Micronutrient status and effects of supplementation in anemic pregnant women in rural China

Aiguo Ma

Thesis committee

Promotors

Prof. Dr. Evert G. Schouten

Professor of Epidemiology and Prevention, Wageningen University

Prof. Dr. Ir. Frans J. Kok

Professor of Nutrition and Health, Wageningen University

Other members

Prof. Dr. Renger F. Witkamp, Wageningen University

Prof. Dr. Ib C.Bygbjerg, University of Copenhagen, Denmark

Prof. Dr. Guansheng Ma, Center for Disease Control and Prevention, Beijing, China

Dr. YunYun Gong, Leeds University, UK

This research was conducted under the auspices of the Graduate SchoolVLAG (Advanced studies in Food Technology, Agrobiotechnology, Nutrition and Health Sciences).

Micronutrient status and effects of supplementation in anemic pregnant women in rural China

Aiguo Ma

Thesis

submitted in fulfillment of the requirement for the degree of doctor

at Wageningen University

by the authority of the Rector Magnificus

Prof.dr. M.J.Kropff,

in the presence of the

Thesis Committee appointed by the Academic Board

to be defended in public

on Monday 28 January 2013

at 1.30 p.m. in the Aula.

Aiguo Ma
Micronutrient status and effects of supplementation in anemic pregnant women in rural China, 155 pages.

PhD thesis, Wageningen University, Wageningen, NL (2013)
With references, with summaries in Dutch and English

ISBN: 978-94-6173-493-8

ABSTRACT

Background

Iron deficiency anemia (IDA) is a major nutrition related problem in China, especially affecting pregnant women, like in most developing countries. Deficiencies of vitamins also play an important role, such that iron, retinol and riboflavin deficiencies tend to coexist in anemic pregnant women. However, vitamin and/or mineral supplements are not routinely used by pregnant women at or below low income levels. Besides being an essential trace element, iron plays a central role in oxygen radical generation, whereas susceptibility during pregnancy is elevated. There is evidence that both iron deficiency and excess may result in free radical damage.

Objectives

Based on this background, objectives of this thesis were to investigate the current prevalence of anemia among pregnant women in different sites of China, and to describe the micronutrient status of anemic and non-anemic pregnant women in China. In addition, in anemic pregnant women, the effect of retinol and riboflavin supplementation on top of iron plus folic acid on anemia and changes in hematological status was assessed. Also oxidative stress and erythrocyte membrane fluidity were evaluated. Finally, we compared the effect of sodium iron ethylenediaminetetraacetate (NaFeEDTA) and ferrous sulfate on hemoglobin (Hb), iron bioavailability and oxidative stress.

Subjects and Methods

A total of 6413 women in their third trimester of pregnancy were recruited from five rural areas in China (years 2000~2003) for screening anemia or iron deficiency. A random subset was selected for measuring micronutrient status. In three supplementation trials, effects of iron, folic acid, retinol and riboflavin were assessed among anemic pregnant women. Outcomes included hematological status, micronutrient status and parameters of oxidative stress. Samples of fasting blood were collected from subjects before and at the end of the interventions for measurements.

Results

The overall prevalence of anemia was 58.6%, ranging between 48.1%~70.5% in the five areas. Serum concentrations of micronutrients were significantly lower in anemic women than non-anemic women. After the 2-mo intervention, the increase of Hb concentration in the group, supplemented with iron and folic acid combined with retinol and riboflavin, was 5.4g/L greater than in the group with iron and folic acid only (p<0.001). The reductions in the prevalence of anemia (Hb<110g/L) and iron deficiency anemia were significantly greater in the groups supplemented with retinol and/or riboflavin than in the iron and folic acid group. Riboflavin and/or retinol supplementation significantly improved gastrointestinal symptoms and well-being. In addition, supplementation of iron combined with retinol and riboflavin significantly decreased levels of serum malondialdehyde, and increased activities of glutathione peroxidase and erythrocyte membrane fluidity as well. NaFeEDTA supplementation showed superior effectiveness.

Conclusion and recommendations

Deficiency of iron and micronutrients in combination appears to contribute to the high prevalence of anemia in pregnant women in rural China. Supplementation with iron, particularly in combination with vitamins improved hematologic status as well as oxidative stress. NaFeEDTA performed better than ferrous sulfate. Multi-micronutrient supplementation may be worthwhile for pregnant women in rural China. Further studies on food-based or supplement-based approaches are warranted to decrease anemia of Chinese pregnant women in the third trimester.

Contents

Chapter 1

General Introduction

Anemia is the most common disorder during pregnancy with estimates of global prevalence reaching over 40% (1). Iron deficiency anemia (IDA) is the most frequent micronutrient deficiency disease in developing countries like China, heavily affecting pregnant women. Although the diagnosis of anemia is fairly simple and the treatment is inexpensive, the prevalence of anemia remains high especially in poverty-stricken areas of China. Also, it is clear that deficiencies of vitamins play a role in IDA, like folate, vitamin A, and riboflavin (2,3). In fact, retinol and riboflavin deficiencies tend to coexist in anemic pregnant women in developing countries or impoverished settings (4). However, vitamin and/or mineral supplements are not routinely used by pregnant women particularly of low income groups. Iron is an essential trace element but plays a central role in generating harmful oxygen species as well. Its redox cycling promotes the Fenton reaction, producing the potent oxidant hydroxyl radical (5). Pregnancy is a condition exhibiting increased susceptibility to oxidative stress (6). In rats, both iron deficiency and excess result in free radical mitochondrial damage (7). This thesis will therefore focus on iron deficiency, which affects Chinese pregnant women living in poor areas, and on the effect of multiple micronutrient supplementation on anemia and on oxidative stress in anemic pregnant women. In this chapter we will discuss iron homeostasis, factors contributing to poor iron status of pregnant women, iron requirements in pregnancy and the effect of iron and other micronutrient supplements.

IRON HOMEOSTASIS

Most of the iron in the body is distributed within red blood cell (RBC) hemoglobin (65%). Approximately 10% is present in muscle fibers (in myoglobin) and other tissues (in enzymes and cytochromes). The remaining body iron is stored in the liver, macrophages of the reticuloendothelial system, and bone marrow (8). The human body has developed intricate but exquisitely controlled mechanisms to absorb, transport and store iron, thus ensuring a ready supply for cellular growth and function, but limiting its participation in reactions that produce free radicals and its availability to invading pathogens (9).

Iron absorption and metabolism

Iron is found in food as either heme or non-heme iron. Heme iron, which makes up 40% of the iron in meat, poultry, and fish, is well absorbed. Sixty percent of the iron in animal tissue and all the iron in plants are in the form of non-heme iron and are relatively poorly absorbed (9). Virtually all dietary non-heme iron is in the ferric form (Fe^{3+}), which must first be reduced to the ferrous form (Fe^{2+}) before it is transported across the intestinal epithelium by a transporter called divalent metal transporter 1 (DMT-1). But the existence of other uptake pathways cannot be excluded (10). The absorption of non-heme iron is diminished by co-administration of phytates (high-fiber diets), tetracyclines, proton pump inhibitors and antacid medication, calcium, and phenolic compounds (coffee and tea). Heme iron is absorbed into enterocytes by a putative, not totally identified heme carrier protein 1, which is a membrane protein found in the proximal intestine, where heme absorption is greatest (11). Once internalized in the enterocytes, it is likely that most dietary heme iron is released in ferrous form by heme oxygenase to enter a common pathway with dietary non-heme iron before it leaves the enterocytes (12). Supplemental iron is usually provided as a ferrous salt which is absorbed directly. In order to reach the circulation, this metal must cross both the apical and basolateral surfaces of the enterocyte (13).

Iron that is not transported into the circulation is sequestered in the storage protein ferritin, a spherical protein that keeps oxidized iron in a soluble and relatively non-toxic form. The metal that does not enter the circulation is eventually lost as the enterocytes are sloughed into the gut lumen. If the body is in iron balance, only 0.1% of the total body iron is absorbed from the gut daily, since the body has no effective means of excreting iron. The regulation of absorption by the duodenum plays a critical role in iron homeostasis in the body (14).

Dietary factors affecting iron status

The amount of iron that is absorbed in the body can be influenced by a number of enhancers and inhibitors present in the diet. Among those enhancers, vitamin C (ascorbic acid) is one of the most important factors. It is well known that vitamin C can enhance iron absorption by reducing ferric iron to ferrous iron, a form of iron that is more soluble and therefore better absorbed by the body (15). In addition, vitamin C can help to overcome some of the negative effects of iron inhibitors and also has an active role in supporting the body’s iron metabolism by enhancing the solubility of iron (16). The addition of ascorbic acid to a juice enhanced Fe absorption and Fe utilization in ferrous sulfate or micronized dispersible ferric pyrophosphate (poorly soluble) to ferrous sulfate (highly soluble) (17). Recent evidence suggests that regular oily fish consumption may also have a role in improving iron status. The oily fish appears to be acting as an enhancer to release iron from a phytate-rich meal (18). There are also many factors present in the diet that inhibit the absorption of non-heme iron. The first one is phytate. Phytates are components found in plants that can interfere with the body's absorption of nutrients such as iron, generally in a dose-dependent fashion. The typical Chinese diet rich in wholegrain can decrease the rate of iron absorption, thereby increasing the risk of iron deficiency. Eating cereals and foods that are fortified with extra iron may help to compensate for some of these effects (19). Iron-binding phenolic compounds (polyphenols) are important inhibitors of iron absorption too. It is shown that tea drinking, a common practice in China, can limit iron absorption, especially from non-heme sources, which is mainly attributed to its polyphenol content (20). Fruits, vegetables, cereals and dry legumes are also rich in polyphenols. Women, who lose iron through their menses and particularly those who eat a predominantly plant-based diet, may benefit from taking a daily multivitamin and mineral supplement that contains iron (21).

IRON DEFICIENCY

Iron is an essential micronutrient because it plays a vital role in oxygen transport, oxidative metabolism, cellular proliferation and many other physiological processes (9). Iron requirements are met by dietary iron or from existing body iron stores. Increased iron requirements, limited external supply, and increased blood loss may lead to iron deficiency (ID) and iron-deficiency anemia (IDA). Inadequate dietary iron intake eventually results in depleted body iron stores, which is presumed to indicate iron deficiency. ID occurs when iron stores mostly found in the liver, start to become depleted, while IDA arises when the production of red blood cells starts to diminish once the iron stores have been depleted.

Iron deficiency in pregnancy

Anemia in pregnancy, defined as a hemoglobin concentration (Hb) < 110 g/L, affects more than 56 million women globally, two thirds of them being from Asia (22). Some women are already anemic before pregnancy and some others become progressively anemic during pregnancy. The causes of iron deficiency and iron deficiency anemia can be multi-factorial, but common causes are low dietary iron intake, poor absorption of iron from the diet, parasitic infections, which are more common in developing regions, and disease states such as intestinal bleeding. Anemia can be mild, moderate or severe and can cause weakness, tiredness and dizziness. A healthy non-anemic woman could progress to a state of low iron stores, then to iron deficiency (ID) with no anemia and finally to a clinical iron deficiency anaemia (IDA) (23). Pregnancy requirements of iron are low in the first trimester, but progressively increase to reach a maximum in the third trimester. It has been estimated that the daily iron requirement of a 55 kg pregnant woman increases from approximately 0.8 mg in the first trimester to 4–5 mg during the second trimester and >6 mg in the third trimester (24).

IDA can lead to reduced work capacity, intellectual capacity and productivity and increased susceptibility to infection of pregnant women (25). It is estimated that anemia resulted in 3.7% of the cases of maternal death in Africa and 12.8% in Asia (26). Severe anemia is associated with adverse perinatal outcomes such as small for gestational age babies and preterm deliveries. The harmful effects of ID at birth also have long term consequences such as poor physical and mental growth continuing throughout childhood and adolescence, and even permanent neuro-physiological deficiencies (27-28). So, it is important to improve iron status during pregnancy.

Prevention of iron deficiency in pregnancy

In view of high prevalence of iron deficiency in pregnancy, it is postulated that weekly iron (60 mg of ferrous sulphate) and folic acid (3 mg) supplementation for women of reproductive age, including adolescent girls between 10–19 years old could be an effective strategy to achieve good iron stores before a woman becomes pregnant (29). It was demonstrated that a weekly iron and folic acid supplementation program for women of reproductive age has been effective in reducing the prevalence of anemia in some districts in Vietnam, the Philippines and Cambodia (30-32). In order to prevent adverse perinatal outcomes resulting from iron and folic acid deficiency in pregnant women, according to WHO a daily supplement of 60 mg of elemental iron and 400 µg of folic acid should be started as soon as possible by all pregnant women in communities where anemia during pregnancy is a significant problem. This should be given throughout pregnancy and continued for six months postpartum to ensure adequate iron stores in the woman (33). This regimen increases maternal hemoglobin levels and the birth weight of the new born, and reduces the delivery of low birth weight babies.

Ferrous iron, as sulfate or sodium iron EDTA (NaFeEDTA) is mostly used for correction of iron deficiency. Sodium iron EDTA is an iron chelate that was used successfully (34) and is not influenced by the inhibitors of iron absorption. It has a bioavailability 2 - 4 times that of ferrous sulfate (35). Nevertheless, NaFeEDTA and ferrous fumarate at low levels compatible with South African brown fortified bread (10 mg/kg flour for NaFeEDTA and 20 mg/kg flour for ferrous fumarate) did not show effective improvement iron or Hb status in a randomized controlled trial (36,37). But a fortification level of 30 mg/kg $FeSO_4$, and 20 mg/kg NaFeEDTA in wheat flour had a positive effect on iron status in anemic students in China, while NaFeEDTA was more effective than $FeSO_4$ (38). However, few data have evaluated the impact of NaFeEDTA to control iron deficiency anemia (IDA) in anemic pregnant women.

Retinol and IDA

As early as the 1920s, vitamin A deficiency and anemia or other indices of low iron status were reported to be associated in humans and animals (39-40). Observational studies showed that serum retinol was positively associated with hemoglobin, hematocrit, and serum iron (41-42). Some studies also showed that vitamin A may be required for the mobilization and utilization of iron for hemoglobin synthesis, and thus contribute to erythropoiesis (43). A study conducted in Indonesia showed that antenatal iron and vitamin A supplements were better than iron alone (44). However studies in Malawi did not show a similar effect (21). Hodges's study indicated that iron stores are unavailable for erythropoiesis during vitamin A deficiency which implies that vitamin A plays a role in the release of iron from the liver (45]. The data of an experimental study showed that in a population with low socioeconomic status, vitamin A supplementation (3.0mg/d) can improve the hematological condition (hemoglobin increased by 9 g/L) of anemic children (46).

Riboflavin and IDA

It's indicated that iron deficiency is often accompanied by other micronutrient deficiencies including retinol and riboflavin (47-48). Riboflavin deficiency may be one of the most common vitamin deficiencies in regions, like rural areas in China, where diets are predominantly rice based and contain insufficient milk, meat, fish, and fresh fruit and vegetables (49). Some animal studies have shown that iron absorption is impaired when riboflavin is deficient (50-51). Moreover, other studies (52-53) have shown better hematological response when riboflavin was given along with iron than when iron was given alone. A recent study also showed good correlations between blood hemoglobin and serum ferritin and dietary intakes of riboflavin (54). It has been shown that riboflavin has a direct role in the release of iron from ferritin (55-56).

PREGNANCY, MICRONUTRIENT STATUS AND OXIDATIVE STRESS

Pregnancy and oxidative stress

Pregnancy is a condition exhibiting increased susceptibility to oxidative stress, defined here as a disturbance in the prooxidant-antioxidant balance in favor of the former, leading to potential damage (57). It has been suggested that pregnancy is a state of oxidative stress, which is characterized by the placental production of reactive oxygen species including superoxide and hydrogen peroxide (6,58,59); Placental cells are rich in mitochondria, which is a condition that favors oxidative stress.Transition metals, especially iron, which are particularly abundant in the placenta, are important in the production of free radicals (6). Excessive free radical production may cause both lipid and protein oxidation and impair normal endothelial cell function (60). The elevated oxidative stress could alter placenta and fetal skeletal formation as well (61). Pregnancy complications such as spontaneous abortion, recurrent pregnancy loss, and preeclampsia, can also develop in response to oxidative stress (62). Oxidative stress is also strongly involved in the pathogenesis of many preterm newborn diseases, such as the presence or absence of advanced retinopathy of prematurity, grade III intraventricular hemorrhage, respiratory distress syndrome, bronchopulmonary dysplasia, sepsis or severe fungal infection (63). This is due to the low efficiency of neonatal antioxidant systems unable to counteract the harmful effects of free radicals. Altered maternal micronutrients through oxidative stress may reduce neurotrophic factors in preterm pregnancy (64). Antioxidant enzymes such as superoxide dismutase (SOD) and glutathione peroxidase (GSH-px) could be beneficial in maintaining pregnancy by antagonizing the harmful oxygen free radicals (65,66).

Iron status and oxidative stress

Iron deficiency anemia (IDA) can severely impair the outcome of pregnancy in animals. It increases the expression of hypoxia and inflammatory markers in the placenta, and may cause oxidative stress in pregnant animals, fetuses and placentas. Treatment with iron polymaltose complex/folic acid corrected the IDA as well as reduced the levels of oxidative stress and inflammatory markers close to nonanemic control values in all the studied organs (67). The relationships of oxidative stress and DNA damage with the severity of anaemia suggest that both oxidative stress and DNA damage may, in part, have a role in the pathogenesis of IDA (68). A systematic review (69) examined potential adverse effects of iron supplementation, including oxidative stress, inhibition of absorption of other nutrients, neonatal morbidity, the requirement for blood transfusion, and other adverse effects. None of the studies found any evidence that iron supplementation increased oxidative stress in low birth weight infants; however, two studies reported an increased incidence of respiratory tract infection with iron supplementation (70-71). IDA tends to increase the pro-oxidant components, which may result in various complications including peroxidation of vital body molecules resulting in increased risk for pregnant women as well as for the fetus (72). Iron deficiency anemia (IDA) affects not only the hematological parameters but also disturb the oxidative balance of body. In pregnancy, this is much more considerable as oxidative stress is considered to be one of the physiological changes during this period (73).

Micronutrient status and oxidative stress

Increased metabolic burdens during late gestation and lactation cause elevated systemic oxidative stress during these important periods. Increased oxidative stress is related to decreased availability of antioxidants during late gestation and lactation, which begin to normalize towards the end of the lactation period. It may be necessary to increase the vitamin E and A contents in the diet during the gestational period in order to compensate for the substantial loss of these nutrients (74). Serum alpha-tocopherol, retinol, and malondialdehyde concentrations were measured at 7 months of pregnancy in 122 women from low socio-economic background. Preterm delivery was associated with low nutrient status especially vitamin A. The role of antioxidant nutrients, especially vitamin A and oxidative stress in relation to fetal growth and pregnancy outcome among mothers from low socioeconomic settings requires attention (75). Vitamin A deficiency (VAD) is prevalent in developing countries (76), retinol equivalents and iron were the major nutritional problems in China (77). Overall, a few studies reported that pregnancy and abnormal environments, such as low intake of antioxidants or micronutrients, might enhance levels of oxidative stress, and be harmful to the outcome of pregnancy. Although some studies showed that development of pregnancy, and fetus and infant growth, and iron status may be related to oxidative stress, the oxidative stress status of pregnant women with iron deficiency and micronutrient deficiency is unclear. Adequate supplementation of iron for anemic pregnant women living on plant-diets is necessary to improve iron deficiency or iron deficiency anemia, and maintain iron stores; while vitamin supplementation may be important to improve iron absorption and utilization as well as oxidative stress level. Multiple micronutrient supplements might increase hemoglobin and improve nutritional status of pregnant women more than iron supplements alone or iron with folic acid (78). No consistent adverse effects of iron supplementation have been reported, although many studies did not monitor such effects. Therefore, further studies are needed on iron deficiency, and the potential adverse effects of iron supplementation, especially with respect to oxidative stress, in pregnancy.

RATIONALE AND OBJECTIVES

Study rationale

Anemia is the scourge of the third world (79). The overall prevalence of anemia during third trimester of pregnancy was 48.2% during 1995-2000 (80). In fact, most of Chinese pregnant women live in rural areas with low socio-economic levels, and anaemia mainly is a problem there, and reports about the prevalence are limited. Therefore, we have performed a cross sectional study in five rural areas all over China, in order to get a more representative and complete picture of the prevalence and determinants of anemia. Furthermore, we carried out three experimental trials to investigate the effect of iron and micronutrient supplementation during pregnancy on anemia and nutritional status as well as possible effects on oxidative balance.

Study objectives

This thesis is composed of five chapters in two parts: an observational and an experimental part.

Observational part

A cross-sectional study was carried out,

1) to assess the prevalence of anemia in Chinese pregnant women, in five geographically different sites, see **chapter 2**;

2) to describe the micronutrient status of anemic and nonanemic women in a subset of this population**.** In addition to iron deficiency anemia may be due to deficits of other micronutrients, see **chapter 3**.

Experimental part

Three randomized supplementation trials were designed:

1) To explore the effect of retinol and riboflavin supplementation on top of iron plus folic acid on anemia and subjective well being in pregnant women (**chapter 4)**;

2)To investigate changes in hematological status, oxidative stress and erythrocyte membrane fluidity in anemic pregnant women after iron supplementation with and without combined retinol and riboflavin (**chapter 5)**;

3) To evaluate effects of NaFeEDTA and ferrous sulfate on hematological status and oxidative stress in anemic pregnant women (**chapter 6**), in order to get evidence for superiority of one or the other supplement.

STUDY SETTINGS

Anemia remains a major problem in nearly all developing countries, and in the wide countryside and poverty-stricken areas of China. We selected five different geographical areas of China to investigate the iron status and anemia prevalence of pregnant women and micronutrient status. The five areas included rural areas of Qingdao in the east, Fuzhou in the southeast, urban areas of Lanzhou in the northwest and Guilin in the southwest, and a district of Liaocheng in central China.

In our cross-sectional study, we investigated the prevalence of anemia among pregnant women in the third trimester in the five selected rural areas of China. The enrolled women did not experience abnormal bleeding and did not take iron and multivitamin supplements, do not smoke tobacco products or drink alcoholic beverages during pregnancy.

Unlike Western societies, food is not routinely fortified with iron in rural areas of China, and green vegetables were scarce in the subjects’ diets during the winter season. Therefore, we supplemented the anemic women with a capsule of iron, folic acid, retinol and/or riboflavin. Our supplementation trials were designed to be carried out in one of the previous five sites, Shen' county located in the middle of China. The number of subjects in three trials was 366, 164 and 153 anemic pregnant women in the second trimester, respectively, who were randomly enrolled from the population of anemic pregnant women.

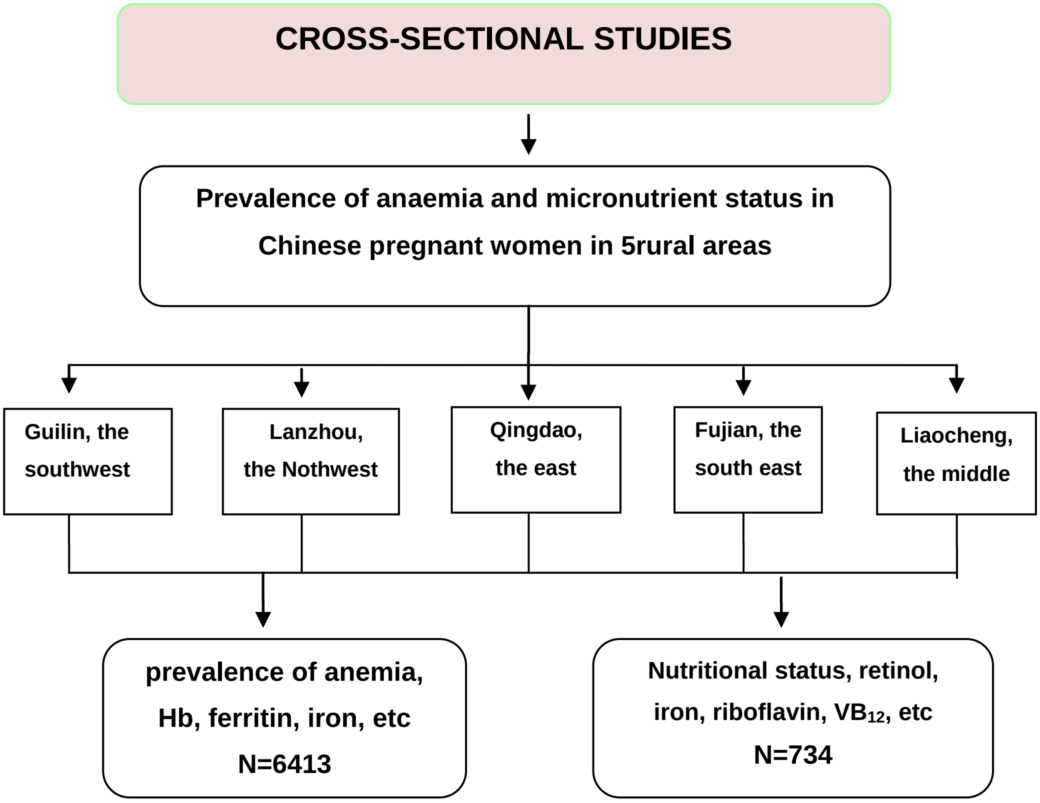

Figure 1.1 Profile of the cross sectional study.

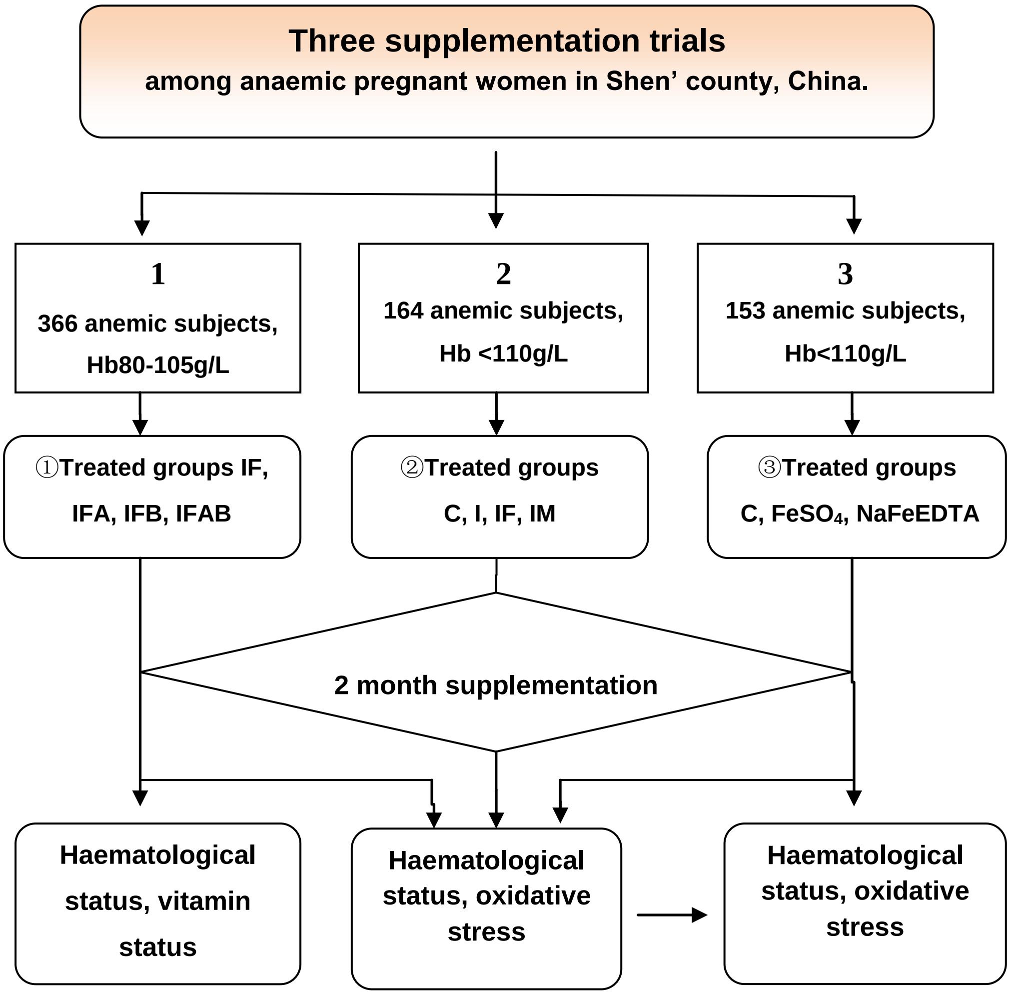

Figure 1.2 Profile of the randomized, double-blind controlled trials.
Aims of the studies are to investigate the effects of supplementations with iron alone and iron combined with vitamins on anemia, micronutrient status and oxidative stress.

①Treated groups: IF, 60 mg ferrous sulfate and 400μg folic acid (reference); IFA, additional 2 mg retinol; IFB, additional 1mg riboflavin; IFAB, additional both 2 mg retinol and 1mg riboflavin daily.
②Treated groups: C, placebo; I, 60mg iron as ferrous sulfate; IF, additional 400μg folic acid; IM, additional 400μg folic acid, 2 mg retinol and 1 mg riboflavin.
③Treated groups: C, placebo; I, 60 mg iron as ferrous sulfate; IE, 60 mg iron as NaFeEDTA.

References

1. McLean E, Egli I, Cogswell M, De Benoist B, Wojdyla D. Worldwide prevalence of anaemia in preschool age children, pregnant women and non-pregnant women of reproductive age. Nutr Anaemia 2007;1:1–12.
2. Ahmed F, Mahmuda I, Sattar A, Akhtaruzzaman M. Anaemia and vitamin A deficiency in poor urban pregnant women of Bangladesh. Asia Pac J Clin Nutr 2003;12: 460-466.
3. Graham JM, Haskell MJ, Pandey P, Shrestha RK, Brown KH, Allen LH. Supplementation with iron and riboflavin enhances dark adaptation response to vitamin A-fortified rice in iron-deficient, pregnant, night-blind Nepali women. Am J Clin Nutr 2007; 85: 1375-1384.
4. Bates CJ, Prentice AM, Paul AA. Seasonal variations in vitamin A, C, riboflavin and folate intakes and status of pregnant and lactating women in a rural Gambian community: some possible implications. Eur J Clin Nutr 1994;48: 660-668.
5. Halliwell B, Gutteridge JC. Oxygen toxicity, oxygen radicals, transition metals and disease. Biochem J 1985;219:1-14.
6. Casanueva E, Viteri FE. Iron and oxidative stress in pregnancy. J Nutr 2003;133:1700S-1708S.
7. Walter PB, Knutson MD, Paler-Martinez A, Lee S, Xu Y, Viteri FE, Ames BN. Iron defic iency and iron excess damage mitochondria and mitochondrial DNA in rats. Proc Natl Acad Sci 2002;99: 2264-2269.
8. Muñoz M, Villar I, García-Erce JA. An update on iron physiology. World J Gastroenterol 2009;15:4617-4626.
9. Nair KM, Iyengar V. Iron content, bioavailability & factors affecting iron status of Indians. Indian J Med Res 2009;130: 634-645.
10. Conrad ME, Umbreit JN. Pathways of iron absorption. Blood Cells Mol Dis 2002;29:336-355.
11. Krishnamurthy P, Xie T, Schuetz JD. The role of transporters in cellular heme and porphyrin homeostasis. Pharmacol Ther 2007;114: 345-358.
12. Andrews NC. Disorders of iron metabolism. N Engl J Med 1999, 341: 1986-1995.
13. Baker JF, Ghio AJ. Iron homoeostasis in rheumatic disease. Rheumatology (Oxford) 2009;48:1339-44.
14. Siah CW, Ombiga J, Adams LA, Trinder D, Olynyk JK. Normal iron metabolism and the pathophysiology of iron overload disorders. Clin Biochem Rev 2006;27: 5-16.
15. Hurrell R, Egli I. Iron bioavailability and dietary reference values. Am J Clin Nutr 2010;91:1461S-1467S.
16. Derbyshire E. Strategies to improve iron status in women at risk of developing anaemia. Nurs Stand 2012;26: 51-57.

