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

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Current treatments of subfertile couples are usually empiric, as the true cause of subfertility often remains unknown. Therefore, we outline the role of nutritional and biochemical factors in reproduction and subfertility. A literature search was performed using MEDLINE, Science Direct and bibliographies of published work with both positive and negative results. The studies showed that folate has a role in spermatogenesis. In female reproduction, folate is also important for oocyte quality and maturation, implantation, placentation, fetal growth and organ development. Zinc has also been implicated in testicular development, sperm maturation and testosterone synthesis. In females, zinc plays a role in sexual development, ovulation and the menstrual cycle. Both folate and zinc have antioxidant properties that counteract reactive oxygen species (ROS). Thiols, such as glutathione, balance the levels of ROS produced by spermatozoa and influence DNA compaction and the stability and motility of spermatozoa. Oocyte maturation, ovulation, luteolysis and follicle atresia are also affected by ROS. After fertilization, glutathione is important for sperm nucleus decondensation and pronucleus formation. Folate, zinc, ROS and thiols affect apoptosis, which is important for sperm release, regulation of follicle atresia, degeneration of the corpus luteum and endometrial shedding. Therefore, the concentrations of these nutrients may have substantial effects on reproduction. In conclusion, nutritional and biochemical factors affect biological processes in male and female reproduction. Further research should identify pathways that may lead to improvements in care and treatment of subfertility.

Key words: antioxidants/folate/MTHFR/ROS/zinc

Introduction

Subfertility is defined as the failure to conceive after 1 year of regular, unprotected intercourse with the same partner. Approximately 10–17% of all couples experience primary or secondary subfertility at some time during their reproductive life (Mosher and Pratt, 1990; Templeton *et al*., 1990; Wallace, 1995; Buckett and Bentick, 1997; Snick *et al*., 1997; Philippov *et al*., 1998). In Table I, various causes of subfertility and the corresponding frequencies are given. 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, such as assisted reproduction techniques (ART), which do not actually treat the cause of the subfertility. 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 vitamin B dependent (Figure 1). DNA synthesis is important for the development of spermatozoa and oocytes. Animal studies have demonstrated that deficiencies of vitamins A, C and D result in diminished fertility in rats and rainbow trout (Kwiecinski *et al*., 1989; van Pelt and de Rooij, 1991; Ciereszko and Dabrowski, 1995). In humans, the effects of nutrition on fertility have scarcely been investigated. Recently, our group found a 74% increase in normal sperm count and a 3% increase in abnormal morphology in subfertile men after supplementation of folic acid in combination with zinc sulphate (Wong *et al*., 2002).

Furthermore, the diet is a source of exogenous antioxidants (vitamins C and E), but several endogenously synthesized antioxidants, such as glutathione, bilirubin and uric acid, are also essential.

Table I. The causes 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 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

Total exceeds 100% as 15% of couples have more than one cause of subfertility.

These antioxidants protect DNA and other important molecules from oxidation and damage, which would otherwise induce apoptosis. A more detailed overview of glutathione metabolism is shown in Figure 1.

Folate, zinc, glutathione and related thiols are implicated in the oxidative pathway. Associations have been described between these nutrients and their derivatives and the occurrence of miscarriages and congenital malformations. Subfertility can be caused by the poor quality of the gametes and conceptus and/or the implantation failure that often present as early miscarriages. Derangements in the oxidative pathway play a role in the pathogenesis of subfertility. From this background, we give an overview of the studies in which folate, zinc, glutathione and related thiols have been investigated in association with subfertility.

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 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 and 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 experimental animal and human studies. The latter studies included case reports, randomized trials and observational studies. As search terms, we used the following words in different combinations: fertilization, subfertility, folate, folic acid, zinc, antioxidants, glutathione, thiols, apoptosis, methylenetetrahydrofolate reductase (MTHFR) and reactive oxygen species (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 methionine (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 its water-soluble character, folic acid inhibits lipid peroxidation (LPO). Therefore, folic acid can protect bio-constituents such as 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

Folic acid administration. Wong *et al*. (2002) found that folic acid administration (5 mg) to subfertile and fertile men for 26 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 intervention with a combination of folic acid and zinc sulphate for 26 weeks was observed. An intervention study by Bentivoglio *et al*. (1993) reported an increase in the number and motility of mature spermatozoa and a decrease in round cell number (the presence of immature cells) after 3 months of supplementation with 15 mg of folinic acid (5-formyl tetrahydrofolate) in 65 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 normozoospermic and oligozoospermic men; however, both the dose and the duration (10 mg for 30 days) were different compared with the study of Bentivoglio *et al*.

