01- 1.6)	(1.1 - 24)	(0.7 - 9.4)	(0.2 -4.4)	(0.5 - 5.4)	(0.6 - 3.9)	(0.3 - 2.9)	(1.0 -7.1)
Cysteinylglycine	9.0	1.6	5.4	8.9	1.8	4.1**	8.3	1.9	5.4†
	(5.3 - 18.1)	(0.3 - 3.3)	(2.6 - 24.3)	(0.7 - 13.0)	(0.3 - 3.2)	(2.3 - 11.6)	(2.5 - 12.0)	(0.2 - 3.2)	(3.3 - 12.7)
Glutathione	842	17	50	827	22	45	757	14	56†
	(539 - 1516)	(2 - 60)	(19 - 496)	(565 - 1303)	(5 - 49)	(16 - 166)	(624 - 993)	(7 - 34)	(28 - 124)

Data are given as median (range) and are expressed as µmol/L.

Ratio = ratio of free to oxidised thiol level.

Statistics:

*: P<0.001 and **: P=0.02 normotensive versus complicated pregnancy

†: P=0.02 preeclampsia versus preeclampsia + HELLP

As reported by Ueland et al. (16) and Droge (17) the ratio of free to oxidised thiol is a measure for the balance between oxidants and antioxidants. Changes of this ratio can therefore be used as a measure of oxidative stress. These ratios were calculated for each thiol in every subject. Median ratios of free to oxidised cysteine, homocysteine, cysteinylglycine and glutathione are summarised in Table 7.2. In the preeclamptic group these ratios were significantly lower for cysteine, homocysteine, and cysteinylglycine compared with the corresponding values in the control group, whereas no significant difference was found for glutathione. No significant differences in free to oxidised ratios were found between HELLP patients and controls. Free to oxidised ratios of cysteinylglycine and glutathione were significantly higher in the HELLP group as compared to the preeclamptic group.

Discussion

Values for free glutathione and cysteine in whole blood are well documented and data in normotensive pregnancies as measured in this study are comparable with those of non-pregnant controls reported elsewhere (9;18;19). However, no whole blood reference values are known for free and oxidised cysteinylglycine and homocysteine levels.

We earlier reported that whole blood glutathione/hemoglobin ratios in women with preeclampsia or HELLP syndrome were lower compared with those in normotensive pregnancy, whereas whole blood glutathione levels tended to be lower but reaching only borderline significance (P=0.05) (14). In this more extensive study we found that free whole blood glutathione levels tended to be lower in the HELLP group, whereas values were not different in women with preeclampsia compared with normotensive pregnant controls. These data appear to contrast with our previous findings. Two reasons for this discrepancy may be valid. At first; in the former report other patient and control populations and smaller groups were studied. The size of the study groups may particularly be important because the whole blood values for glutathione, as reported in literature, vary between 500 and 1500 µmol/L (9;14;18;19). In our study the values in controls showed a similar range (539 –1516 µmol/L). At second; another assay method was used; earlier we used monobromobimane as a fluorescent probe for quantification of glutathione (14), whereas in the present study glutathione and other thiols were estimated simultaneously by using 7-fluorobenzofurazane-4-

sulfonic acid (15). The method using 7-fluorobenzofurazane-4-sulfonic acid is more specific due to absence of breakdown products of monobromobimane and lesser background peaks. In addition the run-time is considerably shortened.

In preeclampsia both free and oxidised levels of cysteine and homocysteine are higher compared to normotensive pregnancy. The ratios of free to oxidised thiol are significantly lower for cysteine, cysteinylglycine, and homocysteine. This points to a shift in redox-balance towards more oxidised levels of cysteine, cysteinylglycine, and homocysteine in women with preeclampsia. As reviewed by Davidge (4) and Walsh (3) preeclampsia can be characterised by an imbalance between oxidants and antioxidants. However, in these former studies oxidative stress was only indirectly demonstrated by assessment of lipid peroxidation products or antioxidant levels (3;4), whereas we now report the direct measurement oxidative stress. It is relevant therefore that Chappell et al. (20) recently showed that oxidative stress could partly be overcome by supplementation with vitamins C and E early in pregnancy, which resulted in lower incidence of preeclampsia in women at high risk for preeclampsia.

Thiols are an important component of the total redox-buffer system (21). Reduced glutathione, particularly, is a well-recognised endogenous scavenger of free radicals and peroxides, maintaining the redox-potential and being highly protective against oxidative stress (10;18;22). In deactivating free radicals and reactive oxygen species the reduced form of glutathione and other thiols is oxidised, thereby changing the ratio of free over oxidised thiol. We anticipated therefore that this ratio for glutathione would be lower in women with preeclampsia, which we did not find. An explanation could be that the oxidised form of glutathione is rapidly reduced by glutathione reductase, thereby consuming nicotinamide adenine dinucleotide phosphate (10) (Figure 7.1), whereas such an efficient reducing system is not present for the other thiols. In addition, when increasing amounts of glutathione are oxidised, some of the oxidised glutathione will undergo renal degradation by γ-glutamyl transpeptidase resulting in an irreversible loss of glutathione (21). As a result of either or both of these mechanisms the free to oxidised ratio might remain unchanged.

Lowered free to oxidised ratios for cysteine, homocysteine, and cysteinylglycine were also expected in preeclamptic women with HELLP syndrome. Although oxidised levels of cysteine and homocysteine were significantly higher, significantly lowered ratios were not found in these women, indicating that the reduced levels of these thiols were elevated in

parallel with the oxidised levels and oxidative stress is probably absent. Hemolysis and especially impairment of liver function might influence whole blood levels of the thiols, since the liver plays an important role in thiol metabolism.

Reduced thiols, specifically cysteine and homocysteine, may combine with the common active nitric oxide radical ($NO^{\cdot}$) yielding S-nitrosothiol intermediates, which may be more functional in vasodilatation than $NO^{\cdot}$ itself (10). Although free levels of cysteine and homocysteine are high in women with preeclampsia, levels of the reduced form of both thiols are low, because free to oxidised ratios are elevated in this group. These lower levels of reduced thiols may possibly lead to a lower nitric oxide mediated vasodilatation resulting in elevation of the blood pressure as seen in these women. In addition, lower levels of reduced cysteine and homocysteine in combination with increased biosynthesis of $NO^{\cdot}$ (23) might result in elevation of oxidative species through reaction of $NO^{\cdot}$ with superoxide, leading to the formation of peroxynitrite. Peroxynitrite is a strong oxidant able to initiate lipid peroxidation (3) which has been reported to be elevated in placentas from women with preeclampsia compared with those from normotensive pregnant women (4).

In this study whole blood thiol levels in normotensive pregnancies and those complicated by preeclampsia with or without HELLP syndrome were investigated for a better understanding of the pathophysiology of both diseases. Some reports suggest that preeclampsia and HELLP syndrome are different clinical syndromes with the same underlying pathology. Our results confirm this view, because both complications of pregnancy exert pressure on the thiol detoxification system. Oxidised levels of cysteine and homocysteine were significantly higher in women with HELLP syndrome. Free to oxidised ratios of cysteine, homocysteine, and cysteinylglycine were significantly lower in preeclampsia compared with normotensive pregnancy, however this was not found in preeclamptic women with HELLP syndrome. The free to oxidised ratios for cysteinylglycine and glutathione were significantly lower in women with preeclampsia than in preeclamptic women with HELLP syndrome. These results suggest that elevated oxidative stress and/or disturbance in detoxification processes may play a role in the etiology or maintenance of preeclampsia or HELLP syndrome.

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Chapter 8

Pregnancy is characterised by oxidative stress, which is more pronounced in preeclampsia.

Maarten T.M. Raijmakers, Eva Maria Roes, Eric A.P. Steegers, and Wilbert H.M. Peters.

Abstract

Background: Oxidative stress plays an important role in the pathophysiology of preeclampsia. In defence against reactive oxygen species glutathione and other thiols can be oxidised, thereby maintaining the intracellular redox status. Therefore, the free-to-oxidised ratio of these thiols can be seen as a measure of oxidative stress.

Aim: To compare the free and oxidised levels as well as the free-to-oxidised ratios of cysteine (Cys) homocysteine (Hcy), cysteinylglycine (CysGly), and glutathione (GSH) in whole blood of women with severe preeclampsia with those in normotensive pregnant women during pregnancy and 6-8 weeks post partum.

Subjects and Methods: At onset of disease and at 6-8 weeks post partum, whole blood of 41 women with severe preeclampsia and of 31 women with normotensive pregnancies was analysed for the free and oxidised levels of thiols by high performance liquid chromatography. The free-to-oxidised ratio for each thiol was calculated. Differences between pregnancy and post-partum values were analysed with the paired t-test, whereas differences between preeclamptic and control pregnancies were analysed using the t-test.

Results: Both in women with severe preeclampsia and control subjects free levels of GSH were lower during pregnancy compared with those post partum ($P<0.0001$ and $P<0.0001$, respectively). Free-to-oxidised ratios of Hcy are lower in preeclampsia ($P<0.01$) as well as normotensive pregnancy ($P<0.01$) compared with those after pregnancy. These ratios were significantly lower in preeclamptic women compared with normotensive controls during as well as after pregnancy ($P\leq0.01$ and $P<0.01$, respectively).

Conclusion: During normotensive pregnancy more oxidative stress is present as compared with the non-pregnant state, whereas in women with severe preeclampsia oxidative stress is higher during the disease as well as in the non-pregnant state afterwards as compared to corresponding values in normotensive pregnant women.

Introduction

Preeclampsia is a severe complication of pregnancy with a prevalence of 5-7% and high maternal as well as foetal mortality and morbidity. It is classically defined by pregnancy induced hypertension and concurrent proteinuria (1). Preeclampsia may be complicated by the haemolysis elevated liver enzymes and low platelets (HELLP) syndrome. The pathogenesis of both these disorders is not yet elucidated, however, it is thought that they have a placental origin (2), since removal of the placenta resolves the clinical symptoms of preeclampsia. Poor trophoblast invasion in early pregnancy may result in a reduced placental perfusion (3). At a certain level the oxidant challenge may exceed the amount of antioxidants, which may trigger placental and maternal oxidative stress.

During uncomplicated pregnancies levels of lipid peroxidation products increase with gestational age, which are counteracted by a parallel increase of antioxidant capacity (4-8). Unless properly controlled, these lipid peroxidation products may result in oxidative damage of cellular membranes, mitochondrial as well as nuclear DNA, and loss of protein function and synthesis (9). This may cause the general dysfunction of vascular endothelium as seen in preeclampsia (1;10;11). For scavenging free radicals and reactive oxygen species (ROS) several enzymatic and non-enzymatic antioxidant defence mechanisms are present. They all react with reactive oxygen species (ROS) or reduce the levels of free transition metals, which are capable to initiate the chain reaction of lipid peroxidation (11). The glutathione / glutathione dependent enzyme system is an important first line defence against oxidative damage (12;13). Glutathione is a non-specific free radical scavenger, which is able to donate its proton to unpaired electrons, thereby "quencing" the free radical. However, it also serves as co-factor in the reaction with glutathione S-transferases and glutathione peroxidases, which scavenge ROS upon oxidation of glutathione (12). To maintain the cellular redox-balance, the oxidised form of glutathione is quickly reduced by glutathione reductase, or it is excreted when excessive amounts are formed (12;14) .

Besides its antioxidant capacity glutathione is important in storage and transport of cysteine. In the two-step synthesis of glutathione a very stable γ-bond instead of a normal peptide bond is formed between cysteine and glutamate. This reaction is immediately followed by the addition of glycine (15;16). Cysteine serves as –SH donor thereby providing the reactive centre of glutathione. The chemistry of cysteine is complex, but it plays an important role in protein synthesis. Cysteine is often seen as an intermediate of homocysteine breakdown, since

homocysteine is irreversible converted into cysteine in the transsulphuration pathway. Further breakdown of cysteine leads to the formation of taurine or inorganic sulphate (15;16).

In a previous study we showed that during preeclampsia higher oxidative stress is present and that free and oxidised levels of cysteine and homocysteine were higher as compared to those in normotensive control pregnancies (17). However, possible due to large individual variation no significant differences were found for glutathione. In this study we investigated the levels of free and oxidised cysteine, homocysteine, cysteinylglycine and glutathione, and the free-to-oxidised ratio of these thiols in women with preeclampsia as well as normotensive pregnant women during and after pregnancy.

Materials and Methods

The Institutional Medical Ethical Review Committee approved the study protocol. Preeclampsia was defined according to the standard of the International Society for the Study of Hypertension in Pregnancy as pregnancy induced hypertension (diastolic blood pressure > 90 mmHg on two or more occasions each more than 4 hours apart) with proteinuria (protein/creatinine ratio >0.30 g/10 mmol). The HELLP syndrome was defined by hemolysis (lactic dehydrogenase enzyme activity >600 IU/L), elevated liver enzymes (both aspartate and alanine aminotransferase enzyme activity >70 IU/L) and low platelets (trombocyte count $<100x10^9$/L). Participants were recruited between 1999 and 2001. Five of the preeclamptic subjects have participated in another study (18), whereas four of those also contributed to the previous study (17). All women gave their informed consent to participate.
At onset of disease we recruited forty-one women with hypertensive disorders of pregnancy; severe preeclampsia (n=21), preeclampsia with HELLP syndrome (n=18), or HELLP syndrome with pregnancy induced hypertension (n=2); and thirty-one women with uncomplicated pregnancy outcome. Characteristics of the study populations are described in Table 8.1.

Whole blood, anti-coagulated with EDTA, was collected by venapuncture immediately after admittance to the University Medical Centre Nijmegen and 6-8 weeks after pregnancy. After venapuncture whole blood was handled within one hour for assessment of free (the sum of

reduced and oxidised non-protein bound) and oxidised levels of cysteine (fCys and oCys), homocysteine (fHcy and oHcy), cysteinylglycine (fCys-Gly and oCys-Gly) and glutathione (GSH and GSSG) as described before by our group (17).

Table 8.1. Pregnancy characteristics of women with severe preeclampsia and normotensive pregnant controls at time of study.

	Normotensive controls (n=31)	Severe preeclamspia (n=41)
Maternal age (years)	33 (27 – 41)	29 (21 – 38)*
Nulliparous	11 (35)	32 (78)*
Birht weigth (gram)	3305 (2335 – 4675)	943 (370 – 3045)†
Gestational age (weeks^{+days})	39^{+1} (37^{+6} – 42^{+1})	28^{+3} (22^{+4} – 37^{+5})†
Hb (mmol/L)	7.3 (6.3 – 8.5)	7.7 (5.0 – 9.0)
Ht	0.34 (0.28 – 0.48)	0.36 (0.28 – 0.45)
Diastolic BP (mmHg; Korotkoff V)	68 (55 – 80)	110 (80 – 135)†
Protein/Creatinine ratio (g/10 mmol)		4.2 (0.1 – 27.4)
LDH (IU/L)		586 (92 – 3870)
ALAT (IU/L)		34 (8 – 936)
ASAT (IU/L)		63 (12 – 1291)
Platelet count (x10^9/L)		121 (20 – 378)
Uric Acid		0.4 (0.2 – 34.0)
Creatinine (µmol/L)		71 (49 – 146)

Data are presented as median (range), except for nulliparous, which is expressed as number (percentage).