17. Haro-Vicente JF, Pérez-Conesa D, Rincón F, Ros G, Martínez-Graciá C, Vidal ML.Does ascorbic acid supplementation affect iron bioavailability in rats fed micronized dispersible ferric pyrophosphate fortified fruit juice?Eur J Nutr 2008 ;47:470-478.
18. Navas-Carretero S, Pérez-Granados AM, Schoppen S, Sarria B, Carbajal A, Vaquero MP. Iron status biomarkers in iron deficient women consuming oily fish versus red meat diet. J Physiol Biochem 2009;65:165-174.
19. Ma G, Jin Y, Piao J, Kok F, Guusje B, Jacobsen E. Phytate, calcium, iron, and zinc contents and their molar ratios in foods commonly consumed in China.J Agric Food Chem 2005;53:10285-10290.
20. Fairweather-Tait SJ. Iron nutrition in the UK: getting the balance right. Proc Nutr Soc2004;63:519-528.
21. Waldmann A, Koschizke JW, Leitzmann C, Hahn A. Dietary iron intake and iron status of German female vegans: results of the German vegan study. Ann Nutr Metab 2004;48:103-108.
22. Goonewardene M, Shehata M, Hamad A. Anaemia in pregnancy. Best Pract Res Clin Obstet Gynaecol 2012;26:3-24.
23. Goonewardene M, Seekkuge J, Liyanage C. Iron stores and its correlation to haemoglobin levels in pregnant women attending an antenatal clinic. Ceylon Med J 1995;40:67–69.
24. Bothwell TH. Iron requirements in pregnancy and strategies to meet them. Am J Clin Nutr 2000;72: 257S–264S.
25. Kandoi A, Bhatia BD, Pandey LK, Pandey S, Sen PC, Satya K. Cellular immunity status in anaemia in pregnancy. Indian J Med Res 1991;94:11–15.
26. Khan KS, Wojdyla D, Say L, Gülmezoglu AM, Van Look PF. WHO analysis of causes of maternal deaths: a systematic review. Lancet 2006;367:1066–1074.
27. Lozoff B, Beard J, Connor J, Barbara F, Georgieff M, Schallert T. Long-lasting neural and behavioral effects of iron deficiency in infancy. Nutr Rev 2006;64:S34–S43.
28. Lukowski AF, Koss M, Burden MJ, Jonides J, Nelson CA, Kaciroti N, Jimenez E, Lozoff B. Iron deficiency in infancy and neurocognitive functioning at 19 years: evidence of long-term deficits in executive function and recognition memory. Nutr Neurosci 2010;13:54-70.
29. World Health Organization. In Vir SC and Cavalli-Sforza T (eds). Weekly iron folic acid supplementation (WIFS) program for women of reproductive age (WRA): an analysis of best program practices. Geneva: World Health Organization, 2011.
30. Berger J, Thanh HT, Cavalli-Sforza T, Smitasiri S, Khan NC, Milani S, Hoa PT, Quang ND, Viteri F. Community mobilization and social marketing to promote Weekly Iron folic acid supplement in women of reproductive age in Vietnam: impact on anaemia and iron status. Nutr Rev 2005;63:S95-S108.

31.Angeles-Agdeppa I, Paulino LS, Ramos AC, Etorma UM, Cavalli-Sforza T, Milani S. Government industry partnership in weekly iron folic acid supplement for women of reproductive age in Philippines: impact on iron status. Nutr Rev 2005; 63:S116-S125.

32.Crape BL, Kenefick E, Cavalli-Sforza T, Busch-Hallen J, Milani S, Kanal K. Positive impact of weekly iron folic acid supplement delivered with social marketing to Cambodian women. Compliance, participation and haemoglobin level increase with higher socio economic status: impact on iron status. Nutr Rev 2005;63:S134-S138.

33.World Health Organization. Iron deficiency anaemia assessment prevention and control: a guide for programme managers. Geneva: World Health Organization, 2001. 132 (WHO/ NHD/01.3).

34. Ballot DE, MacPhail AP, Bothwell TH, Gillooly M, Mayet FG. Fortification of curry powder with NaFe(III)EDTA in an iron deficient population: report of a controlled iron-fortification trial. Am J Clin Nutr 1989;49:162–169.

35. Viteri FE, Alvarez E, Batres R, Toru´n B, Pineda O, Mejı´a LA, Sylvi J. Fortification of sugar with iron sodium ethylenediaminotetraacetate (FeNaEDTA) improves iron status in semirural Guatemalan populations. Am J Clin Nutr 1995;61:1153–1163.

36.Andang'o PEA, Osendarp SJM, Ayah R, West CE, Mwaniki DL, De Wolf CA, Kraaijenhagen R, Kok FJ, Verhoef H. Efficacy of iron-fortified whole maize flour on iron status of schoolchildren in Kenya: a randomised controlled trial. Lancet 2007;69:1799–1806.

37.Bothwell TH, MacPhail AP. The potential role of NaFeEDTA as an iron fortificant. Int J Vitam Nutr Res 2004;74:421–434.

38.Huang J, Sun J, Li WX, Wang LJ, Wang AX, Huo JS, Chen JS, Chen CM. Efficacy of different iron fortificants in wheat flour in controlling iron deficiency.Biomed Environ Sci 2009;22:118-121.

39.Koessler K, Maurer S, Loughlin R. The relation of anemia, primary and secondary, to vitamin A deficiency. JAMA 1926;87:476–482.

40.Blackfan KD, Wolbach SB. Vitamin A deficiency in infants, a clinical and pathological study. J Pediatr 1933;3:679–706.

41.Van Stuijvenberg ME, Kruger M, Badenhorst CJ, Mansvelt EP, Laubscher JA. Response to an iron fortification programme in relation to vitamin A status in 6–12-year-old school children. Int J Food Sci Nutr 1997;48:41–49.

42.Bloem MW, Wedel M, Egger RJ, Speek AJ, Schrijver J, Saowakontha S, Schreurs WH. Iron metabolism and vitamin A deficiency in children in Northeast Thailand. Am J Clin Nutr 1989;50:332–338.

43.Bloem MW, Wedel M, van Agtmaal EJ, Speek AJ, Saowakontha S, Schreurs WH. Vitamin A intervention: short term effects of a single, oral, massive dose on iron metabolism. Am J Clin Nutr 1990; 51:76-79.

44.Suharno D, West CE, Muhilal, Karyadi D, Hautvast JG. Supplementation with vitamin A and iron for nutritional anaemia in pregnant women in West Java, Indonesia. Lancet 1993;342:1325-1328.

45.Hodges RE, Rucker RB, Gardner RH. Vitamin A deficiency and abnormal metabolism of iron. Ann N Y Acad Sci 1980;355:58–61.

46.Mejía LA, Chew F. Hematological effect of supplementing anemic children with vitamin A alone and in combination with iron. Am J Clin Nutr 1988;48:595-600.

47.Ahmed F, Khan MR, Banu CP, Qazi MR, Akhteruzaman M. The coexistence of other multiple micronutrient deficiencies in anaemic adolescent schoolgirls in rural Bangladesh. Eur J Clin Nutr 2008;62:365–372.

48.Dijkhuizen MA, Wieringa FT, West CE, Muherdiyantiningsih, Muhilal. Concurrent micronutrient deficiencies in lactating mothers and their infants in Indonesia. Am J Clin Nutr 2001;73:786–791.

49.Ma AG, Chen XC, Zheng MC, Wang Y, Xu RX, Li JS. Iron status and dietary intake of Chinese pregnant women with anaemia in the third trimester. Asia Pac J Clin Nutr 2002;11:171–175.

50.Powers HJ, Wright AJ, Fairweather-Tait SJ. The effectof riboflavin deficiency in rats on the absorption anddistribution of iron. Br J Nutr 1988;59:381–387.

51.Powers HJ, Weaver LT, Austin S, Wright AJ, Fairweather-Tait SJ. Riboflavin deficiency in the rat: effects on ironutilization and loss. Br J Nutr 1991;65:487–496.

52.Powers HJ, Bates CJ, Lamb WH, Singh J, Gelman W,Webb E. Effects of a multivitamin and iron supplementon running performance in Gambian children. HumNutr Clin Nutr 1985;39:427–437.

53.Buzina R, Jusić M, Milanović N, Sapunar J, BrubacherG. The effects of riboflavin administration on iron metabolismparameters in a school-going population. Int JVitam Nutr Res 1979;49:136–143.

54.Thankachan P, Muthayya S, Walczyk T, Kurpad AV, HurrellRF. An analysis of the etiology of anemia and irondeficiency in young women of low socioeconomic statusin Bangalore, India. Food Nutr Bull 2007;28:328–336.

55.Sirivech S, Frieden E, Osaki S. The release of iron from horse spleen ferritin by reduced flavins. Biochem J 1974;143:311–315.

56.Crichton RR, Roman F, Wauters M. Reductive mobilisation of ferritin iron by reduced nicotinamide adenine dinucleotide via flavin mononucleotide. Biochem Soc Trans 1975;3:946–948.

57.Gveric-Ahmetasevic S, Sunjic SB, Skala H, Andrisic L, Stroser M, Zarkovic K, Skrablin S, Tatzber F, Cipak A, Jaganjac M, Waeg G, Gveric T, Zarkovic N. Oxidative stress in small-for-gestational age (SGA) term newborns and their mothers. Free Radic Res 2009;43:376-384.

58. Myatt L, Cui X. Oxidative stress in the placenta. Histochem Cell Biol 2004;122:369–382.
59. Chen X, Scholl TO. Oxidative stress: changes in pregnancy and with gestational diabetes mellitus. Curr Diab Rep 2005;5:282–288.
60. Serdar Z, Gur E, Colakoethullary M, Develioethlu O, Sarandol E. Lipid and protein oxidation and antioxidant function in women with mild and severe preeclampsia. Arch Gynecol Obstet 2003;268:19–25.
61. Prater MR, Laudermilch CL, Liang C, Holladay SD. Placental oxidative stress alters expression of murine osteogenic genes and impairs fetal skeletal formation. Placenta 2008;29:802–808.
62. Agarwal A, Aponte-Mellado A, Premkumar BJ, Shaman A, Gupta S. The effects of oxidative stress on female reproduction: a review. Reprod Biol Endocrinol 2012 ;10:49.
63. Fereshtehnejad SM, Poorsattar Bejeh Mir K, Poorsattar Bejeh Mir A, Mohagheghi P. Evaluation of the possible antioxidative role of bilirubin protecting from free radical related illnesses in neonates. Acta Med Iran 2012;50:153-163.
64. Dhobale M, Joshi S. Altered maternal micronutrients (folic acid, vitamin B(12)) and omega 3 fatty acids through oxidative stress may reduce neurotrophic factors in preterm pregnancy.J Matern Fetal Neonatal Med 2012;25:317-323.
65. Bedaiwy MA, Falcone T, Goldberg JM, Attaran M, Miller K, Agarwal A. Assessment of the predictive value of follicular fluid IL-6 in IVF cycles. Fertility Sterility 2003;80:S96.
66. Oyawoye O, Abdel GA, Garner A, Constantinovici N, Perret C, Hardiman P. Antioxidants and reactive oxygen species in follicular fluid of women undergoing IVF: relationship to outcome. Hum Reprod 2003;18:2270–2274.
67. Toblli JE, Cao G, Oliveri L, Angerosa M. Effects of iron deficiency anemia and its treatment with iron polymaltose complex in pregnant rats, their fetuses and placentas: oxidative stress markers and pregnancy outcome. Placenta 2012;33:81-87.
68. Aslan M, Horoz M, Kocyigit A, Ozgonül S, Celik H, Celik M, Erel O. Lymphocyte DNA damage and oxidative stress in patients with irondeficiencyanemia. Mutat Res 2006;601:144-149.
69. Friel JK, Andrews WL, Aziz K, Kwa PG, Lepage G, L'Abbe MR. A randomized trial of two levels of iron supplementation and developmental outcome in low birth weight infants. J Pediatr 2001;139:254–260.
70. Miller SM, McPherson RJ, Juul SE. Iron sulfate supplementation decreases zinc protoporphyrin to heme ratio in premature infants. J Pediatr 2006;148:44–48.
71. Braekke K, Bechensteen AG, Halvorsen BL, Blomhoff R, Haaland K, Staff AC. Oxidative stress markers and antioxidant status after oral iron supplementation to very low birth weight infants. J Pediatr 2007;151:23–28.

72. Tiwari AK, Mahdi AA, Zahra F, Chandyan S, Srivastava VK, Negi MP. Evaluation of oxidative stress and antioxidant status in pregnant anemic women.Indian J Clin Biochem 2010;25:411-418.

73. Khalid S, Ahmad SI. Correction of iron deficiency anemia in pregnancy and its effects on superoxide dismutase.Pak J Pharm Sci 2012;25:423-427.

74.Berchieri-Ronchi CB, Kim SW, Zhao Y, Correa CR, Yeum KJ, Ferreira AL. Oxidative stress status of highly prolific sows during gestation and lactation. Animal 2011;5:1774-1779.

75.Lohia N, Udipi S, Ghugre P, Deshpande K.Serum retinol, alpha-tocopherol, and lipid peroxide concentrations in low-income indian pregnant mothers. Int J Vitam Nutr Res 2009;79:142-151.

76.Williams IO, Essien EU, Eka OU. Socioeconomic factors and vitamin a status of pregnant women in Calabar urban, southeastern Nigeria. Matern Child Health J2011;15:943-948.

77.Wang J, Zhao L, Piao J, Zhang J, Yang X, Yin S. Nutrition and health status of pregnant women in 8 provinces in China]. Wei Sheng Yan Jiu 2011;40:201-203.

78.Allen LH, Peerson JM. Maternal Micronutrient Supplementation Study Group. Impact of multiple micronutrients versus iron-folic acid supplements on maternal anemia and micronutrient status in pregnancy. Food Nutr Bull 2009;30:S527-S532.

79.Stevens RD. Anaemia--the scourge of the ThirdWorld. Health Millions 2000 ;26:21-23.

80.Wang J, Ren AG, Ye RW, Zheng JC, Li S, Liu JM, Yang RL, Zhang FR, Zhang T, Zhang JB, Li Z. Study on the third trimester hemoglobin concentrations and the risk of low birth weight and preterm delivery.Zhonghua Liu Xing Bing Xue Za Zhi. 2007;28:15-18.

Chapter 2

Anemia prevalence among pregnant women and birth weight in five areas in China

Ma AG, Schouten E, Wang Y, Xu RX, Zheng MC

, Li Y, Sun YY, Wang QZ.

Med Princ Pract. 2009;18(5):368-72.

Abstract

Objectives: To investigate the current prevalence of anemia among pregnant women in different sites of China and the association with birth weight and educational level.

***Methods*:** A total of 6413 women aged 24~37 in the third trimester of pregnancy were randomly selected from all gravidas who gave a birth in the hospitals during 1999 to 2003. Blood hemoglobin concentration (Hb) was measured by the cyanomethaemoglobin method. Hb<110 g/L result was considered as anemia.

Results: The overall prevalence of anemia was 58.6%, ranging 48.1%~70.5% in the five areas. There was a significant difference in the prevalence of anemia between women who have mental jobs and those who have physical jobs (52.3% versus 61.1%, $p<0.01$). The prevalence of anemia according to the level of education was also significantly different with 52.9%, 62.4% and 66.5%, respectively for college, secondary school and primary education.The association of Hb with birth weight showed a range of higher levels of birth weights during 90 to 140g/L of Hb. However, the lower birth weight was located below 80g/L and above140 g/L of hemoglobin concentrations.

Conclusions: The prevalence of anemia in Chinese pregnant women before delivery was high in rural areas and towns. Resident areas, education levels, kinds of job all have an influence on the prevalence of anemia. The low maternal hemoglobin concentrations influenced the birth weight.

Introduction

Anemia is the scourge of the third world (1). Anemia remains a major problem in nearly all developing and many industrialized countries (2). Pregnant women are at risk especially during the last trimester of pregnancy. The prevalence of anemia (Hb<110g/L) in Chinese pregnant women in the third trimester was 48% during 1995-2000 (3), and the prevalence of iron deficiency (ID) and iron deficiency anemia (IDA) was 42% and 19%, respectively (4). Notwithstanding the fact that the diagnosis of anemia is fairly simple and the treatment is cheap, the prevalence of anemia remains high in developing towns, as well as in the wide countryside and poverty-stricken areas of China.

In pregnant women, a favourable iron status is a prerequisite for a good course of pregnancy. There is evidence that iron deficiency, even in the absence of iron deficiency anaemia (IDA), may have a negative impact in non-pregnant women, e.g. in relation to decreased cognitive ability and physical performance (5). It may impair development of fetus and infants (6). Low maternal hemoglobin levels are associated with increased risk of preterm delivery and low birth weight (7,8). Prenatal prophylactic iron supplementation deserves further examination as a measure to improve birth weight and potentially reduce health care costs (9) A randomized controlled trial suggested that prophylactic iron supplementation begun in early pregnancy can reduce third-trimester anemia and improve birth outcomes (10). However, a recent study showed that anemia during early pregnancy was not associated with increased risk of adverse perinatal outcomes, and anemia in later pregnancy was inversely associated with preterm birth and low birth weight (11). Therefore, the relationship between hemoglobin concentrations and birth weight is still not clear. The purpose of this study is to investigate the current prevalence of anemia in geographically different locations in pregnant women in China, and describe the associations of hemoglobin concentrations with birth weights.

Subjects and methods

Subjects

A cross-sectional study was designed. Because China has a large population and larger area, the 5 sites were randomly selected from a number of pregnant centers nationwide, which was rural areas of Qingdao in the east, Fuzhou in the southeast, urban areas of Lanzhou in the northwest and Guilin in the southwest, and a suburban district of Liaocheng in the middle of China during 1999 to 2003. Also 6413 subjects were randomly enrolled by the order of registration numbers, 1, 5, 10, 15, 20, etc. for regularly pregnant examination from the local population of pregnant women. With respect to geographical and other conditions like lifestyle, they can be regarded as representatives of the large variation present in China, except for remote areas. Subjects included in the study were healthy pregnant women whose delivery ended between 35 and 42 weeks of gestation without delivery complications, such as hypertension. Furthermore, subjects did not experience abnormal bleeding, take iron and multivitamin supplements, smoke tobacco products, or drink alcoholic beverages during pregnancy. Demographic data were collected by questionnaires that were used routinely nation-wide at admittance to hospital for delivery. 6413 samples of venous blood were collected within one or two weeks before delivery for hemoglobin analysis.

The protocol was approved by the ethical committees of local hospitals in five sites. Informed consent was obtained from each of pregnant women recruited.

Method and Criteria for diagnosis of anemia

Blood hemoglobin concentration was measured by the cyanomethemoglobin method within 2~4h (12). Based on the report of the International Anemia Consultative Group (13), Hb<110g/L was considered as anemia.

Statistical analysis

Differences were tested using Student's t-test and the Chi-Square test. The SPSS (11.0) of statistical software was used. The percentile distributions of hemoglobin concentrations were compared among five sites. The association of hemoglobin concentrations and birth weight was described by simple line (summarizes for groups of cases). Two-sided p <0.05 were considered statistically significant.

Results

Among the 6413 pregnant women screened, the overall prevalence of anemia was 58.6%. The prevalence within the five centers ranged from 48.1%~70.5% in **Table 2.1**. Compared with sites of Liaocheng, Lanzhou and Guilin, there were higher rates of anemia in Fuzhou and Qingdao (p<0.001). The prevalence of anemia is shown according to the type of jobs among 6140 and to educational levels among 4624 of the women. There was a significant difference in the prevalence of anemia between women has mental (52.3%) and physical jobs (61.1%, $p<0.01$). Moreover, the prevalence of anemia was significantly different according to educational level: 52.9%, 62.4% and 66.5%, respectively for college, secondary and primary education (**Table 2.2**).

Table 2.1 Prevalence of anemia and Hb distribution in five sites

Sites	Total number	Anemia (%)	Hb (g/L) distribution		
			70 ~ ≤90	>90~ <110	≥110
Qingdao	1085	69.9	19.6	50.3	30.1
Fuzhou	1023	70.5	20.3	50.2	29.4
Liaocheng	2363	55.6	11.1	44.5	44.4
Lanzhou	1003	51.3	7.7	43.6	48.7
Guilin	939	48.1	5.2	42.9	59.1
Total	6413	58.6	12.7	46.4	40.9

Table 2.2 Prevalence of anemia according to work type and educationin pregnant women

Items	total number	anemic number	prevalence (%)	*p*
Work types (n=6140)				
Mental	1339	700	52.3	<0.001
Physical	4801	2942	61.1	
Educational level (n=4624)				
College	665	352	52.9	
Secondary school	2307	1439	62.4	<0.005
Primary school	1652	1098	66.5	

The mean ages in the anemic and non-anemic group were similar (27.0 y and 27.1 y). **Fig 2.1** showed that the association of Hb with birth weight showed a range of higher levels of birth weights during 90 g/L to 140 g/L of Hb. However, the lower birth weight was located below 80 g/L and above 140 g/L of hemoglobin concentrations. The distribution of haemoglobin levels in the five centers. The distributions of hemoglobin concentrations of Qingdao, Fuzhou and Liaocheng were less favorable than those of Lanzhou and Guilin in **Fig 2.2**.

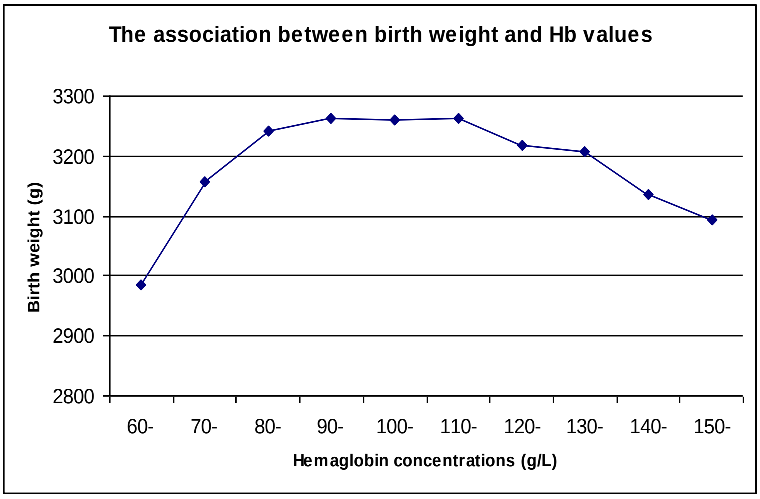

Figure 2.1 The association of average points of hemoglobin concentrations (Hb) with birth weight in 6413 pregnant women was shown on the curve. There was a range of higher levels of birth weights during 90 g/L to 140 g/L of hemoglobin concentrations. However, the lower birth weight on the curve was located below 80 g/L and above 140 g/L of hemoglobin concentrations.

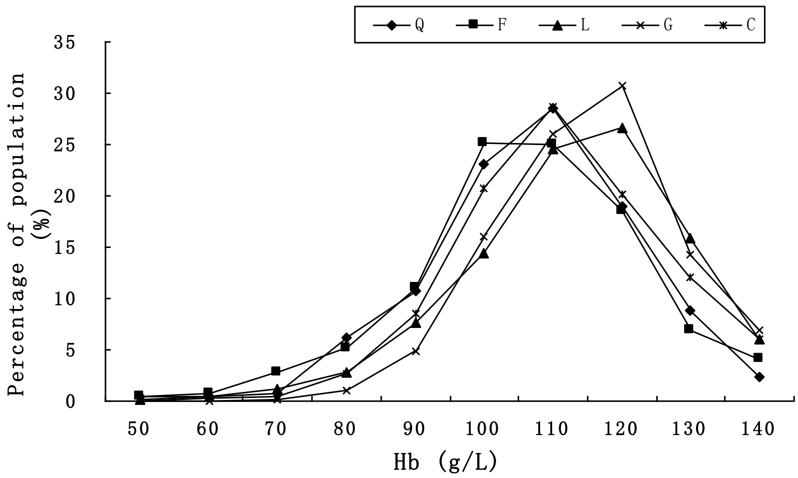

Figure 2.2 The figure shows the distribution of hemoglobin on five curves. The peaks of percentage of hemoglobin in Lanzhou (L) and Guilin (G) are at about 115 g/L, while other three peaks of hemoglobin in Qingdao (Q), Fuzhou (F) and Liaochen (C) are at about 95 g/L ~ 105 g/L. The difference among the peaks is about 10g/L ~ 20g/L.

Discussion

In this study, we observed a very high prevalence of anemia in the third trimester of pregnancy. The results indicated that anemia in pregnant women was still a severe nutrition related problem nowadays. We hypothesized that reasons for the difference in prevalence among the five sites were geographic factors, unbalanced diets and poor nutritional education (14). People living in the countryside have lower incomes and less varied diets. Our study seems to indicate that there is still a high rate of anemia in rural areas. In more remote parts of China, it may be even worse.

Anemia during pregnancy was defined according to WHO guidelines (Hb < 110 g/L), while nutritional anemia should include iron-deficiency anemia (IDA), cobalamin deficiency anemia, folate deficiency anemia, copper deficiency anemia, etc. The prevalence of nutritional anemia is considered to be related to deficiencies of multi-nutrients (15), and more than 85% of the nutritional anemia is IDA alone, or of iron combined with folate or other nutrient deficiencies (16). The purpose of our study was expected to investigate the prevalence of overall anemia, which might show the status of multinutrients in addition to iron deficiency. A study showed that the subjects with iron-deficiency anemia had much higher rates of vitamin C, folate and vitamin B_{12} deficiencies than those in the non anemic subjects, and especially the deficient rates of ascorbic acid and folate in the anemia (Hb <110g/L) group reached 64.0% and 22.7% respectively. And the decreasing hemoglobin concentrations were accompanied by the decreases of serum levels of vitamin A, ascorbic acid, folate and vitamin B_{12}. Somultiple vitamin deficiencies, especially ascorbic acid, retinol and folic acid, may be associated with anemia or iron deficiency in pregnant women in the last trimester. So the study suggested that anemic pregnant women in China should be supplemented with iron and multiple vitamins simultaneously (17).

The level of education may influence the health and nutritional status of individuals. Abel et al (18) reported that combination of education and iron supplementation could improve the hematological status among each of the three trimesters of pregnant women in a rural community. They reported a significant decrease in the prevalence of anemia among women in all trimesters in the intervention areas. Some other researchers reported that there were no statistically significant differences in IDA perceptions and iron-related dietary practices based on education, and relatively few based on where participants live, their available financial resources, or their position (pregnant women or young women) (19). Bondevik (20) found that the risk of anemia increased with duration of pregnancy. Work within the service professions, higher education and higher body mass index were associated with a lower risk of anemia. A similar study, about the effect of nutritional education on anemia and malnutrition of infants and children in rural China, was carried out in the past five years. After one year, the education group mothers showed significantly higher nutrition knowledge and reported better infant feeding practices than the control group. Type of job was also related to the prevalence of anemia. A likely explanation might be that women with mental job have better education, incomes and living conditions.

Strong evidence exists for an association between maternal hemoglobin concentration and birth weight as well as between maternal hemoglobin concentration and preterm birth (21). Although high hemoglobin (>120 g/L) during the second and third trimester significantly increased the risk of low birth weight (RR=3.11) (22), mild and moderate anemia did not increase the risk of preterm delivery and low birth weight statistically (23). The lowest incidence of preterm delivery and low birth weight was found among pregnant women with Hb levels at 90g/L-~99 g/L. The risk for preterm delivery and low birth weight increased with either increasing or decreasing hemoglobin concentrations. Women with severe anemia (Hb< 70 g/L) had 80% higher risk of preterm delivery and a 4-fold higher risk of low birth weight compared with women with an Hb value of 90g/L ~99 g/L. In addition, women with a high Hb concentration (Hb>130 g/L) had 20% higher of preterm delivery and 50% higher risk of low birth weight (23). Moreover, in this study, the distribution of hemoglobin concentrations showed that both lower (Hb<80g/L) and higher hemoglobin concentrations (Hb>140 g/L) might result in deviating from the normal birth weight (2500g~4000g). Birth weight correlates negatively with maternal hemoglobin concentration. Anemia (by causing hypoxia) and iron deficiency (by increasing serum norepinephrine concentrations) can induce maternal and fetal stress, which stimulates the synthesis of corticotropin-releasing hormone (CRH). Elevated CRH concentrations are a major risk factor for preterm labor, pregnancy-induced hypertension and eclampsia, and premature rupture of the membranes. CRH also increases fetal cortisol production, and cortisol may inhibit longitudinal growth of the fetus. An alternative mechanism could be that iron deficiency increases oxidative damage to erythrocytes and the fetoplacental unit. Iron deficiency may also increase the risk of maternal infections, which can stimulate the production of CRH and are a major risk factor for low birth weight and preterm delivery (24). Association between maternal hemoglobin concentration and birth weight needs more attention because reducing the incidence of low birth weight not only lowers infant mortality rates but also has multiple benefits over the life cycle.

Conclusion

The high prevalence of anemia in pregnant women is still a national wide nutritional problem in China. In rural areas the situation seems to be less favorable than in urban areas, partly because of lower socioeconomic status, low education and training. Although this study showed higher hemoglobin concentration might cause birth weight decrease or increase, it deserves to be confirmed in the further study. Therefore, it is suggested that the normal hemoglobin concentration is important to pregnant women, fetus growth and birth weight as well.

Acknowledgements

We thank the Nestle Research Foundation for the grant supporting this project. We sincerely acknowledge professors Joseph Hautvast, Chen Xuecun and Li Juesheng for their kind help. We are grateful to the entire field staff for their teamwork and persistent efforts. The authors also thank Zhang Xiuzhen, Liang Hui, Du Wei, Xu Hongwei and Zhang Shehua for measurements and technical assistance.

References

1. Stevens RD. Anemia —the scourge of the Third World. Health Millions 2000; 26: 21-23.
2. Toteja GS, Singh P, Dhillon BS, Saxena BN, Ahmed FU, Singh RP, Prakash B, Vijayaraghavan K, Singh Y, Rauf A, Sarma UC, Gandhi S, Behl L, Mukherjee K, Swami SS, Meru V, Chandra P, Chandrawati, Mohan U. Prevalence of anemia among pregnant women and adolescent girls in 16 districts of India. Food Nutr Bull 2006; 27:311-315
3. Wang J, Ren AG, Ye RW, Zheng JC, Li S, Liu JM, Yang RL, Zhang FR, Zhang T, Zhang JB, Li Z. Study on the third trimester hemoglobin concentrations and the risk of low birth weight and preterm delivery. Zhonghua Liu Xing Bing Xue Za Zhi 2007; 28:15-18
4. Liao QK. Prevalence of iron deficiency in pregnant and premenopausal women in China: a nationwide epidemiological survey. Zhonghua Xue Ye Xue Za Zhi 2004; 25:653-657
5. Nils Milman. Prepartum anaemia: prevention and treatment. Ann Hematol 2008; 87: 949-59
6. Baron MA, Solano L, Pena E, Del Real S. Iron stores status at early pregnancy. Invest Clin 2005; 46: 121-130
7. Joanne Katz, Parul Christian, Francesca Dominici and Scott L. Zeger. Treatment Effects of Maternal Micronutrient Supplementation Vary by Percentiles of the Birth Weight Distribution in Rural Nepal. J Nutr 2006; 136:1389-1394
8. Lee HS, Kim MS, Kim MH, Kim YJ, Kim WY. Iron status and its association with pregnancy outcome in Korean pregnant women. Eur J Clin Nutr 2006; 60: 1130-1135
9. Cogswell ME, Parvanta I, Ickes L, Yip R, Brittenham GM. Iron supplementation during pregnancy, anemia, and birth weight: a randomized controlled trial. Am J Clin Nutr 2003; 78: 773-781.
10. Siega-riz Am, Hartzema Ag, Turnbull C, Thorp J, McDonald T, Cogswell ME. The effects of prophylactic iron given in prenatal supplements on iron status and birth outcomes: a randomized controlled trail. Am J Obstet Gynecol 2006; 194: 512-519
11. Xiong X, Buekens P, Fraser WD, Guo Z. Anemia during pregnancy in a Chinese population. Int J Gynaecol Obstet 2003; 83: 159-164.
12. Lynch MJ, Raphael SS, Mellor LD. Medical laboratory technology and clinical pathology, 2nd edu. Philadelphia: W. Saunders 1969
13. International Anemia Consultative Group (INACG). Iron deficiency in women. Washington, D.C.: The Nutrition Foundation 1981; 5-36.
14. Liu LJ, Lai YH, Hu ZH, Ji W, Wu YJ and Liu CQ. Investigation of anemia of 158 pregnant women in Jilin city. JiLin YiXueYuan XueBao 1998; 18: 45-47.
15. Carmel R. Nutritional anemias and the elderly. Semin Hematol. 2008; 45: 225-234.
16. Viteri FE. A new concept in the control of iron deficiency: community-based preventive supplementation of at-risk groups by the weekly intake of iron supplements. Biomed

Environ Sci 1998;11:46-60

17. Ma AG, Chen XC, Wang Y, Xu RX, Zheng MC, Li JS. The multiple vitamin status of Chinese pregnant women with anemia and nonanemia in the last trimester. J Nutr Sci itaminol (Tokyo) 2004; 50:87-92.
18. Abel R, Rajaratnam J, Kalaimani A, Kirubakaran S. Can iron status be improved in each of the three trimestre? A community based study. Eur J Clin Nutr 2000; 54: 490-493
19. Jarrah SS, Halabi JO, Bond AE, Abegglen J. Iron deficiency anemia (IDA) perceptions and dietary iron intake among young women and pregnant women in Jordan. J Transcult Nurs. 2007; 18: 19-27
20. Bondevik GT, Ulstein M, Lie RT, Rana G, Kvåle G. The prevalence of anemia in pregnant Nepali women a study in Kathmandu. Acta. Obstet. Gynecol. Scand. 2000; 79:341-349
21. Lone FW, Qureshi RN, Emanuel F. Maternal anaemia and its impact on perinatal outcome. Trop Med Int Health 2004; 9: 486-490.
22. Wang J, Ren AG, Ye RW, Zheng JC, Li S, Liu JM, Yang RL, Zhang FR, Zhang T, Zhang JB, Li Z. Study on the third trimester hemoglobin concentrations and the risk of low birth weight and preterm delivery. Zhonghua Liu Xing Bing Xue Za Zhi 2007; 28:15-18.
23. Chang SC, O'Brien KO, Nathanson MS, Mancini J, Witter FR. Hemoglobin concentrations influence birth outcomes in pregnant African-American adolescents. J Nutr 2003; 133:2348-2355.
24. Allen LH. Biological mechanisms that might underlie iron's effects on fetal growth and preterm birth. J Nutr 2001;131: 581S-589S.