MTHFR. 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 MTHFR, one of the key enzymes in folate metabolism. MTHFR converts 5,10-methylenetetrahydrofolate into 5-methyl tetrahydrofolate, which subsequently donates a methyl group to cobalamin to form methylcobalamin and tetrahydrofolate. Methylcobalamin serves as a cofactor in the conversion of 5-methyl tetrahydrofolate to tetrahydrofolate, in which homocysteine is remethylated to form 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, although 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.

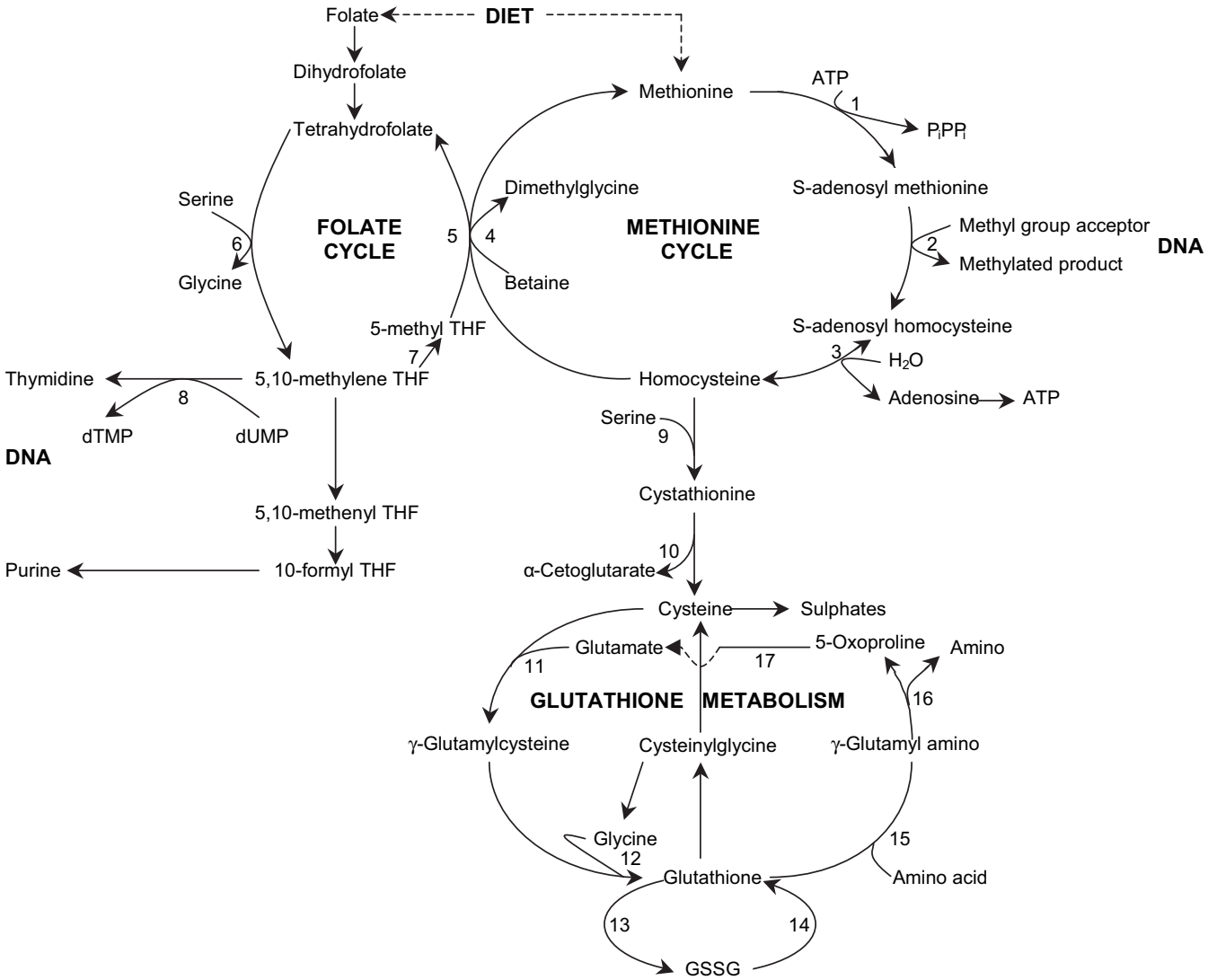

Figure 1. DNA synthesis, the folate cycle and glutathione metabolism. 1. Methionine-adenosyltransferase; 2. Methyltransferase; 3. *S*-adenosylhomocysteine hydrolase; 4. Betaine-homocysteine-methyltransferase (BHMT) (zinc dependent); 5. Methionine-synthase (vitamin B_{12} and zinc dependent); 6. Serineoxidase; 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. glutathione disulphide (GSSG) reductase; 15. γ-Glutamyl transpeptidase; 16. γ-Glutamyl cyclotransferase; 17. 5-Oxoprolinase. dTMP, deoxythymidine monophosphate; dUMP, deoxyuridine monophosphate; GSSG, glutathione disulphide; P_i, orthophosphate; PP_i, pyrophosphate; THF, tetrahydrofolate.

MTHFR and subfertility. 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 therefore 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).

Zinc and MTHFR. Animal *in vivo* and *in vitro* studies have shown that zinc deficiency decreases the absorption and metabolism of dietary folate (Ghishan *et al*., 1986; Quinn *et al*., 1990; Favier *et al*., 1993) because of its function as a cofactor for the folate-metabolizing enzymes dihydrofolate reductase and γ-glutamyl hydrolase. Zinc itself, however, is not a cofactor of the MTHFR enzyme; however, it is a cofactor for methionine synthetase and for betaine-homocysteine methyltransferase (Figure 1).

Folate in females

Little 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 have not been reported. A study on homocysteine concentrations in follicular fluid (FF) in women opting for 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 oocytes and a higher degree of mature oocytes compared with women who did not receive folic acid supplementation (Szymanski and Kazdepka-Zieminska, 2003). Moreover, it has been shown that folic