Abbreviations: Hb – haemoglobin, Ht – haematocrit, BP – blood pressure, LDH – lactate dehydrogenase enzyme activity, ALAT – alanine aminotransferase enzyme activity, ASAT – aspartate aminotransferase.

Statistics:

*: P<0.001 and †: P<0.0001 normotensive controls versus severe preeclampsia

In short, for assay of free thiol levels, 500µL whole blood was added to 500µL ice-cold 12% perchloric acid (PCA) containing 2.0mM bathophenanthrolinedisulfonic acid (BPDS; Sigma Chemicals). For assay of oxidised thiols, another 500 µL whole blood was added to 500 µL 12% PCA containing 2.0 mM BPDS and 40 mM N-ethylmaleimide (NEM). After thorough

mixing and centrifugation at 16,000xg for 20 minutes and 4 °C, supernatants were collected and stored at -80 °C. Samples were analysed within two weeks. The excess of NEM was removed by adding 70µL KOH (2.0M) followed by 60µL HCl (0.01M in 0.3M 3-(N-morpholino)-propanesulfonic acid buffer) to 100 µL of sample. In the assay 100µL sample or standard was reduced by adding 10µL tris(2-carboxyethyl)phospine (Fluka, Bornem, The Netherlands;10% (w/v) in 0.9% sodiumchloride / 4.0 mM EDTA) and incubation at room temperature for 30 min. After reduction, 100µL PCA (0.6M with 1.0mM EDTA) was added and subsequently samples were mixed and centrifuged for 5 min at 10,000xg. Clear supernatant (100µL) was incubated for 1 hour and 60°C with 240µL reaction-mix containing 20µL NaOH (1.55M), 200µL boratebuffer (125mM $K_2B_4O_7{\cdot}4H_2O$ and 4.0 mM EDTA, pH 9.0), and 20µL 7-fluorobenzofurazane-4-sulfonic acid (5mg/mL in borate buffer). Of the derivatised sample 20µL was injected into the high performance liquid chromatography system (16). Data obtained were analysed with the Chromeleon chromatography data-system (Gynkotek, München, Germany). The free-to-oxidised ratio for each thiol (rCys, rHcy, rCys-Gly and rGSH) was calculated from the free and oxidised thiol data.

Characteristics of pregnancy between patients and controls were compared with the Wilcoxon-Mann-Whitney test. Differences in pregnancy as well as post-partum levels between patients and controls were calculated with the Student' s t-test, whereas differences between pregnancy and post-partum levels in the patient and control group were calculated with the paired-Student' s t-test. In view of the multiple testing a significant difference was reached if the P-value < 0.01. All statistical tests were performed using the Astute Statistical Add-In for Microsoft Excel 5 version 1.50.

Results

Women with severe preeclampsia were significantly younger, were more often nulliparous and had offspring with a lower birth weight as compared with control women (Table 8.1). Gestational age at time of study of preeclamptic women was lower compared with that of control women. Table 8.2 shows the results of the free and oxidised concentrations as well as the free-to-oxidised ratios of cysteine (fCys, oCys and rCys, respectively), homocysteine

(fHcy, oHcy and rHcy), cysteinylglycine (fCys-Gly, oCys-Gly, rCys-Gly) and glutathione (GSH, GSSG and rGSH).

Table 8.2. Whole blood free and oxidised levels, and free-to-oxidised ratios of cysteine, homocysteine, cysteinylglycine and glutathione in women with severe preeclampsia and normotensive pregnant controls during pregnancy and in their post-partum period.

	Severe preeclampsia		Controls	
	Pregnancy	Post-partum	Pregnancy	Post-partum
Cysteine				
Free (µmol/L)	106.3 ± 29.8	88.6 ± 19.7*	68.8 ± 10.3$^{\#}$	76.0 ± 8.7*,††
Oxidised (µmol/L)	47.7 ± 13.6	38.6 ± 10.0*	31.2 ± 6.1$^{\#}$	35.7 ± 4.6*
Ratio	2.3 ± 0.4	2.3 ± 0.3	2.2 ± 0.3	2.1 ± 0.2‡
Homocysteine				
Free (µmol/L)	2.7 ± 1.6	3.0 ± 2.1	2.7 ± 1.2	3.4 ± 1.4*
Oxidised (µmol/L)	1.4 ± 0.6	1.2 ± 0.7	1.1 ± 0.5	1.2 ± 0.7
Ratio	2.1 ± 0.9	2.6 ± 0.9†	2.7 ± 0.9††	3.1 ± 0.8‡,††
Cysteinylglycine				
Free (µmol/L)	7.8 ± 2.7	10.1 ± 2.4*	8.5 ± 1.5	11.2 ± 7.9
Oxidised (µmol/L)	1.6 ± 0.6	2.2 ± 0.9*	1.9 ± 0.6	2.4 ± 0.7*
Ratio	5.4 ± 2.4	5.7 ± 3.8	4.7 ± 0.9	5.0 ± 3.7
Glutathione				
Free (µmol/L)	830 ± 179	966 ± 176**	788 ± 124	949 ± 161**
Oxidised (µmol/L)	18.3 ± 9.2	19.0 ± 8.2	17.5 ± 5.5	24.2 ± 9.4*,††
Ratio	54.8 ± 24.2	60.8 ± 33.2	48.6 ± 14.0	46.2 ± 21.3

Data are given in mean ± range.

Statistics:

†: P<0.01, *: P<0.001, **: P<0.0001 pregnancy versus post-partum.

††: P<0.01, ‡: P<0.001, $^{\#}$: P<0.0001 severe preeclampsia versus normotensive controls.

In women with severe preeclampsia fCys as well as oCys levels are higher during pregnancy as compared to the levels after pregnancy, in contrast with controls, who showed lower fCys and oCys levels during pregnancy. This resulted in an unchanged rCys in both groups. During pregnancy women with severe preeclampsia had higher level of fCys and oCys, of which the

fCys level remained higher after pregnancy as compared with corresponding values in control women. After pregnancy also rCys was higher in women with severe preeclampsia than in controls.
During pregnancy fHcy was lower in control women, whereas rHcy was lower in both patients and controls as compared with corresponding values after pregnancy. In women with severe preeclampsia rHcy was lower compared with that of controls. This higher oxidative status of homocysteine remained higher after pregnancy since women with severe preeclampsia had also lower post partum rHcy as compared with control women.

In women with severe preeclampsia both fCysGly and oCysGly were lower, whereas in control subjects only oCysGly was lower during pregnancy as compared with corresponding post partum levels. No differences were found between patient and control subjects during pregnancy or in the post partum period.

During pregnancy free levels of GSH were lower as compared with post partum values in both women with severe preeclampsia and control subjects. Furthermore GSSG levels were elevated in control women after pregnancy and were also higher compared with post partum values of women with severe preeclampsia. No differences between pregnancy and post-partum values were found for rGSH in both groups.

Discussion

Until now only few studies have described the increase of oxidative stress with gestational age in normotensive pregnancies as well as pregnancies complicated with preeclampsia. However, these studies only indirectly showed oxidative stress by measuring products of oxidative damage or antioxidant capacities (19), or performed no measurements in the non-pregnant state (7;8;20) . In contrast, we directly measured oxidative stress by quantification of the free-to-oxidised ratios of cysteine, homocysteine, cysteinyglycine and glutathione. We now demonstrate a transient increase of oxidative stress in normotensive pregnancy, which was even higher in women with preeclampsia as measured by the lower rHcy. Furthermore, in normotensive women as well as women with preeclampsia the levels of glutathione were

transiently decreased during pregnancy, which may point to a higher utilisation of glutathione as also described in women with pregnancy-induced hypertension or mild preeclampsia (19).

In non-pregnant women only reference values for free levels of both cysteine and glutathione in whole blood (21) or only for free levels of glutathione (22;23) are documented. Values as described in these studies are similar to the data in the post partum period reported here. However, no data on whole blood levels of free as well as oxidised homocysteine and cysteinylglycine have been described so far. During uncomplicated pregnancies total plasma volume expands, whereas in preeclampsia a reduction of plasma volume is reported, which might be up to 40% lower in severe cases (24;25). This could explain the transient lower thiol levels during normotensive pregnancy and higher fCys and oCys levels during preeclampsia.

The free and oxidised levels as well as the free-to-oxidised ratios for the thiols during pregnancy in the present study are in line with those described in a previous study of our group (17). Except for the fHcy and oHCy levels in control subjects, which seem to be higher as compared with those in the previous study (2.7±1.2 versus 1.2 (0.2-21.1) µmol/L and 1.1±0.5 versus 0.4 (0.01-1.6) µmol/L, respectively), however, the rHcy was similar. It is known that amounts of lipid peroxides increase with gestational age (5;7), which also results in parallel oxidation of homocysteine. In addition, high levels of free homocysteine are easily (auto)oxidised to homocystine or mixed disulphides, which may induce oxidative stress (26;27). The difference in gestational age between the former (17) and the present study (39^{+1} (37^{+6}-42^{+1}) versus 32^{+0} (28^{+0}-38^{+2}) weeks) may explain the higher levels of oxidised homocysteine in the control group here presented. Therefore, the trend towards a higher oHcy level in women with preeclampsia (P=0.05) suggests that in women with severe preeclampsia a more pronounced oxidative stress is present as compared to that in normotensive pregnant controls. Furthermore, it is interesting, that despite the rather large difference in gestational age between both control groups, we did not find any difference in whole blood values of the other thiols (17). This might indicate that in the third trimester the concentrations of cysteine, cysteinylglycine and glutathione are relatively stable.

In discrepancy with the results of an earlier study (19), we could not confirm the significant higher rGSH 6 weeks post partum in both the patient and control groups. However, in the previous study a different patient population was studied (mild hypertensive diseases versus severe preeclampsia in this study) and only a small number of subjects was investigated.

However, the significant lower ratio for GSH during pregnancy in the previous study emphasises the presence of a transient elevation of oxidative stress in pregnancy. Other studies have only assessed the presence of oxidative stress indirectly by measurement of antioxidant capacity or products of oxidative damage. Davidge et al. (20) reported a higher ratio of malondialdehyde level over antioxidant capacity in women with preeclampsia compared with control subjects, which points to more oxidative stress in preeclampsia. This ratio decreased within 24 hours after delivery in both groups. Furthermore, Little et al.(20) described increasing levels of lipid peroxides with gestational age and a drop of these levels after birth. For vitamin E a longitudinal increase with gestational age was shown by Cikot et al (6), which normalised to preconceptional values within 6 weeks after pregnancy. Our results are in line with those of the above-mentioned studies, which all indicate that during pregnancy oxidative stress increases that normalises after birth.

Barden et al. (28) reported that most biochemical and haematological parameters normalised within 6 weeks after pregnancy. The results of the free-to-oxidised ratios of homocysteine, cysteinylglycine and glutathione in women with preeclampsia and normotensive controls after pregnancy were similar to those in a study of women with severe preeclampsia or normotensive pregnancy who were analysed at least 6 months after their last pregnancy (this thesis, chapter 9). The significant difference for rCys as described here was not present anymore 6 months post partum, which might point to a pregnancy-induced effect. The significant difference for rHcy remained present even after subsequent successful pregnancies and an extended post partum period (pregnancy (this thesis, chapter 9). This might indicate that in women who develop preeclampsia an underlying disturbance in homocysteine redox status is present, which might also predispose to vascular related diseases in later life (9).

In summary, we conclude that during pregnancy more oxidative stress exists as compared with non-pregnant women, whereas in preeclampsia even more oxidative stress is present. This higher level of oxidative stress in women with preeclampsia may be caused by a disorder in homocysteine metabolism, which may also predispose them to vascular related diseases in later life.

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Chapter 9

Thiol status and antioxidant capacity in women with a history of severe preeclampsia.

Maarten T.M. Raijmakers, Eva Maria Roes, Petra L.M. Zusterzeel, Eric A.P. Steegers and Wilbert H.M. Peters.

Abstract

Recent studies suggest that women with a history of preeclampsia are at higher risk to develop cardiovascular diseases (CVD), since the aetiology of both diseases have common characteristics including oxidative stress and activation of vascular endothelium. Therefore, we investigated total plasma levels of cysteine (tCys), homocysteine (tHcy), cysteinylglycine (tCysGly) and glutathione (tGSH), the free-to-oxidised ratio in whole blood of these thiols, the glucose 6-phosphate dehydrogenase enzyme activity and antioxidant capacity in non-pregnant women with a history of severe preeclampsia (n=131) and control women with an uncomplicated obstetrical history (n=94) more than 6 months after their last pregnancy.

Former patients showed a higher level (mean ± SD) of tHcy (13.1 ± 5.0 versus 11.5 ± 4.8 µmol/L; P<0.03) and tCysGly (37.5 ± 5.6 versus 34.0 ± 5.8 µmol/L; P<0.0001) compared to controls, whereas tCys was lower (232 ± 31 versus 242 ± 39; P<0.002). The lower free-to-oxidised ratio of homocysteine (2.3 ± 0.82 versus 2.9 ± 1.0, P<0.001) among women with a history of severe preeclampsia as compared to control subjects might indicate a higher oxidant status for homocysteine. Former patients had also a higher antioxidant capacity as compared to controls (0.79 ± 0.14 versus 0.74 ± 0.11 mmol Fe^{2+}/L, P=0.002). Since women with a history of severe preeclampsia showed elevated total homocysteine levels, which is an independent risk factor for CVD by (auto)oxidation of homocysteine, these women may have an enhanced risk for the subsequent development of cardiovascular-related problems in later life.

Introduction

Preeclampsia is classically defined as pregnancy induced hypertension with proteinuria (1). This severe complication of pregnancy has a prevalence of 5-7% and is accompanied by substantial perinatal as well as maternal morbidity and mortality (2). In the last decade, more and more evidence is found for the role of oxidative stress and endothelial damage in the development of this disease (3-5). Oxidative stress may be counteracted by enzymatic as well as non-enzymatic antioxidants. First line defence against oxidative damage by free radicals, superoxide, lipid peroxides, hydrogen peroxides and other reactive oxygen species (ROS) is provided by the glutathione / glutathione dependent enzyme systems (6;7). Glutathione itself can serve as an antioxidant, in addition to its role as co-factor in the reactions of glutathione S-transferases and glutathione peroxidases, the latter scavenging ROS upon oxidation of glutathione (6;8). To maintain the cellular redox-balance, the oxidised form of glutathione is either quickly reduced by glutathione reductase, or it is excreted when excessive amounts are formed (8).