Chapter 3

Micronutrient status in anemic and non-anemic Chinese women in the third trimester of pregnancy

Ma AG, Schouten EG, Wang Y, Xu RX,
Zheng MC, Li Y, Wang Q, Sun Y.

Asia Pac J Clin Nutr. 2009;18(1):41-47.

ABSTRACT

Background: Anemia is a major nutrition related problem in China. In addition to iron deficiency, this may be due to deficits of other micronutrients.

Objective: To describe the micronutrient status of anemic and non-anemic pregnant women in China**.**

Subjects and Methods: 734 clinically normal pregnant women in the third trimester aged 20~35 were randomly recruited in four provinces of China.Serum concentrations of vitamins A, B_{12} and C, iron and zinc status parameters, and vitamin B_2 in urine were determined. Subjects were categorized according to the presence or absence of anemia and compared according to micronutrient status.

Results: Serum concentrations of iron status and micronutrients were significantly lower in anemic women than nonanemic women: ferritin 13.8µg/L versus 19.6µg/L; vitamin C 308.9µg/dL versus 388.1µg/dL, retinol 50.0µg/dL versus 59.3µg/dL and vitamin B_2 in urine 131.2µg/g creatinine versus 164.9µg/g creatinine. Iron and zinc concentrations were also lower in anemic women. Serum subnormal iron (<700µg/L) and iron depletion (ferritin <12 µg/L) were significantly more frequent in anemic than in nonanemic subjects, as were subnormal vitamin A and ascorbic acid. Subnormal vitamin B_2 and B_{12} were frequent in both anemic and nonanemic groups.

Conclusion: Subnormal concentrations of iron and micronutrients in combination may contribute to this situation. Further studies on food-based or supplement-based approaches trying to increase intake of iron and certain vitamins are warranted to decrease anemia of Chinese pregnant women in the third trimester.

INTRODUCTION

Anemia in pregnancy is a common and worldwide problem that deserves more attention. In many developing countries, prevalence is reported even as higher as 75%. Often, anemia is severe in these situations, contributing significantly to maternal mortality and morbidity[1] and to low birth weight as well [2]. Anemia is also a major nutrition related problem among pregnant women in China. Prevalence of anemia differs in different areas of China. Studies show that the prevalence of anemia during pregnancy is 10% to 20%. It is even 42% among pregnant women in the third trimester in Xi'an city[3], and 55% in Jilin's city in 1997. It was hypothesized that the main probable cause was unbalanced diet lack of protein, iron and certain vitamins[4].

Anemia during pregnancy has been attributed not only to increased iron requirements during the second and the third trimester of gestation[5], but also to micronutrient deficiency. Deficiencies of iron and vitamin A were among the major contributory factors [6]. Several studies in humans and animals have shown that iron deficiency is accompanied with other micronutrient deficiencies like vitamin A and ascorbic acid[7,8]. Studies also have shown that supplementation with these vitamins may improve iron status as measured by hematological indices [9]. However, data on iron status and multivitamin levels in pregnant women with anemia in China are insufficient. In this study, we assessed and compared the micronutrient status of pregnant women with anemia and those without anemia.

SUBJECTS AND METHODS

Subjects

The cross-section study was designed and conducted between November 1999 and April 2001. 734 clinically normal pregnant women aged 20-35 in the third trimester of pregnancy were randomly recruited for hematologic and micronutrient measurements, who were from several local clinics and hospitals in Gansu, the northwest of China; Guangxi, the southwest,Shandong, the northeast and Fujian, the southeast of China. The subjects were healthy pregnant women who did not experience abnormal bleeding and did not smoke or drink any alcoholic beverages, and no dietary supplements during 2 months.

The study was approved by the Research and Ethics Committee of the Institute of

Human Nutrition, Medical College of Qingdao University. The informed consent was obtained from all subjects prior to the trial.

Sample collection and biochemical analysis

Approximately 5 ml of venous blood and 10 ml of urine samples were taken on the day of the prenatal examination, and stored in ice for transport to the local laboratories of the four sites. Hematocrit and hemoglobin concentrations were measured in heparinized blood. Serum was separated from the remainder of the blood by centrifugation at 2000 × g for 15 min at room temperature upon arrival. Serum samples were stored separately at -80 ^{0}C in the dark and transported by air or train to the laboratory of the Institute of Human Nutrition, Medical College of Qingdao University for analyses of ferritin, vitamin A, ascorbic acid, riboflavin, vitamin B_{12} and folate.

Hemoglobin concentration was measured by the cyanomethemoglobin method and hematocrit by the micromethod. A standard hemoglobin cyanide solution was used for the quality control of hemoglobin measurements. Measurements of serum ferritin were performed by radioimmunoassay [10], described by the manufacturer (The North Biol.Tec, Institute Beijing, China). Transferrin (TRF) was determined by a commercially available kit (Yadu Biotech Co. Shanghai, China). Serum retinol concentrations were measured by reversed-phase high-performance liquid chromatography (HPLC) (Beckman 5000 with detector of 168, USA)[11] and the within-assay and between assay CVs were 3% and 8%, respectively. The nutritional status of riboflavin was determined by the ratio of urine riboflavin/creatinine, and the erythrocyte glutathione reductase activity coefficient (EGRAC) was measured for assessing riboflavin status[12]. Urinary riboflavin was measured by fluorometric procedures. Under conditions of adequate intake, the amount excreted per day is more than 80 μg per gram of creatinine. Folic acid and vitamin B_{12} were measured by radioimmunoassay method (Diagnostic Products Corporation DPC, USA) [13]. Serum concentrtions of iron, zinc and copper were measured by 710-ES ICP Optical Emission Spectrometer (Varian Medical System, USA).

Based on the report of the International Anemia Consultative Group [14], the criterion for a diagnosis of anemia was Hb<110 g/L. The following results were considered abnormal: hematocrit <33%, ferritin <12 μg/L, transferrin <2.1 g/L. The following values were considered subnormal: serum iron <700 μg/L, vitamin A <30μg/dL, ascorbic acid <400 μg/dL, riboflavin/creatinine <80 μg/g in urine, folic acid <3.0 ng/mL and vitamin B_{12}<200pg/mL[15].

Statistical analysis

The significance of differences was determined by Independent Samples t-test and by the Chi-Square (χ^2) test. The SPSS (10.0) of statistical software was used. The percentile distributions of serum iron, ferritin, folic acid, retinol, ascorbic acid and vitamin B_2 were compared between anemic and nonanemic women. Two-sided *p* values <0.05 were considered statistically significant.

RESULTS

Results with respect to iron status in **Table 3.1** showed that there were significant differences of serum hemoglobin (-24%), ferritin (-30%), transferrin (-6%) and serum iron (-18%) between anemic and nonanemic pregnant women. Mean concentrations of serum vitamin C (-20%), zinc (-5%), retinol (-16%) and vitamin B_2 in urine (-21%) in the anemia group were significantly lower. There were no significant differences of serum copper levels, vitamin B_{12} and folate.

Table 3.1 Iron status and micronutrient levels in anemic and nonanemic pregnant women

Items	**Hb<110g/L**		**Hb≥110g/L**		**Difference (%)**	***p***
	n	**mean±SD**	**n**	**mean±SD**		
Haemoglobin (g/L)	403	97.0±8.5	331	127.6±11.7	-24.0	0.000
Haematocrit (l/L)	403	30.9±5.1	331	37.1±5.3	-16.7	0.000
Ferritin (μg/L)	403	13.8±9.2	331	19.6±16.0	-29.6	0.000
Transferrin(g/L)	403	3.3±0.5	331	3.5±0.5	-5.7	0.000
Retinol (μg/dL)	403	50.0±15.6	331	59.3±13.9	-15.7	0.000
Vitamin C(μg/dL)	403	308.9±258.8	331	388.1±318.5	-20.4	0.001
Vitamin(B_{12}(pg/mL)	403	439.6±274.2	331	433.3±255.6	1.4	0.749
Folate (ng/mL)	403	5.9±5.8	331	6.0±5.2	-1.7	0.914
vitaminB_2/creatinine(μg/g)	403	131.2±132.7	331	164.9±191.5	-20.5	0.005
Iron (μg/L)	403	909.2±479.7	331	1108.9±748.9	-18.0	0.000
Zinc (μg/L)	403	706.7±197.3	331	744.8±213.7	-5.1	0.012
Copper(μg/L)	403	1758.9±543.1	331	1814.9±499.3	-3.1	0.149

Table 3.2 Prevalence of subnormal micronutrients in anemic and nonanemic women

Items	Subnormal range	Anemia		Non-anemia		Total		*p*
		n	%	n	%	n	%	
Serum iron	<700μg/L	160	39.7	97	29.3	257	35.0	0.003
Ferritin	<12μg/L	212	52.6	116	35.0	328	44.7	0.000
Vitamin C	<400μg/dL	279	69.2	202	61.0	481	65.5	0.020
Retinol	<30μg/dL	91	22.6	24	7.3	115	15.7	0.000
Vitamin B_{12}	<200 pg/mL	56	13.9	37	11.2	93	12.7	0.271
Folate	<3.0 ng/mL	108	26.8	78	23.6	186	25.3	0.316
vitaminB_2/ creatinine	<80 μg/g	159	39.5	121	36.6	280	38.1	0.421

The frequency of subnormal serum iron and micronutrients is shown in **Table 3.2**. Prevalence of subnormal serum iron (<700μg/L) and iron depletion (ferritin <12 μg/L) was 40% and 53% in the anemic group, compared to 29% and 35% in the nonanemic group (p<0.003, p<0.001). Subnormal vitamin A (23% versus 7%) and subnormal ascorbic acid (69% versus 61%) were also significantly more frequent in anemic pregnant women (p<0.001, p<0.02). Nevertheless, with 38% and 25%, frequencies of subnormal riboflavin and folate were not significantly different.

The percentile distribution of iron status and vitamin concentrations according to presence or absence of anemia is presented in **Figure 3.1** and showed more differentiated pictures of serum ferritin, serum iron, serum ascorbic acid, serum retinol, and vitamin B_2/creatinine in urine. Moreover, the curves of Hb<110g/L and Hb≥110g/L in ferritin, serum iron, vitamin C and vitamin B_2/creatinine overlap in the lower end of the distribution, while the upper end is distinct. However, the retinol distributions were entirely distinct for non-anemic and anemic subjects, and there is no difference in serum folate between non-anemic and anemic subjects.

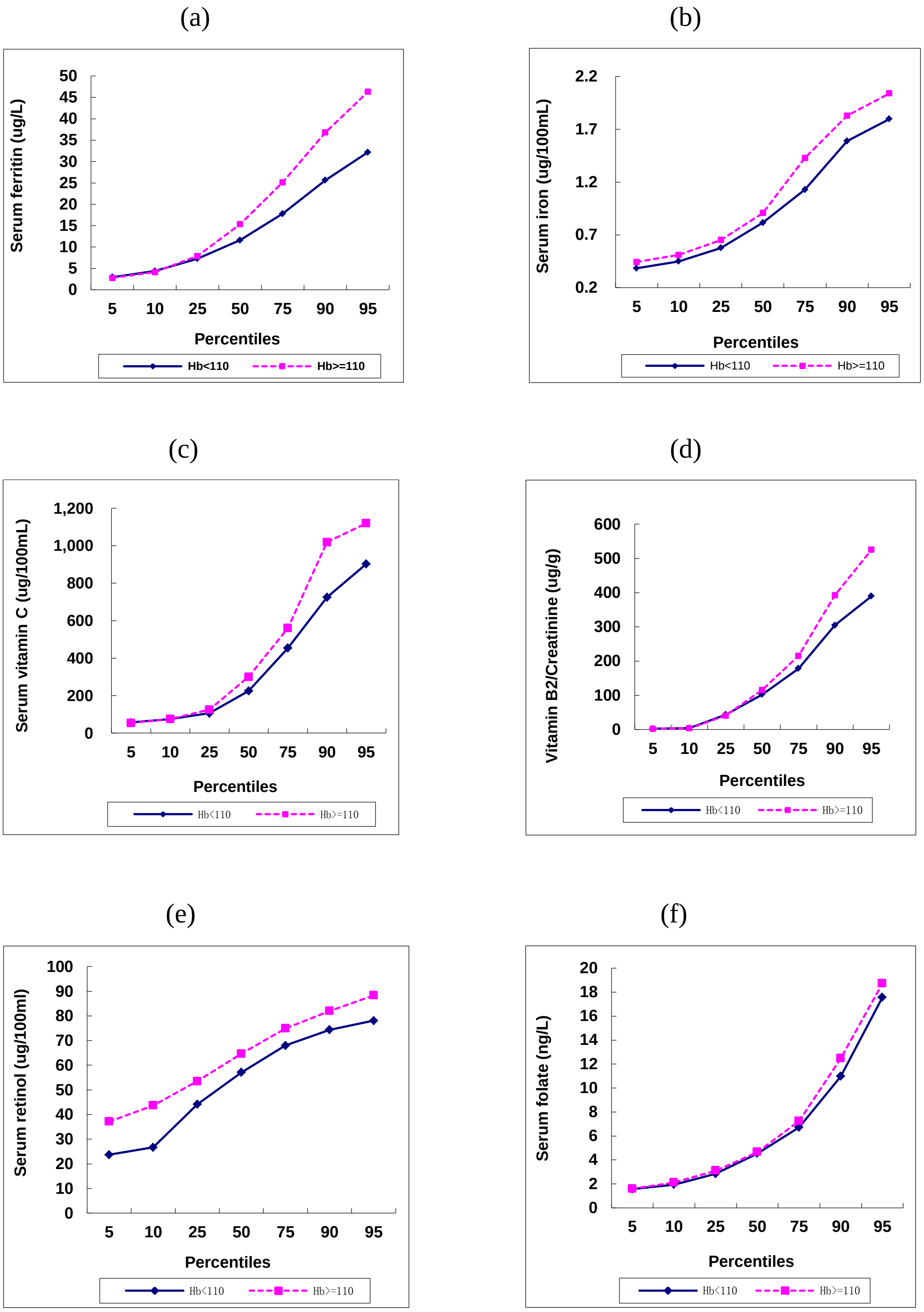

Figure 3.1 The distributions of concentrations of ferritin (a), serum iron (b), serum vitamin C (c), vitamin B_2 in urine (d), serum retinol (e) and serum folate (f) according to presence or absence of anemia are presented both in Hb<110 and Hb≥110.

DISCUSSION

The results investigated in part of Chinese rural areas and low-economic towns showed that anemic pregnant women (Hb<110g/L) had a lower serum iron concentration, ferritin and transferrin levels than those in non-anemic group; moreover, micronutrients were significantly lower in anemic women than non anemic women in serum vitamin C, serum retinol and vitamin B_2 in urine. Serum subnormal vitamin A and ascorbic acid levels were significantly more frequent in anemic than in non anemic subjects.

The study has several advantages over previous studies that examined micronutrient status during pregnancy. Apart from having a large sample size, a wide range of micronutrients was examined simultaneously in Chinese rural areas. In addition, we reported the extent to which multiple deficiencies coexist, data that are scarce in a mixture of rural developing country settings and towns. The results in our study also have the potential to provide valuable reference values for assessing nutritional status. However, the assessment of vitamin and mineral status during pregnancy is complicated because there is a general lack of pregnancy-specific laboratory indices for nutritional evaluation[16], and pregnancy itself may alter.

However, this study was a pilot study on the small scale by a small grant. The subjects selected were from the population living in low or middle socio-economic levels and undeveloped areas, which was expected to be on behalf of an average status of micronutrients and anemia in part nationwide. The information of the characteristics and rates of anemia and iron deficiency of the subjects has been prepared for publication in another paper; while the stratified study may need a larger population by areas, which will be designed on the larger scale and supported by a big grant we expected.

Although the most common cause of anemia is iron deficiency, deficiencies of vitamin B_{12}, folate, vitamin A and even zinc contribute either singly or in combination to maternal anemia[17]. Women in developing countries have a high prevalence of iron deficiency but also tend to be deficient in other micronutrients such as zinc (5), vitamin A, folate and vitamin B_{12}. However, iron deficiency rarely occurs in isolation and is often accompanied by other micronutrient deficiencies[18]. Makola[19] confirmed that micronutrient deficiencies are prevalent in the female population of Tanzania and the prevalence of anemia (63%). In our study, low levels of serum vitamin C, retinol and riboflavin, occurred both in the anemia and non anemia populations, but the three vitamin marginal deficiencies were more severe in pregnant women with anemia than non anemia, which should be contributed to the extra consumption by the fetus and pregnant women, and low intakes. The subjects in the investigation could not get enough green vegetables and animal foods[20]. Unlike Western societies, food is not routinely fortified with iron in rural areas of China. Moreover, green vegetables were scarce in the subjects' diets during the winter season. Therefore, the low intakes and shortage of heme iron and fresh vegetables may contribute to low serum average levels of retinal, ascorbic acid and low iron in anemic women in our study. Retinol status is a putative factor for improved iron status or iron absorption. Deficiency of this vitamin may result also in anemia in humans and animals that can be reversed only by vitamin A supplementation[21]. Vitamin A and β-carotene may form a complex with iron, keeping it soluble in the intestinal lumen and preventing the inhibitory factors on iron absorption [22]. Ascorbic acid is considered a promoter of non-heme-iron absorption. In the Chinese general diet, vegetables are commonly stratified, and fresh fruits are seldom eaten with a meal. Therefore, the amount and availability of vitamin C present in the diet are even more compromised by heat susceptibility explaining low serum concentrations of vitamin C both in anemic and non anemic women. Simultaneous occurrence of both vitamin C and iron in the gut is necessary for effective interaction[23]. In this study, there were 11.2% and 13.9 % of subnormal vitamin B_{12} (<200 pg/mL) in anemic and non anemic pregnant women respectively, which may have confounded the enhancing effect of ascorbic acid on iron status[24]. Vitamin B_2 in urine, estimated by a ratio of vitamin B_2 and creatinine, was also found to be lower in anemic women than in non anemic women. Subnormal folate was not prevalent in this study though it is associated with anemia and other micronutrient deficiencies. It may also be the result of low intake decreased intestinal absorption, or increased demand[25,26].

The serum zinc and iron concentrations were positively related with maternal hemoglobin[27]. The distributions of hemoglobin concentration to zinc and iron indicated that deficiencies of the two elements were common and more severe in anemic pregnant women. The possible reason is not only an expansive blood volume and an increasing need of zinc and iron for pregnant women, but also the poor intake and low bioabsorption of zinc and iron. Zinc supplementation may improve pregnancy outcomes for chronically deficient pregnant women. Prophylactic doses of 20~25 mg elemental zinc per day have generally been used in pregnant women in developing countries[28]. But we should also pay attention to the supplementation because iron can interfere with the absorption of zinc. Adverse effects on zinc metabolism were observed after ingestion of 100 mg Fe/d. An increase in the efficiency of zinc absorption was observed during late pregnancy[29].

In conclusion, in this multi-center cross sectional study we observed a very high prevalence of anemia in the third trimester of pregnancy in rural areas and suburban area as well. There was a high prevalence of anemia in Chinese pregnant women, and the prevalence of iron deficiency (ID) and iron deficiency anemia (IDA) was 42% and 19%, respectively[30]. These women often show poor nutritional status, lacking sufficient dietary intake of multiple micronutrients. The present study indicated that anemia in pregnant women was still a severe possibly nutrition related problem nowadays. It was hypothesized that the possible reasons for the difference in prevalence between the four sites were geographic factors, unbalanced diets and poor nutritional education[4]. Furthermore, concentrations of serum ferritin, iron, retinol, zinc and urinary excretion of riboflavin were lower in anemic women than in non-anemic women. This may be the consequence of an unbalanced diet with a low amount of iron and micronutrients. Therefore, the supplementation of a combination of iron and other micronutrients should be encouraged for pregnant women and be more beneficial to anemic pregnant women in the third trimester as well.

ACKNOWLEDGEMENTS

We sincerely acknowledge professors Joseph Hautvast and Dr Paolo Suter for their kindly help. The authors also thank Zhang Xiuzhen, Liang Hui, Du Wei, Xu Hongwei, and Zhang Shehua for measuring and technical assistance. Thank Wang Xin for managing the contacts with subjects. There are no conflicts of interest and the manuscript has been read and approved by all authors. We sincerely acknowledge the Nestlé Foundation for the grant to this study.

References

1. van den Broek N.Anemia and micronutrient deficiencies. Br Med Bull. 2003; 67: 149-160.
2. Ramakrishnan U. Nutrition and low birth weight: from research to practice. Am J Clin Nutr. 2004; 79 (1): 17-21.
3. Jin H, Han Q, Liang CA, Li L, Wu YZ, Zhao Q. The epidemiologic study of iron deficiency in pregnang women. Journal of Xi'an Medical University. 1995;16(4): 437-439.
4. Liu LJ, Lai YH, Hu ZH. Investigation of anemia of 158 pregnant women in Jilin city. JiLin YiXueYuan XueBao. 1998;18(1): 45-47.
5. International Anemia Consultative Group (INACG). Iron deficiency in women. Washington, D.C. The Nutrition Foundation 1981: 5-36.
6. Brabin BJ, Prinsen-Geerligs PD, Verhoeff FH, Fletcher KA, Chimsuku LH, Ngwira BM, Leich OJ, Broadhead RL. Haematological profiles of the people of rural southern Malawi: an overview. Ann Trop Med Parasitol. 2004; 98(1): 71-83.
7. Mejia LA, Hodges RE, Rucker RB. Clinical signs of anemia in vitamin A deficient rats. Am J Ciin Nutr. 1979; 32(7): 1439-1444.
8. Makola D, Ash DM, Tatala SR, Latham MC, Ndossi G, Mehansho H.A micronutrient-fortified beverage prevents iron deficiency, reduces anemia and improves the hemoglobin concentration of pregnant Tanzanian women. J Nutr. 2003; 133(5): 1339-1346.
9. Bloem MW, Wedel M, van Agtmaal EJ, Speek AJ, Saowakontha S, Schreurs WH. Vitamin A intervention: short-term effects of a single, oral, mass dose on iron metabolism. Am J Clin Nutr.1990; 51(1): 76-79.
10. Chu SL, Chen YZ, Xue JZ, Ni QG, Wang YL. Measurement of clinical application of ferritin by radioimmunoassay. Chin J Hematol. 1983; 4: 296-298.
11. Handelman GJ, Shen B and Krinsky NI. High resolution analysis of carotenoids in human plasma by high-performance liquid chromatography. Methods Enzymol 1992; 213:336–46.
12. Sauberlich HE, Judd JH, Jr. Nichoalds GE, Broquist HP, Darby WJ. Application of the erythrocyte glutathione reductase assay in evaluating riboflavin nutritional status in a high school student population. Am J Clin Nutr. 1972;25:756–62.
13. Magnus EM, Bache-Wiig JE, Aanderson TR, Melbostad E. Folate and vitamin B12 (cobolamin) blood levels in elderly persons in geriatric homes. Scand J Haematol. 1982; 28(4): 360-366.
14. WHO. Maternal health and safe motherhood program. The prevalence of anemia in women: A tabulation of available information.2nd ed. Geneva，Switzerland: World Health Organization, 1992. (WHO/MCH/MSN/92.2).
15. Shile ME, Olson JA, Shike M. Modern Nutrition in health and disease. 2000; Eighth Edition: 287-450.
16. Jiang T, Christian P, Khatry SK, Wu L, West LP. Micronutrient Deficiencies in Early

Pregnancy Are Common, Concurrent, and Vary by Season among Rural Nepali Pregnant Women1J Nutr. 2005;135:1106–1112.

17. Van den Broek NR, Letsky EA. Etiology of anemia in pregnancy in South Malawi. Am J Clin Nutr. 2000; 72: 247S–256S.
18. Azais-Braesco V, Pascal G. Vitamin A in pregnancy: requirements and safety limits. Am J Clin Nutr 2000; 71: 1325S–1333S.
19. Makola D, Ash DM, Tatala SR, Latham MC, Ndossi G and Mehansho H. A Micronutrient-Fortified Beverage Prevents Iron Deficiency, Reduces Anemia and Improves the Hemoglobin Concentration of Pregnant Tanzanian Women. J Nutr. 2003;133:1339–1346.
20. Ma AG, Chen XC, Zheng MC, Wang Y, Xu RX, Li JS. Iron status and dietary intake of Chinese pregnant women with anemia in the third trimester. Asia Pac J Clin Nutr. 2002;11(3): 171-175
21. Bloem MW, Wedel M, Egger R, Speek A J, Shrisjver J. Ironmetabolism and vitamin A deficiency in children in the NorthwestThailand. Am J ClinNutr. 1989; 50(2): 332-338.
22. Semba RD, Bloem MW. The anemia of vitamin A deficiency: epidemiology and pathogenesis. Eur J Clin Nutr. 2002; 56(4): 271-281.
23. Lynch SR, Dassenko SA. Morck TA, Beard JL, Cook JD. Soy protein products and heme iron absorption in humans. Am J Clin Nutr.1985; 41(1): 13-20.
24. Garcia OP, Diaz M, Rosado JL, Allen LH. Ascorbic acid from lime juice does not improve the iron status of iron-deficient women in rural Mexico. Am J Clin Nutr. 2003; 78(2): 267-273.
25. Czeizel AB. Folic acid in prevention of neural tube defects. J Pediatr Gastroenternol Nutr. 1995; 20(1): 4-16.
26. Ladipo OA. Nutrition in pregnancy: mineral and vitamin supplement. Am J Clin Nutr. 2000; 72 (suppl): 280s-290s.
27. Hotz C, Lowe NM, Araya M, Brown KH. Assessment of the trace element status of individuals and population: The example of zinc and copper. J Nutr. 2003;133 (5 Suppl 1): 1563s-1568s.
28. Gibson RS, Ferguson EL. Nutrition intervention strategies to combat zinc deficiency in developing countries. Nutr Res Rev. 1998; 11:115-131.
29. Linda J Harvey, Jack R Dainty, Wendy J Hollands, Victoria J Bull, John H Beattie, Tony I Venelinov, et al. Effect of high-dose iron supplements on fractional zinc absorption and status in pregnant women. Am J Clin Nutr. 2007; 85(1): 131-136.
30. Liao QK. Prevalence of iron deficiency in pregnant and premenopausal women in China: a nationwide epidemiological survey. Zhonghua Xue Ye Xue Za Zhi. 2004;25:653-657.

Chapter 4

Retinol and riboflavin supplementation decreases the prevalence of anemia in Chinese pregnant women taking iron and folic Acid supplements

Ma AG, Schouten EG, Zhang FZ, Kok FJ, Yang F

, Jiang DC, Sun YY, Han XX.

J Nutr. 2008 Oct;138(10):1946-50.

Abstract

In rural China many pregnant women in their third trimester suffer from anemia (48%) and iron deficiency (42%), often with coexisting deficiencies of retinol and riboflavin. We investigated the effect of retinol and riboflavin supplementation on top of iron plus folic acid on anemia and subjective well being in pregnant women. The study was a 2-mo double-blind randomized trial. Subjects (n=366) with anemia [Hemoglobin (Hb) ≤105g/L] were randomly assigned to four groups, all receiving daily 60 mg iron and 400μg folic acid. The iron+folic acid (IF) group (n=93) served as reference, the iron+folic acid+retinol (IFA) (n=91) was treated with 2000 μg retinol, the iron+folic acid+riboflavin (IFB) (n=91) with 1.0 mg riboflavin and the iron+folic acid+retinol+riboflavin (IFAB) (n=91) with retinol and riboflavin. After the 2-mo intervention, the Hb concentration increased in all 4 groups (p<0.001). The increase in the IFAB group was 5.4±1.1 g/L greater than in the IF group (p<0.001). The reductions in the prevalence of anemia (Hb<110g/L) and iron deficiency anemia were significantly greater in the groups supplemented with retinol and /or riboflavin than in the IF group. Moreover, gastrointestinal symptoms were less prevalent in the IFA group than in the IF group (p<0.05) and improved well being was more prevalent in the groups receiving additional retinol and/or riboflavin than in the IF group (p<0.05). Thus, a combination of iron, folic acid, retinol and riboflavin was more effective than iron plus folic acid alone. Multimicronutrient supplementation may be worthwile for pregnant women in rural China.

Introduction

The prevalence of anemia (hemoglobin (Hb) concentration<110g/L) in Chinese pregnant women in the third trimester was 48% during 1995-2000 (1), and the prevalence of iron deficiency (ID) and iron deficiency anemia (IDA) was 42% and 19%, respectively (2). Combined iron and folate therapy showed a better therapeutic response: the increase in Hb concentrations was 14.2 g/L for women treated with both compounds vs 8.0 g/L for those given iron only (3). But overall there are not enough data to determine with certainty that routine supplementation with iron alone or in combination with folic acid has substantial benefits among populations where anemia is common (4). Also deficiencies of vitamins play a role, especially those of folate, vitamin A, and riboflavin (5,6). A study in China showed that 35% of pregnant women had RBC folate deficiency (7). Therefore, routine supplementation of 400 µg folic acid/d should be recommended for pregnant women for preventing neural tube defects (8), as well as preterm delivery and low birth weight (9).

It was reported that there is a low prevalence of parasitic infection in the rural population (hookworm, 0.65%; ascaris lumbricoides 5.16%) in recent years, and this has been never considered as an important factor of anemia in rural China (10). In fact, retinol and riboflavin deficiencies tend to co-exist in anemic pregnant women in developing countries or impoverished settings (11). 44% of women were deficient in at least one B vitamin (9). Biochemical evidence of riboflavin deficiency was documented during the third trimester of pregnancy (12). Serum concentration of retinol <0.70 µmol/L is associated with an increased risk of preterm delivery and maternal anemia (13). Supplementation with iron and riboflavin enhances dark adaptation response to vitamin A-fortified rice in iron-deficient, pregnant, nightblind Nepali women (6). Retinol or riboflavin plus iron supplementation could improve hematological status better than iron alone (14). In a previous unpublished study in 2001, we observed that about 55% of pregnant women suffered from anemia, and the mean vitamin A intake was only 54% of the Chinese recommended daily allowance (RDA). Riboflavin deficiency was widely prevalent among pregnant women. This prompted us to perform the present study, with the objective to investigate the efficacy of vitamin A and/or riboflavin supplementation on top of iron and folic acid on hematologic status, subjective well being and the occurrence of possibly iron related gastrointestinal symptoms.

Subjects and Methods

Study design and subjects.

The study was a 2-mo double-blind randomized trial of retinol and riboflavin on a background of iron and folic acid supplementation, in a two-by-two factorial design. It was carried out in a rural area of China. A total of 633 pregnant women, 12-24-wk gestation, age range 20-35y, were anemic with Hb <110g/L. The cutoff value of anemia in pregnant women was later decided to be ≤105g/L in the study; therefore, 178 pregnant women did not meet the inclusion criteria, and a further 89 women with Hb≤105g/L refused to take any “drugs” during pregnancy. Finally, 366 women remained [Hb: >80 and ≤105g/L, no dietary supplements during 2 months, no abnormal pregnancy response and receiving daily folic acid (400μg)]. They were allocated to the four intervention groups in the order of enrollment. Registration numbers 1, 5, 9, 13 etc. all were allocated to one group. To the groups the four treatments were randomly assigned, leaving the key in a sealed envelope with an independent person in the Institute. IF (n=93) as a reference group were supplemented with 60 mg iron as ferrous sulfate, IFA (n=91) with 2 mg retinol (as retinyl palmitate) and 60 mg iron, IFB (n=91) with 1.0 mg riboflavin and 60 mg iron, and IFAB group (n=91) with 2000 μg retinol, 1.0 mg riboflavin and 60 mg iron daily, respectively (**Figure 4.1**). The sample size was calculated based on difference between the groups, not on interaction effects. An increase of 3 g/L (δ) in IFA and IFB compared with the IF and an additional increase of 3 g/L compared with the IFA and the IFB group. Considering a 5% significance level and a power of 0.80, the total required number of subjects for the study, was 340 anemic pregnant women (85 subjects for each group). The capsules were colored in red, yellow, green and blue and manufactured by Hurun’ company (a Chinese food-additive company, Beijing). Trial participants and the research team were unaware of the treatment assignment. The trial was deblinded after the analysis of the primary outcomes.

After obtaining written consent, the pregnant women had a baseline interview to obtain data on dietary habits, alcohol intake, and tobacco use, and information on household socioeconomic status. Information on well-being, gastrointestinal side effects, appetite and physical functions, were assessed at the end of the treatment period by use of the Short Form 36 (SF-36) (15), a self-administered questionnairewhich was translated from English to Chinese and used in China(16).

During the trial, a village nurse was responsible for recruitment of the subjects and the distribution of the supplements. After ascertainment of eligibility, consenting women were enrolled in the study to receive their allocated supplements daily for a period of two months. Women were home-visited once each week by the village nurse to replenish supplements and to monitor compliance by counting and recording the number of supplements that were taken. Village nurses provided counseling about possible side effects of taking the supplement. The average number of capsules taken was 62 in IF, 60 in IFA, 61 in IFB and 62 in IFAB group during the two months' supplementation.

The study was approved by the ethical review committee of the Medical College of Qingdao University. Written consent was given by each subject at the start of the trial.

Sample collection and laboratory analyse.

Samples of blood (5mL) and urine samples (10mL) were collected from subjects, and transported on dry-ice and stored frozen at -80 °C until analysis. The baseline and final samples were analyzed in duplicate during the same analytic run.