acid present in mouse preimplantation embryos is absolutely essential for early embryo development, probably because of its essential role in the synthesis of thymidine, which is required for DNA synthesis and repair. Furthermore, all the folic acid necessary for this process is 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 80 metalloenzymes involved in DNA transcription and protein synthesis (Figure 1). Because DNA transcription is a major 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), and zinc also 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 counteract the oxidation by binding sulphydryl groups in proteins and 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 (Oteiza *et al*., 1995; Bagchi *et al*., 1998), and zinc salts have been shown to protect against oxidative damage and glutathione depletion in mice (Bagchi *et al*., 1998). It is also important to report that some trace metals can catalyse the reactions that lead to the formation of ROS. There is a study by Lloyd showing *in vitro* negative effects on salmon sperm DNA, determining that at concentrations of 20–50 mM and above, these metal ions cause maximum DNA strand breaks. No such studies are available in humans, and the *in vivo* dosage required for these concentrations in seminal plasma is still unknown (Lloyd *et al*., 1998).

Zinc in males

Zinc is important in several aspects of male reproduction (Table II). Zinc concentrations are very high in the male genital organs compared with other tissues and body fluids (Mann, 1964), particularly 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 (Marmar et al., 1975; Caldamone et al., 1979). Furthermore, it was shown that men with sperm counts <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).

Table II. The functions of zinc in male reproduction

Aspect of male reproduction	References
Testicular steroidogenesis	Favier (1992), Hamdi *et al*. (1997)
Testicular development	Hamdi *et al*. (1997)
Oxygen consumption of spermatozoa	Eliasson *et al*. (1971), Huacuja *et al*. (1973)
Nuclear chromatin condensation	Kvist (1980)
Acrosome reaction	Riffo *et al*. (1992)
Acrosin activity	Steven *et al*. (1982)
Sperm chromatin stabilization	Kvist *et al*. (1990)
Testosterone synthesis	Leake *et al*. (1984)
Conversion of testosterone to 5α-dihydrotestosterone	Netter *et al*. (1981)

