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Role of micronutrients in the periconceptional period

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BACKGROUND: Micronutrient deficiencies have been associated with significantly high reproductive risks, ranging from infertility to fetal structural defects and long-term diseases. In this review we focus on the reproductive risks related to some micronutrients during the periconceptional period, a critical step in determining fetal development and health due to the potential onset of several disorders.

METHODS: Embase Medline and PubMed databases, Google-indexed scientific literature and periodics from on-line University of Milan Bibliotecary Service were searched to identify relevant publications. *In vivo* human studies were mainly searched for, but when needed animal studies as well as *in vitro* and cell culture experiments were also considered.

RESULTS: Fertility, conception, implantation, fetal organogenesis and placentation are the critical stages potentially affected by nutrition during the periconceptional period. Reactive oxygen species (ROS) and total homocysteine (tHcy) plasma levels are factors involved in the respective mechanisms. The preconceptional period is particularly important since it affects both fertility and the early stages of gestation. Micronutrients' dietary intake and maternal status affect the different phases of the onset and development of pregnancy as well as of the conceptus.

CONCLUSION: Although human studies are scarce, and conclusive evidence is provided solely for periconceptional folate and prevention of neural tube defects (NTDs), the overall data indicate that micronutrients may affects fertility, embryogenesis and placentation, and the prophylactic use of some micronutrients may be useful in preventing several adverse pregnancy outcomes. Efforts to increase awareness of a healthy diet should be strengthened not only throughout pregnancy but also before. However, further researches in humans are necessary to optimise periconceptional micronutrient requirements.

Key words: periconceptional period / micronutrients / ROS / preconception nutrition / pregnancy outcomes

Introduction

Diet is recognized as one of the major environmental factors influencing the development of embryo and fetus, as well as maternal health (Keen *et al.*, 2003). Particularly, micronutrient deficiencies have been associated with significantly high reproductive risks, ranging from infertility to fetal structural defects and long-term diseases (McArdle and Ashworth, 1999; Ashworth and Antipatis, 2001; Black, 2001; Andersen *et al.*, 2006). There is evidence that indicates a role for micronutrients supplementation in preventing some pregnancy disorders (Ladipo, 2000; Bendich, 2001; Díaz *et al.*, 2003). Among these, increasing calcium and magnesium intake can reduce the risk of pregnancy-induced hypertensive disorders; ensuring adequate intake of iron, zinc, iodine, calcium and folic acid during pregnancy can improve pregnancy outcomes; increasing the intake of folic acid before pregnancy can reduce birth defects; increasing folic acid and vitamin B$_{12}$ may reduce megaloblastic anaemia in mothers; providing zinc supplements during pregnancy can improve birthweight and reduce prematurity. Moreover, since micronutrients such as vitamin A, iron and zinc are also involved in the function of the immune system, their deficiency can lead to potentially harmful infections, and enhancing vitamin A intake may reduce maternal mortality (West *et al.*, 1999). Furthermore, improved maternal intake of many nutrients directly enhances the quality of breast milk (Picciano, 2003; Allen, 2005). However, the biological mechanisms responsible for these association are not completely clear.

Since pregnancy is characterized by different stages that represent a continuum, the timing of a nutritional insult impacts differently both on the overall outcome of pregnancy and on the nature of adult diseases by programming the post-natal pathophysiology, and having the potential to affect cell numbers or differentiation in the developing embryo (Newnham *et al.*, 2002; Fleming *et al.*, 2004; Rhind, 2004; Buckley *et al.*, 2005; De Boo and Harding, 2006). Each stage in embryonic and fetal development is indeed strongly influenced by maternal nutrients and hormones (Ashworth and Antipatis, 2001; Sacks, 2004; Gluckman *et al.*, 2008), and the placental–maternal—fetal somatotropic axes are fundamental in modulating this interaction (Gluckman and Pinal, 2002). As Bloomfield *et al.* (2004) demonstrated, periconceptional undernutrition in sheep from 60 day before to 30 day after mating alters the development of the fetal hypothalamic-pituitary-adrenal axes (HPAA) inducing accelerated maturation many months later, suggesting that events around the time of conception strongly affect fetal development in late gestation. The authors found significantly higher baseline cortisol levels and a significant positive association between plasma cortisol and adrenocorticotropic hormone concentration in fetuses by undernourished ewes compared with controls. In addition, Edwards and McMillen (2002) demonstrated that the detrimental effect on fetal HPAA of the maternal nutrient-restriction before and in the first week of gestation was not reversed by provision of a maintenance diet from the second week of pregnancy. Similarly, nutrient restriction of ewes, during perimplantation embryonic development, programs long-term cardiovascular dysfunction in adult offspring (Gardner *et al.*, 2004). Interestingly, a maternal low-protein diet (LPD) in the first days of dams' pregnancy reduced cell number in both the inner cell mass and the mid/late blastocyst, and reduced insulin and essential amino acid levels in maternal serum, suggesting that long-term programming of post-natal growth and physiology (i.e. metabolic stress) may be induced irreversibly during the preimplantation period by maternal protein undernutrition through restriction of early embryonic proliferation (Kwong *et al.*, 2000). Similarly, mouse embryo transfer experiments revealed a correlation between maternal preimplantation LPD and offspring's increased weight from birth, hypertension and abnormal anxiety-related behavior, indicating that increases in perinatal weight due to a maternal preimplantation LPD was induced within blastocysts, with the visceral yolk sac endoderm as a proposed mediator (Watkins *et al.*, 2008).

Moreover, in human pregnancies studies in the offspring of women exposed to the Dutch Winter Famine showed that the nutrient environment in the first trimester of pregnancy was linked to increased prevalence of coronary heart disease, raised lipids and obesity (Ravelli *et al.*, 1999; Roseboom *et al.*, 2000, 2001), whereas famine occurring during late gestation led to decreased glucose tolerance in adult life (Ravelli *et al.*, 1998).

In particular, the periconceptional period is critical in determining fetal development and health. The onset of several malformations and pregnancy related disorders (i.e. congenital abnormalities, fetal loss, miscarriage, insufficient fetal growth, premature birth and preeclampsia) may indeed occur during this period (Steegers, 2005).

The present review aims to summarize the recent evidence about the reproductive risk factors related to micronutrients during the periconceptional period. Human *in vivo* studies were evaluated. The majority of these are observational and retrospective, and deal with energy and macronutrient intake. Data on the specific relationship between individual micronutrients and reproductive outcomes are often scarce, inconsistent or contradictory (Osrin *et al.*, 2000; Shah and Sachdev, 2004). Therefore, animal, *in vitro* and cell culture studies were also considered to investigate the effects of micronutrients during the different phases of the onset and development of pregnancy and development of the conceptus.