Plasma retinol concentrations were measured by HPLC (17). Folic acid in plasma was measured by radioimmunoassay method. The nutritional status of riboflavin was determined by the ratio of urine riboflavin/creatinine, and the erythrocyte glutathione reductase activity coefficient (EGRAC) was measured for assessing riboflavin status (18).

Hemoglobin concentration was measured by using HemoCue. Measurements of serum ferritin were performed by radioimmunoassay (19), as described by the manufacturer (The North Biological.Technology Institute, Beijing, China). The cutoff value for mild to moderate anemia was Hb ≤105 g/L in the second and third trimester of pregnancy. The erythrocyte protoporphyrin (EP) was measured with a hematofluorometer. The sTfR assay was performed using a commercial kit (R&D Systems, Minneapolis, MN). According to the instruction of the kit, the central 95th percentile of the reference distribution of soluble transferrin receptor (sTfR) concentration is 4.0 mg/L to 9.1 mg/L.The cutoff value of iron deficiency was >9.1 mg/L. All sTfR assays were performed in duplicate. Plasma iron concentrations were analyzed by atomic absorption spectrometry on an Analyst 3100 Analyzer (Perkin

Elmer Life Sciences, Wellesley, MA). Total iron-binding capacity (TIBC) was determined by a turbidimetric method.

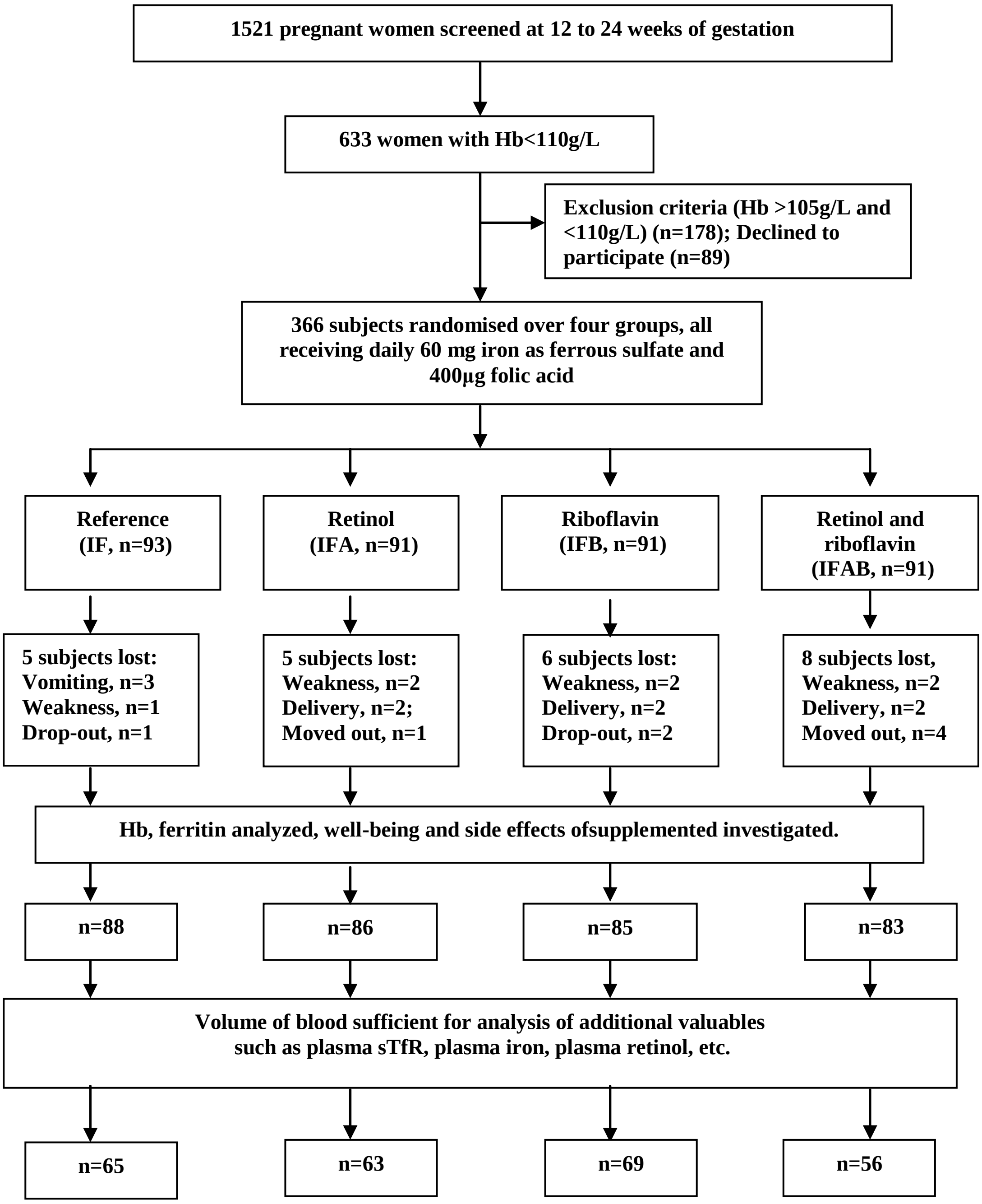

Figure 4.1 The trial profile.
The study was conducted in Shen' county with 960 000 population from 2002 to 2005. At the start of the enrollment, there were 633 pregnant women with Hb concentration <110g/L. However, the cutoff value of Hb ≤105g/L was considered as anemia in the study, and 178 pregnant women did not meet the inclusion criteria, and 89 women refused to take any "drugs" during pregnancy. During the trial, the reasons for subject losses mainly were preterm delivery, weakness, moved out of the trial area, drop-out, etc; some subjects did not complete all measurements because of insufficient plasma sample volume. Weakness was defined as physically too tired to participate, assessed through questionnaire.

Statistical analysis

Categorical data are presented as frequencies, such as prevalence of anemia, whereas continuous data are presented as means±SD or means±SEM. Baseline iron, folate and anemia status of pregnant women were compared across treatment groups. The differences in mean change over the intervention period between intervention groups and reference group and 95% confidence intervals (CI) were estimated for hematologic indicators and vitamin levels using a general linear model ANOVA. Prevalence ratios (PR) and 95% CI's for mild to moderate anemia (Hb <105g/L), all anemia (Hb<110g/L) and iron deficiency anemia (IDA, Hb<110g/L and ferritin <12μg/L), and for well-being and gastrointestinal symptoms were estimated using a binomial model. Since there was no indication of interaction between retinol and riboflavin in 2x2 factorial analysis (p=0.702 for Hb, p=0.777 for ferritin, p=0.746 for sTfR), we calculated variables for each treatment (retinol and riboflavin) using one-way ANOVA followed by Bonferroni correction. Therefore, the effects on changes in Hb concentration, ferritin and sTfR were then assessed by including them in a general linear model. A α of 0.05 was taken as significance level for all tests.

Results

In the trial, 366 pregnant women with Hb 80-105g/L were enrolled. Of them 342 finished the trial and supplied blood for the Hb and ferritin analyses. For 89 subjects, plasma volumes were insufficient for the measurement of all parameters (**Figure.4.1**). Therefore, statistical analyses for a number of variables were based on smaller numbers.

There were no substantial differences between the groups in age, gestational stage, parity, hematological status and vitamin levels at baseline (p>0.05) (**Table 4.1**). After the 2-mo trial, there were significant increases of Hb, plasma ferritin and iron concentrations, and decreases in sTfR, EP and TIBC in all four groups **(Table 4.2)**.

Compared with the reference group, there was a greater increase in Hb concentration in the IFA and in the IFAB group (Table 4.1). Moreover, changes in plasma iron, EP and TIBC differed significantly between the IFAB group and the reference group. Except for a significantly greater decrease in sTfR, the IFA and IFB groups did not differ from the IF group (Table 4.1). The changes in plasma folate and retinol, and riboflavin in urine and EGRAC were as expected **(Table 4.3)**. Moreover, in the IFAB group, the prevalence of mild to moderate anemia, all anemia and IDA were reduced to 7.2%, 15.7% and 6.0%, respectively, significantly lower than in the IF group (**Table 4.4**). Retinol (IFA and IFAB) and riboflavin (IFB and IFAB) treatments both increased Hb concentrations (3.62g/L, $p<0.01$ and 1.78g/L,$p<0.05$, respectively) and decreased sTfR levels compared to no retinol (IF and IFB) or riboflavin (IF and IFA) treatment, respectively; the change of ferritin was greater in women treated with retinol (IFA and IFAB) than in those that were not (IF and IFB) ($p<0.05$) (**Table 4.5).**

The percentage of people who reported subjective well-being was higher in IFAB, IFA, and IFB groups than the IF group **(Table 4.6)**. The percentages of women who felt better, less pale and had better appetites, were 75%, 49% and 41% of subjects in the IFAB group, and 71%, 46% and 42% in IFB group, respectively, significantly higher than in the other groups. In contrast, the percentages of women with gastrointestinal symptoms, including nausea, abdominal discomfort, vomiting, were much significantly lower in the IFAB (2%), IFB (5%) and IFA (6%) groups than in the IF group (21%).

Table 4.1 Baseline characteristics of anemic pregnant women according to treatment[1]

Items	IF[2]		IFA[2]		IFB[2]		IFAB[2]	
Characteristics	n[3]		n[3]		n[3]		n[3]	
Age, *y*	88	28.1 ± 3.6	86	28.2 ± 3.6	85	28.0 ± 3.7	83	28.1 ± 3.5
Gestational age, *wk*	88	20.9 ± 5.6	86	21.1 ± 5.6	85	20.7 ± 5.4	83	20.7 ± 5.5
Parity, *n*	88	1.3 ± 0. 5	86	1.3 ± 0. 5	85	1.3 ± 0. 4	83	1.3 ± 0. 4
Indicators								
Hemoglobin, *g/L*	88	96.6 ± 7.3	86	96.8 ± 6.4	85	96.9 ± 7.1	83	97.0 ± 7.2
Plasma Ferritin[3], *µg/L*	88	14.9 ± 8.4	86	15.0 ± 6.6	85	14.9 ± 6.1	83	14.9 ± 7.0
Plasma sTfR ,*mg/L*	65	11.9 ± 4.6	63	11.9 ± 4.9	69	12.0 ± 4.3	56	11.9 ± 4.1
Plasma iron, *µmol/L*	65	12.9 ± 1.7	63	12.9 ± 1.9	69	13.0 ± 1.9	56	12.9 ± 2.0
EP, µmol/mol heme	65	80.4 ± 83.1	63	82.2 ± 97.7	69	80.4 ± 74.2	56	83.5 ± 63.4
Total iron binding capacity, *µmol/L*	65	67.4 ± 10.5	63	66.9 ± 10.2	69	67.8 ± 9.4	56	65.9 ± 11.2
Plasma retinol, *µmol/L*	65	1.25 ± 1.11	63	1.20 ± 1.11	69	1.19 ± 0.96	56	1.24 ± 0.95
Plasma Folic acid, *nmol/L*	65	8.38 ±3.62	63	8.61 ± 4.30	69	7.93 ± 3.40	56	8.38 ± 3.62
Urine Riboflavin, *mmol/mol*	65	0.22 ± 0.17	63	0.23 ± 0.22	69	0.22 ± 0.20	56	0.21 ± 0.19

[1]Values are means ± SD; [2] See Figure 4.1 for groups, missing values or insufficient plasma analysis;[3] Geometric means ± SD.

Table 4.2 Changes in hematologic indicators in anemic pregnant women during intervention with retinol and riboflavin[1].

Indicators	Groups[2]	n[2]	Baseline	Change from baseline	Difference from IF	*P* –value for difference
Hemoglobin, *g/L*						
	IF	88	96.6 ± 7.3	17.2 ± 1.01*	--	-
	IFA	86	96.8 ± 6.4	21.2 ± 0.97*	3.92 (1.71, 6.13)	0.001
	IFB	85	96.9 ± 7.1	19.3 ± 1.02*	2.08 (-0.13, 4.30)	0.065
	IFAB	83	97.0 ± 7.2	22.6 ± 1.07*	5.39 (3.16 7.62)	<0.001
Plasma Ferritin, *μg/L*						
	IF	88	14.9 ± 8.4	5.6 ± 1.88*	--	-
	IFA	86	15.0 ± 6.6	9.4 ± 1.71*	3.82 (-0.68, 8.40)	0.096
	IFB	85	14.9 ± 6.1	8.1 ± 1.98*	2.51 (-2.05, 7.06)	0.280
	IFAB	83	14.9 ± 7.0	11.0 ± 1.68*	5.43 (0.83, 10.02)	0.021
Plasma sTfR ,*mg/L*						
	IF	65	11.9 ± 4.6	-3.0 ± 0.63*	--	-
	IFA	63	11.9 ± 4.9	-4.0 ± 0.72*	-0.95 (-1.93, 0.02)	0.054
	IFB	69	12.0 ± 4.3	-4.0 ± 0.59*	-0.98 (-1.93, -0.03)	0.043
	IFAB	56	11.9 ± 4.1	-4.7 ± 0.73*	-1.71 (-2.71, -0.70)	0.001
Plasma iron, *μmol/L*						
	IF	65	12.9 ± 1.7	5.4 ± 0.61*	-	-
	IFA	63	12.9 ± 1.9	6.0 ± 0.55*	0.70 (-0.88, 2.27)	0.384
	IFB	69	13.0 ± 1.9	6.3 ± 0.56*	0.99 (-0.55, 2.53)	0.207
	IFAB	56	12.9 ± 2.0	7.8 ± 0.75*	2.41 (0.79, 4.04)	0.004
EP, *μmol/mol heme*						
	IF	65	80.4 ± 83.1	-34.3 ± 11.67*	-	-
	IFA	63	82.2 ± 97.7	-47.7 ± 12.75*	-13.36 (-28.18, 1.45)	0.053
	IFB	69	80.4 ± 74.2	-41.4 ± 11.32*	-7.10 (-22.71, 8.52)	0.293
	IFAB	56	83.5 ± 63.4	-49.9 ± 9.11*	-15.57 (-30.93, -0.20)	0.029
Total iron binding capacity, *μmol/L*						
	IF	65	67.4 ± 10.5	-12.0 ± 1.92*	-	-
	IFA	63	66.9 ± 10.2	-15.2 ± 1.73*	-3.16 (-6.79, 0.47)	0.089
	IFB	69	67.8 ± 9.4	-13.4 ± 1.83*	-1.39 (-5.39, 2.60)	0.442
	IFAB	56	65.9 ± 11.2	-17.3 ± 1.88*	-5.29 (-8.95, -1.63)	0.006

[1] Values are means ± SEM, or means (95% CI). * Significant change from baseline, $p<0.05$.
[2] See Figure 4.1 for groups and sample size; values due to missing values or insufficient plasma samples.

Table 4.3 Changes in vitamin status in anemic pregnant women during intervention with retinoland riboflavin[1].

Indicators	Group[2]	n[2]	Baseline	Change from baseline	Difference from IF	*P*-value for difference
Plasma retinol, *μmol/L*						
	IF	65	1.25±1.11	-0.04± 0.15	--	-
	IFA	63	1.20± 1.11	0.80±0.18*	0.84 (0.58, 1.10)	<0.001
	IFB	69	1.19±0.96	0.02 ± 0.13	0.03 (-0.24, 0.29)	0.840
	IFAB	56	1.24±0.95	0.85±0.17*	0.89 (0.62, 1.16)	<0.001
Plasma Folic acid, *nmol/L*						
	IF	65	8.38±3.62	5.32 ±1.79*	--	-
	IFA	63	8.61±4.30	8.09 ±1.47*	1.76 (-1.68, 7.25)	0.220
	IFB	69	7.93±3.40	6.32±1.34*	1.00 (-3.56, 5.57)	0.667
	IFAB	56	8.38±3.62	6.50 ±1.34*	1.18 (-3.53, 5.89)	0.624
Urine Riboflavin, *mmol/mol*						
	IF	65	0.22±0.17	0.03± 0.03	--	-
	IFA	63	0.23±0.22	0.01± 0.03	-0.01 (-0.07, 0.04)	0.612
	IFB	69	0.22±0.20	0.12 ±0.03*	0.09 (0.04, 0.14)	<0.001
	IFAB	56	0.21±0.19	0.13±0.03*	0.10 (0.05, 0.15)	<0.001
EGRAC						
	IF	65	2.06±0.99	-0.17± 0.16	--	-
	IFA	63	1.89 ± 0.77	-0.22 ±0.14	-0.05 (-0.21, 0.31)	0.698
	IFB	69	1.90±0.78	-0.71±0.12*	-0.54 (-0.81, -0.28)	<0.001
	IFAB	56	1.85±0.76	-0.68±0.13*	-0.51 (-0.79, -0.23)	<0.001

[1] Values are means ± SEM, or means (95% CI). * Significant change from baseline, $p<0.05$.

[2] See Figure 4.1 for groups and sample size; values due to missing values or insufficient plasma samples.

Table 4.4 Prevalence of anemia in anemic pregnant women after Interventionwith retinol and/or riboflavin

anemia[1]	Groups	n[2]	%	PR (95% CI)[3]
Moderate anemia				
	IF	88	18.2	--
	IFA	86	9.3	0.51 (0.10, 0.92)*
	IFB	85	11.8	0.65 (0.17, 1.12)
	IFAB	83	7.2	0.39 (0.04, 0.75)*
All anemia				
	IF	88	29.6	--
	IFA	86	18.6	0.63 (0.28, 0.97)*
	IFB	85	23.5	0.79 (0.40, 1.19)
	IFAB	83	15.7	0.53 (0.21, 0.84)*
Iron deficiency anemia				
	IF	88	15.9	--
	IFA	86	8.1	0.51 (0.07, 0.95)*
	IFB	85	10.6	0.67 (0.15, 1.19)
	IFAB	83	6.0	0.38 (0.01, 0.75)*

[1]Moderate anemia: hemoglobin 80-105g/L; all anemia: Hb<110g/L;
Iron deficiency anemia: Hb<110g/L and plasma ferritin<12μg/L; [2]see Figure 4.1 for groups and sample size; [3]PR, prevalence ratio; CI, Confidence Interval, are calculated using the IF group as the reference.

Table 4.5 Effects of retinol and riboflavin in anemic pregnant women, evaluated with factor analysis

Hematologic indicator	Retinol[1]			riboflavin[2]		
	n	Estimated effects (95% CI)	*P* value	n	Estimated effects (95% CI)	*P* value
Hb, *(g/L)*	169	3.62 (2.03, 5.20)	<0.001	173	1.78 (0.16, 3.40)	0.031
Plasma Ferritin, *(μg/L)*	169	3.39 (0.15, 6.63)	0.040	173	2.03 (-1.22, 5.29)	0.220
Plasma sTfR, *(mg/L)*	125	-0.80 (-1.50, -0.10)	0.024	119	-0.84 (-1.53, -0.14)	0.019

[1]Retinol treatment including IFA and IFAB groups, n=169 (125 in sTfR);
[2]Riboflavin treatment including IFB and IFAB groups, n=173 (119 in sTfR).

Table 4.6 Prevalence of subjective symptoms in anemic pregnant women after retinol and riboflavin intervention

Item[1]	Group[2]	n[2]	%[3]	PR (95% CI) [4]
Feeling better				
	IF	89	50.6	--
	IFA	86	62.8	1.24 (0.92, 1.57)
	IFB	83	71.1	1.40 (1.06, 1.75)*
	IFAB	83	74.7	1.48 (1.12, 1.83)*
Less pale				
	IF	89	24.7	-
	IFA	86	37.2	1.51 (0.82, 2.20)
	IFB	83	45.8	1.85 (1.05, 2.65)*
	IFAB	83	49.4	2.00 (1.15, 2.85)*
Appetite better				
	IF	89	20.2	--
	IFA	86	24.4	1.21 (0.54, 1.88)
	IFB	83	42.2	2.09 (1.08, 3.10)*
	IFAB	83	41.0	2.03 (1.04, 3.02)*
Stronger, and other improvements				
	IF	89	27.5	--
	IFA	86	39.5	1.44 (0.82, 2.05)
	IFB	83	33.7	1.23 (0.67, 1.78)
	IFAB	83	38.6	1.40 (0.80, 2.01)
GI side effects (such as vomiting, nausea, abdominal discomfort)				
	IF	89	21.4	--
	IFA	86	5.8	0.27 (0.02, 0.53)*
	IFB	83	4.8	0.22 (0.01, 0.46)
	IFAB	83	2.4	0.11 (0.05, 0.27)

[1]Well being, including feeling better, less pale (self-reported by subjects who often see themselves in mirror), appetite better, stronger and other improvements as physical functions, such as doing housework, farm-work, walking, etc; GI: Gastrointestinal; gastrointestinalside effects: including nausea, abdominal discomfort, vomiting, etc. [2]See Figure 4.1 for groups and sample size. [3] Proportions of well being or side-effects in four groups. [4]PR, prevalence ratio; CI, confidence Intervals; PR and 95% CI are calculated using the IF group as the reference; *significant change from baseline, p<0.05.

Discussion

During the trial all four groups of pregnant women with anemia significantly improved in Hb concentration and anemia prevalence. The increase in Hb concentration in the IFAB group was 5.4 g/L greater (p<0.001) compared with in the IF group. This way the residual prevalence of anemia after supplementation with iron and folic acid was halved by adding retinol and riboflavin. Moreover, gastrointestinal symptoms were less prevalent and improved well being was more prevalent in the intervention groups than in the reference group.

Compared with the few similar studies carried out so far (14, 20), ours had a relatively large study population. It was carried out under difficult circumstances in a poor rural part of China, where nutritional problems, especially among pregnant women are frequent. However, compliance was excellent because study subjects were motivated by the offer of free of charge medical care, and all women were weekly visited by village nurses, who counted left-over capsules, provided new supplies and gave support in case of any problems or questions related to the study.

Unfortunately, there was a small proportion (6.6%) of women who dropped out after randomization. This drop out and the underlying reasons were evenly distributed over the groups. Due to restriction to the amount of blood that we were allowed to draw we had missing data on a number of hematologic variables for about 80 participants. These were reasonably evenly distributed over the groups and the remaining groups were still balanced with respect to important characteristics.

The quality of the supplements was assured by storage at -20 ^{0}C and micronutrient levels were examined every three months, showing no appreciable changes. Although the four treatment capsules had different colors, blinding of both participants and study personnel stayed intact, since there was no need to break the code before the main analyses had been carried out. In our trial, most subjects only knew their own supplement which was provided by their village nurse. Since they lived in a remote area, contacts with other trial participants were rare.

Riboflavin deficiency may be one of the most common vitamin deficiencies in regions, like rural areas in China, where diets are predominantly rice-based and contain insufficient milk, meat, fish, fresh fruit or vegetables (21). The riboflavin combined with iron was also considered to be beneficial to anemic pregnant women (14) and significantly reduced the prevalence of riboflavin deficiency and iron deficiency anemia (6). Our results further confirmed the positive effect of the combination of retinol and riboflavin on anemia in Chinese pregnant women from rural areas (22, 23).

We found evidence for an additive effect of retinol and riboflavin on hematologic parameters and anemia on top of iron and folic acid. This may be explained by the co-existing deficiencies of these micronutrients in our population. The effects of retinol and riboflavin appeared to be additive and we did not find evidence for interaction. This may indicate that the beneficial effects of each of the vitamins on erythropoiesis are independent. It is well known that retinol plays an important role in reducing anemia and improving iron status. Retinol enhances iron utilization by stimulating erythropoiesis and iron metabolism by raising mean red cell volume, plasma Fe concentration and total Fe-binding capacity (23,24). Improvement of poor appetite was observed in 42.2% and 41.0% of the IFB group and IFAB group, respectively. Riboflavin is thought to improve iron uptake and absorption by improving the gastrointestinal function (25) and better appetite, which may have resulted in significantly enhanced concentration of serum iron (26). Consistently, women who took riboflavin had better appetites (subjectively) than those who did not take any riboflavin. At the same time, riboflavin is also necessary for synthesis of the globin component of hemoglobin (27).

In our study, the combination of iron and folic acid had already a strong beneficial effect and the study was designed to demonstrate possible additional efficacy of retinol and/or riboflavin. Such additional benefits are expected to be modest.

In conclusion, these women often show poor nutritional status, lacking sufficient dietary intake of multiple micronutrients. Surprisingly, almost none of the subjects took dietary supplements, including iron. Our study showing additional benefits of vitamins in tackling the anemia problem has important public health implications. It underlines the need for a comprehensive nutritional policy for this target population. Besides improving their diet, multimicronutrient supplementation may be worthwile for pregnant women in rural China.

Acknowledgments

We thank professors Chen Xuecun and Li Juesheng. We thank Zhang Xiuzhen, Liang Hui, Du Wei, Xu Hongwei, Zhang Shehua and Shi Xuexiang for measurements and technical assistance in hematologic indicators, vitamin A, folate in plasma and riboflavin in urine analyses, and we also thank Wang Xin and Li Yong for working around village nurses and subjects as qualified supervisors.

References

1.Wang J, Ren AG, Ye RW, Zheng JC, Li S, Liu JM, Yang RL, Zhang FR, Zhang T. Study on the third trimester hemoglobin concentrations and the risk of low birth weight and preterm delivery. Zhonghua Liu Xing Bing Xue Za Zhi. 2007;28:15-18.

2. Liao QK.Prevalence of iron deficiency in pregnant and premenopausal women in China: a nationwide epidemiological survey. Zhonghua Xue Ye Xue Za Zhi. 2004;25:653-657.

3. Juarez-Vazquez J, Bonizzoni E, Scotti A.Iron plus folate is more effective than iron alone in the treatment of iron deficiency anaemia in pregnancy: a randomized: a double blind clinical trial. BJOG. 2002;109:1009–1014.

4. Pena-Rosas JP, Viteri FE. Effects of routine oral iron supplementation with or without folic acid for women during pregnancy. Cochrane Database Syst. Rev. 2006;19,3: CD004736.

5. Ahmed F, Mahmuda I, Sattar A, Akhtaruzzaman M. Anaemia and vitamin A deficiency in poor urban pregnant women of Bangladesh. Asia Pac J Clin Nutr. 2003;12:460-466.

6. Graham JM, Haskell MJ, Pandey P, Shrestha RK, Brown KH, Allen LH. Supplementation with iron and riboflavin enhances dark adaptation response to vitamin A-fortified rice in iron-deficient, pregnant, nightblind Nepali women. Am J Clin Nutr. 2007;85:1375-1384.

7. Ren A, Zhang L, Li Z, Hao L, Tian Y, Li Z.Awareness and use of folic acid, and blood folate concentrations among pregnant women in northern China--an area with a high prevalence of neural tube defects. Reprod Toxico. 2006;22:431-36.

8. Centers for Disease Control Prevention (CDC). Use of vitamins containing folic acid among women of childbearing age--United States, MMWR Morb. Mortal Wkly. Rep. 2004;53:847-850.

9. Shaw GM, Carmichael SL, Nelson V, Selvin S, Schaffer DM. Occurrence of low birthweight and preterm delivery among California infants before and after compulsory food fortification with folic acid. Public Health Rep. 2004;119:170-173.

10. Chen YN, Wan GQ, Liu X, Zhao CL, Wang YB. Investigation on hookworm infection in humans of Shangdong province. Chin J Schisto Control. 2008;20:142.

11. Bates CJ, Prentice AM, Paul AA. Seasonal variations in vitamin A, C, riboflavin and folate intakes and status of pregnant and lactating women in a rural Gambian community: some possible implications. Eur J Clin Nutr. 1994;48:660-668.

12. Pongpaew P, Saowakontha S, Schelp FP, Rojsathaporn K, Phonrat B, Vudhivai N, Supawan V, Intarakhao C, Mahaweeravat U, Lumbiganon P, et al. Vitamin B_1, B_2 and B_6during the course of pregnancy of rural and urban women in northeast Thailand. Int J Vitam Nutr Res. 1985;65:111–116.

13. Radhika MS, Bhaskaram P, Balakrishna N, Ramalakshmi BA, Devi SB, Kumar B. Effects of vitamin A deficiency during pregnancy on maternal and child health. BJOG. 2002;109: 689-693.
14. Suprapto B, Widardo, Suhanantyo. Effect of low-dosage vitamin A and riboflavin on iron-folate supplementation in anaemic pregnant women. Asia Pac J Clin Nutr. 2002;11:263–267.
15. Robert JF, Amy BR, Mark S. Use of the Reliable Change Index to evaluate clinical significance.in SF-36 outcomes. Quality of Life Research. 2002;11:509–516.
16. Li L, Wang HM&Shen Y. (2003) Chinese SF-36 Health Survey: translation, cultural adaptation, validation, and normalisation. J Epi Comm Health.;57:259-263
17. Handelman GJ, Shen B, Krinsky NI. High resolution analysis of carotenoids in human plasma by high-performance liquid chromatography. Methods Enzymol. 1992;213:336–346.
18. Sauberlich HE, Judd JH, Jr. Nichoalds GE, Broquist HP, Darby WJ. Application of the erythrocyte glutathione reductase assay in evaluating riboflavin nutritional status in a high school student population. Am J Clin Nutr. 1972;25:756–762.
19. Chu S. Measurement of clinical application of ferritin by radioimmunoassay. Chin J Blo Med. 1983;296.
20. Iannotti LL, O'Brien KO, Chang SC, Mancini J, Schulman-Nathanson M, Liu S, Harris ZL, Witter FR. Iron Deficiency Anemia and Depleted Body Iron Reserves Are Prevalent among Pregnant African-American Adolescents. J Nutr. 2005;135: 2572–2577.
21. Ma AG, Chen XC, Zheng MC, Wang Y, Xu RX, Li JS. Iron status and dietary intake of Chinese pregnant women with anaemia in the third trimester. Asia Pac J Clin Nutr. 2002;11:171-175.
22. Semba RD, Kumwenda N, Taha TE, Mtimavalye,L, Broadhead R, Miotti EPG, Chiphangwi JD. Impact of vitamin A supplementation on anaemia and plasma erythropoietin concentrations in pregnant women: a controlled clinical trial. Eur J Haematol. 2001;66: 389-395.
23. Roodenburg AJ, West CE, Hovenier R, Beynen AC. Supplemental vitamin A enhances the recovery from iron deficiency in rats with chronic vitamin A deficiency. Br J Nutr. 1996;75:623-636.
24. Fishman SM, Christian P, West KP. The role of vitamins in the prevention and control of anaemia. Public Health Nutr. 2000;3:125–150.
25. Yates CA, Evans GS, Pearson T, Powers HJ. Absence of luminal riboflavin disturbs early postnatal development of the gastrointestinal tract .Digestive Diseases and Sciences. 2003;48:1159–1164.

26. Zhao L, Wang S, Jia J, Jia F, Liu C, Liu A, Ping B, Zhai F. Impact of supplementation of vitamins and minerals on nutritional status of women in suburb Wei Sheng Yan Jiu.2003;32:459-461.

27 Fawzi WW, Msamanga GI, Kupka R, Spiegelman D, Villamor E, Mugusi F, Wei R, Hunter D. Multivitamin supplementation improves hematologic status in HIV-infected women and their children in Tanzania. Am J Clin Nutr. 2007;85:1335-134.

Chapter 5

Supplementation of iron alone and combined with vitamins improves haematological status, erythrocyte membrane fluidity and oxidative stress in anaemic pregnant women

Ma AG, Schouten EG, Sun YY, Yang F, Han XX,
Zhang FZ, Jiang DC, Kok FJ.

Br J Nutr. 2010;104(11):1655-1661

ABSTRACT

Pregnancy is a condition exhibiting increased susceptibility to oxidative stress and iron plays a central role in generating harmful oxygen species. The objective of this study is to investigate change in hematological status, oxidative stress and erythrocyte membrane fluidity in anemic pregnant women after iron with and without combined vitamin supplementation. The study was a 2 months double-blind randomized trial. Pregnant women (n=164) were allocated to four groups: C: placebo; I: daily 60 mg iron (ferrous sulfate); IF: in addition 400µg folic acid; IM: in addition 2 mg retinol and 1 mg riboflavin, respectively. After the 2-months' trial, Hemoglobin (Hb) significantly increased with15.8, 17.3 and 21.8 g/L , and ferritin with 2.8, 3.6 and 11.0µg /L in the I, IF and IM groups compared with placebo. Polarisation (ρ) and microviscosity (η) decreased significantly in comparison with placebo, indicating increase in membrane fluidity. Significant decreases of ρ and η values compared with C were 0.033 and 0.959 for I, 0.037 and 1.074 for IF, and 0.064 and 1.865 for IM, respectively. In addition, significant increases of glutathione peroxidase activities and decreases of malondialdehyde were shown in all treated groups, as well as increases of plasma retinol and urine riboflavin in IM. The findings show that supplementation with iron and particularly in combination with vitamins could improve hematologic status as well as oxidative stress and erythrocyte membrane fluidity.

Introduction

Iron is an essential trace element that is frequently deficient in infants and in women of reproductive age from developing countries. Prevalence of anemia is still high in rural areas in China[1]. Iron deficiency is associated with anemia[2], and impairment of iron-dependent enzymes and proteins[3].Iron supplementation is almost universally recommended during pregnancy to correct or prevent deficiency[4]. However, pathological accumulation of the metal within tissues aggravates the generation of reactive oxygen species (ROS) and elicits toxic effects, which are mainly related to oxidative stress[5]. Iron plays a central role in generating harmful oxygen species. Its redox cycling promotes the Fenton reaction producing the potent oxidant hydroxyl radical[6]. ROS can alter the chemical and physical properties of cell membranes leading to a structural alteration, which could modify membrane activity and cause a reduction in membrane fluidity that can be assessed in the erythrocyte[7]. Several studies have reported that free radicals are relatively low in normal conditions due to active defense systems, including chemical scavengers or antioxidant molecules, superoxide dismutase (SOD), and glutathione peroxidase (GSH-Px). In rats, both iron deficiency and excess result in free radical mitochondrial damage[8]. The excessive superoxide produced by abnormal redox reactions can also cause biochemical modification of red blood cell (RBC) membrane protein. This would result in changes of membrane structure or trans-membrane transport and exert a variety of cytotoxic effects[9], and would affect membrane viscoelasticity.