Zinc administration. Several intervention studies investigating the effect of zinc on (sub)fertility have been conducted (Table III). Ronaghy *et al*. (1974) found a higher, although not statistically significant, proportion of already developed genitalia in malnourished schoolboys after zinc supplementation compared with boys not receiving extra zinc. The induction of mild zinc deficiency in five 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 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 estrous cycles in female rats (Swenerton and Hurley, 1968). In addition, the effects of zinc deficiency in two species of monkeys have been investigated, and it was found that normal reproduction was impaired in both the species. The pregnancy rate in zinc-deficient monkeys was significantly lower compared with the original stock colony because of a cessation in 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 two women aged 19 and 20 years, suffering from nutritional dwarfism with delayed sexual maturation. These women had no breast tissue or pubic hair and had infantile external genitalia with extremely low blood plasma

Table III. Effects of zinc supplementation on subfertility

Effect of zinc supplementation	References
Accelerate the onset in sexual function	Halsted *et al*. (1972)
Improves sperm count	Marmar *et al*. (1975), Hartoma *et al*. (1977), Mahajan *et al*. (1982), Wong *et al*. (2002)
Improves sperm motility and morphology	Marmar *et al*. (1975), Caldamone *et al*. (1979)
Improves testosterone concentration	Antoniou *et al*. (1977), Hartoma *et al*. (1977), Mahajan *et al*. (1982)
Improves sexual potency	Antoniou *et al*. (1977), Mahajan *et al*. (1982)

and erythrocyte zinc levels. After zinc supplementation, these women experienced their first menstrual period and developed breast tissue as well as pubic hair growth. Jameson (1976) reported longstanding infertility in seven, 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 two 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 and 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 reflects neither the follicular status nor the status of the oocyte.

ROS, 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. Although several studies have demonstrated the positive effects of *in vitro* antioxidants on semen quality, others have failed to show such an effect because of varying doses and therapy duration (Rolf and Cooper, 1999).

As mentioned before, dietary folate and zinc are also important for several antioxidant functions. These antioxidants can provide protection of cells against oxidative and electrophilic stress caused by ROS, which would lead to damage of DNA or other important structures such as 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 functions (Riley and Behrman, 1991b; de Lamirande and Gagnon, 1994). Animal studies have shown that antioxidants present in nutrition are important to maintain normal semen parameters and to achieve pregnancy (Kwiecinski *et al*., 1989; Ciereszko and Dabrowski, 1995).

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 two consecutive steps catalysed by γ-glutamylcysteine synthetase and glutathione synthetase. The enzyme γ-glutamyl transpeptidase is involved in the breakdown of glutathione, thereby cleaving the γ-bond resulting in glutamate and cysteinylglycine (Figure 1). Glutathione and the GSH-related enzymes, glutathione-S-transferases (GSTs), 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). Glutathione 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).

ROS and antioxidants in males

Spermatozoa and ROS. Human spermatozoa are capable of generating ROS (Alvarez *et al*., 1987; Aitken *et al*., 1989a; Alvarez and Storey, 1989; D'Agata *et al*., 1990; Iwasaki and Gagnon, 1992). ROS exert many effects on spermatozoa, which are summarized in Table IV. From this table, one can conclude that 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 the 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 GST, 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. There is evidence supporting the use of antioxidants in infertile male patients with elevated oxidative stress. Current evidence supports treatment of selected cases of infertility as well as *in vitro* media supplements during sperm preparation.

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*., 1989a,b; Sharma and Agarwal, 1996; Zini *et al*., 2000). Studies are available showing positive

Table IV. Effects of reactive oxygen species (ROS) on spermatozoa

ROS regulate	References
Epididymal maturation and physiological sperm concentrations	Cornwall *et al*. (1988)
Rate of hyperactivation	de Lamirande and Gagnon (1993a,b)
Ability to undergo the acrosome reaction	Burkman (1990), de Lamirande *et al*. (1993)
Attachment to oocytes	Aitken *et al*. (1989a, 1993)

effects of antioxidants on sperm DNA damage and pregnancy outcome (Comhaire, 2000; Lamond *et al*., 2003). LPO 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 (Aitken *et al*., 1989a; Rao *et al*., 1989; D'Agata *et al*., 1990; Iwasaki and Gagnon, 1992; Agarwal *et al*., 1994; Aitken and Fisher, 1994; Alkan *et al*., 1997).

