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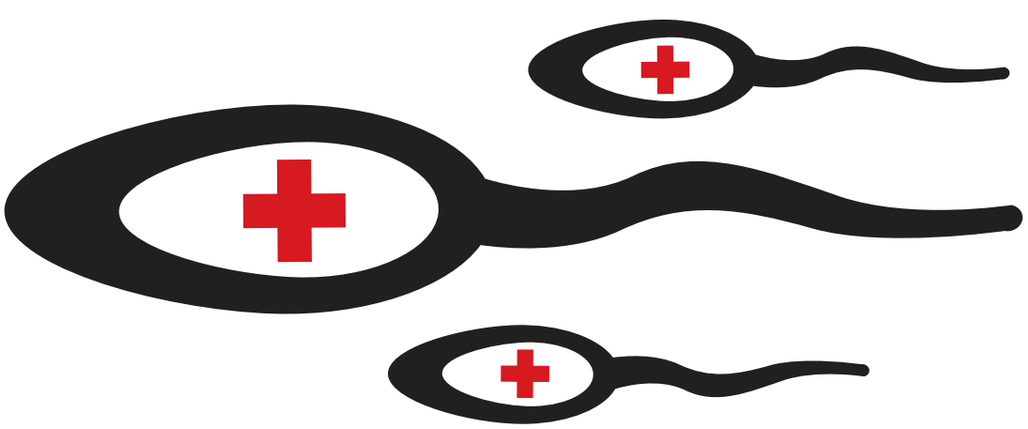
human subfertility: explorative studies on some pathophysiologic factors in semen and follicular fluid

inge ebisch human subfertility



Human subfertility: explorative studies on some pathophysiologic factors in semen and follicular fluid

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Human subfertility: explorative studies on some pathophysiologic factors in semen and follicular fluid

een wetenschappelijke proeve op het gebied
van de Medische Wetenschappen

Proefschrift

Ter verkrijging van de graad van doctor aan de Radboud Universiteit Nijmegen
op gezag van de Rector Magnificus, Prof. Dr. C.W.P.M. Blom,
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door

Inge Maria Wilhelmus Ebisch

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The study presented in this thesis was conducted at the Department of Obstetrics and Gynaecology, (Head: Prof. Dr. D.D.M. Braat), the Department of Chemical Endocrinology, (Head: Prof. Dr. C.G.J. Sweep), and the Department of Epidemiology and Biostatistics, (Head: Prof. Dr. Ir. G.A. Zielhuis) , Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands.

Voor mijn ouders

Contents

List of abbreviations

ANOVA	Analysis of variance
Anx A5	Annexin A5
ART	Artificial reproduction techniques
BTB	Blood testis barrier
CC	Cumulus cells
CC	Wild-type carrier state of the C677T MTHFR polymorphism
cDNA	Complementary deoxyribonucleic acid
CGS	Cysteinylglycine
CI	Confidence interval
COC	Cumulus-oocyte complex
Conc	Concentration
CT	Heterozygous carrier state of the C677T MTHFR polymorphism
CV	Coefficient of variance/variability
Cys	Cysteine
DNA	Deoxyribonucleic acid
E_2	17β-Estradiol
ECM	Extracellular matrix
ELISA	enzyme-linked immunosorbent assay
EM	Endometriosis
FF	Follicle fluid
FFS	Female factor subfertility
Flt-1	Fms-like tyrosine kinase 1 (VEGF receptor 1)
Fol-ser	Serum folate
FSH	Follicle stimulating hormone
FTP	Fallopian tube pathology
GnRH	Gonadotropin releasing hormone
GSH	Glutathione
GSSG	Glutathione disulphide
hCG	Human chorionic gonadotropin
Hcy	Homocysteine
HPLC	High performance liquid chromatography
HPT	Hypothalamic-pituitary-testis
HSG	Hysterosalpingogram
ICSI	Intracytoplasmic sperm injection
IS	Idiopathic subfertility
IU	International unit
IVF	*In vitro* fertilization
KDR	Kinase-insert domain containing receptor (VEGF receptor 2)

LH	Luteinizing hormone
LPO	Lipid peroxidation
MFS	Male factor subfertility
MMPs	Matrix metalloproteins
mRNA	Messenger ribonucleic acid
MTHFR	Methylenetetrahydrofolate reductase
n	Number
NAC	N-acetylcysteine
OAT	Oligoasthenoteratozoospermia
OHSS	Ovarian hyperstimulation syndrome
OR	Odds ratio
PA	Plasminogen activator
PAI-1	Plasminogen activator inhibitor type 1
PAI-2	Plasminogen activator inhibitor type 2
PAI-3	Plasminogen activator inhibitor type 3
PCR	Polymerase chain reaction
PN	Pronucleus
PN-I	Protease nexin I
Prog	Progesterone
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SHBG	Sex hormone binding globulin
T	Testosterone
TAC	Total antioxidant capacity
TBS/HSA	Tris buffered salt/human serum albumin
tPA	Tissue type plasminogen activator
tRNA	Transport ribonucleic acid
TSH	Thyroid stimulating hormone
TT	Homozygous carrier state of the C677T MTHFR polymorphism
uPA	Urokinase type plasminogen activator
uPAR	Urokinase type plasminogen activator receptor
VEGF	Vascular endothelial growth factor
v/v	Volume per volume
VPF	Vascular permeability factor
WHO	World Health Organisation
w/v	weight per volume
Zn-ser	Serum zinc

Chapter 1

General Introduction

Human subfertility

Subfertility, defined as the failure to conceive after 1 year of regular, unprotected intercourse with the same partner, is a prevalent disorder affecting approximately 10-17% of all couples in the Western world (Buckett and Bentick, 1997; Philippov *et al*. 1998; Snick *et al*. 1997).

Zargar *et al*. reported in 1997 that in approximately 22% of the patients the cause of subfertility was predominantly originating from the male, and in 58% mainly of female origin. In 5% of the patients fertility abnormalities were found in both partners, while in the remaining 15% no clear cause of subfertility was identified. An overview of several causes for subfertility in men and women, ranging from congenital, genetic, endocrine, infective and environmental causes, is given in Table I. Although there are many known causes, in up to 40% of men with abnormal sperm parameters or abnormal sperm function, the aetiology remains unknown. This group is called idiopathic subfertile.

Since involuntary childlessness is a heavy burden for most subfertile couples (Verhaak *et al*. 2002; Whiteford and Gonzalez 1995), it is not surprising that many couples seek help by means of artificial reproduction techniques (ART) like in vitro fertilization (IVF) or intracytoplasmic sperm injection (ICSI) to achieve pregnancy.

Although these techniques have improved the treatment of subfertile couples, the success rate is still low, being around 20% ongoing pregnancies per started cycle (Kremer *et al*. 2002), and with a cumulative probability of achieving an ongoing pregnancy of approximately 55% after five IVF or ICSI cycles (Stolwijk *et al*. 2000). Furthermore, hardly any alternative treatment exists. Moreover, ART is a symptomatic, rather than a causal treatment for subfertility.

Targeted treatment and, possibly, prevention of subfertility is indicated. To that end we need more knowledge and understanding of the pathophysiology and causal determinants of (the large group of idiopathic) subfertility.

Objectives of the thesis

Research has been performed on many of the variables presented in Table I. However, many couples with idiopathic subfertility still persist of which the aetiology is not clear. Our general aim was to increase the knowledge and understanding of the pathophysiology of human subfertility by performing explorative studies with emphasis on some biochemical and biological aspects.

Of particular interest are the environmental and lifestyle factors implicated in subfertility, because unlike genetic causes these can be targeted for curative or preventive measures. A significant but largely neglected lifestyle factor is nutrition. Micronutrients like folate and zinc, present in nutrition, are essential for DNA synthesis. In turn, DNA synthesis is important for the development of spermatozoa and oocytes. Therefore, nutritional deficiencies may be an important neglected cause of human reproductive impairment. Moreover, both zinc and folate have been implicated in human reproduction (Favier 1992; Steegers-Theunissen 1995). From this background a double-blind, placebo-controlled randomised intervention study has been conducted by Wong *et al.* (2002) investigating the effect of folic acid and/or zinc sulphate supplementation on sperm parameters in fertile and subfertile men. The results of this study understated the importance of these 2 micronutrients, since our group demonstrated a 74% increase in total normal sperm count in subfertile men supplemented with both folic acid and zinc sulphate for 26 weeks. However, the underlying mechanism of this beneficial effect of folic acid and zinc sulphate was not clear.

This resulted in the first objective of this thesis:

1. To investigate the underlying mechanism of increased sperm count after folic acid and zinc sulphate intervention, observed in our previous study.

Table I Aetiology of human subfertility

MEN	WOMEN
Pretesticular disorders	**'Pre-ovarian disorders'**
Endocrine	*Age*
Diabetes	*Endocrine*
Kallmann syndrome	Diabetes
Pituitary adenomas	Thyroid disease
Coital disorders	Cushing syndrome
Impotence	Pituitary adenomas
Ejaculatory failure	Panhypopituitarism
	Sheehan syndrome
Testicular disorders	Kallmann syndrome
Genetic	Anorexia or bulimia nervosa
Klinefelter syndrome	*Coital disorders*
Y-chromosome deletions	Decreased libido
Kartagener syndrome	Lubrication disorders
Androgen insensitivity	Vaginism
Autosomal rearrangements	Idiopathic
Spermatocytic arrest	
Congenital	**Pelvic disorders**
Anorchism	*Endometriosis*
Cryptorchidism	*Infective*
Infective	Pelvic inflammatory disease
Orchitis	*Neoplastic*
Neoplastic	
Testicular cancer	**Ovarian disorders**
Environmental agents	*Genetic*
Temperature	Turner syndrome
(Ir)radiation	Swyer syndrome
Chemotherapy	*Congenital or functional*
Medications	Resistant ovary syndrome
Occupational exposures	Premature ovarian failure
	Polycystic ovarian syndrome

Table I Aetiology of human subfertility (continued)

MEN	WOMEN
Stimulants	*Infective*
drugs	Adnexitis
alcohol	*Neoplastic*
tobacco abuse	*Environmental agents*
Nutritional deficiency	(Ir)radiation
trace elements	Chemotherapy
vitamins	Medications
Vascular	Occupational exposures
Torsion	Stimulants
Varicocele	drugs
Immunological	alcohol
Idiopathic	tobacco abuse
	Nutritional deficiency
Epididymal disorders	trace elements
Obstructive	vitamins
Congenital	
Infective	**Tubal disorders**
Epididymal hostility	*Congenital*
	Mayer-Rokitansky-Küster syndrome
Vasal disorders	*Infective*
Genetic	Salpingitis
Cystic fibrosis	*Neoplastic*
Acquired	*Acquired*
Vasectomy	Previous extra uterine pregnancy
	Sterilisation
Accessory gland disorders	
Infective	**Uterine abnormalities**
	Congenital
Immunological	Mayer-Rokitansky-Küster syndrome
Post-vasectomy	
Idiopathic	**Cervical mucus defects / dysfunction**

(Modified from Skakkebaek *et al*. 1994 and de Kretser 1997).

Bezold *et al*. (2001) investigated the involvement of the C677T polymorphism in methylenetetrahydrofolate reductase (MTHFR), one of the key enzymes in folate metabolism. This polymorphism is common and is accompanied by an altered folate metabolism, resulting in an increased folate need. The authors reported a higher frequency of the 677TT genotype in infertile males, and suggested that these males in particular may benefit from folic acid supplementation. We hypothesized that differences in prevalence of the C677T MTHFR polymorphism could explain the beneficial effect of folic acid and zinc sulphate in subfertile men.
Another of our hypotheses was that folic acid and zinc sulphate may affect endocrine parameters, for instance by stimulating the function of the Sertoli cells. These cells provide the essential microenvironment for normal germ cell production and could therefore be relevant. Sertoli cells are the main producers of inhibin B in the human body and the inhibin B concentration reflects the quality of the Sertoli cell function and spermatogenesis and as such can be used as a sensitive marker of spermatogenesis in humans.
Finally, it is known from literature that both folate and zinc are related to apoptosis, a programmed and physiological mode of cell death. Both folate and zinc deficiency results in significant apoptosis in several cell types, and zinc possesses anti-apoptotic properties, indicated by the observation that zinc supplementation can prevent apoptosis. Therefore, the final hypothesis we investigated to explain the beneficial effect of folic acid and zinc sulphate on sperm count was the effect of these 2 micronutrients on apoptosis.

Other observations reported in literature regarding folate and zinc were that both these nutrients are important for several antioxidant functions. Antioxidants can provide protection of cells against oxidative stress caused by reactive oxygen species (ROS), leading to damage of DNA, proteins or cell membranes. Another important antioxidant system in human tissues is the endogenous thiol glutathione (GSH) and the glutathione-related enzyme system, quantitatively one of the most important protective systems in human physiology. Many investigators have shown evidence for the role of both ROS and thiols in the physiology and pathology of both male and female reproductive function.

Overproduction of ROS results in a pro-oxidant state in which the vascular endothelial growth factor (VEGF) is expressed. VEGF is a protein produced by many different tissues in response to hypoxia, with the endothelial cell as its main target. VEGF is best known for its actions in angiogenesis, but it also seems to have important functions in male and female reproduction. In the female reproductive organs angiogenesis is necessary for the physiological periodical growth and regression of tissues. In the male very high concentrations of VEGF have been found in seminal plasma. The exact role of this growth factor in the male is still unknown, but the presence of very high concentrations in seminal plasma argues for an important role in male fertility and reproduction.
Finally, for angiogenesis to occur, matrix degradation is necessary. One of the systems involved in matrix degradation in humans is the plasminogen activator (PA)-system. The components of the PA-system occur in a variety of different cell types and are involved in several biological processes. Degradation of the extracellular matrix resulting from plasminogen activation is important in cell migration, necessary for morphogenesis, tissue repair, neovascularization or invasion of malignant cells. One of the interesting areas in which the PA-system appears to play an important role is reproduction.

These observations in literature resulted in the second objective of this thesis:

2. To systematically investigate the role of thiols, the PA-system and VEGF in human (sub)fertility by a) measuring the levels of these different parameters in the ejaculate, purified spermatozoa and follicular fluid of couples undergoing assisted reproduction techniques, and b) to determine associations between the concentrations of thiols, PA components and VEGF and fertility outcome parameters.

Outline of the thesis

The objectives are described in part I and part II, respectively.

PART I

Folic acid, zinc sulphate and human subfertility

Chapter 2 gives a review of the literature regarding the involvement of folate, zinc, antioxidants, and apoptosis on human subfertility. The following studies described in part I are an extension of a previous double-blind, placebo-controlled randomised intervention study conducted by our research group (Wong *et al*. 2002). This study evaluated the effects of a daily intake of 5 mg of folic acid, 66 mg of zinc sulphate, a combination of these 2 micronutrients or placebo treatment for 26 weeks on semen quality in 103 subfertile and 107 fertile males. The results of this study indicated a 74% increase in total normal sperm count for the subfertile group supplemented with the combination treatment. The same trend was found for the fertile group although no significance was reached.

Since the underlying mechanism for this beneficial effect of folic acid and zinc sulphate intervention on semen quality is unclear, we investigated possible influences of folate related gene polymorphisms, hormones, and apoptosis. *Chapter 3* investigated the frequency of the C677T methylenetetrahydrofolate reductase (MTHFR) polymorphism in this male population, and the MTHFR-dependent response of sperm concentration after folic acid and zinc sulphate intervention. *Chapter 4* evaluated pre- and post-intervention serum endocrine parameters, and in *Chapter 5* we determined seminal plasma annexin A5 concentrations, which is a marker of apoptosis, before and after the intervention treatment.

PART II

VEGF and determinants of the oxidative and plasminogen pathway in human subfertile couples

The literature on the involvement of the plasminogen activator system and vascular endothelial growth factor in human subfertility is reviewed in *Chapter 6*. To further explore these 2 processes as possible underlying causes of human subfertility, besides nutritional ‘deficiencies’ of folic acid and zinc sulphate discussed in part I, we collected various fluids from 156 couples

participating in an IVF or ICSI procedure in the Radboud University Nijmegen Medical Centre in Nijmegen, The Netherlands. These couples had several different indications for ART, varying from male factor subfertility characterised by oligoasthenoteratozoospermia (*n* = 52), female factor subfertility divided into women with Fallopian tube pathology (*n* = 26) or endometriosis (*n* = 26), and idiopathic subfertile couples (*n* = 52).
We measured concentrations of the endogenous antioxidant family of thiols in the ejaculate, and purified spermatozoa of the men, and in follicular fluid of the women. We compared these concentrations between the different diagnostic subgroups, and investigated possible associations between these thiol concentrations and fertility outcome parameters after ART (*Chapter 7*). Similar investigations were conducted, by measuring in the same study population different components of the plasminogen activator system involved in matrix degradation (*Chapter 8*), and vascular endothelial growth factor (*Chapter 9*).

In the general discussion (*Chapter 10*) we reflect on all these different results in light of the methodological strengths and weaknesses; in relation to literature we try to synthesise what knowledge about the pathophysiology of subfertility has been achieved and what the links can be between all the different hypotheses and results described in the separate chapters.
This enables us to propose recommendations for new research and potential clinical aspects.

PART I

Folic acid, zinc sulphate and human subfertility

Chapter 2

The importance of folate, zinc, and antioxidants in the pathogenesis and prevention of subfertility, a review

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Didi D.M. Braat, and Régine P.M. Steegers-Theunissen

Human Reproduction update, in press

Abstract

Background: Many subfertile couples have to be treated by artificial reproduction techniques for having children. This is, however, not a causal treatment for subfertility. In this review we outline the importance of nutritional and biochemical factors like folate and zinc, implicated in underlying processes such as detoxification and apoptosis in (sub)fertility.
Method: A literature search was performed using MEDLINE, Science Direct and bibliographies of published works.
Results: Evidence on the role of folate determined by food intake, synthetic folic acid intake, and folate related enzymes on sperm parameters is inconclusive. More data are available on the importance of folate on female reproduction, known to be important for the quality and maturation of oocytes, and in the prevention of early abortion, placenta abruption or infarction, foetal growth retardation, and neural crest related congenital malformations.
Zinc in males is implicated in testicular development, chromatin decondensation and stabilization, acrosome reaction, synthesis of testosterone and the conversion of testosterone into 5α-dihydrotestosterone. In the female, zinc plays a role in sexual development, ovulation, and the menstrual cycle.
Folate and or zinc have antioxidant properties. Reactive oxygen species (ROS) are produced by spermatozoa and play a role in hyperactivation of spermatozoa, acrosome reactions, and binding to the zona pellucida of the oocyte. Thiols like glutathione are important to balance the level of ROS and function in DNA compaction, tail structure stability and motility of spermatozoa. An excess of ROS is detrimental to the motility, morphology, and function of spermatozoa. In the female, ROS affect oocyte maturation, luteolysis, progesterone production by the corpus luteum, ovulation, and follicle atresia, while glutathione in the oocyte is important for sperm nucleus decondensation and male pronucleus formation.
An underlying process influenced by folate, zinc, ROS and thiols is apoptosis. Apoptosis in males is important for the release of sperm. Disorders in apoptosis contribute to an excess of abnormal spermatozoa possibly resulting in subfertility. In women apoptosis regulates follicle atresia, degeneration of the corpus luteum, and the cyclic shedding of endometrium during menstruation.

Conclusions: Nutrition and biochemical factors play an important role in (sub)fertility, and affect biological processes in male and female reproduction. The parameters reviewed in this paper have been shown to be involved in reproduction, but their relative contribution to subfertility in humans is not clear. Therefore, this review may stimulate further research in humans to unravel underlying mechanisms and identify amendable environmental factors to improve the diagnosis and treatment of subfertility.

Introduction

Subfertility is defined as the failure to conceive after 1 year of regular, unprotected intercourse with the same partner. Approximately 10 to 17% of all couples experience primary or secondary subfertility at some time during their reproductive life (Buckett and Bentick, 1997; Mosher and Pratt, 1990; Philippov *et al*., 1998; Snick *et al*., 1997; Templeton *et al*., 1990; Wallace, 1995). In Table I various causes of subfertility and the corresponding frequencies are summarized.

Table I The cause of subfertility and their approximate frequencies (Modified from Cahill and Wardle, 2002)

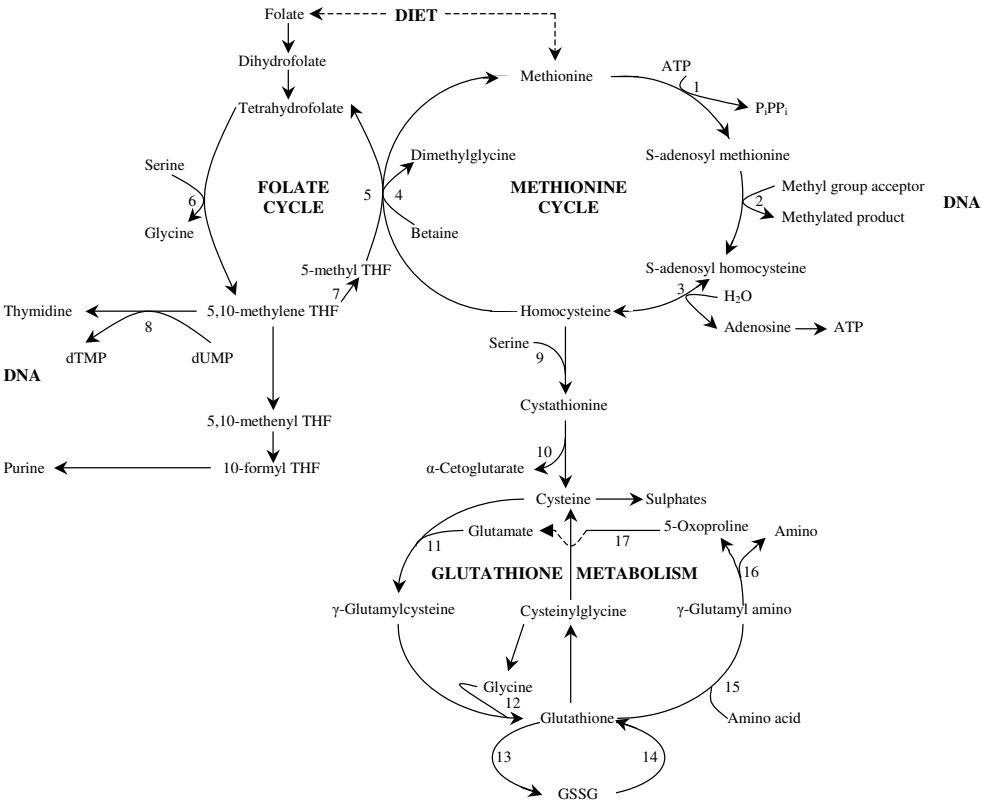
CAUSE	FREQUENCY (%)
Male factor subfertility	
Sperm defects or dysfunction	30
Female factor subfertility	
Ovulation failure (amenorrhoea or oligomenorrhoea)	25
Tubal infective damage	20
Endometriosis	5
Cervical mucus defects or dysfunction	3
Uterine abnormalities (such as fibroids or abnormalities of shape)	(< 1)
Unexplained subfertility	25
Coital failure or infrequency	5

Note: total exceeds 100% as 15% of couples have more than one cause of subfertility

Subfertility resulting in permanent childlessness is very difficult for couples to cope with (Downey *et al*., 1989; Whiteford and Gonzalez, 1995) and, therefore, subfertile couples try to conceive with all possible techniques that, however, do not treat the cause of the subfertility, such as artificial reproduction techniques (ART). Therefore there is a need to unravel the multifactorial origin of subfertility in which many genetic and environmental factors act together.

Of particular interest are the environmental and lifestyle factors implicated in subfertility, because unlike genetic causes, these can be targeted for curative or preventive measures. A significant but largely neglected lifestyle factor is nutrition. Nutrition is important for DNA synthesis, because most of its essential compounds are derived from the diet. Moreover, several enzymes involved in DNA synthesis are zinc or B-vitamin dependent (Figure 1).

Figure 1 DNA synthesis, the folate cycle and glutathione metabolism



Numbers: 1. Methionine-adenosyltransferase; 2. Methyltransferase; 3. S-adenosylhomocysteine hydrolase; 4. Betaine-homocysteine-methyltransferase (BHMT) (zinc dependent); 5. Methionine-synthase (vitamin B_{12} and zinc dependent); 6. Serineoxidase; 7. Methylenetetrahydrofolate reductase (MTHFR; vitamin B_2 dependent); 8. Thymidylate-synthase; 9. Cystathionine-β-synthase (vitamin B_6 dependent); 10. γ-Cystathionase (vitamin B_{12} dependent); 11. γ-Glutamyl-cysteine-synthase; 12. Glutathione-synthase; 13. Glutathione peroxidase; 14. GSSG reductase; 15. γ-Glutamyl transpeptidase; 16. γ-Glutamyl cyclotransferase; 17. 5-Oxoprolinase.
Abbreviations: ATP, adenosine triphosphate; DNA, deoxyribonuleic acid; dTMP, deoxythymidine monophosphate; dUMP, deoxyuridine monophosphate; GSSG, glutathione disulphide; H_2O, water; P_i, orthophosphate; PP_i, pyrophosphate; THF, tetrahydrofolate.

DNA synthesis is important for the development of spermatozoa and oocytes. Animal studies have demonstrated that deficiencies of vitamins A, C and D resulted in diminished fertility in rats and rainbow trout (Ciereszko and Dabrowski, 1995; Kwiecinski *et al.*, 1989; van Pelt and de Rooij, 1991). In

humans the effects of nutrition on fertility have scarcely been investigated. Recently, our group found a 74% increase in normal sperm count in subfertile men after supplementation of folic acid in combination with zinc sulphate (Wong *et al*., 2002).

The diet is a source of exogenous antioxidants (vitamins C and E), but also several endogenously synthesized antioxidants are essential, such as glutathione, bilirubin and uric acid. These antioxidants protect DNA and other important molecules from oxidation and damage, which otherwise would result in apoptosis. A more detailed overview of the glutathione metabolism is displayed in Figure 1.

The role of folate, zinc, glutathione and related thiols in biological processes involved in (sub)fertility are described in this review. We are aware that the pathways discussed here are only part of the complex processes implicated in reproduction, but we hope that this review will stimulate research into these issues in order to contribute to a better diagnosis and treatment of subfertility in humans in the future.

Materials and Methods

A thorough literature search was performed on MEDLINE, Science Direct, and via bibliographies of published works. The literature reviewed consisted of papers in English and Dutch published between 1950 and 2005, discussing both animal and human studies. The following words combined in different ways were used as search terms: fertilization, subfertility, folate, folic acid, zinc, antioxidants, glutathione, thiols, apoptosis, methylenetetrahydrofolate reductase (MTHFR), and ROS.

Folate

The micronutrient folate is present in a wide variety of foods, such as green-leafy vegetables, liver, bread, yeast and fruits. Folate is important for the synthesis of DNA, transfer RNA, and the amino acids cysteine and methonine (Figure 1). DNA synthesis plays an important role in germ cell development and therefore it is obvious that folate is important for reproduction. It has also been reported that folic acid, the synthetic form of folate, effectively scavenges oxidizing free-radicals, and as such can be regarded as an antioxidant (Joshi *et al*., 2001). Despite of its water soluble property, folic acid inhibits lipid

peroxidation. Therefore, folic acid can protect bio-constituents like cellular membranes or DNA from free-radical damage (Joshi *et al*., 2001). Only limited knowledge is available on the impact of dietary folate and synthetic folic acid on (sub)fertility.

Folate in males

Wong *et al.* (2002) found that folic acid administration (5 mg) to subfertile and fertile men for twenty-six weeks resulted in a significant increase of folate concentrations in seminal plasma, but no effect of this intervention was observed on sperm count or motility of spermatozoa. The percentage of normal sperm morphology, as determined according to strict criteria (Menkveld *et al*., 1990) even decreased after folic acid intervention. However, a 74% increase in total normal sperm count after the combination intervention with folic acid and zinc sulphate for twenty-six weeks was observed. An intervention study by Bentivoglio *et al.* (1993) reported an increase in number and motility of mature spermatozoa, and a decrease in round cell number (the presence of many immature cells) after 3 months of supplementation with 15 mg folinic acid (5-formyl tetrahydrofolate) in sixty-five men of infertile couples with round cell idiopathic syndrome. Another intervention study by Landau *et al.* (1978) did not observe a beneficial effect of folic acid supplementation on sperm count in 40 normo- and oligozoospermic men, however, both the dose and the duration was different as compared to the study of Bentivoglio *et al.* (10 mg during 30 days).

When folic acid is administrated, it has to be converted into the biologically active form 5-methyl tetrahydrofolate to exert its functions. This conversion is carried out by methylenetetrahydrofolate reductase (MTHFR), one of the key enzymes in folate metabolism. MTHFR converts 5,10-methylenetetrahydro-folate into 5-methyltetrahydrofolate, which subsequently donates a methyl group to cobalamin to form methylcobalamin and tetrahydrofolate. Methyl-cobalamin serves as a cofactor in the conversion of 5-methyltetrahydro-folate to tetrahydrofolate, in which homocysteine is remethylated to the essential amino acid methionine in humans (Figure 1). In the MTHFR-gene, a common polymorphism, resulting from a cytosine to thymine substitution (C677T) may be present. The prevalence of heterozygosity or homozygosity for this variant is approximately 40% and 10% in Caucasians, respectively (Ueland *et al.*,

2001), and these values vary between populations. This polymorphism is associated with an increased thermolability and reduced specific activity of MTHFR *in vivo*, resulting in a residual enzyme activity of 65% for heterozygous carriers and of only 30% for homozygous carriers (Frosst *et al*., 1995; Van der Put *et al*., 1995). Individuals with the homozygous genotype have mildly, though significantly elevated plasma homocysteine concentrations compared with heterozygotes and wild-types, especially when folate concentrations are low (Malinow *et al*., 1997).

The C677T polymorphism in the MTHFR-gene is accompanied by an altered folate metabolism and an impaired homocysteine remethylation, resulting in an increased folate need. The effect of supplementation with folic acid on semen parameters therefore is likely to be dependent on the MTHFR genotype.

Information on the effects of the MTHFR genotype on biological parameters is scarce. Stern *et al.* (2000) found that the 677T-homozygous genotype was associated with lower DNA methylation capacity compared with the 677C-homozygous genotype. This was explained by the reduced availability of 5-methyl tetrahydrofolate, required for S-adenosylmethionine biosynthesis. Bezold *et al.* (2001) reported a higher frequency of the homozygous 677T MTHFR genotype in infertile men, and suggested that products of MTHFR may have a role in the pathogenesis of male infertility. They furthermore stated that homozygous men in particular may benefit from folic acid supplementation. We reported that, in contrast to 677T MTHFR heterozygotes and homozygotes, sperm concentration in 677C homozygotes significantly improved after folic acid and zinc sulphate intervention, and concluded for this reason that the residual MTHFR activity in homozygote and heterozygote patients for the C677T MTHFR polymorphism might be insufficient to improve spermatogenesis compared with wild-type individuals (Ebisch *et al*., 2003). Animal *in vivo* and *in vitro* studies have shown that zinc deficiency decreases the absorption and metabolism of dietary folate (Favier *et al*., 1993; Ghishan *et al*., 1986; Quinn *et al*., 1990), due to its function as a cofactor for the folate metabolising enzymes dihydrofolate reductase and γ-glutamyl hydrolase. Zinc in itself, however, is not a cofactor of the MTHFR enzyme, but a cofactor for methionine synthetase and betaine-homocysteine methyltransferase (Figure 1).

Folate in females

Not much is known about the effects of folate or folic acid on oocyte development. A study by Lo *et al.* (1991) demonstrated the existence of an endogenous carrier-mediated uptake system for folate in frog oocytes. Similar studies on the existence of such an uptake system in human oocytes were not found. A study on homocysteine concentrations in follicular fluid (FF) in women opting for in vitro fertilization (IVF) treatment reported that women receiving folic acid supplementation had significantly lower homocysteine concentrations in their FF (the microenvironment surrounding the oocyte) (Steegers-Theunissen *et al*., 1993; Brouns *et al*., 2003; Szymanski and Kazdepka-Zieminska, 2003). The latter authors found that women receiving folic acid supplementation (and subsequent lower homocysteine FF concentrations) had a better quality and a higher degree of maturity of oocytes compared with women who did not receive folic acid supplementation (Szymanski and Kazdepka-Zieminska, 2003). Moreover, it was shown that folic acid present in mouse preimplantation embryos was absolutely essential for early embryo development, probably because of its essential role in the synthesis of thymidine required for DNA synthesis and repair. Also, all of the folic acid necessary for this process was present within the gametes at the time of fertilization (O'Neill, 1998).

Zinc

Zinc is a micronutrient abundantly present in meat and seafood. Zinc serves as a cofactor for more than eighty metalloenzymes involved in DNA transcription and protein synthesis (Figure 1). Since DNA transcription is a main part of germ cell development, zinc is likely to be important for reproduction. Furthermore, zinc finger proteins are implicated in the genetic expression of steroid hormone receptors (Favier, 1992; Freedman, 1992), while also zinc has anti-apoptotic (Chimienti *et al*., 2003), and antioxidant properties (Zago and Oteiza, 2001). Two mechanisms for this antioxidant function have been described. Zinc can bind sulfhydryl groups in proteins to counteract oxidation, and lipid peroxidation is prevented by occupying binding sites for iron and copper in lipids, proteins and DNA (Bray and Bettger, 1990; Zago and Oteiza, 2001). To substantiate these antioxidant effects of zinc, evidence was found for oxidative damage of proteins, lipids and DNA in zinc deficient rat and mice

(Bagchi *et al*., 1998; Oteiza *et al*., 1995), whereas zinc salts could protect against oxidative damage and glutathione depletion in mice (Bagchi *et al*., 1998).

Zinc in males

Zinc directly acts on testicular steroidogenesis (Favier, 1992; Hamdi *et al*., 1997). It has been shown that zinc is important in testicular development (Hamdi *et al*., 1997) and influences the oxygen consumption of spermatozoa in seminal plasma (Eliasson *et al*., 1971; Huacuja *et al*., 1973), nuclear chromatin condensation (Kvist, 1980), acrosome reaction (Riffo *et al*., 1992) and acrosin activity (Steven *et al*., 1982). Zinc also is important in sperm chromatin stabilization (Kvist *et al*., 1990), and in the synthesis of testosterone in Leydig cells and the conversion of testosterone to the biologically active 5α-dihydro-testosterone (Leake *et al*., 1984; Netter *et al*., 1981).

Zinc concentrations are very high in the male genital organs as compared to other tissues and body fluids (Mann, 1964), in particular in the prostate gland which is largely responsible for the high zinc content in seminal plasma. Spermatozoa themselves also contain zinc, which is derived from the testis. The relationship between zinc concentrations in seminal plasma and semen fertility parameters, however, is not clear. Kvist *et al*. (1990) found that zinc concentrations in seminal plasma were lower in men with idiopathic subfertility, compared with fertile controls. Low seminal zinc levels have been correlated with decreased fertility potential (Caldamone *et al*., 1979; Marmar *et al*., 1975). Furthermore, it was shown that men with sperm count of less than 20 million cells per millilitre, had slightly lower seminal plasma zinc concentrations compared with men with normal sperm counts (Saaranen *et al*., 1987). However, other authors reported that total seminal plasma zinc concentrations were similar between normozoospermic and oligoasthenozoospermic patients (Carpino *et al*., 1998).

Several intervention studies indicated that zinc supplementation accelerates the onset in sexual function in zinc-deficient males (Halsted *et al*., 1972). Furthermore, it seems to improve sperm count (Hartoma *et al*., 1977; Mahajan *et al*., 1982; Marmar *et al*., 1975; Wong *et al*., 2002), sperm motility and morphology (Caldamone *et al*., 1979; Marmar *et al*., 1975), testosterone concentration (Antoniou *et al*., 1977; Hartoma *et al*., 1977; Mahajan *et al*.,

1982) and sexual potency (Antoniou *et al*., 1977; Mahajan *et al*., 1982) in subfertile men. Ronaghy *et al.* (1974) found a higher, though not statistically significant proportion of already developed genitalia in malnourished school boys after zinc supplementation, as compared to boys not receiving extra zinc. The induction of mild zinc deficiency in 5 healthy, fertile men resulted in a reversible drop in sperm concentration and count, sexual drive, and testosterone in all study subjects, explained by a reduction in Leydig cell function (Abbasi *et al*., 1980). The underlying mechanisms of zinc on the fertility parameters, however, are not yet clear.

Zinc in females

In females, zinc also seems to be important in reproduction, however, only relatively few investigations have been performed. Shaw *et al.* (1974) found that zinc deficiency in female rabbits resulted in disinterest in their male counterparts and therefore in failure of ovulation. Furthermore, the endometrium of these rabbits was pale and inactive, and these rabbits were unable to conceive. However, the authors could not exclude that the observed effects were related to deficiencies in other critical nutrients (Shaw *et al*., 1974). Zinc deficiency also led to abnormal oestrous cycles in female rats (Swenerton and Hurley, 1968). In addition, the effects of zinc deficiency in 2 species of monkeys have been investigated, and it was found that normal reproduction was impaired in both species. The pregnancy rate in zinc deficient monkeys was significantly lower compared with the original stock colony, because of cessartion of the seasonal menstrual cycles. However, some zinc deficient monkeys were still able to conceive (Swenerton and Hurley, 1980).

Studies on the effects of zinc deficiency in women are scarce. Ronaghy and Halsted (1975) described 2 women of 19 and 20 years old, suffering from nutritional dwarfism with delayed sexual maturation. These women had no breast tissue, pubic hair and had infantile external genitalia with extremely low plasma and erythrocyte zinc levels. After zinc supplementation these women experienced their first menstrual period, developed breast tissue as well as pubic hair growth. Jameson (1976) reported longstanding infertility in 7, normal sexually developed women with celiac disease. These women all had normal menstrual cycles, but low serum zinc levels. Soltan and Jenkins (1983) measured plasma zinc concentrations in 48 infertile and 35 control women and

found no differences between these 2 groups. It was therefore concluded that zinc deficiency is not a significant cause of subfertility.
Finally, Ng *et al.* (1987) investigated the zinc levels in FF of 33 women undergoing IVF in Singapore, and did not observe any correlation between FF zinc concentration, follicle volume, the presence of oocytes in the follicle, or the eventual fertilization of the oocyte. Therefore these authors conclude that the FF zinc content does neither reflect the follicular status nor the status of the oocyte.

Reactive oxygen species, antioxidants and fertility

Antioxidants such as vitamin E (α-tocopherol), vitamin C (ascorbic acid), and vitamin A (carotenoids) present in nutrition are important in restoring or maintaining the oxidant-antioxidant balance in tissues. As mentioned before, folate and zinc present in nutrition are also important for several antioxidant functions. These antioxidants can provide protection of cells against oxidative and electrophilic stress caused by ROS, leading to damage of DNA or other important structures like proteins or cell membranes. Many investigations have shown evidence for the role of ROS in the physiology and pathology of both male and female reproductive function (de Lamirande and Gagnon, 1994; Riley and Behrman, 1991b). Animal studies have shown that antioxidants present in nutrition are important to maintain normal semen parameters and to achieve pregnancy (Ciereszko and Dabrowski, 1995; Kwiecinski *et al*., 1989).
Another system involved in restoring or maintaining the proper pro-oxidant-antioxidant balance in human tissues is the glutathione (GSH)/glutathione-related enzyme system, quantitatively one of the most important protective systems in humans. Glutathione is a tripeptide composed of glutamate, cysteine and glycine (L-γ-glutamyl-L-cysteinyl-glycine). It is synthesized in 2 consecutive steps catalysed by γ-glutamylcysteine synthetase and glutathione synthetase. The enzyme γ-glutamyltranspeptidase is involved in the break-down of glutathione, thereby cleaving the γ-bond resulting in glutamate and cysteinylglycine (Figure 1). Glutathione and the GSH-related enzymes, glutathione S-transferases, are involved in the metabolism and detoxification of many cytotoxic and carcinogenic compounds, whereas GSH and glutathione peroxidases are pivotal for elimination of ROS (Beckett and Hayes, 1993). GSH itself also plays an important role in the protection of cells against

oxidative and electrophilic stress caused by ROS and radiation (Shan *et al*., 1990).

Reactive oxygen species and antioxidants in males

Human spermatozoa are capable of generating ROS (Aitken *et al*., 1989a; Alvarez *et al*., 1987; Alvarez and Storey, 1989; D'Agata *et al*., 1990; Iwasaki and Gagnon, 1992), which are involved in regulating epididymal maturation of spermatozoa (Cornwall *et al*., 1988), the rate of hyperactivation (de Lamirande and Gagnon, 1993a; de Lamirande and Gagnon, 1993b), as well as the ability of the spermatozoa to undergo the acrosome reaction (Burkman, 1990; de Lamirande *et al*., 1993). They also have a physiologic role in mediating the attachment of spermatozoa to oocytes (Aitken *et al*., 1989a; Aitken *et al*., 1993). Thus, the presence of ROS seems very important in the process of fertilization of oocytes.