Methods

Embase Medline and PubMed databases (all publication years), Google-indexed scientific literature as well as periodics from on-line University

of Milan Bibliotecary Service were searched for all relevant articles written in English. Search terms included micronutrient intake, micronutrient supplements, micronutrient deficiency, periconceptional period, preconceptional period, food intake, diet, embryogenesis, placentation, fetus outcomes, maternal nutrition status, pregnancy outcomes, oxidative stress (OS) and fertility. Human, animal, in vitro and cell culture experiments were considered. Abstracts were critically evaluated to identify relevant articles focusing on periconceptional critical stages potentially affected by nutrition and effects of micronutrients' intake and maternal status during the different phases of the onset and development of pregnancy and conceptus. The reference lists of articles included in this review were examined for additional potential articles and books were also considered.

Role of specific dietary micronutrients

Malnutrition is the state produced by an inadequate intake of a good quality diet. This can mean intake of too few macronutrients, i.e. undernutrition (protein-energy malnutrition and/or vitamin and mineral deficiency), or of too many macronutrients, i.e. overnutrition (obesity), or excessive amounts of inappropriate substances (alcoholism). Interestingly, vitamin and mineral deficiency may occur in both undernutrition (mainly an issue in developing and transitional countries) and overnutrition (Young et al., 2004). For example, the globalized high-energy and low-nutrient density Western dietary patterns and trends, typified by snacking, breakfast skipping, fast foods, soft drinks and convenience foods, are nutritionally unbalanced, and intake of micronutrients in general fails to meet recommended daily allowance (RDA) values (Nicklas et al., 2001; Paeratakul et al., 2003; Drewnowski and Spencer, 2004; Cordain et al., 2005).

For this reason, micronutrient malnutrition represents an important topic of public health worldwide, mainly in vulnerable population groups such as infants, elderly, pregnant and lactating women. There is sound evidence that adequate intake of micronutrients can prevent many serious birth defects, reduce the risk of premature and low birthweight (LBW) infants, and support maternal health. Micronutrients seem to affect pregnancy outcomes through alterations in maternal and fetal metabolism due to their role/involvement in enzymes, signal transduction and transcription pathways and OS (McArdle and Ashworth, 1999). Here, we deal with micronutrients for which there is sufficient information and major public health concern; Table I shows their main functions.

Antioxidants

Complex antioxidant defenses are distributed within various cellular compartments and in the external milieu; plasma contains a wide range of substances with antioxidant properties.

Antioxidants are necessary for homeostasis; an imbalance in the equilibrium of antioxidants and pro-oxidants can result in OS damage, a key element in the pathogenesis of several diseases (Agarwal et al., 2008), via effects on redox status and/or redox-sensitive signaling pathways and gene expression (reviewed by Ruder et al., 2008). By both direct and indirect contributions, antioxidants enhance many aspects of the immune response (Bendich, 2001; Arrigoni and De Tullio, 2002) and limit pathological aspects of the cytokine-mediated response.

Antioxidants are present in both enzymatic (i.e. superoxide dismutase (SOD), catalase, glutathione (GSH) peroxidase, and GSH reductase) and non-enzymatic (i.e. vitamin C, vitamin E, selenium, zinc, taurine, hypotaurine, GSH, β-carotene and carotene) forms (Agarwal et al., 2008). Many antioxidants are obtained from the diet, such as vitamin E and other tocopherols, vitamin C, β-carotene, whereas proteins and peptides, such as glutathione, ceruloplasmin and metallothionein, are synthesized endogenously (Grimble, 1998). Vitamin E is a chain-breaking antioxidant that prevents the propagation of free radical damage in biologic membranes, thus defending polyunsatured fatty acid (PUFA) from auto-oxidation (Traber, 2006). Vitamin C or L-ascorbic acid, present mainly in vegetables and fruit, exhibits a protective effect against free-radical-induced oxidative damage by acting as a reducing agent (Levine et al., 2006). It is also important in the synthesis of collagen, carnitine, catecholamines. Other water-soluble vitamins, such as vitamin B_{12}, folic acid, vitamin B_6, riboflavin, are crucial for oxidative defense. Carotenoids and polyphenols are potent antioxidants. Other micronutrients such as magnesium, copper, zinc, manganese and selenium are involved in the antioxidant defense as cofactors of enzymes (i.e. zinc in metallothionein, copper in ceruloplasmin, copper$-$selenium$-$zinc in SOD, selenium in GSH peroxidase) (Fang et al., 2002).

Folate

Folate is widely distributed in foods (green-leafy vegetables, fruits, liver, bread etc.). An inadequate dietary folate intake results in a reduction of DNA biosynthesis and thereby of cell division, leading to anemia, leucopenia and thrombocytopenia etc. (FAO/WHO Report, 2004a). A decrease in the methylation cycle results in elevation of plasma total homocysteine (tHcy), or hyperhomocysteinemia (HHCY). HHCY is implicated in the etiology of several diseases such as arterial and/or venous thrombosis, vascular dementia by demyelization, and certain neuropathies, such as sub-acute combined degeneration of the spinal cord and peripheral nerves. In a meta-analysis of randomized trials it was demonstrated that daily supplementation with both 0.5$-$5 mg folic acid and about 0.5 mg vitamin B_{12} would be expected to reduce blood homocysteine concentrations by about a quarter to a third (Homocysteine Lowering Trialists' Collaboration, 1998). Moreover, as an effective scavenger of oxidizing free radicals, folic acid acts as antioxidant and can protect bio-constituents such as cellular membranes or DNA from free radical damage (Joshi et al., 2001).

Vitamin B_{12} or cobalamin

Bacteria and algae synthesize vitamin B_{12}, and it enters the human food chain through incorporation into food of animal origin such as liver, milk, meat, oocytes (FAO/WHO Report, 2004b). Low vitamin B_{12} levels are related to HHCY and high methylmalonic acid. Anaemia, myelopathy and neuropathy are the main clinical manifestations of vitamin B_{12} deficiency (Charmel, 2006). Intriguingly, the Pune Maternal Nutrition Study showed that at 6 years old, children born to mothers with low maternal vitamin B_{12} status at 18 weeks of gestation and high folate status at 28 weeks were the most insulin resistant (Yajnik et al., 2008). Moreover, higher maternal erythrocyte folate concentrations at 28 weeks were associated with higher fat mass and per cent body fat in the offspring. The authors speculated