Women with a history of severe preeclampsia are thought to be at higher risk to develop cardiovascular diseases (CVD) in later life (9;10). It has been proposed that similar pathophysiological pathways lead to the development of both preeclampsia and CVD. Underlying disorders including trombophilia, chronic hypertension and pre-existing endothelial dysfunction, all increase the risk for the development of preeclampsia and are also associated with an increased risk for CVD (11-15). Another common feature in both diseases is the presence of lipid peroxidation (3;16). Hyperhomocysteinemia, which is an independent risk factor for CVD (17;18), may initiate the process of lipid peroxidation by autooxidation of homocysteine, whereby ROS are formed (16).

The goal of our study was to investigate whether risk factors for the development of CVD such as increased levels of homocysteine or oxidative stress are present in non-pregnant women with a history of severe preeclampsia.

Materials and Methods

Patient and Control Subjects

The Institutional Medical Ethical Review Committee approved the study protocol. Preeclampsia was defined according to the standard of the International Society for the Study of Hypertension in Pregnancy as pregnancy induced hypertension (diastolic blood pressure (BP) > 90 mmHg on two or more occasions each more than 4 hours apart) with proteinuria (urinary protein/creatinine ratio >0.30 g/10 mmol). Severe preeclampsia was defined as preeclampsia with a diastolic BP over 110mmHg or the presence of the HELLP syndrome. The HELLP syndrome was defined by hemolysis (lactic dehydrogenase (LDH) enzyme activity >600 IU/L), elevated liver enzymes (both aspartate aminotransferase (ASAT) and alanine aminotransferase (ALAT) enzyme activity >70 IU/L) and low platelets (platelet count $<100x10^9$/L). We recruited 131 women (19), who had experienced severe preeclampsia with or without HELLP syndrome, or HELLP syndrome with or without gestational hypertension (for characteristics see Table 9.1) and 94 women with uncomplicated pregnancies only, at least 6 months after their last pregnancy. All women gave their informed consent. After their index pregnancy 78 (60%) of the former-preeclampsia patients became pregnant again once (n=60; 77%) or more than once (n=18; 23%). Some of these women developed recurrent preeclampsia with or without the HELLP syndrome (27%) or pregnancy induced hypertension (30%), whereas the others remained normotensive (40%) or had a miscarriage (3%). Median (interquartile range) of the time period between blood sampling and last pregnancy did not differ between former-preeclampsia patients and controls (28 (14-54) versus 28 (9-72) months).

Blood Sampling

Whole blood was obtained by venapuncture in sterile vacutainer tubes anti-coagulated with EDTA. Blood was transported without delay to the laboratory at room temperature, where it was handled for analyses of free and oxidised thiols and where the immediate analysis of glucose-6-phoshate dehydrogenase (G6PDH) enzyme activity was performed. The remainder was centrifuged at 1,500xg for 10 min and room temperature. Clear plasma was stored in aliquots at –30°C until analysis.

Table 9.1. Characteristics of women with a history of severe preeclampsia during pregnancy.

Gestational age at delivery (weeks^{+days})	31^{+3} (28^{+6} – 35^{+3})
Systolic BP (mmHg; Korotkoff V)	165 (150 – 180)
Diastolic BP (mmHg; Korotkoff V)	110 (100 – 115)
Protein/Creatinine ratio (g /10 mmol)	2.9 (0.4 – 6.5)
LDH (IU/L)	631 (377 – 1148)
ASAT (IU/L)	84 (28 – 230)
ALAT (IU/L)	75 (21 – 230)
Platelet count (*10^9 / L)	94 (56 – 165)
Hb (g/dL)	7.3 (6.8 – 8.0)
Ht	0.35 (0.31 – 0.38)
Serum creatinine (µmol/L)	76 (69 – 83)
Serum uric acid (µmol/L)	0.40 (0.34 – 0.46)

Note: Values are presented as median (interquartile range).

Abbreviations: Hb – haemoglobin, Ht – haematocrit, BP – blood pressure, LDH – lactate dehydrogenase enzyme activity, ALAT – alanine aminotransferase enzyme activity, ASAT – aspartate aminotransferase.

Biochemical Analyses

For the measurement of the G6PDH enzyme activity the method as described by Fairbanks et al. was used (20). In short; hemolysates were prepared by adding 900µL hemolysis buffer (5% (v/v) Triton-X 100, EDTA pH 7.0 (2.7 mM), and dithiotreitol (4 mM) in milliQ) to 100µL whole blood followed by thoroughly mixing. 20-µL sample or blank (hemolysis buffer without Triton-X 100) was added to a reaction tube containing 880µl reaction mix (100 µL Tris (1 M) / EDTA-pH 8.0 (5 mM), 100µL $MgCl_2$ (100 mM), and 100µL NADP (2.0 mM) in milliQ) and incubated for 10 min at 37°C. Hereafter, samples were mixed with 100µL glucose-6-phosphate (0.6 mM) in Tris/EDTA buffer) and transferred to a disposable cuvet. The increase of absorbance at 340nm was measured during 10 min at 37°C. Slopes were used to calculate the G6PDH enzyme activity and expressed as U/L.

For the measurement of free and oxidised thiols whole blood was handled within one hour as described earlier by our group (8). In short, for assay of the free thiol levels (both reduced and oxidised non-protein bound thiols), 500µL whole blood was added to 500µL ice-cold 12% perchloric acid (PCA) containing 2.0 mM bathophenanthrolinedisulfonic acid (BPDS). For

assay of oxidised free thiols, another 500 µL whole blood was added to 500 µL 12% PCA containing 2.0 mM BPDS and 40 mM N-ethylmaleimide (NEM). After thorough mixing and centrifugation at 16,000xg for 20 min and 4 °C, supernatants were collected and stored at -80 °C until analysis. Before analysis the excess of NEM was removed by adding 70µL KOH (2.0 M) followed by 60µL HCl (10 mM in 300 mM 3-[N-morpholino]-propanesulfonic acid buffer) to 100 µL of sample.

Whole blood as well as plasma samples were analysed for levels of cysteine (Cys), homocysteine (Hcy), cysteinylglycine (CysGly), and glutathione (GSH) by high performance liquid chromatography with fluorescent detection as described previously (8;21). In short, 100µL sample or standard was reduced by adding 10µL tris(2-carboxyethyl)phospine (10% (w/v) in NaCl (0.9%)/ EDTA (4.0 mM)) and followed by incubation at room temperature for 30 min. After reduction, 100µL PCA (0.6 M with 1.0 mM EDTA) was added and subsequently samples were mixed and centrifuged for 5 min at 10,000xg. Clear supernatant (100µL) was incubated with 240µL reaction-mix containing 20µL NaOH (1.55 M), 200µL boratebuffer ($K_2B_4O_7 \cdot 4H_2O$ (125 mM) and EDTA (4.0 mM); pH 9.0), and 20µL 7-fluorobenzofurazane-4-sulfonic acid (5mg/mL in borate buffer) for 1 hour and 60°C. Of the derivatised sample 20µL was injected into the high performance liquid chromatography system. Data obtained were analysed with the Chromeleon chromatography data-system, Gynkotek (München, Germany). Thiol levels were calculated using four-point calibration curves for each thiol and were expressed in µmol/L. As a measure of the oxidant status we calculated the free-to-oxidised ratio, which has no dimension.

The antioxidant capacity of each subject was measured using the Ferric Reducing Ability of Plasma (FRAP) assay as described by Benzie and Strain (22). Briefly; 33µL sample, blank (H_20) or Fe^{2+}-standard was added to 1.0mL FRAP solution (25mL sodiumacetaat-pH 3.6 (300 mM), 2.5mL 2,4,6-tripyridyl-s-triazine (40mM), and 2.5mL $FeCl_3 \cdot 6H_20$ (20 mM)) and incubated for 3 min at 37°C. Absorbance against the blank was read at 593nm. The antioxidant capacity was calculated using a seven-point calibration curve of known amounts of Fe^{2+} and expressed as mmol Fe^{2+}/L.

Statistical Analysis

Differences in biochemical parameters between the patient and control population were calculated with the Student' s t-test using the Astute Statistical Add-In for Microsoft Excel 5 version 1.50. A difference was considered significant when $P<0.05$.

Table 9.2. Whole blood glucose 6-phosphate dehydrogenase enzyme activity, plasma antioxidant capacity, total plasma concentrations and free-to-oxidised ratios in whole blood of thiols in women with uncomplicated pregnancy or with a history of severe preeclampsia.

	History of uncomplicated pregnancy	History of severe preeclampsia
G6PDH enzyme activity (U/L)	3.5 ± 0.6	3.4 ± 0.6
FRAP (mmol Fe^{2+}/L)	0.74 ± 0.11	0.79 ± 0.14†
Cysteine:		
Plasma (µmol/L)	242 ± 39	232 ± 31‡
Ratio free-to-oxidised	2.3 ± 0.3	2.4 ± 0.5
Homocysteine:		
Plasma (µmol/L)	11.5 ± 4.8	13.1 ± 5.0‡
Ratio free-to-oxidised	2.9 ± 1.0	2.3 ± 0.8*
Cysteinylglycine:		
Plasma (µmol/L)	34.0 ± 5.8	37.5 ± 5.6*
Ratio free-to-oxidised	4.7 ± 2.3	5.0 ± 3.7
Glutathione:		
Plasma (µmol/L)	8.3 ± 1.9	8.3 ± 1.7
Ratio free-to-oxidised	51.1 ± 20.1	56.4 ± 22.3

Data are given as mean ± SD, Number of subjects (normotensive – preeclampsia) investigated are G6PDH enzyme activity (84 – 125), FRAP (86 – 124) and thiol measurements (94 – 131).

Abbreviations: G6PDH – glucose 6-phosphate dehydrogenase enzyme activity; FRAP – ferric reducing ability of plasma.

Statistics: *: $P<0.0001$, †:$P< 0.002$, ‡: $P<0.03$ normotensive versus preeclamptic pregnancy.

Results

Table 9.2 shows the results of the whole blood G6PDH enzyme activity, plasma antioxidant capacity, total concentrations of cysteine (tCys), homocysteine (tHcy), cysteinylglycine (tCysGly) and glutathione (tGSH) in plasma, and free-to-oxidised ratios of these thiols in whole blood in both study groups.

The enzyme activity of G6PDH was similar in both groups. Plasma levels of tCys were significantly lower in women with a history of severe preeclampsia as compared with those in control women, whereas tHcy and tCysGly levels were higher. No significant differences were found for tGSH levels between former severe preeclampsia patients and control women.

We did not find any differences between patient and control subjects for the free-to-oxidised ratio of Cys, CysGly and GSH. Only the free-to-oxidised ratio of Hcy was significantly lower in women with a history of severe preeclampsia as compared with that of control women, which indicates the presence of a higher oxidative Hcy status in former severe preeclampsia-patients. Former severe preeclampsia-patients showed a significant higher antioxidant capacity, as measured by the FRAP assay, in comparison to control women.

Discussion

Preeclampsia and cardiovascular diseases (CVD) have common characteristics including genetic thrombophilia, endothelial dysfunction and oxidative stress (3;4;12;23;24). Furthermore, in follow-up studies a clear association between preeclampsia and the subsequent development of CVD was found (9;10). Previous studies have looked at these common characteristics in former patients with preeclampsia respectively 10 weeks (25), 3 months (26), or 5 months (11) after the index pregnancy. In our study we investigated the thiol status and antioxidant capacity in former severe preeclampsia-patients at least 6 months after their last pregnancy. Our main finding was that both plasma tHcy concentrations and the homocysteine oxidant status in whole blood were higher in former severe preeclampsia-patients as compared with control subjects, whereas also antioxidant capacity was higher in women with a history of severe preeclampsia.

Hyperhomocysteinemia is an independent risk factor for the development of CVD, including those of coronary, cerebral as well as peripheral arteries (16;27), which affects females more than males (18). The pathways how Hcy may lead to endothelial dysfunction and CVD are still unclear, however, it has been hypothesised that oxidative stress might play a role, since (auto)oxidation of Hcy, indicated by a decrease of tCys levels (28) as noticed in our study, results in the formation of several types of reactive oxygen species (ROS) (17). Furthermore, the induction of endothelial dysfunction by oral methionine loading (27;28), which results in temporal elevated homocysteine levels, could be prevented by pre-treatment with vitamin C (29). In the defence against oxidative damage by ROS glutathione plays an important role. In scavenging ROS, glutathione is oxidised (GSSG) either by autooxidation or in a reaction catalysed by glutathione peroxidases (8). Since increasing amounts of intracellular GSSG may cause metabolic perturbations, including demolished protein synthesis (30), two different ways to preserve intracellular redox state exist. GSSG can quickly be reduced by glutathione reductase in a NADPH dependent reaction (8;31), or GSSG can be excreted from the erythrocyte to plasma for which a Mg-ATP dependent transporter is described (30;32;33). However, the efflux of intracellular glutathione is the initial step of glutathione breakdown by γ-glutamyltranspeptidase, which might cleave the γ-glutamyl bond of glutathione resulting in glutamate and cysteinylglycine (33). Therefore, in the prevention of intracellular oxidative stress the plasma levels of cysteinylglycine might increase. Indeed in our study former severe preeclampsia patients showed lower plasma cysteine and higher cysteinylglycine levels as compared with control subjects.

Surprisingly, the antioxidant capacity, as measured by the FRAP assay, was higher in former severe preeclampsia patients as compared with those in control subject. Since glutathione is a very potent antioxidant, we also expected a higher plasma glutathione concentration in women who had severe preeclampsia. However, this was not found which could be explained by the fact that during homeostasis glutathione disulfide is quickly reduced by glutathione reductase (8;31) instead of secreted from the cell. Since the FRAP assay does not measure –SH containing antioxidants (22), this indicates that other non thiol-related antioxidants are probably elevated. The higher antioxidant capacity may be an adaptive mechanism to prevent oxidative damage in these women during their non-pregnant state, however due to continuous generation of ROS oxidative damage may slowly accumulate, which could result in problems during later life. The antioxidant capacity might not be sufficient during pregnancy, because

the level of oxidative stress progressively increases during gestation (34) and is even more elevated in preeclampsia (3).