In relation to pregnancy, there was a report that supplementation with vitamin C (1000 mg/d) and vitamin E (400 mg/d) might be beneficial in pregnant women with increased risk of preeclampsia, a complication in which oxidation is an important feature[10]. Two large randomized trials showed, however, that supplementation with vitamin C and E during the second trimester did not reduce the risk[11,12]. The administration of an iron supplement with vitamin C to 27 women during the third trimester of pregnancy significantly increased maternal iron status as well as an indicator of lipid peroxidation, compared with controls[13]. Increased oxidative stress was reported to occur in uncomplicated pregnancy and to be counteracted by a high level of plasma vitamin E[14].

Thus, provision of a moderate dose of iron (100mg/d) might be deleterious in certain and not in other circumstances with respect to the possible generation of oxy-free radicals. Therefore, the purpose of the study was to investigate the effect of supplementation with iron only, and iron combined with folic acid or with folic acid, retinol and riboflavin, on hemotological status, oxidative stress parameters, such as SOD, GSH-Px and malondialdehyde (MDA) level, and erythrocyte membrane fluidity in anemic pregnant women during the trial.

Materials and Methods

Participants

The study was a 2 months intervention trial of the effect of 1) iron, 2) iron and folic acid, and 3) iron, folic acid, retinol and riboflavin on indicators of oxidative stress, compared with placebo. Participants were recruited between March 2004 and September 2006 from the community hospitals of Shen's county in the central area of China. 366 pregnant women consented to participate and fulfilled the other eligibility criteria of no dietary supplements during the past 2 months and no abnormal pregnancy response. Finally, a random sample of 164 anemic pregnant women (80g/L< Hb <110 g/L), 12 to 24 weeks gestation, and 20 - 35 years old, were randomly allocated to four groups in the order of recruitment: group C (n=41) was the placebo control group, group I (n=41) was supplemented daily with 60 mg iron as ferrous sulfate, IF (n=41) with 60 mg iron and 400μg folic acid, IM (n=41) with 60 mg iron, 400μg folic acid, 2 mg retinol and 1 mg riboflavin. The sample size was calculated based on a difference in hemoglobin (Hb) change (δ) of 5 g/L between the intervention groups and placebo and a standard error of 6.67 g /L was derived from the study reported by Suharno[15]. Considering a 5% (α=0.05) significance level and a power of 0.80 (β=0.20), the total number of subjects required for the study was 164. Treatments were blindly assigned to the groups, leaving the key in a sealed envelope with an independent person in the Institute. The capsules were labelled in red, yellow, green and blue color and manufactured by Hurun company (a Chinese food-additives company, Beijing, China). Trial participants and the research team were unaware of the treatment assignment. The trial was deblinded after analysis of the primary outcomes.

Pregnant women were enrolled after obtaining written informed consent and had a baseline interview on characteristics, such as age, gestation, previous pregnancies, etc. In each community, a local female community health worker called "village nurse", was responsible for recruitment and distribution of the supplements. After ascertainment of eligibility, consenting women were enrolled in the study, had a baseline interview and started with their allocated supplements to be taken daily for a period of two months. Women were home-visited once each week by the village nurse to replenish supplements and to monitor compliance by counting and recording the number of supplements that were taken. The nurse also provided counseling about possible side effects.

This study was conducted according to the guidelines laid down in the Declaration of Helsinki and all procedures involving human subjects were approved by the ethics committee of Medical College of Qingdao University (QY-ETC2002821).

Sample collection and laboratory analyses

Before and at the end of the intervention overnight fasting (>12h) blood samples were collected between 6 and 8 am. Moreover, daily sample collection was evenly distributed over each of the four groups. The urine first pass was discarded and the next 10ml was collected in dark containers for riboflavin and creatinine analysis, which were immediately aliquoted. The samples were transported on dry-ice and stored frozen at -80 °C until analysis. The baseline and final samples were analyzed in duplicate during the same analytic run.

Plasma retinol concentrations were measured by reversed-phase high-performance liquid chromatography (HPLC) (Beckman 5000 with detector of 168, USA) and the within-assay and between assay CVs were 3% and 8%, respectively[16]. Folic acid in plasma was measured by radioimmunoassay method. The nutritional status of riboflavin was determined by the ratio of urine riboflavin/creatinine (Cr) [17].

Hemoglobin concentration was measured by the cyanomethemoglobin method by using HemoCue for confirmation. The cutoff value of anemia was Hb<110g/L. Measurements of serum ferritin were performed by radioimmunoassay[18], as described by the manufacturer (The North Biological.Technology Institute, Beijing, China). Plasma iron concentrations were analyzed by atomic absorption spectrometry on an Analyst 3100 Analyzer (Perkin Elmer Life Sciences, Wellesley, MA).

The activities of superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) were determined in plasma[19] as U/ml and IU/ml, respectively. The MDA concentration was determined by using the thiobarbituric acid reaction, and calculated by comparing the absorbance values of the samples with those of standard MDA solutions. Results were expressed as nmol/ml blood. Additionally, routine parameters were also studied in plasma from the subjects[20].

The erythrocyte membrane fluidity can be measured by fluorescence polarization (ρ) and microviscosity (η). To evaluate membrane fluidity by fluorescence spectroscopy, cell suspensions were incubated with the fluorescence probes (final concentration 10^{-6} M). Erythrocytes were washed by the physiological saline, and suspended in the phosphate buffered saline (PBS) solution (pH = 7.4) at 0.01mol/L. To the erythrocytes suspension, 1,6-diphenyl-1,3,5-hexatriene (DPH, 2 $\times 10^{-6}$ mol/L) was added before being incubated in a water bath at 25^0C for 15 min[21]. Samples were illuminated with the linear (vertically – v or horizontally – h) polarized monochromatic light (λ_{ex}) and the emitted fluorescence intensities (I - in arbitrary units) parallel or perpendicular to the direction of the excitation beam were recorded. The suspension was examined by spectrofluorophotometer (Perkin Elmer fluorescence spectrometer, LS-50) with the excitation wavelength of 320 nm and emission wavelength of 430 nm[22,23], ρ and η of the erythrocyte membrane was calculated by the following formulae:

$$\rho = I_{VV} - GI_{VH} / I_{VV} + GI_{VH};\ \eta = 2\rho/0.46 - \rho$$

Indices V and H denote the vertical and horizontal position of the polarizer in the excitation and the fluorescence beam, respectively. G is an instrumental correction factor equal to (I_{VV}/I_{VH}), The subscript H refers to the horizontally polarized excitation beam. I_{VV} and I_{VH} represent the components of the corrected polarized emission parallel and perpendicular to vertical direction, respectively[24,25].

Statistical analysis

Categorical data are presented as frequencies, such as prevalence of anemia, Continuous data, hematologic indicators, vitamin concentrations and oxidative parameters, microviscosity and polarization values, were normally distributed and presented as mean ± SD. Baseline variables were compared across treatment groups using a general linear model ANOVA. Mean changes over the intervention period and differences between groups were tested with Student t-tests for hematologic indicators, vitamin levels, oxidative indicators and microviscosity and polarization values. A two-sided $p<0.05$ was considered as the significant level for all tests.

Results

Complete data were available for 145 of the original 164 pregnant women. 19 women did not complete the trial for the following reasons: moved to other villages 5; stopped taking supplements 11; not willing to provide a second blood sample 3. There were no substantial differences between the remaining groups in any of the baseline characteristics (**Fig 5.1 and Table 5.1**). Compared with the means of hematologic status at the start of the trial, significant decreases of Hb, plasma iron and ferritin values were found in the placebo group two months later; while the levels of hematologic status were significantly increased in the I, IF and IM groups after two months supplementation (**Fig 5.2.**).

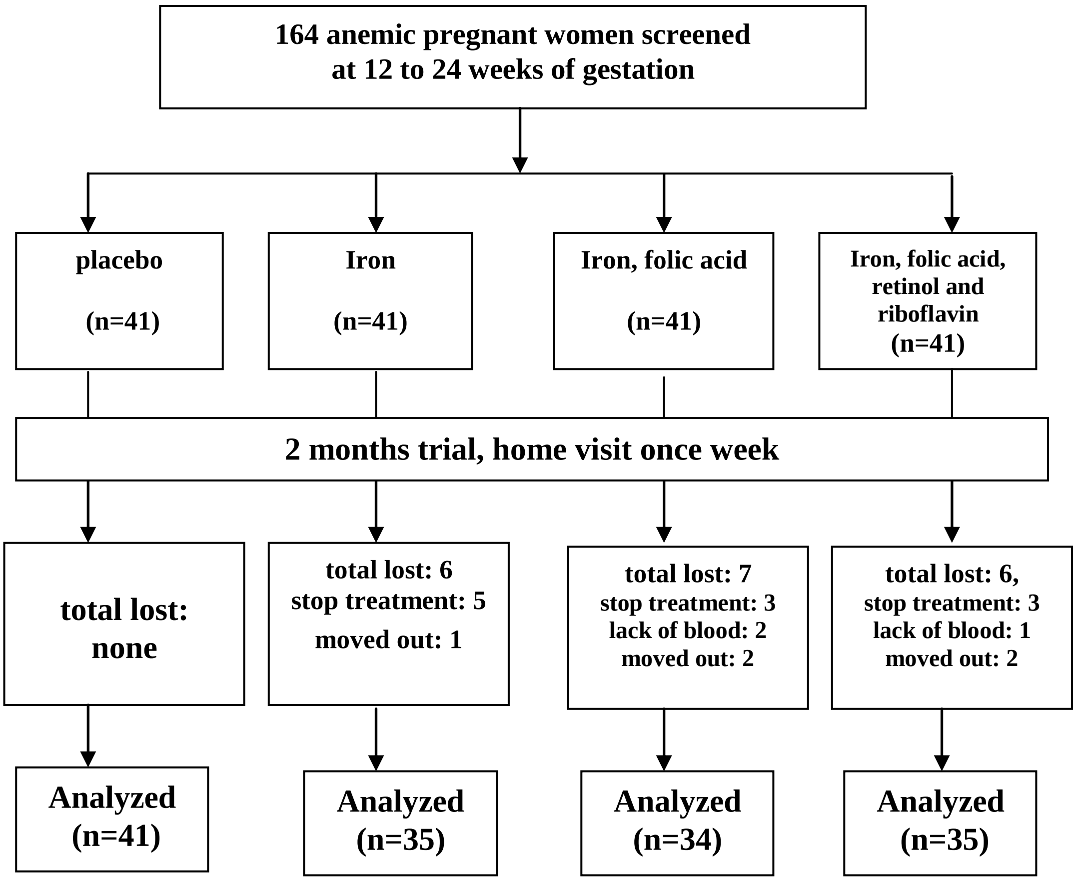

Figure 5.1 The trial profile. The trial enrollment was conducted from 2004 to 2006. Of a total of 366 women were eligible, whom we took a random sample of 164, and they were allocated to the intervention groups by order of randomization. In the intervention study, complete data were available on 145, which is 88.4% of the original number of 164 pregnant women. 19 women did not complete the trial. However, there were no substantial differences between the groups in any of the baseline characteristics.

After the 2-months supplementation, there were considerable increases in hematologic indicators in I, IF and IM groups compared with C: 15.8 g/L, 17.3 g/L and 21.8 g/L for Hb (all p values <0.001); 4.7 μmol/L, 5.5 μmol/L and 9.7μmol/L for plasma iron (all p values <0.001), and 2.8 μg/L, 3.6 μg/L and 11.0μg/L for ferritin (all p values <0.001). The increases of plasma retinol and urine riboflavin in IM were 26.75μg /dL (p<0.001) and 61.59 mg/g creatinine (p<0.001), respectively. For plasma folic acid the increase was 4.06μg/L in the IF group (P<0.001) and 4.65μg/L in the IM group (P<0.001), all compared with C (**Table 5.2**). The increases in all hematologic and vitamin indicators in IM were significantly greater than in I (all p values <0.001).

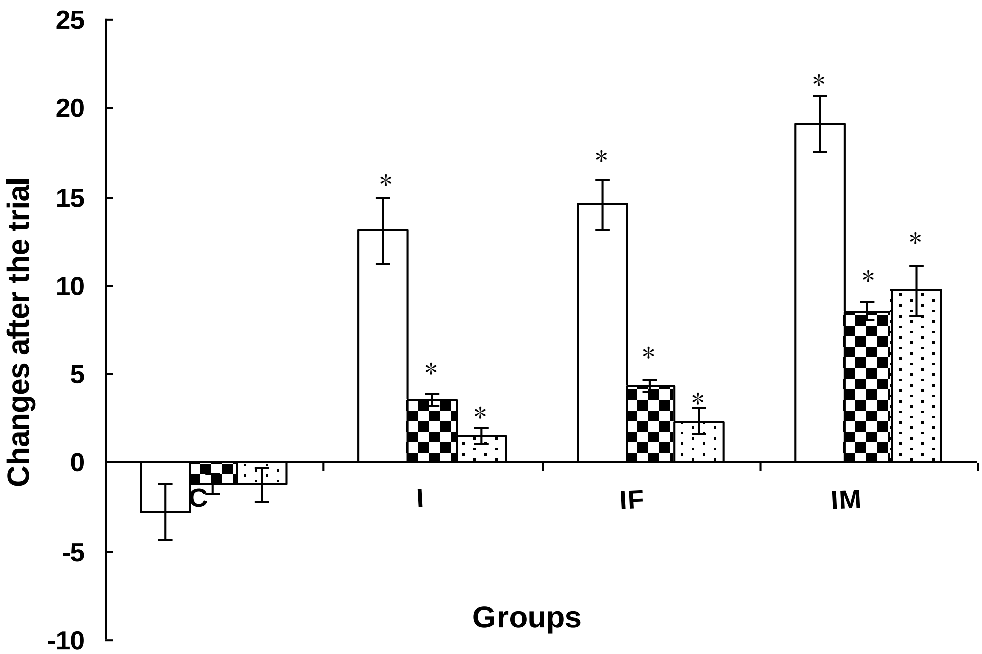

Fig. 5.2 A comparison of hematologic status between before and after the trial. The blank bars represent the mean and standard deviation of hemoglobin concentration (Hb), the square bars for plasma iron concentration and the dot bars for ferritin with significant changes between groups; group C as placebo, group I supplemented daily with 60 mg iron, IF with 60 mg iron and 400 μg folic acid and IM with 60 mg iron, 400 μg folic acid, 2 mg retinol and 1 mg riboflavin, respectively. * Mean value was significantly different from that of the control group (p<0.05).

Erythrocyte membrane fluidity was evaluated using fluorescence polarization (ρ) and microviscosity (η); lower values indicate better membrane fluidity. After the trial, the ρ and η values compared with C decreased by 0.033 and 0.959 for I, 0.037 and 1.074 for IF, and 0.064 and 1.865 for IM, respectively. The ρ and η values in the IM compared with those in the I group decreased by 0.031 and 0.906 (all p values <0.05), (**Table 5.3)**.

The changes in oxidative stress parameters are presented in **Table 5.3**. Compared with the C, increases of GSH-Px activities were 37.7, 43.4 and 87.9 IU/ml in the I, IF and IM groups, respectively; the levels of MDA decreased by 0.55, 1.06 and 2.56μmol/L.. Moreover, the increase of GSH-Px activity and decease of MDA level were 50.2 IU/ml and 2.01 μmol/L greater in the IM group compared with the I group (all p values <0.01); however, there were no important changes of SOD activities during the trial.

Table 5.1 Baseline characteristics of Chinese anemic pregnant women in four groups[§]

Characteristics	All baseline		C		I		IF		IM		*F*	*P*[*]
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD		
Subjects (*n*)	145		41		35		34		35			
Age (years)	27.4	3.4	27.3	3.4	27.8	3.5	27.4	3.1	27.0	3.7	0.62	0.601
Gravidity (*n*)	1.3	0.4	1.3	0.5	1.3	0.5	1.3	0.4	1.2	0.4	0.86	0.464
Gestational stage (wk)	20.9	4.7	21.1	4.8	21.9	4.4	20.0	4.8	20.3	4.5	1.28	0.285
Hb (g/L)	99.8	6.5	101.7	8.7	99.5	5.6	99.0	4.8	98.8	5.7	0.76	0.519
Plasma iron (μmol/L)	12.2	2.1	12.8	2.8	11.6	2.3	11.7	1.5	12.3	1.2	1.88	0.136
Ferritin (μg/L)	12.1	3.8	12.6	5.1	12.8	2.6	11.9	3.3	10.9	3.6	0.76	0.521
Folate (μg/L)	3.90	1.84	4.10	1.86	3.68	1.65	4.13	1.95	3.66	1.92	0.21	0.891
Retinol (μg/dL)	29.68	6.75	30.69	7.95	30.13	6.22	29.78	6.13	27.93	6.20	0.28	0.842
Riboflavin (mg/g creatinine)	41.95	15.39	42.29	17.64	42.98	14.10	42.52	14.75	39.96	14.90	0.08	0.969
MDA(μmol/L)	4.97	1.34	4.95	1.10	5.38	1.77	4.68	1.11	4.88	1.28	1.57	0.200
SOD (U/ml)	73.3	26.2	73.3	35.1	72.3	22.4	73.8	23.3	73.7	20.7	0.02	0.995
GSH-px (IU/ml)	116.6	29.0	108.3	26.7	116.3	27.1	124.2	30.7	119.4	30.2	2.06	0.108

[§]group C: placebo, group I: 60 mg ferrous sulfate; group IF in addition 400 μg folic acid; group IM in addition 2 mg retinol and 1 mg riboflavin.

*One-way ANOVA.

Table 5.2 Comparison of hematologic and vitamin status between control and supplemented groups after the trial§

Indicators	**C**		**I**		**IF**		**IM**		***F***	***P****
	Mean	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**		
Subjects (*n*)	41		35		34		35			
Hb (g/L)	99.0 [a]	5.5	114.8[b]	8.6	116.3[c]	6.0	120.8[d]	6.1	80.38	0.000
Plasma iron (μmol/L)	11.6 [a]	1.9	16.3 [b]	0.5	17.1 [c]	0.8	21.3 [d]	1.5	344.95	0.000
Ferritin (μg/L)	11.4 [a]	3.4	14.2 [b]	1.2	15.0 [c]	2.2	22.4 [d]	6.4	55.14	0.000
Folic acid (μg/L)	3.52 [a]	1.50	4.12	1.29	7.58 [c]	1.67	8.17 [d]	1.74	83.44	0.000
Retinol (μg/dL)	29.05 [a]	6.34	31.31	5.58	31.48	4.93	55.79 [d]	9.00	129.68	0.000
Riboflavin (mg/g creatinine ratio)	39.79 [a]	15.56	47.17	20.03	47.05	19.16	101.38 [d]	46.40	37.91	0.000

§group C: placebo, group I: 60 mg ferrous sulfate; group IF in addition 400 μg folic acid; group IM in addition 2 mg retinol and 1 mg riboflavin.*One-way ANOVA.[a,b,c,d] Mean values within a row with unlike superscript letters were significantly different ($p<0{\cdot}05$; independent t-test).

Table 5.3 Comparison of membrane fluidity and oxidative stress status between control and supplemented groups after the trial§

Indicators	**C**		**I**		**IF**		**IM**		***F***	***P****
	Mean	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**		
Subjects (*n*)	41		35		34		35			
Membrane fluidity										
ρ	0.330 [a]	0.052	0.297 [b]	0.012	0.293 [c]	0.008	0.266 [d]	0.009	30.81	0.000
η	4.593 [a]	2.088	3.634 [b]	0.419	3.519 [c]	0.243	2.728 [d]	0.178	17.12	0.000
Oxidative stress										
GSH-Px (IU/ml)	115.1 [a]	23.5	152.8 [b]	60.0	158.5 [c]	63.8	203.0 [d]	88.5	12.62	0.000
SOD (U/ml)	72.5	35.9	76.0	19.6	75.1	17.7	72.9	18.3	0.17	0.915
MDA (μmol/L)	5.04 [a]	1.12	4.49 [b]	0.57	3.98 [c]	0.60	2.48 [d]	0.46	78.34	0.000

§group C: placebo, group I: 60 mg ferrous sulfate; group IF in addition 400 μg folic acid; group IM in addition 2 mg retinol and 1 mg riboflavin.*One-way ANOVA.[a,b,c,d] Mean values within a row with unlike superscript letters were significantly different ($p<0{\cdot}05$; independent t-test).

Discussion

After 2 months supplementation with iron and/or vitamins, anemic pregnant women significantly improved in erythrocyte membrane fluidity and antioxidative markers, in addition to increases of hemoglobin concentration, plasma iron, ferritin, retinol and urine riboflavin concentrations. Most effects were greatest in the iron combined with multivitamin (IM) group.

Up to now, few studies evaluated changes of both hematologic status and erythrocyte membrane fluidity after iron supplementation in anemic pregnant women, possibly because erythrocyte membrane preparation is quite laborious. Our study had a relatively large study population and it was carried out under difficult circumstances in a poor rural part of China. Unfortunately, there were a small proportion of women who dropped out after treatment allocation, only in the supplement groups. Some subjects stopped treatment because of possible side effects, including nausea, abdominal discomfort, vomiting[(26)]. For other subjects data were not complete because of an insufficient amount of plasma, refusing to give a second blood sample or moving out of the area. This did not result in imbalances with respect to important baseline characteristics. Compliance of the remaining participants was almost complete, probably because study subjects were motivated by the offer of free of charge medical care, and by weekly visits by village nurses.

In fact, retinol and riboflavin deficiencies tend to coexist in anemic pregnant women in developing countries or impoverished settings[(27)]. In a previous study [(1)], high percentages of subnormal vitamin A and subnormal riboflavin were 23% and 38% in anemic pregnant women. Vitamin A deficiency may also result in anemia in humans and animals that can be reversed only by vitamin A supplementation[(28)]. Biochemical evidence of riboflavin deficiency was documented during the third trimester of pregnancy[(29)]. Retinol or riboflavin plus iron supplementation could improve hematological status better than iron alone[(30)]. The reduced prevalence of anemia (Hb<110g/L) and ID anemia were significantly greater in the groups supplemented with retinol and /or riboflavin than in the iron group. Retinol status is a putative factor for improved iron status or iron absorption. Vitamin A and β-carotene may form a complex with iron, keeping it soluble in the intestinal lumen as well as preventing the inhibitory factors on iron absorption[(31)]. Moreover, gastrointestinal symptoms were less prevalent in the group supplemented with iron, folic acid and retinol than in the iron group, and improved well-being was more prevalent in the groups receiving additional retinol and/or riboflavin than in the iron group.

Although fluorescence polarization was used to assess membrane fluidity in past decades, now it is frequently applied in oxidative stress[32], erythrocyte aggregation[33], understanding of membrane protein function[34], etc. Especially the noninvasiveness of the method makes it suitable for clinical applications[35]. Pregnancy is a condition exhibiting increased susceptibility to oxidative stress. In normal pregnancy, the implantation process, proliferation, differentiation, and trophoblast invasion, produce reactive oxygen species[36,37]. Transitional metals, especially iron, which are particularly abundant in the placenta, are important in the production of these free radicals[38]. Increased iron levels may be responsible for placental oxidative stress and abnormalities in antioxidants[39]. Iron overload could promote the generation of free radicals and result in cellular damage[12], and iron supplementation with 100mg/d or overload might be potential risk to increase MDA levels or oxidative stress in the maternal plasma and the placenta during pregnancy[40]. But low doses of oral ferrous iron (36mg/d) did not unfavorably change the physiological pattern of parameters of oxidation[41]. However, in our stduy, hemotological status has been improved and MDA levels decreased in anemic pregnant women after iron supplementation daily with 60 mg iron, which might be attributed to the favorate supplementation with low dose of iron and a combination of three vitamins. Erythrocyte membrane fluidity improved as well, which indicates that the iron supplementation did not deteriorate, but even improved antioxidant capacity. There are no documented side effects of iron supplements below 100 mg/day[42]. Iron combined with multivitamin supplementation showed a more favorable effect than iron only in our study, and this dose of iron supplementation did not increase hemoglobin higher than the optimal concentration needed for oxygen delivery[43]. Daily supplementation of 100mg iron as ferrous sulfate was recommended during the second half of pregnancy to address the corresponding iron requirements[2], and risks associated with oxidative stress were not observed in women supplemented with 120mg iron once or twice per week[38]. Suggested guidelines are ferritin <30μg/L: 80-100mg ferrous iron daily, for which there are no documented side effects[42].

Several studies have shown that micronutrient supplementation improves the glutathione peroxidase (GSH-Px) activities and decreases levels of malondialdehyde(MDA)[44,20]**,** and antioxidant supplementation was associated with better maternal and perinatal outcome in pregnant women with low antioxidant status than supplementation with iron and folate alone[45]. However, we did not find any change of SOD activity during the trial. Some studies showed similar results[46] and even a decrease of SOD activity after antioxidant supplementation[47]. This aspect deserves further study. In our study, antioxidant defenses and oxidative stress appear to be favorably modulated by iron supplementation alone. Such findings have not been reported in anemic pregnant women before, and further mechanistic studies are to be expected.

Taken together, subjects had more antioxidative capacity, i.e. lower levels of circulating lipid peroxidation products (MDA), and higher GSH-Px activity and higher vitamin concentrations after 2 months of supplementation. The improvement of erythrocyte membrane fluidity, as apparent in decreases of polarization (ρ) and microviscosity (η), indicates that the RBCs accumulate less oxidative lesions. Moreover, the increase in the plasma antioxidant status could contribute to the prevention of polyunsaturated fatty acid peroxidation of erythrocytes. This evidence could also explain the lower peroxidation found in subjects supplemented with iron and vitamins in comparison with the control. Iron combined with multivitamin supplementation showed a more favorable effect than iron only. We could not find any indication of an increase of oxidative stress after iron supplementation. On the contrary, a moderate dose of iron, preferably combined with multivitamin supplementation may be beneficial both by improving anemia and decreasing the status of oxidative stress during pregnancy.

Acknowledgements

We thank Nestle Foundation and Danone Nutrition Institute China for the financial support. A.G. M. and E.G.S designed the intervention study. Y.Y.S., F. Y., F. Z. Z. and D. C. J. conducted investigation in field sites, data collection, analyses and interpretation, laboratory analyses. X.X.H. analyzed data. A.G.M., E.G.S. and F.J.K. wrote the paper.

There are no conflicts of interest.

References

1. Ma AG, Schouten EG, Wang Y, et al.(2009) Anemia prevalence among pregnant women and birth weight in five areas in China. Med Prin Pract 18, 368-372.
2. Schümann K, Ettle T, Szegner B, et al.(2007) On risks and benefits of iron supplementation recommendations for iron intake revisited. J Trace Elem Med Biol21,147-168.
3. Beard JL& Dawson HD. Iron. In: O'Dell BL, Sunde RA, editors (1997) Handbook of nutritionally essential elements. New York: Marcel Dekker Inc.
4. Stoltzfus RJ& Dreyfuss ML. (1998) Guidelines for the Use of Iron Supplements to Prevent and Treat Iron Deficiency Anemia. Washington, DC: International Nutritional Anemia Consultative Group, International Life Sciences Institute.
5. Galaris D&Pantopoulos K. (2008) Oxidative stress and iron homeostasis: mechanistic and health aspects. Crit Rev Clin Lab Sci45, 1-23.
6. Halliwell B& Gutteridge JC. (1985) Oxygen toxicity, oxygen radicals,transition metals and disease. Biochem J219, 1–14.
7. Lutz HU. Erythrocyte clearence (1990) In: Blood Cell Biochem, Vol.1. New York: Plenum; p81–90.
8. Srigiridhar K, Nair KM, Subramanian R, et al.(2001) Oral repletion of iron induces free radical mediated alterations in the gastrointestinal tract of rat. Mol Cell Biochem219, 91−98.
9. Uyesaka N, Hasegawa S & Schechter AN. (1992) Effects of superoxide anions on red cell deformability and membrane proteins (Abstract). Biorheology 29, 217–229.
10. Chappell L.C, Seed PT, Briley AL, et al. (1999) Effect of antioxidants on the occurrence of pre-eclampsia in women at increased risk: a randomised trial. Lancet354, 810–816.
11. Poston L, Briley AL, Seed,PT, et al.(2006) Vitamin C and vitamin E in pregnant women at risk for pre-eclampsia (VIP trial): randomised placebocontrolled trial. Lancet367, 1145–1154.
12. Rumbold AR, Crowther CA, Haslam RR, et al.(2006) Vitamins C and E and the risks of preeclampsia and perinatal complications. N Engl J Med354, 1796–1806.
13. Lachili B, Hininger I, Faure H, et al. (2001) Increased lipid peroxidation in pregnant women after iron and vitamin C supplementation. Biol Trace Elem Res83, 103−110.
14. Cargnoni AA, Gregorini G, Ceconi C,et al.(1994) Lipid peroxidation in normal pregnancy and preeclampsia. Adv Exp Med Biol366, 420–423.
15. Suharno D, West CE, Karyadi MD, et al.(1993) Supplementation with vitamin A and iron for nutritional anemia in pregnant women in west Java, Indonesia. Lancet 342, 1325-1328.
16. Handelman GJ, Shen B& Krinsky NI (1992) High resolution analysis of carotenoids in

human plasma by high-performance liquid chromatography. Methods Enzymol213, 336–346.

17. Sauberlich HE, Judd JHJr, Nichoalds GE, et al.(1972) Application of the erythrocyte glutathione reductase assay in evaluating riboflavin nutritional status in a high school student population. Am JClinNutr25, 756–762.
18. Liu ZW, Han QY, Zhang N, et al.(2004) Sequential changes of serum ferritin levels and their clinical significance in lamivudine-treated patients with chronic viral hepatitis B.World J Gastroentero10, 972-976.
19. Durak I, Canbolat O, Kavutcu M,et al.(1996) Activities of total, cytoplasmic and mitochondrial superoxide dismutase enzymes in sera and pleural fluids from patients with lung cancer. J Clin Lab Anal10, 17–20.
20. Avci A, Atli T, Ergüder IB, et al.(2008)Effects of garlic consumption on plasma and erythrocyte antioxidant parameters in elderly subjects. Gerontology54, 173–176.
21. Xue ML, Hou JM, Zhang XZ, et al. (2006) Effect of Kiwifruit extracts on plasma lipids and erythrocyte membrane fluidity in hyperlipidemic rats. Acta Nutrimenta Sinica 28, 350-351.
22. Sun YY, Ma AG & Zhang XZ. (2005) Affects of Vitamin C on the antioxidant ability and erythrocyte membrane fluidity in guinea pig.Wei Sheng Yan Jiu34, 329-330.
23. Ortiz GG, Pacheco-Moisés F, El Hafidi M, et al. (2008) Detection of membrane fluidity in submitochondrial particles of platelets and erythrocyte membranes from Mexican patients with Alzheimer disease by intramolecular excimer formation of 1,3 dipyrenylpropane. Dis Markers24, 151–156.
24. Tang X, Xia Z & Yu J. (2007) An experimental study of hemolysis induced by onion (Allium cepa) poisoning in dogs. J vet Pharmacol Therap 31, 143–149.
25. Marczak A (2009) Fluorescence anisotropy of membrane fluidity probes in human erythrocytes incubated with anthracyclines and glutaraldehyde. Bioelectrochemistry 74, 236-239.
26. Ma AG, Schouten EG, Zhang FZ, et al. (2008) Retinol and riboflavin supplementation decreases the prevalence of anemia in Chinese pregnant women taking iron and folic Acid supplements. J Nutr138, 1946-1950.
27. Bates CJ, Prentice AM &Paul AA. (1994) Seasonal variations in vitamin A, C, riboflavin and folate intakes and status of pregnant and lactating women in a rural Gambian community: some possible implications. Eur J Clin Nutr 48, 660–668.
28. Bloem MW, Wedel M, Egger R, et al. (1989) Iron metabolism and vitamin A deficiency in children in the Northwest Thailand. Am J Clin Nutr 50, 332-338.
29. Pongpaew P, Saowakontha S, Schelp FP, et al.(1995) Vitamin B1, B2 and B6 during the course of pregnancy of rural and urban women in northeast Thailand. Int J Vitam Nutr Res 65,111–116.