Table I Main functions of micronutrients involved in the periconceptional period

Micronutrient	Function	References
Folate	Involvement in the DNA replication (cell cycle); methylation cycle (amino acids cysteine and methionine cycle)	FAO/WHO Report (2004a), Scholl and Johnson (2000)
Vitamin B_{12}	Conversion of homocysteine to methionine as cofactor of the methionine synthase	Ryan-Harshman and Aldoori (2008)
Vitamin B_6	Metabolism of amino acids, lipids, one-carbon units and glycogen as co-enzyme; pathways of gluconeogenesis, heme and neurotransmitter biosynthesis	Mackey *et al.* (2006)
Vitamin A	Growth and differentiation of a number of cells and tissues	Ross (2006)
Antioxidants	Defence systems against free radical species	Fang *et al.* (2002)
Iron	Haematopoiesis; nucleic acid metabolism; carrier of oxygen to the tissues by red blood cell haemoglobin; transport medium for electrons within cells; integrated part of important enzyme systems	Cook *et al.* (1992); Beard (2003), FAO/WHO Report (2004c), Wood and Ronnenberg (2006)
Zinc	Structural, regulatory and catalytic functions as cofactor for numerous metalloenzymes	McCall *et al.* (2000), Hambidge (2000)
Copper	Neurotransmission, neuroropeptide maturation, oxidative phosphorylation, defence from free radical damage as cofactor for numerous cuproenzymes and copper-binding proteins	Turnlund (2006)

that vitamin B_{12} deficiency prevents the generation of methionine from homocysteine by trapping folate as 5-methyltetrahydrofolate, and subsequently reduces protein synthesis and lean tissue deposition. Moreover, the increased lipogenesis might be caused by the inhibition of β-oxidation, owing to elevated concentrations of methylmalonic-co-enzyme A. In fact, it can be hypothesized that imbalances of adequate folate methyl donor and poor vitamin B_{12} cofactor lead to further depletion of mitochondrial cobalamin stores, thereby to dysfunction of other B_{12}-dependent reactions (Rosemberg, 2008). On the whole, these results postulate that the defects in the one-carbon metabolism plays a crucial role in intrauterine programming of adult diseases.

Vitamin B_6

Foods of animal origin are rich in vitamin B_6; wholegrains and many vegetables are also good sources. Deficiencies of vitamin B_6 may lead to HHCY (Mackey *et al.*, 2006). Moreover, low plasma levels of vitamin B_6 are an independent risk factor for thrombosis (Saibeni *et al.*, 2003).

Vitamin A

Vitamin A can be obtained from food either as pre-formed vitamin A, in the form of retinol or retinyl-esters which come from animal sources, or as provitamin A form from plants, i.e. provitamin A-carotenoids such as β-carotene. The highest concentration of vitamin A is found in liver and fish liver oil. Yellow and green leafy vegetables provide provitamin A-carotenoids (Ross, 2006). Retinol is its endogenous metabolite and retinoic acid (RA), a vitamin A derivative, is a morphogenetically active compound. Vitamin A plays a key role in vision and ocular retinoid metabolism as well as in cellular differentiation (related to embryonic development and immunity). Carotenoids exert antioxidant properties. Vitamin A deficiency is linked to xerophthalmia and Vitamin A-deficiency anemia whereas hypervitaminosis seems to be involved in teratogenesis, liver abnormalies and bone mineral loss. Interestingly, it has been observed that in rodents the control of neural patterning and differentiation are disrupted

when RA concentrations are lowered, whereas inappropriately high concentrations of RA result in abnormal development of cerebellum and hindbrain nuclei. Thereby, it has been supposed that even the adult brain may be susceptible to an imbalance of RA, particularly the hippocampus (McCaffery *et al.*, 2003).

Iron

Nutritional sources of iron are meat, poultry, fish, cereals, bread and green vegetables (FAO/WHO Report, 2004c). Worldwide, iron deficiency represents the most common nutritional deficit (Scholl, 2005), that can exist with or without anemia. Although iron deficiency is a common cause of anemia, anemia may also result from other causes (i.e. deficiencies of folate, vitamin B_{12} and vitamin B_6). Studies in both humans and animals have shown that iron-deficient anemia in early life is linked to altered behavioral and neural development, and is suggested to result in irreversible effects on neurochemistry and neurobiology (reviewed by Beard, 2003, 2007). This may be explained by considering alterations in morphology, neurochemistry and biogenetics within the central nervous system. In fact, data on human infants are consistent with altered myelination of white matter, changes in monoamine metabolism in striatum and functioning of the hippocampus (Beard, 2008).

Zinc

Zinc is abundantly present in meat, seafood, pulses, legumes and whole-grain cereals (FAO/WHO Report, 2004d; King and Cousins, 2006). Zinc has antioxidant properties, by counteracting oxidation through binding sulphydryl groups in proteins and by occupying binding sites for iron and copper in lipids, proteins and DNA (Zago and Oteiza, 2001). Evidence has been found for oxidative damage in zinc-deficient rats and mice (Oteiza *et al.*, 1995), although zinc salts have been shown to protect against oxidative damage and glutathione depletion in mice (Bagchi *et al.*, 1998). Moreover, zinc is present in the brain, bound to proteins, and it is important for its structure and function (Bhatnagar and Taneja, 2001).
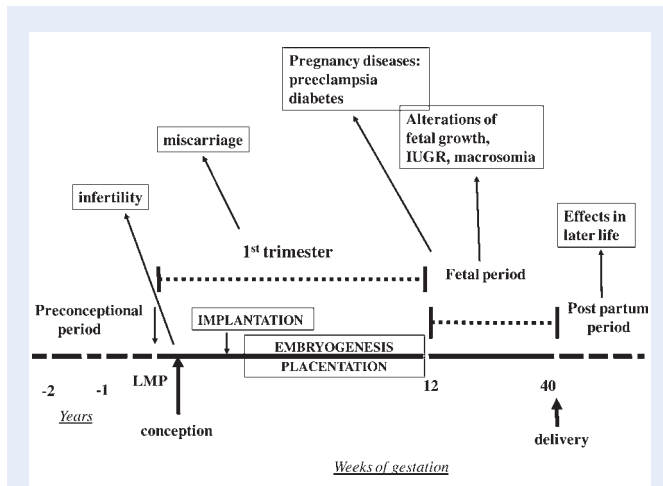
Figure 1 Different pregnancy stages that represent a continuum, from the preconceptional to the post-partum period.
Several and specific malformations and pregnancy related disorders may originate during each phase. Preconception, conception, implantation, placentation and embryo- or organogenesis are stages of the periconceptional period, which represents a critical step in determining fetus development. From the nutritional point of view, firstly preconceptional nutrition affects the overall health of childbearing-age women, thereby their reproductive potential. Subsequently, periconceptional maternal nutrition influences both the onset and the early stages of pregnancy, affecting both maternal health and conceptus development throughout the entire pregnancy. LMP: last menstrual period.

Copper

The richest dietary sources of copper include shellfish, nuts, seeds, legumes, grains' bran and germ, liver and organ meats. Copper exhibits several biological roles being involved in connective tissues formation, iron metabolism, cardiac function, immune function (Turnlund, 2006) and central nervous system development (Prohaska, 2000; Gybina and Prohaska, 2003). Interestingly, new insights are emerging into the role of iron and copper in neurocognitive and neurobehavioral development during the last two thirds of gestation, and in long-term consequences of their perinatal deficiency (Beard et al., 2003; Penland and Prohaska, 2004; Gybina and Prohaska, 2006; Beard, 2008). Balances between copper and iron are recognized to assure the proper brain development, as iron-deficiency results in hypomyelination (Prohaska and Gybina, 2005) to suggest that iron accumulation in rat brain during perinatal growth depends on adequate copper nutrition of dams.

Critical stages and mechanisms potentially affected by nutrition in the periconceptional period

The periconceptional period consists of preconception, conception, implantation, placentation and embryo- or organogenesis stages (Fig. 1), and specific cellular events that occur during the distinct stages of embryogenesis (Hirschi and Keen, 2000). Besides genetics, each of these steps may be affected by maternal nutrition and, specifically, by micronutrient imbalances (Finnell et al., 2004; Allen, 2005). In

particular, these may interfere with fetal organogenesis (embryogenesis) as well as with placentation.