After their index pregnancy 60% of the former severe preeclampsia patients had one or more subsequent pregnancies. Of those pregnancies 40% remained normotensive, whereas in 57% a recurrence of hypertensive complications being either pregnancy induced hypertension or preeclampsia / HELLP was reported. Women with recurrent preeclampsia may represent a different subset of women with a history of severe preeclampsia with regard to underlying pathophysiological mechanism. We could not demonstrate any significant differences, however, for all the parameters investigated between former patients without or with recurrent preeclampsia after the index-pregnancy (Table 9.3).

Table 9.3. Whole blood glucose 6-phosphate dehydrogenase enzyme activity, plasma antioxidant capacity, total plasma concentrations and free-to-oxidised ratios in whole blood of thiols in preeclamptic women with recurrent hypertensive disorders of pregnancy after the index-pregnancy and those without further complications.

	No complications after index-pregnancy (n=31)	Recurrent hypertensive disorders of pregnancy (n=45)
G6PDH enzyme activity (U/L)	3.4 ± 0.6	3.3 ± 0.5
FRAP (mmol Fe^{2+}/L)	0.79 ± 0.15	0.80 ± 0.14
Cysteine:		
Plasma (µmol/L)	227 ± 30	235 ± 32
Ratio free-to-oxidised	2.3 ± 0.5	2.4 ± 0.3
Homocysteine:		
Plasma (µmol/L)	13.4 ± 6.0	12.9 ± 4.1
Ratio free-to-oxidised	2.2 ± 0.8	2.3 ± 0.7
Cysteinylglycine:		
Plasma (µmol/L)	37.8 ± 5.4	37.3 ± 5.9
Ratio free-to-oxidised	5.0 ± 3.0	5.1 ± 4.1
Glutathione:		
Plasma (µmol/L)	8.1 ± 1.5	8.4 ± 1.9
Ratio free-to-oxidised	56.8 ± 24.6	56.5 ± 19.3

Data are given as mean ± SD

Furthermore, in most former patients blood was collected in the period after pregnancies, which were not complicated by severe preeclampsia. This may indicate that the differences found in former patients compared to controls may represent underlying factors instead of being initiated or influenced by pregnancy. Therefore, we postulate that the higher redox-status of homocysteine in women with a history of severe preeclampsia as compared to uncomplicated pregnancy outcome may be a pre-existing factor, which predisposes these women to develop preeclampsia and eventually CVD in later life. Consequently our results indicate that homocysteine-lowering therapy, in addition to antioxidant supplementation that showed to decrease the risk of preeclampsia in a high-risk population (35), might be beneficial in prevention of preeclampsia.

In conclusion, the higher oxidative homocysteine status in whole blood, the higher tHcy and tCysGly and lower tCys levels in plasma of non-pregnant women with a history of severe preeclampsia compared to corresponding values in women after uncomplicated pregnancies, may be involved in the pathophysiological mechanisms contributing to the increased risk for the subsequent development of CVD. The higher antioxidant capacity might be an adaptive mechanism to prevent general oxidative stress in these women.

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Part IV

Genetic contributors to the pathogenesis of pregnancy disorders

Chapter 10

The C242T-polymorphism of the NADPH/NADH oxidase gene p22phox subunit is not associated with preeclampsia.

Maarten T.M. Raijmakers, Eva Maria Roes, Eric A.P. Steegers, and Wilbert H.M. Peters.

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Abstract

Background: Preeclampsia is a pregnancy related multisystem disorder characterised by elevation of blood pressure and proteinuria, in which oxidative stress may play an important role. Blood pressure is partly controlled by O_2^- production by NADPH/NADH oxidase and recently it was shown that a C242T substitution in the p22phox gene was associated with coronary artery disease, in which elevated blood pressure and oxidative stress are also important pathophysiologic features.

Aim: To study the prevalence of the C242T polymorphism in the NADPH/NADH oxidase gene in women with preeclampsia and / or hemolysis, elevated liver enzymes and low platelets (HELLP) syndrome as compared to women with a normotensive pregnancy.

Methods and Results: DNA from control women (n=78), women with preeclampsia (n=40), HELLP syndrome (n=9) or women with HELLP complicated by pregnancy induced hypertension or preeclampsia (n=46) was tested for the presence of the C242T polymorphism by polymerase chain reaction followed by restriction fragment length polymorphism. The prevalence of the homozygous CC-genotype was similar in the patient groups compared with controls. The allele frequency of the T-allele was 31% in both control and patient groups.

Conclusions: The C242T polymorphism in the p22phox subunit of the NADPH/NADH oxidase gene is not associated with preeclampsia. Therefore, oxidative stress generated by NADPH/NADH oxidase probably does not play a role in the development of preeclampsia.

Introduction

Preeclampsia is a pregnancy related multi-system disorder with considerable maternal and foetal mortality and morbidity (1). It is clinically characterised by an elevated blood pressure and proteinuria. Although the pathogenesis of the disorder is not yet elucidated, it is thought that oxidative stress and damage of the endothelium lining the blood vessel wall are important contributing factors (2). Regulation of blood pressure is partly controlled by NADPH/NADH oxidase, which releases the superoxide anion O_2^- upon angiotensin II activation, resulting in vasoconstriction and elevation of blood pressure (3). The localisation of NADPH/NADH oxidase, mainly on the cell membrane of neutrophils and endothelial cells, suggests that this enzyme may play an role in the pathophysiology of disorders in which oxidative stress is involved. Recently it was shown that a C242T substitution in the gene of the p22phox subunit of NADPH/NADH oxidase, changing histidine-72 to tyrosine in the heme-binding site, which reduces the superoxide production (4), might reduce the susceptibility to coronary artery disease (CAD) in Japanese populations (5-6). Although a controversy exists between the reports in Caucasian populations (7-8), the C242T polymorphism was associated with progression of CAD (9). Since the aetiology of CAD seems to be comparable to that of preeclampsia and the higher risk of preeclamptic women to develop vascular related diseases in later life (10-11), we studied the prevalence of the CC-genotype, which is associated with highest superoxide production, in women with preeclampsia and/or hemolysis, elevated liver enzymes, low platelets (HELLP) syndrome and compared it with controls.

Materials and Methods

The Institutional Medical Ethical Review Committee approved the study protocol. After informed consent was obtained, whole blood from women hospitalised for preeclampsia (n=40), women with the HELLP syndrome only (n=9) or both HELLP syndrome and preeclampsia or pregnancy induced hypertension (n=46) was collected. Preeclampsia was defined according to the ISSHP criteria, being a pregnancy induced hypertension (diastolic blood pressure ≥90 mmHg on two or more occasions, each more than 4 hours apart) with proteinuria (protein/creatinine ratio ≥0.30 g/10 mmol). The HELLP syndrome was biochemically characterised by the simultaneous occurrence of hemolysis (lactic

dehydrogenase level >600 IU/L), elevated liver enzymes (both aspartate and alanine aminotransferase activity >70 IU/L) and low platelets (thrombocyte count under $100x10^9$/L). Women with at least one completed normotensive pregnancy served as control subjects (n=78). The characteristics of the women studied are depicted in Table 10.1.

DNA was isolated using the Puregene genomic DNA isolation kit (Gentra Systems, Minneapolis, USA), according the instructions of the manufacturer. DNA was analysed for the C242T polymorphism in the p22phox subunit of the NADPH/NADH oxidase gene using PCR/RFLP as described previously (5). In short, a 348 bp DNA fragment containing the polymorph C242T site was amplified from genomic DNA using sterile H_2O as a negative control. The PCR product was digested with RsaI. When an allele bears a T instead of a C at position 242 this results in a RsaI digestion site yielding two fragments of 160 bp and 188 bp. Otherwise, when a C is present, the 348 bp PCR product is not digested. The presence of both heterozygous or homozygous polymorph subjects indicates that the digestion in the RFLP analysis was successful. Genotypes at position 242 were assigned as follows: CC, homozygous wildtype; CT: heterozygous; TT: homozygous polymorph.
Differences between patient characteristics were analysed with the non-parametric Wilcoxon-Mann-Whitney test and significance was reached at $P<0.05$. Prevalence of the different genotypes was calculating using Chi-square test. Both statistical tests were performed using the Astute Statistical Add-In for Microsoft Excel 5 version 1.50.

Results

A similar distribution of the three possible C242T polymorph genotypes in the p22phox gene of NADPH/NADH oxidase was found in the different patient and control groups (Table 10.2). This resulted in non-significant odds ratios (95% CI) of 0.95 (0.44-2.04), 0.87 (0.42-1.81) and 1.9 (0.44-8.14) for the women with preeclampsia, HELLP with preeclampsia or pregnancy induced hypertension and HELLP solely, respectively. Also the prevalence of the 242 C- or T-genotypes for the total patient population were found to be similar when compared to the control group. The odds ratio (95% CI) of the total patient population compared with controls was 0.97 (0.53-1.77).
The T-allele frequency was 0.31 for both the control and the total patient population.

Table 10.1. Characteristics of the women studied.

	Controls (n=78)	Preeclampsia (n=40)	PE/PIH + HELLP (n=46)	HELLP (n=9)	All patients (n=95)
Age (y)	32 [22 – 42]	31 [20 – 37]	30 [19 – 41]	30 [25 – 34]	30 [19 – 41]
Gestational age (wk^{+days})		33^{+4} [24^{+4} – 38^{+0}]	30^{+6} [26^{+3} – 39^{+5}]	30^{+0} [27^{+6} – 40^{+4}]	31^{+3} [24^{+4} – 40^{+4}]
Diastolic BP (mmHg; Korotkoff V)		115 [95 – 140]	115 [90 – 140]	80 [80 – 95]†,‡	115 [80 – 140]
Protein/creatinine ratio (g/10mmol)		3.3 [0.1 –33.8]	6.9 [0.01 – 47.0]	0.1 [0.03 – 0.23]†,‡	5.2 [0.01 – 47.0]
Alanine aminotransferase (IU/L)		18 [6 – 596]	206 [40 – 1760]*	455 [220 – 727]*,‡	137 [6 – 1760]
Aspartate aminotransferase (IU/L)		27 [12 – 593]	238 [73 – 1780]*	518 [338 – 768]*,‡	152 [12 – 1780]
Lactic dehydrogenase (IU/L)		464 [292 – 1974]	1179 [319 – 5980]*	1349 [715 – 1927]†	780 [292 – 5980]
Thrombocyte count ($x10^9$)		185 [101 – 573]	52 [15 – 98]*	58 [39 – 98]*	72 [15 – 573]

Abbreviations: BP – blood pressure; PE – preeclampsia; PIH – pregnancy-induced hypertension; HELLP – hemolysis, elevated liver enzymes and low platelets syndrome

Statistics:

*: P<0.0001 Preeclampsia versus PE/PIH + HELLP and Preeclampsia versus HELLP

†: P<0.001 Preeclampsia versus HELLP

‡: P<0.03 PE/PIH + HELLP versus HELLP

Table 10.2. Distribution of C242T polymorhism in the p22phox subunit of the NADPH/NADH-oxidase gene.

	Controls (n=78)	Preeclampsia (n=40)	PE/PIH + HELLP (n=46)	HELLP (n=9)	All patients (n=95)
CC	40 (51.3)	20 (50.0)	22 (47.8)	6 (67.7)	48 (50.5)
TC + TT	38 (48.7)	20 (50.0)	24 (52.2.)	3 (33.3)	47 (49.5)
Odds Ratio (95% CI)		0.95 (0.44 – 2.04)	0.87 (0.42 – 1.81)	1.9 (0.44 – 8.14)	0.97 (0.53 – 1.77)
T allele frequency	0.31	0.30	0.35	0.17	0.31

Abbreviations: PE – preeclampsia; PIH – pregnancy-induced hypertension; CI – confidence interval;
HELLP – hemolysis, elevated liver enzymes and low platelets syndrome

Percentages of the prevalence of the different genotypes are given in parenthesis.

Polymorpic genotypes at position 242 were assigned as follows:

CC: homozygous wildtype; both alleles bearing cytosine

CT: heterozygous; bearing cytosine at one allele and thymidine at the other

TT: homozygous mutant; both alleles bearing thymidine

Discussion

Previously it was shown by Inoue et al. (5) that the C242T polymorphism in the p22phox subunit of NADPH/NADH oxidase gene decreased the susceptibility to coronary artery diseases (CAD) in a Japanese population. They postulated that this polymorphism in the heme-binding site might have an effect on activity and regulation of NADPH/NADH oxidase. Individuals with the CT or TT genotypes might have lower oxidative stress as result of lower O_2^- production as compared to individuals bearing the CC genotype (4). Since mechanisms contributing to CAD may also be important in the aetiology of preeclampsia (2) we hypothesised that women who develop preeclampsia may have a higher prevalence of the CC genotype. Although our subgroups are small and as a consequence the power to detect differences is relatively low, the similar distribution of the CC-genotype in the patient and control populations provide evidence that the CC genotype was not associated with preeclampsia in a Dutch study population. Our findings in a Caucasian population are in line with the findings of Guzik et al. (4), Gardemann et al. (7) Li et al. (8), and Cahilly (9), who found similar prevalences of the CC-genotype (45.4%, 44.3%, 45.6%, and 43.5%, respectively) and T-allele frequency (33%, 32%, 34%, and 34%, respectively) in large Caucasian populations. However, in Japanese control subjects the prevalence of the CC genotype was 73.6% (5) or 86.7% (6) compared with 51.3% in our Caucasian population. Racial differences between the two study populations could explain these different findings for the prevalence of the C242T-polymporhic variants in controls.

In conclusion, since preeclampsia is a multi-factorial condition (1), in which oxidative stress seems to plays a central role, the finding of a similar distribution of the C242T polymorph variants between patients and controls suggests that superoxide radical production by NADPH/NADH oxidase does not play a role in development of preeclampsia (2).

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Chapter 11

Haptoglobin genotype and its association with the HELLP syndrome.

Maarten T.M. Raijmakers, Eva Maria Roes, René H.M. te Morsche, Eric A.P. Steegers, Wilbert H.M. Peters.

Abstract

Background: Haptoglobin (Hp) is a glycoprotein that consists of three phenotypes, Hp1-1, Hp1-2 and Hp2-2, which are encoded by different alleles. Hp is protective against oxidative damage by its capacity to bind free haemoglobin for which Hp2-2 has the lowest affinity. In the pathogenesis of preeclampsia and the haemolysis, elevated liver enzymes, low platelets (HELLP) syndrome, oxidative stress may play an important role, which might be exaggerated by elevated levels of free haemoglobin generated by haemolysis.

Aim: To investigate the prevalence of the *Hp2-2* genotype in women with a history of severe preeclampsia with or without HELLP syndrome as compared to women with uncomplicated pregnancies only.

Materials & Methods: Haptoglobin genotypes were assessed in genomic DNA samples of women with severe preeclampsia with or without HELLP syndrome (n=131) and control women (n=166) using polymerase chain reaction. A subset of patients with HELLP syndrome (n=90) was analysed separately. Data were analysed using the Chi-square test.