30. Suprapto B, Widardo & Suhanantyo. (2002) Effect of low-dosage vitamin A and riboflavin on iron-folate supplementation in anaemic pregnant women. Asia Pac J Clin Nutr 11, 263–267.
31. Semba RD & Bloem MW. (2002)The anemia of vitamin A deficiency: epidemiology and pathogenesis. Eur J Clin Nutr 56, 271-281.
32. Soto-Arriaza MA, Sotomayor CP&Lissi EA.(2008) Relationship between lipid peroxidation and rigidity in L-alpha-phosphatidylcholine-DPPC vesicles. J Colloid Interface Sci323, 70-74.
33. Spengler MI, Bertoluzzo SM, Catalani G, et al.(2008) Study on membrane fluidity and erythrocyte aggregation in equine, bovine and human species. Clin Hemorheol Microcirc 38, 171-176.
34. Periasamy N, Teichert H, Weise K, et al.(2009)Effects of temperature and pressure on the lateral organization of model membranes with functionally reconstituted multidrug transporter LmrA. Biochim Biophys Acta1788, 390-401.
35. Kaneko T, Matsui H, Shimokawa O, et al.(2007) Cellular membrane fluidity measurement by fluorescence polarization in indomethacin-induced gastric cellular injury in vitro. J Gastroenterol42, 939-946.
36. Gitto E, Pellegrino S, Gitto P, et al.(2009) Oxidative stress of the newborn in the pre- and postnatal period and the clinical utility of melatonin. J Pineal Res 46, 128-139.
37. Dennery PA. (2007) Effects of oxidative stress on embryonic development. Birth Defects Res C Embryo Today81, 155-162
38. Casanueva E& Viteri EF. (2003) Iron and oxidative stress in pregnancy. J Nutr 133, 1700S−1708S.
39. Vaughan JE&Walsh SW. (2002) Oxidative stress reproduces placental abnormalities of preeclampsia. Hypertens Pregnancy21, 205-223.
40. Devrim E, Tarhan I, Ergüder IB, et al.(2006) Oxidant/antioxidant status of placenta, blood, and cord blood samples from pregnant women supplemented with iron.J Soc Gynecol Investig 13, 502-505.
41. Rehema A, Zilmer K, Klaar U, et al. (2004)Ferrous iron administration during pregnancy and adaptational oxidative stress (Pilot study). Medicina (Kaunas)40, 547-552.
42. Milman N. (2006)Iron prophylaxis in pregnancy--general or individual and in which dose?Ann Hematol 85, 821-828.
43. Yip R. (2000) Significance of an abnormally low or high hemoglobin concentration during pregnancy: special consideration of iron nutrition.Am J Clin Nutr 72, 272S-279S.
44. Zhang M, Ma AG, Zhang XZ, et al.(2005) Effect of multiple micronutrients supplementation on anti-oxidative activity and oxidized DNA damage of lymphocytes in children. Zhonghua Liu Xing Bing Xue Za Zhi26, 268-272.
45. Rumiris D, Purwosunu Y, Wibowo N, et al.(2006) Lower rate of preeclampsia after

antioxidant supplementation in pregnant women with low antioxidant status. Hypertens Pregnancy25, 241-253.

46. Oh HY, Lim S, Lee JM, et al. (2007) A combination of soy isoflavone supplementation and exercise improves lipid profiles and protects antioxidant defense-systems against exercise-induced oxidative stress in ovariectomized rats. Biofactors29, 175-185.

47. Ahmed RS, Suke SG, Seth V, et al. (2006) Impact of oral vitamin E supplementation on oxidative stress & lipid peroxidation in patients with polymorphous light eruption. Indian J Med Res 123, 781-787.

Chapter 6

Moderate NaFeEDTA and ferrous sulfate supplementation can improve both hematologic status and oxidative stress in anemic pregnant women

Han XX, Sun YY, Ma AG, Yang F, Zhang FZ, Jiang DC, Li Y.

Asia Pac J Clin Nutr. 2011;20(4):514-20.

Abstract

Background: Iron is an important element for our well being, to prevent or treat anemia, and is a cofactor of many enzymes in the antioxidant process. The effect of sodium iron ethylenediaminetetraacetate (NaFeEDTA) and ferrous sulfate on iron bioavailability and oxidative stress in anemic pregnant women was evaluated. **Methods:** The study was designed as a 2 month randomized controlled trial. 153 anemic pregnant women, with 80≤Hb<110g/L, were randomly allocated to three groups: group C (n=51) was the placebo control group, group I (n=51) was supplemented daily with 60 mg iron as ferrous sulfate, IE (n=51) with 60 mg iron as NaFeEDTA. Samples of blood were collected from subjects before and at the end of the intervention for measurements of hematological indices and oxidative stress parameters. **Results:** After the trial, considerable increases of hematologic indicators were: 20.5 g/L and 21.8 g/L for Hb (both*p* values <0.001); 4.81 μmol/L and 7.19 μmol/L for plasma iron (both*p* values <0.001), 2.63μg/L and 8.99μg/L for ferritin (both *p* values <0.05) in I and IE groups, respectively, compared with the control group. Glutathione peroxidise (GSH-Px) activities increased by 32.6 IU/ml and 75.3 IU/ml, and malondialdehyde (MDA) levels decreased by 0.70μmol/L and 1.12μmol/L in I and IE groups than in the C (all *p* values <0.05); moreover, differences of plasma iron, ferritin and GSH-Px activity were 2.38 μmol/L, 6.36μg /L and 42.7 IU/ml also significantly greater in IE than in I group. **Conclusions:** Moderate iron supplementation may be beneficial to improving iron deficiency and oxidative stress, and NaFeEDTA is better than ferrous sulfate.

INTRODUCTION

Iron deficiency is the most common and widespread nutritional deficiency in the world[1]. Prevalence of anemia is still high in rural areas in China [2]. The main causative factors are poor iron content, low bioavailability of iron, or both in the largely plant-based diets that are typically consumed in many low-income countries[3,4]. Therefore, administration of iron supplements may be indicated if iron stores are inappropriately low at the start of pregnancy or impairment of the expected increase in hemoglobin mass in the mother is to be avoided[5].

Ferrous iron, such as ferrous sulfate ($FeSO_4$) and sodium iron ethylenediamine-tetraacetate (NaFeEDTA) as two forms of iron supplements, is mostly used for correction of iron deficiency. $FeSO_4$, such as inorganic iron has been used to cure anemia since 1831. Its absorptivity is very good but it is irritating to the stomach, causing gastrointestinal side effects, such as upper abdominal discomfort, nausea, and constipation [6,7]. NaFeEDTA is an iron chelate that has been used successfully as a dietary fortifier in several trials in the developing world[8-10], as in this form, the iron is protected from inhibitors of iron absorption.

It has a bioavailability 2~ 4 times that of ferrous sulfate, especially in meals with high phytate content[11]. Therefore, the study will be expected effectively to improve both of iron deficiency and gastrointestinal side effects for iron supplementation in anemic pregnant women.

Pregnancy is a condition exhibiting increased susceptibility to oxidative stress, with both mothers and babies being exposed to oxidative stress during and after delivery [12]. Oxidative stress and damage have also been found in iron deficiency; however, increased MDA levels in the maternal plasma and the placenta in the iron-supplemented group suggests that iron supplementation may contribute to increased oxidative stress in women taking iron supplements during pregnancy [13].

Thus, pregnant women have a higher iron requirement, pregnancy or iron deficiency is a condition exhibiting increased susceptibility to oxidative stress and iron might play a role in generating harmful oxygen species. Therefore, the objective was to investigate the effect of iron supplements on hematological status and indicators of antioxidant status such as superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) and malondialdehyde (MDA) levels in anemic pregnant women during the second and third trimester of pregnancy. It was hypothesized that

NaFeEDTA would be better than ferrous sulfate at improving iron status and reducing oxidative stress

SUBJECTS AND METHODS

Subjects

The study was a 2 month double-blind randomized trial. Participants were recruited between June 2003 and December 2005 from the communities of Shen county, Shandong province, China. At the start, pregnant women, 12 to 24 -wk gestation, age range 20 - 30 y, were examined for eligibility. Finally, 153 anemic pregnant women with 80≤Hb<110 g/L, no dietary supplements during the previous 2 months and no abnormal pregnancy response, were allocated to the three groups in the order of enrollment. Group C (n=51) was the placebo control group, group I (n=51) was supplemented daily with 60 mg iron as ferrous sulfate, group IE (n=51) with 60 mg iron as NaFeEDTA. The capsules were labeled in red, yellow and blue color and manufactured by Hurun's company (a Chinese food-additive company, Beijing). Trial participants and the research team were unaware of the treatment assignment. The trial was deblinded after analysis of the primary outcomes.

After ascertainment of eligibility, consenting women were enrolled in the study, had a baseline interview and started with their allocated supplements to be taken daily for a period of two months. Women were home-visited once each week by the village nurse to replenish supplements and to monitor compliance by counting and recording the number of supplements that were taken.

The study was approved by the ethical review committees of the Medical College of Qingdao University. Written consent was given by each subject at the start of the trial. The subjects in the placebo group in our study have been given iron supplementation with NaFeEDTA or food rich in iron, such as the hemachrome-iron being from animal foodstuff, such as meat, fish and sea food, immediately after the trial.

Sample collection and laboratory analyses

Before and at the end of the intervention, overnight fasting (>12 h) blood samples (about 5mL) were collected from subjects by venipuncture into heparinized tubes between 6 and 8 o'clock in the morning; moreover, daily sample collection was evenly distributed over each of the groups. Hemoglobin concentrations were measured in heparinized blood. Plasma was separated from the remainder of the blood by centrifugation at 2000 × g for 15 min at 4°C. The samples were transported on dry-ice and stored frozen at -80 °C until analysis for measurements of hematological indices and oxidative stress parameters. The baseline and final samples were analyzed in duplicate during the same analytic run.

Hemoglobin concentration was measured by the cyanomethemoglobin method by using HemoCue for confirmation. A cutoff value of anemia was Hb<110g/L. Measurements of serum ferritin were performed by radioimmunoassay [14]. Plasma iron concentrations were analyzed by atomic absorption spectrometry on an Analyst 3100 Analyzer (Perkin Elmer Life Sciences, Wellesley, MA). The soluble transferrin receptor (sTfR) assay was performed using a commercial kit (R&D Systems, Minneapolis, MN). According to the instruction of the kit, the central 95th percentile of the reference distribution of sTfR concentration is 4.0 mg/L to 9.1 mg/L. The cutoff value of iron deficiency was >9.1mg/L. All sTfR assays were performed in duplicate. Total iron-binding capacity (TIBC) was determined by a turbidimetric method. A standard curve for each analyte was constructed from authentic standards. Standard concentrations were calculated on the basis of their known extinction coefficients.

The activities of superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) were determined in plasma as U/ml and IU/ml, respectively[15]. One unit of SOD activity was defined as the amount of protein causing 50% inhibition of the nitroblue tetrazolium salt reduction rate. The malondialdehyde (MDA) concentration was determined by using the thiobarbituric acid reaction. The MDA, an end product of fatty acid peroxidation, reacts with thiobarbituric acid to form a colored complex that has maximum absorbance at 532 nm. MDA concentrations were calculated by comparing the absorbance values of the samples with those of standard MDA solutions. Results were expressed as μmol/L blood[16]. All oxidative indicators were calculated from their standard curves.

Statistical analysis

Continuous data are presented as mean±SD or mean±SEM. Baseline variables were compared across three groups using a general linear model ANOVA. Mean changes over the intervention period and differences between groups and 95% confidence intervals (CI) were estimated for hematologic indicators and oxidative indicators and tested with Student *t*-tests. The SPSS 18.0 package was used for all analyses. A $p < 0.05$ was considered as a significance level for all tests.

RESULTS

In the intervention study, complete data were available on 147, which is 96.1% of the original number of 153, pregnant women. 6 women did not complete the trial for the following reasons: 4 moved to other villages; 2 stopped taking supplements during the trial. Moreover, compliance was excellent, because study subjects were motivated by the offer of free medical care and all women were visited weekly by village nurses, who counted leftover capsules, provided new supplies, and gave support in case of any problems or questions related to the study. At the end of the trial, there were no substantial differences between the groups in any of the baseline characteristics (**Figure 6.1 and Table 6.1**).

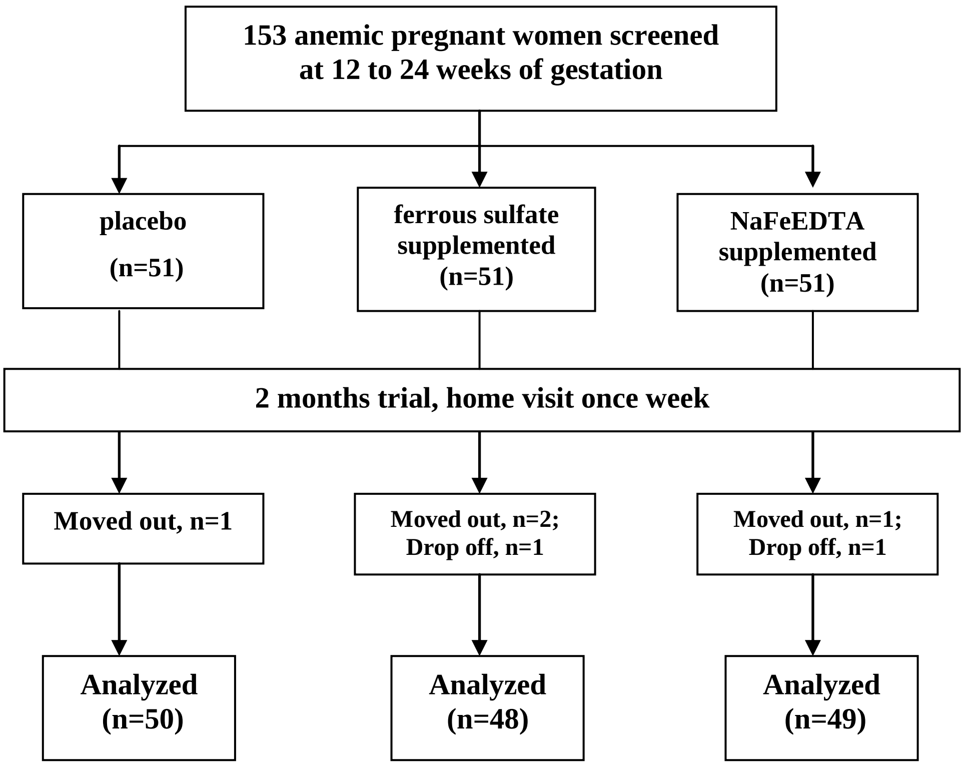

Figure 6.1 The trial profile. After ascertainment of eligibility, 153 consenting pregnant women were enrolled in the study. After 2 month trial, complete data were available on 147.There were no substantial differences between the groups in any of the baseline characteristics.

After the 2-months supplementation, significant changes in the placebo group were -3.64 g/L in Hb concentration, 0.87 mg/L in sTfR, -1.23 μmol/L in plasma iron and -1.59 μg /L in ferritin compared with baselines (all *p* values <0.05). Considerable increases in hematologic indicators were: 20.5 and 21.8 g/L for Hb (all *p* values <0.001); 4.8 and 7.2 μmol/L for plasma iron (both *p* values <0.001), 2.6 and 9.0 μg /L for ferritin (both *p* values <0.05); and decreases in TIBC: 31.4% and 26.6% (both values *p*<0.001) in I and IE groups, respectively, compared with the control group (**Table 6.2**). The differences of plasma iron and ferritin were 2.38 μmol/L and 6.37 μg /L, also significantly greater in the IE group than in the I group (both *p* values <0.001).

The changes in oxidative stress parameters are presented in **Table 6.3**. Compared with C, increases of GSH-px activities were 32.6 IU/ml and 75.3 IU/ml in I and IE groups(both *p* values <0.05), respectively; the levels of MDA decreased by 0.70 and 1.11 μmol/L in the twotreated groups (both *p* values <0.05), respectively. Moreover, the increase of GSH-Px activity was 42.7 IU/ml greater in IE group compared with I group (*p*<0.001).

Table 6.1 Characteristics of subjects at baseline

Indicator[1]	All baseline		C[2]		I[2]		IE[2]		*p*[3]
	mean	SD	mean	SD	mean	SD	mean	SD	
n	153		51		51		51		
Age, years	28.1	3.6	27.6	3.3	28.4	3.5	28.4	3.6	0.394
Gravidity	1.33	0.47	1.29	0.46	1.37	0.49	1.33	0.48	0.707
Gestational stage, wk	21.4	4.69	21.2	4.75	21.5	4.48	21.5	4.92	0.936
Hb, g/L	100.0	7.9	102.0	9.0	99.9	6.1	99.8	8.0	0.164
sTfR, mg/L	10.5	2.2	10.2	2.8	11.0	1.78	10.5	2.20	0.132
PI, µmol/L	12.2	2.93	12.7	3.13	11.9	2.00	11.9	3.43	0.275
Ferritin, µg/L	12.7	4.78	13.1	5.28	13.1	2.58	11.8	5.83	0.313
TIBC, %	80.7	11.4	78.7	10.5	81.8	10.6	81.7	11.4	0.312
MDA, µmol/L	5.21	1.53	5.11	1.25	5.54	1.88	4.97	1.34	0.137
SOD, U/ml	76.9	28.7	73.5	34.1	75.9	23.4	81.2	27.5	0.378
GSH-Px, IU/ml	117.0	26.5	111.0	26.3	119.0	26.7	121.0	25.8	0.131

[1]Indicators and abbreviations: Hb, hemoglobin concentration; PI, plasma iron; sTfR, soluble transferrin receptor; TIBC, total iron-binding capacity; MDA, malondialdehyde; SOD, superoxide dismutase; GSH-Px, glutathione peroxidase. [2]group C as placebo, group I supplemented with 60 mg iron as ferrous sulfate; group IE with 60 mg iron as NaFeEDTA; [3]p-value of the difference across treatment groups tested by one way ANOVA.

Table 6.2 Changes and differences of indicators for hematological status in three groups

Indicator[1]	Group[2]	n	Changes[3]			Difference[4]			Difference[5]		
			mean	95%CI	*P* value	mean	95%CI	*p* value	mean	95%CI	*p* value
Hb, *g/L*	C	50	-3.6	-6.6, -0.4	0.026	--	--	--	--	--	--
	I	48	19.4	16.3, 22.8	<0.001	20.5	17.3, 23.8	<0.001	--	--	--
	IE	49	20.8	17.6, 24.1	<0.001	21.8	18.9, 25.1	<0.001	1.30	-1.98, 4.58	0.435
sTfR, *mg/L*	C	50	0.87	0.14, 1.60	0.019	--	--	--	--	--	--
	I	48	-4.37	-5.10, -3.36	<0.001	-5.20	-5.94, -4.46	<0.001	--	--	--
	IE	49	-5.61	-6.35, -4.87	<0.001	-5.70	-6.44, -4.95	<0.001	0.49	-1.24, 0.26	0.196
PI, *μmol/L*	C	50	-1.23	-2.12, -0.34	0.007	--	--	--	--	--	--
	I	48	4.40	3.50, 5.30	<0.001	4.81	3.91, 5.72	<0.001	--	--	--
	IE	49	6.75	5.86,7.65	<0.001	7.19	6.29, 8.09	<0.001	2.38	1.47, 3.29	<0.001
Ferritin,*μg /L*	C	50	-1.59	-3.15, -0.02	0.047	--	--	--	--	--	--
	I	48	0.98	-0.61, 2.56	0.226	2.63	1.04, 4.22	0.001	--	--	--
	IE	49	8.62	7.05, 10.2	<0.001	8.99	7.41, 10.6	<0.001	6.36	4.76, 7.96	<0.001
TIBC, *%*	C	50	3.11	-0.31, 6.52	0.075	--	--	--	--	--	--
	I	48	-31.3	-34.8, -27.9	<0.001	-31.4	-34.8, -27.9	<0.001	--	--	--
	IE	49	-26.4	-29.8, -23.0	<0.001	-26.6	-30.0, -23.1	<0.001	4.80	1.13, 8.29	0.007

[1]Indicators and abbreviations: Hb, Hemoglobin concentration; PI, plasma iron; sTfR, soluble transferrin receptor; TIBC, total iron-binding capacity. [2]group C as placebo, group I supplemented with 60 mg iron as ferrous sulfate; group IE with 60 mg iron as NaFeEDTA; [3]Changes: value at end of trial subtracted from baseline value within group; [4]Differences: changes in the group I (or IE)- changes in the control group, espectively;[5]Differences:changes in the group IE- changes in the group I.

Table 6.3 Changes and differences of oxidative stress in MDA, GSH-Px and SOD in three groups

Indicator[1]	Group[2]	n	Changes[3]			Difference[4]			Difference[5]		
			mean	95%CI	*P* value	mean	95%CI	*p* value	mean	95%CI	*p* value
MDA, μmol/L	C	50	0.17	-0.33, 0.66	0.502	--	--	--	--	--	--
	I	48	-1.11	-1.61, -0.61	<0.001	-0.70	-1.20, -0.20	0.006	--	--	--
	IE	49	-0.91	-1.40, -0.41	<0.001	-1.12	-1.61, -0.62	<0.001	-0.41	-0.92, 0.09	0.108
SOD, U/ml	C	50	1.19	-9.87, 12.3	0.832	--	--	--	--	--	--
	I	48	0.22	-11.0, 11.4	0.696	1.47	-9.76, 12.7	0.797	--	--	--
	IE	49	-11.7	-22.8, -0.6	0.039	-5.10	-16.3, 6.07	0.370	-6.57	-17.9, 4.7	0.253
GSH-Px, IU/ml	C	50	10.6	-4.23, 25.5	0.160	--	--	--	--	--	--
	I	48	29.5	14.2, 44.3	<0.001	32.6	17.5, 47.7	<0.001	--	--	--
	IE	49	66.2	51.3, 81.1	<0.001	75.3	60.3, 90.3	<0.001	42.7	27.5, 57.9	<0.001

[1]Indicators and abbreviations: MDA, malondialdehyde; SOD, superoxide dismutase; GSH-Px, glutathione peroxidase. [2]group C as placebo, group I supplemented with 60 mg iron as ferrous sulfate; group IE with 60 mg iron as NaFeEDTA; [3]Changes: value at end of trial subtracted from baseline value within group; [4]Differences: changes in the group I (or IE)- changes in the control group, respectively; [5]Differences:changes in the group IE- changes in the group I.

DISCUSSION

After 2 months trial, both NaFeEDTA and ferrous sulfate supplementation in anemic pregnant women did not only significantly improve hematologic indicators, but also reduced oxidative stress, as shown by increased GSH-Px activities and decreased levels of MDA. An additional effect was found in iron status and GSH-Px activity for NaFeEDTA compared to ferrous sulfate supplementation.

Normal human pregnancy is considered to be a state of enhanced oxidative stress, which may play an important role in embryo development, implantation, placental development and function, foetal development, and labour in pregnancy [17]. However, pathologic pregnancies including gestational diabetes mellitus (GDM), obesity and hypertensive disorders are associated with a heightened level of oxidative stress [18-20]. Maternal undernutrition, particularly at a time that includes pregnancy, results in reduced offspring ovarian follicle numbers and may be mediated by increased ovarian oxidative stress coupled with a decreased ability to repair the resultant oxidative damage [21]. Untreated iron deficiency is deleterious, while iron overload could promote the generation of free radicals and result in cellular damage[22]. It is, therefore, important to maintain optimal iron intake [23]. Low doses of oral ferrous iron (36mg/d) did not unfavorably change the physiological pattern of parameters of oxidation[24]. Daily supplementation of 100mg iron as ferrous sulfate during the second half of pregnancy was recommended to address the corresponding iron requirements[25], and risks associated with oxidative stress were not observed in women supplemented with 120mg iron once or twice per week[26]. Suggested guidelines are 80-100mg ferrous iron daily, for which there are no documented side effects[5]. In our study, the moderate supplementation with 60mg/d iron has been beneficial to improving hematological status and oxidative stress parameters. We found that MDA levels, a marker of lipid peroxidation, were significantly decreased and GSH-Px activity increased in two supplemented groups, which indicates that the moderate iron supplementation improved oxidative status without increased deleterious effects.

Unfortunately, the hematologic status of pregnant women in the second or third trimester of pregnancy in the placebo group deteriorated after the trial, and the changes of Hb concentration, ferritin and plasma iron levels decreased, which might be contributing to blood volume expansion and iron deficiency. During the period of pregnancy, plasma volume expands by 25% to 80% of pre-pregnancy volumes[27].

Pregnant women have an increased demand for iron to expand about 30% of their erythrocyte mass and generate the iron supply to the growing fetus[28], which results in a decrease of hematocrit of about 3% to 5% between gestational weeks 20 and 30[29]. Therefore, 80-100 mg ferrous iron/day for women with iron deficiency (ID) and iron deficiency anemia (IDA) should be recommended[30].

With respect to changes of iron supplementation and oxidative stress parameters in this study, subjects also had significantly higher glutathione peroxidase activity and low level of plasma MDA after iron supplementation. This finding is in accordance with previously reported data in human and animal models[31,32]. Ferrous iron is the form that is mostly used for correction of iron deficiency, and a central pro-oxidant that propagates free radical reactions through Fenton chemistry both locally (in the gastrointestinal tract) and systemically. An excess of pro-oxidants over antioxidants results in oxidative stress, but there was no increase in the markers of oxidation or inflammation studied. Moreover, we could not find any indication of an increase of oxidative stress after iron supplementation. On the contrary, the moderate dose of iron may be beneficial both in improving anemia and decreasing the status of oxidative stress during pregnancy.

NaFeEDTA might be a better form of iron supplements than electrolytic iron [33]. Iron absorption from NaFeEDTA might be two to three times higher than from electrolytic iron [34]. NaFeEDTA increased both Hb concentration and serum ferritin concentration substantially in iron deficient populations [35]. At least 10 mg/d iron as NaFeEDTA would be necessary to prevent iron deficiency anemia, even in those populations relying for their subsistence on vegetable food only, although those suffering from severe anemia would require more than 10 mg/d [36].

Several studies showed that NaFeEDTA is more suitable than electrolytic iron in children [10,37], but few reports were related to anemic pregnant women. The study showed that iron deficiency among pregnant women untreated in the middle trimester of pregnancy might deteriorate in the third trimester in both hematologic status and oxidative stress; and moderate iron supplementation has been beneficial to improving iron deficiency and oxidative stress, and NaFeEDTA is better than ferrous sulfate. Thus, based on the results from this study, we suggest that iron supplementation with NaFeEDTA should be recommended for anemic pregnant women. Surprisingly, almost none of the subjects with poor nutritional status took dietary supplements, including iron. Our study has important public health implications. It underlines the need for a comprehensive nutritional policy for this target population. Besides improving their diet, NaFeEDTA supplementation may be worthwhile for pregnant women in rural China.

ACKNOWLEDGEMENT

This work was supported by a grant from Danone Nutrition Institute China for the financial support. We thank Han XX and Ma AG for study design and management at the field site, data collection, analyses and interpretation, laboratory analyses, and writing of the manuscript; Sun YY and Li Y for laboratory analyses, data collection in the sites, preparing the manuscript; Zhang FZ and Yang F for co-investigator at the field site and data collection; Jiang DC for overall management of the study, Shen county, China. And all authors have read and approved the final manuscript.

AUTHORS DISCOSURES

Han XX, Sun YY, Ma AG, Yang F,Zhang FZ, Jiang DC and Li Y, no conflicts of interest.

References

1. World Health Organization: Iron deficiency anemia. assessment, prevention and control. Geneva, Switzerland: WHO;2001.
2. Ma AG, Schouten E, Wang Y, Xu RX, Zheng MC, Li Y, Sun YY, Wang QZ. Anemiaprevalence among pregnant women and birth weight in five areas in China. Med Prin Pract. 2009;18:368-372.
3. Ma AG, Chen XC, Zheng MC, Wang Y, Xu RX, Li JS. Iron status and dietary intake of Chinese pregnant women with anemia in the third trimester. Asia Pac J Clin Nutr. 2002;11:171-175.
4. Mendoza C, Viteri FE, Lönnerdal B, Raboy V, Young KA, Brown KH.Absorption of iron from unmodified maize and genetically altered, low-phytate maize fortified with ferrous sulfate or sodium iron EDTA. Am J Clin Nutr. 2001;73:80-85.
5. Milman N.Iron prophylaxis in pregnancy--general or individual and in which dose?Ann Hematol. 2006;85:821-828.
6. Hallberg L, Ryttinger L, Solvell L. Side-effects of oral iron therapy. A double-blind study of different iron compounds in tablet form. Acta Med Scand Suppl 1966;459:3–10.
7. Solvell L. Oral iron therapy-side effects. In: Hallberg L, Harwerth HG, Vannotti A, eds. Iron deficiency: pathogenesis, clinical aspects, therapy. 1st ed. London: Academic Press, 1970:573–583.
8. Ballot DE, MacPhail AP, Bothwell TH, Gillooly M, Mayet FG. Fortification of curry powder with NaFe(III)EDTA in an iron deficient population: report of a controlled iron-fortification trail. Am J Clin Nutr. 1989;49:162–169.
9. Viteri FE, Alvarez E, Batres R, Torún B, Pineda O, Mejía LA, Sylvi J.. Fortification of sugar with iron sodium ethylenediaminotetraacetate (FeNaEDTA) improves iron status in semirural Guatemalan populations. Am J Clin Nutr. 1995;61:1153–1163.
10. Andang'o PEA, Osendarp SJM, Ayah R, West CE, Mwaniki DL, De Wolf CA, Kraaijenhagen R, Kok FJ, Verhoef H. Efficacy of iron-fortified whole maize flour on iron status of schoolchildren in Kenya: a randomised controlled trial. Lancet 2007; 69:1799–1806.
11. Bothwell TH, MacPhail AP. The potential role of NaFeEDTA as an iron fortificant. Int J Vitam Nutr Res. 2004;74:421–434.
12. Gveric-Ahmetasevic S, Sunjic SB, Skala H, Andrisic L, Stroser M, Zarkovic K, Skrablin S, Tatzber F, Cipak A, Jaganjac M, Waeg G, Gveric T, Zarkovic N. Oxidative stress in small-for-gestational age (SGA) term newborns and their mothers. Free Radic Res. 2009;43:376-384.

13. Devrim E, Tarhan I, Ergüder IB, Durak I. Oxidant/antioxidant status of placenta, blood, and cord blood samples from pregnant women supplemented with iron. J Soc Gynecol Investig. 2006;13:502-505.

14. Liu ZW, Han QY, Zhang N, Kang W. Sequential changes of serum ferritin levels and their clinical significance in lamivudine-treated patients with chronic viral hepatitis B.World J Gastroenterol. 2004;10:972-976.

15. Durak I, Canbolat O, Kavutcu M, Öztürk HS,Yurtarslani Z. Activities of total, cytoplasmic and mitochondrial superoxide dismutase enzymes in sera and pleural fluids from patients with lung cancer. J Clin Lab Anal. 1996;10:17–20.

16. Ohkawa H, Ohishi N, Yagi K. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal Biochem. 1979; 95:351–358.

17. Burton GJ, Jauniaux E. Oxidative stress.Best Pract Res Clin Obstet Gynaecol.2011; 25:287-299.

18.Lappas M, Hiden U, Froehlich J, Desoye G, Haugel-de Mouzon S, Jawerbaum A. The role of oxidative stress in the pathophysiology of gestational diabetes mellitus.Antioxid Redox Signal. 2011;15:3061-3100.

19. Zavalza-Gómez ABArch. Obesity and oxidative stress: a direct link to preeclampsia?Gynecol Obstet.2011;283:415-422.

20. Fabry IG, Richart T, Chengz X, Van Bortel LM, Staessen JA.Diagnosis and treatment of hypertensive disorders during pregnancy.Acta Clin Belg.2010;65:229-236.

21. Bernal AB, Vickers MH, Hampton MB, Poynton RA, Sloboda DM. Maternal Undernutrition Significantly Impacts Ovarian Follicle Number and Increases Ovarian Oxidative Stress in Adult Rat Offspring.PLoS One. 2010;5:e15558.

22. Rumbold AR, Crowther CA, Haslam RR, Dekker GA, Robinson JS. Vitamins C and E and the risks of preeclampsia and perinatal complications. N Engl J Med 2006;354:1796–1806.

23. Walter PB, Knutson MD, Paler-Martinez A, Lee S, Xu Y, Viteri FE, Ames BN. Iron deficiency and iron excess damage mitochondria and mitochondrial DNA in rats. Proc Natl Acad Sci USA. 2002; 99:2264-2269.

24.Rehema A, Zilme, K, Klaar U, Karro H, Kullisaar T, Zilmer M.Ferrous iron administration during pregnancy and adaptational oxidative stress (Pilot study). Medicina (Kaunas). 2004; 40:547-552.

25.Schümann K, Ettle T, Szegner B, Elsenhans B, Solomons NW. On risks and benefits of iron supplementation recommendations for iron intake revisited. J Trace Elem Med Biol. 2007;21:147-168.

26. Casanueva E, Viteri EF. Iron and oxidative stress in pregnancy. J Nutr. 2003; 133:1700S−1708S.

27. Pirani BBK, Campbell DM, MacGilivray I. Plasma volume expansion during normal first pregnancy. J Obstet Br Commonw. 1973;80:804-8.

28. Bothwell TH. Iron requirements in pregnancy and strategies to meet them. Am J Clin Nutr. 2000;72: 257S–264S.

29.von Tempelhoff GF, Heilmann L, Rudig L, Pollow K, Hommel G, Koscielny J. Mean maternal second-trimester hemoglobin concentration and outcome of regnancy: a population-based study. Clin Appl Thromb Hemost. 2008;14:19-28.

30.Milman N, Byg KE, Bergholt T, Eriksen L, Hvas AM. Body iron and individual iron prophylaxis in pregnancy--should the iron dose be adjusted according to serum ferritin? Ann Hematol. 2006;85:567-573.

31.Cazzola R, Russo-Volpe S, Cervato G, Cestaro B. Biochemical assessments of oxidative stress, erythrocyte membrane fluidity and antioxidant status in professional soccer players and sedentary controls. Eur J Clin Invest . 2003;33:924-930.