Embryogenesis

The association between maternal folate status and fetal neural tube defects (NTDs) is well recognized, as demonstrated by several interventional trials and observational studies (reviewed by de Bree et al., 1997; Pitkin, 2007). Neural tube develops into the spine and NTDs occur when the brain and skull and/or the spinal cord and the protective spinal column do not develop properly within the first 4 weeks after conception. Folate functions as a co-enzyme in single-carbon transfers in the metabolism of aminoacids and nucleic acids. Moreover, folate is the substrate donor in the remethylation of homocysteine into methionine, catalyzed by methionine synthase and 5,10-methylenetetrahydrofolatereductase (MTHFR). Altered homocysteine metabolism leading to HHCY has been proposed as the mechanism involved in NTDs given that higher tHcy levels were found in plasma or amniotic fluid of NTD infants and their mothers with respect to non-NTD individuals (Locksmith and Duff, 1998; Tamura and Picciano, 2006). Moreover, HHCY depends on inherited and acquired conditions (genetic polymorphisms of MTHFR 677 C→T and 1298 A→C); methionine synthase (MTR) 2756 C→G; methionine synthase reductase (MTRR) 66 A→G) (Guéant et al., 2003) and folate and/or vitamin B_6/B_{12} deficiencies due to deregulation of their normal metabolism and/or low dietary intake (Steen et al., 1998).

Implantation and placentation

The process of implantation and placentation may also be affected by maternal nutrition. Placental function is critical for nourishing the fetus throughout pregnancy (Cross and Mickelson, 2006; Jansson and Powell, 2006; Pafilis et al., 2007). Specifically, the placenta forms a highly branched villous structure thus providing nutrients and oxygen to the fetus (Cetin et al., 2005) to assure appropriate fetal growth (Sparks et al., 1998). In particular, intrauterine growth restriction (IUGR) is associated with a range of alterations in placental transport functions, whereas accelerated fetal growth, in association with maternal diabetes, is characterized by increased activity of placental systems (Sacks, 2004; Cetin and Alvino, 2009). Nutrition may play a role in altering the development of the placenta. In fact, despite an initially normal growth trajectory, fetuses may have impaired growth in the second part of gestation subsequent to nutrient deprivation occurring early in gestation. Early in pregnancy the muscular maternal spiral arteries are transformed to fibrinoid lined vessels, and after further invasion by endovascular cytotrophoblasts, these vessels bath the chorionic villi in maternal blood bearing oxygen and nutrients for fetal development. Defects in this process have been reported in pregnancies complicated by IUGR (Sibley et al., 2005; Cetin and Alvino, 2009). Proposed mechanisms include lowered number and surface area of arterioles of tertiary villi, secondary vascular obliteration (Gagnon, 2003), increased susceptibility of trophoblasts to apoptosis by inflammatory cytokines or oxygen reduction (Crocker et al., 2003). Also, impaired release of the vasoactive agents nitric oxide (NO) and carbon monoxide by the invasive trophoblast cells seems to be responsible for the reduction in spiral artery transformation (Lyall, 2003).

Diabetes

Blastocyst development and subsequent implantation are affected by high concentrations of D-glucose in diabetic mothers in a mouse model (Leunda-Casi *et al.*, 2001, 2002). This has also been demonstrated in culturing rat embryos, as OS occurs in diabetes (Cedeberg *et al.*, 2001; Ornoy, 2007). Diabetes is associated with an imbalance between pro-oxidant and antioxidant defenses in favor of pro-oxidants, resulting in oxidative structural and functional modifications of biomolecules. These modifications may result in secondary trophoblast dysfunction (Jauniaux *et al.*, 2006). Scavenging of free radicals is achieved through enzymatic and non-enzymatic reactions.

Reactive oxygen species

Reactive oxygen species (ROS), reactive nitrogen species and reactive chlorine species are produced in humans and animals under physiological and pathological conditions (reviewed by Fang *et al.* 2002). Free radicals play a key role in the origin of life and biological evolution, such as signal transduction and gene transcription, regulating platelet aggregation, leukocyte adhesion and angionesis, mediation of the immune response. As oxidants and inhibitors of iron-sulfur center enzymes, ROS cause oxidation of biomolecules such as DNA, lipids, proteins, leading to cell injury and death (cytotoxic effect). Furthermore, ROS are involved in female reproduction, that is physiological processes such as folliculogenesis, oocyte maturation, ovulation, corpus luteum formation, endometrial cycle, luteolysis, implantation, embryogenesis and pregnancy (reviewed by Taylor, 2001, Agarwal *et al.*, 2005, 2008). As demonstrated in embryo culture systems and in pregnant animals with induced diabetes, ROS are suggested to be involved in diabetic teratogenesis and to contribute to the prostaglandin imbalance, resulting from myoinositol and arachidonic pathways, linking together potentially teratogenic mechanisms (Akazawa, 2005). *In vitro*, high glucose concentration impaired rat preimplantation embryo development or induced degeneration of the embryos due to apoptosis of blastocysts (Kos and Vogel, 2005) by suppression of insulin and glucokinase expression, decreased mitochondrial function, increased ROS formation and accelerated apoptosis, as well as to activation of common stress signaling pathways, which could affect proliferative, metabolic and neuroendocrine axes during later development (Evans *et al.*, 2002). Thus, imbalance in homeostatic control of ROS exposure causes OS, which can affect fertilization and induce apoptosis, resulting in embryo fragmentation, implantation failure or abortion (Agarwal *et al.*, 2008) or impair metabolic activity within embryos for fetal and post-natal development. OS during embryogenesis and in the placenta seems to be implicated in adverse pregnancy outcomes such as birth defects, early pregnancy failure, miscarriage and pre-eclampsia (Evers *et al.*, 2004; Jauniaux *et al.*, 2006; Agarwal *et al.*, 2005; Forges *et al.*, 2007). Interestingly, NTDs in a mouse model of diabetic embryopathy have been demonstrated to be associated with deficient expression of Pax3, a gene required for neural tube closure, and hyperglycemia-induced OS was responsible (Li *et al.*, 2005).