It should be realized that the production of ROS by spermatozoa is a normal physiological process. However, the amount of ROS produced has to be carefully controlled because an imbalance between generation and scavenging of ROS may lead to damage of DNA or other important structures. Spermatozoa are particularly susceptible to peroxidative damage, because most of their cytoplasm is removed during the final stages of spermatogenesis. Therefore, the spermatozoa have almost no cytoplasmic defensive enzymes, such as catalase, glutathione peroxidase or glutathione S-transferase, which are involved in the protection of most cell types from peroxidative damage induced by ROS. Furthermore, the plasma membranes of spermatozoa contain large amounts of unsaturated fatty acids, which are particularly vulnerable to free-radical attack (Jones and Mann, 1973). Thus, high concentrations of ROS are detrimental to sperm.

Membrane fluidity of spermatozoa is necessary for sperm motility, the acrosome reaction, and the ability of the spermatozoa to fuse with the oocyte (Aitken *et al*., 1989b; Aitken *et al*., 1989a; Sharma and Agarwal, 1996; Zini *et al*., 2000). Lipid peroxidation caused by high concentrations of ROS may lead to peroxidation of plasma membrane lipids, resulting in altered membrane fluidity, and sperm dysfunction, which may be a cause of male subfertility. This is supported by the observations that subfertile men have higher concentrations of ROS in seminal plasma, whereas an inverse correlation exists

between ROS concentration and motility, as well as a normal morphology of the spermatozoa (Agarwal *et al*., 1994; Aitken and Fisher, 1994; Aitken *et al*., 1989a; Alkan *et al*., 1997; D'Agata *et al*., 1990; Iwasaki and Gagnon, 1992; Rao *et al*., 1989).

Because of the involvement of GSH in the protection of cells against oxidative stress caused by ROS, this tripeptide may also be important in fertility. Raijmakers *et al.* (2003) found that considerable amounts of GSH were present in the seminal plasma of both fertile and subfertile men. However, the median GSH concentrations were significantly higher in fertile men compared with subfertile men. Furthermore, the GSH concentrations in seminal plasma were positively correlated with sperm motility, and inversely correlated with sperm morphology. These results indicate that the concentrations of GSH in seminal plasma play a role in fertility. This was also suggested by Lenzi *et al.* (1993) who investigated the effects of intra-muscular injection of 600 mg GSH for 2 months. These authors observed a significant increase in sperm motility, in particular forward motility, and a significant reduction in the percentage of abnormal spermatozoa (Lenzi *et al*., 1993). Another intervention study conducted by Kodama *et al.* (1997) showed that a combination therapy of 400 mg GSH, 200 mg vitamin C and 200 mg vitamin E for 2 months significantly improved sperm concentration and reduced the level of oxidative DNA damage in the spermatozoa. However, Ochsendorf *et al*. (1998) could not find a difference in seminal plasma GSH concentration between azoospermic, oligozoospermic-, or normospermic men.

GSH and cysteine are also present in spermatozoa themselves. Free sulfhydryl groups in protamines of sperm chromatin are important in the decondensation of the sperm nucleus after fertilization (Rousseaux and Rousseaux-Prevost, 1995). Garrido *et al.* (2004) did not find a difference in GSH content in spermatozoa between fertile semen donors and infertile men attending an infertility centre, while their wives had no demonstrable cause of infertility. However, in this study only semen samples with more than 50 million spermatozoa were used. These authors also observed that sperm GSH concentrations were low in semen samples with less than 5% spermatozoa of normal morphology (Garrido *et al*., 2004). Ochsendorf *et al.* (1998) also investigated GSH content in spermatozoa and found that the mean intracellular GSH concentration in oligospermic patients was significantly lower compared

with that of normal volunteers. Furthermore, these authors investigated the relationship between sperm GSH concentration and sperm function evaluated by the ability of the spermatozoa to penetrate bovine cervical mucus and observed a small but significant positive correlation between sperm GSH concentration and penetration of mucus (Ochsendorf *et al*., 1998).

Reactive oxygen species and antioxidants in females

The role of ROS at the level of the oocyte and female infertility is not clear. The intensive metabolism of granulosa cells and the high numbers of macrophages and neutrophilic granulocytes in the follicle wall at ovulation may point at active generation of ROS. ROS levels in FF within certain physiologic ranges may be necessary for the normal development of the oocyte and subsequent embryo growth. However, as in many other systems, high levels may indicate oxidative stress.

ROS are involved in oocyte maturation (Riley and Behrman, 1991b), luteolysis (Riley and Behrman, 1991a; Sugino *et al*., 2000), progesterone production by the corpus luteum (Musicki *et al*., 1994; Sawada and Carlson, 1996; Shimamura *et al*., 1995; Sugino *et al*., 1993) and ovulation. Inhibition of ROS actually hinders ovulation (Miyazaki *et al*., 1991), Furthermore, Margolin *et al*. (1990) observed that ROS are involved in the loss of sensitivity of granulosa cells to gonadotropic hormones and in loss of steroidogenic function, both of which are characteristics of follicular atresia. These results suggest that ROS may be involved in the atretic regression of the cohort of newly grown follicles to leave only one follicle that is destined for ovulation (Margolin *et al*., 1990). One definite consequence of an excess of ROS in the ovary is damage of plasma membranes, but what is the effect of this damage on female fertility? Several investigators studied the involvement of ROS/oxidative stress in the FF and its consequences on several outcome parameters of assisted reproduction procedures. Pasqualotto *et al.* (2004) measured lipid peroxidation (LPO) as a marker for oxidative stress, and the total antioxidant capacity (TAC) in pooled FF from women undergoing IVF treatment and found that both markers were positively correlated with pregnancy rate. These authors therefore concluded that high levels of LPO in FF are not impairing oocyte and embryo development and quality, and that a certain amount of oxygen is critical for oocyte maturation (Pasqualotto *et al*., 2004). Furthermore, they state that the

higher TAC levels in these patients can buffer the higher LPO levels. No correlations were found between LPO and TAC levels and oocyte maturity, embryo quality, fertilization and cleavage (Pasqualotto *et al*., 2004).

Similar results were obtained in a study by Attaran *et al.* (2000) who investigated FF levels of ROS and TAC in women undergoing IVF. These authors observed that granulosa cells were the primary source of FF ROS, and that patients who became pregnant after IVF had significantly higher FF ROS levels compared with patients who did not become pregnant, ROS being a marker for obligatory minimal metabolic activity within the follicle. Unlike Pasqualotto *et al.* (2004) Attaran *et al*. (2000) did not observe a difference in TAC levels between patients who became pregnant and those who did not. Also, results off Bedaiwy *et al.* (2002) indicated that women with higher FF ROS levels were more likely to become pregnant after IVF compared with women having lower FF ROS levels. Oyawoye *et al.* (2003) reported that the mean TAC levels in fluid from follicles that yielded successfully fertilized oocytes were significantly higher than the mean TAC levels from FF associated with oocytes eventually not becoming fertilized. Conversely, the TAC concentration in FF eventually yielding oocytes that gave rise to a surviving embryo was significantly lower compared with TAC levels in fluid from follicles eventually not yielding viable embryos at time of transfer. These authors therefore concluded that ROS may have different effects at different stages of embryonic development (Oyawoye *et al*., 2003). Finally, Wiener-Megnazi (2004) refined the results and concluded that extreme reductive or oxidative states of the FF do not favour the occurrence of pregnancy.

Some reports deal with the role of ROS and TAC in female fertility, but the specific role of GSH in female reproduction is not investigated so far. It is known, however, that after fertilization GSH in pig oocytes is very important for the reduction of disulphide bonds in the sperm nucleus, and also is involved in sperm nucleus decondensation and male pronucleus formation (Yoshida *et al*., 1993). Furthermore, GSH in bovine oocytes is also important for male pro-nucleus formation, and for pronuclear apposition during fertilization (Sutovsky and Schatten, 1997). This role of GSH in the oocyte is further endorsed by the observation that oocytes matured in the presence of a GSH antagonist were not capable of decondensing the sperm nucleus and did not support pronucleus apposition after IVF, while these changes could be reversed by the

addition of thiols (Sutovsky and Schatten, 1997). Furthermore, supplementation of components of GSH during *in vitro* maturation of oocytes resulted in improved male pronucleus formation, normal fertilization, and embryo development (Jeong and Yang, 2001; Rodriguez-Gonzalez *et al*., 2003; Sawai *et al*., 1997). Also, enhancement of GSH or components of GSH in the oocyte and in culture medium of embryos during IVF or ICSI procedures was likely to improve fertilization rates and embryo development (Ali *et al*., 2003; Fukui *et al*., 2000; Kim *et al*., 1999; Takahashi *et al*., 1993).

Folate, zinc, antioxidants and apoptosis

Apoptosis is a programmed and physiological mode of cell death, essential in morphogenesis and normal tissue remodelling. In contrast to necrosis, the apoptotic process does not elicit an inflammatory response nor does it damage adjacent cells.

Folate is related to apoptosis. Several investigators observed that folate deficiency resulted in significant apoptosis in ovarian cells of the Chinese hamster (James *et al*., 1994), human hepatoma Hep G2 cells (Chern *et al*., 2001), human T lymphocytes (Courtemanche *et al*., 2004a; Courtemanche *et al*., 2004b), human trophoblasts (Steegers-Theunissen *et al*., 2000), and human erythrocytes (Koury and Ponka, 2004). Chern *et al.* (2001) furthermore reported that the mechanism whereby folic acid deficiency resulted in apoptosis was the severe disruption of methionine metabolism, leading to enhanced accumulation of homocysteine. This latter compound was shown to be responsible for an increase in oxidative stress through the overproduction of hydrogen peroxide. As a result of this oxidative stress situation, a redox-sensitive transcriptional factor (NK-κB) was found to be hyper-activated, resulting in apoptosis through expression of death genes (Chern *et al*., 2001). Other authors observed apoptotic effects of homocysteine on vascular smooth muscle cells, while folic acid administration to the cell culture diminished apoptosis, probably through reduction of homocysteine concentrations (Buemi *et al*., 2001).

As mentioned briefly in the section about zinc, this micronutrient also possesses anti-apoptotic properties. The inhibitory effects of zinc on apoptosis have been postulated to involve 2 mechanisms: early in the apoptotic pathways, zinc may inhibit caspases (proteases involved in programmed cell

death), whereas later in the apoptotic chain of events, zinc may suppress calcium and magnesium-dependent endonucleases, which cause apoptotic DNA fragmentation (Chai *et al*., 1999; Chimienti *et al*., 2003; Perry *et al*., 1997; Truong-Tran *et al*., 2000). However, excessively high concentrations of zinc may be toxic and can induce apoptosis and even necrosis (Chimienti *et al*., 2003; Truong-Tran *et al*., 2000). Further evidence for the involvement of zinc in apoptosis is indicated by the observations that zinc depletion can induce apoptosis in several cell types whereas zinc supplementation can protect cells against diverse pro-apoptotic molecules, preventing programmed cell death (Chimienti *et al*., 2003; Sunderman, Jr., 1995).

As mentioned above, folic acid and zinc both have antioxidant properties. This is still another mechanism whereby these micronutrients can affect apoptosis, since oxidative stress is known to influence apoptosis. Buttke and Sandstrom state that 'many agents which induce apoptosis are either oxidants or stimulators of cellular oxidative metabolism. Conversely, many inhibitors of apoptosis have antioxidant activities or enhance cellular antioxidant defences, and they discuss several studies supporting this statement (Buttke and Sandstrom, 1994). Decreasing the ability of a cell to scavenge or detoxify ROS is another way by which oxidative stress can induce apoptosis. Several studies have indicated a relationship between intracellular GSH depletion and apoptosis. Chiba *et al.* (1996) observed that intracellular GSH modulated Fas-mediated apoptosis in malignant and normal human T lymphocytes (Fas is a death factor that controls apoptosis). They found that Fas-resistant cells contained higher concentrations of GSH, and that pre-treatment of these resistant cells with buthionine sulfoximine (an inhibitor of GSH synthesis) or depletion of intracellular GSH reversed their resistance to Fas-mediated apoptosis (Chiba *et al*., 1996). Furthermore, Beaver and Waring (1995) studying apoptosis in murine thymocytes reported that a reduction in intracellular GSH preceded the onset of apoptosis, while pre-treatment of thymocytes with GSH before inducing apoptosis resulted in inhibited apoptosis. Therefore, they conclude that a decrease in intracellular GSH or an increase in GSSG (oxidized glutathione), or perhaps a change in the ratio of GSH to GSSG, constitutes a trigger for apoptosis (Beaver and Waring, 1995). Finally, Iwata *et al.* (1997) found that GSH depletion in the culture medium of human lymphocytes induced apoptosis because of an increase of ROS in the cultured

cells, whereas the addition of GSH to the depleted medium protected the lymphocytes from apoptosis (Iwata *et al*., 1997).

Apoptosis in males

Apoptosis in certain classes of germ cells is a normal feature of spermatogenesis in a variety of mammalian species, including the human being, and may be important in regulating sperm release (Barroso *et al*., 2000; Hikim *et al*., 1998). However, little information is available on the biological significance of apoptosis in spermatogenesis or its possible role in male fertility (Oosterhuis *et al*., 2000).

Apoptosis has been related to some forms of male factor subfertility. The number of spermatozoa expressing the cell surface protein Fas is low in men with normal sperm numbers and higher in men with abnormal sperm parameters (Sakkas *et al*., 1999). The expression of Fas in ejaculated spermatozoa may be linked to abnormal apoptosis, as these spermatozoa are destined for apoptosis, but still appear in the ejaculate. This can result in abnormal spermatozoa in the ejaculate possibly contributing to male subfertility.

Furthermore, intracellular abnormalities such as various nuclear alterations including an abnormal chromatin structure, microdeletions in chromosomes, aneuploidy and DNA strand breaks are observed in spermatozoa from infertile men and possibly induce apoptosis (Barroso *et al*., 2000). ROS are known to cause oxidative damage to DNA in spermatozoa (Hughes *et al*., 1996; Kodama *et al*., 1997; Twigg *et al*., 1998a; Twigg *et al*., 1998b), possibly leading to a higher proportion of spermatozoa undergoing apoptosis. This ROS-induced apoptosis can be inhibited by various antioxidants (Hockenbery *et al*., 1993; Kane *et al*., 1993), suggesting a significant role of oxidative stress as well as antioxidant activity in inducing or inhibiting apoptosis. As mentioned above, the micronutrients folate and zinc, reported to increase sperm count (Wong *et al*., 2002), also have antioxidant properties (Joshi *et al*., 2001; Zago and Oteiza, 2001). Therefore, these 2 micronutrients also may have an effect on apoptosis.

Apoptosis in females

Throughout female foetal and postnatal life 99.9% of follicles undergo atresia by means of programmed cell death (Tilly *et al*., 1991; Tilly, 2001). Why so

many oocytes do not survive to become fertilized by a spermatozoon is not understood as yet. Tilly (2001) states that oocyte apoptosis can be considered as an utterance to the survival-of-the-fittest theory of evolution, whereby the ongoing deletion of inferior oocytes through the apoptotic pathway maximizes the chance of reproductive success. Yuan and Giudice (1997) investigated the role of apoptosis in different stages of follicular development and observed that programmed cell death occurred in human ovaries, and particularly in the granulosa cell layer. Furthermore they state that apoptosis commences as soon as follicles begin to leave their resting state, since primordial, primary, and secondary follicles in the human ovary are not affected by apoptosis, while from the preantral stage onwards, the growing follicles show increasing amounts of apoptotic granulosa cells (Yuan and Giudice, 1997). Interestingly, dominant follicles do not have apoptotic granulosa cells (Yuan and Giudice, 1997). Similar results were found in the ovaries from rats (Hughes, Jr. and Gorospe, 1991; Palumbo and Yeh, 1994).

Yuan and Giudice also reported that oestrogen-dominant follicles did not display any apoptosis, in contrast to androgen-dominant follicles in which apoptosis was a characteristic feature. Therefore, these authors state that apoptosis in follicles is a function of follicular androgen dominance (Yuan and Giudice, 1997). In a review Hsueh *et al.* (1994) discussed the hormonal control of apoptosis in follicular atresia. They stated that gonadotropins and estrogens in combination with several ovarian growth factors and regulatory peptides, like insulin-like growth factor 1 (IGF-1), epidermal growth factor (EGF)/transforming growth factor-α (TGFα) and basic fibroblast growth factor (bFGF), are involved in the escape from follicular apoptosis, while androgens and atretogenic factors like interleukin-6 (IL-6) and GnRH are involved in the induction of apoptosis in follicles.

Apoptosis furthermore seems to be important in the degeneration of corpora lutea in the ovary (Shikone *et al*., 1996; Tilly *et al*., 1991; Yuan and Giudice, 1997) and the cyclic shedding of the endometrium in the uterus (Spencer *et al*., 1996).

Another possible role suggested for apoptosis is the prevention of fertilization of potentially defective oocytes after ovulation (Fujino *et al*., 1996). However, Van Blerkom and Davis (1998) investigated the extent of apoptosis in newly ovulated mouse and human oocytes and found no indication of significant

apoptosis. Furthermore, these authors showed that penetration and fertilization failure of human oocytes were not related to apoptosis. In addition, apoptosis of oocytes, if it occurred at all, was patient specific and not related to maternal age (Van Blerkom and Davis, 1998). The presence of apoptosis in oocytes from specific patients could have consequences regarding their fertility (Van Blerkom and Davis, 1998).

Likewise, İdil *et al.* investigated the role of granulosa cell apoptosis in the aetiology of idiopathic subfertility and found that the apoptotic rate in women with unexplained infertility was significantly higher compared with that in women with tubal pathology. Therefore, these authors state that granulosa cell apoptosis may have a role in the aetiology of unexplained infertility (Idil *et al*., 2004). Nakahara *et al.* (1997a) also investigated the rate of apoptosis per patient in relation to different causes of female infertility, and found that the amount of apoptosis in mural granulosa cells was significantly higher compared with that of cumulus cell masses. In addition, they investigated the difference in apoptotic rates between women with tubal infertility, endometriosis, male infertility and idiopathic infertility. The results showed no difference in mural granulosa cell apoptosis, however, patients with endometriosis showed a significantly higher incidence of apoptotic bodies in the cumulus cells compared with patients in the other groups (Nakahara *et al*., 1997a). Other authors also suggested involvement of deranged apoptosis in endometriosis patients (Garcia-Velasco *et al*., 2002; Garcia-Velasco and Arici, 2003; Nakahara *et al*., 1998). However, not all authors could demonstrate such a connection (Cahill, 1998).

Finally, Nakahara *et al.* (1997a) investigated the relationship between apoptosis and parameters associated with the IVF program. Results indicated that significantly more apoptosis was found in both mural and cumulus granulosa cells in patients with less than 6 retrieved oocytes compared with patients with more than 6 retrieved oocytes (Nakahara *et al*., 1997a). Also, the apoptotic rates in mural granulosa cells were significantly higher in non-pregnant compared with pregnant patients, indicating that a higher incidence of apoptosis could be indicative of a poor prognosis for IVF (Nakahara *et al*., 1997a). Similar results were observed by Oosterhuis *et al.* (1998) who found that women with less than 13% apoptotic granulosa-lutein cells were far more likely to become pregnant as compared to women with more than 13%

apoptotic cells.
In an additional study investigating apoptosis per follicle, Nakahara *et al.* (1997b) reported that significantly more apoptotic granulosa cells were present in follicles containing no oocyte compared with follicles with oocyte, whereas follicles from fertilized oocytes contained significantly less apoptotic granulosa cells as compared to follicles from non-fertilized oocytes. In addition, follicles containing oocytes from which good embryos developed did contain significantly less apoptotic granulosa cells compared with follicles with oocytes from which morphologically poor embryos developed (Nakahara *et al*., 1997b). These findings also suggest that a higher incidence of apoptosis might be a poor prognostic factor for IVF outcome (Nakahara *et al*., 1997b).
Finally, Seifer *et al.* (1996) observed a possible role for apoptosis in women with decreased ovarian reserve like in the premature ovarian failure syndrome.

Conclusions

Many environmental and biochemical factors are involved in male and female reproduction. The importance of many of these factors is not yet clearly understood. Still numerous couples face unexplained subfertility and can only be treated by assisted reproduction technologies. However, these treatments do not abolish the cause of subfertility, for which no therapies are available. A better understanding of underlying mechanisms in (sub)fertility is important to improve diagnosis and treatment. In addition to other possible underlying mechanisms of subfertility, the pathways in which nutrition, antioxidants, and apoptosis are involved as reviewed in this paper need to be further investigated.
Many investigations in the field of subfertility reviewed here were performed on laboratory animals or cell cultures, which is a comprehensive first research design. However, it is very important to verify the results in human subjects. Also, most investigations actually performed in human beings involve couples participating in ART procedures. This is the only way to obtain research material in humans. The disadvantage is that all couples experience sub-fertility, and since subfertility is known to be a combination of female and male factors, no information on truly fertile couples is available. It would be a great advantage to investigate the same biochemical parameters in fertile couples.

Chapter 3

C677T methylenetetrahydrofolate reductase polymorphism interferes with the effects of folic acid and zinc sulphate on sperm concentration

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Abstract

Objective: To determine the frequency of C677T methylenetetrahydrofolate reductase (MTHFR) polymorphism in fertile and subfertile males, and the MTHFR-dependent response of sperm concentration after folic acid and/or zinc sulphate intervention.
Design: Double-blind, placebo-controlled randomised intervention study.
Setting: Two outpatient fertility clinics and nine midwifery practices in The Netherlands.
Patients: One hundred thirteen fertile and 77 subfertile males.
Interventions: Daily capsules of folic acid (5 mg) and/or zinc sulphate (66 mg), or placebo for 26 weeks.
Main outcome measures: Prevalence of C677T MTHFR polymorphism and the response of sperm concentration related to MTHFR carriership after intervention treatment.
Results: The C677T methylenetetrahydrofolate reductase genotypes were comparable in fertile and subfertile males. Independent of fertility state, sperm concentration significantly increased in wild-types after folic acid and zinc sulphate treatment only. Heterozygotes and homozygotes did not significantly benefit from either treatment.
Conclusions: C677T methylenetetrahydrofolate reductase polymorphism is not a risk factor for male factor subfertility. In contrast to heterozygotes and homozygotes for C677T MTHFR polymorphism, sperm concentration in wild-types significantly improved after folic acid and zinc sulphate intervention. A stronger role of other folate genes on spermatogenesis is suggested.

Introduction

Gene-environment interactions are involved in the reported decrease of semen quality in the last decades (Wong *et al*. 2000; Auger *et al*. 1995; Skakkebaek *et al*. 1994). Although nutrition is thought to have an effect on spermatogenesis, data on the influence of specific nutrients on spermatogenesis in humans are scarce. Recently, our group demonstrated in a randomised controlled trial that total normal sperm count significantly increased after intervention with folic acid and zinc sulphate in subfertile males (Wong *et al*. 2002). The beneficial effect of both nutrients could be explained by their role in transport ribonucleic acid (tRNA) and deoxyribonucleic acid (DNA) synthesis. Moreover, as a cofactor of dihydrotestosterone reductase, zinc is involved in testosterone synthesis, and thus is important in testicular development, spermatogenesis, and sperm motility. There is some evidence that zinc in seminal plasma influences the oxygen consumption of spermatozoa, acrosin activity, and stabilization of sperm chromatin (Riffo *et al*. 1992; Kvist *et al*. 1990).

Methylenetetrahydrofolate reductase (MTHFR) is one of the key enzymes in folate metabolism. The C677T MTHFR polymorphism is common, and its prevalence varies among populations. The prevalence of the heterozygous and homozygous state is approximately 40% and 10% in Caucasians, respectively (Ueland *et al*. 2001). This polymorphism is associated with an increased thermolability and reduced specific activity of MTHFR in vivo, resulting in a decreased residual activity of 65% for heterozygous carriers and of 30% for homozygous carriers (van der Put *et al*. 1995; Frosst *et al*. 1995).

The methylenetetrahydrofolate reductase-enzyme converts 5,10-methylene-tetrahydrofolate to the biologically active form 5-methyltetrahydrofolate; subsequently, 5-methyltetrahydrofolate donates its methyl group to cobalamin to form methylcobalamin and tetrahydrofolate. Methylcobalamin serves as a cofactor in the conversion of 5-methyltetrahydrofolate to tetrahydrofolate, in which homocysteine is remethylated to the essential amino acid methionine in humans. The C677T polymorphism in the MTHFR-gene is accompanied by an altered folate metabolism and an impaired homocysteine remethylation, resulting in an increased folate need. Individuals with the homozygous (TT) genotype have mildly, albeit significantly, elevated plasma homocysteine concentrations compared with heterozygotes (CT) and wild-types (CC), especially when folate concentrations are low (Malinow *et al*., 1997).

Animal in vivo and *in vitro* studies have shown that zinc deficiency alters the absorption and metabolism of dietary folate, because of its function as a cofactor for dihydrofolate reductase and γ-glutamyl hydrolase (Ghishan *et al*. 1986; Quinn *et al*. 1990; Favier *et al*. 1993). Both enzymes are involved in folate metabolism. Zinc is not a cofactor of the MTHFR enzyme. However, the product of MTHFR, 5-methyltetrahydrofolate, is converted to tetrahydrofolate by zinc-dependent methionine synthase (Tamura *et al*. 1987).

Information on the effects of the MTHFR genotype on biological parameters is scarce. Stern *et al*. (2000) found that the 677TT genotype is associated with decreased DNA methylation compared with the 677CC genotype. This is explained by the reduced availability of 5-methyltetrahydrofolate required for S-adenosylmethionine biosynthesis. Bezold *et al*. (2001) reported finding a higher frequency of the 677TT genotype in infertile males, and suggested that these males in particular may benefit from folic acid supplementation.

As an extension of our previous study (Wong *et al*. 2002), we determined the prevalence of the MTHFR C677T polymorphism in a Caucasian population of fertile and subfertile males and evaluated whether this polymorphism interferes with the response of folic acid and/or zinc sulphate intervention on sperm concentration.

Materials and Methods

Patients

The present study is based on the previously described double-blind, placebo-controlled randomised intervention study in which the effect of folic acid and/or zinc sulphate intervention on semen parameters was studied in fertile and subfertile males (Wong *et al*. 2002). In summary, fertile men were recruited from 9 midwifery practices in the area of Nijmegen in The Netherlands. These healthy men, who had no history of fertility problems, had a pregnant partner at the moment of enrolment who had conceived spontaneously within 1 year of regular, unprotected intercourse. The fertile men had a sperm concentration of more than 20 million cells/ml at study entry. Subfertile men were recruited form the fertility clinics of the Radboud University Nijmegen Medical Centre and the Canisius Wilhelmina hospital in Nijmegen. Subfertility was defined as failure of the female partner to conceive after 1 year of regular, unprotected intercourse,

and the man having a sperm concentration of less than 20 million cells/ml at study entry. All the participants gave their written informed consent and were assigned to four different intervention groups by computer-generated random numbers. The intervention consisted of 26 weeks of a daily dose of folic acid (5 mg) and placebo, zinc sulphate (66 mg) and placebo, zinc sulphate and folic acid (combined), or placebo and placebo. This study was approved by the institutional review board and the medical ethical committee of the Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands.
Methylenetetrahydrofolate reductase polymorphism was determined in both fertile and subfertile men, and the frequencies of the three genotypes were compared. The wild-type (CC), heterozygous (CT), and homozygous (TT) carrier-state of C677T MTHFR was evaluated together with the effect of intervention treatment (placebo-placebo, zinc-placebo, folic acid-placebo or zinc-folic acid supplementation) on sperm concentration.

Semen samples

Before and after intervention, one standardized semen sample was obtained from every participant for semen analysis according to World Health Organization (WHO) guidelines (WHO 1992; Menkveld *et al*. 2001; Guzick *et al*. 2001). The semen samples were produced by the participants via masturbation after an abstinence period of 3-5 days. These samples were delivered within 1 hour of production to the fertility laboratory. After liquefaction, an aliquot of semen was centrifuged at 1,400 x *g* (Hettich 16A, 1323 rotor) for 10 minutes. The supernatant seminal plasma was frozen without preservatives and stored at -80°C. The sperm concentration evaluated in this study was determined with a Makler counting chamber.

Methylenetetrahydrofolate reductase

The prevalence of the C677T polymorphism was investigated by polymerase chain reaction (PCR) of genomic DNA, followed by restriction enzyme digestion with *Hinf*I according to the method of Frosst *et al*. (1995).

Statistical analyses

The Chi-square Test for independence was performed to compare the frequencies of the MTHFR polymorphism in the fertile and subfertile groups;

$P < 0.05$ was considered statistically significant. The results were analysed for statistical significance with the Wilcoxon Signed-Ranks Test for comparing 2 dependent variables, and the Mann-Whitney *U*-test for comparing 2 independent variables. Nonparametric tests were used because of the skewed distributions of the determinants. The p values were two-tailed; $P < 0.05$ was considered statistically significant.

Results

Table I shows the prevalence of the C677T MTHFR polymorphism in fertile and subfertile men. The frequency of the MTHFR genotypes in fertile and subfertile men was not statistically different ($P = 0.35$).

Table I The distribution of the MTHFR C677T polymorphism in fertile and subfertile men

	MTHFR C677T genotype, % (n)			
Patients	CC	CT	TT	Total
Fertile men	44.2 (50)	42.5 (48)	13.3 (15)	(113)
Subfertile men	54.2 (42)	36.4 (28)	9.1 (7)	(77)
Total	48.4 (92)	40.0 (76)	11.6 (22)	(190)

Note: CC = wild-type carriers, CT = heterozygous carriers, and TT = homozygous carriers.
Chi-square Test, $P = 0.35$

We compared the change in sperm concentration before intervention minus after intervention delta, between fertile and subfertile men in the four different intervention groups, subdivided according to genotype. This revealed no statistically significant differences in delta sperm concentration between fertile and subfertile men in any of the four intervention treatment groups for the three different MTHFR genotypes (data not shown). This indicated that fertile and subfertile men in the three polymorphism groups did not respond differently to the intervention treatments. We thus pooled the subfertile and fertile men.

A statistically significant increase in sperm count was found only in the combined folic acid and zinc sulphate intervention group. Also, statistically significant increases in sperm concentration were found in wild-types for the C677T MTHFR polymorphism only ($P = 0.005$) (Fig. 1A). No statistically significant increase in sperm concentration was found in other intervention groups or in the heterozygous (see Fig. 1B) and homozygous (see Fig. 1C)

carriers.

To study the effects of the different intervention treatments on sperm concentration, we pooled the heterozygous (CT) and homozygous (TT) men (see Fig. 1D). We pooled these groups because of the small sample size of the TT group (only 10% of the study population, which is in line with the prevalence in the Dutch population) and the reduced MTHFR activity in both genotypes. No statistically significant difference in sperm concentration was found after intervention with any of the treatments in the latter group (see Fig. 1D).

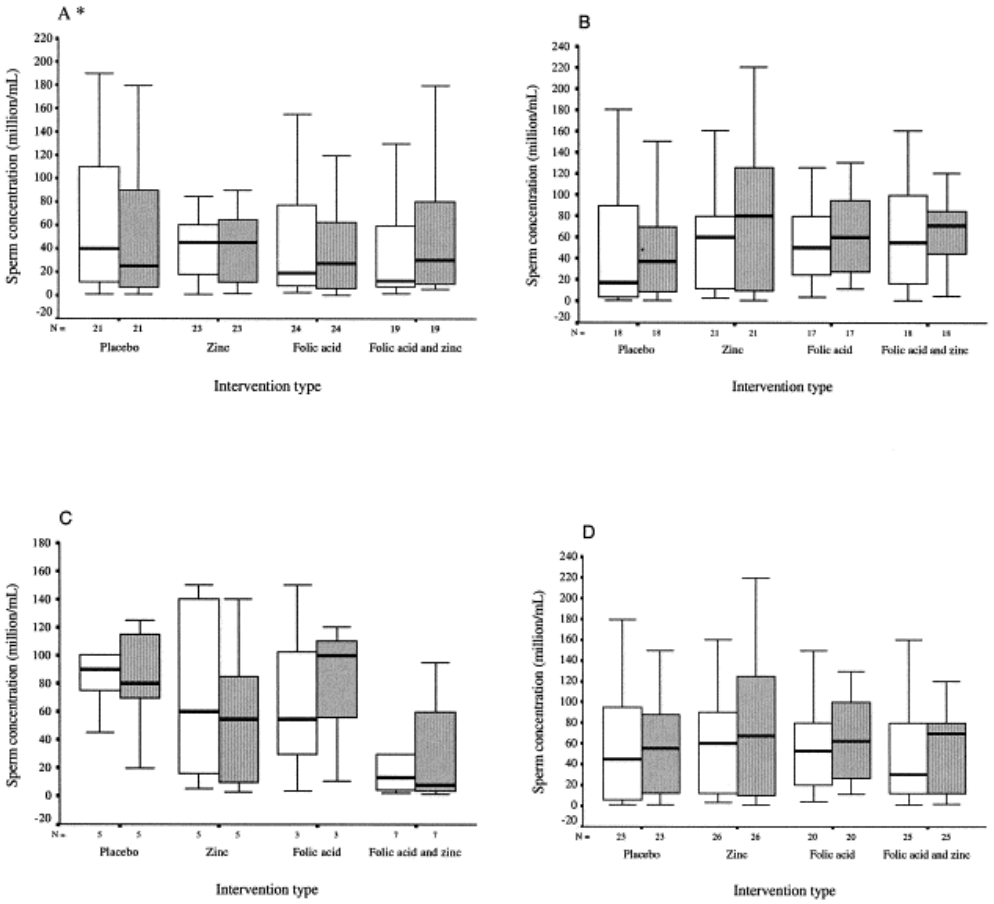
Discussion

The present study does not provide evidence that C677T MTHFR polymorphism is a risk factor for male factor subfertility. The homozygote prevalence rate in subfertile and fertile men was not statistically different: 9.1% and 13.3%, respectively. Furthermore, we found a statistically significant increase in sperm concentration after the combined folic acid and zinc sulphate intervention in wild-types for the C677T MTHFR polymorphism, but no such increase was found in heterozygous or homozygous carriers having compromised enzyme activity.

Regarding our finding that the prevalence of the three different genotypes for the C677T MTHFR polymorphism are comparable between fertile and subfertile men, we conclude that it is reasonable to believe that homozygosity for the C677T MTHFR polymorphism is not a risk factor for subfertility. However, Bezold *et al*. (2001) found an almost twofold higher prevalence rate of C677T MTHFR homozygotes in infertile men compared with fertile men (18.8% and 9.5%, respectively) and concluded that homozygosity for the C677T MTHFR polymorphism could be a risk factor for male factor infertility. However, their report did not provide information on how their study had defined male factor infertility (for comparison with our study of male factor subfertility), and did not elaborate on the inclusion criteria for the fertile and infertile groups. This makes it difficult to compare study results and explain the discrepancies. In addition to their findings concerning the significantly higher frequency of the homozygous C677T MTHFR polymorphism in infertile men, they suggested a beneficial effect of folic acid on sperm concentration.

Although our data did not reveal the MTHFR polymorphism to be a risk factor for male factor subfertility, we did demonstrate that combination treatment with

Figure 1 Sperm concentration before and after intervention



Note: (**A**) wild-type, CC genotype, (**B**) heterozygote, CT genotype, (**C**) homozygote, TT genotype, and (**D**) heterozygote and homozygote genotype. **P*=.005, comparing sperm concentration before and after folic acid and zinc sulphate intervention in wild-type individuals. White columns = Sperm concentration before intervention. Gray columns = Sperm concentration after intervention.

folic acid and zinc sulphate had a statistically significant beneficial effect on sperm concentration. However, the effect was observed solely in wild-type carriers; intervention treatment in heterozygote and homozygote carriers did not show any effect on sperm concentration. We have concluded, therefore, that the residual MTHFR activity in homozygote and heterozygote patients might be insufficient to improve spermatogenesis compared with wild-type individuals. However, other polymorphisms in folate enzymes other than

MTHFR also seem to be involved.

The study by Wong *et al*. (2002) found a 74% increase in normal sperm count specifically in subfertile men but not in fertile men after folic acid and zinc sulphate intervention treatment. In our current analysis we pooled fertile and subfertile men because wild-type, heterozygous, or homozygous men did not respond differently to the intervention treatment regardless of their fertility state. However, it remains to be investigated if the observed significant increase in sperm concentration in wild-type carriers after folic acid and zinc sulphate intervention is due to changes in sperm concentration in fertile men, subfertile men, or both. A larger scale, randomised, placebo-controlled study should be performed to further investigate sperm concentration and the influence of fertility state in wild-type carriers of the MTHFR polymorphism who receive folic acid and zinc sulphate.

There are several factors of concern in studying semen parameters. These factors comprise the biology of spermatogenesis itself, methods used to assess these parameters, and the analysis of semen parameters in different groups of men. It is well-known that semen analyses must be carefully interpreted, because data can always be biased by intra-individual biological fluctuations in semen variables, limitations and inaccuracies of the methods used, and intraobserver and interobserver variability (Tielemans *et al*. 1997; Neuwinger *et al*. 1990). This bias will in particular impact the semen parameters in fertile men, because of their broad ranges. In addition, the use of one semen sample to distinguish between fertile and subfertile men may introduce some random misclassification. However, this would equally affect either group.

Our results cannot be explained by regression to the mean, because a placebo group was included among both fertile and subfertile men. It would have been favourable to obtain more semen samples from every participant before and after intervention, but this was not feasible in our study, particularly from the fertile men.

Male factor subfertility is a multifactorial disorder. Although heterozygous and homozygous carriers of the MTHFR C677T polymorphism do not seem to be more susceptible to subfertility, our findings emphasize the importance of nutrient-gene interactions. Unlike genetic factors, nutritional factors are amendable. Although we found a beneficial effect of the intake of folic acid and

zinc sulphate supplements on sperm count in 677T MTHFR wild-types, a higher percentage of abnormal spermatozoa independent of genotype was also found after this intervention treatment (Wong *et al*. 2002). Therefore, a large scale, randomised, placebo-controlled study on the efficacy and safety of folic acid and zinc sulphate intervention should be performed to further investigate the possible interfering effect of the three different C677T MTHFR genotypes and other folate enzymes.

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Chapter 4

Does folic acid and zinc sulphate intervention affect endocrine parameters and sperm characteristics in men?

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Abstract

Background: To investigate the underlying mechanism of increased sperm concentration after folic acid and zinc sulphate intervention, we evaluated pre- and post-intervention endocrine and semen parameters in a double-blind, placebo-controlled randomised intervention study.

Method: A total of 47 fertile and 40 subfertile males participated in a 26-weeks intervention study consisting of a daily treatment with folic acid (5 mg/day) and zinc sulphate (66 mg/day), or placebo. Pre- and post-intervention semen parameters, serum folate, zinc, FSH, testosterone and inhibin B concentrations were measured.

Results: The results indicated that intervention treatment significantly increased sperm concentration in subfertile males. Other semen and endocrine parameters were not affected by intervention treatment. At baseline, positive correlations were found between serum zinc and sperm concentration, motility, and inhibin B. Serum zinc and FSH were inversely correlated. As (already) well-known from previous research, inhibin B positively correlated with sperm concentration, motility, and morphology, and was inversely correlated with FSH. The latter was positively correlated with testosterone. In addition, testosterone and inhibin B were inversely correlated. Striking was that after intervention, the correlations with zinc disappeared.

Conclusions: We conclude that the increase in sperm concentration after folic acid and zinc sulphate intervention is not due to alterations in FSH, testosterone or inhibin B concentrations. Although zinc and folate have several effects on spermatogenesis, the underlying mechanisms involved are not clear.

Introduction

Approximately 15% of all couples in the Western world are involuntarily childless, which has a great influence on the quality of life (Wallace, 1995). Male factor subfertility plays a role in about 50% of subfertile couples (World Health Organization, 1987). In up to 40% the aetiology of male subfertility remains unknown (de Kretser, 1997). Additional knowledge on the cause of male factor subfertility is crucial for better treatments of couples who are involuntarily childless.

Unlike genetic causes of male factor subfertility, environmental causes of subfertility are of particular interest, because of the possibility of curative or preventive measures. A significant amendable environmental factor is nutrition. Animal studies have demonstrated the importance of effects of nutrition on spermatogenesis (Ciereszko & Dabrowski, 1995; Kwiecinski *et al.*, 1989; van Pelt & de Rooij, 1991).

In a first double-blind, placebo-controlled randomised intervention study, in which fertile and subfertile males were included, we recently demonstrated a 74% increase in normal sperm concentration in the subfertile males after folic acid and zinc sulphate intervention (Wong *et al.*, 2002). The fertile and subfertile males were assigned to four intervention groups, comprising daily doses of folic acid (5 mg) and placebo, zinc sulphate (66mg) and placebo, a combined dose of folic acid and zinc sulphate, or placebo-placebo throughout 26 weeks. The significant increase of more than 70% in sperm concentration was only observed in subfertile males receiving the combined treatment with folic acid and zinc sulphate. In an extended study, we showed that MTHFR 677CC carriers benefit significantly more from this intervention treatment with regard to the sperm concentration than MTHFR CT/TT carriers (Ebisch *et al.*, 2003).