Glutathion and subfertility. 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 i.m. injection of 600 mg of 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 of GSH, 200 mg of vitamin C and 200 mg of 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 sulphydryl 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 whose wives had no demonstrable cause of infertility. However, in this study, only semen samples with >50 million spermatozoa were used. These authors also observed that sperm GSH concentrations were low in semen samples with <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 oligozoospermic 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).

ROS 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 to active generation of ROS. ROS levels in within certain physiological 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 functions. 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 (Sugino *et al*., 1993; Musicki *et al*., 1994; Shimamura *et al*., 1995; Sawada and Carlson, 1996) 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 gonadotrophic hormones and in the 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).

Oxidative stress. One definite consequence of an excess of ROS in the ovary is damage of plasma membranes, but the question of how this damage affects female fertility remains. Several investigators have 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 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 or 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, indicating that ROS is a marker for obligatory minimal metabolic activity within the follicle. Unlike Pasqualotto *et al*. (2004), these authors did not observe a difference in TAC levels between patients who became pregnant and those who did not (Attaran *et al*., 2000). The results of Bedaiwy *et al*. (2002) also indicated that women with higher FF levels of ROS 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 that were not 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 that did not yield 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.

ROS and (sub)fertility. Some reports deal with the role of ROS and TAC in female fertility, but the specific role of glutathione in female reproduction has not as yet been investigated. It is known, however, that after fertilization, glutathione in pig oocytes is very important for the reduction of disulphide bonds in the sperm nucleus, involved in sperm nucleus decondensation and male pronucleus formation (Yoshida *et al*., 1993). Furthermore, glutathione in bovine oocytes is also important for male pronucleus formation and for pronuclear apposition during fertilization (Sutovsky and Schatten, 1997). This role of glutathione in the oocyte is further endorsed by the observation that oocytes matured in the presence of a glutathione 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 glutathione during *in vitro* maturation of oocytes resulted in improved male pronucleus formation, normal fertilization and embryo development (Sawai *et al*., 1997; Jeong and Yang, 2001; Rodriguez-Gonzalez *et al*., 2003). Also, enhancement of glutathione or components of glutathione in the oocyte and in culture medium of embryos during IVF or ICSI procedures has been shown to improve fertilization rates and embryo development (Takahashi *et al*., 1993; Kim *et al*., 1999; Fukui *et al*., 2000; Ali *et al*., 2003).

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 and apoptosis

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,b), 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 results in apoptosis is 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. Because of this oxidative stress situation, a redox-sensitive transcriptional factor [natural killer (NK)-κB] was found to be hyper-activated, resulting in apoptosis through expression of death genes (Chern *et al*., 2001). Other authors have 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).

Zinc and apoptosis

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 two 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 (Perry *et al*., 1997; Chai *et al*., 1999; Truong-Tran *et al*., 2000; Chimienti *et al*., 2003). However, excessively high concentrations of zinc may be toxic and can induce apoptosis and even necrosis (Truong-Tran *et al*., 2000; Chimienti *et al*., 2003). 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 (Sunderman, 1995; Chimienti *et al*., 2003).

Antioxidants and apoptosis

As mentioned above, folic acid and zinc both have antioxidant properties. This is still another mechanism whereby these micronutrients can affect apoptosis, because 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 these authors discuss several studies supporting this statement (Buttke and Sandstrom, 1994). Inhibiting 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 glutathione depletion and apoptosis. Chiba *et al*. (1996) observed that intracellular glutathione 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 glutathione and that pre-treatment of these resistant cells with buthionine sulphoximine (an inhibitor of glutathione synthesis) or depletion of intracellular glutathione 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 glutathione preceded the onset of apoptosis, while pre-treatment of thymocytes with glutathione before inducing apoptosis resulted in inhibited apoptosis. Therefore, they conclude that a decrease in intracellular glutathione or an increase in glutathione disulphide (GSSG) (oxidized glutathione), or perhaps a change in the ratio of glutathione to GSSG, constitutes a trigger for apoptosis (Beaver and Waring, 1995). Finally, Iwata *et al*. (1997) found that glutathione depletion in the culture medium of human lymphocytes induced apoptosis because of an increase of ROS in the cultured cells, whereas the addition of glutathione to the depleted medium protected the lymphocytes from apoptosis (Iwata *et al*., 1997).