Pre-eclampsia and IUGR

The pathogenesis of pre-eclampsia is established in the first trimester when an inadequate remodeling of the spiral arteries results in a decreased perfusion of the placenta in the third trimester. OS and inflammatory mediators are also involved in the abnormal implantation associated with pre-eclampsia and IUGR. In fact, not only endothelial dysfunction is causally important in the disorder but also alterations in function predate clinically-evident pre-eclampsia, suggesting an interaction of reduced perfusion with maternal factors (Roberts *et al.*, 2003). Abnormal placentation leads in fact to placental ischemia, resulting in generation of placental OS and increased levels of lipid peroxidation (reviewed by Agarwal *et al.*, 2008). HHCY has been implicated in adverse pregnancy outcomes such as placental abruption or infarction and pre-eclampsia (Goddijn-Wessel *et al.*, 1996; Tamura and Picciano, 2006; Braekke *et al.*, 2007). In this context, maternal vitamin B_6 status was observed to influence reproductive events throughout the entire course of pregnancy (Ronnenberg *et al.*, 2007). The authors reported that this relationship was also found in a case–control study (Wouters *et al.*, 1993) demonstrating significantly lower plasma concentrations of vitamin B_6 in women with histories of recurrent spontaneous miscarriage compared with control women. Furthermore, significantly lower plasma vitamin B_6 was detected among women with placental abruption or infarction compared with control women (Goddijn-Wessel *et al.*, 1996). In addition, some studies have documented associations between low vitamin B_6 status and inflammatory responses (Friso *et al.*, 2001; Saibeni *et al.*, 2003), and inflammation has been linked to early pregnancy loss (Thellin and Heinen, 2003).

Influence of the preconceptional maternal diet and health status

Preconceptional maternal nutrition plays a key role in reproductive health (Allaire and Cefalo, 1998). This affects both fertility and the early stages of gestation. Insufficient energy stores may negatively affect ovulation, menses and challenge the beginning of pregnancy. On the other hand, excessive fat stores may inhibit conception by affecting ovulation because of insensitivity to insulin, excess of male sex hormones and overproduction of leptin (The ESHRE Capri Workshop Group). It is also well known that maternal overweight and obesity before conception increase the risk of maternal complications during pregnancy such as gestational hypertension, gestational diabetes, Cesarean delivery, macrosomia, as well as birth defects such as NTDs, other neurological abnormalities, congenital heart disease, intestinal malformations and multiple congenital anomalies (ADA, 2002; Watkins *et al.*, 2003; Bartley *et al.*, 2005; Catalano and Ehrenberg, 2006; Oken, 2009).

Interestingly, the Nurses' Health Study II, a prospective cohort study of more than 116,000 women aged 24–42 years, demonstrated that a high 'fertility diet' score was characterized by a lower intake of *trans* fat with a simultaneous greater intake of mono-unsaturated fat, a lower intake of animal protein with greater vegetable protein intake, a higher intake of high-fiber and low glycemic carbohydrates, a greater preference for high fat dairy products and a higher non-heme iron intake (Chavarro *et al.*, 2007a). In particular, the dietary *trans* unsaturated fats may increase the risk of ovulatory infertility when consumed instead of carbohydrates or unsaturated fats commonly found in non-hydrogenated vegetable oils (Chavarro *et al.*, 2007b) and replacing animal sources of protein with vegetable sources of protein may reduce the risk of infertility because of anovulation (Chavarro *et al.*, 2008). Significantly higher preconceptional dietary intakes of saturated fat was found in Dutch mothers of children with outflow tract defects (OTD), compared with controls (Smedts *et al.*, 2008). In a case–control study, the use of the maternal Western

diet, e.g. high intakes of organ meat, red meat, processed meat, pizza, legumes, potatoes, French fries, condiments, and mayonnaise but low intakes of fruits, namely low fiber, iron, vitamins, antioxidants and complex carbohydrate intakes and high amounts of saturated fatty acid, protein, sugar and sodium, increased the risk of offspring with a cleft lip or cleft palate approximately 2-fold compared with a Prudent diet, e.g. high intakes of fish, garlic, nuts and vegetables (Vujkovic et al., 2007). These factors are involved in OS and inflammation pathways triggering fertility status. The pathological effects are exerted by various mechanisms including lipid and DNA damage, inhibition of protein synthesis, and depletion of ATP (Agarwal et al., 2005). Nutrients modulate cytokine production by influencing tissue concentrations of the molecules involved in cytokine biology. Aberrant cytokine production is involved in atherosclerosis. The association of dietary fat intake with atherosclerosis has been the subject of extensive studies. In particular, the intake of saturated fat has been suggested to be positively associated with atherosclerosis and coronary heart disease, and sources of saturated fat in the diet mainly derived from animal sources and contain cholesterol. Cholesterol may enhance cytokine production.

Infants of women with pregestational diabetes are reported to have about 3-fold increased incidence of birth defects compared with those of non-diabetic population (Sheffield et al., 2002). Casele and Laifer (1998) have shown that diabetic women frequently enter pregnancy with poor glycemic control.

Some observational studies show an association between short interpregnancy interval and increased risks of adverse outcomes probably due to maternal depletion of nutrients (Smith et al., 2003). As suggested by Smits and Essed (2001), depletion of maternal folate seems to be mainly involved, with particular regard to the risk of fetal growth restriction (van Eijsden et al., 2008).

Another potential problem is that women of reproductive age and pregnant women enter pregnancy without adequate iron reserves or are already iron deficient (Viteri and Berger, 2005; Milman, 2006). Preconceptional anemia, particularly iron-deficiency anemia, was found to be associated with reduced infant growth and increased risk of adverse pregnancy outcomes (Scholl and Hediger, 1994; Ronnenberg et al., 2004; Buckley et al., 2005). Plausibly, it may be supposed that either anemia in the periconceptional period has an independent effect on infant growth by influencing hormone synthesis (Allen, 2001) or the moderate preconceptional anemia turns to a more severe anemia during pregnancy (Breymann, 2002) leading to the observed growth deficits. In a prospective observational study in China, it was observed that the risk of preterm delivery and LBW was increased more than 2-fold in moderately anemic women and more than 3-fold in those with severe anemia during early pregnancy (4–8 weeks) (Zhou et al., 1998).

Effects of periconceptional micronutrients on pregnancy outcomes

The preconceptional period

Preconceptional maternal nutritional status and diet impact on reproductive health. Evidence on the preconceptional involvement of micronutrients upon pregnancy outcomes in humans is reported in Table II.

Despite some dietary investigations that refer to periconceptional period, data reflect the maternal nutrition status in the preconceptional period.

Preconceptional folic acid supplementation is a goal of public health to prevent NTDs (reviewed by Pitkin, 2007). A low iron status was suggested to adversely affect important regulators of growth and development in rats (Gambling et al., 2002). A relationship was observed between low ferritin (<12 µg/l), a marker of depleted iron stores, and reduced birthweight, as well as between elevated ferritin (≥ 60 µg/l), a biomarker of acute or chronic inflammation, and increased risk of LBW and fetal growth restriction (Ronnenberg et al., 2004). An open question is whether iron supplementation, which is generally started in the second half of pregnancy, could reduce the rates of preterm LBW, or whether anemia plays a role in placental development solely during early pregnancy and therefore must be prevented prior to conception. Interestingly, prophylactic iron supplementation given from about 12 weeks of gestation to the third trimester among low income women in a randomized controlled trial in North Carolina evidenced that the birthweight was significantly higher in the supplemented groups than in controls (Siega-Riz et al., 2006).