Natural folate is important for the synthesis of DNA, transfer RNA-, and proteins, and is therefore suggested to be important in spermatogenesis. Zinc serves as a cofactor for more than eighty metalloenzymes involved in DNA transcription, expression of steroid receptors, and protein synthesis. Furthermore, it has been shown that zinc is important in testicular development, spermatogenesis and sperm motility, as reviewed by Wong *et al.* (2000).

The underlying mechanisms involved in the beneficial effects of synthetic folic

acid and zinc sulphate on spermatogenesis are not clarified. Therefore, we hypothesized that folic acid and zinc sulphate may affect endocrine parameters, for example by stimulating the function of the Sertoli cells. These cells provide the essential microenvironment for normal germ cell production and could therefore be relevant.

Sertoli cells are the main producers of inhibin B in the human body. The serum inhibin B concentration positively correlates with sperm concentration, testicular volume and the state of the spermatogenetic epithelium (Pierik *et al.*, 1998; von Eckardstein *et al.*, 1999). Moreover, inhibin B appears to be a marker of advanced stages of spermatogenesis (Anderson & Sharpe, 2000; Andersson, 2000). Thus, the inhibin B concentration reflects the quality of the Sertoli cell function and spermatogenesis and as such can be used as a sensitive marker of spermatogenesis in humans.

From this background, the aims of the present study were to assess the effects of folic acid and zinc sulphate intervention on semen parameters and the hormonal levels of inhibin B, FSH, and testosterone, and to determine associations between baseline and post-intervention folic acid and zinc concentrations, and the endocrine and semen parameters.

Materials and Methods

Patients

This study is part of our previously described randomised trial on the effects of folic acid and/or zinc sulphate administration on semen parameters of fertile and subfertile males (Wong *et al.*, 2002). Every participant received 2 capsules per day, took the capsules after dinner and for 26 weeks. The randomisation schedule was: folic acid-placebo, folic acid-zinc sulphate, zinc sulphate-placebo, or placebo-placebo. The content of the folic acid capsule was 5 mg per capsule per day, and for zinc sulphate 66 mg per capsule per day. In the current study we evaluated the serum concentrations of folate, zinc, FSH, testosterone, and inhibin B, in the available serum specimens, and the semen parameters in the fertile and subfertile males, before and after intervention with folic acid and zinc sulphate or placebo.

For the inhibin B determinations a total of 47 serum samples were available from the original group of 49 fertile males, of which 23 males had received

placebo-placebo, and 24 had the combination treatment. From the subfertile males 40 serum samples were available from the original group of 49 subjects, of which 22 males had received the placebo-placebo, and 18 the combination treatment. The data of the other hormones and folate and zinc concentrations were available from the original data set.

The fertile group comprised healthy males without a history of fertility problems whose partners were pregnant at the start of the recruitment and who conceived spontaneously within 1 year of regular, unprotected intercourse. Subfertility was defined as failure of the female partner to conceive after 1 year of regular, unprotected intercourse and a sperm concentration between 5 and 20 x 10^6 spermatozoa/ml at the first routine semen analysis after referral to the fertility clinic. We excluded subfertile males with a sperm concentration of less than 5 million spermatozoa/ml semen, thereby excluding males with a severe, possibly genetically caused, deranged spermatogenesis, who are not very likely to benefit form intervention treatment. The cut-off value of 20 million spermatozoa/mL semen was deducted from the WHO guidelines for fertility. Also, no further investigations were conducted regarding the fertility of the female partners, since it was not our aim to study the outcome measure 'pregnancy'. Recruitment of these males was performed as described previously by Wong *et al.* (2002).

The study was approved by the Institutional Review Board of the Radboud University Nijmegen Medical Centre, and all participants gave written informed consent before participation.

Semen samples

Before and after intervention one semen sample was obtained from every participant for standardised semen analysis according to WHO guidelines (World Health Organization, 1992). The semen samples were produced by the participants via masturbation after an abstinence period of at least 3 days. These samples were delivered at the fertility laboratory within 1 hour after production. After liquefaction, the semen parameters volume, sperm concentration, motility, and morphology were determined.

Sperm concentration was determined with a Makler counting chamber. Motility was expressed as the percentage of motile spermatozoa, and morphology was determined according to WHO criteria (1992), after incubation of the sample

with trypsin (10 minutes at room temperature), staining with methylene blue/eosin, feathering, and fixation by flame.

Folate and zinc measurements

Serum folate concentrations were measured using radioassays (Dualcount Solid Phase Boil radioassay; Diagnostic Products Corp., Los Angeles, CA, USA). Serum zinc was measured using flame atomic absorption spectrophotometry (Perkin Elmer 4100; Norwalk, CT, USA).

Hormone determinations

FSH was quantitatively determined in serum (AxSYM; Abbott Laboratories, Abbott Park, IL, USA). Serum testosterone was routinely measured by specific dextran-coated radioimmunoassay after extraction of serum specimens with diethylether and subsequent isolation of the T fraction by Sephadex LH-20 (Pharmacia) column chromatography, using an antiserum raised in rabbits and directed against T-3-(o-carboxymethyl)oxime-BSA (Dony *et al.*, 1985). Inhibin B was determined using kits purchased from Serotec Limited, Oxford, UK. The intra-assay coefficient of variance (CV) was less than 9%, and the inter-assay CV was less than 15%. The lowest detectable inhibin B concentration was 5 ng/l, based on the mean value of the zero-dose standard plus twice the standard deviation. A value of 2.5 ng/l (i.e. half of the value of the undetectable range) was assigned to test results below 5 ng/l.

Statistical analyses

Results are expressed as medians (25^{th}-75^{th} percentile) and were analysed for statistical significance using non-parametric tests, because of the skewed distribution of the semen parameters and hormone concentrations.

Results are expressed as medians (25^{th}-75^{th} percentile) and were analysed for

The Mann-Whitney *U*-test was used to compare age, folate, zinc, and hormone concentrations, and the deltas (post-intervention value minus pre-intervention value) between the fertile and subfertile males. To compare endocrine and semen parameters in fertile and subfertile males before and after intervention, the Wilcoxon Signed-Ranks Test was used. Spearman's test was used to calculate correlation coefficients between the biochemical and semen parameters.

Furthermore linear regression models were used to analyse the influence of folic acid and zinc sulphate on inhibin B, FSH, testosterone and sperm concentration. Statistical analyses were performed using SPSS 11.0 for Windows software (SPSS Inc, Chicago, IL, USA).

Results

In Table I the baseline characteristics of the endocrine and semen parameters are depicted in the fertile and subfertile groups. The semen parameters were by indication significantly different between the groups. Although serum folate was not significantly different, serum zinc concentrations were significantly higher in fertile males compared with subfertile males ($P < 0.05$). The median FSH concentration was higher in subfertile males compared with fertile males ($P < 0.05$), while the median inhibin B concentration was lower in subfertile males than in fertile males ($P < 0.05$).

Table I Baseline characteristics of subfertile and fertile males

	Pre-intervention			
	Subfertile males ($n = 40$)		Fertile males ($n = 47$)	
Age (y)	35.0	(32.3-37.0)	34.0	(31.0-38.0)
Folate (nmol/l)	18.0	(15.0-22.5)	18.0	(15.0-21.0)
Zinc (µmol/l)	18.3	(17.3-20.4)	21.1	(18.9-23.4)[c]
FSH (IU/l)	5.3	(4.0-10.6)	3.9	(2.7-6.7)[c]
Testosterone (nmol/l)	19.6	(14.0-24.4)	19.5	(16.5-23.0)
Inhibin B (ng/l)	197.0	(154.5-273.0)	255.0	(182.0-338.0)[c]
Sperm concentration (10^6/ml)[a]	7.0	(3.2-16.8)	75.0	(40.0-120.0)[b]
Motility (%)[a]	20.0	(15.0-40.0)	50.0	(40.0-60.0)[b]
Abnormal morphology (%)[a]	79.0	(70.0-83.0)	63.0	(50.0-70.0)[b]

Note: data are given in median (25^{th}-75^{th} percentile). Folate and zinc and the endocrine parameters are all measured in serum. [a] Determination according to WHO (1992). Normal reference values are sperm concentration of $20x10^6$/ml, motility > 50%, and morphology < 70% abnormal, [b] $P < 0.001$, [c] $P < 0.05$

The effects of intervention with folic acid and zinc sulphate treatment versus placebo treatment on the serum levels of folate and zinc, the hormones of the hypothalamic-pituitary-testis (HPT) axis and on the sperm concentrations in both fertile and subfertile males are presented in Table II. After intervention with folic acid and zinc sulphate treatment the serum folate concentrations increased in both groups ($P < 0.001$), but the serum

zinc concentrations did not significantly increase in fertile and subfertile males (P = 0.06 and P = 0.72, respectively). The sperm concentration increased significantly in subfertile males, but not in fertile males, P = 0.007 and P = 0.45, respectively. After adjusting for the placebo-effect by comparing the delta sperm concentration for subfertile males receiving placebo with the delta sperm concentration for subfertile males receiving folic acid and zinc sulphate intervention, sperm concentration in subfertile males having the combination treatment was significantly higher (P = 0.006).

In order to investigate whether this increase in sperm concentration was due to a lower production of prostate or seminal vesicle fluids, we examined the change in sperm count (ejaculate volume x spermatozoa concentration). The sperm count appeared significantly higher in subfertile males after intervention with folic acid and zinc sulphate compared with the baseline sperm count. The median baseline sperm count in the subfertile males was 28.8 (8.5-46.2) x 10^6 spermatozoa, and after folic acid and zinc sulphate intervention it was 44.4 (13.6-151.8) x 10^6 spermatozoa (P = 0.009). From these data we can conclude that the observed increase in sperm concentration after the combined intervention is due to an effect on spermatogenesis, because intervention resulted in a higher production of spermatozoa.

It is not likely that folate and zinc affected the endocrine parameters, because FSH, testosterone, and inhibin B concentrations were not significantly changed by the intervention treatment in the fertile and subfertile groups.

In Table III the correlation coefficients pre-intervention (italics) and post-intervention (bold) in the pooled group of fertile and subfertile males are shown for serum concentrations of folate, zinc, endocrine parameters, and semen parameters. Before intervention, significant correlations were observed between serum zinc and sperm concentration, motility, FSH, and inhibin B, which disappeared after intervention. In addition, at baseline inhibin B was significantly correlated with sperm concentration, motility, morphology and the endocrine parameters FSH and testosterone.

The FSH concentration was inversely correlated with testosterone. The three semen parameters themselves were also positively correlated with each other. The post-intervention correlations were comparable to the pre-intervention correlations although the correlation coefficients appeared to be slightly lower.

However, the correlations between zinc and the semen and endocrine

Table II Effects of folic acid and zinc sulphate treatment on serum levels of folate and zinc, endocrine parameters and sperm concentration in males

Intervention	Placebo (Subfertile *n* = 22, Fertile *n* = 23)			Zinc and Folic acid (Subfertile *n* = 18, Fertile *n* = 24)		
	Pre-intervention	Post-intervention	Delta	Pre-intervention	Post-intervention	Delta
Subfertile (*n* = 40)						
Folate (nmol/l)	18.0 (15.0-23.0)	17.0 (12.0-24.0)	-1.0 (-3.2 - 1.0)	18.0 (13.0-22.8)	105.0 (86.5-122.5) [a]	86.0 (69.0 - 100.0) [b]
Zinc (µmol/l)	18.8 (18.0-20.0)	20.3 (16.9-22.4)	-0.2 (-2.4 - 4.5)	18.2 (16.2-20.5)	19.9 (18.9-22.5)	2.1 (-1.8 - 4.9)
FSH (IU/l)	5.6 (4.0-9.7)	6.3 (4.9-9.0)	-0.4 (-1.2 - 0.8)	5.2 (4.0-11.3)	5.6 (4.0-9.6)	0.2 (-0.6 - 0.9)
T (nmol/l)	18.3 (14.0-23.1)	19.0 (14.0-24.0)	1.0 (-4.7 - 3.3)	21.0 (13.0-27.8)	18.0 (13.0-24.0)	2.3 (-4.3 - 8.2)
Inhibin B (ng/l)	183.0 (159.8-300.3)	200.0 (157.8-288.0)	-7.5 (-29.8 - 28.0)	204.5 (137.5-256.5)	191.0 (124.8-283.0)	6.5 (-18.0 - 19.0)
Sperm conc (10^6/ml)	7.5 (1.5-25.0)	7.0 (3.8-18.3)	-2.8 (-7.3 - 5.3)	7.0 (4.0-14.5)	8.0 (5.0-30.0) [a]	5.0 (1.0 - 17.0) [b]
Fertile (*n* = 47)						
Folate (nmol/l)	8.0 (15.0-21.8)	16.0 (13.0-23.0)	-1.0 (-4.0 - 1.0)	18.0 (15.0-21.0)	110.0 (77.0-13.0) [a]	9.0 (57.0 - 99.0) [b]
Zinc (µmol/l)	21.3 (18.9-24.1)	19.6 (18.4-21.9)	-1.9 (-5.6 - 3.2)	20.7 (18.2-23.0)	20.5 (18.1-23.1)	0.5 (-3.0 - 4.8)
FSH (IU/l)	3.7 (2.4-6.5)	3.7 (2.7-6.7)	-0.3 (-1.0 - 0.3)	4.3 (2.7-6.8)	4.5 (3.1-5.9)	-0.3 (-1.1 - 1.0)
T (nmol/l)	20.5 (17.8-24.7)	21.0 (17.0-25.0)	-1.0 (-4.1 - 5.0)	18.0 (13.2-21.3)	18.0 (13.3-24.0)	0.2 (-2.9 - 2.3)
Inhibin B (ng/l)	253.0 (163.0-287.0)	246.0 (187.0-271.0)	18.0 (-17.0 - 63.0)	261.5 (182.8-379.3)	247.0 (174.3-355.8)	7.0 (-13.0 - 26.0)
Sperm conc (10^6/ml)	85.0 (45.0-130.0)	80.0 (40.0-110.0)	7.0 (-30.0 - 25.0)	65.5 (34.0-111.3)	77.5 (55.0-95.0)	9.0 (-22.3 - 43.0)

Note: data are medians (25^{th}-75^{th} percentile), deltas are calculated as post-intervention value minus pre-intervention value. [a] $P < 0.01$ for comparison between pre- and post-intervention values (Wilcoxon Signed-Ranks test), [b] $P < 0.01$ for comparison between “delta placebo” and “delta zinc and folic acid” (Mann-Whitney *U*-test).

Table III Spearman correlation coefficients between serum folate (Fol-ser) and zinc concentrations (Zn-ser), semen and serum endocrine parameters, of the pooled data of 47 fertile and 40 subfertile males

	Fol-ser	Zn-ser	Sperm conc	Motility	Morph	FSH	T	Inh B
Fol-ser		*-0.11*	*-0.06*	*-0.03*	*-0.02*	*0.02*	*-0.13*	*0.07*
Zn-ser	**0.16**		*0.36* [a]	*0.22* [c]	*-0.002*	*-0.27* [b]	*-0.18*	*0.29* [b]
Sperm conc	**0.008**	**0.004**		*0.52* [a]	*0.52* [a]	*-0.50* [a]	*-0.09*	*0.49* [a]
Motility	**-0.02**	**0.08**	**0.62**[a]		*0.48* [a]	*-0.34* [b]	*-0.16*	*0.29* [b]
Morph	**-0.02**	**-0.05**	**0.64**[a]	**0.48**[a]		*-0.26* [c]	*-0.02*	*0.25* [c]
FSH	**-0.03**	**< 0.001**	**-0.44**[a]	**-0.29**[b]	**-0.22**[c]		*0.23* [c]	*-0.65* [a]
T	**-0.11**	**0.11**	**0.01**	**0.08**	**0.07**	**0.11**		*-0.26* [c]
Inh B	**0.08**	**-0.001**	**0.30**[a]	**0.23**[c]	**0.13**	**-0.66**[a]	**-0.05**	

Note: the r-values *in italics* indicate correlations observed before intervention and the r-values **in bold** indicate correlations observed after intervention. [a] $p \leq 0.001$, [b] $p \leq 0.01$, [c] $p \leq 0.05$

parameters, between inhibin B and morphology, between FSH and testosterone, and between testosterone and inhibin B disappeared.

Because at baseline the semen parameters correlated with most endocrine parameters, we analysed the data by linear regression analysis to adjust for these variables and to find valid correlations. To find the independent prognostic variables predicting the inhibin B concentration we used the linear regression model including the dependent variables: serum zinc concentration, sperm concentration, motility and morphology, the FSH and the testosterone concentration. We found that the FSH concentration is the only independent prognostic factor for the inhibin B concentration in serum (standardized r = -0.60; $P < 0.001$). We used the same linear regression model mentioned above,but in each case used a different independent variable and found that the FSH concentration itself was also predicted by the inhibin B concentration (standardized regression coefficient -0.55; $P < 0.001$). Moreover, the sperm concentration was predicted by sperm motility (standardized r = 0.22; $P = 0.03$), sperm morphology (standardized r = -0.32; $P = 0.002$) and inhibin B concentration (standardized r = 0.24; $P = 0.03$). We evaluated the possible confounders age and duration of sexual abstinence in regard to the prediction of sperm concentration, but found that they in fact are no confounders in our

study population. Therefore they do not need to be included in the regression analysis. Folic acid and zinc sulphate concentrations did not influence inhibin B, FSH, testosterone and sperm concentrations.
After intervention, the results from the linear regression models remained the same for the predicting variables of inhibin B and FSH concentrations, but the sperm concentration was significantly predicted by sperm motility (standardized r = 0.31; $P = 0.001$), sperm morphology (standardized r = -0.42; $P < 0.001$), and FSH concentration (standardized r = -0.30; $P = 0.004$).

Discussion

In this study we confirm our previous data on the beneficial effect of folic acid and zinc sulphate intervention on sperm concentration in subfertile males in a subset of both study groups. We could not demonstrate an effect of this intervention of FSH, testosterone and inhibin B concentrations, reflecting the endocrine status of subfertile and fertile males. By interpreting these data we have to take into account that only an 18% increase in sperm concentration of subfertile males after intervention was now observed.
Although, this is a true effect, because significance remained after adjusting for the placebo-effect, compared with the 74% increase described in the original paper by Wong *et al.* (2002), this increase is much smaller. This may certainly have affected the negative findings on the endocrine parameters. Particularly the data on inhibin B should be interpreted with caution, because this hormone could only be determined in samples of 47 fertile and 40 subfertile males from the original study.
The baseline serum inhibin B concentrations were significantly lower in subfertile males compared with fertile males. Only one study also made a direct comparison of inhibin B concentrations between fertile and subfertile males (Lee *et al.*, 2001), and reported that the mean inhibin B concentration in subfertile and fertile males were 163 and 146 ng/l, respectively, and were not significantly different. The relatively high values we found may be due to the timing of blood collection. The serum inhibin B concentration shows a diurnal rhythm in which the levels in the early morning are 30% higher than in the late afternoon (Andersson, 2000).
Another explanation for the different inhibin B levels is the different selection of subfertile males. In contrast to the group of Lee *et al.* (2001), we excluded

subfertile males with a known cause for infertility and with a sperm concentration of less than 5 million spermatozoa/ml semen, thereby excluding males with a severe, possibly genetically caused, deranged spermatogenesis and concomitant low inhibin B levels. Analytical differences of the immunoassay procedures might explain the differences in inhibin B concentration as well. Finally, the difference in inhibin B levels could be explained by the wide range of sperm concentration with a 10-fold increase between the fertile and subfertile groups.

Furthermore, it remains to be established which specific type of germ cell determines inhibin B secretion. It has already been suggested that pachytene spermatocytes or early spermatids are the most important determinants of circulating inhibin B (Andersson, 2000; Foresta *et al.*, 1999; Pierik *et al.*, 2003). Because no significant change in circulating inhibin B concentration was found after intervention, it is possible that the effects of the intervention on sperm concentration are exerted in later stages of spermatogenesis (e.g., late spermatids, spermiogenesis), thereby not influencing circulating inhibin B concentrations.

We demonstrated a significantly positive effect on sperm concentration in subfertile males after 26 weeks of folic acid and zinc sulphate treatment. It is well-known that natural folate plays an important role in the synthesis of purines and pyrimidines for tRNA and DNA, which are both important in spermatogenesis. The small study Bentivoglio *et al.* (1993) showed that oral folinic acid supplementation improved sperm concentration and motility in infertile males presenting with a high sperm concentration of immature germ cells, e.g. spermatids and spermatocytes. In our study we could not demonstrate any correlations between serum folate concentrations and semen parameters.

Of interest is the significantly positive correlation between serum zinc concentration and sperm concentration. This is supported by findings of others. The zinc content in the adult testis is high, and the prostate has an even higher concentration of zinc than any other organ in the human body (Mann, 1964; Zaichick *et al.*, 1997). Moreover, hypogonadism is a clinical feature of zinc deficiency in humans (Prasad, 1985a; Prasad, 1985b; Sandstead *et al.*, 1967) and sperm defects have been observed in zinc-deficient rats (Hamdi *et al.*, 1997). It has also been shown that zinc improved spermatogenesis in animals

(Hamdi *et al*., 1997), and increased sperm concentration (Hartoma *et al*., 1977; Stankovic & Mikac-Devic, 1976), motility (Kynaston *et al*., 1988), and morphology in subfertile males (Tikkiwal *et al*., 1987).

In this study we observed a significantly higher serum zinc concentration in fertile males compared with subfertile males, however, both fertile and subfertile males had serum zinc concentrations within the normal ranges. In our previous study by Wong *et al.* (2002) no difference was observed regarding serum zinc concentrations between these 2 groups. This could be explained by the smaller sample size in the present study.

Another striking result is the lack of rise in serum zinc concentrations after intervention with folic acid and zinc sulphate, also observed in our previous study. Possible explanations for this phenomenon are the absence of zinc deficiencies, the relatively low dose of zinc used to avoid gastrointestinal side-effects, or lack of compliance. The latter explanation is not very likely considering the marked increase of folate concentration after the combined intervention with folic acid and zinc sulphate.

Conclusion

Despite the absence of an effect of folic acid and zinc sulphate on endocrine parameters, we conclude that the correlations between zinc and sperm concentration, and the increase in sperm concentration after intervention in subfertile males, should stimulate research onto nutrition and environmental factors in the pathogenesis and prevention of fertility disorders.

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Chapter 5

Seminal plasma annexin A5 concentration is not associated with male subfertility and cannot be influenced by folic acid and zinc sulphate treatment

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Abstract

Background Annexin A5 (anxA5) is abundantly present in seminal plasma, however, its endogenous function in seminal plasma is not known. Recently, we demonstrated that folic acid and zinc sulphate intervention increased sperm count. To explore the involvement of anxA5 in human (sub)fertility, we measured anxA5 concentrations in seminal plasma before and after folic acid and zinc sulphate intervention in fertile and subfertile males.

Methods In a double-blind, placebo-controlled randomised intervention study with folic acid (5 mg/day) and/or zinc sulphate (66 mg/day) in 86 fertile and 78 subfertile males, pre- and post-intervention seminal plasma anxA5 concentrations were determined by sandwich ELISA.

Results Seminal plasma anxA5 concentrations at baseline were not significantly different between fertile and subfertile males, (median) 5.2 mg/l (25^{th} –75^{th} percentile: 4.2-7.2), and 5.6 mg/l (4.3-6.7), respectively. The various treatments did not affect seminal plasma anxA5 concentrations.

Conclusions Seminal plasma anxA5 concentration is not associated with male factor subfertility and the observed increase in sperm count after folic acid and zinc sulphate treatment cannot be explained by a change in the seminal plasma anxA5 concentration. Further studies are needed to elucidate the mechanisms responsible for the beneficial effect of this intervention treatment on sperm count.

Introduction

Subfertility is a common disorder with a prevalence of about 15% in all couples in the Western world (Mosher and Pratt, 1990; Templeton *et al*., 1990; Wallace, 1995). In about half the number of cases a male factor is identified, defined as male factor subfertility. In most cases, subfertility is regarded as idiopathic, however, gene-environment interactions are suggested to be involved (Wong *et al*., 2003). The environmental causes are particularly of interest, because they are better amendable to curative and/or preventive measures than genetic factors. A significant but largely neglected environmental factor is nutrition. It is well-known that nutrition plays an important role in reproduction (Steegers-Theunissen, 1995). The vitamin folate is known to contribute to the prevention of neural tube defects when taken periconceptionally (Steegers-Theunissen, 1995; Wald *et al*., 1991). Folate plays an important role in the synthesis of transport ribonucleic acid (tRNA) and deoxyribonucleic acid (DNA) and methylation of proteins.

Zinc is an essential nutritional compound, serving as a cofactor for more than 80 metalloenzymes, and also as a cofactor in the synthesis of macromolecules such as DNA and tRNA. It has been shown that zinc is essential in testicular development (Hamdi *et al*., 1971). Also, seminal plasma zinc concentrations influence the oxygen consumption of spermatozoa (Eliasson *et al*., 1971; Huacuja *et al*., 1973), nuclear chromatin condensation (Kvist, 1980), acrosome reaction (Riffo *et al*., 1992), and acrosin activity (Stevens *et al*., 1982). Furthermore, the synthesis of testosterone in the Leydig cells and the conversion of testosterone to 5α-dihydrotestosterone by the 5α-reductase enzyme is dependent on zinc supply (Netter *et al*., 1981).

Recently, we conducted an intervention study supplying both folic acid and zinc sulphate to fertile and subfertile men, and found that after 26 weeks of intervention treatment, subfertile men had a 74% increase in normal sperm count (Wong *et al*., 2002). Despite the knowledge that zinc and folate are essential for the synthesis of genetic material, the precise underlying mechanism by which these micronutrients affect spermatogenesis is not clear.

Annexin A5 is a member of the protein family of annexins which contains more than ten members. These proteins (especially anxA5) are characterized by their high affinity for negatively charged phospholipids present in cell membranes (Andree *et al*., 1990). Annexin A5 is primarily known because of its

world-wide use to detect apoptosis *in vitro* and also experimentally in vivo (Hofstra *et al*., 2000; Martin *et al*., 1995). Due to the affinity to negatively charged phospholipids, anxA5 is a potent inhibitor of blood coagulation and inflammation (van Heerde *et al*., 1995). It is also known that anxA5 is abundantly present in seminal plasma (Christmas *et al*., 1991). Because our research group has not yet identified the underlying mechanism of the beneficial effect of folic acid and zinc sulphate on spermatogenesis, and because of the observation that anxA5 is abundantly present in seminal plasma, we explored the possibility that endogenous anxA5 is directly involved in male factor subfertility. Furthermore, we evaluated the effect of intervention treatment with folic acid and zinc sulphate on anxA5 concentrations in seminal plasma.

Materials and methods

From the double-blind, placebo-controlled randomised intervention study designed to study the effect of folic acid and zinc sulphate on semen parameters (Wong *et al*., 2002; Ebisch *et al*., 2003), samples before and after intervention from 86 fertile and 78 subfertile males were available for the determination of endogenous anxA5 concentrations.

In the referred study, fertile males were recruited from 9 midwifery practices in the surrounding areas of Nijmegen, The Netherlands. These healthy men, without a history of fertility problems at the moment of enrolment, had a pregnant partner who conceived spontaneously within 1 year of regular, unprotected intercourse.

Subfertile males were recruited from the fertility clinics of the Radboud University Nijmegen Medical Centre and the Canisius Wilhelmina Hospital in Nijmegen. Subfertility was defined as failure of the female partner to conceive after 1 year of regular, unprotected intercourse and a sperm concentration between 5 and 20 million spermatozoa per mL on the first routine semen analysis after referral to the fertility clinic. The females of these subfertile males were not further evaluated, because the main focus was on the effect of folic acid and zinc sulphate treatment on semen parameters in subfertile males. The fertile and subfertile males were included after having given their written informed consent and assigned to the four intervention groups by computer-generated random numbers. The interventions consisted of a daily dose of folic

acid (5 mg) and placebo, zinc sulphate (66 mg) and placebo, or a combined dose of folic acid and zinc sulphate, or placebo-placebo throughout 26 weeks.
Before and after intervention one standardized semen sample was obtained from every participant for semen analysis according to World Health Organization (WHO) guidelines (WHO, 1992). The semen samples were produced by the participants via masturbation after an abstinence period of at least 3-5 days. These samples were delivered within 1 hour after production to the fertility laboratory. In this hour the participants were advised to keep the sample at room temperature. After liquefaction, an aliquot of semen was centrifuged at 1,400 x g (Hettich 16A, 1323 rotor) for 10 minutes. The supernatant seminal plasma was frozen without preservatives and stored at –80 °C until assayed. Sperm concentration was determined with a Makler counting chamber.
The Medical Ethical Committee and the Institutional Review Board of the Radboud University Nijmegen Medical Centre approved of this trial.

Annexin A5

The anxA5 concentration was investigated by sandwich enzyme-linked immunosorbent assay (ELISA) (ZYMUTEST anxA5, Hyphen BioMed, Andrésy, France) as described by van Heerde *et al.* (2003). The antibodies used in this test are affinity purified rabbit polyclonal antibodies specific for human anxA5 ($F(ab')_2$ fragments) and an horse radish peroxidase coupled affinity purified rabbit polyclonal antibody against anxA5. The substrate used is ortho-phenylene diamine (OPD) in presence of hydrogen peroxide. After colour development adsorption is measured in a micro ELISA plate reader at 492 nm (Easy reader, SLT Labinstruments Austria).

Quantitative real time annexin A5 RT-PCR

The seminal plasma anxA5 concentration may originate from different sites of synthesis, e.g. from testis, prostate or seminal vesicles. To determine whether the prostate or the testis is the main producer of anxA5, anxA5 mRNA was measured and the anxA5 antigen was stained in human prostate and testis sections. Complementary DNA was synthesized by using 1 µg RNA of prostate tissue and testis (Clontech, Palo Alto). Testis messenger RNA (mRNA) was isolated out of whole normal testes pooled from 45 Caucasians (age 14-64)

who deceased suddenly. Prostate mRNA was isolated from 47 Caucasians (age 14-57) who also deceased suddenly. The mRNA was mixed with 625 µM dNTPs, 5 mg/l random hexamer primer DTT, RNAsin (20 U) and M-MLV RT (200 U) in a total volume of 15 µl to obtain cDNA. The mixture was incubated for 10 minutes at 20°C, followed by 45 minutes at 42°C and 10 minutes at 95°C. Primer-probe combinations for the anxA5 cDNA were designed using PRIMER-EXPRESS software. De forward primer CCACAGTCTGGTCCTGCT-TC, the reverse primer AGTCACAGTGCCTCTGAGAACCT, and the minor groove binding probe CTGACCTGAGTAGTCGC were mixed with 50 ng cDNA, 1.25 U AmpliTaq Gold DNA polymerase with 250 µmol/l dNTPs. 1 X Taqman buffer A in a total volume of 50 µl. Samples were heated at 95°C for 10 minutes and amplified for 45 cycles of 15 sec at 95°C and 60 sec at 60°C (ABI/Prism 7700 Sequence detector, Applied Biosystems). The expression of porphobilinogendeaminase (PBGD), a low copy number housekeeping gene, was measured in duplicate onto each sample to normalize for PCR and cDNA input variations (Mensink *et al*., 1998). The anxA5 mRNA concentrations were measured in duplicate and analysed with Taqman software. The results were expressed as delta cycle threshold (δCt) in which δCt = Ct (PBGD) minus Ct (anxA5). The relative difference in expression is calculated by $2^{-\delta\delta Ct}$.

Immunohistochemistry

Post mortem human paraffin-embedded prostate and testis tissue sections were stained with a polyclonal antibody directed against human anxA5. The sections were macroscopically and microscopically checked by the pathologist as being normal prostate and testis tissues. The sections were routinely processed to remove the paraffin and to rehydrate the tissues. Next, the sections were blocked with human serum albumin (0.1%) containing 50mM tris-buffered salt buffer, pH 7.4 (TBS/HSA) to which 20% normal swine serum was added. After 30 minutes the sections were washed in TBS and incubated for another 2 hours with a polyclonal antibody against human anxA5 (1000 X diluted in TBS/HSA)(Hyphen Biomed, Andrésy, France). The sections were washed again and incubated for 90 minutes with a biotin-conjugated swine anti-rabbit polyclonal antibody (1000-fold diluted in TBS/HSA)(Dako, Glostrup, Denmark). Finally, after washing the sections were incubated with alkaline phosphatase-conjugated streptavidin-biotin complex (DakoCytomation,

Glostrup, Denmark) for 1 hour. After extensive washing the sections were stained by using the alkaline-phosphatase substrate kit containing 2mM levamisole (Sigma, St Louis, MI) to block the endogenous alkaline phosphatase activity, according to the manufacturers procedure (Vector, Burlingame, CA, USA). The presence of anxA5 is notified by a red colour. The nucleus was counterstained blue with Mayer's Haematoxylin (Merck, Darmstadt, Germany).

Statistical analyses

The results were analysed for statistical significance using non-parametric tests, because of the skewed distributions of the determinants. Concentrations of endogenous anxA5 are given as median and 25^{th} –75^{th} percentile. Baseline seminal plasma anxA5 concentrations were compared between fertile and subfertile males using the Mann-Whitney *U*-test. The effect of the four interventions in fertile and subfertile males was investigated by comparison between the baseline and post-intervention seminal plasma anxA5 concentration by the Wilcoxon Signed-Ranks test. We corrected for a possible placebo-effect by comparing the delta anxA5 concentration for males receiving placebo with the delta anxA5 concentration for males receiving the folic acid, zinc sulphate, or combined intervention treatment. The p values were two-tailed and $p \leq 0.05$ was considered statistically significant. Statistical analyses were performed using SPSS 10.0 for Windows software (SPSS Inc, Chicago, IL, USA).

Results

Annexin A5 concentration in seminal plasma

Although anxA5 could be determined in seminal plasma, it did not show an association with male fertility. The median (25^{th}-75^{th} percentile) baseline seminal plasma anxA5 concentrations was 5.2 mg/l (4.2-7.2) in fertile (Table I) and 5.6 mg/l (4.3-6.7) in subfertile males ($P = 0.96$, Table II).

Effect of intervention on seminal plasma annexin A5 concentration

Tables I and II show the effects of all interventions on seminal plasma anxA5 concentration in both fertile and subfertile males, respectively. We observed no

Table I The effect of interventions on seminal plasma anxA5 concentrations (mg/l) in fertile males

	N	Pre-intervention anxA5 (mg/l)	Post-intervention anxA5 (mg/l)	Delta AnxA5 (mg/l)
All subjects (baseline)	86	5.2 (4.2-7.2)	--	--
Intervention:				
Placebo	21	5.2 (4.0-5.9)	5.2 (4.2-6.9)	-0.5 (-1.1-1.6)
Folic acid	20	5.6 (4.3-7.6)	5.4 (3.7-7.2)	-0.3 (-2.1-1.6)
Zinc sulphate	22	5.4 (4.3-8.1)	5.5 (3.2-6.9)	-0.7 (-2.1-0.8)
Folic acid and zinc sulphate	23	4.9 (3.6-7.1)	3.4 (1.8-6.6)	-1.3 (-2.3-1.0)

Note: data are the median (25^{th}-75^{th} percentile). Deltas are calculated as post-intervention – pre-intervention value. AnxA5 = annexin A5

Table II The effect of interventions on seminal plasma anxA5 concentrations (mg/l) in subfertile males

	N	Pre-intervention anxA5 (mg/l)	Post-intervention anxA5 (mg/l)	Delta AnxA5 (mg/l)
All subjects (baseline)	78	5.6 (4.3-6.7)	--	--
Intervention				
Placebo	23	5.7 (4.3-7.6)	5.5 (2.6-8.9)	-0.5 (-2.6-2.9)
Folic acid	16	5.4 (3.8-6.6)	5.2 (3.0-6.4)	-0.01 (-1.7-1.2)
Zinc sulphate	18	5.2 (4.4-6.7)	4.9 (3.5-6.9)	-1.1 (-2.0-1.6)
Folic acid and zinc sulphate	21	5.6 (2.5-6.1)	3.7 (2.1-5.7)	-0.4 (-1.6-0.9)

Note: data are the median (25^{th}-75^{th} percentile). Deltas are calculated as post-intervention – pre-intervention value. AnxA5 = annexin A5

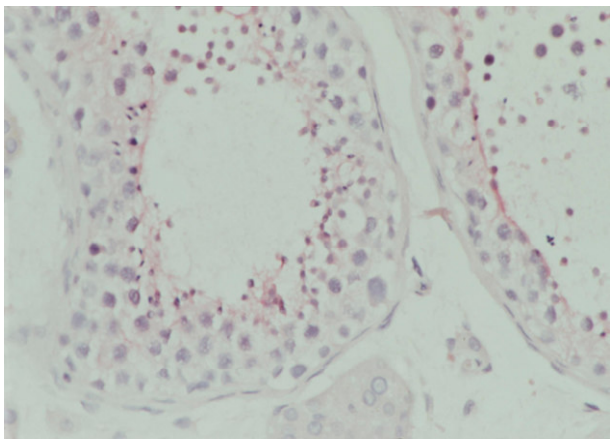
effect of the combination treatment with folic acid and zinc sulphate on anxA5 concentration in both fertile and subfertile males (from 4.9 mg/l (3.6-7.1) to 3.4 mg/l (1.8-6.6) in fertile males and from 5.6 mg/l (2.5-6.1) to 3.7 mg/l (2.1-5.7) in subfertile males). All the other intervention types (placebo, folic acid, and zinc sulphate intervention) also did not significantly affect the anxA5 concentrations, neither in fertile, nor in subfertile males.

Annexin A5 site of synthesis

To get some global insight in the synthesis of anxA5, the anxA5 mRNA concentrations and immunohistochemical localization of anxA5 protein were determined. Annexin A5 mRNA concentrations appeared to be approximately twice as high in the prostate (δCt 5.0) as compared to the testis (δCt 3.8).

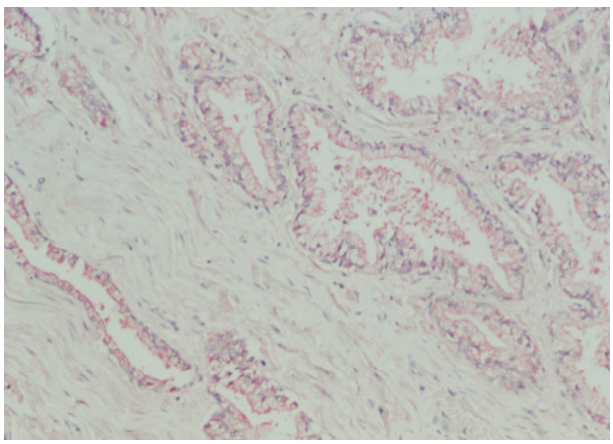
Figure 1

a. Seminiferous tubules of the testis (x40)



Immunohistochemical staining with a polyclonal antibody directed against anxA5 counterstained by Haematoxylin. Annexin A5 is stained red whereas the nucleus is blue. In the testis a gradual staining of anxA5 was observed. The highest intensity is nearby the spermatocytes. The spermatogonia are not stained.

b. Prostate (x20)



In the prostate mainly the glandular epithelium is stained.

Furthermore, as shown in Figure 1, immunohistochemical staining of anxA5 revealed strong staining in the prostate and some staining in the testis. In the prostate mainly the glandular epithelium is stained. In the testis a gradual staining of anxA5 was observed. The highest intensity is nearby the spermatocytes. The spermatogonia are not stained.

Discussion

This study was performed to explore if anxA5 is associated with male factor subfertility and to find an underlying mechanism for the intriguing observation that folic acid and zinc sulphate intervention increases the sperm count in sub-

fertile males (Wong *et al*., 2002; Ebisch *et al*., 2003). Remarkable was the finding that the endogenous seminal plasma anxA5 concentrations are at least 1000-fold higher compared with the concentrations of anxA5 in blood plasma of healthy volunteers, in which anxA5 concentrations as low as 5 ng/ml are found (van Heerde *et al*., 2003; Flaherty *et al*., 1990).

The baseline seminal plasma anxA5 concentration, however, was not significantly different between fertile and subfertile males. This strongly suggests that it is not very likely that anxA5 is associated with sperm concentration. Therefore, anxA5 is probably not involved in male factor subfertility.

Our data suggest that anxA5 is not produced by the spermatozoa themselves, since the immunohistochemical results indicate that the spermatocytes themselves are not stained. Furthermore, the real time mRNA analysis shows that the prostate is the most important organ in the production of anxA5 in seminal plasma.