Sperm DNA fragmentation and apoptosis in males

Causes of DNA fragmentation. 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 (Hikim *et al*., 1998; Barroso *et al*., 2000). Apoptosis is one of the causes of DNA fragmentation. Other causes include (i) chromatin remodelling during the process of spermiogenesis; (ii) DNA damage during sperm transport through the seminiferous tubules; (iii) activation of sperm caspases and endonucleases and (iv) radio and chemotherapy. The activation of caspases and endonucleases in sperm is different from that described for the classic apoptosis pathway, as described

by Sakkas *et al*. Caspase activity has been identified in the post-acrosomal and midpiece regions of human spermatozoa (Paasch *et al*., 2004) and, in addition to oxygen radicals, can be activated by high temperature and toxic factors. Moreover, DNA damage during sperm transport through the epididymis is increasingly being recognized as one of the main mechanisms of DNA damage of human spermatozoa and is mainly related to ROS-induced damage (Ollero *et al*., 2001; Greco *et al*., 2005).

Apoptosis and subfertility. 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 levels of 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 these 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,b), 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. Of interest is that the administration of antioxidants improves cellular redox equilibrium. The use of isolated antioxidants such as vitamin C, however, can paradoxically induce DNA damage (Halliwell, 1999). 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 two micronutrients also may have an effect on apoptosis.

Apoptosis in females

Apoptosis in the ovary. Throughout female fetal and post-natal 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, as primordial, primary and secondary follicles in the human ovary are not affected by apoptosis, while from the pre-antral 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 and Gorospe, 1991; Palumbo and Yeh, 1994). Apoptosis furthermore seems to be important in the degeneration of corpora lutea in the ovary (Tilly *et al*., 1991; Shikone *et al*., 1996; Yuan and Giudice, 1997) and the cyclic shedding of the endometrium in the uterus (Spencer *et al*., 1996).

Apoptosis and hormones. Yuan and Giudice (1997) reported that estrogen-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. In a review, Hsueh *et al*. (1994) discussed the hormonal control of apoptosis in follicular atresia. They stated that gonadotrophins and estrogens, in combination with several ovarian growth factors and regulatory peptides, such as insulin-like growth factor-I (IGF-I), epidermal growth factor (EGF)/transforming growth factor-α (TGF-α) and basic fibroblast growth factor (bFGF), are involved in the escape from follicular apoptosis, while androgens and atretogenic factors such as interleukin-6 (IL-6) and GnRH are involved in the induction of apoptosis in follicles.

Apoptosis and (sub)fertility. A 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 only from specific patients may have consequences regarding their fertility (Van Blerkom and Davis, 1998).

Likewise, Idil *et al*. (2004) 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 degree of apoptosis in mural granulosa cells was significantly higher compared with that of cumulus cell masses. In addition, they investigated the differences in apoptotic rates between women with tubal infertility, endometriosis, no infertility (being treated because of male factor 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 (Nakahara *et al*., 1998; Garcia-Velasco *et al*., 2002; Garcia-Velasco and Arici, 2003). However, not all authors could demonstrate such a connection (Cahill, 1998). Finally, Seifer *et al*. (1996) observed a possible role for apoptosis in women with decreased ovarian reserve such as in premature ovarian failure syndrome.

Apoptosis and outcome parameters of fertility. Nakahara *et al*. (1997a) investigated the relationship between apoptosis and parameters associated with the IVF programme. Results indicated that significantly more apoptosis was found in both mural and cumulus

granulosa cells in patients with less than six retrieved oocytes compared with patients with more than six 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 <13% apoptotic granulosa-lutein cells were far more likely to become pregnant compared with women with >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 an oocyte, whereas follicles associated with fertilized oocytes contained significantly less apoptotic granulosa cells compared with follicles associated with non-fertilized oocytes. In addition, follicles containing oocytes from which good embryos developed contained 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).

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 ART. However, these treatments do not address the cause of subfertility, for which no therapies are available. A better understanding of underlying mechanisms in (sub)fertility and better study results clarifying the effectiveness of nutritional and biochemical factors are important to improve diagnosis and treatment. Such aspects include patient selection, study duration, sample size and dosage of the treatment. In addition to other possible underlying mechanisms of subfertility, the pathways in which nutrition, genetics, antioxidants and apoptosis are involved as reviewed in this article 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 such couples experience subfertility, and because 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.

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