Maternal vitamin B_6 status was observed to influence reproductive events from the start of pregnancy (Ronnenberg et al., 2007). Conceivably, the effects of poor prepregnancy maternal vitamin B_6 status on the early gestational events could be explained firstly through the involvement of vitamin B_6 dependent-co-enzymes in the metabolism of amino acids, lipids, nucleic acids and glycogen. Then, by considering the association between vitamin B_6 deficiency and impairment of enzymes involved in the structural integrity of arterial walls, it could be supposed to affect implantation and early placental development. Thereby, assessing hematological indices before conception should be of great utility, as they are likely to reflect status in the periconceptional period. On the contrary, biomarker concentrations assessed at various times throughout pregnancy are affected by plasma volume expansion, consequently the interpretation of the relation between these measures and birth outcomes can be challenging (de Weerd et al., 2003a).

Despite the paucity of studies dealing with preconceptional dietary patterns, it is clear that there is a strong relation between imbalance of micronutrients before conception and the successful onset, as well as healthy development of pregnancy as listed below.

Fertility

Analysis of data from the Nurses' Health Study II evidenced that the consumption of iron supplements and non-heme iron from foods may decrease the risk of ovulatory infertility (Chavarro et al., 2006). Folate seems to be important for oocyte quality and maturation (Ebisch et al., 2007). Zinc plays a role in ovulation and the menstrual cycle. Oocyte maturation, ovulation, luteolysis and follicle atresia are affected by ROS unbalance. OS and apoptosis are involved in folliculogenesis, follicular atresia and luteal regression. In particular, OS leads to detrimental effects on second meiotic division progression, diminished gonadotrophin, antisteroidogenic actions, DNA damage and inhibited protein ATP production (Ruder et al., 2008). For this reason, a positive role of the antioxidant status may be explained by counteracting ROS effects (Forges et al., 2007; Ruder et al., 2008). Similarly, folate, zinc, ROS and thiols affect apoptosis, which is

Table II Human studies about the impact of preconceptional micronutrients on pregnancy outcomes

Reference	Type of study	Micronutrient	Outcome	Results
Groenen *et al.* (2004)	Case control study. The Netherlands. Food frequency questionnaire	Preconceptional intake of several vitamins and minerals	Spina bifida	Intakes of iron, magnesium and niacin are associated with a 2- to 5-fold increased risk of spina bifida
Velie *et al.* (1999)	Population-based case–control. California. A 98-item food frequency questionnaire	Preconceptional supplemental and dietary zinc intake	NTDs	Risk of NTDs in infants and fetuses decreased with increasing maternal zinc intake
Krapels *et al.* (2004a)	Case–control study. The Netherlands. Food frequency questionnaire	Preconceptional dietary intake	OFC	Increased intake of iron, magnesium, ascorbic acid reduced the risk of offspring affected by OFC
Smedts *et al.* (2008)	Case control study. The Netherlands. Semiquantitative food frequency questionnaire	Dietary intake of riboflavin and nicotinamide	OTD	Dietary intakes of riboflavin and nicotinamide were lower in mothers of a child with an OTD than in controls ($P < 0.05$). Low dietary intakes of both riboflavin (<1.20 mg/day) and nicotinamide (<13.5 mg/day) increased more than 2-fold the risk of a child with an OTD
Verkleij-Hagoort *et al.* (2006)	Case control study. The Netherlands. Food frequency questionnaire	Dietary intake of B-vitamins	Congenital hearth defect (CHD)	A diet low in vitamin B_{12} is associated with an increased risk of a child with a CHD
Ronnenberg *et al.* (2002)	Prospective case–control study. China. Haematological assessment	Preconception B vitamin status	Preterm birth, LBW, small-for-gestational-age	Risk of preterm birth was 60% lower among women with vitamin $B_{12} \geq 258$ pmol/l than among vitamin B_{12}-deficient women ($P < 0.05$) and was 50% lower among women with vitamin $B_6 \geq 30$ nmol/l than among vitamin B_6-deficient women
Ronnenberg *et al.* (2007)	Prospective study. China. Haematological assessment	Preconceptional B vitamin status	Conception, early pregnancy loss, clinical pregnancy	Poor vitamin B_6 status decreased the probability of conception and contributed to the risk of early pregnancy loss in conceptive cycles

important for regulation of follicle atresia (Hussein, 2005), degeneration of the corpus luteum and endometrial shedding. The concentrations of these nutrients may therefore have substantial effects on reproduction. Poor folate status and HHCY are mainly crucial due to their involvement in cell division (e.g. of oogonia or of granulose cells), inflammatory cytokine production, OS, apoptosis and defective methylation reactions (Forges *et al.*, 2007). In this context, DNA synthesis is important for the development of oocytes, and several enzymes involved in DNA synthesis are zinc- or vitamin B-dependent (Ebisch *et al.*, 2007). Deficiencies of vitamins A, C and D have been demonstrated to result in diminished fertility in rats and rainbow trout as reported by Ebisch *et al.* (2007). However, though addition of vitamins C and E has been shown to strengthen antioxidant defenses in *in vitro* media, oral antioxidant supplementation in human studies does not provide definite, conclusive evidence.

Conception and implantation

The interaction of nutrients with the epigenetic system may lead to variations associated with chromatin remodeling and regulation of gene expression that underlie the developmental programming of pathological consequences in adulthood (reviewed by Junien, 2006). After fertilization, the genome of the zygote undergoes rapid demethylation at coding sequences and at repetitive sequences. After implantation, the embryonic ectoderm and mesoderm

genome is hypermethylated, through *de novo* methylation, whereas the extra-embryonic cell genome, such as the primary endoderm and trophoblast, remains hypomethylated. These processes might be enhanced by methyl donors provided by the folate-methionine pathway. In this context, experiments on mice and rats demonstrated that methyl-supplements and zinc adequate diets during pregnancy affected phenotypic modifications in offspring (Wolff *et al.*, 1998; Cooney *et al.*, 2002; Prasolova *et al.*, 2006; Maret and Sandstead, 2008). Interestingly, Sinclair *et al.* (2007) provided the first clinical evidence about the extent to which the periconceptional supply of folate, vitamin B_{12} and methionine in mature female sheep affected epigenetic alterations to DNA methylation and adult health-related phenotype in offspring. The methyl-deficient diet around the time of conception resulted in significant changes in the methionine cycles within ovarian follicles of ewes, and led to adult offspring being heavier and fatter, insulin-resistant, eliciting altered immune responses and with elevated blood pressure. These clinical outcomes were associated with modification in methylation status of 4% of 1400 gene-associated cytidine-guanosine (CpG) islands, mainly in male fetuses, to indicate that periconceptional specific dietary inputs to the methionine cycles may affect a significant part of genome in offspring with long-terms implications for adult health.