Only one other study could be found in which the authors investigated the concentration of anxA5 in seminal plasma. These authors obtained semen samples from normal or vasectomised patients and found anxA5 concentrations of approximately 20 mg/l (Christmas *et al*., 1991). It is remarkable that these authors found an almost 4 times higher anxA5 concentration in seminal plasma compared with our findings. A possible explanation for this difference in anxA5 concentration is the method used to measure these concentrations. Christmas *et al*. (1991) pooled all there seminal plasma, purified the annexins present in the seminal plasma using an affinity column coated with phospholipids to which the annexins bind, and thereafter used SDS-PAGE and immunoblot analysis to determine if anxA5 was present in seminal plasma. The concentrations of anxA5 in seminal plasma were estimated by comparing the immunostaining intensity of immunoreactive bands with known standards of placental anxA5. In comparison to our ELISA procedure the method used by Christmas *et al.* (1991) is only semi-quantitative which may explain the higher yields of anxA5 from seminal plasma. Christmas *et al.* (1991) further state that the annexins in seminal plasma are actively secreted by the prostate, it being the main producer of seminal plasma anxA5, which is in agreement with our findings. They exclude the epididymis as a possible source of annexin because the seminal plasma annexin

concentrations are not changed by vasectomy. This is consistent with our finding that the anxA5 concentrations were not significantly different between fertile and subfertile males at baseline.

The question remains what the possible link can be between increases in sperm count in subfertile males after folic acid and zinc sulphate intervention and the seminal plasma anxA5 concentration. An interesting hypothesis is the effect of both nutrients on the control of apoptosis. Spontaneous death of certain classes of germ cells has been shown to be a constant feature of normal spermatogenesis in a variety of mammalian species, including man (Barroso *et al*., 2000; Hikim *et al*., 1998). Scarce information is available on the biological significance of apoptosis in spermatogenesis or its possible role in male fertility (Oosterhuis *et al*., 2000). Since it is known that endogenous anxA5 binds to apoptotic cells in vivo, possibly the seminal plasma anxA5 concentration reflects the degree of apoptosis of spermatozoa and other cell types involved in spermatogenesis and seminal fluid production.

Another possible link between seminal plasma anxA5 concentration and sperm concentration is related to the function of anxA5 as an inhibitor of inflammation. It is well-known that subfertile men have higher leukocyte numbers in their semen compared with their fertile counterparts, the frequency of leukocytospermia (> 10^6 white blood cells/ml semen) being between 10-20% among infertile males (Wolff, 1995). Sperm damage by white blood cells can amongst others be mediated by proteases and cytokines, released during inflammation reactions. Since anxA5 inhibits inflammation it could have a protective effect in these situations, keeping sperm counts up.

In conclusion, the results presented in this paper do not support that anxA5 is associated with male factor subfertility. Intervention with folic acid and zinc sulphate does not affect seminal plasma anxA5 concentration. Therefore, it is not very likely that the observed increase in sperm count after intervention can be attributed to a possible decrease in apoptosis rate of cells involved in spermatogenesis or protection to inflammation by endogenous seminal plasma anxA5 concentration. Further research is needed to clarify the underlying mechanisms responsible for the observed increase in sperm count after folic acid and zinc sulphate intervention.

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PART II

VEGF and determinants of the oxidative and plasminogen pathway in human subfertile couples

Chapter 6

Review on the role of the plasminogen activation system and VEGF in subfertility

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Abstract

Background: Fertilization in subfertile couples often is successfully achieved by artificial reproduction techniques. Nevertheless, this is not a causal treatment of the subfertility. From this background we outline the importance of the plasminogen activator (PA)-system and vascular endothelial growth factor (VEGF) in subfertility.

Method: A literature search was performed using MEDLINE, Science Direct and bibliographies of published works.

Results: The PA-system in the male is involved in the passage of spermatozoa precursor cells over the blood-testis-barrier, the epididymal maturation of spermatozoa, the modifications of the sperm surface for capacitation, the acrosome reaction and zona pellucida attachment, and finally the facilitation of spermatozoa to move into the Fallopian tubes. In the female, the PA-system plays a role during ovulation in the release of the oocyte from the follicle, the facilitation of oocyte movement into the Fallopian tubes, and the extracellular matrix degradation, important for angiogenesis in the ovary.

The function of VEGF during fertilization is largely unknown. It has been suggested that VEGF is important for the fluid and proteins in semen and fluid secretion in the female genital tract, thereby influencing sperm motility and survival. The latter could be due to an effect of VEGF on testicular microvasculature through which an adequate microenvironment for spermatogenesis is provided. In the female, VEGF is involved in the regulation of the cyclic ovarian angiogenesis, the development and/or selection of follicles, the accumulation of follicular fluid, and corpus luteum angiogenesis.

Conclusions: The role of the PA-system and VEGF in reproduction most likely is of great interest. However, much of the data are derived from experimental animal studies. So far, information on the importance of these systems in humans is scarcely investigated. Therefore, further research is required to elucidate the role of the PA-system and VEGF in the pathogenesis and prevention of male and female subfertility. Eventually, this will contribute to the improvement of the diagnosis of subfertility and may possibly lead to targeted therapeutic management of subfertility.

Introduction

Subfertility is a disorder occurring in approximately 10-17% of all couples, either as primary or secondary subfertility at some time during reproductive life (Philippov *et al*., 1998; Snick *et al*., 1997). Subfertility is defined as the failure to conceive after 1 year of regular, unprotected intercourse with the same partner. Various causes and frequencies of subfertility are summarized in Table I.

Table I Causes and frequencies of human subfertility (Modified from Cahill and Wardle, 2002)

CAUSE	FREQUENCY (%)
Male factor subfertility	
Sperm defects or dysfunction	30
Female factor subfertility	
Ovulation failure (amenorrhoea or oligomenorrhoea)	25
Tubal infections	20
Endometriosis	5
Cervical mucus defects or dysfunction	3
Uterine abnormalities	(< 1)
Unexplained subfertility	25
Coital failure or infrequency	5

Note: total exceeds 100% as 15% of couples have more than one cause of subfertility

Zargar *et al.* (1997) reported that in 22% the cause was predominantly originating from the male, and in 58% mainly of female origin. In 5% fertility abnormalities were found in both partners, while in the remaining 15% no clear cause of subfertility was identified.

Several methods are used to assess (sub)fertility in men and women. The WHO designed guidelines giving cut-off values for abnormal sperm parameters, including sperm concentration, motility and morphology, which can be used to estimate fertility in men. According to these guidelines, a sperm concentration of less than 20 million cells per millilitre, a progressive motility of less than 50% and a proportion of less than 30% cells with normal morphology in the ejaculate is regarded as abnormal semen (WHO, 1992). Another more holistic approach to evaluate spermatozoa morphology is by using the strict Tygerberg criteria (Menkveld *et al*., 1990; Menkveld *et al*., 2001). Also, the evaluation of endocrine parameters like follicle stimulating hormone (FSH),

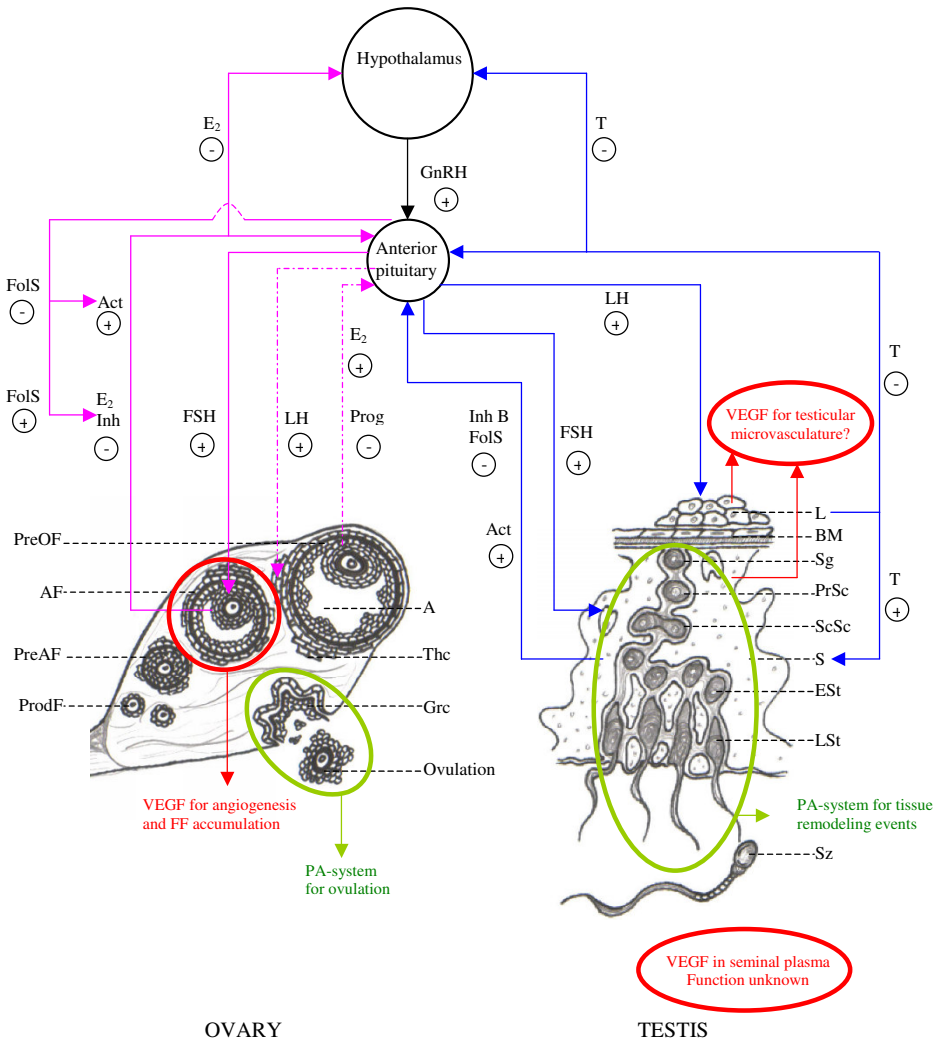
luteinizing hormone (LH), testosterone and inhibin B concentrations can contribute to the characterization of male fertility (Figure 1). Furthermore, in men with sperm concentrations below 5 million per millilitre, karyotyping and search for microdeletions of the Y-chromosome are often performed.

In women with regular cycles a basal body temperature curve chart is used to assess the menstrual cycle for ovulation disorders. Furthermore, in women with oligo-amenorrhea serum concentrations of FSH, LH, 17β-estradiol, progesterone, testosterone, sex hormone binding globulin (SHBG), prolactin, and thyroid stimulating hormone (TSH) are assessed to detect the origin of anovulation (Figure 1). Evaluation for possible tubal damage is performed by using Chlamydia serology. More specifically, a hysterosalpingogram (HSG) can be made to assess the uterine cavity and the tubal patency. Laparoscopy can be performed to assess tubal patency, pelvic adhesions and endometriosis. Since both male and female factors determine the chance of conception, the Sims-Hühner test is also performed, because it takes into account the interaction of both partners. This assay tests the quality of the cervical mucus, assesses the occurrence of coital problems, the buffer capacity of the semen, and the motility of sperm in the cervical mucus.

Subfertile couples can have difficulties coping with their involuntary childlessness (Whiteford and Gonzalez, 1995), and therefore are often eager to conceive by artificial reproduction techniques (ART). In most cases these treatments, however, do not treat the underlying cause of the subfertility. This is because subfertility often is a multifactorial condition in which many and mostly unknown genetic and environmental factors are involved.

It is hypothesized that derangements in matrix degradation by the plasminogen activator system and vascularization induced by the vascular endothelial growth factor (VEGF) are causally involved in subfertility (Figure 1). In this paper we will review the evidence in literature for the involvement of these 2 systems in reproduction. Figure 2 displays the plasminogen activator system involving molecules like tissue-type plasminogen and urokinase-type plasminogen activators, together with plasminogen activator inhibitors, which are involved in the process of matrix degradation and fibrinolysis. For sperm progenitor cells to be able to pass the tight junctions between the Sertoli cells, and for the oocyte to undergo ovulation, matrix degradation has to occur. Finally, the micro-environment in which the germ cells develop affects the

Figure 1 The Hypothalamus-Pituitary-Gonadal axis in men and women

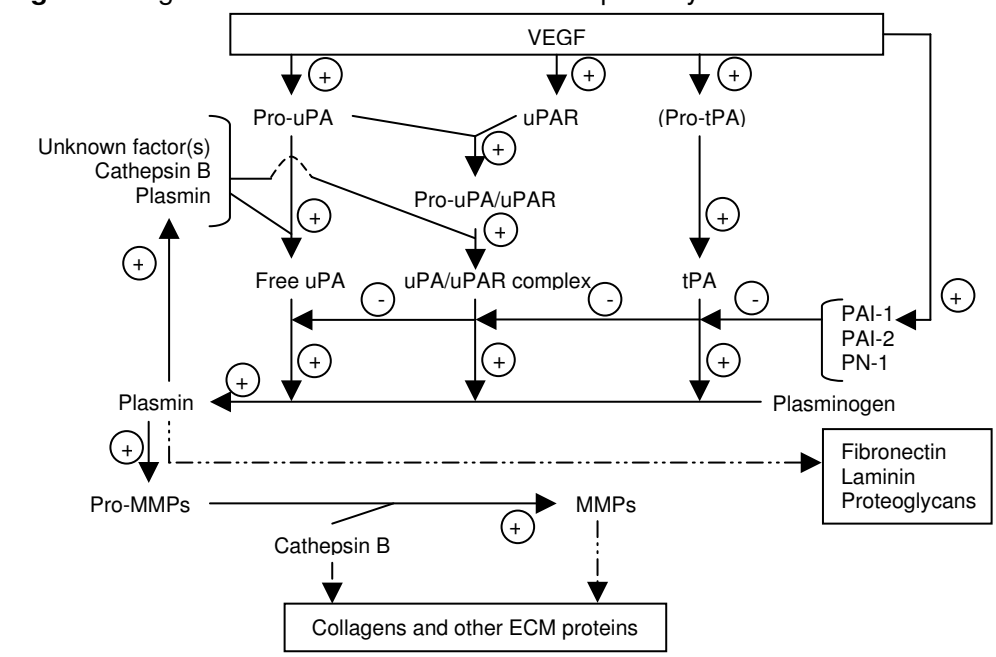


Abbreviations : A, antrum; Act, Activin; AF, antral follicle; BM, basement membrane; E_2, estradiol; Est, early spermatid; FolS, Follistatin; FSH, follicle stimulating hormone; GnRH, Gonadotropin releasing hormone; Grc, Granulosa cells; Inh B, inhibin B; L, Leydig cells; LH, luteinizing hormone; Lst, late spermatids; PreAF, preantral follicle; PreOF, preovulatory follicle; ProdF, primordial follicle; Prog, progesterone; PrSc, primary spermatocytes; S, Sertoli cells; ScSc, secondary spermatocytes; Sg, spermatogonium; Sz, spermatozoa; T, testosterone; Thc, theca cells.

reproductive capacity of these cells. Essential for the micro-environment is the blood flow for the provision of nutrients and growth factors and the removal of (toxic) metabolites. A significant factor involved in vascularization is the vascular endothelial growth factor. Interestingly, vascular endothelial growth factor induces the expression of tissue- and urokinase plasminogen activators and plasminogen inhibitor type 1 (Mandriota *et al.*, 1995; Pepper *et al.*, 1991), indicating that both systems interact (Figure 2).

We are aware that fertilization is a complex process and that the 2 systems outlined in this review are only part of the puzzle. Research into these specific fields, however, will lead to more insight into the pathogenesis, treatment, and prevention of subfertility in men.

Figure 2 Regulation and control of extracellular proteolysis



Abbreviations: ECM, extracellular matrix; MMP, matrix metalloproteases; PAI, plasminogen activator inhibitor; PN-1, protease nexin-1; Pro-uPA, enzymatically inactive form of urokinase plasminogen activator; tPA, tissue-type plasminogen activator; uPA, urokinase-type plasminogen activator; uPAR, urokinase plasminogen activator receptor.

Materials and Methods

A thorough literature search was performed on MEDLINE, Science Direct, and via bibliographies of published works. The literature reviewed consisted of papers in English published between 1950 and 2005, discussing both animal and human studies. The following words combined in different ways were used as search terms: fertilization, subfertility, tissue-type plasminogen activator (tPA), urokinase-type plasminogen activator (uPA), plasminogen activator inhibitor type 1 (PAI-1) and vascular endothelial growth factor (VEGF).

The Plasminogen Activator (PA)-system

The PA-system (Figure 2), mostly known for its role in fibrinolysis, tumour invasion and metastasis, is involved in the activation of the inactive proenzyme plasminogen into the active serine proteinase, plasmin. Plasmin is capable of readily degrading extracellular matrix (ECM) proteins. The inactive plasminogen is abundantly present in blood and in most other body fluids (Saksela and Rifkin, 1988). The PA-system is present in a variety of different cell types and body fluids (Dano *et al*., 1985). The combination of plasminogen and plasminogen activators serves as an almost unlimited supply of proteolytic capacity.

The PA-system involves 2 plasminogen activators, the urokinase-type plasminogen activator (uPA) and the tissue-type plasminogen activator (tPA). The homology between these 2 PAs at the amino acid level is only 40% (Degen *et al*., 1986), however, both enzymes are highly similar in their basic structures. Both uPA and tPA are secreted as single-chain polypeptides. The single-chain form of uPA is an inactive proenzyme, while the two-chain form is the active enzyme (Eaton *et al*., 1984; Wun *et al*., 1982). The two-chain form of tPA also is the active enzyme, although conflicting results as to the enzyme activity of the single-chain form of tPA have been reported (Andreasen *et al*., 1984; Rijken *et al*., 1982).

Because PAs occur in a variety of different cell types in the organism, it is likely that PAs are involved in several biological processes. Furthermore, the different distribution of uPA and tPA in the organism makes it feasible that these 2 activators have different biological functions, uPA being implicated in processes of cell migration and tissue remodelling, while tPA is thought to be

involved in thrombolysis (see for reviews: Dano *et al*., 1985; Schmitt *et al*., 1997).
PA-activity is effectively controlled by specific inhibitors produced by a number of cell types. These inhibitors belong to the serine proteinase inhibitor (serpin) gene superfamily and involve plasminogen activator inhibitor type-1 (PAI-1), plasminogen activator inhibitor type-2 (PAI-2), type-3 (PAI-3 or protease C inactivator) and protease nexin I (PN I). Furthermore, an uPA receptor (uPAR) exists, which is a membrane-anchored binding protein for (pro-)uPA, concentrating uPA-activity at the cellular surface (Vassalli *et al*., 1985).
Degradation of the ECM resulting from plasminogen activation is important in cell migration, necessary for morphogenesis, tissue repair and remodelling, neovascularization and invasion of (malignant) cells.

The PA-system in the male reproductive system
Germinal cell development is a continuous cyclic process of the seminiferous epithelium. The interaction between Sertoli cells and germ cells is important for normal spermatogenesis. The Sertoli cells form junctional complexes that constitute the blood testis barrier (BTB). During spermatogenesis, preleptotene and leptotene spermatocytes must cross this BTB, moving from the basal compartment of the seminiferous epithelium to the adluminal compartment for further development into spermatids. This necessary passage across the BTB indicates that the interactions between the Sertoli cells themselves and between Sertoli cells and germ cells are dynamic and require a rapid rate of tissue remodelling (Griswold, 1995). Protease activities, including that of the PA-system, seem to be important for several aspects of the passage of spermatocytes across the BTB and release of mature spermatozoa into the lumen of the seminiferous tubule (Zhang *et al*., 1997b).
Sertoli cells are generally considered the main source of tPA in rodent testis (Lacroix *et al*., 1977). The secretion of tPA is controlled by different hormones (Liu *et al*., 1995; Tolli *et al*., 1995), by cell-cell interactions between Sertoli and Leydig cells (Liu *et al*., 1996a; Tolli *et al*., 1995), and by the interaction with neighbouring preleptotene primary spermatocytes (Vihko *et al*., 1984). The same control mechanisms are operational for tPA production in monkey testis (Zhang *et al*., 1997b). Furthermore, rat, mouse and monkey testes are all capable of producing PAI-1 (Lacroix *et al*., 1977; Vihko *et al*., 1986; Zhang *et*

al., 1997b), and germinal cells are the cells producing the highest intracellular levels of PAI-1 mRNA (Zhou and Liu, 1996), however Sertoli cells in these species are also capable of producing PAI-1 mRNA (Magueresse-Battistoni *et al*., 1998; Liu *et al*., 1993; Liu *et al*., 1995). Treatment with FSH decreased PAI-1 mRNA and expression (Zhang *et al*., 1997a; Magueresse-Battistoni *et al*., 1998).

It was shown in rat testis that under basal conditions uPA was secreted by Sertoli cells (Hettle *et al*., 1986; Vihko *et al*., 1986). In contrast to the unchanged uPA mRNA concentration (Vihko *et al*., 1989), treatment with FSH increased tPA mRNA levels (Nargolwalla *et al*., 1990). Other authors, however, reported a decrease in uPA production in response to FSH treatment, and considering the complex regulation of uPA and tPA production by rat Sertoli cells in response to the same hormonal stimulus, they hypothesized that the 2 PAs have different functions (Tolli *et al*., 1995).

It has been suggested that uPA is involved in the extensive tissue remodelling taking place during the stages VII-VIII of spermatogenesis, mostly because uPA mRNA levels are highest at these stages (Vihko *et al*., 1989). The tissue remodelling includes the release of preleptotene spermatocytes from basement membranes (Hettle *et al*., 1986), spermiation (Le Blond and Clermont, 1952), and the detachment of residual bodies from the mature spermatids (Morales *et al*., 1986). On the contrary, tPA mRNA levels were highest at stages IX-XII (Penttila *et al*., 1994), and therefore tPA has been suggested to release the tight junctions between neighbouring Sertoli cells during stages X-XII, when zygotene spermatocytes migrate to the adluminal compartment of the seminiferous tubules (Ailenberg and Fritz, 1989).

Conversely, PAI-1 of Sertoli cells, secreted in the adluminal compartment of the seminiferous tubules, can limit the net protease activity during passage of preleptotene spermatocytes across the BTB, thereby preventing damage to the Sertoli cell barrier. Furthermore, secreted PAI-1 can prevent the premature release of the new elongated spermatids at spermiation (Magueresse-Battistoni *et al*., 1998).

The PA-system is not only active in the testis. Urokinase PA is present in the stereociliated epithelial cells, in the lumen of the vas deferens of mice (Huarte *et al*., 1987; Larsson *et al*., 1984), in the seminal vesicles and to a lesser extent in the epididymis of mice (Huarte *et al*., 1987) and monkeys (Zhang *et al*.,

1997a). In addition, evidence for the presence of tPA mRNA in monkey epididymis was presented (Zhang *et al*., 1997a). Most spermatozoa are stored in the cauda epididymis and vas deferens awaiting ejaculation. Proteases like uPA and tPA in the epididymal and vas deferens luminal fluid may play an important role in epididymal maturation, involving the loss or alteration of sperm surface molecules (Eddy *et al*., 1985; Tulsiani *et al*., 1995). These alterations are necessary for sperm maturation, motility and fertilizing ability of the spermatozoa. Furthermore, ejaculated spermatozoa express uPA activity on their cell surface, particularly around the head region (Huarte *et al*., 1987). However, spermatozoa stored in the vas deferens, or spontaneously drained from dissected vas deferens possess almost no uPA immunoreactivity. This suggests that the secretion of uPA by the male accessory organs may be stimulated by ejaculation, followed by the binding of uPA to the cell surface of the spermatozoa, possibly via its specific plasma membrane receptor (Huarte *et al*., 1987).

It has been suggested that a trypsin-like enzyme is involved in the limited proteolysis necessary for sperm surface modifications occurring during capacitation (Talbot and Chacon, 1981), and in the stimulation of the acrosome reaction (Pillai and Meizel, 1991). Huarte *et al*. (1987) observed a proteolytic cascade around the head region of the ejaculated spermatozoa, which was triggered by uPA. Therefore, uPA could very well be the aforementioned trypsin-like enzyme involved in sperm surface modification. Another possible function of the uPA mediated proteolysis is the facilitation of spermatozoa movement towards the ampulla of the Fallopian tubes by preventing adhesion of spermatozoa to fibrin deposits on the tubal mucosa. In the ampulla of the Fallopian tubes the actual fertilization takes place. To establish this fertilization, spermatozoa have to attach to the zona pellucida of the oocyte. In this regard, trypsin enzyme inhibitors were shown to prevent binding of mouse spermatozoa to the zona pellucida, probably by their action on a sperm-derived protease (Saling, 1981). These data suggest that spermatozoa-bound uPA may participate in the proteolytic events that accompany capacitation and fertilization.

Other investigators also have shown the presence of both uPA and tPA in ejaculated spermatozoa and seminal plasma of various species, including humans (Smokovitis *et al*., 1987). The observed tPA was demonstrated to be

synthesized in the prostate gland (Reese *et al*., 1988). Smokovitis *et al*. (1992) observed a species difference in the presence of tPA and/or uPA on the outer acrosomal membrane and plasma membrane of the spermatozoa. Tissue PA and uPA, present on different locations of sperm membranes, may have distinct roles in regulating sperm functions. Tissue PA and uPA located on the outer acrosomal membrane of human spermatozoa may participate in the attachment too, and penetration of the spermatozoon to the zona pellucida, whereas the PAs located on the plasma membrane might be involved in spermatozoa maturation, capacitation, binding to the zona pellucida, and acrosome reaction (Smokovitis *et al*., 1992).

In addition, PAI-1 is also found outside the testis. High concentrations of PAI-1 mRNA were expressed in the caput of the epididymal epithelial cells of adult monkeys, while in the initial and caudal regions of the epididymis PAI-1 mRNA levels were much lower (Zhang *et al*., 1997a). The authors hypothesize that PAI-1 is important in some aspects of sperm maturation and since PAI-1 concentrations are higher in the caput of the epididymis, they hypothesize that the caput is more important in sperm maturation than the other regions. However, the precise mechanism of action is unclear.

Furthermore, PAI-1 antigens are located on the surface of the sperm head, midpiece and tail of human spermatozoa, which indicates that human spermatozoa are capable of binding PAI-1 (Liu *et al*., 1996b). Smokovitis *et al*. also demonstrated PAI-1 on both plasma and outer acrosomal membranes of human spermatozoa (Smokovitis *et al*., 1992). Since both uPA and tPA can also be found on spermatozoa, PAI-1 may function as a counterbalancing factor, thereby coordinating the proteolysis induced by the 2 activating factors.

The PA-system in the female reproductive system

It is known from animal experiments that around ovulation, tPA and uPA are produced by rat granulosa cells that line the interior of the follicle, and by rat thecal cells in the interstitium of preovulatory follicles (Liu *et al*., 1987; Ny *et al*., 1985). Mouse granulosa cells, however, do not produce tPA but rather secrete uPA (Canipari *et al*., 1987). This indicates that similar ovarian cell types from these closely related species produce different PAs which could have similar functions.

Synthesis of tPA and uPA in the granulosa and thecal cells is under

gonadotropin control (Canipari *et al*., 1987; O'Connell *et al*., 1987). In the rat ovary, the regulation of the 2 PAs by gonadotropins differs between granulosa and theca cells. Hormonal stimulation of the rat ovary results in an increased uPA and tPA activity in both granulosa and theca cells. However, only tPA mRNA was up-regulated in both cell types, while uPA mRNA was up-regulated in the thecal cells and down-regulated in granulosa cells (Macchione *et al*., 2000). This discrepancy in uPA activity and mRNA levels in granulosa cells was explained by a shift from the soluble form of uPA in the FF to the cell-surface of the granulosa cell where it binds to a specific uPA-receptor. Furthermore, the down-regulation of uPA mRNA indicates that the increase in uPA activity is not the result of newly formed uPA molecules by these cells themselves. The redistribution of the uPA molecule from the fluid phase to the cell membrane as a result of binding to its specific receptor, while tPA is active in the fluid phase, strongly suggests that these 2 PAs may have different functions in ovulation (Macchione *et al*., 2000).

Because of the redistribution of uPA to the cell-surface of granulosa cells, this factor seems to be involved in the degradation of the ECM between mural granulosa cells, important for the escape of the cumulus-oocyte complex (COC) from the follicle. Tissue PA, in solution in the FF, is more likely to be involved in the degradation of the core protein of proteoglycans present in the FF, responsible for the high viscosity of the FF. Around the time of ovulation the FF liquefies, thereby facilitating the release of the COC from the inside of the follicle. Another possible role for tPA is the prevention of blood clotting during rupture and subsequent bleeding of the highly vascularized theca cell layers of the follicle during ovulation, thereby preventing entrapment of the COC inside the follicle (Canipari and Strickland, 1985).

Proof for the involvement of tPA in ovulation was found by Tsafriri *et al*. (1989). These authors observed a significant decrease in ovulation rate after a single intrabursal injection of tPA antibodies in the rat ovary at the time of human chorionic gonadotropin (hCG) injection. Ovulation was, however, not completely inhibited. Morphologic evaluation revealed that there were no pathologic changes in the ovary after the injection, and several follicles with oocytes entrapped within their antrum were observed (Tsafriri *et al*., 1989). No significant decrease in ovulation rate was detected if the tPA antibodies were injected 4 hours after hCG injection. Therefore, the authors conclude that tPA

is involved in the early stages of ovulation (Tsafriri *et al*., 1989).
Not only granulosa and theca cells express PA activity, also COCs and denuded oocytes are reported to synthesize PAs. Liu *et al*. (1986) demonstrated that the COCs and denuded oocytes collected from preantral follicles of hypophysectomized immature rats contained tPA. This tPA was present in the cytoplasm of the primary oocytes. The tPA enzyme activity in COCs was stimulated by FSH, and even uPA activity was observed after FSH stimulation. The tPA enzyme activity in denuded oocytes, however, was not enhanced by treatment with FSH. Thus, these authors suggest that cumulus cells may stimulate tPA activity at the oocyte level (Liu *et al*., 1986). Furthermore, Liu and Hsueh (1987) found that after hCG treatment, tPA activity in the COCs responded similarly as found after FSH treatment, but denuded oocytes showed an increase in tPA activity after treatment with hCG. Liu and Hsueh not only reported that the intracellular content of tPA was increased after gonadotropin treatment, but also that the secretion of tPA by the COC was upregulated during the preovulatory period. The oocyte content of tPA even continued to increase after ovulation, but the secretion of tPA by the COC decreased after ovulation (Liu and Hsueh, 1987). The precise function of tPA in the primary oocyte/COC is not yet clear. Tissue PA concentrations secreted by the COC could facilitate liquefying of the FF, thereby enabling ovulation in a way similar to tPA produced by granulosa and theca cells. In addition, tPA from the oocyte could prevent entrapment of the released oocyte in fibrin deposits present in the oviducts, or could be involved in the breakdown of the communication between cumulus cells (CC), and between CC and the oocyte.
In the preovulatory period, the CCs which normally are tightly packed around the oocyte, start to lose contact with each other and with the oocyte. However, the COC does not disintegrate, because a mucoelastic ECM is formed keeping the different cell types together. Because of loosening of the tightly packed CCs, and formation of the ECM the volume of the COC significantly increases, hence this process is referred to as cumulus expansion. This CC-matrix is important, for instance in oocyte release from the follicle and pickup of the oocyte by the tubal fimbriae, and also this matrix provides an appropriate microenvironment for successful fertilization. After ovulation, the CC and matrix are gradually shed from the COC, thereby reducing the chance of oocyte fertilization. This shedding of CCs is probably designed to promote fertilization

of freshly ovulated oocytes and to prevent fertilization of aged oocytes. During the shedding of the CC the aforementioned matrix is degraded, and it is hypothesized that components of the PA-system are involved in this proteolysis. D'Allessandris *et al*. (2001) showed that uPA production in CCs of mice differs from uPA production of mural granulosa cells during the preovulatory period. Urokinase PA production in CCs does not significantly increase during this period, when ECM deposition of the COC occurs. They concluded that the decreased uPA production by CCs compared with the mural granulosa cells is due to a paracrine control of the oocyte on the response of surrounding cumulus cells to gonadotropin stimuli. However, this inhibitory action of the oocyte does not last, and uPA synthesis by cumulus cells abruptly rises after full expansion of the CC-matrix. This rise in uPA synthesis is reflected in concomitant matrix degradation and cumulus dispersion, resulting in a less fertilizable oocyte.

In this review we presented on outline of the production of PAs by granulosa and theca cells, the COC and the oocyte itself, but also other cells of the ovary synthesize PAs. Ovarian surface epithelial cells produce uPA, thereby contributing to ECM degradation resulting in ovulation (Colgin and Murdoch, 1997). Also, endothelial cells in the ovary express uPA in a transient fashion. In the ovary, unvascularized preantral follicles only can mature into Graafian follicles if they develop their own capillary sheath, indicating that angiogenesis is necessary. To form new blood vessels, endothelial cells must be released from the basement membrane, which is achieved by targeted proteolysis. Also, the subsequent migration of endothelial cells to form new blood vessels requires proteolysis of the ECM. The observation that endothelial cells in the ovary express uPA provides support for a role of this enzyme in controlling the angiogenesis-associated proteolysis (Bacharach *et al*., 1992).

Finally, some gene function studies were conducted to investigate the role of the PA-system in reproduction. Carmeliet *et al*. (1994) found that both single and double-knock-out (uPA and tPA) male and female mice were able to reproduce. However, double-knock-out mice were significantly less fertile compared with control mice, indicated by their significantly less frequent litters and smaller litter size. Whether this decrease in ability to reproduce was related to the poor general health of these double-knock-out mice, or to more specific fertility reducing consequences related to their uPA or tPA

deficiency was not clear.

Leonardsson *et al*. (1995) quantified the effects of PA deficiencies on ovulation. They found that ovulation efficiency was not different between wild-type mice and mice that were heterozygous or homozygous for mutations in the tPA or uPA gene. However, they found a reduced ovulation efficiency in mice with a combined deficiency of tPA and uPA, thereby suggesting that the PA-system is involved in ovulation, although, there is no unique requirement for either uPA or tPA. The data suggest that there is a redundancy in plasminogen activator activity regulating ovulation, and that one of the PAs (uPA, tPA) can functionally compensate for the loss of the other (Leonardsson *et al*., 1995). It is obvious however, that the PA-system is not the only system involved in ECM degradation during ovulation, because in the absence of PAs ovulation still occurs, although with a reduced efficiency. This indicates that other proteolytic active proteases like matrix metalloproteins (MMPs) contribute to ovulation (for review on MMPs and ovulation see: Ny *et al*., 1993).

Not only uPA and tPA, but also PAI-1 is expressed in the female reproductive tract. In the mouse ovary, PAI-1 is expressed throughout the periovulatory period, however in much lower concentrations compared with uPA and tPA (Leonardsson *et al*., 1995). Therefore these authors conclude that it is possible that other protease inhibitors can reduce plasminogen activation in the mouse ovary. PAI-1 levels were slightly induced around the time of ovulation, induced by hCG treatment (Leonardsson *et al*., 1995). Hägglund *et al*. (1996) observed similar results in mouse ovaries, and they also defined the cells expressing PAI-1 mRNA as being thecal-interstitial cells of postovulatory follicles. These authors recognize the importance of other protease inhibitors suggested by Leonardsson *et al*. since they observed high expression of the protease nexin-1 by granulosa cells and cumulus cells in the entire periovulatory period (Hägglund *et al*, 1996). Furthermore, Leonardsson *et al*. (1995) found that there was no difference in ovulation efficiency between wild-type control mice and mice with heterozygous or homozygous mutations in PAI-1 genes. In the rat ovary a different expression of PAI-1 is observed, compared with the expression in the mouse. The intensity of PAI-1 increased with follicular development, reaching peak levels in the preovulatory period (Li *et al*., 1997). The distribution of PAI-1 was uniformly distributed between granulosa and theca interna cell compartments, but the PAI-1 transcript levels were highest in

granulosa cells. These authors hypothesize that PAI-1 is important in preventing premature rupture of the ovulatory follicle until the expected time of ovulation when PAI-1 expression and activity are down-regulated (Li *et al*., 1997). Since changes in the PAI-1 expression followed a similar temporal pattern as tPA Li *et al.* (1997) suggest that the synchrony in the synthesis of these proteins may be important in focussing the tPA action primarily to the site of follicle rupture as well as keeping the functional proteolytic activity low.

After ovulation the corpus luteum begins to form. Also, in this process PAI-1 expression is found. In the monkey PAI-1 mRNA is equally expressed throughout the luteal tissues, with the highest PAI-1 expression found in 10-day-old corpus luteum, decreasing to 50% on day 13 (Liu *et al*., 1997). A similar pattern was observed for uPA in the corpus luteum and therefore these authors conclude that proteolysis mediated by uPA and regulated by PAI-1 could play a role in luteal maintenance, while tPA may participate in luteal regression, since this protein increased at the time of luteal regression (Liu *et al*., 1997). Other authors investigating the expression of PAI-1 in the corpus luteum of rats found that during corpus luteum formation PAI-1 mRNA was expressed inside the collapsed postovulatory follicle in a subpopulation of cells localized at the innermost layers of the proliferating granulosa cells (Bacharach *et al*., 1992). Again, a physical proximity was observed between PAI-1 and a plasminogen activator being uPA in rats. Urokinase PA was mainly detected in capillary sprouts within the developing corpus luteum, indicating that uPA is important in the neovascularization of this structure, while PAI-1 was mainly found in cells in the proximity of capillaries. Therefore, these authors conclude that PAI-1 may protect these cells from degradation by uPA bound to the surface of nearby cells (Bacharach *et al*., 1992).

Finally, Jones *et al*. (1989) observed that follicular fluid (FF) from human preovulatory follicles contained a relative abundance of PAI-1 in relation to tPA and uPA, resulting in low levels of uncomplexed free and thus active plasminogen activators in FF. They further state that granulosa cells may be the primary producers of FF PAI-1 (Jones *et al.*, 1989).

Vascular endothelial growth factor (VEGF)

Vascular endothelial growth factor is a protein with anti-apoptotic, mitogenic and permeability-increasing activities. VEGF mRNA is produced by non-

malignant and malignant cells in response to hypoxia and inflammation. VEGF is a member of a family of growth factors comprising VEGFA, -B, -C, -D, and -E, placental growth factors and platelet derived growth factors. Alternative splicing of VEGF A pre-mRNA leads to production of a number of variants consisting of 121, 145, 165, 183, 189, and 206 amino acid residues. As mentioned before, VEGF has been shown to inhibit apoptosis in several cell types including endothelial cells (Spyridopoulos *et al*., 1997), multiple myeloma cells (Le Gouill *et al*., 2004), smooth muscle cells (Yamanaka *et al*., 2005), human podocytes (Foster *et al*., 2005), endometrial stroma cells (Berkkanoglu *et al*., 2004), and haematopoietic stem cells (Gerber *et al*., 2002). The mechanisms involved in this survival function of VEGF includes the up-regulation of members of the anti-apoptotic Bcl-2 protein family (Gerber *et al*., 1998; Le Gouill *et al*., 2004; Nor *et al*., 1999), and the decrease of Fas ligand expression (a mediator of apoptosis) (Berkkanoglu *et al*., 2004).
Furthermore, it is reported that the expression of VEGF is increased by homocysteine and other thiol-containing reductive compounds, due to activation of VEGF transcription (Roybal *et al*., 2004). Therefore, folate deficiencies resulting in hyperhomocysteinemia could hypothetically result in increased VEGF expression. Some authors found that reactive oxygen species (ROS) are dose- and time-dependent inducers of VEGF gene and protein expression in vascular smooth muscle cells, human retinal pigment epithelial cells, human melanoma cells and glioblastoma cells (Bassus *et al*., 2001; Kuroki *et al*., 1996). Interesting to note is that the antioxidant *N*-acetylcholine (NAC) can suppress VEGF induction (Chua *et al*., 1998; Redondo *et al*., 2000).
Finally, VEGF is known to induce the expression of uPA, tPA, uPAR, and PAI-1 in cultured bovine microvascular endothelial cells in a dose dependent manner (Mandriota *et al*., 1995; Pepper *et al*., 1991). These results suggest that the PA-system is important in the ECM degradation and endothelial cell migration during angiogenesis, induced by VEGF (Mandriota *et al*., 1995; Pepper *et al*., 1991). The induction of PAI-1 by VEGF is necessary to maintain a proper balance between proteolytic PA-activity and inhibition, necessary for normal capillary morphogenesis (Pepper and Montesano, 1990).

VEGF in the male reproductive system

Brown *et al.* (1995) showed that VEGF is present in seminal plasma in very

high concentrations, even exceeding the concentrations of VEGF measured so far in malignant effusions (Yeo *et al*., 1993). They also demonstrated that VEGF mRNA and protein were strongly expressed in both prostate and seminal vesicle epithelium. This was supported by the observation that post-vasectomy ejaculates still contained high concentrations of VEGF. These results indicate that prostate and/or seminal vesicles, but not testes or epididymis, are the major sources of VEGF in seminal plasma (Brown *et al*., 1995). However, it was also shown that VEGF is synthesized and secreted by Leydig and Sertoli cells, and not by germ cells (Ergun *et al*., 1997).