Results: Women with severe preeclampsia tended to have a lower prevalence of *Hp2-2* genotype as compared with that of control women (27% versus 35%; χ^2=1.9 and P=0.2), however the difference was significant in the subset of women with HELLP syndrome (20% versus 35%; χ^2=6.2 and P=0.01).

The *Hp2* allele frequency in women with preeclampsia was similar to that of control women (0.52 and 0.57, respectively), whereas that of women with HELLP syndrome was lower (0.44; χ^2=6.3 and P=0.01).

Conclusion: Women with the *Hp2-2* genotype have a lower risk to develop HELLP syndrome.

Introduction

Haptoglobin (Hp) is an acute-phase α_2-sialoglycoprotein, which is characterised by molecular heterogeneity (1). Due to a genetic polymorphism different Hp phenotypes exist of which Hp1-1, Hp1-2 and Hp2-2 are the three major isoforms in humans. Hp consists of two different polypeptide chains, the heavy β-chain, which is identical in all haptoglobins, whereas modifications in the light α-chain lead to the different Hp phenotypes. The α^1-chain can be divided in a slow (α^{1S}) and fast (α^{1F}) migrating chain. These two chains differ in one amino acid; at position 54 the lysine of the α^{1F}-chain is substituted for a glutamic acid in the α^{1S}-chain. The α^2-chain is the result of a fusion of the α^{1S}- and the α^{1F}-allele (2).

The most important function of Hp is capturing haemoglobin, thereby preventing iron loss and subsequent oxidative damage generated by free iron in the vascular system of the kidneys. Binding of haemoglobin to Hp is beneficial for the human body in several other ways. Hp is protective against cell damage by scavenging free radicals, such as the hydroxyl radical, of which the formation is promoted by the presence of free haemoglobin. Furthermore, the Hp-haemoglobin complex inhibits the vasodilatory effect of nitric oxide and provides a non-specific defence against bacterial invasion, since free haeme iron is necessary for bacterial growth. Furthermore, Hp itself was identified as a serum angiogenic factor and plays a role in proliferation and differentiation of vascular endothelium. Hp2-2 has stronger angiogenic functionality than Hp1-1, whereas Hp1-1 has the highest affinity for haemoglobin and is therefore associated with antioxidant capacity and other functional properties of Hp (1).

Preeclampsia, which is characterised by pregnancy-induced hypertension and concurrent proteinuria, can be complicated by the haemolysis, elevated liver enzymes and low platelets (HELLP) syndrome, which may also occur solely (3). The pathogenesis of preeclampsia and HELLP is largely unknown, although, it is postulated that maladaptation of throphoblast invasion may results in poor placental perfusion and local oxidative stress (4), which could subsequently affect maternal circulation. Systemic maternal oxidative stress may result in the clinical manifestations as seen in women with preeclampsia including dysfunction of the vascular endothelium (5).

A previous study associated a higher incidence of the Hp2-2 phenotype with the occurrence of pregnancy induced hypertension (6). Since Hp2-2 has the lowest antioxidant capacity by poor affinity for haemoglobin and therefore may be less capable to prevent oxidative damage induced by free haemoglobin present after haemolysis, we hypothesised that occurrence of the *Hp2-2* genotype was associated with the HELLP syndrome. Therefore, we investigated the prevalence of the *Hp2-2* genotype in patients with a history of severe preeclampsia with or without HELLP syndrome as compared to women with uncomplicated pregnancies only.

Materials and Methods

The Institutional Review Board approved the study protocol. After informed consent was obtained whole blood was collected from 131 women, who had experienced severe preeclampsia with or without HELLP syndrome, or HELLP syndrome solely (7). Since haptoglobin is associated with the binding of free haemoglobin we separately analysed a subset of 90 women with HELLP syndrome with or withoud gestational hypertension. Characteristics of the total patient group and the subgroup of women with HELLP syndrome are depicted in Table 11.1. Preeclampsia was defined as a diastolic blood pressure >90 mmHg on two or more occasions each more than 4 hours apart, with proteinuria (protein/creatinine ratio >0.30 g/10 mmol) according to the standard of the International Society for the Study of Hypertension in Pregnancy. HELLP was defined as haemolysis (lactic dehydrogenase level >600 IU/L), elevated liver enzymes (both aspartate and alanine aminotransferase activity >70 IU/L) and low platelets (platelet count $<100x10^9$/L). As a control group 166 women, who experienced uncomplicated pregnancies only, were recruited.

DNA was isolated using the Puregene genomic DNA isolation kit (Gentra Systems, Minneapolis, USA), according to the instructions of the manufacturer. Genomic DNA was analysed for the presence of the three different main genotypes encoding for haptoglobin using polymerase chain reactions with primer sets and conditions exactly as described before by Yano et al. (2).

The differences between the two study groups were calculated with the Chi-square test using Astute Statistical Add-In for Microsoft Excel 5 version 1.50. Odds ratios (OR) with 95%

confidence interval (95% CI) were calculated for the *Hp2-2* genotype versus the other genotypes.

Table 11.1. Characteristics of women with severe preeclampsia and the subset of women with HELLP syndrome.

	Severe Preeclampsia (n=131)	HELLP Syndrome (n=90)
GA at delivery (weeks^{+days})	31^{+3} (29^{+3} – 35^{+3})	31^{+1} (29^{+0} – 33^{+4})
Systolic BP (mmHg; Korotkoff V)	160 (150 – 175)	160 (150 – 180)
Diastolic BP (mmHg; Korotkoff V)	110 (100 – 115)	110 (100 – 115)
Protein/Creatinine ratio (g /10 mmol)	3.5 (0.4 – 6.0)	1.4 (0.3 – 4.6)
LDH (IU/L)	735 (506 – 1295)	845 (614 – 1732)
ASAT (IU/L)	143 (55 – 320)	202 (94 – 397)
ALAT (IU/L)	140 (51 – 270)	202 (85 – 362)
Platelet count (*10^9 / L)	78 (52 – 142)	65 (50 – 95)
Haemoglobin (g/dL)	7.6 (6.8 – 8.0)	7.4 (6.7 – 7.9)
Haematocrit	0.35 (0.32 – 0.48)	0.35 (0.31 – 0.37)
Serum creatinine (µmol/L)	75 (68 – 83)	74 (68 – 83)
Serum uric acid (µmol/L)	0.40 (0.34 – 0.46)	0.41 (0.34 – 0.46)

Data are given as medians (25th – 75th percentiles)

Abbreviations: GA – gestational age; BP – blood pressure; LDH – lactic dehydrogenase; ASAT – aspartate aminotransferase enzyme activity; ALAT – alanine aminotransferase enzyme activity

Results

The distribution of the different Hp genotypes is summarised in Table 11.2. In one woman from the HELLP group none of the specific Hp alleles could be amplified and this patient therefore most probably bears the rare Hp null-genotype (*Hp0-0*) (1). The prevalence of the *Hp2-2* genotype in women with a history of severe preeclampsia was not different as compared with that of control women (27% versus 35%, respectively; $\chi^2 = 1.9$ and $P = 0.2$), which resulted in an odds ratio (OR) (95% CI) of 1.0 (0.6 – 1.6). In the subset of women with HELLP syndrome, however, a lower prevalence of the *Hp2-2* genotype (20%) was noticed as compared to controls ($\chi^2 = 6.2$ and $P = 0.01$) resulting in an OR (95% CI) of 2.1 (1.2 – 3.9).

Table 11.2. Distribution of haptoglobin genotypes.

Genotype	Severe Preeclampsia (n=131)	HELLP syndrome (n=90)	Controls (n=166)
Hp2-2	36 (27%)	18 (20%)[a]	58 (35%)
Hp2-1	65 (50%)	43 (48%)	73 (44%)
Hp1-1	29 (23%)	28 (31%)	35 (21%)
Hp0-0	1 (0.8%)	1 (1%)	
Frequency of *Hp2*-allele	0.52	0.44 [a]	0.57

Percentages are given between parenthesis.

Statistics:

[a]: P=0.01 complicated pregnancy versus control women.

Genotypes: *Hp1-1*: Both alleles encoding for α^{1S} or α^{1F}

Hp1-2: One allele encoding for α^{1S} or α^{1F} and the other for α^{2}

Hp2-2: Both alleles encoding for α^{2}

Hp0-0: No amplification of one of the alleles

The *Hp2*-allele frequency was 0.57 in the control women. Women with a history of severe preeclampsia had a similar allele frequency (0.52; OR (95% CI) of 1.1 (0.8 – 1.6)), whereas in women with HELLP syndrome the *Hp2* allele was less frequently present (0.44; $\chi^2 = 6.3$, P = 0.01). This resulted in an OR (95% CI) of 1.6 (1.1 – 2.3).

Discussion

The pathogenesis of preeclampsia and cardiovascular related diseases, e.g. essential hypertension and coronary artery diseases, share common characteristics including thrombophilia, endothelial damage and oxidative stress (5,8-11). Furthermore, follow-up studies show that women who had preeclampsia during their pregnancy are at higher risk to develop vascular related diseases (12,13). Higher incidence of the Hp 2-2 phenotype was associated with an increased risk for pregnancy induced hypertension (6), severity of cardiovascular diseases like established essential hypertension (14) or coronary artery disease (15). Therefore, we hypothesised that a higher incidence of the *Hp 2-2* genotype could be present in women with severe preeclampsia as compared to control women. In contrast,

however, we found a tendency to a lower incidence of the *Hp 2-2* genotype in women with severe preeclampsia, whereas in a subset of women with the HELLP syndrome, a significant lower incidence of the *Hp2-2* genotype was present as compared to control women. Also the *Hp2*-allele frequency was lower in women with HELLP syndrome than in control women. However, control women showed a similar *Hp2*-allele frequency as reported in other control populations by other investigators (1,14). Therefore, our results indicate that women with the *Hp2-2* genotype have a lower risk to develop the HELLP syndrome.

This unexpected finding of the lower prevalence of the *Hp2-2* genotype in women with HELLP syndrome as compared with that in control women may be explained by a combination of factors. Firstly, preeclampsia and HELLP syndrome are multifactorial diseases in which placental development and maternal defence against oxidative stress among many other factors may play an important role (3,9,16). Secondly, Hp1-1 and Hp2-2 phenotypes have different efficiency for the various functional properties of Hp (1), therefore each phenotype may be protective at another stage of the disease. Hp has angiogenetic properties with Hp2-2 being the most potent polymorph variant, consequently Hp2-2 may be beneficial for placental development. However, during the clinical stage of preeclampsia the antioxidant properties of Hp, i.e. binding of free haemoglobin and direct scavenging of free radicals, may be more important. Since Hp1-1 has the highest affinity to bind haemoglobin, it may provide the best protection against oxidative stress (1). Our results might suggest that Hp is important as one of the factors regulating placental development during early pregnancy, where absence of Hp2-2 could lead to a disturbed placental development. This could explain the lower incidence of the *Hp2-2* genotype in women with the HELLP syndrome. Thus, the angiogenetic role of Hp2-2 during early pregnancy might be more important than the antioxidant capacity of binding free haemoglobin during a later stage of gestation of Hp1-1.

In conclusion, the *Hp2-2* genotype is associated with a lower risk for the development of the HELLP syndrome.

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Chapter 12

Parental association of the Tyr113His polymorphism in the epoxide hydrolase gene with preeclampsia.

Eva Maria Roes, Maarten T.M. Raijmakers, Hennie M.J. Roelofs, René H.M. Te Morsche, Petra L.M. Zusterzeel, Wilbert H.M. Peters, Eric A.P. Steegers.

Abstract

Background: A genetic predisposition is known to be involved in the development of preeclampsia. In previous studies maternal protein levels as well as genotype of biotransformation enzymes, such as glutathione S-transferase P1-1 and epoxide hydrolase (EPHX), were associated with the development of preeclampsia. In addition, for the polymorphism in glutathione S-transferase P1-1 also a paternal and foetal contribution to the risk for preeclampsia could be demonstrated.

Objective: To investigate the association of the Tyr113His polymorphism in *EPHX* in families (mother, father and offspring) with a history of preeclampsia as compared to control families who experienced uncomplicated pregnancies only

Study-design: Genomic DNA of families who experienced preeclampsia (n=134) and control families with a normotensive obstetrical history (n=126) was analysed for the presence of the Tyr113His polymorphism in *EPHX* by polymerase chain reaction / restriction fragment length polymorphism.

Results: In former preeclamptic women the incidence of the His^{113}/His^{113} genotype was higher as compared with that of controls (23% versus 11%; $\chi^2=4.4$ and $P<0.05$), whereas a similar distribution was found for paternal as well as foetal genotype between cases and controls. The transmission disequilibrium test showed that the Tyr^{113} allele was more often transmitted to offspring born after preeclampsia than could be expected by chance ($\chi^2=4.7$ and $P<0.05$).

Conclusion: The His^{113}/His^{113} genotype of *EPHX* in mothers was associated with a higher susceptibility to develop preeclampsia, but not in fathers and offspring.

Introduction

Preeclampsia is characterised by pregnancy-induced hypertension and concurrent proteinuria (1). Despite extensive research the pathogenesis of preeclampsia and HELLP is still largely unknown. Although, it is postulated that insufficient throphoblast invasion may result in poor placental perfusion and local oxidative stress (2), which could subsequently affect maternal circulation resulting in dysfunction of the vascular endothelium (3). A genetic susceptibility for preeclampsia has been proposed years ago (4) and a wide variety of involved genes have been previously reviewed by Broughton-Pipkin (5).

Biotransformation enzymes, such as glutathione S-transferase P1-1 (GSTP1-1) and microsomal epoxide hydrolase (EPHX), are important in scavenging free radicals and detoxification of xenobiotics (6,7). EPHX mainly metabolises reactive epoxide intermediates to more water-soluble transdihydrodiol derivatives. However, under certain circumstances, the chemical products resulting from cytochrome P450 and microsomal EPHX interactive metabolism include highly reactive electrophiles (7). Polymorphisms of genes encoding for these enzymes have been associated with an increased susceptibility for preeclampsia (8,9). Homozygosity for Tyr113His polymorphism in exon three of the *EPHX* gene has been associated with low enzyme activity (7), therefore prolonged exposure to reactive intermediates due to malfunctioning of the EPHX enzyme might contribute to maternal endothelial damage. Also a local effect on placental development and growth of reactive intermediates or other toxic compounds can be assumed (10).