The involvement of one-carbon metabolism has been suggested in genetic disorders such NTD. Significantly, the MTHFR genotype has been shown to alter homocysteine metabolism mainly when folate

status is low, suggesting a genetic-nutrient interactive effect in disease risk (Guéant et al., 2003). Similarly, it has therefore been suggested that gene–nutrient interactions affecting one-carbon metabolism may influence the chance/risk of survival and the risk of trisomy (Wilson et al., 1999). Interestingly, notwithstanding weakly, polymorphisms of folate-related genes are reported to be associated with the prevalence of placental abruption (reviewed by Tamura and Picciano, 2006). Even retinoids represent an example of a gene–nutrient interaction (reviewed by Finnell et al., 2004). This issue raised in 1969 when differential teratogenic responses to retinoids in three rat strains were observed. Then, the large amount of data from several experimental over the past two decades seems to indicate that the biological activity of retinoids is mediated by a retinoid receptor superfamily encoded by different genes and regulating cell proliferation and differentiation (reviewed by Finnell et al., 2004). Namely, the expression of receptors and the availability of retinoid compounds in the adequate concentrations contribute to the normal homeostasis and development of organism. Thereby, alteration in retinoids' concentrations interacting with the embryonic genotype can results in defects such as congenital malformations.

Placentation

In a prospective study of 1650 pregnancies, low iron status early in pregnant women was found to be inversely related to placental size (Hindmarsh et al., 2000). An inverse relation between serum ferritin concentration and overall measures of peripheral villous capillarization was observed. The surface area of capillaries involved in gas exchange in the mature intermediate and terminal villi was strongly and inversely related to serum ferritin suggesting that anaemia influences the pattern of placental vascularization.

The use of antioxidants seems to be useful to reduce the damage on placenta, thereby the risk of pre-eclampsia because of its limited antioxidant enzyme capacity in the first trimester (Jauniaux et al., 2004). In fact, given that the risk factors for pre-eclampsia are also risk factors for atherosclerosis, other similarities can be suggested (Gratacós, 2000). OS has been hypothesized to be important in altered endothelial function leading to atherosclerosis (Navab et al., 2004). Consequently, it has been assumed that reduced placental perfusion generates free radicals generating systemic oxidative damage, as supported by evidence of OS in the circulation and tissues of women with pre-eclampsia (Roberts et al., 2003). Gratacós et al. (1998) and Jain and Wise (1995) confirmed that serum lipid peroxide levels are significantly higher and serum vitamin E levels significantly lower in women with pre-eclampsia than in women with normal pregnancies. In this regard, the reduction in maternal and placenta antioxidant defense in pre-eclampsia is linked to an excessive depletion of antioxidants through the increased generation of oxygen free radicals (Jauniaux et al., 2006). Moreover, abnormal placentation leads to increased levels of placental lipid peroxidation, probably due to the presence of NADPH oxidase in the placental syncytial microvillus membrane, producing increased amounts of the superoxide radical (reviewed by Agarwal et al., 2008). At present, nutrition has not been assessed during the periconceptional period or during the early pregnancy in women that later develop pre-eclampsia. However, we may hypothesize that nutrients can affect OS by increasing or decreasing free radicals or antioxidants or by providing

substrates for the formation of ROS. Moreover, they could modify inflammatory response in the period when placentation occurs. In particular, adequate intake of vitamin C and E during the second trimester of pregnancy is recognized to improve the biochemical incidence of OS (Jauniaux et al., 2006). Similarly, the postulated involvement of deficiencies of trace elements in pre-eclampsia relates to the fact that they are present in metallothionein (zinc), ceruloplasmin (copper), SOD (copper, selenium, zinc) and glutathione peroxides (selenium). In addition, a potential benefit of n-3 fatty acids in preventing pre-eclampsia has been suggested in a prospective cohort study enrolling 1718 women (Oken et al., 2007). Theoretically, n-3 fatty acids may alter prostanoids in favor of vasodilators eicosanoids. Interestingly, lower n-3 fatty acid levels were demonstrated in erythrocytes of women with pre-eclampsia (reviewed by Roberts et al., 2003). Moreover, in an observational study in an Icelandic community with traditional fish and cod liver oil consumption, an inverse association was found between the proportion of long-chain n-3 PUFA in red blood cells of women at the 11th to 15th week of pregnancy and placental weight (Magnusardottir et al., 2009).

Some 'placenta events' are postulated to arise from deficiencies of either folate and/or vitamin B_{12} or defects within the methionine–homocysteine metabolic pathways (Goddijin-Wessel et al., 1996; Ray and Laskin, 1999). In this context, HHCY has been shown to provoke vascular inflammation, to decrease the bioavailability of NO that is an important endothelium vasodilatator, and seems to be associated with the production of ROS (Forges et al., 2007). This means that folate-deficiency or HHCY may underlie endothelian dysfunction, and therefore placental endovasculature (Ray and Laskin, 1999), a theory supported by the observation that elevated serum homocysteine concentrations have been associated with an increased risk of diseases, such as atherosclerotic, thromboembolic and neurodegenerative disorders (reviewed by Diaz-Arrastia, 2000; Kuo et al., 2005; Folstein et al., 2007; Forges et al., 2007).

Embryogenesis

There is consistent scientific evidence that folic acid (the synthetic form of the vitamin folate) is of critical importance both pre- and periconceptionally in protecting against NTDs in the developing fetus (MRC, 1991; Czeizel and Dudás, 1992; Bendich, 2001; Moore et al., 2003; Czeizel et al., 2004; Shah and Sachdev, 2004; Padmanabhan, 2006). In general, women are advised to take 0.4 mg/day when planning a pregnancy whereas they are recommended to take 4.0 mg/day if they experienced a previous pregnancy affected by NTD (de Bree et al., 1997; Geisel, 2003; Pitkin, 2007). Involved in DNA synthesis and cell division, folic acid plays a vital role in fetal development. Moreover, low folate status seems to increase risk of preterm delivery, LBW and fetal growth restriction as well as other fetal malformations (Czeizel et al., 1999; Tamura and Picciano, 2006). Low B_{12} levels are related to HHCY and high tHcy level is associated with NTDs (Mills et al., 1995; Ray and Laskin, 1999; Ray and Blom, 2003) and CHDs (Verkleij-Hagoort et al., 2008). Neural crest cells are involved not only in the embryogenesis of the neural tube, lip and palate, but also in cardiovascular development. The migration and differentiation of neural crest cells is influenced by homocysteine, and vitamin B_{12} is an important determinant in the homocysteine pathway, thereby contributing to the embryogenesis of the heart in the first weeks after

conception (Verkleij-Hagoort et al., 2008). In a case–control study in the Netherlands, it was observed that the periconceptional intake of thiamine, niacin and pyridoxine (vitamin B_6) seems to contribute to the prevention of orofacial cleft (OFC) defects (Krapels et al., 2004b). This result may be explained by considering vitamin B_6 involvement into the homocysteine pathway.

Regarding vitamin A, retinoids are thought to be involved in the development of several embryonic systems (reviewed by Finnell et al., 2004). In particular, several embryonic anomalies were observed in vitamin A-deficient quail embryos developing in the absence of RA (Maden et al., 1996). Thus, RA plays a crucial role in central nervous system development (Maiden et al., 1996), i.e. neural crest survival, neuritis outgrowth and hindbrain patterning. Vitamin A and RA are part of the normal regulatory system required. Nevertheless, excessive intake (>10 000 IU/day) has been shown to be teratogenic in animals (Williamson, 2006). Malformations were also seen in the human embryo exposed to RA due to treatment of the mother with the acne drug Accutane (13-cis RA) (reviewed by McCaffery et al., 2003). However, other results are contrasting (Dolk et al., 1999). In fact, although the overall recommendation is that vitamin A or retinol intake below 3000 μg (10 000 IU)/day is safe, some have argued that vitamin A intake up to 9000 μg (30 000 IU)/day represents harmless levels (Miller et al., 1998). In this context, in a population-based case–control study carried out in Norway no evidence of an increased risk of clefting was found with an intake of total vitamin A or retinol of more than 3000 μg (10 000 IU) (Johansen et al., 2008).