Since VEGF is produced in very high quantities by prostate and seminal vesicles, an important role for this growth factor in fertility is emphasized. However, the function of VEGF in the male genital tract is yet unclear. Brown *et al*. (1995) suggested several possible functions for VEGF in reproduction. VEGF, as a regulator of microvascular permeability, could play an important role in determining the fluid and protein composition of semen. Furthermore, they suggest that VEGF present in semen could alter microvascular permeability and may influence fluid secretion in the female genital tract when deposited there during coitus. This effect of VEGF on the amount and composition of fluids from both the male and female genital tract may be of influence on fertility by affecting sperm motility or survival. The stimulatory effect of VEGF on endothelial cell growth and angiogenesis could play a role in early angiogenic events that are critical for the implantation of the blastocyst in the endometrium. Another possible function of VEGF in the male genital tract was provided by Ergün *et al*. (1997) who stated that VEGF, produced by Sertoli and Leydig cells, has a paracrine effect on testicular microvasculature, providing an adequate microenvironment in seminiferous, tubular, and interstitial compartments of the testes. These authors also reported the presence of VEGF receptors on Sertoli and Leydig cells, and suggested that VEGF may have an autocrine regulatory effect on the activity of both cell types. The function of this autocrine effect until now is still unclear.

Obermair *et al.* (1999) showed that seminal plasma VEGF concentrations correlated with pregnancy rates in patients undergoing *in vitro* fertilization (IVF) or intracytoplasmic sperm injection (ICSI) procedures. However, VEGF concentrations in seminal plasma were not associated with male infertility, semen parameters or oocyte fertilization rate. Also, expression of VEGF

receptors on spermatozoa was demonstrated, suggesting that seminal plasma VEGF may target the spermatozoa themselves. Furthermore, Obermair *et al*. (1999) propose a possible effect of seminal plasma VEGF in the female genital tract, however, sexual intercourse is often discouraged during an IVF procedure.

Overexpression of $VEGF_{121}$ in mice resulted in markedly reduced fertility or even infertility. The testes of these mice were macroscopically atrophic, showing partial or complete sperm maturation arrest, with disorganization of germ cells in the seminiferous tubules (Huminiecki *et al*., 2001). A loss of adhesion between differentiating spermatocytes and Sertoli cells was observed. Also, in the epididymis large quantities of immature precursors and apoptotic spermatocytes were present (Huminiecki *et al*., 2001). Similar observations were made by Korpelainen *et al*. (1998), who found that overexpression of $VEGF_{165}$ resulted in male infertility and no offspring was produced by these transgenic mice. The infertility in these male mice is probably due to effects of VEGF on both the testis and the epididymis. The rete testis was remarkably dilated and the capillary density was increased. Also, a sperm maturation arrest at the elongated phase of the spermatids was observed. Furthermore, enlarged and swollen epididymes were observed.

The presence of VEGF receptors on the spermatozoa themselves (on mid-pachytene spermatocytes and round spermatids) suggests that VEGF may act directly on spermatogenic cells. The expression of VEGF receptors on round spermatids is of particular interest since the observed spermatogenic arrest occurs at this point (Korpelainen *et al*., 1998). Korpelainen *et al*. also suggest that the observed infertility could result from an effect of VEGF on Leydig cells, which also express the VEGF receptor. The Leydig cells are the main source of testosterone, necessary for normal spermatogenesis. Furthermore, a rise in testicular temperature due to increased capillary density and size, resulting from the vascular effects of VEGF overexpression in testis, could also contribute to the infertile phenotype (Korpelainen *et al*., 1998).

VEGF in the female reproductive system

The female reproductive organs contain some of the few tissues that exhibit periodic growth and regression. Thus, it is not surprising that female reproductive tissues belong to the few tissues in which angiogenesis takes

place as a regular physiological process.

For the cyclic growth and regression of ovarian, endometrial and placental structures, programmed angiogenesis is necessary. In the ovary, this growth and regression is regulated by cyclic and pulsatile secretion of gonadotropins, which leads to ovulation and steroidogenesis within the ovary. Therefore, it is suggested that the angiogenic waves involved in the cyclic growth within the female reproductive system are likely to be coordinated by gonadotropins and/or locally produced steroids (Geva and Jaffe, 2000). This suggestion is supported by *in vivo* and *in vitro* studies, which show that FSH, LH and hCG modulate the expression of VEGF mRNA and protein levels (Christenson and Stouffer, 1997; Laitinen *et al*., 1997).

Several studies have indicated that VEGF is involved in the physiological regulation of ovarian angiogenesis (Fujimoto *et al*., 1998; Otani *et al*., 1999; Ravindranath *et al*., 1992). VEGF is expressed and secreted by the human ovary in strong association with the cyclic angiogenesis and permeability patterns observed in the ovary. This suggests a role for VEGF in these 2 processes, both of which are critical for ovarian folliculogenesis and normal reproductive function.

Among the four VEGF isoforms, the $VEGF_{121}$ and $VEGF_{165}$ transcripts are predominantly expressed in human ovaries (Fujimoto *et al*., 1998; Otani *et al*., 1999). In situ hybridisation studies in female rats showed that VEGF mRNA is expressed in the ovary along with follicular growth and maturation (Shweiki *et al*., 1993). In preantral and small antral follicles, VEGF mRNA is expressed in the interstitial tissue and theca layers. Later in the development of the follicles, the cumulus cells engulfing the oocyte are the first cells to express VEGF inner to the theca cell layer. The granulosa cells express high levels of VEGF mRNA only at the immediate preovulatory stage. Finally, shortly after ovulation, when the formation of the corpus luteum begins, the main site of VEGF mRNA expression is the granulosa-lutein cell (Shweiki *et al*., 1993).

In human ovaries, VEGF expression was found to be related to follicular maturation. Otani *et al*. (1999) observed by immunohistochemistry that granulosa cells in primordial follicles did not express VEGF, while the corresponding oocytes did express this growth factor. In the small antral follicle, VEGF mRNA expression was observed in granulosa cells facing the follicular antrum in the oocytes themselves, but not in theca cells (Otani *et al*.,

1999). Only theca cells in medium sized follicles expressed VEGF, and in this stage the VEGF expression was higher than in granulosa cells. Like in the rat, human preovulatory follicles express high levels of VEGF in both granulosa and theca cells and the intensity of immune staining for VEGF gradually increased as the follicles matured (Otani *et al*., 1999). Also, the granulosa and theca lutein cells in the human corpus luteum expressed VEGF in the early, mid- and late-luteal phase. The VEGF expression was highest in the mid-luteal phase (Otani *et al*., 1999).

VEGF expressed by theca and granulosa cells of the follicle probably acts primarily on the nearby endothelial cells (Shweiki *et al*., 1993), thereby stimulating vascular endothelial proliferation in the theca cell layer (Otani *et al*., 1999). VEGF also can increase vascular permeability, leading to accumulation of antral fluid, thereby regulating gonadotropin accumulation in the different follicles. Therefore, highly vascularized theca cell layers are probably critical for gonadotropin-dependent follicle growth. Thus, the follicle with the highest vascularized theca cell layer probably receives most gonadotropins, resulting in more pronounced growth. This indicates that VEGF might play a role in the selection of the dominant follicle. Furthermore, it is known that VEGF induces uPA, tPA and PAI-1 mRNA and activity (Olofsson *et al*., 1998; Pepper *et al*., 1991), components that are involved in ovulation as described above. Finally, an essential role for VEGF in corpus luteum angiogenesis was demonstrated (Ferrara *et al*., 1998).

Several authors investigated the possibility of predicting artificial reproduction outcome by measuring VEGF concentrations in FF. Friedman *et al*. (1998) and Asimakopoulos *et al*. (2005) observed that elevated VEGF concentrations in FF were associated with poor conception rates in artificial reproduction technique (ART) cycles. Women with elevated VEGF concentrations in FF had fewer oocytes retrieved at the ART procedure, lower peak serum estradiol concentrations, and reduced pregnancy rates (Friedman *et al*., 1998). These findings could imply that VEGF in the FF may be a marker of ovarian senescence or decreased ovarian reserve. This is in accordance with Lee *et al*. (1997) who reported that VEGF concentrations in FF correlated with age of women. Ocal *et al.* (2004) also observed that VEGF levels in FF were associated with poor conception rates in IVF. Elevated VEGF concentrations in FF were associated with fewer follicles, fewer oocytes retrieved, fewer mature

oocytes, and fewer embryos, consistent with ovarian aging. However, no correlation was observed between VEGF and chronological age of the women in this study (Ocal *et al*., 2004). In addition, it was found that fertilized oocytes developing into high quality embryos originated from follicles containing low levels of VEGF (Tozer *et al*., 2004).

In contrast with these findings, Kim *et al*. (2004) could not verify the relationship between the VEGF concentration in FF and age or pregnancy outcome. Also, no correlation was found between VEGF concentration in FF and the cause of infertility (Kim *et al*., 2004). Similarly, Attar *et al*. (2003) also could not find a correlation between VEGF concentration in FF and age of the women, number of oocytes retrieved, number of mature follicles, oocyte number, fertilization rate, or IVF success rates.

A possible side effect of ART cycles is the ovarian hyperstimulation syndrome (OHSS) due to exaggerated gonadotropin induced follicular stimulation. The OHSS is characterized by hyperpermeability of ovarian capillaries and several studies have implicated VEGF to be involved in the pathogenesis of OHSS (Geva and Jaffe, 2000; Ludwig *et al*., 1999; Meldrum, 2002). Women at risk of developing OHSS in an artificial reproduction program are treated by either discontinuation of the cycle or by withholding gonadotropin and delaying hCG administration. It is thought that coasting diminishes the functional granulosa cell cohort through increased granulosa cell apoptosis. Tozer *et al*. (2004) investigated the effect of withholding gonadotropins during controlled ovarian stimulation on FF VEGF concentration and observed a significantly lower FF VEGF concentration in the coasted group of women compared with the control group, possibly reflecting the diminished capacity of the remaining granulosa cells to produce VEGF.

Finally, VEGF seems to be involved in the aetiology of endometriosis (for review, see McLaren, 2000). Elevated concentrations of VEGF were found in FF of women with endometriosis compared with patients with Fallopian tube pathology (Attar *et al*., 2003). Furthermore, the expression of VEGF in endometriotic lesions appeared to be associated with the extent of their neovascularization, and the imbalance in VEGF expression in the endometrium could play a role in the development of endometriosis (Tan *et al*., 2002).

Conclusions

Subfertility is a problem which numerous couples have to face. Despite the available assisted reproduction techniques, such as IVF or ICSI, most subfertile couples remain involuntarily childless. This is at least in part because the causes of the subfertility are not tackled.

It is known that subfertility mostly is a multifactorial condition in which many factors of genetic, endocrine, environmental and life style origin are involved. The importance of these factors and their interplay is not clearly understood. To improve diagnosis and treatment of subfertility, a better understanding of several processes involved in reproduction like ovulation, spermatogenesis, fertilization and embryo implantation is required.

In order to further investigate the underlying mechanisms of subfertility, this review emphasises the importance of the PA-system and VEGF in reproduction. Further investigations in this field may lead to targeted therapies for subfertility. In males the PA-system is involved in tissue remodelling events in the seminiferous tubules, epididymal maturation of spermatozoa, and events important for fertilization like the acrosome reaction and attachment to the oocyte. In females, the PA-system components are involved in angiogenesis in the ovary and ovulation, and movement of both oocyte and spermatozoa in the Fallopian tubes. The function of VEGF in the male reproductive tract is still largely unknown, but VEGF seems to be involved in influencing the testicular microvasculature and the composition of seminal plasma. In the female reproductive tract VEGF is likely implicated in the cyclic ovarian angiogenesis, follicle development and selection, the accumulation of FF, and corpus luteum angiogenesis. Also, VEGF can influence the composition of fluid secretions in the female genital tract, influencing the motility and survival of sperm present in the female genital tract after sexual intercourse.

Most investigations discussed in this review involve experimental animal studies and the data clearly show that these systems are relevant. Therefore, it is justified to extend these results in humans. To obtain materials, blood, semen, spermatozoa, FF, or tissue, in order to study the involvement of the PA-system and VEGF in human fertility, te ART procedures are an important tool.

Chapter 7

Homocysteine, glutathione and related thiols affect fertility parameters in the (sub)fertile couple

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Abstract

Background: Thiols are scavengers of reactive oxygen species (ROS). We aim to investigate associations between thiols in various fluids in (sub)fertile couples and fertility outcome parameters.

Methods: In 156 couples undergoing assisted reproduction techniques we measured the concentrations of glutathione (GSH), cysteine (Cys), homocysteine (Hcy), and cysteinylglycine (CGS) and fertility outcome parameters in the ejaculate, purified spermatozoa and follicular fluid (FF).

Results: All thiols were detectable in most ejaculates, spermatozoa and FFs, of which Cys concentrations were highest. Thiol concentrations in the ejaculate were similar in fertile and subfertile men. However, Hcy in FF was higher in women with endometriosis compared with women in the idiopathic subfertile group ($P = 0.04$). The GSH, Cys, Hcy and CGS concentrations in spermatozoa of subfertile men were significantly higher compared with men in the idiopathic subfertile group and fertile men ($P < 0.001$). Most notably, Hcy concentrations in both the ejaculate and FF were negatively associated with embryo quality on culture day 3 in the IVF/ICSI procedure.

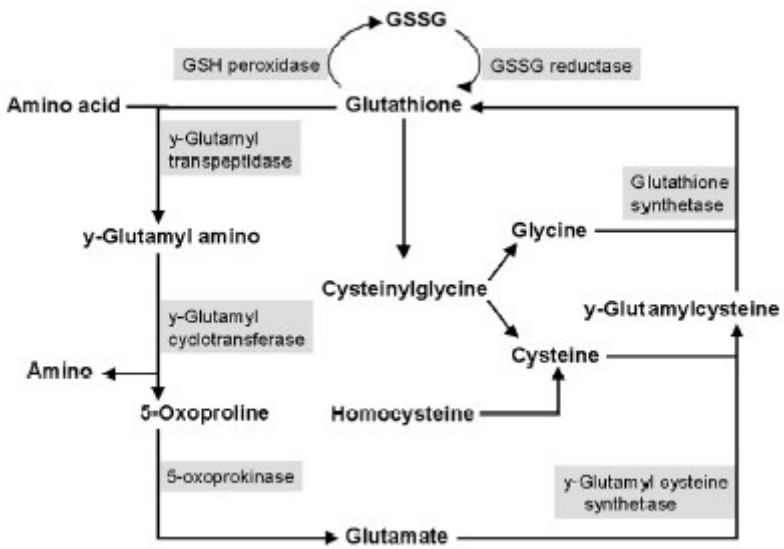
Conclusions: Spermatozoa of subfertile men contain significantly higher thiol concentrations as compared to those of fertile men. The detrimental effect on embryo quality of a high Hcy concentration in the ejaculate and in FF is intriguing and may suggest that Hcy is inversely associated with fertility outcome.

Introduction

Subfertility is a prevalent disorder occurring in approximately 10% of all couples during reproductive life. In about 30% of these couples, no cause for the subfertility can be found (Snick *et al.*, 1997).

In humans the thiol glutathione (GSH) (L-γ-glutamyl-L-cysteinyl-glycine) functions as the most important endogenous antioxidant involved in maintaining the pro-oxidant-antioxidant balance in human tissues. Other endogenous thiols are cysteine (Cys), homocysteine (Hcy) and cysteinylglycine (CGS). Cys is a precursor amino acid of GSH and both are taken up by food or are formed as a metabolic product of Hcy. CGS is composed of cysteine and glycine and is a main intermediate in the transport or synthesis of GSH (Figure 1).

Figure 1 Schematic summery of the glutathione metabolism



Schematic summary of glutathione metabolism. Glutathione is a tripeptide (L-γ-glutamyl-L-cysteinyl-glycine) synthesized from glutamate, cysteine and glycine in 2 consecutive steps catalyzed by γ-glutamylcysteine synthetase and glutathione synthetase. The enzyme γ-glutamyltranspeptidase is involved in the breakdown of glutathione, thereby cleaving the γ-bond resulting in glutamate and cysteinylglycine. Abbreviations: GSH, glutathione (reduced glutathione); GSSG, glutathione disulphide (oxidized glutathione).

GSH is also involved in the metabolism and detoxification of cytotoxic and carcinogenic compounds, and in the elimination of reactive oxygen species (ROS) (Shan *et al*., 1990).

Thiols and ROS are implicated in human reproduction. In humans, spermatozoa generate ROS which are known to affect hyperactivation of spermatozoa, the acrosome reaction, and the attachment of spermatozoa to oocytes thereby contributing to the fertilization of oocytes (de Lamirande *et al*., 1993; de Lamirande and Gagnon, 1993a; 1993b; Aitken *et al*., 1989a). Thiols are scavenging ROS and are therefore suggested to be important in sperm function and fertilization as well. The DNA in the spermatozoa head is intensely compacted as a result of disulphide bridges between oxidized Cys residues in protamine molecules important during the maturation of spermatozoa in the epididymis. The oxidation of thiols is also important for the stabilization of the tail structure, sperm motility, and the protection of sperm DNA against physical or chemical damage. After fertilization of the oocyte, the compacted sperm nucleus is decondensed to form the male pronucleus (PN). The decondensation depends on the presence of a small amount of free Cys capable of initiating a thiol-disulphide exchange (Rousseaux and Rousseaux-Prevost, 1995). Besides the beneficial effects of ROS, an excess of ROS is detrimental to spermatozoa and leads to damage of the DNA and plasma membrane through lipid peroxidation. Because spermatozoa have discarded most of their cytoplasm during the final stages of spermatogenesis, the availability of cytoplasmic defensive enzymes is limited, and therefore, these cells in particular are susceptible to ROS. Increased lipid peroxidation of spermatozoal plasma membranes may lead to altered membrane fluidity, which can render sperm dysfunction through impaired metabolism, acrosome reaction reactivity and ability of the spermatozoa to fuse with the oocyte (Cummins *et al*., 1994). This may result in abnormal sperm concentrations, loss of motility and abnormal morphology of the spermatozoa, leading to loss of fertility (Agarwal *et al*., 1994; Aitken *et al*., 1989b; Aitken *et al*., 1991; Sharma and Agarwal, 1996).

Little is known about the role of ROS and thiols at the level of the oocyte and female fertility. At the time of ovulation, the intense metabolism of granulosa cells and the high numbers of macrophages and neutrophilic granulocytes in the follicle wall may point at an active generation of ROS. Physiologic ranges of ROS are involved in oocyte maturation, luteolysis, progesterone production by

the corpus luteum, atretic regression of the cohort of newly grown follicles to leave only one follicle destined for ovulation and the ovulation itself (Riley and Behrman, 1991b; Miyazaki *et al*., 1991; Margolin *et al*., 1990; Sawada and Carlson, 1996). In similarity with males, one consequence of an excess of ROS in the ovary may be plasma membrane damage of the oocytes. The significance of such damage for female fertility, however, is unknown. It has been shown that follicular fluid (FF) contains free-radical scavengers to keep the ROS level in balance and to protect the oocyte and embryo (Jozwik *et al*., 1999; Pasqualotto *et al*., 2004). It is suggested from animal studies that the concentration of GSH in the oocyte is important to reduce the disulphide bonds during sperm nucleus decondensation and enable PN formation, decapitation, formation of the zygotic centrosome, and pronuclear apposition (Sutovsky and Schatten, 1997). This is endorsed by the observation that GSH antagonists disturbed the maturation of oocytes by compromising the decondensation of the sperm nucleus and thus preventing PN apposition. Furthermore, supplementation of Cys or GSH during *in vitro* maturation of oocytes resulted in improved male PN formation, normal fertilization, and embryo development (Jeong and Yang, 2001; Rodriguez-Gonzalez *et al*., 2003; Sawai *et al*., 1997). Also, enhancement of GSH or GSH-building blocks in the oocyte and in culture medium of embryos during IVF or ICSI procedures seems to improve fertilization rates and embryo development (Ali *et al*., 2003; Fukui *et al*., 2000; Kim *et al*., 1999; Takahashi *et al*., 1993). On the contrary, it is also known that high levels of ROS in the culture medium of embryos results in low blastocyst and low cleavage rates and high embryonic fragmentation (Bedaiwy *et al*., 2004). However, excessive scavenging of ROS by thiols has a negative effect on IVF, in particular fertilization, indicating that physiological levels of ROS are essential for normal fertilization (Ali *et al*., 2003; Bedaiwy *et al*., 2004; Kim *et al*., 1999).

Given the effects of ROS and thiols on physiological and pathological processes involved in fertility as described above, our goal was to systematically investigate the role of thiols in human fertility. Therefore, the aims of the present study were: (i) to measure the levels of GSH, Cys, Hcy, and CGS in the ejaculate, purified spermatozoa and FF at the day of oocyte retrieval of couples undergoing assisted reproduction techniques (ART); and (ii) to determine associations between thiol concentrations in semen,

spermatozoa and FF, and the fertility outcome parameters proportion of fertilized oocytes, proportion of cleaved embryos, mean embryo quality and pregnancy.

Materials and Methods

Patient selection

From April 2002 until May 2003 all patients visiting the fertility clinic of the Radboud University Nijmegen Medical Centre were considered for participation. The selection criteria for participation comprised the following diagnostic categories: (i) idiopathic subfertility, absence of abnormalities in both man and woman regarding semen and endocrine analyses, menstrual cycle, Chlamydia serology and hysterosalpingogram, but no spontaneous conception within 1 year of unprotected intercourse; (ii) female factor subfertility consisting of endometriosis or Fallopian tube pathology (any condition leading to an impediment of oocyte pick-up or transport); or (iii) male factor subfertility (MFS) defined by the presence of at least one of the sperm anomalies oligozoo-, asthenozoo-, and/or teratozoospermia. Couples with both a male and a female factor explaining their subfertility and patients first visiting other hospitals for fertility treatment were excluded from the study. The group of fertile men was defined as the male partners without abnormal semen parameters of the women suffering from Fallopian tube pathology or endometriosis. The group of fertile women without tube and menstrual cycle abnormalities comprised the partners of the men with MFS.

All materials collected were anonymized. The patients were notified about the study by brochures available in the waiting room of the IVF treatment unit. Information was given on the background and objectives of the study, inclusion criteria, study periods and other relevant procedures. It was acknowledged that the decision whether or not to participate in the study would neither interfere nor have consequences for the IVF or ICSI treatment.

The materials have been collected in accordance with the guidelines of the ethical and institutional board of the Radboud University Nijmegen Medical Centre.

A total of 156 couples were enrolled in the study and information concerning age, type of treatment (IVF or ICSI), 17β-estradiol (E_2) concentration in serum

of the women and FSH dose (Puregon, Organon, Oss, The Netherlands) administered for ovarian stimulation treatment was made available from the medical records.

Ejaculate collection

Participants provided the ejaculates in polypropylene containers produced via masturbation at home or in the hospital after an abstinence period of 3-5 days. After liquefaction for 20 minutes, the total volume was measured and the main part of the sample was prepared for IVF or ICSI. Two hundred microlitres of the remaining ejaculate was obtained for semen analysis and preservation. Semen analysis was performed according to the guidelines of the World Health Organization (Guzick *et al*., 2001; World Health Organization, 1992). Thus, sperm concentration was determined using a Makler counting chamber, designed in a 10-by-10 compartments frame format. Motility was expressed as the proportion of motile spermatozoa, and morphology was determined after incubation of the sample with trypsin (10 minutes at room temperature), staining with methylene blue/eosin, feathering, and fixation by flame. The sample was frozen without preservatives and stored at –80 °C until assay for the thiol concentrations.

Spermatozoa collection

The ejaculate provided for the IVF or ICSI procedure (minus the 200 microlitre obtained for this study) was purified by means of centrifugation on 80% Pure Sperm reagent (Nidacon International AB, Sweden). After isolation, the concentration and motility of spermatozoa was determined according to the WHO guidelines. In the following step, the spermatozoa were diluted with culture medium (Human Tubal Fluid, Cambrex company, Belgium) to the concentrations required for the IVF or ICSI procedures. Residual concentrated sperm was collected, frozen without preservatives and stored at –80 °C until assay for the thiol concentrations.

Follicular fluid collection

After oocyte retrieval for the IVF or ICSI procedure a sample of the follicular fluid (diluted with 10 IU/ml heparin and phosphate buffer, pH 7.4) was centrifuged for 10 minutes at 2000 *g* to separate red blood cells, leukocytes

and granulosa cells from the follicular fluid. The follicular fluid was frozen without preservatives and stored at –80 °C until assay for the thiol concentrations, 17β-E_2, progesterone and total protein content.

Fertility outcome parameters

The number of follicles and the number of follicles > 15 mm were determined using ultrasound 2 days before the follicles were punctured. One day after the IVF or ICSI procedure, fertilization was determined by counting the number of pronuclei in the oocyte. The proportion of fertilized oocytes was calculated by dividing the number of oocytes with two pronuclei by the total number of oocytes retrieved (IVF), or by the total number of oocytes injected (ICSI).
On the following 2 days, the embryos were examined once a day for development. The proportion of cleaved embryos was calculated following division of the number of cleaved embryos on day 3 by the total number of oocytes retrieved (IVF), or the total number of oocytes injected (ICSI). Furthermore, embryo quality was established by judgement of fragmentation and cleavage activity of the embryos after 3 days of culturing, and was denoted as low, moderate, reasonable or high.
An hCG-based pregnancy test was performed in first-morning voided urine from the woman on day 15 after the embryo transfer. The result of this test was communicated by phone to the personnel of the fertility clinic.

17β-Estradiol and progesterone in FF

The concentrations of 17β-E_2 and progesterone in FF were measured by a specific procedure described previously (Thomas *et al.*, 1977). Fifty microlitres of a FF specimen was extracted twice with diethylether, and after drying, the residue containing 17β-E_2 and progesterone was further purified by chromatography on Sephadex LH-20 columns. The steroids were quantified by specific radioimmunoassays.

Total protein content in FF

The FFs were assayed for protein concentration using the Pierce BCA protein assay reagent kit (Pierce, Rockford, IL). Absorptions were read at 540 nm in an automatic microtiter plate reader (Multiskan Ascent, Labsystems). The protein

content of the FF was determined as a biomarker for the maturity of the follicle (Spitzer *et al*., 1996).

Thiol assays

For the analysis of the thiol contents of the spermatozoa, these cells first had to be lysed. This was accomplished by freezing in liquid nitrogen and subsequent thawing for five times. Subsequently, the thiol assay was similar for the spermatozoa, the ejaculate and the FF. The samples were diluted six times with 12% (v/v) perchloric acid and centrifuged for 5 minutes at 10 000 *g*. Next, 10 µl 10% (w/v) Tris (2-carboxyethyl) phospine (Fluka Chemie AG, Bornem, The Netherlands) was added to 100 µl of each sample. After reduction for 30 minutes at room temperature, samples were neutralized by adding 76 µl, 2 mol/l NaOH. Subsequently, 100 µl of the neutralized sample was derivatized with 7-fluorobenzofurazane-4-sulfonic acid (SBDF; Fluka Chemie AG) for 1 h at 60 °C by adding 60 µl of derivatization 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 mol/l) (Raijmakers *et al*., 2001). Of each derivatized sample, 20 µl was injected and thiols were eluted with an isocratic eluent (2.0% methanol in 0.1 mol/l acetic acid, pH 5.0) at flow rates of 350 µl/minutes for 5 minutes, 600 µl/minutes for another 5 minutes and 300 µl/minutes for 15 minutes. Thiols were separated by high-performance liquid chromatography (HPLC) with fluorescent detection using an autosampler (Model Marathon, Spark, Holland), solvent delivery system (High Precision Pump model 480; Gynkotek, Munich, Germany), and fluorescent detector (Intelligent Spectrofluorometric Detector model 821-FP; Jasco, H.I. Ambacht, The Netherlands) operating at an excitation wavelength of 385 nm and an emission wavelength of 515 nm. The separating column (Inertsil ODS-2; 100 x 3 mm, 5-µm particle size) and guard column (R2; 10 x 2 mm) were from Chrompack (Middelburg, The Netherlands). Data obtained were analysed with the Chromeleon chromatography data system (Gynkotek, Munich, Germany). Concentrations of thiols were determined using calibration curves for all thiols, which were run in parallel with the samples. These calibration curves were prepared by diluting stock solutions with 0.9% sodium chloride/4mmol/l EDTA that were stored in small aliquots at –30 °C. All samples were analysed in

duplicate.

Statistical analyses

The fertility outcome parameters were normally distributed and the results are expressed as means and standard deviation (SD). The proportion of fertilized oocytes, cleaved embryos and mean embryo quality in the IVF and ICSI groups were compared using the independent sample *t*-test. The number of clinical pregnancies occurring in IVF and ICSI groups was compared using the Chi-square test.

Thiol concentrations in the ejaculate were normally distributed and the results are expressed as means and SD. Thiol concentrations in spermatozoa and FF were skewed; therefore natural logarithmic transformations were performed and data are expressed as geometrical means and 25^{th}-75^{th} percentiles. The thiol concentrations between groups were compared using one-way analysis of variance (ANOVA) and further analysed using *post hoc* Bonferroni comparisons.

In the pooled group of men, Pearson correlations were calculated between thiol concentrations in the ejaculate and the logarithmic transformed characteristics of the study population age, sperm concentration, motility percentage and percentage abnormal cells. In the pooled group of women, Pearson correlations were calculated between the logarithmic transformed thiol-protein ratios in FF and the study population characteristics described in Table II.

Linear regression analyses were performed in the pooled group of men or women to find associations between the fertility outcome parameters, being the proportion of oocytes fertilized and the proportion of cleaved embryos on culture day 3 as the independent variables and GSH, Cys, Hcy and CGS concentrations in the ejaculate, in spermatozoa and in FF as the independent variables. Also, logistic regression analyses were carried out in the pooled group of men or women to find associations between the dependent variables embryo quality on culture day 3 (expressed as low-moderate, or reasonable-high) and achievement of pregnancy (yes or no) and the independent continous variables such as GSH, Cys, Hcy and CGS concentrations in ejaculate purified spermatozoa and FF. Potential confounders were included in the linear or logistic regression models, and the factors that substantially affected the association between the dependent variable and the thiol variable

(causing a change in the regression coefficient of more than 10%) were maintained in the final models.

A *P*-value of ≤ 0.05 was considered statistically significant. Statistical analyses were performed using SPSS 12.0 for Windows software (SPSS Inc, Chicago, IL, USA).

Results

A total of 156 couples undergoing ART were evaluated in this study of which 67.9% received IVF treatment and 32.1% underwent an ICSI procedure. Most idiopathic subfertile couples and almost all couples experiencing female factor subfertility underwent IVF treatment, 84.6% and 96.2%, respectively. Most couples experiencing MFS however, received ICSI treatment (e.g. 76.9%).

The basic characteristics of the male study group are summarized in Table I. Patient's age and volume of the ejaculate were comparable between the groups. As expected, the sperm concentration and motility in the ejaculate and purified samples, and morphology in the ejaculate were different in the MFS group compared with fertile men and men in the idiopathic subfertile group.

The basic characteristics of the female study group are summarized in Table II. The only difference between the four diagnostic subgroups was age. Women with endometriosis were slightly younger as compared to women in the idiopathic subfertile group.

The outcome parameters in the IVF, ICSI, and total group of couples are presented in Table III. The proportion of fertilized oocytes and the proportion of cleaved embryos were significantly higher in the ICSI group, compared with the IVF group, $P = 0.04$ and $P = 0.01$, respectively. However, the mean embryo quality and the number of clinical pregnancies are comparable between the two treatment groups.

The concentrations of the thiols measured in the ejaculate and spermatozoa are presented in Table IV and V, respectively. All thiols could be determined in both specimens. However, because measurement of the ejaculate samples gave considerable problems by congesting the separating column of the HPLC apparatus, only 77 of the total number of 156 samples could be evaluated. Of the thiols measured, Cys was present in the highest concentrations in the total ejaculate samples. The concentrations of the four different thiols in ejaculate were comparable between the three diagnostic subgroups. Furthermore, thiol

concentrations in the ejaculate were much higher than the concentrations in spermatozoa (results not shown).

Table I Characteristics of men undergoing an IVF/ICSI procedure

Characteristics	Fertile (*n* = 52)	IS (*n* = 52)	MFS (*n* = 52)
Age (years)	35.0 (32.0-37.7)	35.7 (34.0-38.0)	34.7 (32.0-37.7)
Volume ejaculate (ml)	2.8 (2.1-3.5)	2.9 (2.2-4.4)	3.3 (2.5-4.8)
Ejaculate Sperm conc (x10^6/ml)	66.1 (45.5-100.0)	55.6 (31.3-100.0)	3.8 (1.2-14.5)
Motility (%)	56.2 (50.0-65.0)	54.0 (50.0-63.8)	18.9 (10.0-30.0)
Abnormal cells (%)	78.7 (72.0-87.0)	81.1 (75.0-87.8)	93.0 (89.0-97.0)
Purified sperm (x10^6/ml)			
Sperm conc (x10^6/ml)	19.9 (10.0-53.7)	11.9 (5.0-30.0)	2.2 (0.7-5.0)
Motility (%)	75.8 (70.0-90.0)	67.9 (60.0-85.0)	29.8 (20.0-50.0)

Note: values are given as geometrical mean and (25^{th} –75^{th} percentile). IS = idiopathic subfertility, MFS = male factor subfertility.

Table II Characteristics of women undergoing an IVF/ICSI procedure

Characteristics	Fertile (*n* = 52)	IS (*n* = 52)	FTP (*n* = 26)	EM (*n* = 26)
Age (years)	32.8 (30.3-36.0)	34.3 (32.0-36.0)	33.2 (31.0-38.0)	31.5 (29.0-35.3)
Puregon dose (IU)	179 (150-250)	173 (150-200)	182 (150-300)	170 (144-263)
E_2 in blood (pmol/l)	5114 (3725-7575)	5499 (3925-8350)	5545 (5025-7025)	5603 (4050-8500)
E_2 in FF (nmol/l)	871 (660-1350)	924 (683-1300)	971 (763-1400)	849 (540-1425)
Prog in FF (µmol/l)	24.7 (19.0-36.8)	27.3 (22.5-35.8)	22.2 (16.0-32.3)	23.5 (19.0-33.0)
Protein in FF (µg/l)	38.2 (34.0-43.5)	39.1 (35.8-45.8)	38.7 (33.6-47.2)	38.9 (32.7-49.1)
Follicles (n)	12.0 (9.0-20.8)	14.1 (10.0-21.0)	13.3 (10.8-17.0)	14.4 (13.0-20.3)
Follicles> 15mm (n)	6.2 (5.0-10.0)	6.5 (5.0-10.0)	7.1 (6.0-9.0)	6.9 (4.8-11.3)
Oocytes (n)	8.5 (6.0-13.0)	9.2 (7.0-13.8)	8.6 (6.0-13.0)	8.2 (5.8-13.0)

Note: values are given as geometric mean and (25^{th} –75^{th} percentile). E_2 = 17β-estradiol, EM = endometriosis, FF = follicular fluid, FTP = Fallopian tube pathology, IS = idiopathic subfertility, Prog = progesterone.

Table III Outcome parameters of IVF and ICSI procedures

	IVF group (*n* = 106)	ICSI group (*n* = 50)	Total group (*n* = 156)
Fertilization rate (%)	55.9 (26.6)	65.1 (25.7)[a]	58.9 (26.6)
Cleavage rate (%)	65.9 (27.5)	76.2 (19.7)[a]	69.2 (25.7)
Mean embryo quality	2.2 (0.7)	2.0 (0.8)	2.1 (0.8)
No. of pregnancies (%)	33 (31.1)	17 (34.0)	50 (32.1)

Note: values are given as mean (SD). a: $P < 0.05$

[†] Embryo quality is scored on a scale of 0 to 3; 0 = low, 1 = moderate, 2 = reasonable and 3 = good embryo quality.

Table IV Thiol concentrations in ejaculate of men undergoing an IVF/ICSI procedure

Thiol concentrations (µmol/l)	Fertile (*n* = 28)	IS (*n* = 26)	MFS (*n* = 23)
Glutathione	7.7 (2.4)	7.2 (2.9)	6.2 (2.9)
Cysteine	36.9 (17.2)	32.5 (13.4)	31.0 (14.9)
Homocysteine	5.9 (3.1)	5.8 (3.4)	4.2 (3.0)
Cysteinylglycine	7.9 (3.4)	7.9 (3.5)	7.9 (2.9)

Note: values are given as mean and (SD). IS = idiopathic subfertility, MFS = male factor subfertility.

Table V Thiol concentrations in spermatozoa of men undergoing an IVF/ICSI procedure

Thiol concentrations (pmol/million cells)	Fertile (*n* = 52)	IS (*n* = 52)	MFS (*n* = 52)
Glutathione	0.90 (0.42-2.25)	1.60 (0.42-4.00)	7.29 (3.10-20.0)[a,b]
Cysteine	60.8 (34.1-103.0)	106.5 (32.8-257.2)	426.1 (172.1-946.9)[a,b,c]
Homocysteine	12.4 (6.35-20.0)	20.9 (8.83-44.3)	58.8 (26.3-155.0)[a,b]
Cysteinylglycine	8.9 (3.72-15.5)	17.3 (5.7-40.5)	86.1 (39.0-222.3)[a,b,c]

Note: values are given as geometrical mean and (25^{th}-75^{th} percentile). *Post hoc* Bonferroni comparisons a: between the fertile and male factor subfertility (MFS) group, $P < 0.001$; b: between the idiopathic subfertile (IS) and MFS group, $P < 0.001$; c: between the fertile and IS, $P \leq 0.05$.

The GSH concentrations in spermatozoa were below the detection limit in 122 out of 156 samples tested. These 122 samples were allocated a value of 0.01 µmol/l (half of the lowest detectable value for GSH in spermatozoa). Thiols measured in the purified spermatozoa samples were eventually expressed as pmol thiol/million spermatozoa to compare the thiol concentrations between the different diagnostic subgroups. Again the concentrations of Cys were much higher compared with the other thiols measured. All thiol concentrations in spermatozoa differed between the three diagnostic subgroups (ANOVA, $P < 0.001$). *Post hoc* Bonferroni comparisons revealed that spermatozoa of men in the MFS group contained significantly higher GSH (mean difference 2.0 pmol (95% CI 1.37-2.62); $P < 0.001$), Cys (mean difference 1.95 pmol (95% CI 1.39-2.50); $P < 0.001$), Hcy (mean difference 1.56 pmol (95% CI 0.99-2.12); $P < 0.001$), and CGS (mean difference 2.27 pmol (95% CI 1.69-2.85); $P < 0.001$) per million spermatozoa as compared to those in fertile men. Similarly, spermatozoa of men in the MFS group contained significantly higher GSH (mean difference 1.51 pmol (95% CI 0.89-2.14); $P < 0.001$), Cys (mean

difference 1.39 pmol (95% CI 0.83-1.95); $P < 0.001$), Hcy (mean difference 1.04 pmol (95% CI 0.48-1.60); $P < 0.001$), and CGS (mean difference 1.60 pmol (95% CI 1.02-2.18); $P < 0.001$) per million spermatozoa as compared to these thiol concentrations in spermatozoa of men in the idiopathic subfertile group. Furthermore, *post hoc* Bonferroni comparisons revealed that Cys concentrations in spermatozoa of men in the idiopathic subfertile group were significantly higher compared with those in fertile men (mean difference 0.56 pmol per million spermatozoa (95% CI 0.001-1.12); $P = 0.049$). Also, CGS concentrations in spermatozoa of men in the idiopathic subfertile group were significantly higher compared with those in fertile men (mean difference 0.67 pmol per million spermatozoa (95% CI 0.09-1.25); Bonferroni $P < 0.02$).

The concentrations of the thiols in FFs are presented in Table VI. All thiols were detectable in the FF samples. The Hcy concentrations in the FF were below the detection limit in 43 out of 156 samples tested. These 43 samples were allocated a value of 0.1 µmol/l (half of the lowest detectable value for Hcy in FF). Thiols measured in FF were eventually expressed as µmol thiol/mg protein to adjust for the maturity of the follicle. In similarity to the ejaculate samples, Cys was present in the highest concentrations as compared to the other thiols measured. In the women, FF Hcy concentrations differed between the diagnostic subgroups (ANOVA, $P = 0.02$). The Hcy concentrations in the FF of endometriosis patients were significantly higher compared with those in women in the idiopathic subfertile group (mean difference 0.71 µmol/mg protein (95% CI 0.02-1.39); Bonferroni, $P = 0.04$).

Table VI Thiol concentrations in follicular fluids of women undergoing an IVF/ICSI procedure

Thiol concentrations (µmol/mg protein)	Fertile ($n = 52$)	IS ($n = 52$)	FTP ($n = 26$)	EM ($n = 26$)
GSH	0.31 (0.24-0.43)	0.29 (0.24-0.43)	0.25 (0.18-0.44)	0.28 (0.23-0.47)
Cys	1.40 (1.0-2.0)	1.32 (1.0-1.9)	1.14 (1.0-1.4)	1.37 (1.0-2.1)
Hcy	14.7 (9.9-26.7)	9.2 (2.6-27.1)	9.8 (2.9-23.2)	18.8 (14.4-44.4)[a]
CGS	0.21 (0.16-0.26)	0.20 (0.14-0.25)	0.17 (0.13-0.22)	0.22 (0.17-0.31)

Note: values are given as geometrical mean and (25^{th} and 75^{th} percentile). a: $P = 0.02$

CGS = cysteinylglycine, Cys = cysteine, EM = endometriosis, FTP = Fallopian tube pathology, GSH = glutathione, Hcy = homocysteine, IS = idiopathic subfertility.