32. Oh HY, Lim S, Lee JM, Kim DY, Ann ES, Yoon S. A combination of soy isoflavone supplementation and exercise improves lipid profiles and protects antioxidant defense-systems against exercise-induced oxidative stress in ovariectomized rats. Biofactors. 2007;29:175-785.

33. Zou K, Gong JS, Yanagisawa K, Michikawa M.A novel function of monomeric amyloid beta-protein serving as an antioxidant molecule against metal-induced oxidative damage.J Neurosci. 2002;22:4833-4841.

34. Hurrell RF. Fortification: overcoming technical and practical barriers.J Nutr 2002;132:806S-812S.

35. Wang B, Zhan S, Xia Y, Lee L.Effect of sodium iron ethylenediaminetetra-acetate (NaFeEDTA) on haemoglobin and serum ferritin in iron-deficient populations: a systematic review and meta-analysis of randomised and quasi-randomised controlled trials. Br J Nutr. 2008;100:1169-1178.

36. Martínez-Torres C, Romano EL, Renzi M, Layrisse M. Fe(III)-EDTA complex as iron fortification. Further studies. Am J Clin Nutr. 1979;32:809-816.

37. Huang J, Sun J, Li WX, Wang LJ, Wang AX, Huo JS, Chen JS, Chen CM. Efficacy of different iron fortificants in wheat flour in controlling iron deficiency.Biomed Environ Sci. 2009;22:118-121.

Chapter 7

General Discussion

According to a World Health Organization (WHO) review of nationally representative surveys from 1993 to 2005, 42% of pregnant women have anemia worldwide. Almost 90% of them reside in Africa or Asia (1). Chinese pregnant women are probably seriously at risk of anemia (2) and micronutrient deficiency (3), especially in the rural areas; but information is still insufficient, and the optimal treatment should be determined (4). Although being an essential trace element, iron plays a central role in generating harmful oxygen species. In rats, both iron deficiency and excess result in free radical mitochondrial damage (5), however, whether iron deficiency or iron supplementation in anemic pregnant women result in an increase of oxidative stress is unclear.

The general aim of this thesis was to investigate the prevalence of anemia, and micronutrient status in Chinese pregnant women, and to examine effects of supplementation with iron and other micronutrients on anemia and micronutrient status, and on oxidative stress. In this final chapter, we will firstly provide a brief overview of the main findings of the research; secondly, methodological issues, including epidemiological considerations, internal validity and external validity, will be discussed. Finally, conclusions, research recommendations and the implications for public health will be given.

MAIN FINDINGS

Anemia and micronutrient status

In order to have a representative picture of anemia in Chinese pregnant women in rural areas, we carried out a cross-sectional study among 6413 pregnant women aged 24～37 in the third trimester of pregnancy in five rural areas in four provinces, situated in the east, the southeast, the northwest and the southwest of China. The overall prevalence of anemia was 58.6%, ranging 48.1%~70.5% in the five areas. In a random subset of 734 women micronutrient status was determined. We found that serum ferritin levels were lower in anemic than in nonanemic women. Subnormal serum iron, iron depletion and subnormal vitamin A and ascorbic acid were significantly more frequent in anemic subjects. Subnormal concentrations of vitamin B_2 in urine and B_{12} in serum were frequent in both anemic and non-anemic groups. These results indicate that anemia is still a severe health problem in the Chinese rural area, and deficiency or subnormal status of iron and other micronutrients are even more frequent.

Supplementation studies

Anemia

Because of the findings in the cross sectional study, we investigated the effect of supplementation with riboflavin (1mg) and retinol (2000μg) and their combination on a background of iron and folic acid in a 2 months randomized double blind trial. Hemoglobin (Hb) concentration increased in all 4 groups ($p<0.001$). The increases in the combination, retinol and riboflavin groups were respectively 5.4 g/L, 3.62 g/L and 1.78 g/L greater than in the reference group (iron and folic acid), and also the reductions in the prevalence of anemia (Hb<110g/L) and iron deficiency anemia were significantly greater in the three groups. The literature reports that ferrous sulphate supplements may cause gastrointestinal side effects, such as upper abdominal discomfort, nausea, and constipation (6,7). Our study showed that subjective well being was better, and gastrointestinal symptoms were less in the riboflavin and combination groups than in retinol and the reference group. These findings imply that retinol and riboflavin have additional effects on anemia, and that riboflavin can prevent or improve gastrointestinal side effects of the iron supplement.

Oxidative stress

Pregnancy, subnormal hematological status and iron supplementation may all increase oxidative stress. Free radicals that are produced in this situation can attack the erythrocyte membrane and decrease its fluidity. The trial we designed investigated such changes in anemic pregnant women. We found that GSH-px activities increased, and levels of MDA decreased in both iron (I), and iron combined with vitamin groups (IM), but more in IM. Erythrocyte membrane fluidity was evaluated using fluorescence polarization (ρ) and microviscosity (η), lower values of which indicate better membrane fluidity (8, 9). After the trial, the ρ and η values compared with placebo, decreased in I, IF and IM groups. The decreases were greatest in the IM group. This trial showed that a combination of retinol and riboflavin, and iron not only improves hematological status, but also oxidative stress.

NaFeEDTA supplement

Both ferrous sulphate and sodium iron ethylenediaminetetraacetate (NaFeEDTA) are used as iron supplements (10) and NaFeEDTA is thought to have superior bioavailability. We designed a trial to compare effects of NaFeEDTA and ferrous sulfate on improving hematological status and oxidative stress. During the trial, considerable increases of hematologic indicators, such as Hb, plasma iron, ferritin, were observed both in ferrous sulfate and NaFeEDTA groups, compared with the control group. Glutathione peroxidase (GSH-Px) activities were increased, and malondialdehyde (MDA) levels decreased in ferrous sulfate and NaFeEDTA groups, compared with placebo. Moreover, the effects were much better in NaFeEDTA than ferrous sulphate groups. The results indicate that deteriorating anemia in the second trimester can be improved by iron supplementation, whereas NaFeEDTA has superior effectiveness compared with ferrous sulfate.

METHODOLOGICAL ISSUES

In this section, we will discuss a few important methodological issues, in addition to what was already discussed in previous chapters. Aspects regarding study population and design in relation to internal and external validity will be addressed.

Internal validity

Cross-sectional study design

The five field sites we selected in the cross sectional study, were geographically located in the east, northwest, southeast, southwest and middle of China. Their economic level ranges from middle to poor and poverty-stricken. We believe that our results, prevalence of anemia and micronutrient status, are representative for healthy pregnant women in these five areas.

All subjects were randomly enrolled from the local population of pregnant women reporting for regular pregnancy examination. Information was collected on maternal socio-demographic characteristics and clinical history at enrollment. Duration of pregnancy was calculated on the basis of the woman's reported date of last menses as well as by clinical indication.

Intervention study design

In order to investigate effects of iron supplement ($FeSO_4$ or NaFeEDTA), and combined multiple micronutrients on hematological status, anemia and oxidative stress, we designed three supplementation trials, all carried out in Shen County, an area in the centre of China with 960,000 inhabitants (see **Figure 1.1** in **chapter 1**).

The first study (trial 1 in **Figure 4.1**) was a 2-mo double-blind randomized supplementation trial of retinol and riboflavin on a background of iron and folic acid in a two-by-two factorial design, conducted in 2002-2005 and reported in **chapter 4**. A cutoff value of Hb ≤105g/L was taken for anemia in the study, since this was expected to give a larger effect size. A total number of 366 anemic pregnant women with 80g/L<Hb≤105g/L, were selected. Of them 342 finished the trial and supplied blood for the Hb and ferritin analyses.

The recruitment for the first subject began in April, 2003. After recruitment of 172 subjects from 128 villages distributed in 8 communities, unfortunately, the trial had to be stopped because of the SARS epidemic. We were not able to follow them up according to protocol and had to give up the 172 subjects, and start all over again, including finding additional financial support. Finally, the Nestlé Foundation agreed to supplement an additional grant.

In **chapter 5**, a second intervention study (trial 2 in **Figure 5.1**) was described to examine the effects of the supplements on hematological status and oxidative stress in anemic pregnant women; which was carried out from 2004-2006. A total of 164 pregnant women, at 12 to 24 weeks gestation, anemic with hemoglobin <110g/L, were allocated to the intervention groups by order of enrollment. In the intervention study, complete data were available on 145, which is 88.4% of the original number of 164 pregnant women.

The third trial (trial 3 in **Figure 6.1**) aimed to assess whether moderate dose NaFeEDTA and ferrous sulfate supplementation can improve both hematologic status and oxidative stress in anemic pregnant women, and to confirm whether NaFeEDTA was superior (**Chapter 6**). It was conducted from 2002 to 2005. 153 anemic pregnant women, with 80≤Hb<110g/L, were randomly allocated. Finally, complete data were available on 147 women, which is 96.1% of the original number of 153.

Selection of subjects

All three trials were conducted in Shen county, China, which has a population of pregnant women of about 10.000 yearly, 60% of whom have anemia in the third trimester of pregnancy. In all three trials we used a similar study design, i.e. a 2 months randomized double-blind controlled study. The sample sizes were calculated given a two-sided significance level of 0.05 and a power of 0.80, by the formula, $n=2(((t_{2\alpha}+t_{2\beta})*SE)^2/\delta)$, ($t_{2\alpha}$=1.645, $t_{2\beta}$=1.282). Sample sizes per group are calculated for minimal relevant differences between treatment and reference. An increase of 3 g/L (δ) in IFA and IFB compared with the IF and an additional increase of 3 g/L compared with the IFA and the IFB group in **chapter 4**; and the sample size was calculated based on a difference in hemoglobin (Hb) change (δ) of about 5 g/L between the intervention groups and placebos in **chapter 5 and chapter 6**. Moreover, an addition of 5% to 10% subjects was considered for dropping out per group. In the trials in **Chapter 4, 5 and 6,** subjects were allocated to the intervention groups by registration number, for example, in **chapter 4**, subjects 1, 5, 10, 15, 20, etc. were allocated to the group receiving iron and folic acid. We assumed that the order of recruitment was random.

Compliance and dropping out

During the trials in **chapter 4, 5 and 6**, we found that 24, 19 and 6 subjects, respectively, did not complete the study for the following reasons: moved to other villages, preterm delivery. A few stopped taking supplements or dropped out without reasons. This drop out and the underlying reasons were evenly distributed over the groups. Due to restriction to the amount of blood (some subjects were not willing to give more than 3 ml of blood), we had missing data on a number of hematologic variables or antioxidative parameters. These were reasonably evenly distributed over the groups and the remaining groups were still balanced with respect to important characteristics. At the end of the trials, there were no substantial differences between the groups in any of the baseline characteristics.

The small percentages of drop out indicate excellent compliance of the subjects, probably because they were motivated by the offer of free medical care and all women were visited weekly by village nurses. During the trial, a village nurse was responsible for recruitment of the subjects and the distribution of the supplements. After ascertainment of eligibility, consenting women were enrolled in the study to receive their allocated daily supplements for a period of two months. Women were home-visited once each week by the village nurse to replenish supplements and to monitor compliance by counting and recording the number of supplements that were taken. The village nurses also provided counseling about possible side effects of taking the supplement. So we believe that compliance in our studies was excellent and that they were well supervised.

Treatments

In the trials of chapter 4, 5 and 6, the supplements were prepared in colored capsules and manufactured by Hurun' company (a Chinese food-additive company, Beijing). Trial participants and the research team were unaware of the treatment assignment. The trial was deblinded after the analysis of the primary outcomes. Moreover, the quality of the supplements was assured by storage at -20^{0}C and micronutrient levels were examined every three months, showing no appreciable changes. Although the treatment capsules unfortunately had different colors for different groups, blinding of both participants and study personnel stayed intact, since there was no need to break the code before the main analyses had been carried out. In our trial, most subjects only knew their own supplement which was provided by their village nurse. Since they lived in a remote area, contacts with other trial participants were rare, and we did not find any change of supplements between subjects in the groups.

Sample collection and analyses

Overnight fasting (>12h) blood samples in the cross sectional study, and before and at the end of the intervention trials were collected between 6 and 8 am. Moreover, daily sample collection was evenly distributed over each of the groups. Urine samples (10mL) were collected for riboflavin and creatinine analyses, and immediately aliquoted. The samples were transported on dry-ice and stored frozen at -80 °C until analysis. The baseline and final samples were analyzed in duplicate during the same analytic run in all studies reported in **chapters 2, 3, 4, 5 and 6**.

Erythrocyte membrane fluidity was evaluated using fluorescence polarization (ρ) and microviscosity (η); lower values indicate better membrane fluidity in **chapter 5**. Although fluorescence polarization was used to assess membrane fluidity in past decades, now it is frequently applied in oxidative stress (11), erythrocyte aggregation (12), understanding of membrane protein function (13), etc. Especially the noninvasiveness of the method makes it suitable for clinical applications (14). Up to now, few studies evaluated changes of both hematologic status and erythrocyte membrane fluidity after iron supplementation in anemic pregnant women, possibly because erythrocyte membrane preparation is quite laborious. However, it could be an effective biomarker for evaluating hematological status and oxidative stress simultaneously.

External validity

China has a high prevalence of anemia in pregnant women in undeveloped areas. In some counties, the prevalence of anemia may be a serious public health problem. The overall prevalence of anemia among pregnant women was 39.6 % from 1993 to 2005, and anemiaprevalence increased from the first (29.6%) to the second (33.0%) and third (56.2%) trimesters in South-EastChina, (15). These results are quite similar to our findings. Therefore, the findings of this thesis are expected to be valid for the population in other rural areas.

This cross-sectional study was conducted in geographically different areas nationwide, i.e. the east (Laixi, Qingdao), the southeast (Fuqing, Fujian), the northwest (Lanzhou), the southwest (Guiling), and the middle (Shen county) of China. These five counties are less developed rural areas. The population sizes are 0.96, 0.73, 1.23, 0.98 and 1.87 millions in Shen county, Laixi county, Qingdao; Fuqing county, Fujian; Guilin; Lanzhou; respectively. Compared with the few similar studies carried out so far (16,17), ours had a relatively wide distribution and large source population with a total of 5.77 millions. Similar to other rural areas in China, farming remains the main occupation of the local population, mainly living on a plant food diet. In line with these findings, we observed that low education or training was strongly associated with a high prevalence of anemia. The prevalence was 66.5% in primary school women in rural areas. Compared to women villagers, pregnant women in cities get more health improvement information from hospitals or medical centers, books, television, and even internet (18). In line with this, it was reported by others that the prevalence of anemia in the third trimester (56.2 %) was higher in women in rural areas who were farmers and had less education than women in towns or cities of south-east China (19). Iron deficiency is a major cause of anemia and the most prevalent nutrient deficiency among pregnant women in developing areas (20). Similarly, we found that poor heme iron diet or plant food diet in rural areas was one of most important factors causing anemia, iron deficiency, and micronutrient deficiencies as well in our previous study (21). The results of our supplementation trials are comparable to most previous supplementation trials conducted in other regions in China, showing that supplementation with iron, micronutrients and a combination significantly decreased the prevalence of anemia and improved hematological status (22) and oxidative stress (23,24).In rural areas, people typically consume a low iron diet with a lot of grain and small amounts of animal food. They also seldomly eat fresh fruits (25). Therefore iron and micronutrients supplements are necessary to prevent iron deficiency and anemia. Thus, the supplementation with a combination of iron (ferrous sulfate or NaFeEDTA) and multiple vitamins is not only effective, but also more feasible for anemic pregnant women in poor rural areas or remote areas nationwide.

In conclusion, based on internal and external comparisons, we conclude that our study population was representative for Chinese rural areas, and our findings are close to those findings in other rural regions with high prevalence of anemia in China. Moreover, supplementation trials with iron (ferrous sulfate or NaFeEDTA) and/or multiple vitamins, were beneficial to decreasing anemia and improving oxidative stress in anemic pregnant women in one of five research sites. Considering the study population, our findings from the supplementation trials should not be extrapolated to urban areas and other populations without iron and micronutrient deficiencies.

INTERPRETATION OF FINDINGS

Nutritional anemia and iron deficiency

The overall prevalence of anemia in pregnant women, was 58.6% in **chapter 2,** and anemia during pregnancy was defined according to WHO guideline (Hb<110g/L). We know that serum ferritin should be measured as a better indicator for diagnosing iron deficiency or iron deficiency anemia, but we were not able to accomplish that because the measurement of ferritin by radioimmunoassay was too expensive to be carried out in so many samples (6413). So our study aimed to investigate the prevalence of overall anemia, which includes iron-deficiency anemia (IDA), cobalamin-deficiency anemia, folate deficiency anemia, copper deficiency anemia, etc. The prevalence of nutritional anemia is considered to be related to deficiencies of multi-nutrients, and more than 85% of the nutritional anemia is iron deficiency related, or of iron combined with folate or other nutrient deficiencies.

Anemia and micronutrients

In **chapter 3**, the results showed that micronutrient status was significantly lower in anemic women than non anemic women for serum vitamin C, serum retinol and vitamin B_2 in urine. Serum subnormal vitamin A was significantly more frequent in anemic than in non anemic subjects. Although the most common cause of anemia is iron deficiency, deficiencies of vitamin B_{12}, folate, vitamin A and even zinc contribute either singly or in combination to maternal anemia (26). Women in developing countries have a high prevalence of iron deficiency but also tend to be deficient in other micronutrients such as vitamin A, folate, etc. (27). This implies that iron deficiency rarely occurs in isolation and is often accompanied by other micronutrient deficiencies (28). Makola (29) confirmed that micronutrient deficiencies are prevalent in the female population of Tanzania as well as the prevalence of anemia (63%). In our study, for retinol and ascorbic acid there were frequent marginal deficiencies in pregnant women with anemia. The extra requirement of the fetus and the mother and low intake will both have contributed. These women often show poor nutritional status, lacking sufficient dietary intake of multiple micronutrients, such as from green vegetables and animal foods. The present study indicates that anemia in pregnant women is still a severe nutrition related problem nowadays. It is hypothesized that the reasons for the difference in prevalence between the five sites were geographic differences, in unbalanced diets and poor nutritional education (30).

Role of retinol and riboflavin in anemia

An additive effect of retinol and riboflavin on hematologic parameters and anemia on top of iron and folic acid was found in **chapter 4**. This may be explained by the co-existing deficiencies of these micronutrients in our population. The effects of retinol and riboflavin appeared to be additive and we did not find evidence for interaction. This may indicate that the beneficial effects of each of the vitamins on erythropoiesis are independent. It is well known that retinol plays an important role in reducing anemia and improving iron status. Retinol enhances iron utilization by stimulating erythropoiesis and iron metabolism by raising mean red cell volume, plasma Fe concentration and total Fe-binding capacity (31,32).

Improvement of poor appetite was observed in 42.2% and 41.0% of the IFB group and IFAB group, respectively in **chapter 4**. Riboflavin is thought to improve iron uptake and absorption by improving the gastrointestinal function (33) and better appetite, which may have resulted in significantly enhanced concentration of serum iron (34). Consistently, women who took riboflavin had better appetites (subjectively) than those who did not take any riboflavin. At the same time, riboflavin is also necessary for synthesis of the globin component of hemoglobin (35).

After 2 months of supplementation in **chapter 5,** anemic pregnant women had more antioxidative capacity, i.e. lower levels of circulating lipid peroxidation products (MDA), and higher GSH-Px activity and higher vitamin concentrations, such as retinol, riboflavin, etc. Several studies have shown that micronutrient supplementation improves the glutathione peroxidase (GSH-Px) activities and decreases levels of malondialdehyde (MDA) (36,37), and antioxidant supplementation, such as vitamins A, B_{12}, C, E, folic acid, etc., was associated with better maternal and perinatal outcome in pregnant women with low antioxidant status than supplementation with iron and folate alone (38).

Iron supplementation and oxidative stress

Transitional metals, especially iron, which are particularly abundant in the placenta, are important in the production of free radicals (39). Increased iron levels may be responsible for placental oxidative stress and abnormalities in antioxidants (40). Iron overload could promote the generation of free radicals and result in cellular damage (41), but low doses of oral ferrous iron (36mg/d) did not unfavorably change the physiological pattern of parameters of oxidation (42). The question that should be addressed is whether presently recommended iron supplementation schemes and doses reduce or increase oxidative stress by correcting iron deficiency. The evidence for this is almost nonexistent. Daily supplementation of 100mg iron as ferrous sulfate was recommended during the second half of pregnancy to address the corresponding iron requirements (43), and risks associated with oxidative stress were not observed in women supplemented with 120mg iron once or twice per week. In our study in **chapter 5 and 6**, the daily dose of iron (ferrous sulphate and/or NaFeEDTA) was 60 mg. We found that the MDA levels, a marker of lipid peroxidation, were significantly decreased and GSH-Px levels increased in all supplemented groups. Erythrocyte membrane fluidity improved as well, which indicates that the iron supplementation did not deteriorate, but even improved oxidative status.

CONCLUSIONS

The prevalence of anemia in pregnant women is still high in China especially in rural areas, and no important changes in anemia among this vulnerable group have been observed in the last decade. These women often show poor nutritional status, lacking sufficient dietary intake of multiple micronutrients. Surprisingly, almost none of the subjects took dietary supplements, including iron. Our study showing additional benefits of vitamins in tackling the anemia problem has important public health implications. It underlines the need for a comprehensive nutritional policy for this target population. Besides improving their dietary intake, multiple and moderate micronutrient supplements in pregnant women in rural China should be encouraged.

Iron deficiency is prone to be accompanied by multiple micronutrients deficiency. Our study showed that hematological indicators of Hb concentration, plasma iron and ferritin, significantly decreased, and GSH-px activity decreased and MDA level increased in anemic pregnant women in the third trimester in the placebo group, compared with baseline two months before. These findings imply that iron deficiency among pregnant women untreated in the middle trimester of pregnancy might deteriorate in the third trimester in both hematologic status and oxidative stress. Iron supplementation did not increase oxidative stress in the form and supplemental levels as reported in this thesis. A moderate dose of iron, preferably combined with multivitamin supplementation is beneficial both in improving hematological status and in decreasing the status of oxidative stress during pregnancy. NaFeEDTA, as a choice of iron supplement for anemic pregnant women, showed better effect and less side effects than ferrous sulfate (44).

FURTHER RESEARCH

In this study in **chapter 2**, the distribution of hemoglobin concentrations showed that both lower (Hb <80g/L) and higher hemoglobin concentrations (Hb>140g/L) might result in deviating from the normal birth weight (2500g~4000g). Women with a high Hb concentration (Hb>130 g/L) had 20% higher risk of preterm delivery and 50 % higher risk of low birth weight (45). But this deserves to be confirmed in further studies.

We reported the extent to which multiple deficiencies coexist in **chapter 3**, data that are scarce in rural developing country settings. The results in our study also have the potential to provide valuable reference values for assessing nutritional status. However, the assessment of vitamin and mineral status during pregnancy is complicated because there is a general lack of pregnancy-specific laboratory indices for nutritional evaluation (46). To improve this situation, networks for monitoring nutritional status of pregnant women should be established in larger rural areas nationwide.

In our study in **chapter 4**, the combination of iron and folic acid had already a strong beneficial effect. The effects of retinol and riboflavin appeared to be additive and we did not find evidence for interaction. Further studies may focus ona possible additional efficacy of higher dosages of retinol and/or riboflavin.

We did not find any change of SOD activity during the trial. Some studies showed similar results (47) and even a decrease of SOD activity after antioxidant supplementation (48, 49). This aspect deserves further study. In our study, antioxidant defenses and oxidative stress appear to be favorably modulated even by iron supplementation alone. Such findings have not been reported in anemic pregnant women before, and further mechanistic studies are to be expected.

IMPLICATIONS AND RECOMMENDATIONS

Comprehensive anemia control for pregnant women should include different strategies applied in an integrated and coordinated way. Monitoring, health education, diet improvement, and supplementation are all part of such an approach.

Network for monitoring anemia

This thesis found that anemic pregnant women often have lower levels of retinol, zinc, riboflavin, etc., than nonanemic women. Thus, once a pregnant woman is found with ID or IDA, the status of other micronutrients should be examined. Therefore, anemia in pregnant women, as a severe public health problem in rural areas, deserves urgent attention, for instance in the form of a network for monitoring anemia in pregnant women nationwide, so that cases can be treated earlier.This network can initiate large scale prevalence investigations and interventions in order to improve the situation.

Health education and training

The level of education may influence the health and nutritional status of individuals. In this thesis, we found that the prevalence of anemia was significantly different according to educational level: 52.9%, 62.4% and 66.5%, respectively for college, secondary and primary education in Table 2 in **Chapter 2**. Subjects with better education can easily get health information from reading, communicating, and internet; however, most villagers only have primary education level, and have less access to health information. Thus, a comprehensive community-based intervention including iron supplementation, health education and communication through effective strategies may improve the haematological status of pregnant women in each trimester (50), in rural areas China.

Balanced diet and supplementation

Staple grains vary from rice to wheat to corn and the main source of iron in rural areas in China is vegetables. Such diets include abundant amounts of cereals, legumes and vegetable components, such as phytate, fibres and soybean protein, which are inhibitory to non-heme iron absorption (51-53). Subjects selected in the cross sectional study were from five sites, which were developing or poor areas of the countryside. Therefore, one of the nutritional problems related to nutrient adequacy in their diets is iron deficiency, especially if soybean products are used as protein sources instead of animal products. Nevertheless, no subjects in this study took any supplement containing iron. This may imply that the general population in the areas studied was not aware of the risk of iron deficiency. Unlike Western societies, food in China is not routinely fortified with iron. Therefore, a balanced diet with food rich in iron, such as, meat or other animal food, should be supplied for pregnant women in rural areas. If this is not feasible, iron and micronutrient supplementation should be considered.

Our experimental study showed that iron combined with folic acid supplementation decreased all anemia to 29.6% and iron deficiency anemia (IDA) to 15.9%; while iron combined with folic acid, retinol and riboflavin further decreased to 15.7% all anemia and 6% IDA in **Table 4.4**; which should be referred to the additional effects of retinol and riboflavin. Moreover, retinol and riboflavin treatments increased Hb concentrations with 3.62g/L and 1.78g/L, respectively; and the change of ferritin was greater in women treated with retinol than in those that were not. A successful intervention program needs a wonderful compliance. Oral supplementation with ferrous sulfate is an effective therapy, but gastrointestinal side effects may impair treatment compliance. A few studies tried to limit side effects by substituting a ferrous sulfate prolonged release formulation (PRF) for ferrous sulfate, and by using iron supplementation by intravenous route for replacing the oral (54-56). PRF effects still need to be confirmed and the price is relatively high. Parenteral administration is not feasible for the large population in poor rural areas. However, this study supplied the evidence that riboflavin could decrease gastrointestinal side effects caused by iron supplementation alone, and NaFeEDTA may has a similar effect. Therefore, adding riboflavin to iron supplementation may be a promising scheme for treating anemia, while NaFeEDTA may be a promising substitute for sulfate supplement. Based on this evidence, our studies suggest that anemic pregnant women nationwide should take moderate dose iron and multiple micronutrients, sunch as folic acid, retinol, riboflavin, etc. supplementation in combination, which will be beneficial by reducing anemia and oxidative stress, and by increasing their well-being as well.

In conclusion, eradicating anemia from rural areas will require a more comprehensive effort including establishing a network for monitoring anemia, health education, enhancing economic development, and supplementation programmes.

References

1. Sanghvi TG, Harvey PW, Wainwright E. Maternal iron-folic acid supplementation programs: evidence of impact and implementation.Food Nutr Bull 2010;31:S100-S1007.
2. Coad J, Conlon C. Iron deficiency in women: assessment, causes and consequences.Curr Opin Clin Nutr Metab Care. 2011;14(6):625-34
3. Yang Z, Huffman SL. Review of fortified food and beverage products for pregnant and lactating women and their impact on nutritional status.Matern Child Nutr. 2011 ;7 Suppl 3:19-43.
4. Pasricha SR. Should we screen for iron deficiency anaemia? A review of the evidence and recent recommendation.Pathology 2012;44:139-147.
5. Srigiridhar K, Nair KM, Subramanian R, Singotamu L.Oral repletion of iron induces free radical mediated alterations in the gastrointestinal tract of rat. Mol Cell Biochem 2001;219:91−98.
6. Palacios S. The management of iron deficiency in menometrorrhagia.Gynecol Endocrinol 2011;27 Suppl 1:1126-1130.
7. Oriji VK, Enyindah CE, Nyeche S. Factors determining compliance to routine iron supplementation in pregnancy at the University of Portharcout Teaching Hospital.Niger J Med 2011;20:131-134.
8. Rauchová H, Vokurková M, Koudelová J. Hypoxia-induced lipid peroxidation in the brain during postnatal ontogenesis.Physiol Res 2012 ;61 Suppl 1:S89-S101;
9. Han XX, Zhang M, Ma AG, Ge S, Shi XX, Sun YY, Wang QZ, Liang H. Antioxidant micronutrient supplementation increases erythrocyte membranefluidity in adults from a rural Chinese community.Br J Nutr 2011;106:1676-1682.
10. Orozco MN, Arriaga C, Solomons NW, Schümann K. Equivalent effects on fecal reactive oxygen species generation with oral supplementation of three iron compounds: ferrous sulfate, sodium iron EDTA and iron polymaltose.Ann Nutr Metab 2012;60:108-114.
11. Soto-Arriaza MA, Sotomayor CP, Lissi EA. Relationship between lipid peroxidation and rigidity in L-alpha-phosphatidylcholine-DPPC vesicles. J Colloid Interface Sci 2008;323:70-74.
12. Spengler MI, Bertoluzzo SM, Catalani G, Rasia ML. Study on membrane fluidity and erythrocyte aggregation in equine, bovine and human species. Clin Hemorheol Microcirc. 2008;38:171-176.
13. Periasamy N, Teichert H, Weise K, Vogel RF, Winter R. Effects of temperature and pressure on the lateral organization of model membranes with functionally reconstituted multidrug transporter LmrA. Biochim Biophys Acta 2009;1788:390-401.

14.Kaneko T, Matsui H, Shimokawa O, Nakahara A, Hyodo I. Cellular membrane fluidity measurement by fluorescence polarization in indomethacin-induced gastric cellular injury in vitro. J Gastroenterol 2007;42:939-946.

15 Wang J, Ren AG, Ye RW, Zheng JC, Li S, Liu JM, Yang RL, Zhang FR, Zhang T, Li Z. Study on the third trimester hemoglobin concentrations and the risk of low birth weight and preterm delivery. Zhonghua Liu Xing Bing Xue Za Zhi 2007;28:15-18.

16.Suprapto B, Widardo, Suhanantyo. Effect of low-dosage vitamin A and riboflavin on iron-folate supplementation in anaemic pregnant women. Asia Pac J Clin Nutr 2002;11:263–267.

17.Iannotti LL, O'Brien KO, Chang SC, Mancini J, Schulman-Nathanson M, Liu S, Harris ZL, Witter FR. Iron Deficiency Anemia and Depleted Body Iron Reserves Are Prevalent among Pregnant African-American Adolescents. J Nutr 2005;135: 2572–2577.

18. Wang J, Zhao L, Piao J, Zhang J, Yang X, Yin S. Nutrition and health status of pregnant women in 8 provinces in China.Wei Sheng Yan Jiu. 2011;40:201-203

19. Jin L, Yeung LF, Cogswell ME, Ye R, Berry RJ, Liu J, Hu DJ, Zhu L. Prevalence of anaemia among pregnant women in South-East China, 1993-2005.Public Health Nutr. 2010;13:1511-8.

20. Choudhury N, Aimone A, Hyder SM, Zlotkin SH. Relative efficacy of micronutrient powders versus iron-folic acid tablets in controlling anemia in women in the second trimester of pregnancy.Food Nutr Bull. 2012 ;33:142-149.

21. Ma AG, Chen XC, Wang Y, Xu RX, Zheng MC, Li JS. The multiple vitamin status of Chinese pregnant women with anemia and nonanemia in the last trimester.J Nutr Sci Vitaminol (Tokyo). 2004;50:87-92.

22. Hovdenak N, Haram K. Influence of mineral and vitamin supplements on pregnancy outcome. Eur J Obstet Gynecol Reprod Biol. 2012;164:127-132.

23, Rumiris D, Purwosunu Y, Wibowo N, Farina A, Sekizawa A. Lower rate of preeclampsia after antioxidant supplementation in pregnant women with low antioxidant status.Hypertens Pregnancy. 2006;25:241-253.

24. Serdar Z, Gür E, Develioğlu O. Serum iron and copper status and oxidative stress in severe and mild preeclampsia.Cell Biochem Funct. 2006 ;24:209-215.

.25. Ma A, Chen X, Zheng M, Wang Y, Xu R, Li J. Iron status and dietary intake of Chinese pregnant women with anaemia in the third trimester.Asia Pac J Clin Nutr. 2002;11:171-175.

26.Van den, Broek NR, Letsky EA. Etiology of anemia in pregnancy in South Malawi. Am J Clin Nutr 2000;72:247S–256S.

27.International Anemia Consultative Group (INACG). Iron deficiency in women. Washington, D.C. The Nutrition Foundation 1981: 5-36.

28.Azais-Braesco V and Pascal G. Vitamin A in pregnancy: requirements and safety limits. Am J Clin Nutr 2000;71:1325S–1333S.

29.Makola D, Ash DM, Tatala SR, Latham MC, Ndossi G and Mehansho H. A Micronutrient-Fortified Beverage Prevents Iron Deficiency, Reduces Anemia and Improves the Hemoglobin Concentration of Pregnant Tanzanian Women1. J Nutr 2003;133:1339–1346.