Animal models have shown that severe maternal zinc deficiency in early pregnancy results in impaired implantation, abortions and fetal malformations, including cleft lip and palate, brain and eye malformations, numerous abnormalities of the heart, lung and urogenital systems (Keen et al., 2003). Biochemical and functional abnormalities can occur as a result of a zinc deficit (Maret and Sandstead, 2008). Interestingly, Keen et al. (2003) reported that even transitory periods of zinc deficiency (5–6 days) can be teratogenic in rodent models, and 3 days of periconceptional zinc deficiency can adversely affect embryonic development. The consideration that zinc deficiency is a teratogenic risk in humans may be supported by the correlation of low plasma zinc concentrations in the first and third trimesters of pregnancy with an increased risk for malformations and LBW, respectively. Zinc deficiency is thought to influence embryonic and fetal development through reduced cell proliferation, or reduced protein synthesis or reductions in rates of tubulin polymerization rather than increased rates of cellular oxidative damage or increased rates of apoptosis and reduced binding of hormones and transcription factors dependent on zinc-finger regions (Jankowski-Hennig et al., 2000; Mackenzie et al., 2002).

A copper deficiency-associated teratogenicity has been suggested (Keen et al., 1998). Embryos obtained from copper-deficient dams and cultured for 48 h in either copper-adequate or copper-deficient serum displayed numerous abnormalities, such as swollen hindbrains, blisters, blood pooling and distention of the large vessels (Hawk et al., 1998). Since SOD activity was lowest in embryos cultured in copper-deficient serum, and antioxidant (copper–zinc-SOD or GSH peroxidase) supplementation reduced the teratogenicity of the copper-deficient serum, it may be postulated that dysmorphology is due in part to free radical induced damage occurring secondary to an impaired oxidant defense system. Moreover, the copper-deficient embryos also had low cytochrome c oxidase activity compared with control embryos suggesting that multiple factors contribute to copper deficiency-induced abnormalities. Similar results were found in 'Xenopus laevis' embryos from frogs administered a low-Cu diet for 120 day (Fort et al., 2000). On the contrary, although an effect of copper deficiency on the human conceptus has been suggested, no causal relation between low fetal copper concentrations and malformations has been demonstrated. In other words, it has not yet been proven whether the effects of copper deficiency on the embryo or fetus are due directly to a deficiency of the micronutrient, or whether they indirectly occur through the copper deficiency-modulated metabolism of the mother (for example as a consequence of maternal anemia, production of pregnancy hormones or growth factors etc.) (Keen et al., 1998).

In a study aimed to evaluate the effects of ethanol on fetal development, 'Xenopus laevis' embryos were treated with ascorbic acid 2 h prior to ethanol exposure, and then transferred to medium in the presence or absence of 100 mM ethanol for 12 h. The ethanol-induced ROS production and NF-kB activation were inhibited and the ethanol-treated embryos were protected against microencephaly and growth retardation, suggesting a potential use of ascorbic acid as an effective protective agent for human fetal alcohol syndrome (Peng et al., 2005). Furthermore, antioxidative treatment of streptozotocin-induced diabetic rats with vitamins E and C decreased fetal malformation rate and diminished oxygen radical related tissue damage (Cedeberg et al., 2001). A prophylactic effect of vitamin E against diabetic embryopathy was also demonstrated by Sivan et al. (1996). Inclusion of vitamin E in culture medium, which stimulates development both before and after embryo transfer, was demonstrated to suppress ROS damage in bovine embryos (Olson and Seidel, 2000). On the whole, these data suggest that antioxidants exert beneficial effects on embryo development, possibly by a reduction in the incidence of apoptosis (Agarwal et al., 2008). Similarly, the use of a dietary PUFA that specifically increases arachidonic levels significantly reduced the incidence of diabetic embryopathy in rats (Reece et al., 1996). A significant reduction in the incidence of maternal diabetes-related fetal malformations and IUGR has been reported in diabetic rats also with the supplementation of lipoic acid (Al Gafli et al., 2004).

Conclusions

The achievement of a successful pregnancy represents one of the fundamental functions of existence. Although scant, data suggest a significant role of micronutrients in the periconceptional period. The nutritional status of women must be a goal for preventing detrimental nutritional unbalances. In particular, diet during the first trimester may be more important to development and differentiation of various organs. Moreover, also preconceptional nutrition is crucial for an optimal onset and development of pregnancy. Unfortunately, nutritional intake of childbearing-age women appears to be inadequate during the preconceptional period (de Weerd et al., 2003b; Mouratidou et al., 2006) mainly in terms of micronutrients, but also considering the global shift towards the Western diet (Popkin and Gordon-Larsen, 2004). Thus, efforts to increase awareness of a healthy diet and lifestyle should be strengthened not only throughout

pregnancy but also before, given that pregnancies are often unplanned. A meta-analysis evidenced the relationship between preconceptional care and reduced risk of congenital anomalies in the offspring of women with pregestational diabetes mellitus (Ray *et al.*, 2001). Moreover, the role of preconceptional folic acid supplementation in the prevention of NTDs has been documented. In this context, considering the relatively high frequencies of both the MTRR and the MTHFR polymorphisms in the general population, maintenance of adequate vitamin levels (cobalamin and folate) should be stressed for pregnancy (Wilson *et al.*, 1999).

However, conclusive evidence was provided solely for periconceptional folate and prevention of NTDs, and prospective studies related to the association between periconceptional maternal nutrition and pregnancy outcomes are scarce. Interestingly, intakes of magnesium and ascorbic acid above the RDA at $375.9 - 502.9$ and $97.9 - 295.8$ mg/day, respectively, were found to substantially decrease OFC risk (Krapels *et al.*, 2004a). By considering that RDAs for pregnant women are $300 - 360$ mg/day magnesium and 90 mg/day ascorbic acid, these results seem to indicate that the RDAs for these nutrients are insufficient for women of reproductive age. Moreover, although the majority of data indicate that vitamin A intake above 3000 μg/day should be avoided in the periconceptional period, this has been recently discussed (Miller *et al.*, 1998; Johansen *et al.*, 2008), and similarly, there is disagreement on the need for iron prophylaxis early in pregnancy.

In particular, causes or at least potential mechanisms between micronutrient status in the periconceptional period and pregnancy outcomes remain still to be well-defined/understood. Specifically, the timing of the potential insults need to be studied in depth both *in vitro* and in animal models. Moreover, there is a dramatic need for further experimental and interventional human studies, in order to optimize dietary recommendation and thus maternal periconceptional nutritional status.

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