Furthermore, we calculated correlations between the thiol levels in the ejaculate versus the various basic characteristics. No significant correlations were observed for GSH, Hcy or CGS in the ejaculate, however, Cys in the ejaculate was inversely correlated with the proportion of abnormal spermatozoa (r = -0.29; P = 0.01). Likewise, in women correlations were calculated between the thiol-protein ratios in the FF and the fertility (outcome) parameters. The GSH-protein concentrations were positively correlated with the number of retrieved oocytes (r = 0.17; P = 0.03), and an inverse correlation was determined between the Cys-protein concentrations and progesterone levels in FF (r = -0.30; $P \leq 0.001$). No other significant correlations were observed. Linear and logistic regression analyses were performed to investigate whether the thiol concentrations in the ejaculate and spermatozoa were associated with the proportion of fertilized oocytes, the proportion of cleaved embryos, embryo quality or pregnancy. A significant association was observed between the Hcy concentration in the ejaculate and embryo quality on culture day 3, expressed as reasonable/high or moderate/low quality (OR = 0.83, 95% CI 0.70-0.98). None of the other characteristics confounded this association.

For the female parameters similar regression analyses were performed, and again Hcy concentrations in FF and embryo quality on culture day 3 were significantly associated (OR = 0.58, 95% CI 0.35-0.97) without confounding by other characteristics.

Finally, we combined the male and female data in one logistic regression model to assess the association between embryo quality on culture day 3 and Hcy concentrations. Included in the analyses were Hcy in the ejaculate, Hcy in spermatozoa, Hcy in FF and as confounders spermatozoa concentration and proportion motile spermatozoa in the purified spermatozoa sample, proportion motile spermatozoa in the ejaculate, and the number of oocytes retrieved. These regression analyses yielded an adjusted OR of 0.81 (95% CI 0.65-1.00; P = 0.05) for Hcy concentrations in the ejaculate and an adjusted OR of 0.42 (95% CI 0.18-0.94; P = 0.04) for Hcy concentrations in the FF in relation to the embryo quality on culture day 3 after IVF or ICSI procedures.

Discussion

This study demonstrates that women with endometriosis have higher FF Hcy concentrations compared with women in the idiopathic subfertile group. Furthermore, thiol concentrations in the ejaculate were higher as compared to those in spermatozoa indicating the important antioxidant function of the seminal plasma for the protection of spermatozoa. The thiol concentrations were significantly higher in purified spermatozoa of men with MFS as compared to fertile men and men in the idiopathic subfertile group. Unique and most interesting is that for the first time an association has been found between embryo quality in an IVF/ICSI procedure and Hcy concentrations in the total ejaculate and FF of the couple. It reveals that a 1 µmol/l decrease of the Hcy concentrations in the ejaculate was associated with a 1.2 fold higher chance of achieving a reasonable/high quality embryo in an IVF or ICSI procedure. Similarly, a 1 µmol/l decrease of the Hcy concentration in FF was associated with a significant 2.4 fold higher chance of a reasonable/high quality embryo in an IVF or ICSI procedure. These findings are supported by *in vitro* studies in which exposure of chicken embryos to Hcy concentrations varying from physiologic to toxic amounts leads to developmental defects, such as neural tube defects and cardiac abnormalities, growth retardation and lethality (Boot *et al*., 2004a; Rosenquist *et al*., 1996).

So far not much is known about specific individual thiol concentrations in ejaculate, spermatozoa and FF. More literature is available on the total non-enzymatic antioxidant capacity (TAC) of seminal plasma and spermatozoa. Most authors report significantly lower TAC levels in seminal plasma of subfertile men as compared to fertile men (Lewis *et al*., 1995; Lewis *et al*., 1997; Smith *et al*., 1996). More specifically, Raijmakers *et al*., (2003) reported significantly higher seminal plasma GSH concentrations in fertile men compared with subfertile men. In accordance with this finding, Alkan *et al*. (1997) observed lower levels of sulphydryl groups in seminal plasma of subfertile patients compared with fertile men. In contrast we could not observe any difference in total ejaculate thiol concentrations between fertile and subfertile men in our study group, which was comparable to the results of Lewis *et al*. (1997) and Ochsendorf *et al*. (1998).

Furthermore, other authors observed positive correlations between TAC or GSH levels in seminal plasma and sperm motility, whereas inverse correlations

were found between TAC or GSH levels and sperm morphology (Raijmakers *et al*., 2003; Smith *et al*., 1996). The latter observations are in accordance with the negative association between Cys concentrations in the ejaculate and the proportion of abnormal spermatozoa, as reported in this study. However, none of the other thiols were related to sperm morphology in our study group.

Measurement of thiols in spermatozoa by other research groups yielded conflicting results. Garrido *et al*. (2004) observed that the GSH concentrations in spermatozoa from fertile and idiopathic subfertile men were comparable, which is similar to our findings. However, these authors did observe significantly lower GSH concentrations in spermatozoa of samples with less than 5% normal morphology, whereas our results indicate that spermatozoa of men in the MFS group contain significantly higher concentrations of GSH. Also, Ochsendorf *et al*. (1998) found that spermatozoa of oligozoospermic patients contained much lower GSH concentrations than those of normozoospermic men. Similar to our results, Lewis *et al*. (1997) observed significantly higher thiol concentrations in spermatozoa of asthenozoospermic men. According to their explanation, this may be due to the contribution of ROS produced by spermatozoa in this group leading to the up-regulation of thiol synthesis in order to protect the spermatozoa from oxidative damage. These authors speculate that the high thiol concentrations caused the reduced motility in these spermatozoa, because of a decrease in disulphide bonding during sperm maturation in the epididymis. The different data could be explained therefore by differences in the cause and amount of triggering of the (anti)oxidant system.

So far, thiol concentrations in FF and the association with fertility parameters have not been studied before. More data, however, are available on the relationship between TAC and fertility. Although Pasqualotte *et al*. (2004) observed a positive association between TAC and pregnancy rate, in FF the TAC was not associated with oocyte maturity, fertilization, cleavage and embryo quality. Therefore, they concluded that a certain amount of oxidative stress is necessary for the establishment of pregnancy. In contrast, Oyawoye *et al*. (2003) reported that the mean TAC level in FF from follicles yielding oocytes that were successfully fertilized, was significantly higher than the TAC levels from FF associated with oocytes that were not fertilized. Furthermore, they observed that the TAC level of FF from follicles whose oocytes gave rise to an embryo that survived until the moment of transfer was significantly lower

compared with the FF TAC level resulting in non-viable embryos. Therefore, these authors conclude that ROS may have different effects at different stages of embryonic development, and that the role of ROS prior to ovulation differs from that in relation to fertilization and embryo viability. Similar to our results, Attaran *et al*. (2000) did not find an association between TAC levels in FF and pregnancy rates. However, they observed a positive correlation between the ROS levels in FF and pregnancy rate, which is supported by others (Bedaiwy *et al*., 2002).

Direct comparisons of the results of other studies is often difficult, because of the differences in the methods used for the processing and analysis of semen. Most researchers use the guidelines of the WHO to classify the diagnostic subgroups and semen samples. Others, such as Garrido *et al*. (2004) also used Tygerberg strict criteria for sperm morphology. In addition centrifugation, the use of Percoll gradients or swim-up techniques for the purification of spermatozoa are often applied. The methods used to measure (anti)oxidants or (anti)oxidant activity vary significantly in the various studies cited. In most studies TAC was measured by using enhanced chemoluminescence assays; total sulfhydryl groups were measured after 5,5'-dithiobis (2-nitrobenzoic acid) derivation and subsequent spectrophotometry or lipid peroxidation tests were performed by using thiobarbituric acid tests. The only authors specifically measuring GSH also used 5,5'-dithiobis (2-nitrobenzoic acid) derivation and subsequent spectrophotometry (Ochsendorf *et al*., 1998) or biochemical reactions and spectrophotometry methods (Garrido *et al*., 2004). Only Raijmakers *et al*. (2003) used the same HPLC method we used. However, they measured GSH in seminal plasma after centrifugation of the total ejaculate, in which spermatozoa and other cell materials were removed.

The only study focused on the association between embryo quality and antioxidants in an IVF or ICSI setting published so far was performed by Paszkowski and Clarke (1996). They found that incubation of poor quality embryos was associated with a decline in TAC in the preimplantation embryo culture medium, which was significantly larger than that observed in good and fair embryos. These authors therefore stated that impaired embryo development may be associated with an increased generation of ROS by the embryo. No studies were done so far investigating the role of thiols in the ejaculate or FF, in association with the subsequent embryo quality after an IVF

or ICSI procedure.

The findings of the higher FF Hcy concentrations in women with endometriosis and the inverse association between FF Hcy levels and embryo quality are supported by previous prospective and retrospective studies. These clinical trials demonstrate a decreased oocyte and embryo quality in women with endometriosis, which is suggested to be due to an altered intrafollicular milieu in endometriosis. We found a biochemical difference in the FF Hcy level that can be due to environmental factors, such as nutrition and lifestyle, and genetic variations in for example folate genes. If the detrimental effects of endometriosis are non-genetic in origin, modulation of the process of folliculogenesis may become feasible to treat the disease and cure the infertility (Garrido *et al*., 2002).

Some limitations of the present study have to be addressed. The separation of spermatozoa from seminal plasma in IVF or ICSI procedures, the washing, centrifugation, vortexing and also freeze-thawing may have generated ROS in the spermatozoa sample in contrast to the ejaculate. As a consequence, GSH concentrations in the spermatozoa samples may have decreased. Thus, it is very likely that the thiol concentrations measured underestimate the in vivo antioxidant capacity of the spermatozoa (Gadea *et al*., 2004). This may also explain the many samples with an undetectable GSH concentration in our study. However, because all samples were treated exactly in the same manner, we believe that the differences observed between fertile and subfertile men are real. Next, it is also possible that due to some leukocyte contamination of the ejaculate samples, the ROS production was increased and counteracted by thiols that consequently decreased in particular in the subfertile population (Aitken *et al*., 1992). Because the thiol concentrations in the ejaculate were comparable between fertile and subfertile men in our study and higher than the concentrations in subfertile men reported by others, we do not believe that the presence of leukocytes was a factor of significance. With regard to the purified spermatozoa samples, similar problems with contaminating leukocytes could arise. We believe the purification of spermatozoa with the Pure Sperm gradient had discarded almost all present leukocytes; however, we did not verify the efficiency of this purification. Therefore, it is possible that purified spermatozoa samples of subfertile men in particular still contained some leukocytes. This would result in higher ROS concentrations in these samples leading to a

depletion of thiols. Because our results show that spermatozoa of subfertile men contain significantly higher thiol concentrations, we do not believe that contamination with leukocytes has interfered with our results.

Furthermore, the FF samples collected were pooled samples from different follicles. Therefore, it may be incorrect to directly correlate the FF thiol concentrations with the development of a specific embryo. However, we could not change the method of FF collection because we had to adhere to the regular IVF and ICSI protocols.

Finally, because we examined several variables and their associations with fertility outcome parameters, multiple testing could be an issue in our study. Because all the thiols measured were higher in the spermatozoa of subfertile men, and both the Hcy concentrations in ejaculates as well as in the FFs were negatively associated with embryo quality, in concordance with the toxicity of Hcy reported in the literature, we believe that the results obtained in this study do not merely reflect chance.

The strengths of our study is the relatively large number of couples undergoing an IVF or ICSI procedure that were investigated. Furthermore, our study group is homogeneous and subfertile, and fertile men or women are very comparable because we applied strict selection criteria. We only included couples who were unable to achieve a spontaneous conception within 1 year of regular, unprotected intercourse. Women with menstrual disorders were excluded, as were couples with both a male and a female factor explaining their subfertility or patients with multiple causes for subfertility within one individual.

In conclusion, thiol concentrations in spermatozoa of subfertile men are significantly higher compared with the concentrations in spermatozoa of fertile men. These high thiol concentrations may result in a diminished motility, because of decreased disulphide bonding during sperm maturation in the epididymis and perhaps may also lead to excessive scavenging of ROS to levels below those physiologically necessary for normal sperm function. However, we did not measure in parallel the ROS levels and therefore further research is necessary to verify this explanation. Intriguing are the high Hcy concentrations in the ejaculate and FF which are associated with moderate/low embryo quality, and the corresponding higher FF Hcy concentration in women with endometriosis, known to suffer from low embryo quality. This strongly suggests a predictive value for embryo quality in artificial reproduction of the

Hcy concentration in these biological fluids.

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Chapter 8

Possible role of the plasminogen activator system in human subfertility

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Abstract

Objective: To correlate components of the plasminogen activator (PA)-system with fertility outcome parameters in participants of an IVF/ICSI procedure.
Design: Case-control study.
Setting: Outpatient clinic for IVF/ICSI treatment at the Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands.
Participants: 156 couples undergoing an IVF/ICSI procedure .
Interventions: None
Main Outcome Measures: Urokinase-type plasminogen activator (uPA), tissue-type plasminogen activator (tPA), plasminogen activator inhibitor 1 (PAI-1) and tPA-PAI-1 complex concentrations in the ejaculate, spermatozoa and follicular fluid (FF).
Results: Concentrations of tPA were higher in spermatozoa of the male factor subfertility group (geometrical mean 77.1 pg/million spermatozoa, 25^{th}-75^{th} percentile (31.8-211.2) compared with fertile (1.91 (0.74-5.79) and idiopathic subfertile men (3.14 (0.97-9.97), $P < 0.001$). Furthermore, the concentration of tPA in the spermatozoa is significantly associated with pregnancy (OR 0.995, $P = 0.03$). Likewise, a trend was shown for higher tPA concentrations in FF of women with Fallopian tube pathology (geometrical mean 18.5 pg tPA/mg protein, 25^{th}-75^{th} percentile (11.4-25.7) or endometriosis (18.8 (11.4-27.1) compared with fertile (14.3 (10.3-17.6) and idiopathic subfertile women (13.9 (9.5-17.8), $P = 0.07$. Also, tPA in FF is associated with the proportion of cleaved embryos (regression coefficient 0.16, $P < 0.001$). The concentrations of uPA, PAI-1 and tPA-PAI-1 complex were comparable between the diagnostic subgroups in both men and women.
Conclusions: The tPA concentrations in spermatozoa and in follicular fluid tend to be higher in human subfertility and seem to be associated with some fertility outcome parameters.

Introduction

Subfertility is a prevalent disorder occurring in approximately 10-17% of all couples during reproductive life (Snick *et al*., 1997). In many of these couples no cause for the subfertility can be found.

The components of the plasminogen activator (PA)-system are involved in the activation of the inactive proenzyme plasminogen into the active serine proteinase, plasmin. Plasmin degrades extracellular matrix (ECM) proteins. The PA-system involves 2 plasminogen activators, the urokinase-type plasminogen activator (uPA) and the tissue-type plasminogen activator (tPA). Urokinase PA and tPA activities are effectively controlled by specific inhibitors like plasminogen activator inhibitor 1 (PAI-1) (Andreasen *et al*., 1997; Dano *et al*., 1985; Schmitt *et al*., 1997). The components of the PA-system occur in a variety of different cell types and are involved in several biological processes. Degradation of the ECM resulting from plasminogen activation is important in cell migration, necessary for morphogenesis, tissue repair, neovascularization or invasion of malignant cells. One of the interesting areas in which the PA-system appears to play an important role is reproduction.

In the male, uPA and tPA are produced by Sertoli cells (Hettle *et al*., 1986; Lacroix *et al*., 1977; Vihko *et al*., 1986). Proteolysis mediated by these 2 components is suggested to be involved in the release of preleptotene spermatocytes from the basement membranes (Hettle *et al*., 1986), passage of spermatocytes across the blood testis barrier (Zhang *et al*., 1997b), spermiation (LeBlond and Clermont, 1952), and the detachment of residual bodies from the mature spermatids (Morales *et al*., 1986). The PA-system seems also involved in the release of mature spermatozoa into the lumen of the tubules (Zhang *et al*., 1997b). It has been reported that in the epididymis the PA components play a role in the alteration of sperm surface molecules (Eddy *et al*., 1985; Tulsiani *et al*., 1995). These alterations are necessary for sperm maturation and motility, and the fertilizing ability of the spermatozoa. The PA-system is also involved in sperm surface modifications involved in capacitation (Talbot and Chacon, 1981; Talbot and Franklin, 1978), stimulation of the acrosome reaction (Dravland *et al*., 1984; Pillai and Meizel, 1991), and attachment to, and penetration of the spermatozoon into the zona pellucida (Smokovitis *et al*., 1992). In the female, a variety of ovarian tissues (granulosa, theca (Beers *et al*., 1975; Canipari and Strickland, 1985; Liu *et al*., 1987; Ny *et*

al., 1985), cumulus, oocyte (Liu *et al*., 1986) and ovarian surface epithelial cells (Colgin and Murdoch, 1997)) are known to produce PA components, which are under gonadotropin control (Canipari *et al*., 1987; Canipari and Strickland, 1985; O'Connell *et al*., 1987). Of its many functions, these components are involved in angiogenesis-associated proteolysis, such as the transition of the unvascularized preantral follicles into mature Graafian follicles (Bacharach *et al*., 1992) and in the regulation of follicular fluid (FF) viscosity, prevention of blood clotting during rupture of the follicular wall, thereby preventing entrapment of the cumulus-oocyte complex inside the follicle (Canipari and Strickland, 1985). Also, the PA-system plays a part in the changing interactions between oocyte and cumulus cells important for cumulus expansion, oocyte pick-up, fertilization and eventually the ageing of the oocyte (D'Alessandris *et al*., 2001).

Thus, the PA-system is involved in a variety of reproductive processes in both males and females. With the possibilities of the assisted reproductive techniques our aim was to gain more insight into the role of the PA-system by: (i) measuring uPA, tPA and PAI-1 concentrations in human FF at the day of oocyte retrieval, ejaculate and spermatozoa of couples undergoing an IVF or ICSI treatment; and (ii) exploring associations between FF and semen PA component concentrations and various fertility parameters and the pregnancy rate, respectively.

Materials and methods

Patient selection

From April 2002 until May 2003 all patient couples visiting the fertility clinic of the Radboud University Nijmegen Medical Centre were considered for participation. The selection criteria for participation comprised the following diagnostic categories: (i) idiopathic subfertility, absence of abnormalities in both man and woman regarding semen and endocrine analyses, menstrual cycle, Chlamydia serology and hysterosalpingogram, but no spontaneous conception within 1 year of unprotected intercourse; (ii) female factor subfertility (FFS) consisting of endometriosis or Fallopian tube pathology (any condition leading to an impediment of oocyte pick-up and transport); or (iii) male factor subfertility

(MFS) defined by the presence of at least one of the sperm anomalies oligozoo-, asthenozoo-, and/or teratozoospermia. Couples with both a male and a female factor explaining their subfertility and patients first visiting other hospitals for fertility treatment were excluded from the study. Fertile men were the partner of a women with Fallopian tube pathology or endometriosis, without abnormalities in semen parameters. Fertile women comprised partners of the men with MFS, without tube and menstrual cycle abnormalities.
All material collected was made anonymous in accordance with the guidelines of the ethical and institutional board of the Radboud University Nijmegen Medical Centre. The patients were notified about the study by brochures available in the waiting room of the IVF treatment unit. The materials have been collected in accordance with the guidelines of the ethical and institutional board of the Radboud University Nijmegen Medical Centre. A total of 156 couples were enrolled in the study.

Ejaculate and spermatozoa collection

Participants provided semen samples produced via masturbation, after an abstinence period of 3-5 days. The main part of the sample was prepared for IVF or ICSI. Two hundred microlitres of the remaining semen sample was obtained for semen analysis performed according to the guidelines of the World Health Organization (Guzick *et al*., 2001; World Health Organization, 1992). The sample was frozen without preservatives and stored at –80 °C until assay for uPA, tPA and PAI-1 concentrations.
The ejaculate provided for the IVF or ICSI procedure (minus the 200 microlitre obtained for study purposes) was purified and after isolation the concentration and motility of spermatozoa was determined according to the guidelines of the WHO and the cells were diluted with culture medium (Human Tubal Fluid, Cambrex Company, Belgium). Residual concentrated sperm was collected, frozen without preservatives and stored at –80 °C until assay for uPA, tPA, PAI-1, and tPA-PAI-1 complex concentrations.

Follicular fluid collection

After oocyte retrieval for the IVF or ICSI procedure, a sample of the follicular fluid (diluted with 10 IU/ml heparin and phosphate buffer, pH 7.4) was

centrifuged for 10 minutes at 2000 *g* to separate cells from the fluid. The FF was frozen without preservatives and stored at –80 °C until assay for uPA, tPA, PAI-1, 17β-estradiol (E_2), progesterone and protein concentrations.

Fertility outcome parameters

The number of follicles grown and the number of follicles > 15 mm were determined using ultrasound 2 days before the follicles were punctured. One day after the IVF or ICSI procedure, fertilization was determined by counting the number of pronuclei in the oocyte. The percentage of fertilized oocytes was calculated by dividing the number of oocytes with two pronuclei by the total number of oocytes retrieved (IVF), or by the total number of oocytes injected (ICSI).

On the following 2 days, the embryos were examined once a day for development. On day 3 the proportion of cleaved embryos (vs. arrested at 2 pronuclei) was calculated by dividing the number of cleaved embryos by the total number of oocytes retrieved (IVF), or the total number of oocytes injected (ICSI). Furthermore, embryo quality was established by judgement of fragmentation and cleavage activity of the embryos after 3 days of culturing, and was denoted as low, moderate, reasonable or high.

An hCG-based pregnancy test was performed in first-morning voided urine from the woman on day 15 after the embryo transfer.

17β-Estradiol, progesterone and protein concentrations in FF

The concentrations of 17β-E_2 and progesterone in FF were measured by specific radioimmunoassays described previously by Thomas *et al*. (1977). The protein content of the FF was determined as a biomarker for the maturity of the follicle (Spitzer *et al*., 1996) using the Pierce BCA protein assay reagent kit (Pierce, Rockford, IL).

uPA, tPA, PAI-1 and tPA-PAI-1 complex concentrations

For the measurement of uPA, tPA and PAI-1 in follicular fluid, ejaculate and spermatozoa samples, and for the measurement of tPA-PAI-1 complexes in a subset of spermatozoa samples we used ELISAs developed by our department for components of the plasminogen activation system (Grebenchtchikov *et al.*,

1997). To increase the sensitivity of the assays, the colour detection was replaced by a fluorometric detection using the biotin-streptavidin-labelled β-Galactosidase detection as previously described by Manders *et al.* (2004). The spermatozoa samples were frozen in liquid nitrogen and subsequently thawed for five times to lyse the spermatozoa before the ELISA was performed. All samples were analysed in duplicate.

Statistical analyses

The fertility outcome parameters were normally distributed and the results are expressed as means and standard deviation (SD). The proportion of fertilized oocytes, cleaved embryos and mean embryo quality between the different diagnostic subgroups were compared using the one-way analysis of variance (ANOVA). The number of clinical pregnancies occurring in the different subgroups was compared using the Chi-square test.

Because of the skewed distributions of the PA-component variables measured in this study, natural logarithmic transformations were performed and data were subsequently expressed as geometrical means and (25^{th}-75^{th} percentiles). The uPA, tPA and PAI-1 concentrations between the groups were compared using ANOVA. The tPA and tPA-PAI-1 complex concentrations within the different subgroups of men were compared using the Paired-Samples T Test. Further analyses were carried out by *post hoc* Bonferroni comparisons.

In the pooled group of men, Pearson correlations were calculated between the concentrations of the PA-system components in the ejaculate and the logarithmic transformed semen characteristics of the study. In the pooled group of women, Pearson correlations were calculated between the logarithmic transformed PA-system component-protein ratios in FF and the study population characteristics described in Table II.

Linear regression analyses were performed in the pooled group of men or women to find associations between the dependent fertility outcome parameters and the continuous independent uPA, tPA and PAI-1 concentrations in the ejaculate, in spermatozoa and in FF. Also, logistic regression analyses were carried out in the pooled group of men or women to find associations between the dependent variables embryo quality on culture day 3, expressed as low-moderate or reasonable-high, achievement of pregnancy

(yes or no), and uPA, tPA and PAI-1 concentrations in ejaculate, purified spermatozoa and FF as the independent continuous variables. Potential confounders were included in the linear and logistic regression models; factors that substantially affected the association between the dependent variable and the PA-system components (causing a change in the regression coefficient of more than 10%) were maintained in the final models.

A *P*-value of ≤ 0.05 was considered statistically significant. Statistical analyses were performed using SPSS 12.0 for Windows software (SPSS Inc, Chicago, IL, USA).

Results

Basic characteristics

The basic characteristics in men and women are summarized in Table I and II, respectively. As expected, sperm concentrations and the proportion motility were substantially lower in the MFS group as compared to the fertile and idiopathic subfertile men. Furthermore, the MFS group displayed a substantially higher proportion of abnormal spermatozoa in the ejaculate compared with fertile and idiopathic subfertile men. Women with endometriosis were slightly younger as compared to idiopathic subfertile women. In Table III the fertility outcome parameters for the different diagnostic subgroups are summarized. All the fertility outcome parameters were comparable between the different subgroups.

Table I Characteristics of the male study population

Characteristics	Fertile (*n* = 52)	IS (*n* = 52)	MFS (*n* = 52)
Age (years)	35.0 (32.0-37.7)	35.7 (34.0-38.0)	34.7 (32.0-37.7)
Volume ejaculate (mL)	2.8 (2.1-3.5)	2.9 (2.2-4.4)	3.3 (2.5-4.8)
Sperm conc. ($x10^6$/ml)	66.1 (45.5-100.0)	55.6 (31.3-100.0)	3.8 (1.2-14.5)
Motility (%)	56.3 (50.0-65.0)	54.0 (50.0-63.8)	18.9 (10.0-30.0)
Abnormal cells (%)	78.7 (72.0-87.0)	81.1 (75.0-87.8)	93.0 (89.0-97.0)

Note: values are given as geometric mean and (25^{th} –75^{th} percentile). IS = idiopathic subfertility, MFS = male factor subfertility.

Table II Characteristics of the female study population

Characteristics	Fertile (*n* = 52)	IS (*n* = 52)	FTP (*n* = 26)	EM (*n* = 26)
Age women (years)	32.8 (30.3-36.0)	34.3 (32.0-36.0)	33.2 (31.0-38.0)	31.5 (29.0-35.3)
Puregon dose (IU)	179 (150-250)	173 (150-200)	182 (150-300)	170 (144-263)
E_2 in blood (pmol/l)	5114 (3725-7575)	5499 (3925-8350)	5545 (5025-7025)	5603 (4050-8500)
E_2 in FF (nmol/l)	871 (660-1350)	924 (683-1300)	971 (763-1400)	849 (540-1425)
Prog in FF (µmol/l)	24.7 (19.0-36.8)	27.3 (22.5-35.8)	22.2 (16.0-32.3)	23.5 (19.0-33.0)
Protein in FF (µg/l)	38.2 (34.0-43.5)	39.1 (35.8-45.8)	38.7 (33.6-47.2)	38.9 (32.7-49.1)
Follicles (n)	12.0 (9.0-20.8)	14.1 (10.0-21.0)	13.3 (10.8-17.0)	14.4 (13.0-20.3)
Follicles > 15mm (n)	6.2 (5.0-10.0)	6.5 (5.0-10.0)	7.1 (6.0-9.0)	6.9 (4.8-11.3)
Oocytes (n)	8.5 (6.0-13.0)	9.2 (7.0-13.8)	8.6 (6.0-13.0)	8.2 (5.8-13.0)

Note: values are given as geometric mean and (25^{th} –75^{th} percentile). E_2 = 17β-estradiol, EM = endometriosis, FF = follicular fluid, FTP = Fallopian tube pathology, IS = idiopathic subfertility.

Table III Fertility outcome parameters

	MFS (*n* = 52)	IS (*n* = 52)	FTP (*n* = 26)	EM (*n* = 26)
Fertilization rate (%)	57.5 (28.8)	56.9 (24.5)	60.3 (27.3)	63.9 (25.9)
Cleavage rate (%)	67.1 (26.3)	67.4 (26.9)	75.5 (20.9)	71.0 (26.5)
Mean embryo quality	2.06 (0.82)	2.19 (0.72)	2.08 (0.72)	2.08 (0.72)
No. of pregnancies (%)	15 (30.6)	17 (35.4)	5 (19.2)	13 (52.0)

Note: values are given as mean and (SD) unless otherwise indicated. EM = endometriosis, FTP = Fallopian tube pathology, IS = idiopathic subfertility, MFS = male factor subfertility.

† Embryo quality is scored on a scale of 0 to 3; 0 = low, 1 = moderate, 2 = reasonable and 3 = good embryo quality.

uPA, tPA, PAI-1 and tPA-PAI-1-complex concentrations in ejaculate and spermatozoa

The ejaculate showed a much higher tPA concentration compared with the uPA and PAI-1 concentrations (Table IV). The concentrations of these three PA components in ejaculate were not significantly different between fertile men, idiopathic subfertile men or men with MFS. No PA components could be detected in the collected suspension of purified spermatozoa, but after freeze-thawing for five times tPA could be detected in small amounts, while uPA and PAI-1 remained below the lower level of detection in spermatozoa.

The spermatozoa of men with MFS contained significantly higher concentrations of tPA compared with fertile and idiopathic subfertile men, geometrical mean: 162.7 pg/ml (25^{th}-75^{th} percentile (105.0-321.0), 38.0 pg/ml (25.5-99.9) and 37.2 pg/ml (23.9-97.5), respectively, $P < 0.001$). Because men with MFS had much lower sperm concentrations, we corrected for the number

of spermatozoa in the purified semen sample resulting in an increased difference between subfertile and fertile men. Subfertile and fertile men showed concentrations of approximately 70 pg tPA/million spermatozoa and 2 pg tPA/million spermatozoa ($P < 0.001$) (mean difference 3.70, (95% CI 2.92-4.48); Bonferroni, $P < 0.001$), respectively. In idiopathic subfertile men the concentration was around 3 pg tPA/million spermatozoa ($P < 0.001$) (mean difference 3.20, (95% CI 2.42-3.98); Bonferroni $P < 0.001$).

Because the ELISA we used for the determination of tPA in the spermatozoa does not differentiate between active tPA and inactive tPA-PAI-1 complexes, it is possible that the tPA measured is an inactive complexed form. To investigate this further we measured the tPA-PAI-1 complexes with a specific ELISA. The tPA-PAI-1 complex concentrations were comparable in fertile and idiopathic subfertile men, however, spermatozoa of men with MFS contained significantly more tPA-PAI-1 complex (Table V) Furthermore, spermatozoa of subfertile men contained significantly higher total tPA concentrations as compared to the tPA-PAI-1 complex concentrations.

Table IV Plasminogen activation component concentrations in men

		Fertile ($n = 52$)	IS ($n = 52$)	MFS ($n = 52$)
Total ejaculate	uPA	24.0 (18.0-30.0)	25.6 (21.7-30.3)	22.6 (19.3-27.8)
(ng/ml)	tPA	178.6 (100.4-254.6)	203.9 (110.8-312.0)	169.4 (95.4-262.9)
	PAI-1	3.99 (3.14-4.74)	4.14 (3.13-5.51)	3.60 (2.31-5.23)
Spermatozoa	uPA	n.d.	n.d	n.d.
(pg/million cells)	tPA	1.91 (0.74-5.79)	3.14 (0.97-9.97)	77.1 (31.8-211.2)[a]
	PAI-1	n.d.	n.d.	n.d.

Note: values are given as geometric mean and (25^{th} and 75^{th} percentile). [a] $P < 0.001$ in Oneway ANOVA. IS = idiopathic subfertility, MFS = male factor subfertility, n.d. = non detectable (below detection limit).

Table V Tissue-type PA and tPA-PAI-1 complex concentrations in spermatozoa

PA-components	Fertile ($n = 11$)	IS ($n = 10$)	MFS ($n = 9$)
pg tPA/million cells	2.12 (1.30-3.00)	3.39 (2.41-5.18)	70.5 (56.2-85.1)[a]
tPA-PAI-1 complex (pg/ml)	56.1 (49.0-78.0)	58.9 (41.3-89.5)	61.9 (45.5-78.5)[b]
pg tPA-PAI-1 complex/million cells	2.09 (0.98-3.67)	3.07 (1.92-4.65)	24.8 (15.5-46.8)[a,b]

Note: values are given as geometric mean and (25^{th} and 75^{th} percentile). [a] $P < 0.001$ in Oneway ANOVA, for comparison between the three diagnostic subgroups. [b] $P = 0.003$ in Paired-Samples T Test, for comparison between tPA and tPA-PAI-1 complex concentrations within the three diagnostic subgroups. IS = idiopathic subfertility, MFS = male factor subfertility.

uPA, tPA, and PAI-1 concentrations in FF

In the FF, PAI-1 concentrations are much higher than the uPA and tPA concentrations (Table VI). Although, the uPA and PAI-1 concentrations were not significantly different between the four diagnostic subgroups, a trend was shown for higher tPA concentrations in FF of women with Fallopian tube pathology or endometriosis (one-way ANOVA, $P = 0.07$).

Table VI Plasminogen activation component concentrations in follicular fluid

PA-components	Fertile ($n = 52$)	IS ($n = 52$)	FTP ($n = 26$)	EM ($n = 26$)
pg uPA/mg protein	6.48 (5.04-6.89)	6.74 (4.77-8.86)	5.96 (4.88-6.98)	8.03 (4.97-9.70)
ng PAI-1/mg protein	7.19 (5.69-10.1)	7.78 (5.90-10.7)	6.70 (4.94-8.98)	6.94 (5.02-8.71)
pg tPA/mg protein	14.3 (10.3-17.6)	13.9 (9.5-17.8)	18.5 (11.4-25.7)	18.8 (11.4-27.1)

Note: values are given as median and (25^{th} and 75^{th} percentile). EM = endometriosis, FTP = Fallopian tube pathology, IS = idiopathic subfertility.

Correlations between the PA-system components and basic characteristics

No significant correlations were observed between uPA and tPA in the ejaculate and age, spermatozoa concentration, motility percentage and percentage abnormal cells. However, PAI-1 in the ejaculate was correlated with age ($r = 0.21$; $P = 0.01$) and with sperm concentration ($r = 0.19$; $P = 0.02$).
The uPA concentration in FF was inversely correlated with age, number of follicles grown, number of follicles larger than 15 mm, number of collected oocytes and the concentration of progesterone in FF (Table VII). The PAI-1 concentration was inversely correlated with the Puregon dose received, positively correlated with the number of follicles grown, and the number of collected oocytes. Finally, tPA was inversely correlated with age, number of collected oocytes, and the concentration of progesterone in FF.

Associations between the PA-system components and outcome parameters

None of the components of the PA-system were significantly associated with oocyte fertilization, cleavage or embryo quality, investigated with linear and logistic regression analyses. However, tPA concentration in the spermatozoa was significantly associated with pregnancy. Confounders included in the logistic regression model were the three semen parameters, age, the propor-

Table VII Pearson correlation coefficients between the natural logarithms of the components of the PA-system in FF and basic characteristics of the pooled data of 156 women

	uPA (pg/mg protein)	tPA (pg/mg protein)	PAI-1 (ng/mg protein)
Age (years)	**-0.20 (0.01)**	**-0.34 (< 0.001)**	-0.05 (0.52)
Puregon dose (IU)	0.04 (0.65)	-0.15 (0.06)	**-0.21 (0.01)**
E_2 in serum (pmol/l)	-0.13 (0.10)	-0.01 (0.88)	0.01 (0.91)
E_2 in FF (nmol/l)	-0.13 (0.12)	-0.15 (0.07)	-0.11 (0.16)
Prog in FF (µmol/l)	**-0.40 (< 0.001)**	**-0.56 (< 0.001)**	0.10 (0.21)
Follicles (n)	**-0.18 (0.02)**	0.01 (0.90)	**0.24 (0.003)**
Follicles > 15mm (n)	**-0.16 (0.04)**	0.001 (0.99)	0.13 (0.12)
Oocytes (n)	**-0.29 (< 0.001)**	**-0.16 (0.05)**	**0.26 (0.001)**

Note: data are given as r-value (*P*-value). E_2 = 17ß-estradiol; FF = follicular fluid.

tion of fertilized oocytes, the proportion of cleaved embryos, mean embryo quality and the artificial reproduction method IVF or ICSI. The OR for tPA was 0.995 with a 95% confidence interval (0.991-1.000) (P = 0.03). Because tPA is a continuous variable in the logistic regression analyses, the height of the OR is dependent on the dimension of tPA and can be illustrated as follows: A mean decrease in the spermatozoa tPA concentration of around 125 pg/ml (the difference between the fertile and subfertile group) results in a nearly 2-fold increased probability to achieve pregnancy after an IVF or ICSI procedure, calculated using the formula $OR = e^{\beta \times 0.125}$ with $\beta = -4.621$.

Also, for the female parameters tPA seemed to be important. The concentration of tPA in the FF was significantly associated with the proportion of cleaved embryos on day 3. Confounders included in this linear regression model were the proportion of fertilized oocytes and the progesterone concentration in FF. The adjusted regression coefficient for tPA and proportion of embryo cleavage was 0.16 (P < 0.001). The tPA concentration in FF, however, was not significantly associated with the probability to become pregnant after an IVF or ICSI procedure.

Discussion

Male study population We demonstrate that the mean tPA concentration in spermatozoa of men with MFS and undergoing an IVF or ICSI procedure is significantly higher than in fertile and idiopathic subfertile men. Moreover, spermatozoa of subfertile men possessed higher concentrations of tPA-PAI-1 complexes compared with the other 2 groups thereby suggesting increased complex formation in response to the overload of tPA. As the tPA concentrations measured in fertile and idiopathic subfertile men were predominantly in the inactive complex form, spermatozoa of men in the MFS group contained some inactive tPA-PAI-1 complexes, but most of the tPA measured was in the free and therefore active form. Even more interesting, fertile men with lower tPA concentration in spermatozoa have about a 2-fold increased chance of pregnancy after an IVF or ICSI procedure.

Our observations are supported by those of others. Maier *et al.* observed significantly higher tPA concentrations in spermatozoa of patients with oligo-asthenoteratozoospermia (OAT-syndrome) than in normozoospermic men (Maier *et al*., 1991). Moreover, the tPA concentrations in the ejaculate were also significantly higher compared with uPA, and the tPA and uPA concentrations in the ejaculate were comparable between normozoospermic and oligozoospermic men (Maier *et al*., 1991). This is in accordance with the findings of Arnaud *et al.* (1994).

Considering the tPA and uPA activity in seminal plasma, Liu *et al*. (1996b) observed a significantly increased activity of these 2 factors in seminal plasma of 6 infertile compared with 4 fertile men. Furthermore, Maier *et al.* found uPA concentrations and PAI-1 activity in spermatozoa (Maier *et al*., 1991), while we did not detect these 2 components in our spermatozoa samples. However, we did observe tPA-PAI-1 complexes in spermatozoa indicating that PAI-1 has been present in the samples. Other authors report the presence of uPA, tPA and PAI-1 on human spermatozoa outer acrosomal and plasma membranes also using ELISA (Huang *et al*., 1997; Smokovitis *et al*., 1992). The differences between the results in our study in comparison with the results of others could be due to differences in detection limits of the assays, the different methods of spermatozoa washing and lysing, and differences in number and selection of patients and controls.

Regarding the correlations between the PA-components and semen characteristics only the PAI-1 concentration in the ejaculate was positively correlated with the sperm concentration. Other groups, however, found either no correlations between the PA-system components and semen parameters (Arnaud *et al*., 1994), or significant positive correlations between uPA in spermatozoa and sperm motility (Huang *et al*., 1997), and between PA activity, measured as the ability of the purified PAs to lyse fibrin, in seminal plasma, and immotility and azoospermia (Liu *et al*., 1996b).
Although the pregnancy rate was only slightly higher in couples with lower tPA concentrations in spermatozoa, this effect was significant, and extremely interesting, since it may indicate a paternal influence on achieving pregnancy. To our knowledge we are the first reporting the association between tPA in spermatozoa and the chance of pregnancy after artificial reproduction techniques.

Female study population

Our study demonstrates a relative abundance of PAI-1 in FF compared with tPA and uPA concentrations. This is in concordance with observations made by Jones *et al.* (1989). These authors examined the presence of active (i.e., free) tPA and inactive tPA-PAI-1 complexes and found that the tPA measured is only present as tPA-PAI-1 complex (Jones *et al*., 1989). We could not verify this in our samples, but believe that it is very likely that the tPA concentrations measured indeed reflect inactive tPA considering the much higher free PAI-1 concentrations as compared to the total tPA concentrations measured in FF.
Another interesting result of our study is the apparently increased tPA concentration in the FF of women with FFS, Fallopian tube pathology and endometriosis. This has not been reported before and it is interesting to note that there is a trend towards higher FF tPA concentrations in women with Fallopian tube pathology as well as in women with endometriosis, since the pathogenesis of these 2 conditions is very different.
We observed several correlations between uPA and PAI-1 in FF and fertility parameters in women undergoing the IVF treatment. We found a negative correlation between uPA and follicle number, oocyte number, and progesterone concentration in FF as a marker of follicular luteinization. This was unexpected as uPA has a function in angiogenesis-associated proteolysis,

turning unvascularized preantral follicles into mature and highly vascularized Graafian follicles.