Both GSTP1-1 and EPHX have been found in placental tissue (11,12). Since placenta is of foetal origin and therefore is characterised by both a maternal and paternal contribution, the risk for preeclampsia might by modified by maternal as well as paternal genetic variations in detoxification activities. Recently, we demonstrated a paternal as well as foetal contribution of the polymorphism in *GSTP1-1* to the susceptibility to develop preeclampsia (13). The present study was performed to investigate the role of the Tyr113His polymorphism in *EPHX* in families (mother, father and offspring) with a history of preeclampsia as compared to control families with uncomplicated obstetrical history.

Materials and Methods

Patient and Control Subjects

The Institutional Medical Ethical Review Committee approved the study protocol. Preeclampsia was defined according to the standard of the International Society for the Study of Hypertension in Pregnancy as pregnancy induced hypertension (diastolic blood pressure > 90 mmHg on two or more occasions each more than 4 hours apart) with proteinuria (urinary protein/creatinine ratio >0.30 g/10 mmol). The HELLP syndrome was defined by haemolysis (lactic dehydrogenase level >600 IU/L), elevated liver enzymes (both aspartate and alanine aminotransferase activity >70 IU/L) and low platelets (trombocyte count $<100x10^9$/L).

We recruited 134 women, who had experienced severe preeclampsia (diastolic blood pressure > 110 mmHg or the presence of the HELLP syndrome) during singleton pregnancy, or HELLP syndrome with or without gestational hypertension, their male partners and offspring of preeclamptic pregnancies concerned. One-hundred-and-twentysix women who had experienced uncomplicated pregnancies only, their male partners and offspring of their last singleton pregnancy were recruited as control families. All investigated subjects gave written informed consent. One-hundred-and-thirteen families with severe preeclampsia have contributed in another study (13).

DNA analysis

Whole blood from women and their partners was obtained by venapuncture in sterile vacutainer tubes anti-coagulated with ethylenediaminetetra-acetic acid (EDTA) and DNA was isolated using the Puregene genomic DNA isolation kit (Gentra Systems, Minneapolis, USA) according to the instructions of the manufacturer. DNA from offspring was collected from buccal cell samples collected on sterile swaps as described by Richards et al. (14).

Genomic DNA was analysed for the presence of the Tyr113His polymorphism in exon 3 of *EPHX*, according to the polymerase chain reaction / restriction fragment length polymorphism (PCR/RFLP) method as described by Harrison et al. (15). In short, during PCR amplification a digestion site for EcoRV is created in the wild-type allele. After restriction with this enzyme, fragments were separated on 3% agarose resulting in fragments of 140 en 25bp for a wild-type allele and an undigested fragment of 165bp for the polymorph allele.

Statistics

The distribution of the different genotypes of the *EPHX* polymorphism between the two study groups was calculated with the Chi-square test.

Association analysis was performed using the transmission disequilibrium test (TDT), which evaluates the observed number of parent-offspring transmission of alleles, compared with the number of transmissions expected by chance, as described by Spielman et al (16).

All analyses were performed with the Statistical Package for Social Sciences (SPSS Inc, Chicago, USA). A p-value below 0.05 was considered significant.

Table 12.1. Characteristics of women with severe preeclampsia.

GA at delivery (weeks^{+days})	31^{+3} (26^{+0} – 40^{+6})
Systolic BP (mmHg; Korotkoff V)	165 (110 – 220)
Diastolic BP (mmHg; Korotkoff V)	110 (75 – 140)
Protein/Creatinin ratio (g /10 mmol)	2.9 (0 – 27.4)
LDH (IU/L)	631 (65 – 7670)
ASAT (IU/L)	84 (5 – 2640)
ALAT (IU/L)	75 (4 – 1920)
Platelet count (*10^9 / L)	94 (10 – 483)
Haemoglobin (g/dL)	7.3 (3.7 – 9.4)
Haematocrit	0.35 (0.18 – 0.45)
Serum creatinine (µmol/L)	76 (49 – 215)
Serum uric acid (µmol/L)	0.40 (0.22 – 0.80)

Data are given as median and ranges.

Abbreviations: GA – gestational age; BP – blood pressure; LDH – lactic dehydrogenase enzyme activity; ASAT – aspartate aminotransferase enzyme activity; ALAT – alanine aminotransferase enzyme activity

Results

The data for analysis included 127 women, 122 partners, and 110 children of the preeclampsia families and 80 women, 104 partners, and 86 children of the control families. Other DNA samples could not successfully be evaluated. In Table 12.1 clinical data of the former preeclamptic patients are depicted during their disease, whereas Table 12.2 shows the distribution of the three genotypes.

The His^{113}/His^{113} genotype was more often found in women with a history of preeclampsia as compared to that of control women (23% versus 11%; $\chi^2 = 4.4$ and $P<0.05$), however, we could not find a different distribution of this genotype between partners and offspring from the former preeclamptic and those of control women.

TDT analysis revealed that the wildtype Tyr^{113} allele was significantly more often transmitted to offspring than the rare His^{113} allele ($\chi^2=4.7$; $P<0.05$).

Table 12.2. Distribution of polymorph genotypes of *EPHX*.

Genotype	Preeclampsia			Control		
	Mother (n=127)	Father (n=122)	Offspring (n=110)	Mother (n=80)	Father (n=104)	Offspring (n=86)
Tyr^{113}/Tyr^{113}	50 (39%)	65 (54%)	61 (56%)	47 (59%)	51 (49%)	43 (50%)
Tyr^{113}/His^{113}	48 (38%)	36 (30%)	39 (36%)	24 (30%)	31 (30%)	32 (37%)
His^{113}/His^{113}	29 (23%)*	21 (18%)	10 (9%)	9 (11%)	22 (21%)	11 (13%)
OR (95% CI)	2.3 (1.0-5.2)	0.9 (0.5-1.7)	0.7 (0.3-1.7)	1	1	1

Note: The number of DNA samples that successfully could be evaluated are given in parenthesis.

*Statistics: $\chi^2 = 4.4$, $P<0.05$

Discussion

We have demonstrated an association between the Tyr113His polymorphism in *EPHX* and susceptibility to develop preeclampsia, since the prevalence of His^{113}/His^{113} genotype is significantly higher in women with a history of preeclampsia as compared to controls. The rare His^{113}/His^{113} genotype is known to be associated with a lower enzyme activity of epoxide hydrolase as compared with the wildtype genotype (7). Therefore, former patients may have a lower enzyme activity, which might result in an accumulation of highly reactive intermediates (17). In addition, the imbalance between oxidants and antioxidants plays a prominent role in the pathophysiology of preeclampsia (18) and it is proposed that lipid peroxidation might contribute to lipid epoxide formation (19). Thus lower EPHX activity might also result in an accumulation of lipid epoxide and peroxides, which in turn may lead to oxidative stress.

Contradictory, in an earlier study from our group we found an association between the Tyr^{113}/Tyr^{113} genotype of *EPHX* with the development of preeclampsia (9), however, population-based control values of that study were comparable to those found for other Chinese control populations (20,21) whereas present control values, which were selected for uncomplicated pregnancy outcome, are comparable to those found for Caucasians (15, 22-25). In addition, different PCR/RFLP methods were used, in the previous study the rare polymorph allele was digested versus the wild-type allele in the present study, and both studies consisted of different and relatively small study populations. This could have resulted in the difference found between these studies.

Heterozygous parents who had a history of preeclampsia significantly more often transmitted the Tyr^{113} than the His^{113} allele to their offspring. Hassett et al. presumed that alleles at amino acid 113 were not in Hardy-Weinberg equilibrium, since the His^{113} is less often found than expected (7). However, in contradiction, recent studies demonstrated Hardy-Weinberg equilibrium at this position of *EPHX* (22-24). Therefore, it can be presumed that the transmission disequilibrium for the Tyr^{113} allele, as found in this study for the families who experienced severe preeclampsia, might be the result of the disease. Recurrent abortion is associated with a higher frequency of the ^{113}His allele among women (21). Therefore, pregnancies of mothers or foetuses with the homozygous rare genotype might more frequently end in an abortion. However, preeclampsia is a multifactorial complication and therefore heterozygous foetuses may be presumably protected from an early abortion by an adaptive mechanism, which however does not prevent them to develop preeclampsia. This could explain the higher incidence of the His^{113}/His^{113} genotype in former preeclampsia patients as found in this study. Furthermore, in this way foetuses should have received the Tyr^{113} allele from the father, which resulted in a positive TDT-test for this allele.
This could also explain the absence of an association between paternal or foetal genotype and preeclampsia for *EPHX*. Recently, we found a paternal and foetal association between *GSTP1-1* genotype and preeclampsia (13), therefore we hypothesised to find such an association for *EPHX* as well. Another possibility is that EPHX enzyme activity is less important for placental detoxification than GSTP1-1 enzyme activity is (11).

In conclusion, the His^{113}/His^{113} genotype of *EPHX* in mothers was associated with severe preeclampsia, whereas no contribution of paternal or foetal genotype was found.

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Chapter 13

The Tyr113His polymorphism in exon 3 of the microsomal epoxide hydrolase gene is a risk factor for perinatal mortality.

Maarten T.M. Raijmakers, Tanja E.M. de Galan-Roosen, Geurt W. Schilders, Hans M.W.M. Merkus, Eric A.P Steegers and Wilbert H.M. Peters.

Abstract

Background: Microsomal epoxide hydrolase (EPHX) as well as glutathione S-transferase P1 (GSTP1) play an important role in the metabolism of xenobiotics. In previous studies polymorphisms in the genes encoding for these detoxification enzymes were associated with an increased risk for complications of pregnancy. Perinatal mortality is a multifactorial event, in which also a genetic predisposition to impaired detoxification could play a role.

Aim: To study the prevalence of the genetic polymorphism in exon 3 of the *EPHX* gene (Tyr113His) and that of the *GSTP1* gene (Ile105Val) in both women and their partners who experienced perinatal mortality as compared with control couples with uncomplicated obstetrical histories.

Design: Genomic DNA of case couples (79 females and 52 males) and control couples (73 females and 69 males) was analysed for the presence of polymorphisms in both the *EPHX* and *GSTP1* gene by polymerase chain reaction / restriction fragment length polymorphism.

Results: A similar distribution of the *GSTP1* polymorphism was found in all subjects investigated. In women, who experienced perinatal mortality, we demonstrated a higher prevalence of the *EPHX* His^{113}/His^{113} genotype as compared with controls (26% versus 11%; χ^2 of 4.8 and $P<0.03$) with an odds ratio (95% CI) of 3.0 (1.1 – 8.2).

***Conclusion*:** The Tyr113His-polymorphism in the microsomal epoxide hydrolase gene of the mother seems to be a risk factor for perinatal mortality, while there is no association with the paternal genotype.

Introduction

Perinatal mortality rates include both still births and live-born infants dying within the first week of live (1). Although the past decades a strong decline of perinatal mortality in Western Europe is seen, the prevalence of perinatal mortality in the Netherlands is still approximately 7 cases per 1,000 births (2). Perinatal mortality can be regarded as a multifactorial event, main causes being acute or chronic placental pathology, congenital malformations and complications of prematurity (3).

Many carcinogens or mutagens present in tobacco smoke or drugs contain polycyclic aromatic hydrocarbons (PACs) (4,5). In the human body the defence against such compounds is provided by the two-stage process of phase I and phase II biotransformation reactions. In phase I reactions PACs are metabolised by cytochrome monooxygenases to arene and alkene oxides, also called epoxides. Due to their electronic polarisation and ring tension, epoxide metabolites are highly reactive compounds, which often are mutagenic or carcinogenic and therefore may influence normal functioning of the cell. Epoxides can be further metabolised in several distinct pathways (4). One of the main enzymes involved is microsomal epoxide hydrolase (EPHX), which catalyses the hydrolysis of epoxides into their corresponding *trans*-dihydrodiols. Epoxides can also directly be conjugated to glutathione (GSH) either non-enzymatically or catalysed by one of the glutathione S-transferases (GSTs) (4), of which GSTP1 is predominantly expressed in placental tissue.

Several complications of pregnancy, such as preeclampsia and recurrent early pregnancy loss, are associated with the Tyr113His polymorphism in exon 3 of the *EPHX* gene (6) or the Ile105Val polymorphism in the gene for glutathione S-transferase P1 (*GSTP1*) (7,8). Both polymorphisms result in a lower enzyme activity and decreased detoxification capacity. In addition, also the paternal genotype of the polymorphism in *GSTP1* is associated with an increased risk for preeclampsia (9). The aim of the present study is to investigate whether the genetic polymorphisms of the *EPHX* and *GSTP1* genes in both mothers and their partners are risk factors for perinatal mortality.

Materials and Methods

Subjects

Perinatal mortality was defined according to the WHO criteria for standard national perinatal mortality figures using a birthweight of 500 grams or more (10). Between December 1999 and May 2000, seventy-nine women who participated in a prospective study on perinatal mortality, which was performed from 1983 to 1992 (3), were recruited for the present study.

Table 13.1. Pregnancy characteristics of women with perinatal mortality and causes of perinatal mortality.

Pregnancy characteristics:	
Gestational age at delivery (weeks)	35 (23-43)
Parity	3 (2-7)
Smoking	24 (30%)
Causes of perinatal mortality:	
Infection (transamnionic or haematogenous)	6 (8%)
Placental pathology	48 (62%)
Incompatability of blood group	1 (1%)
Congenital disorders	19 (24%)
Complications of premature delivery	4 (5%)

All values expressed as numbers (percentage) except for gestational age and parity, which are expressed in medians (range),

Note: For one woman cause of perinatal mortality was reported unknown.

Pregnancy characteristics and causes of perinatal mortality of the cases are summarised in Table 13.1. Out of 79 male partners 52 could be included in the present study. Reasons for non-inclusion were divorce (n=10), recent death (n=2) or refusal to participate (n=15). Seventy-one control women, with no history of perinatal mortality, and 66 of their partners were recruited by public advertisement. The local medical-ethical committee of the "Reinier de Graaf Hospital", Delft, The Netherlands approved the study protocol and informed consent was obtained from all women and their partners.

Sample collection and analysis

Whole blood was obtained by venapuncture in sterile vacutainer tubes anti-coagulated with EDTA. DNA was isolated from whole blood using the Puregene genomic DNA isolation kit (Gentra Systems, Minneapolis, USA) according to the instructions of the manufacturer. Genomic DNA was analysed for the presence of the Ile105Val polymorphism in the *GSTP1* gene (7) and the Tyr113His polymorphism in exon 3 of the *EPHX* gene (11) exactly as described previously.

Statistics:

Statistical evaluation of the distribution of the polymorpisms between cases and controls was performed with the Chi-square test. A P-value less than 0.05 was considered significant.