Furthermore, our results indicate that tPA concentration in the FF is a significant predictor of the proportion of cleaved embryos on culture day 3. We are aware that the tPA measured in FF is probably tPA-PAI-1 complex, but this may still indirectly reflect the level of the original active tPA. Contrasting results are found regarding the relationship between PA and embryo cleavage. Rom *et al.* observed higher PA-activity in follicles yielding oocytes that did not cleave *in vitro* (Rom *et al*., 1987). Similar to our results is their finding that PA activity in FF cannot distinguish pregnant from non-pregnant cycles in IVF procedures (Rom *et al*., 1987).

Other authors believe that measurement of tPA in FF has potential as a predictive parameter in clinical practice (Deutinger *et al*., 1988; Milwidsky *et al*., 1989). Deutinger *et al.* observed higher levels of tPA in granulosa cell lysates of fertilized oocytes as compared to their unfertilized counterparts (Deutinger *et al*., 1988), while Milwidsky *et al.* (1989) found significantly higher PA-activity in FF from follicles yielding fertilized oocytes in an IVF program as compared to the PA-activity in FF from follicles yielding non-fertilized oocytes. In our material, we could not find any association between FF PA-content and the percent rate of fertilization in the retrieved oocytes.

Some limitations of the present study have to be addressed. The FF samples collected were pooled samples from different follicles. Therefore, it may be incorrect to directly correlate the FF PA concentrations to the development of a specific follicle or embryo which may explain the absence of associations between FF PA concentrations and fertility outcome parameters. Also, data regarding the percentage of fertilized oocytes are influenced by the fact that the number of oocytes retrieved in conventional IVF patients included immature oocytes, whereas in ICSI cases, only the number of mature oocytes are in the denominator. Aside from the fact that in ICSI patients the oocytes are injected with a spermatozoon, the difference in maturity of the oocytes in the denominator of this variable may contribute to the higher fertilization rate in ICSI patients.

The strengths of our study as compared to other studies in literature is the investigation of a relatively large number of couples, man and women, who are

involved in an IVF or ICSI procedure. We only included couples who were unable to achieve a spontaneous conception within 1 year of regular, unprotected intercourse. Women with menstrual disorders were excluded, as were couples with both a male and a female factor explaining their subfertility, or patients with multiple causes for subfertility within 1 individual.

Conclusion

Subfertile men showed significantly higher mean tPA concentrations in spermatozoa compared with fertile men. Interestingly, a positive paternal influence on pregnancy rate was demonstrated in couples by significantly lower tPA concentrations in spermatozoa.

A trend was observed towards higher tPA-protein ratios in FF of women with Fallopian tube pathology and endometriosis as compared to fertile and idiopathic subfertile women. Moreover, the tPA concentration in the FF were significantly associated with the proportion of cleaved embryos on culture day 3. Further studies are needed to investigate the validity and impact of these associations in the prediction of successful artificial reproduction techniques in clinical practice.

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Chapter 9

VEGF and fertility parameters in IVF or ICSI couples

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Abstract

Background: Vascular endothelial growth factor (VEGF) is a mitogenic and permeability increasing growth factor which also seems to have important functions in human reproduction. Our aim was to investigate associations between VEGF concentrations in fluids involved in reproduction in men and women and fertility outcome parameters.

Methods: We enrolled 156 couples undergoing assisted reproduction techniques and determined VEGF concentrations in the ejaculate, purified spermatozoa and follicular fluid (FF) and correlated the concentrations to the fertility outcome parameters percentage of cleaved embryos on culture day 3, embryo quality and pregnancy rate.

Results: VEGF concentrations in ejaculate and FF were comparable between the diagnostic subgroups. However, VEGF concentrations in spermatozoa from men with male factor subfertility (geometrical mean 73.9 pg/million spermatozoa, 25^{th}-75^{th} percentile (37.5-176.2) were significantly higher as compared to those in fertile (4.6 (1.7-9.3) and idiopathic subfertile men (6.4 (1.8-14.0), $P < 0.001$)). High VEGF concentrations in the ejaculate tend to be negatively associated with the percentage of cleaved embryos on culture day 3 ($r = -0.08$, $P = 0.06$). In addition VEGF concentrations in spermatozoa were positively associated with embryo quality (OR = 0.17, $P = 0.09$), and pregnancy rate (OR = 0.07, $P = 0.06$). No significant associations were found between VEGF concentrations in FF and fertility outcome parameters.

Conclusions: VEGF in spermatozoa may be a new biomarker for subfertility. Of interest are the potential associations between VEGF in spermatozoa and ejaculate and various fertility outcome parameters, which supports the need for more research in this field.

Introduction

Vascular endothelial growth factor (VEGF) also called vascular permeability factor (VPF), is a protein produced by many different tissues in response to hypoxia, with the endothelial cell as its main target (Shweiki *et al*., 1992; Keck *et al*., 1989). Its actions are mostly mitogenic and permeability-increasing (Senger *et al*., 1990). At least five different human VEGF isoforms are known, resulting from alternative splicing of the VEGF gene on chromosome 6. These splice variants consist of 121, 145, 165, 189, and 206 amino acids and have surprisingly identical biological activities whereas others have demonstrated that VEGF-A isoforms differ in angiogenic properties (Ferrara and Davis-Smyth, 1997; Küsters *et al*., 2003).

VEGF is best known for its actions in tumour-induced angiogenesis (Kim *et al*., 1993), but it also seems to have important functions in male and female reproduction.

In the male reproductive organs VEGF is expressed by Leydig and Sertoli cells of the testis and in the prostate and seminal vesicle epithelium (Ergun *et al*., 1997; Ergun *et al*., 1998). Prostate and seminal vesicles seem to be the main source of VEGF, resulting in very high concentrations of VEGF in seminal plasma, even exceeding the highest concentration of VEGF measured so far in malignant effusions (Brown *et al*., 1995;Yeo *et al*., 1993). The exact role of VEGF in seminal plasma is currently unknown, but the presence of very high concentrations in seminal plasma argues for an important role in male fertility and reproduction.

One study showed that the concentration of VEGF in seminal plasma correlated with the chance of pregnancy in patients undergoing assisted reproduction techniques (Obermair *et al*., 1999). Furthermore, these authors could localize a VEGF-receptor on the spermatozoa themselves, indicating that spermatozoa could be a target for the high VEGF concentrations in seminal plasma (Obermair *et al*., 1999). Also, VEGF-receptors were localized on Leydig and Sertoli cells (Ergun *et al*., 1997). Other authors found that overexpression of VEGF in male mice resulted in a disruption of sperm maturation and male infertility (Korpelainen *et al*., 1998; Huminiecki *et al*., 2001).

In the female reproductive organs periodical growth and regression of tissues is regarded as a physiological process involved in normal reproduction, in which angiogenesis is necessary (Reynolds *et al*., 1992; Christenson and

Stouffer, 1996). Several studies have indicated that VEGF is involved in angiogenesis. Especially the increase in vascular supply of the ovary, important for folliculogenesis, ovulation of the dominant follicle and subsequent formation of the corpus luteum seems to be mediated by VEGF (Shweiki *et al*., 1993; Koos, 1995; Ferrara *et al*., 1998).

Both granulosa and theca cells of the ovary produce VEGF (Kamat *et al*., 1995; Gordon *et al*., 1996) and the production of VEGF is increased in response to FSH, LH and hCG (Dissen *et al*., 1994; Neulen *et al*., 1995). Therefore, women undergoing assisted reproduction, receiving gonadotropin stimulation demonstrate very high concentrations of VEGF in the follicular fluids (Ferrari *et al*., 2006). Furthermore, the development of ovarian hyperstimulation syndrome (OHSS) has been related to the overproduction of VEGF by the multiple formed corpora lutea (Abramov *et al*., 1997; Ludwig *et al*., 1999).

The significance of VEGF concentrations in follicular fluid (FF) is unknown. Some authors suggest that high VEGF concentrations in FF reflect high vascularization and therefore oxygenation of the follicle, which results in “healthy” oocytes with superior pregnancy potential (Van Blerkom 1998). This is in contrast to others claiming that high VEGF concentrations in FF reflect follicular hypoxia and is therefore a marker of a hostile follicular environment, resulting in oocytes with diminished pregnancy potential (Friedman *et al*., 1998; Barroso *et al*., 1999; Malamitsi-Puchner *et al*., 2001).

From this background, the aims of the current study were: (i) to measure VEGF concentrations in the ejaculate and purified spermatozoa and in FF at the day of oocyte retrieval of a relatively large number of couples undergoing an IVF or ICSI treatment; (ii) to determine whether differences exist in the VEGF concentrations between different diagnostic subfertile subgroups; and (iii) to explore associations between semen and FF VEGF concentrations and fertility outcome parameters.

Materials and Methods

Patient selection

From April 2002 until May 2003, all patient couples visiting the fertility clinic of the Radboud University Nijmegen Medical Centre were considered for participation. The selection criteria for participation comprised the following

diagnostic categories: (i) idiopathic subfertility, absence of abnormalities in both man and woman regarding semen and edocrine analyses, menstrual cycle, Chlamycia serology and hysterosalpingogram, but no spontaneous conception within 1 year of unprotected intercourse; (ii) female factor subfertility (FFS) consisting of endometriosis or Fallopian tube pathology (any condition leading to an impediment of oocyte pick-up or transport); or (iii) male factor subfertility (MFS) defined by the presence of at least one of the sperm anomalies oligozoo-, asthenozoo-, and/or teratozoospermia. Couples with both a male and a female factor explaining their subfertility and patients first visiting other hospitals for fertility treatment were excluded from the study. The group of fertile men was defined as the male partners without abnormal semen parameters of the women suffering from Fallopian tube pathology or endometriosis. The group of fertile women without tubal and menstrual cycle abnormalities comprised the partners of the men with MFS.

All materials collected were anonymized. The patients were notified about the study by brochures available in the waiting room of the IVF treatment unit. Information was given on the background and objectives of the study, inclusion criteria, study periods and other relevant procedures. It was acknowledged that the decision whether or not to participate in the study would neither interfere nor have any consequences for the IVF or ICSI treatment.

The materials have been collected in accordance with the guidelines of the ethical and institutional board of the Radboud University Nijmegen Medical Centre.

A total of 156 couples were enrolled in the study and information concerning age, smoking behaviour, type of treatment (IVF or ICSI), 17β-estradiol (E_2) concentration in serum of the women and FSH dose (Puregon, Organon, Oss, The Netherlands) administered for ovarian stimulation treatment was made available from the medical records.

Ejaculate and spermatozoa collection

Participants provided the ejaculates in polypropylene containers produced via masturbation at home or in the hospital, after an abstinence period of 3-5 days. After liquefaction for 20 minutes, the total volume was measured and the main part of the sample was prepared for IVF or ICSI. Two hundred microlitres of the remaining semen sample was obtained for semen analysis and VEGF

measurements. Semen analysis was performed according to the guidelines of the World Health Organization (Guzick *et al.*, 2001; World Health Organization, 1992). Thus, sperm concentration was determined using a Makler counting chamber, designed in a 10-by-10 compartments frame format. Motility was expressed as the proportion of motile spermatozoa, and morphology was determined after incubation of the sample with trypsin (10 minutes at room temperature), staining with methylene blue/eosin, feathering, and fixation by flame. The sample was frozen without preservatives and stored at –80 °C until assay for VEGF concentrations.

The ejaculate provided for the IVF or ICSI procedure (minus the 200 microlitre obtained for this study) was purified by means of centrifugation on 80% Pure Sperm reagent (Nidacon International AB, Sweden). After isolation, the concentration and motility of spermatozoa was determined according to the guidelines of the WHO. In the following step, the spermatozoa were diluted with culture medium (Human Tubal Fluid, Cambrex Company, Belgium) to the concentrations required for the IVF or ICSI procedure. Residual concentrated sperm was collected, frozen without preservatives and stored at –80 °C until assay for VEGF concentrations.

Follicular fluid collection

After oocyte retrieval for the IVF or ICSI procedure, a sample of the FF (diluted with 10 IU/ml heparin, and phosphate buffer, pH 7.4) was centrifuged for 10 minutes at 2000 *g* to separate red blood cells, leukocytes and granulosa cells from the FF. The FF was frozen without preservatives and stored at –80 °C until assay for VEGF, 17β-E_2, progesterone and protein concentrations.

Fertility outcome parameters

The number of follicles and the number of follicles > 15 mm were determined by using ultrasound 2 days before the follicles were punctured. One day after the IVF or ICSI procedure, fertilization was determined by counting the number of pronuclei formed in the oocyte. The percentage of fertilized oocytes was calculated by dividing the number of oocytes with two pronuclei by the total number of oocytes retrieved (IVF), or by the total number of oocytes injected (ICSI).

On the following 2 days, the embryos were examined once a day for their development. On day 3 the proportion of cleaved embryos was calculated following division of the number of cleaved embryos by the total number of oocytes retrieved (IVF) or the total number of oocytes injected (ICSI). On the following 2 days, the embryos were examined once a day for development. The proportion of cleaved embryos was calculated following division of the number of celaved embryos on day 3 by the total number of oocytes retrieved (IVF) or the total number of oocytes injected (ICSI). Furthermore, embryo quality was established by judgment of fragmentation and cleavage activity of the embryos after 3 days of culturing and was denoted as low, moderate, reasonable or high.
An hCG-based pregnancy test was performed in first-morning voided urine from the woman on day 15 after the embryo transfer. The result of this test was communicated by phone to the personnel of the fertility clinic.

17β-Estradiol and progesterone in FF

The concentrations of 17β-E_2 and progesterone in FF were measured by a specific procedure described previously (Thomas *et al*., 1977). Fifty microlitres of a FF specimen was extracted twice with diethylether, and after drying, the residue containing 17β-E_2 and progesterone was further purified by chromatography on Sephadex LH-20 columns. The steroids were quantified by specific radioimmunoassays.

Protein concentration in FF

The FFs were assayed for protein concentration using the Pierce BCA Protein Assay reagent kit (Pierce, Rockford, IL). Absorptions were read at 540 nm in an automatic microtiter plate reader (Multiskan Ascent, Labsystems). The protein content of the follicle was determined as a biomarker for the maturity of the follicle (Spitzer *et al*., 1996).

VEGF concentrations

Vascular endothelial growth factor concentrations were measured in ejaculate, purified spermatozoa, and FF samples using a four-stage/two-site ELISA as described previously by Manders *et al.* (2003). This assay is based on a combi-

nation of four polyclonal antibodies (raised in four different animal species including duck, chicken, rabbit and goat), which were employed in a sandwich assay form. The polyclonal antibodies raised in chickens and rabbits are specifically directed against VEGF. To increase the sensitivity of the assay, the colour detection was replaced by a fluorometric detection using the biotin-streptavidin-labelled β-Galactosidase detection. The spermatozoa samples were frozen in liquid nitrogen and subsequently thawed, for five times to lyse the spermatozoa (checked under a microscope) before the ELISA was performed. Furthermore, prior to the analysis the total ejaculate samples were diluted 3,200- and 6,400-fold, the spermatozoa samples were diluted 5-fold, and the FF samples were diluted 20- and 40-fold. All samples were analysed in duplicate. In the VEGF assay distinct molecular forms of VEGF are measured. There is no cross-reactivity with VEGF B, VEGF C, VEGF D, Platelet Derived Growth Factor AB (PDGF AB), Insulin Growth Factor type 1 (IGF-1), human Growth Factor (hGH), Placental Growth Factor (PGF), Nerve Growth Factor (NGF), Tumor Necrosis Factor (TNF-alpha) and Transforming Growth Factor (TGF). The analytical sensitivity for VEGF is 0.005 ng/ml, whereas the functional sensitivity for VEGF amount to 0.010 ng/ml. For VEGF the within-run CV and between-run CV are found to be 4.3% and 11.6%, respectively.

Statistical analyses

The fertility outcome parameters were normally distributed and the results are expressed as means and standard deviation (SD). The proportion of fertilized embryos, cleaved embryos and mean embryo quality between the different diagnostic subgroups were compared using the one-way analysis of variance (ANOVA). The number of clinical pregnancies occurring in the different subgroups was compared using the Chi-square test.

Because of the skewed distributions of the VEGF concentrations measured in this study, natural logarithmic transformations were performed and data are expressed as geometrical means and 25^{th}-75^{th} percentiles. The VEGF concentrations between groups were compared using one-way analysis of variance (ANOVA) and further analysed using *post hoc* Bonferroni comparisons.

In the pooled group of men, Pearson correlations were calculated between the natural logarithmic transformed VEGF concentrations in the ejaculate and the

logarithmic transformed characteristics of the study population, age, sperm concentration, motility percentage and percentage abnormal cells. In the pooled group of women, Pearson correlations were calculated between the logarithmic transformed VEGF-protein ratios in FF and the study population characteristics described in Table II.

Linear regression analyses were performed in the pooled group of men or women to find associations between the different fertility outcome parameters, being the proportion of oocytes fertilized and the proportion of cleaved embryos on culture day 3 as the dependent variables and the VEGF concentrations in the ejaculate, in purified spermatozoa and in FF as the independent variables. Also, logistic regression analyses were carried out in the pooled group of men or women to find associations between the dependent variables such as embryo quality on culture day 3 (expressed as low-moderate, or reasonable-high), and achievement of pregnancy (yes or no) and the independent continuous variables such as VEGF concentrations in ejaculate, purified spermatozoa and FF. Potential confounders were included in the linear and logistic regression models, and the factors that substantially affected the association between the dependent variable and the VEGF concentration variable (a change in the regression coefficient of more than 10%) were maintained in the final models.

A *P*-value of ≤ 0.05 was considered statistically significant. Statistical analyses were performed using SPSS 12.0 for Windows software (SPSS Inc, Chicago, IL, USA).

Results

The basic characteristics of the male study group are summarized in Table I. Age of the subjects and the volume of the ejaculate were comparable between the three groups. As expected, sperm concentration, motility and morphology were substantially different in the male factor subfertility (MFS) group as compared to the fertile and idiopathic subfertile men.

The basic characteristics of the female study group are summarized in Table II. The only difference between the four diagnostic subgroups was age. Women with endometriosis were slightly younger as compared to idiopathic subfertile women.

Table I Characteristics of the male study population

Characteristics	Fertile (*n* = 52)	IS (*n* = 52)	MFS (*n* = 52)
Age (years)	35.0 (32.0-37.7)	35.7 (34.0-38.0)	34.7 (32.0-37.7)
Volume ejaculate (mL)	2.8 (2.1-3.5)	2.9 (2.2-4.4)	3.3 (2.5-4.8)
Sperm conc (x10^6/ml)	66.1 (45.5-100.0)	55.6 (31.3-100.0)	3.8 (1.2-14.5)
Motility (%)	56.2 (50.0-65.0)	54.0 (50.0-63.8)	18.9 (10.0-30.0)
Abnormal cells (%)	78.7 (72.0-87.0)	81.1 (75.0-87.8)	93.0 (89.0-97.0)

Note: values are given as geometrical mean and (25^{th} –75^{th} percentile). IS = idiopathic subfertility, MFS = male factor subfertility.

Table II Characteristics of the female study population

Characteristics	Fertile (*n* = 52)	IS (*n* = 52)	FTP (*n* = 26)	EM (*n* = 26)
Age women (years)	32.8 (30.3-36.0)	34.3 (32.0-36.0)	33.2 (31.0-38.0)	31.5 (29.0-35.3)
Puregon dose (IU)	179 (150-250)	173 (150-200)	182 (150-300)	170 (144-263)
E_2 in blood (pmol/l)	5114 (3725-7575)	5499 (3925-8350)	5545 (5025-7025)	5603 (4050-8500)
E_2 in FF (nmol/l)	871 (660-1350)	924 (683-1300)	971 (763-1400)	849 (540-1425)
Prog in FF (µmol/l)	24.7 (19.0-36.8)	27.3 (22.5-35.8)	22.2 (16.0-32.3)	23.5 (19.0-33.0)
Protein in FF (µg/l)	38.2 (34.0-43.5)	39.1 (35.8-45.8)	38.7 (33.6-47.2)	38.9 (32.7-49.1)
Follicles (n)	12.0 (9.0-20.8)	14.1 (10.0-21.0)	13.3 (10.8-17.0)	14.4 (13.0-20.3)
Follicles > 15mm (n)	6.2 (5.0-10.0)	6.5 (5.0-10.0)	7.1 (6.0-9.0)	6.9 (4.8-11.3)
Oocytes (n)	8.5 (6.0-13.0)	9.2 (7.0-13.8)	8.6 (6.0-13.0)	8.2 (5.8-13.0)

Note: values are given as geometric mean and (25^{th} –75^{th} percentile. E_2 = 17β-estradiol, FF = follicular fluid, FTP = Fallopian tube pathology, IS = idiopathic subfertility, EM = endometriosis.

In Table III the fertility outcome parameters for the different subgroups are summarized. All the fertility outcome parameters were comparable between the different subgroups.

Table III Fertility outcome parameters

	MFS (*n* = 52)	IS (*n* = 52)	FTP (*n* = 26)	EM (*n* = 26)
Fertilization rate (%)	57.5 (28.8)	56.9 (24.5)	60.3 (27.3)	63.9 (25.9)
Cleavage rate (%)	67.1 (26.3)	67.4 (26.9)	75.5 (20.9)	71.0 (26.5)
Mean embryo quality †	2.06 (0.82)	2.19 (0.72)	2.08 (0.72)	2.08 (0.72)
No. of pregnancies (%)	15 (30.6)	17 (35.4)	5 (19.2)	13 (52.0)

Note: values are given as mean and (SD) unless otherwise indicated. EM = endometriosis, FTP = Fallopian tube pathology, IS = idiopathic subfertility, MFS = male factor subfertility.

† Embryo quality is scored on a scale of 0 to 3; 0 = low, 1 = moderate, 2 = reasonable and 3 = good embryo quality.

The VEGF concentrations measured in the ejaculate and spermatozoa are presented in Table IV. The VEGF concentrations in ejaculate were comparable between the three diagnostic subgroups. Furthermore, VEGF concentrations in the ejaculate were much higher than the concentrations in spermatozoa. The VEGF concentrations measured in spermatozoa were below the detection limit in 11 fertile men, 14 idiopathic subfertile men, and 8 oligoasthenoteratozoospermic men, thus 33 out of 156 samples.

Table IV VEGF concentrations in ejaculate and spermatozoa

	Fertile (*n* = 52)	IS (*n* = 52)	MFS (*n* = 51)
VEGF in ejaculate (ng/ml)	945 (640-1318)	1025 (755-1288)	905 (693-1176)
VEGF in spermatozoa (ng/ml)	0.09 (0.04-0.23)	0.08 (0.02-0.19)	0.16 (0.11-0.32)[a]
VEGF in spermatozoa (pg/million cells)	4.6 (1.7-9.3)	6.4 (1.8-14.0)	73.9 (37.5-176.2)[b]

Note: values are given as geometrical mean and (25^{th} and 75^{th} percentile).). [a] $P = 0.005$, [b] $P < 0.001$ in Oneway ANOVA. IS = idiopathic subfertility, MFS = male factor subfertility. One missing case in the MFS group.

Spermatozoa of men in the MFS group contained significantly higher VEGF concentrations per million spermatozoa (geometrical mean 73.9 (25^{th}-75^{th} percentile 37.5-176.2) compared with the concentrations in fertile men (4.6 (1.7-9.3), and in idiopathic subfertile men (6.4 (1.8-14.0); ($P < 0.001$ in one-way ANOVA). *Post hoc* Bonferroni comparisons revealed that VEGF concentrations in spermatozoa of men in the MFS group were significantly higher compared with both fertile (mean difference 2.78, (95% CI 2.04-3.51); Bonferroni, $P < 0.001$), and idiopathic subfertile men (mean difference 2.45, (95% CI 1.72-3.19); Bonferroni $P < 0.001$).

The VEGF concentrations measured in FFs are presented in Table V. Vascular endothelial growth factor measured in FF was expressed as pg VEGF/mg protein to adjust for the maturity of the follicle. No significant differences in VEGF concentrations were observed between the four diagnostic subgroups.

Table V VEGF concentrations (pg/mg protein) in follicular fluid

	Fertile (*n* = 52)	IS (*n* = 52)	FTP (*n* = 26)	EM (*n* = 26)
VEGF	86.8 (53.9-148.3)	98.5 (65.7-133.6)	90.6 (73.5-147.2)	79.7 (55.4-119.6)

Note: values are given as median and (25^{th} and 75^{th} percentile). IS = idiopathic subfertility, FTP = Fallopian tube pathology, EM = endometriosis.

In the pooled group of men or women, we investigated whether smoking status influenced the VEGF concentrations and observed that spermatozoa of male smokers contained significantly higher VEGF concentrations (geometrical mean = 35.9 pg VEGF/million cells, 25^{th}-75^{th} percentile (7.4-97.8) compared with non-smokers (18.3 pg VEGF/million cells (5.0-83.0); $P < 0.01$). In the FF comparable results were observed, however the results were of borderline statistical significance (smokers 112.6 pg VEGF/mg protein (82.9-160.5) and non-smokers 87.5 pg VEGF/mg protein (59.8-132.8); $P = 0.06$).
Furthermore, we calculated correlation coefficients between the VEGF concentrations in the ejaculate and spermatozoa versus the various basic characteristics. The VEGF concentration in the ejaculate of all the subjects was inversely correlated with age (r = -0.27; $P = 0.001$). Likewise, correlations were evaluated between the VEGF-protein ratios in the FF versus the various basic characteristics, of which the results are presented in Table VI. The VEGF-protein ratio was positively correlated with age, Puregon dose received, and progesterone concentrations in the FF, while inverse correlations were observed between the VEGF-protein ratio and 17β-E_2 concentration in serum, and the number of follicles grown (both total number of follicles and number of follicles > 15 mm) during the stimulation procedure.

Table VI Pearson correlation coefficients between the natural logarithms of VEGF in FF and basic characteristics of the pooled data of 156 women

	VEGF (pg /mg protein)
Age (years)	**0.22 (0.005)**
Puregon dose (IU)	**0.33 (< 0.001)**
E_2 in serum (pmol/l)	**-0.19 (0.02)**
E_2 in FF (nmol/l)	-0.003 (0.97)
Progesterone in FF (µmol/l)	**0.54 (< 0.001)**
Follicles (n)	**-0.25 (0.002)**
Follicles > 15mm (n)	**-0.27 (0.001)**
Oocytes (n)	-0.06 (0.47)

Note: data are given as r-value (*P*-value).

To investigate whether the VEGF concentrations in the ejaculate and spermatozoa were associated with the proportion of fertilized oocytes, the proportion of cleaved embryos, embryo quality or pregnancy, linear and logistic

regression analyses were performed. No significant associations were found between VEGF concentration in men and these fertility outcome parameters. The only association may exist between the VEGF concentration in the ejaculate and the proportion of cleaved embryos (r = -0.08; *P* = 0.06).

Confounders included in the regression model were the percentage of fertilized oocytes, and the artificial reproduction method IVF or ICSI. Also, VEGF in the spermatozoa may be associated with embryo quality on culture day 3, expressed as reasonable/high or moderate/low quality (OR = 0.17, 95% CI 0.02-1.29; *P* = 0.09). Included confounders in the regression model were both the sperm concentration in the ejaculate and in the purified spermatozoa sample used for insemination, and the proportion of motile spermatozoa in the purified spermatozoa sample.

Finally, VEFG in spermatozoa may be associated with pregnancy (OR = 0.07, 95% CI 0.004-1.13; *P* = 0.06). This regression model included the proportion of motile spermatozoa in the ejaculate, smoking status, age, the sperm concentration in the purified spermatozoa sample and the median embryo quality on culture day 3 as confounders. However, these associations between VEGF and these fertility outcome parameters were not statistically significant.

Finally, similar regression analyses were performed for the female parameters. It revealed that VEGF in FF was not significantly associated with any of the fertility outcome parameters.

Discussion

This study demonstrates that VEGF concentrations in the ejaculate were substantially higher than in spermatozoa. Also, the VEGF concentration was significantly higher in purified spermatozoa of men with MFS, as compared to fertile and idiopathic subfertile men. No significant associations could be found between VEGF concentrations in the ejaculate and in spermatozoa and the fertility outcome parameters in an IVF or ICSI procedure. However, trends could be found for negative associations between VEGF in the ejaculate and the proportion of cleaved embryos in culture day 3, between VEGF in spermatozoa and embryo quality, and between VEGF in spermatozoa and pregnancy rates.

There are only 2 studies in literature on VEGF in the male reproductive tract. Similar to our observations, the study by Brown *et al*. reported that seminal

plasma contained extremely high levels of VEGF (Brown *et al*., 1995). The other study by Obermair *et al*. did not observe a correlation between seminal plasma VEGF and male factor subfertility, which is similar to our observations. Furthermore, these authors state that the VEGF concentration in seminal plasma is a significant predictor for pregnancy. We did not find such a correlation, but we observed a weak correlation between VEGF in spermatozoa and pregnancy rate. No data are available on VEGF concentrations in spermatozoa.

We observed that in women, VEGF concentrations in FF were comparable between fertile and subfertile patients. No significant associations could be found between VEGF concentrations in FF and the fertility outcome parameters in an IVF or ICSI procedure. In literature, several studies report on VEGF concentrations in women and (sub)fertility. We did not find an association between FF VEGF concentrations and subfertility, which is similar to observations by Benifla *et al*. (2001) and Kim *et al*. (2004). However, some authors have found higher or lower VEGF concentrations in the FF of women with endometriosis (Pellicer *et al*., 1998; Attar *et al*., 2003), a finding that we cannot confirm. The correlations we found between VEGF and the different basic characteristics are in line with previous data. Our findings suggest that VEGF concentration in FF is an indirect marker of follicular hypoxia and ovarian aging, which is in line with the results found by several other groups (Friedman *et al*., 1998; Battaglia *et al*., 2000; Ocal *et al*., 2004). However a limitation of our study is that we did not determine direct markers of hypoxia or oxidative stress. In spite of the indications that VEGF concentration in FF is a marker of follicular hypoxia, we did not find any association with the IVF/ICSI outcome parameters. Similar to our results are reports stating that VEGF in FF is not correlated to the fertilization rate of the oocytes after IVF or ICSI (Friedman *et al*., 1998; Barroso *et al*., 1999; Manau *et al*., 2000; Attar *et al*., 2003), nor with embryo quality (Manau *et al*., 2000; Ocal *et al*., 2004), or pregnancy rate (Benifla *et al*., 2001; Dorn *et al*., 2003; Kim *et al*., 2004; Cunha-Filho *et al*., 2005). However, other authors observed inverse correlations with embryo quality (Barroso *et al*., 1999) and pregnancy rate (Friedman *et al*., 1998; Battaglia *et al*., 2000; Ocal *et al*., 2004).

Some limitations of the present study have to be addressed. The FF samples collected were pooled samples from different follicles. Therefore, it may be in-

correct to directly correlate the FF VEGF concentrations to the development of a specific follicle or embryo, which may explain the absence of associations between FF VEGF concentrations and fertility outcome parameters. However, we could not change the method of FF collection, because we had to adhere to the regular IVF and ICSI protocols.

The strengths of our study as compared to other studies in literature is the investigation of a relatively large number of couples, man and woman, who are involved in an IVF or ICSI procedure. Furthermore, our study group is homogeneous and subfertile and fertile men or women are very comparable since we applied strict selection criteria. We only included couples who were unable to achieve a spontaneous conception within 1 year of regular, unprotected intercourse. Women with menstrual disorders were excluded, as were couples with both a male and a female factor explaining their subfertility, or patients with multiple causes for subfertility within one individual.

In conclusion, VEGF concentrations in spermatozoa of subfertile men are significantly higher than those of fertile men. The implications of these high VEGF concentrations for spermatozoa function and fertility are yet unknown, but might be related to embryo quality and pregnancy rate in an IVF/ICSI procedure. Furthermore, VEGF in FF is not suitable as a predictor for IVF/ICSI outcome parameters.

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Chapter 10

General Discussion

The studies described in this thesis were performed to gain more insight into the pathophysiology of human subfertility. The first objective of the studies described in part I was to provide an explanation for the previously demonstrated increase in sperm count after 26 weeks of folic acid and zinc sulphate intervention in subfertile men. The objective of part II was to study the involvement of several determinants of the oxidative pathway as thiol antioxidants and VEGF and of the plasminogen pathway, tPA, uPA and PAI-1 in human subfertility and outcome of the IVF/ICSI procedure.
In this chapter we generally discuss the studies described in this thesis and elaborate on the findings. Furthermore, implications for clinical practice and future research are discussed.

Study population: selection, confounders and generalizability
In part I, subfertile men comprised men with oligozoospermia as determined by the first routine semen analysis in the fertility laboratory. Furthermore, subfertile males with a known cause for subfertility or with a sperm concentration of less than 5 million spermatozoa per mL were excluded, thereby excluding males with a severe, possibly genetically caused, deranged spermatogenesis, since it is unlikely that the sperm parameters of these males will respond to micronutrient interventions. Men in the fertile group, recruited in midwifery practices, where healthy and proven to be fertile. These 2 groups were comparable with regard to age, serum folate concentrations, and serum testosterone concentrations. Serum zinc concentrations were lower in subfertile compared with fertile men, though within the normal range. The data of our study are part of the study described by Wong *et al*. (2002), in which these zinc concentrations appeared to be comparable between subfertile and fertile men. This could be due to the differences in sample size. Fertile and subfertile men considerably differed in serum FSH and inhibin B concentrations, and semen parameters. This is as expected, since we aimed to recruit men with normal and abnormal semen- and endocrine parameters. Furthermore, it is important to realise that the differences between the fertile and subfertile men are not important to answer the research question whether folic acid and zinc sulphate intervention affects sperm parameters, since this is evaluated in the fertile and subfertile group separately.
It is known that participation rates in sperm studies among men in the general

population are very low, which can lead to selection bias. However, the fertile group comprised couples who conceived within 1 year of regular unprotected intercourse, and the men were not aware of their fertility potential. Furthermore, since fertile men served as their own controls regarding the effect of folic acid and zinc sulphate intervention, it is unlikely that selection bias is involved.

In part II, several diagnostic subgroups of subfertility patients are compared with each other and with controls. The strength of this study is the relatively large number of men and women undergoing an IVF or ICSI procedure that were investigated, and also the investigation of both partners in the subfertile couple. Furthermore, the study subgroups are relatively homogeneous and subfertile and fertile men or women are very comparable since we applied strict selection criteria for (sub)fertility. We only included couples who were unable to achieve a spontaneous conception within 1 year of regular, unprotected intercourse. Women with menstrual disorders were excluded, as were couples with both a male and a female factor explaining their subfertility, or patients with multiple causes for subfertility within 1 individual.
The age and semen volume of the men was comparable and sperm concentration, -motility, and -morphology were significantly different between the case and control group. In women, the idiopathic subfertile group is slightly older compared with the endometriosis group. To overcome this difference, we evaluated age as a confounder in the subsequent analyses. The other basic characteristics in women, comprising the stimulation protocols, endocrine parameters, and outcome parameters, were comparable between the diagnostic subgroups. So far the limitations of most studies among subfertile couples is that they are not including and comparing data of the couple, both man and woman, and also no data are available on a proven fertile population. It is very difficult to obtain study material of a fertile population, especially of fertile women. However, Tielemans *et al*. (2002) states that the female partners of men with abnormal semen parameters seem to represent a population with a relatively normal fertility potential, while in couples with female factor subfertility, the male partners fertility potential is relatively normal.
The population of fertility patients in our study are not a representation of the total subfertile population, since not all subfertile couples seek medical advice. Also, because of our exclusion criteria, our study group is not completely

comparable to the subfertile population that does seek medical advice. In clinical practice often multiple causes for subfertility can be found in a couple or in an individual. Therefore caution has to be taken to relate our results to all subfertile couples.

Quality of classification

In part I we describe the intervention study with folic acid and zinc sulphate in fertile and subfertile men, in which the diagnosis of male subfertility was based on the evaluation of 1 semen sample only in couples attending the fertility clinics of 2 hospitals in Nijmegen, The Netherlands, because of 1 year of regular unprotected intercourse without conceiving. It would have been favourable to obtain multiple semen samples per male before and after intervention, however, this was not feasible in our study, particularly from the fertile men. It is well-known that semen parameters must be carefully interpreted because of wide intra-individual biological fluctuations (Tielemans *et al*., 1997; Neuwinger *et al*., 1990), intra- and interobserver variability, and limitations and inaccuracies of the methods used to measure them. Therefore, misclassification could have occurred in this study. However, this misclassification would equally affect both groups, and therefore the results will underestimate the actual effects of folic acid and zinc sulphate supplementation. The effects of folic acid and zinc sulphate intervention cannot be explained by regression towards the mean because a placebo group was included in both the fertile and the subfertile group.

We describe in part II an observational hospital-based case (subfertile) and control (fertile) study in IVF and ICSI couples. In man at least 2 semen analyses were performed and the endocrine status was determined. In the women a basal body temperature curve chart was used to assess the menstrual cycle for ovulation disorders. In addition in these women we determined the endocrine status, Chlamydia serology and a hysterosalpingogram or laparoscopy. The latter on indication only, to assess the uterine cavity and tubal patency. Because of these extensive examinations misclassification of cases and controls is limited.

Quality of measurements

The differences in biochemical parameters found between fertile and subfertile groups in our studies are not due to differences in measurement errors. All semen samples were obtained via a similar method, that is, masturbation into polypropylene containers after an abstinence period of 3-5 days. The semen analyses were performed in a similar fashion by several experienced laboratory technicians using the WHO guidelines (1992). Possibly it would have been more accurate to define sperm morphology according to the strict (Tygerberg) criteria, since Menkveld and Kruger (1995) report that the strict criteria are a better prognosticator of male fertility potential, expected fertilization *in vitro*, and pregnancy rates in assisted reproduction in comparison with the WHO guidelines for sperm morphology. However, evaluation of sperm morphology according to strict criteria needs experience. We had to adhere to the regular IVF and ICSI protocols used in the fertility clinic of the Radboud University Nijmegen Medical Centre, where WHO guidelines are used to evaluate semen samples.

All women were treated according to the same protocol in the IVF or ICSI program. Furthermore, all biological samples obtained were stored at comparable temperatures before analyses of the different biochemical parameters. The measurement of these biochemical parameters was conducted in a random fashion, using the same equipment and standards.

In part II specific concentrations of a biochemical parameter in spermatozoa or follicular fluid (FF) are associated with outcome parameters in the IVF or ICSI program. For the determination of these parameters, FFs of several follicles were pooled, as well as the spermatozoa from which the fertilising spermatozoon originated. Therefore, we do not know the specific concentration of the biochemical parameter in the FF surrounding the oocyte, or in the spermatozoon that lead to the evaluated embryo. This hampered the interpretation of the independent determinant with fertility outcome. The consequence namely was that no direct association could be calculated between the concentration of the parameter in the FF of the oocyte and in the spermatozoon and the successful fertilisation and implantation of those gametes.

Part I Main results

The prevalence of the different C677T MTHFR polymorphism genotypes were comparable in fertile and subfertile men. Furthermore, in the pooled group of men, only wild-types show improved sperm counts after folic acid and zinc sulphate intervention. Men with the heterozygous or homozygous polymorphism did not benefit from folic acid and zinc sulphate intervention (*Chapter 3*). These observations are different from the results of Bezold *et al*. (2001) who found an almost twofold higher prevalence rate of C677T MTHFR homozygotes in infertile men compared with fertile men, and who suggested a beneficial effect of folic acid supplementation on sperm count in infertile men. Apparently, the residual MTHFR activity in homozygote and heterozygote patients is insufficient to improve spermatogenesis after supplementation. Therefore we conclude that a higher prevalence rate of the C677T MTHFR homozygosity in subfertile men, accompanied with an altered folate metabolism, responsive to folic acid (and zinc sulphate) intervention is not the most important underlying mechanism explaining the beneficial effect of this intervention treatment on sperm count found in our previous study (Wong *et al*., 2002).

The observation that the hormones FSH, testosterone and inhibin B did not significantly change after intervention with folic acid and zinc sulphate in both fertile and subfertile men (*Chapter 4*) strongly suggest that the beneficial effect of this intervention on sperm count is not regulated through an effect on the endocrine status of these men.

Finally, the annexin A5 concentration in seminal plasma used as a marker of apoptosis (*Chapter 5*), did not change after folic acid and zinc sulphate intervention in both fertile and subfertile men. Since we did not assess the amount of apoptosis in spermatozoa themselves, we cannot conclude that the beneficial effect of the intervention treatment did not affect the rate of apoptosis in spermatozoa of these men.

Part II Main results

Since the oxidative pathway is important in human reproduction (*Chapter 2*), we hypothesized that the endogenous family of thiol antioxidants could be involved in idiopathic subfertility. Therefore we measured the concentrations of these thiols in several biological fluids of both the men and women participating

in an IVF or ICSI program (*Chapter 7*). It revealed that the spermatozoa of men with oligoasthenoteratozoospermia contain much higher thiol concentrations than in idiopathic subfertile and fertile men. This suggests that the oxidative pathway is more triggered than in men with normal semen parameters. Because the thiol levels are not depleted, we conclude that the trigger of oxidative activation is either mild, taking place during a short period, or a rich source of antioxidants is available in the male reproductive tract. The high thiol concentrations are very likely a result of the counter balance between these antioxidants and reactive oxygen species (ROS) in spermatozoa. We did not measure ROS concentrations in our specimen samples, but there are now enough indications to study the balance between these determinants. Moreover, it is known from literature that the sperm of subfertile men often contains higher ROS levels as compared to those of fertile men (Sharma and Agarwal, 1996; Taylor, 2001). An important question still is whether the amount of thiols in the spermatozoa of these subfertile men is high enough to prevent major damage to their spermatozoa. If not, this could be a cause of defective sperm function and subfertility.

In addition, we observed that a high concentration of the specific thiol homocysteine (Hcy) both in the ejaculate and in FF of all participants was associated with a higher risk of moderate to low embryo quality on culture day 3. This is in line with the known teratogenic effects of hyperhomocysteinemia (Boot *et al*., 2004a; Greene *et al*., 2003; Rosenquist *et al*., 1996) and suggests that Hcy may be a biomarker for fertility outcome in an IVF/ICSI procedure. However, the underlying mechanism of this detrimental effect of Hcy in the ejaculate and FF on embryo quality is not clear yet. Hyperhomocysteinemia can be regarded as an oxidant and in the ejaculate and/or FF may results in damage to membranes or DNA of the spermatozoa or oocytes. It has been shown that auto-oxidation of Hcy results in the generation of the reactive oxygen radical hydrogen peroxide, which in turn leads to oxidative damage to DNA and plasma membranes eventually leading to apoptosis (Huang *et al*., 2001). Szymanski and Kazdepka-Zieminska (2003) concluded that folic acid supplementation reduces the FF concentrations of Hcy, and that women with low compared with high Hcy concentrations in FF have oocytes of better quality and maturity. No detailed information about the effects of Hcy on spermatozoa is available in literature.

The plasminogen activator system (PA-system) plays a role in human reproduction (*Chapter 6*). Therefore, we investigated some of the determinants in the ejaculate, spermatozoa and FF of couples with different causes of subfertility and associated the concentrations of the determinants with fertility outcome parameters of an IVF/ICSI program (*Chapter 8*). We observed that the tissue-type plasminogen activator (tPA) concentration was higher in spermatozoa of men with oligoasthenoteratozoospermia compared with men with normal semen analysis. Furthermore, there was a trend towards higher tPA concentrations in FF of females with Fallopian tube pathology or endometriosis compared with fertile women. Also, tPA in spermatozoa was negatively associated with the chance of pregnancy, and tPA in FF was positively associated with the proportion of cleaved embryos on culture day 3 of the IVF/ICSI program. Literature reveals that tPA in the male has several important functions in sperm maturation and fertilization (*Chapter 6*). It can be concluded from our data, however, that an excess of tPA seems to have detrimental effects on sperm concentration and success of pregnancy. In literature, only the study by Maier *et al*. (1991) also investigated the difference in tPA concentration in lysed spermatozoa between fertile and subfertile males. These authors also observed higher tPA concentrations in men with oligoasthenoteratozoospermia. They postulate that the high concentrations of tPA could result in premature detachment of the spermatozoa from the basal lamina in the seminiferous tubules. This could lead to more precursors of spermatozoa in semen. We cannot verify this in our study, since we did not independently score the number of precursor sperm cells in the ejaculate. Another possible explanation is that the balance between tPA and its inhibitor PAI-1 in spermatozoa is disturbed in subfertile men, leading to an excess of tPA activity (*Chapter 8*), thereby damaging the spermatozoa resulting in a diminished capacity of the subsequent embryo to implant. This hypothesis is consistent with the new finding of a negative association between tPA concentrations in spermatozoa and successful pregnancy we observed. However, we did not observe a correlation between tPA concentration in spermatozoa and embryo quality in culture.

Our observation that the FF of women with female factor subfertility due to Fallopian tube pathology or endometriosis contain slightly higher concentrations of tPA compared with fertile women, and that this FF tPA

concentration is positively correlated with the percentage of cleaved embryos on culture day 3 in an IVF/ICSI program is new and cannot be verified in literature. Rom *et al*. (1987) suggested that a high follicular PA activity, indicative of impending ovulation, could be used as an index of follicular maturation. This idea is consistent with our finding that high FF tPA concentrations are associated with a higher percentage of embryo cleavage in culture. However, Rom *et al*. found opposite results, indicating that follicles with the highest PA activity yielded oocytes that did not cleave *in vitro*. We suggest that as for most biochemical parameters will hold true that there is a Gauss distribution in which too low or too high concentrations are detrimental. Dependent on the place of the curve and background influences the determinants will have a biological effect. We measured protein concentrations in FF as an established measure of follicular maturity (Spitzer *et al*., 1996) and found no difference in these concentrations between the different diagnostic subgroups. Therefore, the difference in tPA concentration observed cannot be explained by a difference in follicle maturity. Since the pathogenesis of Fallopian tube pathology and endometriosis are very different, we do not have an explanation for the higher FF tPA concentrations in these subfertile women as compared to those in fertile women.

Finally, we observed higher VEGF concentrations in spermatozoa of subfertile men compared with fertile men. This finding was substantiated by an inverse trend between VEGF in the ejaculate and the percentage of cleaved embryos on culture day 3. Also, inverse trends between VEGF concentration in spermatozoa and embryo quality on culture day 3, and pregnancy after IVF/ICSI were found. These data are new and cannot be related to that of other authors. On the contrary, it is known that spermatozoa express the Flt-1 and KDR VEGF receptors on their surface (Obermair *et al*., 1999) thereby implying that VEGF acts directly on spermatogenic cells. The function of VEGF in spermatozoa is unknown. Hypoxia and thus oxidative stress triggers the expression of the growth factor VEGF which has mitogenic, permeability increasing, and anti-apoptotic properties. Our results indicate that high VEGF concentrations have a negative effect on fertility outcome parameters in an IVF/ICSI program, and, therefore, may reflect the presence of oxidative stress and subsequent damage to cell structures leading to a diminished fertilizing

potential of spermatozoa.
In women, no differences were observed in VEGF concentrations between the diagnostic subgroups. The correlations between VEGF in FF and basic characteristics correspond with the hypothesis that VEGF in FF is a marker of ovarian aging and hypoxia, resulting in oocytes of diminished quality. However we did not find any correlation between FF VEGF concentrations and fertility outcome parameters, such as embryo quality or pregnancy.

Links between part I and II

This thesis consists of 2 separate studies: A double-blind, placebo-controlled randomised intervention study in part I and an observational hospital-based case-control study in part II. Regarding the different biochemical parameters discussed in part II, Hcy, tPA and VEGF were observed to be associated with fertility outcome parameters in an IVF/ICSI program. It is interesting to note that Hcy induces VEGF transcription and expression *in vitro* (Roybal *et al*., 2004). In turn, VEGF is known to be an inducer of tPA expression *in vitro* (Mandriota *et al*., 1995; Pepper *et al*., 1991).
Several links can be made between Hcy, VEGF and tPA (Part II) and folate and zinc discussed in part I of this thesis. It is well-known that a deficiency in folate results in hyperhomocysteinemia which is implicated in several obstetric and gynaecologic complications (de la Calle *et al*., 2003), however, no specific literature information is available concerning the direct relationship between hyperhomocysteinemia and subfertility. Furthermore, folic acid supplementation is an effective treatment for hyperhomocysteinemia and its intake by woman trying to conceive in order to prevent occurrence of neural-tube defects in their offspring is nowadays widely accepted.
No direct links between tPA and folate are known today, however, folate related hyperhomocysteinemia is also associated with cardiovascular disease, characterised among others by extracellular matrix modifications. Interestingly, abnormal matrix deposition can be provoked in blood vessels of neural crest origin by homocysteine injections into the neural tube lumen of chick embryos (Boot *et al*., 2004a). Possibly, dysregulation of the plasminogen activator system is involved, however, no studies on this direct relationship have been conducted so far. This hypothesis is further supported by the notion that hyperhomocysteinemia results in a defect in fibrillin, an extracellular matrix

protein (Bellamy and McDowell, 1997; Boot *et al*., 2004b). The hypothesis is that fibrillin is especially vulnerable to high levels of homocysteine because of its epidermal growth factor (EGF)-like domain (Krumdieck and Prince, 2000). Interestingly, tPA also contains such an EGF-like domain susceptible to homocysteine attack.
In literature, no direct link is known between VEGF and folate. However, as already mentioned, folate deficiency can result in hyperhomocysteinemia, which in turn is responsible for the induction of VEGF transcription and expression. Furthermore, folate is known to have antioxidant functions (Joshi *et al*., 2001), by clearing reactive oxygen species (ROS). In turn, ROS molecules are inducers of VEGF expression. Hypothetically, a deficiency in folate could result in an oxidant state, resulting in higher VEGF levels.

The relationships between zinc and the different biochemical parameters discussed here are less straightforward. A common characteristic of enzymes involved in methyl transfers to thiols is the essential role of zinc incorporated in these enzymes (Matthews and Goulding, 1997). Methionine synthetase (MS) and betaine-homocysteine methyltransferase (BHMT), 2 enzymes involved in the conversion of homocysteine to methionine, accomplished by the addition of a methyl group to homocysteine (a thiol), are zinc dependent (Goulding and Matthews, 1997; Millian and Garrow, 1998). Surprisingly, Hong *et al*. (2000) observed that dietary zinc deficiency in rats resulted in an increase in hepatic MS activity, subsequently leading to lower plasma homocysteine and folate concentrations in the zinc deficient animals.
In a study investigating the effects of zinc and tPA on neural toxicity in mice and rats, it was observed that zinc is capable of binding strongly and dose-dependently to both active and inactive tPA resulting in an inhibition of its proteolytic activity (Siddiq and Tsirka, 2004).
Finally, no direct link is known between zinc and VEGF. As was mentioned in the paragraph discussing the possible link between folic acid and VEGF, zinc which is a known antioxidant (Zago and Oteiza, 2001) could lower ROS levels, which are inducers of VEGF expression.

Conclusions

Male and female factor subfertility are complex entities. The aim of the present thesis was to gain more insight into the complex pathophysiology of human subfertility. Despite some negative findings we have found indeed some new and intriguing determinants in pathways involved in male and female subfertility. However, the causality of these associations have to be investigated and confirmed in further studies. Also, since subfertility is regarded as a multifactorial condition, of which we tried to elucidate some aspects involved, many other involved facets of this condition remain unknown. There is a need for more basic research into the causes of human subfertility to elucidate these facets, reducing the percentage of couples faced with idiopathic subfertility. This could facilitate the development of more suitable treatments for subfertile couples or even lead to preventative measures for subfertility.

In part I we ascertained that the beneficial effect of folic acid and zinc sulphate cannot be explained by differences in folate related gene polymorphism C677T in the MTHFR gene, neither by an influence on the endocrine status of these men, nor as a result of an effect on annexin A5 concentrations in seminal plasma. The studies in part II show that thiols, tPA and VEGF in males and tPA in females are important in the pathophysiology of subfertility. Whether the difference in these concentrations is a cause or a consequence of impaired semen or oocyte quality and how these mechanisms act cannot be answered yet.

The three biochemical parameters, Hcy, tPA and VEGF are interrelated, and differences or deficiencies in folate and zinc levels can (hypothetically) affect Hcy, tPA and VEGF concentrations. Since we did not find an explanation for the earlier observed increase in sperm count after folic acid and zinc sulphate intervention, along with our observations that the concentrations of endogenous thiols, plasminogen activator system parameters, and VEGF are higher in subfertile men, and inversely associated with fertility outcome parameters, it would have been interesting to evaluate the parameters discussed in part II in the intervention study presented in part I. Possibly, the beneficial mechanism of this intervention is caused by an effect on Hcy, tPA or VEGF levels.

Implications for clinical practice and future research

We did not unravel the underlying mechanism of the beneficial effect of folic acid and zinc sulphate on sperm count. Also, possible side effects are not known yet. For instance Wong et al. (2002) observed next to the 74% increase in the total normal sperm count a 4% increase of abnormal sperm count in subfertile men after folic acid and zinc sulphate intervention. Furthermore, it is not clear yet whether the improvement in total normal sperm count actually leads to an increase in pregnancy rates, since an increase in total normal sperm count of 74% does not necessarily mean that the sperm concentration of these subfertile men increases above 20 million per mL. Furthermore, an increase in sperm quantity alone does not necessarily lead to higher pregnancy rates. The quality of spermatozoa is a very important factor for fertilization. However, the beneficial effect on the quantitative aspect of spermatogenesis could result in an IVF treatment instead of an ICSI treatment for subfertile couples coping with male factor subfertility. An IVF treatment is preferable over an ICSI treatment because the aspect of natural selection of a spermatozoon for fertilization is preserved in an IVF treatment, thereby assuring that a high quality spermatozoon fertilizes the oocyte. Because of the uncertainties outlined above, we do not find it justified to implement the supply of folic acid and zinc sulphate to subfertile males in clinical practice. However, we do believe that improvements of diet, i.e. increased intake of fruits and vegetables, should also be recommended to subfertile men since a healthy lifestyle is advisable to everyone.

Also, the measurement of Hcy, tPA or VEGF in biological fluids of men and women participating in an IVF or ICSI program to assess their chances of successful conceiving is not indicated yet. Further research is needed to confirm that the observed associations in part II are causally related to subfertility and IVF/ICSI outcome. This will be difficult to achieve in men and women participating in these artificial reproductive techniques for obvious ethical reasons, but possibly these relationships could be investigated in laboratory animals, or in *in vitro* experiments. In these experiments one could change concentrations of Hcy, tPA or VEGF and investigate the effects on fertility outcome parameters. If Hcy, tPA and VEGF are causally related to subfertility and IVF/ICSI outcome, an assessment should be made what the contribution of these factors is in predicting IVF/ICSI outcome in different

subtypes of subfertility. If the contribution of these parameters is large enough, the assessment of these variables could be implemented in clinical practice to assess the chance of success for a subfertile couple. A logical and interesting next step would then be to investigate the possible modifying effects of folic acid and zinc sulphate on these parameters in a new randomised, double-blind, placebo-controlled trial. Also, other methods for the lowering of these components could be tested for their ultimate effect on natural or artificial fertility potential and outcome.

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Summary

Subfertility is defined as the failure to conceive after 1 year of regular, unprotected intercourse with the same partner. It is a prevalent disorder affecting approximately 10-17% of all couples in the Western world. The World Health Organization (WHO) reports that in 20% of cases the cause of subfertility is predominantly originating from the male, and in 38% is mainly of female origin. In 27% of cases fertility abnormalities are found in both partners. Although there are many known causes of subfertility, ranging from congenital, genetic, endocrine, infective and environmental factors, in up to 15% of couples the aetiology of subfertility remains unknown. This group is termed idiopathic subfertile.

Since involuntary childlessness is a heavy burden for most subfertile couples, many couples seek help in the form of *in vitro* fertilization (IVF) or intracytoplasmic sperm injection (ICSI) to achieve pregnancy. Although these techniques have improved the treatment of subfertile couples, the success rate is still low (around 25%), and hardly any alternative treatment options exist. Moreover, ART is a symptomatic, rather than a causal treatment for subfertility. Targeted treatment and, possibly, prevention of subfertility is indicated. To that end we need more knowledge and understanding of the pathophysiology and the causal determinants of (the large group of idiopathic) subfertility.

Of particular interest are the environmental and lifestyle factors implicated in subfertility, because these can be targeted for curative or preventive measures.

A significant but largely neglected lifestyle factor is nutrition, being a source of micronutrients, and exogenous antioxidants like vitamins C and E. Also, several endogenously synthesized antioxidants are essential. Furthermore, it is hypothesized in literature that matrix degradation by the plasminogen activator system and vascularization induced by the vascular endothelial growth factor (VEGF) are causally involved in subfertility.

Part I of this thesis focuses on the role of folic acid and zinc sulphate on sperm parameters, while Part II of this thesis discusses the role of antioxidants, the plasminogen activator system and VEGF.

Part I

An overview of the role of the micronutrients folate and zinc, the antioxidants glutathione and related thiols in biological processes involved in (sub)fertility are described In *Chapter 2* of this thesis. In the remaining three Chapters of Part I the results of an extension of our previously conducted double-blind, placebo-controlled intervention study investigating the effects of folic acid and zinc sulphate treatment on semen parameters in fertile and subfertile males are discussed. In this previous study, the participants were assigned to four different intervention groups consisting of 26 weeks of a daily dose of folic acid (5 mg) and placebo, zinc sulphate (66 mg) and placebo, zinc sulphate and folic acid (combined), or placebo and placebo. We demonstrated from this intervention study that total normal sperm count significantly increased after intervention with folic acid and zinc sulphate in subfertile males. However, the precise mechanism of this beneficial effect was not clear.

In *Chapter 3* the prevalence of C677T methylenetetrahydrofolate reductase (MTHFR) polymorphism was determined in 113 fertile and 77 subfertile males, and the MTHFR-dependent response of sperm concentration after folic acid and/or zinc sulphate intervention was evaluated. MTHFR is one of the key enzymes in folate metabolism. The C677T MTHFR polymorphism is common, the prevalence of the heterozygous and homozygous state is approximately 40% and 10% in Caucasians, respectively. The C677T polymorphism in the MTHFR-gene is accompanied by an altered folate metabolism.

The prevalence of the different C677T MTHFR polymorphism genotypes are comparable in fertile and subfertile men, indicating that C677T MTHFR polymorphism is not a risk factor for male factor subfertility. Furthermore, independent of fertility state, only wild-types show improved sperm counts after folic acid and zinc sulphate intervention. Men with the heterozygous or homozygous polymorphism do not benefit from folic acid and zinc sulphate intervention. Apparently, the residual MTHFR activity in homozygote and heterozygote patients is insufficient to improve spermatogenesis after supplementation. In conclusion, a higher prevalence rate of the C677T MTHFR homozygosity in our subfertile group of men, accompanied with an altered folate metabolism, responsive to folic acid (and zinc sulphate) intervention is not the underlying mechanism explaining the beneficial effect of this intervention treatment on sperm count found in our previous study.

In *Chapter 4* we investigated the hypothesis that folic acid and zinc sulphate affect endocrine parameters in 47 fertile, and 40 subfertile males, for example by stimulating the function of the Sertoli cells, thereby facilitating higher sperm production. Sertoli cells provide the essential microenvironment for normal germ cell production and are the main producers of inhibin B in the human body. The serum inhibin B concentration can be used as a sensitive marker for the quality of the Sertoli cell function and spermatogenesis in humans. The observation that the hormones FSH, testosterone and inhibin B did not significantly change after intervention with folic acid and zinc sulphate in both fertile and subfertile men, indicates that the beneficial effect of this intervention on sperm count is not regulated through an effect on the endocrine status of these men.

Because our research group has not yet identified the underlying mechanism of the beneficial effect of folic acid and zinc sulphate on spermatogenesis, and because of the observation that annexin A5 is abundantly present in seminal plasma, we explored the possibility that endogenous annexin A5 is directly involved in male factor subfertility (*Chapter 5*). Annexin A5 is a member of the protein family of annexins, and is primarily known because of its world-wide use to detect apoptosis. We measured annexin A5 concentrations in seminal plasma as a marker of apoptosis before and after folic acid and zinc sulphate intervention in 86 fertile and 78 subfertile males. Seminal plasma annexin A5 concentrations are not significantly different between fertile and subfertile males, indicating that seminal plasma annexin A5 concentration is not associated with male factor subfertility. Intervention with folic acid and zinc sulphate does not affect seminal plasma annexin A5 concentration. Therefore, it is not very likely that the observed increase in sperm count after intervention can be attributed to a possible decrease in apoptosis rate of cells involved in spermatogenesis by endogenous seminal plasma annexin A5 concentration. Since we did not directly assess the amount of apoptosis in spermatozoa themselves, we cannot conclude that the beneficial effect of the intervention treatment did not affect spermatozoal apoptosis in these men.

In conclusions, we still cannot explain why supplementation of folic acid and zinc sulphate is beneficial for sperm count in subfertile men. However, we did find out that this beneficial effect is not likely to be explained by differences in the folate related gene polymorphism C677T in the MTHFR gene, neither by an

influence on the endocrine status of these men, nor as a result of an effect on annexin A5 concentrations in seminal plasma.

Part II

The objective of part II was to study the involvement of the endogenous family of thiol antioxidants, the plasminogen activator system, and VEGF in human subfertility and in the outcome of the IVF or ICSI procedure.

In *Chapter 6* of this thesis the involvement of the plasminogen activation system and VEGF in reproduction are reviewed.

In *Chapters 7, 8 and 9* the results of a case-control study under couples applying for IVF or ICSI procedures are described. Cases consisted of idiopathic subfertile couples, women with Fallopian tube pathology or endometriosis, or men with the presence of at least one of the sperm anomalies oligozoo-, asthenozoo-, and/or teratozoospermia. Two control groups were defined. The male partners without abnormal semen parameters of the women suffering from Fallopian tube pathology or endometriosis, and the female partners without tube and menstrual cycle abnormalities of the men with male factor subfertility. A total of 156 couples were included, 52 idiopathic subfertile couples, 52 couples suffering from male factor subfertility, and 52 couples suffering from female factor subfertility.

In *Chapter 7* we investigated our hypothesis that the endogenous family of thiol antioxidants could be involved in unexplained cases of subfertility. Thiols are scavengers of reactive oxygen species (ROS), and both thiols and ROS are implicated in human reproduction. In humans the thiol glutathione (GSH) functions as the most important endogenous antioxidant. Other endogenous thiols are cysteine (Cys), homocysteine (Hcy) and cysteinylglycine (CGS). Cysteine is a precursor amino acid of GSH. Cysteinylglycine is composed of cysteine and glycine and is a main intermediate in the transport or synthesis of GSH. Our results indicate that the spermatozoa of subfertile men contain much higher thiol concentrations as compared to the spermatozoa of idiopathic subfertile and fertile men. This finding suggests that oxidative defence mechanisms are triggered to a higher extent in subfertile men as those in men with normal semen parameters. These higher thiol concentrations could be a counter mechanism to balance higher ROS concentrations in spermatozoa of subfertile men. Whether the amount of thiols in the spermatozoa of these

subfertile men is high enough to prevent major damage to their spermatozoa is not clear. If not, this could be a cause of their defective sperm function and subfertility. Furthermore, we observed that high concentrations of Hcy both in the ejaculate and in follicular fluid (FF) of couples participating in IVF or ICSI procedures was associated with a higher chance of a moderate/low embryo quality. This result suggests that Hcy is a biomarker for fertility outcome in an IVF/ICSI procedure. However, the underlying mechanism of this detrimental effect of Hcy in the ejaculate and FF on embryo quality is not clear.

In *Chapter 8* we investigated the importance of the plasminogen activator system (PA-system) in human subfertility. The PA-system is important in matrix degradation and is known to be involved in a variety of reproductive processes in both males and females. We observed that tissue-type plasminogen activator (tPA) concentrations were higher in spermatozoa of men in the male factor subfertility group compared with these concentrations in spermatozoa of fertile and idiopathic subfertile men. Furthermore, there was a trend towards higher tPA concentrations in FF of females with Fallopian tube pathology or endometriosis compared with fertile women. Furthermore, tPA in spermatozoa was negatively associated with the chance of pregnancy, and tPA in FF was positively associated with the proportion of cleaved embryos on culture day 3 of the IVF/ICSI program. Therefore we conclude that tPA in both males and females is important in the pathophysiology of subfertility.

Chapter 9 presents the results of our study on the relationship between VEGF and human subfertility. VEGF is a mitogenic and permeability increasing growth factor, which also seems to have important functions in human reproduction. We observed higher VEGF concentrations in spermatozoa from men with male factor subfertility as compared to those in fertile and idiopathic subfertile men. High VEGF concentrations in the ejaculate tend to be negatively associated with the percentage of cleaved embryos on culture day 3. Furthermore, high VEGF concentrations in spermatozoa tend to be negatively associated with embryo quality, and pregnancy rate. These results suggest that VEGF is a new biomarker for subfertility, and that VEGF affects fertility outcome parameters in an IVF/ICSI procedure.

In conclusion, the studies in part II show that thiols, tPA and VEGF in males and tPA in females are important in the pathophysiology of subfertility. Whether the difference in these concentrations are causes or consequences of impaired

semen or oocyte quality and how these mechanisms work cannot be answered yet.

In the general discussion (*Chapter 10*) we reflect on all these different findings in the light of the methodological strengths and weaknesses, and in relation to literature we try to synthesise what knowledge about the pathophysiology of subfertility has been achieved and what the links can be between all the different hypotheses and results described in the separate chapters.
This enables us to formulate recommendations for new research and potential clinical implications.

Samenvatting

Subfertiliteit wordt gedefinieerd als het onvermogen zwanger te worden binnen een jaar van regelmatige, onbeschermde coïtus met dezelfde partner. Het is een veel voorkomende aandoening die ongeveer 10-17% van alle stellen in de westerse wereld treft. De wereldgezondheidsorganisatie rapporteert dat de oorzaak van subfertiliteit in 20% van de gevallen bij de man ligt en in 38% bij de vrouw. In 27% van de gevallen zijn oorzaken voor subfertiliteit te vinden in beide partners. Hoewel er veel bekende oorzaken zijn voor subfertiliteit, variërend van congenitaal, endocrien en infectieus tot omgevingsfactoren, blijft de etiologie voor de subfertiliteit in 15% van de gevallen toch onbekend. Dit wordt idiopathische subfertiliteit genoemd.
Omdat ongewenste kinderloosheid een zware last is voor de meeste subfertiele koppels, zoeken vele van hen hulp in de vorm van *in vitro* fertilisatie (IVF) of intracytoplasmatische sperma injectie (ICSI) om toch zwanger te worden. Hoewel deze technieken de behandeling van subfertiele stellen verbeterd hebben, is het succespercentage nog steeds laag (rond 25%) en alternatieve behandelingsopties bestaan nauwelijks. Bovendien zijn artificiële reproductietechnieken een symptomatische in plaats van een causale behandeling voor subfertiliteit. Doelgerichte behandeling en zo mogelijk preventie van subfertiliteit is geïndiceerd. Om dat doel te bereiken is meer kennis en begrip van de pathofysiologie en de causale determinanten van (de grote groep van idiopathische) subfertiliteit noodzakelijk.
Met name van belang zijn de omgevings- en leefstijlfactoren bij subfertiliteit, omdat deze factoren aangepakt kunnen worden in het kader van curatieve of preventieve maatregelen.

Een significante, maar grotendeels genegeerde leefstijlfactor is voeding, een bron van micronutriënten en exogene antioxidanten zoals vitamine C en E. Ook essentieel zijn verschillende endogeen gesynthetiseerde antioxidanten. In de literatuur komt verder nog de hypothese naar voren dat matrix degradatie door het plasminogeen activator systeem en vascularisatie geïnduceerd door de vaatgroeifactor VEGF causale factoren zijn bij subfertiliteit.
Deel I van dit proefschrift focust op de rol van foliumzuur en zinksulfaat op semenparameters, terwijl in Deel II de rol van antioxidanten, het plasminogeen activator systeem en VEGF bediscussieerd wordt.

Deel I

In *Hoofdstuk 2* van dit proefschrift wordt een overzicht gegeven van de rol van de micronutriënten foliumzuur en zink en de antioxidant glutathion en gerelateerde thiolen in biologische processen van belang bij (sub)fertiliteit. In de overgebleven drie hoofdstukken van Deel I worden de resultaten bediscussieerd van een vervolgstudie op onze eerder uitgevoerde dubbel-blinde, placebo-gecontroleerde interventiestudie over de invloed van foliumzuur en zinksulfaat behandeling op semenparameters in fertiele en subfertiele mannen. In deze interventiestudie, werden de deelnemers ingedeeld in vier verschillende interventiegroepen die respectievelijk een dagelijkse dosis foliumzuur (5 mg) en placebo, zinksulfaat (66 mg) en placebo, zinksulfaat en foliumzuur (gecombineerd), of placebo en placebo, hebben ingenomen gedurende 26 weken. Uit deze interventiestudie hebben we kunnen concluderen dat na inname van foliumzuur en zinksulfaat het totaal aantal normale spermatozoa van subfertiele mannen significant toenam. Het precieze mechanisme van dit positieve effect is echter nog niet duidelijk.

in *Hoofdstuk 3* wordt de prevalentie van het C677T methyleentetrahydrofolaat reductase (MTHFR) polymorfisme bepaald en wordt de MTHFR-afhankelijke respons van de spermaconcentratie na foliumzuur en/of zinksulfaat interventie geëvalueerd in 113 fertiele en 77 subfertiele mannen. MTHFR is een van de belangrijkste enzymen in het foliumzuurmetabolisme. Het C677T MTHFR polymorfisme komt veel voor, met een prevalentie van de heterozygote en homozygote status van respectievelijk ongeveer 40% en 10% in Caucasiërs. Het C677T polymorfisme in het MTHFR-gen gaat hand in hand met een veranderd foliumzuurmetabolisme.

De prevalentie van de verschillende C677T MTHFR polymorfisme genotypes zijn vergelijkbaar in fertiele en subfertiele mannen, wat aangeeft dat C677T MTHFR polymorfisme geen risicofactor is voor mannelijke-factor-subfertiliteit. Voorts, onafhankelijk van fertiliteitstatus, hebben alleen mannen zonder polymorfisme baat bij foliumzuur en zinksulfaat interventie wat betreft hun spermatozoa-aantal. Noch mannen met een heterozygoot als met een homozygoot polymorfisme hebben baat bij foliumzuur en zinksulfaat interventie. Klaarblijkelijk is de restactiviteit van MTHFR in homozygote en heterozygote patiënten onvoldoende om de spermatogenese te verbeteren na suppletie. Concluderend, een hogere prevalentie van C677T MTHFR homozygotie in

onze subfertiele populatie, met als gevolg een veranderd foliumzuur-metabolisme, gevoelig voor foliumzuur (en zinksulfaat) interventie, is niet het onderliggende mechanisme dat het positieve effect van deze interventie op spermatozoa-aantal, gevonden in onze eerdere studie, verklaart.

In *Hoofdstuk 4* hebben we in 47 fertiele en 40 subfertiele mannen de hypothese getest dat foliumzuur en zinksulfaat endocriene parameters beïnvloedt, bijvoorbeeld door stimulatie van de functie van Sertolicellen, waardoor een hogere spermatozoaproductie kan worden gerealiseerd. Sertolicellen verschaffen het essentiële micromilieu voor normale kiemcelproductie en zijn de belangrijkste producenten van inhibine B in het menselijke lichaam. De serum inhibine B concentratie kan gebruikt worden als een gevoelige marker voor de kwaliteit van Sertolicelfunctie en spermatogenese in de mens. De observatie dat er geen verandering optreedt in de concentratie van de hormonen FSH, testosteron en inhibine B na foliumzuur en zinksulfaat interventie in zowel fertiele als subfertiele mannen, geeft aan dat het positieve effect van deze interventie op spermatozoa-aantal niet gereguleerd wordt door een effect op de endocriene status van deze mannen.

Omdat onze onderzoeksgroep nog steeds geen verklaring heeft voor het onderliggende mechanisme van het positieve effect van foliumzuur en zinksulfaat op de spermatogenese en gezien de observatie dat annexine A5 in hoge concentraties aanwezig is in seminaal plasma, hebben we gekeken naar de mogelijkheid dat endogeen annexine A5 direct betrokken is bij mannelijke-factor-subfertiliteit (*Hoofdstuk 5*). Annexine A5 is een lid van de eiwitfamilie van annexines en is met name bekend om zijn wereldwijde gebruik als apoptose detector. Wij hebben annexine A5 concentraties gemeten, als een marker voor apoptose, in het seminaal plasma van 86 fertiele en 78 subfertiele mannen. Seminaal plasma annexine A5 concentraties zijn niet significant verschillend tussen fertiele en subfertiele mannen, wat aangeeft dat de seminaal plasma annexine A5 concentratie niet geassocieerd is met mannelijke-factor-subfertiliteit. Interventie met foliumzuur en zinksulfaat heeft geen invloed op seminaal plasma annexine A5 concentraties. Daarom is het niet erg waarschijnlijk dat de geobserveerde toename in spermatozoa-aantal na interventie, toegeschreven kan worden aan een mogelijke daling van apoptose van cellen van belang voor de spermatogenese door endogene seminaal plasma annexine A5 concentraties. Omdat we niet gekeken hebben naar de

mate van apoptose van de spermatozoa zelf, kunnen we niet concluderen dat het positieve effect van de interventie geen effect heeft gehad op apoptose van spermatozoa in deze mannen.

Concluderend kunnen we nog steeds niet verklaren waarom suppletie van foliumzuur en zinksulfaat een positief effect heeft op het spermatozoa-aantal van subfertiele mannen. Echter, we kunnen wel concluderen dat het niet waarschijnlijk is dat dit positieve effect verklaard wordt door verschillen in het foliumzuur gerelateerde genetische polymorfisme C677T in het MTHFR gen, door invloed op de endocriene status van deze mannen, of als resultaat van een effect op annexine A5 concentraties in seminaal plasma.

Deel II

Het doel van deel II van dit proefschrift was het bestuderen van de betrokkenheid van de endogene familie van thiol antioxidanten, het plasminogeen activator systeem, en VEGF, in subfertiliteit van de mens en in de uitkomst van de IVF/ICSI procedure.

In *Hoofdstuk 6* van dit proefschrift wordt de betrokkenheid van het plasminogeen activator systeem en VEGF in reproductie besproken. In *Hoofdstuk 7, 8 en 9* worden de resultaten besproken van een patiënt-controle-onderzoek in stellen die in aanmerking komen voor een IVF of ICSI behandeling. Patiënten bestaan uit idiopathisch subfertiele stellen, vrouwen met een tubafactor of endometriose of mannen met de aanwezigheid van ten minste een van de sperma-abnormaliteiten oligozoö-, asthenozoö-, en/of teratozoöspermie. We hebben twee controlegroepen gedefinieerd. De mannelijke partners van vrouwen met een tubafactor of endometriose, zonder abnormale spermaparameters, en de vrouwen zonder afwijkingen in eileiders of menstruatiecylcus met een partner met mannelijke-factor-subfertiliteit. Een totaal aantal van 156 stellen werden in het onderzoek geïncludeerd, waarvan 52 idiopatisch subfertiele stellen, 52 stellen lijdend aan mannelijke-factor-subfertiliteit en 52 stellen met vrouwelijke-factor-subfertiliteit.

In *Hoofdstuk 7* hebben we onze hypothese onderzocht dat de endogene familie van thiol antioxidanten een rol speelt in de gevallen van onverklaarde subfertiliteit. Thiolen vangen reactieve zuurstofradicalen (ROS) weg, en zowel thiolen als ROS worden van belang geacht in de reproductie van de mens. In

de mens functioneert de thiol glutathion (GSH) als de belangrijkste endogene antioxidant. Andere endogene antioxidanten zijn cysteïne (Cys), homocysteïne (Hcy) en cysteïnylglycine (CGS). Cysteïne is een voorloper aminozuur van GSH. Cysteïnylglycine is samengesteld uit cysteïne en glycine en is een belangrijke intermediair in het transport of de synthese van GSH. Onze resultaten laten zien dat de spermatozoa van subfertiele mannen veel hogere thiolconcentraties bevatten dan de spermatozoa van idiopathisch subfertiele en fertiele mannen. Deze bevindingen suggereren dat oxidatieve verdedigings-mechanismen in subfertiele mannen in hogere mate gestimuleerd worden dan in mannen met normale semenparameters. Deze hogere thiolconcentraties zouden een verdedigingsmechanisme kunnen zijn ter compensatie van de hogere ROS concentraties in spermatozoa van subfertiele mannen. Of de thiolhoeveelheid in de spermatozoa van deze subfertiele mannen hoog genoeg is om ernstige schade aan de spermatozoa te voorkomen, is niet duidelijk. Is de thiolconcentratie relatief te laag, dan kan dat een oorzaak zijn voor hun slechtere spermafunctie en subfertiliteit. In deze studie hebben we ook gezien dat hogere concentraties van Hcy zowel in het ejaculaat als in de follikelvloeistof (FF) van stellen die deelnamen aan een IVF of ICSI procedure, geassocieerd was met een hogere kans op een matig/slechte embryokwaliteit. Dit resultaat suggereert dat Hcy een biomarker is voor fertiliteitsuitkomst in een IVF/ICSI procedure. Echter, het onderliggende mechanisme van dit negatieve effect van Hcy in ejaculaat en FF op embryo-kwaliteit is niet duidelijk.

In *Hoofdstuk 8* hebben we onderzocht wat het belang is van het plasminogeen activator systeem (PA-systeem) bij subfertiliteit van de mens. Het PA-systeem is van belang bij matrix degradatie en bekend om zijn betrokkenheid bij een veelheid aan reproductieprocessen in zowel mannen als vrouwen. Wij hebben gezien dat tissue-type plasminogeen activator (tPA) concentraties hoger waren in spermatozoa van subfertiele mannen dan in spermatozoa van mannen in de idiopathisch subfertiele en fertiele groep. In aanvulling hierop hebben we een trend gezien richting hogere tPA concentraties in FF van vrouwen met een tubafactor of endometriose in vergelijking met fertiele vrouwen. Een ander resultaat is dat tPA in spermatozoa negatief geassocieerd is met de kans op zwangerschap en tPA in FF positief geassocieerd is met het percentage gedeelde embryo's op kweekdag 3 van het IVF/ICSI programma.

Hieruit concluderen wij dat tPA in zowel mannen als vrouwen belangrijk is in de

pathofysiologie van subfertiliteit.
Hoofdstuk 9 presenteert de resultaten van onze studie naar de relatie tussen VEGF en subfertiliteit in de mens. VEGF is een vaatgroeifactor die ook de doorlaatbaarheid van vaten verhoogt. Deze functies zijn ook van belang in reproductie. Onze resultaten laten zien dat spermatozoa van subfertiele mannen hogere VEGF concentraties bevatten dan spermatozoa van idiopathisch subfertiele en fertiele mannen. Hoge VEGF concentraties in het ejaculaat leken negatief geassocieerd te zijn met het percentage gedeelde embryo's op kweekdag 3. Tevens leken hoge VEGF concentraties in spermatozoa negatief geassocieerd te zijn met embryokwaliteit en zwangerschapskans. Deze resultaten suggereren dat VEGF een nieuwe biomarker is voor subfertiliteit en dat VEGF de uitkomstparameters van een IVF/ICSI procedure beïnvloedt.

Concluderend kunnen we stellen dat de resultaten gepresenteerd in deel II laten zien dat thiolen, tPA en VEGF in mannen en tPA in vrouwen belangrijk zijn in de pathofysiologie van subfertiliteit. Of de verschillen in concentratie van deze stoffen oorzaken of consequenties zijn van verminderde semen- of oocytkwaliteit en hoe deze mechanismen precies werken, is nu nog niet bekend.

In de algehele discussie (*Hoofdstuk 10*) reflecteren we op al de verschillende bevindingen en bekijken we ze in het licht van de methodologische sterke en zwakke punten. In relatie tot de literatuur proberen we de kennis van de pathofysiologie van subfertiliteit verkregen middels dit proefschrift bij elkaar te brengen tot een geheel. Ook bediscussiëren we de samenhang tussen de verschillende hypothesen en resultaten in de verschillende hoofdstukken. Dit stelt ons in staat om aanbevelingen te doen voor toekomstig onderzoek en potentiële klinische implicaties van onze resultaten.

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Curriculum Vitae

Inge Maria Wilhelmus Ebisch was born on February 26th, 1980. From 1992 till 1998 she attended secondary school (VWO) at the Bouwens van der Boijecollege in Panningen. In 1998 she started with the study Biomedical Health Sciences at the current Radboud University Nijmegen Medical Centre. In her practical research training she started with her thesis by performing the studies described in part I of the thesis under the supervision of Dr. R.P.M. Steegers-Theunissen. She graduated in 2002 with a major in pathobiology. From September 2002 until August 2003 she became a PhD student and worked full time on part II of the thesis at the Departments of Obstetrics and Gynaecology, Chemical Endocrinology and Epidemiology and Biostatistics of the Radboud University Nijmegen Medical Centre. In September 2003 she started Medical School at the Radboud University Nijmegen Medical Centre. In the mean time she continued the research and finished her thesis (supervision Dr. R.P.M. Steegers-Theunissen, Dr. C.M.G. Thomas and Prof. Dr. D.D.M. Braat). In August 2005 she started her internships and will finish Medical School in May 2007.