Results

Distribution of the polymorph variants in both *GSTP1* and *EPHX* are shown in Table 13.2. Not all DNA samples available could be successfully evaluated. In exon 3 of *EPHX* the polymorph His^{113}/His^{113} genotype was more prevalent in female cases (26%) than in female controls (11%; $\chi^2 = 4.8$ and $P<0.03$). This resulted in an odds ratio (95% CI) of 2.9 (1.1 – 8.2).

Table 13.2. Distribution of polymorph variants in *GSTP1* and *EPHX*.

	Female Cases	Female Controls	Male Cases	Male Controls
EPHX:	(n=77)[1]	(n=56)	(n=51)	(n=59)
Tyr^{113} / Tyr^{113}	27 (35%)	28 (50%)	19 (37%)	28 (47%)
Tyr^{113} / His^{113}	30 (39%)	22 (39%)	21 (41%)	20 (34%)
His^{113} / His^{113}	20 (26%)*	6 (11%)	11 (22%)	11(19%)
GSTP1:	(n=79)	(n=71)	(n=52)	(n=66)
Ile^{105} / Ile^{105}	44 (56%)	33 (46%)	23 (44%)	32 (48%)
Ile^{105} / Val^{105}	31 (39%)	29 (41%)	24 (46%)	26 (39%)
Val^{105} / Val^{105}	4 (5%)	9 (13%)	5 (10%)	8 (12%)

Data are presented as number (percentage)

[1]Note: The number of DNA samples analysed is given in parenthesis

Statistics: *: $\chi^2 = 4.8$ with $P<0.03$

A similar distribution between cases and controls of the *EPHX* genotypes was found in their partners.
No differences were found in the prevalence of the polymorph Val^{105}/Val^{105} genotype in *GSTP1* gene between cases and controls both for females and males.

Discussion

In a previous report, the His^{113}/His^{113} genotype *EPHX* was associated with an increased risk for spontaneous abortion in a Chinese population (12). In our study we now describe for the first time an association between a polymorphism in the detoxification pathway and perinatal mortality. The His^{113}/His^{113} genotype of *EPHX*, which may result in a lower enzyme activity, appears to be more prevalent in women with perinatal mortality. Although, the prevalence of the His^{113}/His^{113} genotype in the present study (11%) was similar to those described in other Caucasian populations (5,11,13-16), it was much lower than that reported in Chinese controls (42%) (12). Furthermore, we were not able to demonstrate a paternal contribution as was previously found for preeclampsia (9).

The exact contribution of the His^{113}/His^{113} genotype on the multifactorial biochemical mechanisms leading to perinatal mortality remains to be determined. Smoking during pregnancy, which is a risk factor for perinatal mortality (1), could be one of those mechanisms. In cigarette smoke several PACs are abundantly present and in metabolism of PACs reactive and toxic epoxide intermediates are frequently formed (5,15). However, when non-smoking and smoking women who experience perinatal mortality were compared a similar distribution of the His^{113}/His^{113} genotype in EPHX was found in both groups (22% and 25%, respectively). This might indicate that EPHX genotype is a risk factor independent from smoking habits.

The Ile105Val-polymorphism in *GSTP1* has been shown to be associated with several complications of pregnancy including recurrent early pregnancy loss (8) and preeclampsia (7,9). However, in our study we found no association between the Val^{105}/Val^{105} genotype and perinatal mortality in both mothers as well as their partners. The prevalence of the polymorph genotype was in line with the data previously reported for controls by other investigators

(7,9,16,17). Since GSTP1 is the predominant GST isoform in placental tissue (18), we investigated the presence of the Val^{105}/Val^{105} genotype in a subgroup of cases with perinatal mortality related to placental pathology (Table 13.1). The incidence of the Val^{105}/Val^{105} genotype was similar in cases with placental pathology and cases with other cause of perinatal mortality (5% and 7%, respectively). In addition, the prevalence of the Val^{105}/Val^{105} genotype in the subset of cases with placental pathology (5%) was not different to that of control women (13%; χ^2 of 2.1 and P=0.15). Since placental tissue is of foetal origin and its constitution is regulated by maternal as well as paternal genes, an association of paternal genotype with perinatal mortality could be expected, however no such association was found for both investigated genes with the risk for perinatal mortality.

In conclusion, we found no evidence for a contribution of the paternal genotype of the Ile105Val polymorphism in *GSTP1* and the Tyr113His polymorphism of *EPHX* to the risk for perinatal mortality. Women homozygous for the Tyr113His polymorphism of *EPHX* have an increased risk for perinatal mortality and further studies should be performed to elucidate the pathophysiological mechanism involved.

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Summary and Conclusions

Introduction

Oxidative stress is a key factor in the pathogenesis of preeclampsia. Inadequate trophoblast invasion may result in poor placental perfusion and placental oxidative stress. In time also the maternal circulation will be affected, in which defence against oxidative stress is provided by numerous exogenous antioxidants (e.g. vitamins E and C) or endogenous enzyme systems (e.g. catalase and glutathione-related enzymes). When maternal antioxidant capacity is insufficient to deal with the increased load of oxidative compounds during pregnancy, preeclampsia or the hemolysis, elevated liver enzymes and low platelets syndrome may occur. Since 1995 numerous investigations have been performed on the topics described above in a collaboration between the departments of Gastroenterology and Obstetrics & Gynaecology of the University Medical Centre Nijmegen. In this thesis several studies on glutathione and glutathione-related enzymes on male fertility, embryonic, foetal and placental development are presented (**Part II**). In addition, studies on the redox-balance of glutathione and other thiols in preeclampsia (**Part III**), and genetic polymorphisms in association with oxidative stress in preeclampsia (**Part IV**) are described.

Part I

In **Chapter 1** the objectives of the thesis are outlined. Objectives are 1) To investigate the importance of the glutathione / glutathione biotransformation system in male fertility and embryonic, foetal and placental development, 2) To study the role of the aminothiol redox-balance in women with severe preeclampsia, and 3) To investigate the association between polymorphisms in oxidative-stress-related-genes and the pathogenesis of severe preeclampsia.

Chapter 2 provides a schematic overview of the concept of detoxification (**Paragraph 2.1**) and oxidative stress (**Paragraph 2.2**), followed by the description of changes in these metabolic features in both physiological pregnancies and those complicated by (severe) preeclampsia and/or HELLP syndrome (**Paragraph 2.3**). Glutathione S-transferases and glutathione peroxidases as well as their co-factor glutathione play an important role in the detoxification of numerous toxic compounds including xenobiotics, carcinogens, reactive oxygen species (ROS), and other harmful metabolic products. Oxidative stress is defined as a

disturbance in the balance between (pro)oxidants and antioxidants, in favour of the former. During normal pregnancy products of oxidative damage transiently increase, whereas most antioxidants decrease, pointing at increased oxidative stress. In early pregnancy oxidative stress is a key factor in placental development. When oxidative stress is poorly regulated, e.g. by impaired antioxidant defence, this may lead to placental maladaptation and poor placental perfusion, resulting in local as well as maternal oxidative stress and endothelial damage, which may lead to preeclampsia.

Part II

The second part of the thesis focuses on the presence of glutathione and related enzymes in seminal plasma in relation to male fertility (**Chapter 3**) and during pregnancy (**Chapters 4 & 5**).

In seminal plasma variable amounts of GSTP1-1 and GSTA1-1 are found. The levels of glutathione are lower in subfertile males than in fertile males (**Chapter 3**). Additionally, glutathione levels are associated with motility quality and negatively associated with abnormal sperm morphology, indicating that glutathione may play a role in male fertility.

Glutathione and glutathione S-transferases seem to play an important role during foetal development, since they are abundantly present in several embryotic and foetal organs (**Paragraph 4.1**). However, the expression of GSTs in some foetal tissues differs from that in corresponding adult tissues, indicating that several organs may have other functional properties during intra-uterine development. Glutathione and related enzymes, except for GSTAlpha, are homogeneously expressed in term placentas (**Paragraph 4.2**). The high levels of GSTTheta suggest that besides GSTPi, GSTTheta is also an important placental GST isoform.

In **Chapter 5** the assessment of thiol levels in venous and arterial umbilical cord as well as corresponding maternal plasma after both vaginal and caesarean deliveries are described. Both cysteine and homocysteine are transported either by active transport or driven by a concentration gradient from the maternal to the foetal circulation where they are utilised by the developing foetus, as seen by the lower arterial levels in comparison to the venous umbilical cord levels (**Paragraph 5.1**). After vaginal delivery glutathione levels in arterial umbilical cord are higher as compared with those in the venous umbilical cord, whereas no

such difference is found in women with caesarean section, suggesting that vaginal delivery is associated with higher levels of oxidative stress (**Paragraph 5.2**).

Part III

In this part of the thesis a study on maternal plasma thiol levels (**Chapter 6**) and three studies on the aminothiol redox status during and after pregnancy in women with severe preeclampsia (**Chapters 7 – 9**) are described.

Women with preeclampsia have higher plasma levels of cysteine and homocysteine than normotensive pregnant women, who show lower levels as compared with corresponding values in non-pregnant controls (**Chapter 6**). These differences might be explained by the physiological process of hemodilution during pregnancy and the plasma volume reduction in preeclampsia. Plasma glutathione levels are lower during pregnancy than in the non-pregnant state, whereas these levels are even lower in women with preeclampsia.

In **Chapter 7** the redox status for aminothiols is described in women with severe preeclampsia. In comparison to controls, women with preeclampsia have a lower free-to-oxidised ratio for cysteine, homocysteine, and cysteinylglycine indicating that the redox-balance of these thiols has shifted to higher levels of the oxidised thiols. This points at higher levels of oxidative stress in women with preeclampsia. The ratios for both cysteine and homocysteine are still lower 6 weeks after pregnancy (**Chapter 8**). In addition, in women with normotensive pregnancy as well as in patients with severe preeclampsia the free levels of glutathione transiently decrease during pregnancy as compared to corresponding levels 6 weeks after pregnancy. Therefore, during pregnancy oxidative stress is present, which is even more pronounced in women with preeclampsia.

The ratio for homocysteine is lower in women who experienced severe preeclampsia after subsequent pregnancies (**Chapter 9**). Plasma homocysteine levels are elevated in women with a history of severe preeclampsia, whereas the antioxidant capacity is higher as compared to corresponding values in women with an uncomplicated obstetrical history. These findings strongly suggest that the presence of elevated oxidised levels of homocysteine might be an underlying factor for the development of preeclampsia. Since hyperhomocysteinemia and autooxidation of homocysteine are risk factors for the development of cardiovascular

diseases, this may explain why women with a history of severe preeclampsia are at higher risk for cardiovascular diseases in later life.

Part IV

In the last part of the thesis some studies on genes involved in oxidative stress and their contribution to the development of preeclampsia and perinatal mortality are described.
NAD(P)H oxidase is an enzyme that produces O_2^- upon activation by angiotensin II (**Chapter 10**). The C242T-polymorphism in the p22phox subunit of this enzyme results in lower enzyme activity and subsequent lower O_2^- production. Therefore this polymorphism could be protective against preeclampsia, however, a similar distribution of the three genotypes is present in normotensive controls and women with preeclampsia, indicating that this polymorphism is not associated with preeclampsia.
In **Chapter 11** a study on the contribution of haptoglobin to the development of severe preeclampsia and HELLP syndrome is described. Haptoglobin, a glycoprotein with genetic heterogenity resulting in three phenotypes with different structural and functional properties, could be associated with preeclampsia in two different ways: a) haptoglobin 1-1 may prevent oxidative stress by capturing free iron, since it has a strong affinity for free haemoglobin; b) haptoglobin 2-2 is an angiogenic factor and could be beneficial in placental development. The latter function of haptoglobin seems to be most important for the development of the HELLP syndrome since the haptoglobin 2-2 genotype is found more common in patients with the HELLP syndrome.
In **Chapter 12** the parental genetic contribution of the detoxification enzyme epoxide hydrolase (EPHX) to the development of preeclampsia is described. The rare His^{113}/His^{113} genotype in mothers is associated with preeclampsia, whereas no paternal or foetal contribution is found. However, the wild-type Tyr^{113} allele is more often transmitted to offspring than could be expected by chance.
In **Chapter 13** the Tyr113His polymorphism in *EPHX* is identified as a maternal risk factor for perinatal mortality, since the His^{113}/His^{113} genotype is more frequently found in women who experienced perinatal mortality. A similar distribution is found for the Ile105Val polymorphism of *GSTP1* in cases and controls. Furthermore, no paternal contribution in the occurrence of perinatal mortality is found for both polymorphisms.

Conclusions

Summarising, we may conclude that glutathione and related enzymes are correlated with male fertility and are important during the physiology of normotensive pregnancy, whereas disturbances are seen in preeclampsia and the HELLP syndrome.

Disturbances of thiol levels are associated with preeclampsia. Using the free-to-oxidised ratio of thiols we were able to demonstrate the presence of a transient oxidative stress during pregnancy, being even more pronounced in preeclampsia and disappearing after delivery. However, in women with severe preeclampsia a lower free-to-oxidised ratio for homocysteine seems to persist after consecutive pregnancies, following the index-pregnancy. Therefore, the free-to-oxidised ratio for homocysteine may be a predictor for preeclampsia or may serve as indicator for the development of cardiovascular problems in later life.

Oxidative stress during preeclampsia seems not to be associated with polymorphisms in the genes encoding for the p22phox subunit NAD(P)H oxidase or haptoglobin. However, as an angiogenic factor, haptoglobin may play a role during placental development.

Samenvatting en Conclusies

Inleiding

Oxidatieve stress speelt waarschijnlijk een belangrijke rol in de pathogenese van pre-eclampsie. Een verminderde trofoblastinvasie zou kunnen leiden tot een slechte placentaire doorbloeding en placentaire oxidatieve stress. Na verloop van tijd zou dit ook in de moederlijke circulatie op kunnen treden. In de moederlijke circulatie zijn talrijke exogene antioxidanten (bijv. vitamine C en E) of endogene enzymsystemen (bijv. katalase en glutathion gerelateerde enzymen) aanwezig die bescherming bieden tegen oxidatieve stress. Echter indien de moederlijke antioxidantcapaciteit niet toerijkend is om de grotere hoeveelheid oxidanten onschadelijk te maken, dan zou dit kunnen leiden tot het ontstaan van pre-eclampsie of het 'hemolysis, elevated liver enzymes and low platelets' (HELLP) syndroom.

Vanaf 1995 zijn verscheidene onderzoeken uitgevoerd ter opheldering van de hierboven beschreven onderwerpen door een samenwerkingsverband tussen de afdelingen Maag-, Darm- & Leverziekten en Obstetrie & Gynaecologie van het Universitair Medisch Centrum St. Radboud te Nijmegen. In dit proefschrift zijn verschillende studies over glutathion en glutathion-gerelateerde enzymen in relatie tot de vruchtbaarheid van de man en de embryonale, foetale en placentaire ontwikkeling beschreven (**Deel II**). Bovendien staan er studies beschreven over het redox-evenwicht van glutathion en andere thiolen in pre-eclampsie (**Part III**) en over genetische polymorfismen die mogelijk geassocieerd zijn met oxidatieve stress in pre-eclampsie (**Part IV**).

Deel I

In **Hoofdstuk 1** zijn de doelen van het proefschrift uiteen gezet. Deze zijn 1) Onderzoek naar de betekenis van glutathion en het glutathion gerelateerde biotransformatiesysteem bij de vruchtbaarheid van de man en de embryonale, foetale en placentaire ontwikkeling, 2) De rol van het redox-evenwicht van aminothiolen bij vrouwen met een ernstige pre-eclampsie en 3) Onderzoeken wat de associatie is tussen oxidatieve stress-gerelateerde genen en de pathogenese van ernstige pre-eclampsie.

Hoofdstuk 2 geeft een schematisch overzicht over het concept van ontgifting (**Paragraaf 2.1**) en oxidatieve stress (**Paragraaf 2.2**), gevolgd door een beschrijving van de veranderingen in deze metabole processen bij zowel fysiologische zwangerschappen als die gecompliceerd door (ernstige) pre-eclampsie en / of het HELLP syndroom (**Paragraaf 2.3**). Zowel glutathion S-transferasen en glutathion-peroxidasen als hun co-factor glutathion spelen een belangrijke rol in het onschadelijk maken van giftige stoffen zoals xenobiotica, carcinogenen, reactieve zuurstof radicalen en andere schadelijke (metabole) producten. Oxidatieve stress wordt gedefinieerd als een verstoring van het evenwicht tussen oxidanten en antioxidanten in het voordeel van de oxidanten. Gedurende een normale zwangerschap neemt de hoeveelheid oxidatieve schadeproducten tijdelijk toe, terwijl de hoeveelheid van de meeste antioxidanten afneemt, wat op de aanwezigheid van oxidatieve stress wijst. In de vroege zwangerschap speelt deze tijdelijke oxidatieve stress een belangrijke rol bij de aanleg van de placenta. Als deze oxidatieve stress slecht gereguleerd wordt, bijv. door een verminderde antioxidant-capaciteit, kan dit leiden tot een verstoorde placenta aanleg en een verminderde placentaire doorbloeding wat kan leiden tot een lokale of zelfs maternale oxidatieve stress en endotheel-schade hetgeen tot pre-eclampsie kan leiden.

Deel II

Het tweede gedeelte van het proefschrift richt zich op de aanwezigheid van glutathion en gerelateerde enzymen in seminaal plasma in relatie tot mannelijke vruchtbaarheid (**Hoofdstuk 3**) en de zwangerschap (**Hoofdstuk 4 & 5**).

In seminaal plasma zijn variabele concentraties glutathion S-transferase P1-1 en glutathion-S-transferase A1-1 aanwezig. Mannen met een verminderde vruchtbaarheid hebben een lagere glutathion spiegel in semen dan vruchtbare mannen (**Hoofdstuk 3**). Verder zijn de glutathion concentraties gerelateerd aan de kwaliteit van beweging en negatief gecorreleerd aan een abnormale vorm van de spermatozoïden, wat aangeeft dat glutathion mogelijk een rol speelt bij de mannelijke vruchtbaarheid.

Glutathion en glutathion-S-transferasen lijken een rol te spelen in de foetale ontwikkeling, omdat ze overvloedig tot expressie komen in verscheidene embryonale en foetale organen (**Paragraaf 4.1**). Echter in sommige foetale organen is de expressie van de glutathion-S-transferases verschillend van overeenkomstige volwassen organen. Het lijkt erop dat deze

organen andere functionele eigenschappen hebben gedurende de foetale ontwikkeling. Op glutathion-S-transferase A1-1 na, komen glutathion en glutathion-gerelateerde enzymen homogeen tot expressie in de placenta (**Paragraaf 4.2**). De hoge concentratie van glutathion-S-transferase T1-1 suggereert dat dit enzym ook een belangrijke rol in de placentaire ontgifting speelt net als glutathion S-transferase P1-1.

In (**Hoofdstuk 5**) worden de bepalingen van de thiolconcentraties, in zowel veneus en arterieel navelstrengbloed als overeenkomstig maternaal bloed, na een vaginale bevalling of keizersnede beschreven. Cysteine en homocysteine worden beide door actief transport of via een concentratie-gradiënt van de maternale naar de foetale bloedsomloop getransporteerd, waar ze gebruik worden in de ontwikkeling van de foetus (**Paragraaf 5.1**). Na een vaginale bevalling zijn de glutathionwaarden in arterieel navelstrengbloed hoger in vergelijking met de veneuze waarden, terwijl dit verschil niet gevonden wordt na een keizersnede. Dit suggereert dat een vaginale bevalling gepaard gaat met meer oxidatieve stress dan een keizersnede (**Paragraaf 5.2**).

Deel III

In dit gedeelte van het proefschrift worden een studie naar de concentraties van thiolen in maternaal plasma (**Hoofdstuk 6**) en drie studies naar het redox-evenwicht van deze thiolen tijdens en na de zwangerschap bij vrouwen met een ernstige pre-eclampsie beschreven (**Hoofdstuk 7 – 9**).

Vrouwen met pre-eclampsie hebben hogere plasmaconcentraties van cysteine en homocysteine dan normotensieve zwangere vrouwen, die weer lagere waarden laten zien in vergelijking met niet zwangere controles (**Hoofdstuk 6**). Deze verschillen zouden verklaard kunnen worden door het fysiologische proces van haemodilutie tijdens de zwangerschap en indikking van het maternale bloed tijdens pre-eclampsie. De waarden van glutathion zijn lager tijdens de zwangerschap dan in de niet zwangere toestand, terwijl deze waarden nog lager zijn in vrouwen met een pre-eclampsie.

In **Hoofdstuk 7** wordt het redox evenwicht voor de aminothiolen in vrouwen met een ernstige pre-eclampsie beschreven. In vergelijking met controles hebben vrouwen met pre-eclampsie een lagere vrij-over-geoxideerd-ratio voor cysteine, homocysteine en cysteinylglycine, wat erop wijst dat het redox evenwicht verschoven is naar de aanwezigheid van meer geoxideerde

thiolen. Oftewel bij vrouwen met pre-eclampsie is meer oxidatieve stress aanwezig. Voor cysteine en homocysteine waren deze ratio' s 6 weken na de zwangerschap nog steeds lager (**Hoofdstuk 8**). Bovendien wordt in dit hoofdstuk beschreven dat tijdens de zwangerschap, zowel ongecompliceerd als pre-eclamptisch, de hoeveelheid glutathion tijdelijk afneemt in vergelijking met de waarden 6 weken na de zwangerschap. Daarom is het waarschijnlijk dat tijdens een normale zwangerschap oxidatieve stress aanwezig is, die in vrouwen met pre-eclampsie zelfs groter is.

Zelfs na één of meerdere opeenvolgende zwangerschappen na de indexzwangerschap is de ratio voor homocysteine is nog steeds lager in vrouwen die een ernstige pre-eclampsie hebben gehad (**Hoofdstuk 9**). Tevens zijn zowel de antioxidantcapaciteit als de homocysteine-concentratie in plasma hoger in vergelijking met vrouwen die alleen ongecompliceerde zwangerschappen hebben gehad. Deze bevindingen zijn sterke aanwijzingen dat de aanwezigheid van verhoogde concentraties van homocysteine in de geoxideerde vorm een risicofactor voor pre-eclampsie zou kunnen zijn. Omdat hyperhomocysteïnemie en auto-oxidatie van homocysteine tevens risicofactoren zijn voor hart- en vaatziekten, zou dit kunnen verklaren waarom vrouwen, die een pre-eclampsie hebben gehad, een verhoogd risico hebben op het krijgen van cardiovasculaire aandoeningen.

Deel IV

In het laatste gedeelte van het proefschrift staan een aantal studies beschreven over genen, die betrokken zijn bij oxidatieve stress en hun relatie tot pre-eclampsie en perinatale sterfte.

NAD(P)H oxidase is een enzym dat na stimulatie door angiotensine II zuurstofradicalen produceert (**Hoofdstuk 10**). Het C242T-polymorfisme in p22phox subunit van dit enzym resulteert in een verlaging van de enzymactiviteit en daardoor in een verminderde zuurstofradicaalproductie. Hierdoor zou dit polymorfisme beschermend kunnen werken voor het ontstaan van pre-eclampsie. Echter de drie mogelijke genotypen zijn op dezelfde manier verdeeld bij normotensieve controles en vrouwen met pre-eclampsie, wat aangeeft dat dit polymorfisme niet geassocieerd is met pre-eclampsie.

In **Hoofdstuk 11** staat een studie beschreven naar de rol van haptoglobine in de ontwikkeling van pre-eclampsie en het HELLP syndroom. Haptoglobine is een glycoproteine met een genetische heterogeniteit, waardoor er drie verschillende fenotypen bestaan, die zowel

structureel als functioneel verschillend zijn. Haptoglobine kan daarom op twee verschillende manieren betrokken zijn bij het ontstaan van pre-eclampsie en het HELLP syndroom: a) haptoglobine 1-1 kan oxidatieve stress voorkomen door het binden van vrij ijzer, omdat het een sterke affiniteit heeft voor de binding van haemoglobine; b) haptoglobine 2-2 is een factor die betrokken is bij de angiogenese en zou daarom een gunstig effect kunnen hebben op de vroege aanleg van de placenta. Deze laatste functie van haptoglobine lijkt belangrijk te zijn in de ontwikkeling van het HELLP syndroom, omdat de aanwezigheid van het haptoglobine 2-2 genotype veel lager is bij vrouwen met het HELLP syndroom.

Hoofdstuk 12 beschrijft een studie naar de genetische bijdrage van de ouders met betrekking tot het enzym epoxidehydrolase (EPHX). Moeders met het zeldzame His^{113}/His^{113} genotype hebben een verhoogd risico op pre-eclampsie, terwijl er geen associaties zijn gevonden voor het vaderlijke of kinderlijke genotype. Het Tyr^{113} allel werd echter vaker doorgeven aan de kinderen geboren uit een pre-eclamptische zwangerschap dan kan worden verwacht vanuit een normaal overervingpatroon.

Zowel glutathion-S-transferase P1-1 als EPHX zijn betrokken bij het metaboliseren van talloze xenobiotica. In **Hoofdstuk 13** wordt beschreven dat het Tyr113His polymorfisme in EPHX bij vrouwen een risicofactor is voor perinatale sterfte, omdat het His^{113}/His^{113} genotype vaker gevonden wordt bij vrouwen die een perinatale sterfte hebben meegemaakt in vergelijking met controles, terwijl voor het Ile105Val polymorfisme in glutathion-S-transferase P1-1 een gelijke verdeling aanwezig is. Er werd geen vaderlijke bijdrage in beide polymorfismen gevonden met betrekking tot het voorkomen van perinatale sterfte.

Conclusie

Samenvattend kunnen we concluderen dat glutathion en glutathion-gerelateerde enzymen zijn gecorreleerd met mannelijke vruchtbaarheid en dat ze belangrijk zijn tijdens een fysiologische zwangerschap, terwijl verstoringen gezien zijn in pre-eclampsie en het HELLP syndroom.

Veranderingen in de thiolconcentraties zijn geassocieerd met het ontstaan van pre-eclampsie. Door de bepaling van de vrij-over-geoxideerde-ratio van de thiolen hebben we kunnen aantonen dat gedurende de zwangerschap er tijdelijk oxidatieve stress aanwezig is, dat deze nog hoger is in vrouwen met pre-eclampsie, maar na de zwangerschap weer verdwijnt. Echter in vrouwen met een ernstige pre-eclampsie blijft de verlaagde vrij-over-geoxideerde-ratio

zelfs na opeenvolgende zwangerschappen aanwezig. Daarom zou de vrij-over-geoxideerde-ratio voor homocysteine een predictor voor pre-eclampsie kunnen zijn, of zou kunnen dienen als indicator voor het ontstaan van hart- en vaatziekten in het latere leven.

Oxidatieve stress tijdens pre-eclampsie lijkt niet geassocieerd te zijn met polymorfismen in de genen coderend voor de p22phox subunit van NAD(P)H oxidase of haptoglobine. Haptoglobine, als angiogenetische factor, zou een belangrijke rol kunnen spelen bij de aanleg van de placenta.

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Curriculum Vitae auctoris

Maarten Raijmakers werd op 25 februari 1974 geboren te Valkenswaard. In 1991 behaalde hij zijn HAVO-diploma, waarna in 1993 succesvol het VWO werd afgerond op het toenmalige Hertog-Jan college in Valkenswaard. In hetzelfde jaar werd begonnen met de Scheikunde studie aan de Katholieke Universiteit Nijmegen. Tijdens zijn studie is hij als student-assistent werkzaam geweest (eerstejaars scheikunde practicum). Onderzoekservaring werd opgedaan gedurende een bijvakstage op de afdeling Kindergeneeskunde & Neurologie (Universitair Medische Centrum St. Radboud) onder leiding van dr. NMJ van der Put en dr. HJ Blom, een hoofdvakstage op de afdeling Biochemie (Medische faculteit) onder leiding van mw. drs. K van Norren, drs. HPH Hermsen en Prof. Dr. JJHHM de Pont en een tweede bijvak bij Organon Teknika onder leiding van Dr. P Boender en Dr. B van Gemen. In 1998 werd het doctoraalexamen succesvol afgelegd.

Van 1 juli 1998 tot 1 juli 2002 was hij onder supervisie van dr. WHM Peters en dr. EAP Steegers als Junior Onderzoeker werkzaam op de afdelingen Maag-, Darm- en Leverziekten (hoofd: prof. dr. JBMJ Jansen) en Obstetrie & Gynaecologie (hoofd destijds: Prof. Dr. JMWM Merkus) van het Universitair Medisch Centrum St. Radboud in Nijmegen, alwaar hij het onderzoek heeft verricht dat tot het proefschrift 'Oxidative stress and detoxification in reproduction with emphasis on glutathione and preeclampsia' heeft geleid.
Op de '13th World Congress of the International Society for the Study of Hypertension in Pregnancy' (Toronto, 2002) heeft hij tijdens het 'Antioxidants and Preeclampsia'-symposium op uitnodiging een lezing verzorgt en heeft hij een 'Young Investigators Travel Award' in ontvangst mogen nemen.

Per 1 oktober 2002 is hij als postdoc werkzaam op de 'Maternal and Fetal Research Unit' van het 'Centre for Cardiovascular Biology and Medicine', St Thomas' Hospital te Londen.