30.Ma AG, Chen XC, Zheng MC, Wang Y, Xu RX and Li JS. Iron status and dietary intake of Chinese pregnant women with anemia in the third trimester. Asia Pacific J of Clin Nutr 2002;11:171-175.

31.Roodenburg AJ, West CE, Hovenier R, Beynen AC. Supplemental vitamin A enhances the recovery from iron deficiency in rats with chronic vitamin A deficiency. Br J Nutr 1996;75:623-636.

32.Fishman SM, Christian P, West KP. The role of vitamins in the prevention and control of anaemia. Public Health Nutr 2000;3:125–150.

33.Yates CA, Evans GS, Pearson T, Powers HJ. (2003) Absence of luminal riboflavin disturbs early postnatal development of the gastrointestinal tract .Digestive Diseases and Sciences 2003;48:1159–1164.

34. Zhao L, Wang S, Jia J, Jia F, Liu C, Liu A, Ping B, Zhai F. Impact of supplementation of vitamins and minerals on nutritional status of women in suburb. Wei Sheng Yan Jiu2003;32:459-461.

35.Fawzi WW, Msamanga GI, Kupka R, Spiegelman D, Villamor E, Mugusi F, Wei R, Hunter D. Multivitamin supplementation improves hematologic status in HIV-infected women and their children in Tanzania. Am J Clin Nutr 2007;85:1335-1343.

36.Zhang M, Ma AG, Zhang XZ, Ge S, Shi XX. Effect of multiple micronutrients supplementation on anti-oxidative activity and oxidized DNA damage of lymphocytes in children. Zhonghua Liu Xing Bing Xue Za Zhi 2005;26:268-272.

37.Avci A, Atli T, Ergüder IB, Varli M, Devrim E, Aras S, Durak I.Effects of garlic consumption on plasma and erythrocyte antioxidant parameters in elderly subjects. Gerontology 2008;54:173–176.

38.Rumiris D, Purwosunu Y, Wibowo N, Farina A, Sekizawa A. Lower rate of preeclampsia after antioxidant supplementation in pregnant women with low antioxidant status. Hypertens Pregnancy 2006;25:241-253.

39.Casanueva E, Viteri EF. Iron and oxidative stress in pregnancy. J Nutr 2003;133:1700S−1708S.

40.Vaughan JE, Walsh SW. Oxidative stress reproduces placental abnormalities of preeclampsia. Hypertens Pregnancy 2002;21:205-223.

41.Rumbold AR, Crowther CA, Haslam RR, Dekker GA, Robinson JS. Vitamins C and E and the risks of preeclampsia and perinatal complications. N Engl J Med 2006;354:1796–1806.

42.Rehema A, Zilmer K, Klaar U, Karro H, Kullisaar T, Zilmer M. Ferrous iron administration during pregnancy and adaptational oxidative stress (Pilot study). Medicina (Kaunas) 2004;40:547-552.

43.Schümann K, Ettle T, Szegner B, Elsenhans B, Solomons NW. On risks and benefits of iron supplementation recommendations for iron intake revisited. J Trace Elem Med Biol 2007;21:147-168.

44.Huo JS, Yang XG, Piao JH, Gao JQ, Miao H, Yu B, Lu CQ, Chen JS. NaFeEDTA fortifiedsoy sauce showed higher iron absorption rate in Chinese females.Biomed Environ Sci 2007;20:126-130.

45.Chang SC, O'Brien KO, Nathanson MS, Mancini J and Witter FR. Hemoglobin concentrations influence birth outcomes in pregnant African-American adolescents. J Nutr 2003; 133:2348-2355.

46.Jiang T, Christian P, Khatry SK, Wu L and West LP. Micronutrient Deficiencies in Early Pregnancy Are Common, Concurrent, and Vary by Season among Rural Nepali Pregnant Women. J Nutr 2005;135:1106–1112.

47.Oh HY, Lim S, Lee JM, Kim DY, Ann ES, Yoon S. A combination of soy isoflavone supplementation and exercise improves lipid profiles and protects antioxidant defense-systems against exercise-induced oxidative stress in ovariectomized rats. Biofactors 2007;29:175-185.

48.Ahmed RS, Suke SG, Seth V, Jain A, Bhattacharya SN, Banerjee BD. Impact of oral vitamin E supplementation on oxidative stress & lipid peroxidation in patients with polymorphous light eruption. Indian J Med Res 2006;123:781-787.

49.Zhang M, Ma AG, Zhang XZ, Ge S, Shi XX. Effect of multiple micronutrients supplementation on anti-oxidative activity and oxidized DNA damage of lymphocytes in children. Zhonghua Liu Xing Bing Xue Za Zhi 2005;26:268-272.

50. Abel R, Rajaratnam J, Kalaimani A, Kirubakaran S. Can iron status be improved in each of the three trimestre? A community based study. Eur J Clin Nutr 2000; 54: 490-493.

51. Cook JD, Morck TA, Lynch SR. The inhibitory effect of soy products on nonheme iron absorption in man. Am J Clin Nutr 1981;34: 2622–2629.

52 Hallberg L, Rossander L. Effect of soy protein on nonheme iron absorption in man. Am J Clin Nutr 1982; 36: 514–520.

53. Schriker BR, Miller DD, Van Campen D. In vivo estimation of iron availability in meals containing soy products. J Nutr 1982;112:1696–1705.

54. Zaim M, Piselli L, Fioravanti P, Kanony-Truc C. Efficacy and tolerability of a prolonged release ferrous sulphate formulation in iron deficiency anaemia: a non-inferiority controlled trial.Eur J Nutr 2012;51:221-229.

55.Oriji VK, Enyindah CE, Nyeche S. Factors determining compliance to routine iron supplementation in pregnancy at the University of Portharcout Teaching Hospital.Niger J Med 2011;20:131-134.

56. Rizvi S, Schoen RE. Supplementation with oral vs. intravenous iron for anemia with IBD or gastrointestinal bleeding: is oral iron getting a bad rap?Am J Gastroenterol 2011;106:1872-1879.

Summary

Summary

Anemia is the scourge of the third world. Pregnant women are at risk especially during the last trimester of pregnancy. The prevalence of anemia (Hb<110g/L) in Chinese pregnant women in the third trimester was 48% in parts of urban areas during 1995-2000. Notwithstanding the fact that the diagnosis of anemia is fairly simple and the treatment is cheap, the overall prevalence of anemia was 39.6% among pregnant women from 1993 to 2005, and 29.6% in the first, 33.0% in the second and 56.2% in the third trimester in Jiangsu and Zhejiang provinces with top-economic level in China, averaged over a period of 12 years. In rural areas, anemia may remain an even more severe public health problem among pregnant women in the third trimester because of lack of iron intake during the second and the third trimester of gestation, but also due to micronutrient deficiencies. Therefore, a cross-sectional study was carried out to investigate the situation among pregnant women in rural areas of China. Furthermore we conducted three iron and micronutrient supplementation trials, in order to assess the effect on anemia, micronutrient status and to evaluate possible adverse effects.

The prevalence of iron deficiency anemia (IDA), and deficiencies of micronutrients in pregnant women in rural areas nationwide is not well known. From 1999 to 2003, we conducted a large population based study in five rural areas, i.e. Qingdao in the east, Fuzhou in the southeast, Lanzhou in the northwest, Guilin in the southwest, and a suburban district of Liaocheng in the middle of China, which are less developed rural areas with different geographical conditions. In total, 6413 pregnant women were recruited and the overall prevalence of anemia was 58.6%, ranging 48.1%~70.5% in the five areas (**Chapter 2)**.

In addition, we simultaneously evaluated micronutrient status of a random subset of the subjects, in order to confirm whether deficiencies of multiple micronutrients co-exist in anemic pregnant women. The findings showed that micronutrient status was lower in anemic women than non anemic women. Subnormal serum iron (<700µg/L) and iron depletion (ferritin<12µg/L) were 39.7% and 52.6% in anemic women, significantly more frequent than 23.9% and 35.0%, respectively in non-anemic subjects; as were subnormal retinol and subnormal ascorbic acid. Subnormal riboflavin and vitamin B_{12} showed a high prevalence both in anemic and non-anemic pregnant women. Thus, subnormal concentrations of iron and micronutrients in combination may contribute to the higher prevalence of anemia and iron deficiency anemia (**Chapter 3)**.

Based on the findings in **Chapter 2 and 3**, we conducted a 2-mo randomized double-blind trial among 366 anemic pregnant women (Hb<105g/L) in the period from October 2003 to May 2005. They were supplemented with retinol and/or riboflavin on top of iron and folic acid in **Chapter 4.** The increase of Hb concentration was greatest in the combination group (5.4g/L). The residual prevalence of anemia after supplementation with iron and folic acid alone was halved by adding retinol and riboflavin. We also observed favorable effects of riboflavin and retinol on gastrointestinal symptoms like nausea, abdominal discomfort and vomiting often caused by ferrous sulphate and well-being. Thus, a combination of iron, folic acid, retinol and riboflavin was more effective than iron plus folic acid alone.

Pregnancy and iron (over load) might enhance levels of oxidative stress produced by reactive oxygen species (ROS), which is harmful to the fetus and the pregnant woman and may cause a reduction in erythrocyte membrane fluidity. We carried out a 2-mo supplementation trial among 164 anemic pregnant women in 2004-2006. Four groups were compared: placebo; iron; iron plus folic acid; iron plus folic acid, retinol and riboflavin (**Chapter 5**). Considerable improvements of hematological parameters, and significant increase of glutathione peroxidase activities (GSH-px) and decrease of malondialdehyde (MDA) were observed in all three intervention groups compared to the placebo. Moreover, fluorescence polarisation (ρ) and microviscosity (η), indicators of membrane fluidity decreased significantly after 2 months of supplementation. Supplementation with iron and particularly in combination with vitamins improved hematologic status as well as oxidative stress and erythrocyte membrane fluidity.

In any context of iron supplementation in the prenatal prophylaxis or therapeutic dosage range, iron supplements might cause increased oxidation. Therefore, a third trial was conducted among subjects of 153 anemic pregnant women, from 2003 to 2005 (**Chapter 6**). Its aim was to compare effects of moderate amounts of two often used forms of iron supplements, i.e. ferrous sulphate ($FeSO_4$) and NaFeEDTA, in improving hematological status and oxidative stress. We found that iron deficiency among pregnant women untreated in the second trimester of pregnancy

deterioratedin the third trimester both with respect to hematologic status and oxidative stress. However, after iron supplementation, hematologic indicators, such as plasma iron, Hb and ferritin, considerably increased; and also GSH-Px activities increased and MDA levels decreased in both intervention groups compared with placebo. Sodium iron EDTA supplementation induced significantly greater changes in plasma iron, ferritin and GSH-Px activity. Thus, iron supplementation seems to improve both iron deficiency and oxidative stress in anemic pregnant women, and NaFeEDTA performs better than ferrous sulfate.

In conclusion, this thesis is a series of studies, involving descriptive studies and supplementation trials. Anemia appeared to be a severe public health problem in pregnant women in poor rural areas. We observed favorable effects of iron supplementation and even better of iron combined with other micronutrients on anemia and on measures of oxidative stress. Thus, our study has important public health implications. It underlines the need for a comprehensive nutritional policy for this target population. Besides improving their dietary intake, multiple micronutrients in pregnant women in rural China should be recommended.

Samenvating

Anemie (bloedarmoede) is de gesel van de derde wereld. Zwangere vrouwen zijn hier vatbaar voor, vooral in het laatste trimester van de zwangerschap. Tussen 1995 en 2000 was de prevalentie van anemie (Hb<110g/L) in urbane gebieden bij Chinese vrouwen in het derde trimester van de zwangerschap 48%. Ondanks het feit dat de diagnose van anemie vrij eenvoudig is te stellen en de behandeling goedkoop, was de algehele prevalentie van anemie onder zwangere vrouwen in Jiangsu en Zhejiang, provincies met het hoogste economische niveau, over de periode 1993-2005, gemiddeld 39,6%. Dit was 29,6 in het eerste, 33,0 in het tweede en 56,2 in het derde trimester. In rurale gebieden is anemie mogelijk zelfs nog steeds een erstiger volksgezondheidsprobleem bij zwangere vouwen in het derde trimester vanwege onvoldoende ijzerinname, maar ook als gevolg van andere micronutriënt deficiënties. Om die reden hebben we een dwarsdoorsnede-onderzoek uitgevoerd om de situatie bij zwangere vrouwen in rurale gebieden in China te onderzoeken. Bovendien hebben we drie suppletietrials uitgevoerd met ijzer en micronutrienten om het effect hiervan op anemie en micronutriëntenstatus vast te stelen en om mogelijke bijwerkingen te evalueren.

De prevalentie van ijzergebreksanemie en micronutientendeficiënties bij zwangere vrouwen in rurale gebieden op nationale schaal is niet goed bekend. Van 1999 tot 2003 hebben we een grote populatiestudie uitgevoerd in vijf rurale gebieden, namelijk Qingdao in het oosten, Fuzhou in het zuidoosten, Lanzhou in het noordwesten, Guilin in het zuidwesten en een buitengebied van Liaocheng in centraal China. Dit zijn allemaal onderontwikkelde gebieden met verschillende geografische condities. In totaal 6413 zwangere vrouwen werden gerecruteerd, bij wie de prevalentie van anemie overall 58,6 was met een range van 48,1 tot 70.5 in de vijf gebieden (Hoofdstuk 2).

Tevens evalueerden we bij een random subgroep van de deelneemsters de micronutriëntenstatus, om vast te stellen of deficiënties van meerdere nutriënten tezamen voorkomen bij zwangere vrouwen met anemie. De bevindingen lieten zien dat de micronutriëntenstatus van anemische zwangere vrouwen lager was dan die van niet-anemische vrouwen. De prevalenties van verlaagd serum ijzer (<700μg/L) en ijzerdepletie (ferritine<12μg/L) waren bij anemische vrouwen met 39,7% en 52,6% significant hoger dan bij niet-anemische vrouwen met respectievelijk 23,9% en

35,0%, evenals verlaagd serum retinol en ascorbinezuur. Verlaagd riboflavine en vitamine B12 kwamen frequent voor bij zowel anemische als niet-anemische zwangere vrouwen. Derhalve dragen subnormale concentraties ijzer en andere micronutriënten mogelijk bij aan de hoge prevalentie van anemie (Hoofdstuk 3).

Op basis van de bevindingen in Hoofdstuk 2 en 3, hebben we een twee maanden durende dubbelblinde trial uitgevoerd bij 366 anemische zwangere vrouwen (Hb<105g/L) in de periode van oktober 2003 tot mei 2005. De vrouwen kregen supplementen met retinol en/of riboflavine, dan wel placebo, tegen een achtergrond van ijzer en foliumzuur (Hoofdstuk 4). De stijging van de Hb concentratie was het grootst in de combinatiegroep (5,4g/L). De prevalentie van anemie die overbleef na suppletie met alleen ijzer en foliumzuur, werd gehalveerd bij toevoegen van retinol en riboflavine. We vonden ook gunstige effecten van riboflavine en retinol op maag-darmverschijnselen zoals misselijkheid, buikpijn en braken, bekende bijwerkingen van ijzersulfaat, en op welbevinden. Een combinatie van ijzer, foliumzuur, retinol en riboflavine was dus effectiever dan alleen ijzer en foliumzuur.

Zwangerschap en (overdaad aan) ijzer kunnen mogelijk bijdragen aan oxidatieve stress veroorzaakt door zuurstofradicalen, Dit kan schade toebrengen aan de zwangere vrouw en het ongeboren kind en een verlaging van de membraanfluïditeit van erythrocyten veroorzaken. In 2004-2006 voerden wij een twee maanden durende suppletietrial uit bij 164 zwangere vrouwen met anemie. Vier groepen werden met elkaar vergeleken: placebo; ijzer; ijzer plus foliumzuur; en ijzer plus foliumzuur, retinol en riboflavine (Hoofdstuk 5). Aanzienlijke verbeteringen ten opzichte van placebo werden in alle drie de interventiegroepen waargenomen in hematologische prameters, en significante stijgingen in glutathion peroxidase (GSH-px) activiteit en daling van malondialdehyde (MDA). Bovendien daalden fluorescentie polarisatie (ρ) en microviscositeit (η), indicatoren van membraanfluïditeit, significant na twee maanden suppletie. Suppletie met ijzer en vooral in combinatie met de vitaminen, verbeterde zowel de hematologische status als de oxidatieve stres en de membranfluïditeit van de erythrocyten.
Prenatale ijzer suppletie, in profylactische of therapeutische doseringen, zou toegenomen oxidatie kunnen veroorzaken. Om die reden hebben we een derde trial uitgevoerd bij 153 zwangere vrouwen tussen 2003 en 2005 ((Hoofdstuk 6). Het doel

was het vergelijken van de effecten van matige hoeveelheden van twee dikwijls gebruikte ijzer supplementen (ijzersulfaat (FeSo4) en Na FeEDTA) op de hematologische status en op oxidatieve stress. We vonden dat ijzer deficiëntie bij vrouwen die in het tweede trimester van de zwangerschap niet waren behandeld verergerde in het derde trimester, zowel wat betreft hematologische status als wat betreft oxidatieve stress. Na ijzersuppletie stegen hematologische indicatoren zoals plasma ijzer, Hb en ferritine aanzienlijk. Tevens steeg de GSH-Px activiteit en daalde het MDA-niveau in beide interventiegroepen vergeleken met placebo. NaFeEDTA suppletie induceerde significant grotere veranderingen in plasma ijzer, ferritine en GSH-Px activiteit. Derhalve lijkt ijzer suppletie zowel ijzerdeficiëntie als oxidatieve stress bij zwangere vrouwen met anemie te verbeteren. NaFeEDTA doet het daarbij beter dan ijzersulfaat.

Concluderend rapporteert dit proefschrift een serie studies, beschrijvend onderzoek en suppletietrials die zijn uitgevoerd in China. Anemie blijkt een ernstig volksgezondheidsprobleem te zijn bij zwangere vrouwen in arme rurale gebieden. Wij hebben gunstige effecten waargenomen van ijzersuppletie op anemie en op maten van oxidatieve stress. De effecten van ijzer gecombineerd met andere micronutriënten waren bovendien duidelijk beter. Daarmee hebben onze studies belangrijke volksgezondheidsimplicaties. Ze onderstrepen de noodzaak van een alomvattend voedingsbeleid voor deze doelpopulatie. Behalve verbeteren van de voedingsinname van zwangere vrouwen in rurale gebieden van China, dient suppletie met meerdere micronutriënten te worden aanbevolen.

论文总结

(Chinese)

贫血被认为是发展中国家的一种严重营养缺乏病，特别多见于孕晚期妇女。中国一项调查显示在 1995-2000 年期间城市孕晚期妇女贫血患病率高达 48%。尽管贫血的诊断简单、治疗也便宜；但对经济较发达的江苏和浙江两省在过去 12 年（即 1993-2005 年）间孕妇贫血调查发现，贫血总患病率达到 39.6%，其中孕早期为 29.6%，中期为 33.0%，而孕晚期高达 56.2%。在农村，孕晚期妇女贫血患病情况更加严重，可能与当地妇女在孕早期、孕中期铁的摄入和利用减少以及其他微量营养素摄入不足有关。因此，我们设计和开展了三项采用铁剂与其他微量营养素补充剂进行联合补充试验，目的是改善孕妇贫血和微量营养素缺乏以及观察其他生物学效果。

到目前为止，中国农村妇女缺铁性贫血及微量营养素不足营养问题的严重程度了解不多，重视不够。从 1999 年至 2003 年，考虑地域分布我们选择了经济尚不发达甚至比较落后的山东（青岛莱西和聊城莘县）、福建、兰州、桂林作为调查点，随机选择了 6413 名孕晚期妇女进行贫血患病情况及维生素等微量营养素状况调查，结果发现四省五县市孕晚期贫血平均总患病率高达 58.6%，最低为 48.1%，最高达到 70.5%（见论文第 2 章）。此外，我们同时随机抽取了部分被调查的孕妇血样进行了分析，目的是了解被检孕妇是否存在某些微量营养素缺乏。结果发现贫血孕妇机体多种微量营养素水平明显低于非贫血孕妇，血清铁水平不足（血清铁<700μg/L）和铁严重缺乏者分别达到 39.7%和 52.6%，而非贫血妇女仅为 23.9%和 35.0%。另外，分析发现贫血孕妇维生素 A、维生素 C 的水平也是比较低的；而贫血孕妇及非贫血孕妇机体维生素 B_2 和维生素 B_{12} 都明显不足。因此，调查结果说明孕晚期妇女机体铁和微量营养素的营养状况是比较差的（见论文第 3 章）。

本论文在第 2 章和第 3 章调查研究的基础上，设计并在山东莘县开展了三项随机双盲对照试验干预研究。第一项干预研究（见本论文第 4 章）是 2003-2005 年选取 366 名孕中期贫血（Hb<105g/L）孕妇，采用铁+叶酸（IF）、铁+叶酸+维生素 A (IFA)、铁+叶酸+维生素 B_2 (IFB)和铁+叶酸+维生素 A+维生素 B_2(IFAB)进行干预，为期 2 个月，结果显示 IFAB 组贫血孕妇血红蛋白水平升高了 5.4g/L,明显高于其他各组血红蛋白升高的水平。同时，该研究观察到添加维生素 B_2 组（IFB 和 IFAB）的孕妇因

铁剂补充产生的胃肠道不良反应得到明显改善，其中精神状况好转，由硫酸亚铁引起的恶心、呕吐、胃肠不适的表现明显改善。

有报道认为怀孕和铁过量摄入可能提高机体氧化应激水平，甚至影响红细胞膜氧化水平及流动性，对胎儿发育和母体健康均有不利影响。因此，本研究开展了第二个干预试验，在 2004-2006 年期间，选取 164 名孕晚期贫血（Hb<110g/L）妇女，研究设计对照组、铁组、铁+叶酸组、铁+叶酸+维生素 A+维生素 B_2 组，补充干预为 2 个月（见本论文第 5 章）。结果显示除了贫血及相关指标明显好转外，机体氧化应激水平也得到明显改善，谷胱甘肽过氧化物酶（GSH-px）的活性明显升高，脂质过氧化物 MDA 水平明显减低，红细胞的流动性明显升高，而在多种营养素补充组效果最佳；很遗憾在对照组孕中期妇女在 2 个月后到达孕晚期表现贫血患病情况更加严重，氧化应激水平未能得到明显改善。

贫血治疗和预防的制剂较多，如硫酸亚铁（$FeSO_4$），NaFeEDTA 等；但铁剂种类和补充剂量、效果，以及是否会影响机体氧化应激水平，国内外报道较少。因此，设计有 153 名贫血孕妇参与随机双盲对照试验研究，设计对照组、硫酸亚铁组和 NaFeEDTA 组进行补充并比较两者的效果，为期 2 个月（见本论文第 6 章）。结果显示，对照组孕妇贫血在孕晚期有所加重，氧化应激水平也没明显改善；但是在硫酸亚铁组和 NaFeEDTA 组孕妇贫血得到明显改善，氧化应激水平明显好转，尤其是 GSH-px 的活性明显升高， MDA 水平明显减低；与硫酸亚铁的效果相比，NaFeEDTA 制剂效果更明显。

总之，本论文是一个系列研究，由描述性流行病学研究到实验流行病学的改善干预研究，从发现在农村妇女中贫血率较高及其他微量营养素状况较差，到有针对性的加以改善。通过研究我们发现铁剂补充对改善农村孕妇贫血是有效的，如同时补充或改善贫血孕妇其他微量营养素的状况可能对改善贫血及提高农村孕妇健康水平效果更好；因此，建议和推荐农村孕妇应注意膳食平衡，适量补充铁剂和其他微量营养素，以有效地预防和治疗贫血。

Acknowledgements

Acknowledgements

It was a great pleasure to be a sandwich PhD student at Wageningen University, and to complete a PhD thesis under profs Evert G Schouten and Frans J Kok's supervision. So I am very grateful to both of them for their kind help in our research project and in my thesis preparation. It has been a long memorable period during which I learnt a lot.

In 1999, we were pleased to get financial support from Nestle Foundation, for a project titled: a comparison of micro-nutrient status among anaemic and non-anaemic pregnant women in China, which was going to be carried out in four sites, including Qingdao in Shandong; Lanzhou in Gansu; Guilin in Guangxi and Fuzhou in Fujian provence. The research team included Zheng Mingci from Guilin, Wang Yu from Gansu, Xu Rongxian from Fujian, and myself from Shandong. We first had a visit to the Division of Nutrition at Wageningen University, The Netherlands. During the stay, we enjoyed several courses in statistics and epidemology, and an English language course that helped us to communicate better in English. Professors, Chen Xuecun, J Hautvast and Schouten were a tremendous help in preparing the research proposal for the further supplementation trial.

During the trial we again had a lot of support from the Division of Human Nutrition. In 2003, prof. Evert G. Schouten, as one of supervisors of the project, made a supervision visit to the trial site in Shen's county, Shandong, China, meeting study coordinators and personnel and local government officials. He visited a village clinic where fieldwork was in progress, and houses of some of the pregnant women who were taking part in the study. In the report to the Nestle Foundation he concluded that the study was carried out under difficult circumstances, especially when weather conditions are bad, and that the SARS epidemic had caused a serious draw back. After that, we successfully applied for additional financial support from Nestle Foundation for recruiting additional subjects replacing those who were lost to follow up during the SARS epidemic.

In order to keep a more structural research collaboration, including academic exchanges, we estabolished a triangle association, among the Division of Human

Nutrition, Wageningen University; the Institute of Chinese Nutrition and Food hygiene, Chinese CDC (prof Ma Guansheng); and Institute of Human Nutrition, Qingdao University in 2005. Since then, I have had more than 6 travellings between Wageningen and Qingdao, China. I thank my great teachers Profs. Evert Schouten and Frans Kok for their help on manuscript preparation. Frankly speaking, I still had to learn writing English papers. At the beginning, they gave me a lot of training and coaching, scrutinizing every sentence in drafts of the manuscripts I wrote. Finally, I am surprised that I have finished 7 papers, which have been published during the past three years.

We greatly thank the support of the Nestle Foundation for funding the project in China, which is beneficial to Chinese pregnant women, and to improve the research ability and skills at our nutrition department in Qingdao. Furthermore, we like to acknowledge Dr Paolo Suter and Dr Beat Schürch for their kind help. We know Dr Beat Schürch, a very nice person, has passed away some years ago, but we always cherish the memory of his kind help and support.

We also sincerely acknowledge Profs. Xuecun Chen and Juesheng Li for their kind help. Prof Chen is a great Chinese teacher in my life and gave me so great suggestions in the field investigation and preparing the proposal. Prof Li, as a former president of Qingdao Medical College, have been help me since 1996 in research project, academic activity, department affairs, and my livings as well. Although Profs Chen (96y) and Li (85y) are elderly people this year, they still work in their office, and do not feel to too old to learn or do work. Moreover, I would like to thank Dr Zhang Guoxiong and Danone Research Institute China for a couple of grants as a supplement for the trials.

We thank Dianchen Jiang, Guiying Li, Fengzhi Zhang, Fang Yang, etc. as co-investigators for recruiting subjects and monitoring the trial at the research site; and many of field staff (about 49 nurses and village leaders) for their delivery door by door and home visits weekly for the consumption of the supplementation. We also thank Xiuzhen Zhang, Wei Du, Hui Liang, Zhixu Wang, Hongwei Xu, Yongye Sun, Shehua Zhang, Yongli Sun, Peilong Xu, Yan Zhang, Chunjing Dong, Meilan Xue, Ying Liu for measurements; and Han Xiuxia and Song Xuxia for data analysis.

Also, I thank all colleagues in the Division of Human Nutrition, Wageningen University; and special thanks to Lous duym, Eric, Lidwien, Dione, Edith, etc. for their help in my travelling, working, living and paper work in Wageningen. And I have to thank to Chinese colleagues and students in Wageningen, Dr.Du Huaidong, Guangsheng Ma, Ming Wu, Yanping Li, Devin Lu, Suying Chang, and so on, to give my assistances in my stay here.

In the meanwhile, I acknowledge our Medical College and Qingdao University , as well as my family for giving enough time to finish the field work, lab measurements and often travellings between Wageningen, the Netherlands and Qingdao, China, for completing the thesis. Moreover, I have to thank those persons, who gave me a lot of help that I can not write down all the names.

Finally, I thank you all, remember you all and miss you all when I go back to China. And I wish you all best!

致谢

（Acknowledgements）

在论文完成之际，非常感谢 Wageningen 大学人类营养系的 Frans Kok 教授和 Schouten Evert 教授多年来对我科研项目工作和论文撰写过程中给予的帮助，也衷心感谢 Wageningen 大学给我这次机会参加论文撰写和答辩。

此时此刻，让我想起了从项目申请、现场工作到最后总结和论文撰写的漫长的、让人难忘的经历。记得早在 1999 年，我们很高兴获得了瑞士 Nestle Foundation 基金的资助，为了能有较好的国家代表性，拟在中国四省区开展孕妇贫血的调查工作，初步确定为山东青岛（东部地区）或聊城（中部地区）某农村、甘肃（西北地区）兰州某农村、广西（西南地区）桂林某农村，以及福建省福州市（东南地区）某农村现场，四个项目点的负责人分别马爱国、王玉、郑明慈和许榕仙。在项目工作没有开展之前，大家希望能统一学习调查方法和实验分析方法。经过联系，Wageningen 大学人类营养系主任 Frans Kok 教授和 J Hautvast 教授同意我们研究小组一行四人到 Wageningen 大学进行学习培训。在长达一个月学习期间，Frans Kok 教授给我们专门安排了流行病学调查方法和质量控制知识和技能的学习，计算机统计学习，以及英语交流的培训学习。经过培训，我们不仅学习掌握了开展项目工作的基本方法和技能，而且还认识了 Schouten Evert 教授、Paul 教授等专家，并建立了长期的交流和合作。

从那以后，我们保持着很好的联系，针对项目工作问题，Frans 和 Evert 经常给予指导。2003 年 12 月，Evert 教授作为项目观察员专门到山东聊城莘县的项目工作现场进行调研；由于农村道路状况较差，泥泞坑洼，汽车无法通行，甚至雨后自行车也无法通行，Evert 教授宁可步行数十里也要去贫血孕妇家里查看孕妇生活情况、服药情况、项目配合情况等；特别是 2003 年中期，正值 SARS 传染病流行，疫情严重致使我们前期（2002 年底至 2003 年初）收集到的贫血孕妇病例不能完成随访，使得项目工作停滞和项目经费损失。Evert 教授看完现场工作后，对项目工作给予了肯定，对存在的问题和困难提出了很好的建议，包括总结项目进展情况向项目基金会汇报并争取经费补助。后来，在 Evert 教授的帮助下，我们及时递交了申请，修改和调整了项目进度，结果 Nestle Foundation 基金会同意了我们的建议和方案并给了一定数量的经费补助，使我们的项目工作得以保质保量的完成。由此，我们从内心非常感谢 Evert 教授和

Wageningen 大学营养系的领导和老师们的帮助。此外，对 Wageningen 大学营养系的 Lous, Edith，Eric, Lidwien, Dione 等多位荷兰老师在生活、学习和工作中给予的帮助，表示衷心地感谢。

项目工作的完成离不开参与的所有人员的帮助。尤其是要感谢王玉、郑明慈和许榕仙三位教授所负责的三个调查点出色的工作，保证了项目现况调查工作的顺利完成。在二期的营养补充干预研究中，感谢莘县计划生育局的领导和计生站的专家们的积极配合帮助，尤其是要感谢江殿臣、杨芳、张凤芝等，以及参与现场工作的各乡镇的计生干部、村主任、妇联主任、村医等 49 人之多的配合和支持，使我们的营养补充改善工作顺利完成。

此时，也请允许我对瑞士 Nestle Foundation 基金会说声谢谢。尤其是感谢 Suter Paolo 博士和 Beat Schürch 博士对我们两期项目（现况调查和营养补充干预研究）的支持。很遗憾，Beat Schürch 博士几年前已经去世了，但是他的和蔼可亲和对我们项目团队以及我们研究对象贫血孕妇的无私帮助让我们永远难以忘怀。我们会永远记住他。

我们还要特别感谢陈学存教授、李珏声教授，在项目工作中给予的鼓励和支持。陈学存教授指导我们完善项目申请、调查设计、资料整理等工作，2003 年还亲自到莘县工作现场指导和帮助工作，为我们的研究工作作出了巨大的贡献，我们深受感动。李珏声教授不断地指导我们的项目工作，帮助指导研究生课题研究工作和论文撰写指导。

另外，我们需要感谢的是我们的同事、朋友以及我的家人。感谢马冠生教授、武鸣博士、杜怀东博士、李艳萍博士、何宇娜博士、常素英博士等在 Wageningen 期间的帮助和支持。感谢我所在单位的同事们和领导们对我们项目工作的支持和帮助。感谢他们在现场调查工作、样品采集、实验室检测分析、数据整理和统计分析以及项目总结和论文撰写过程中付出的辛勤劳动和给予的支持和无私帮助。

最后，再一次感谢你们，所有帮助我的老师、同事和朋友们；并祝你们身体健康、心情愉快、家庭幸福。

2013 年 1 月写于荷兰

Curriculum vitae

Ma Aiguo, male, born in November 1956, Director and professor of the Institute of Human Nutrition, Medical College of Qingdao University. He is a member of standing committee of Chinese Nutrition Society, members of Chinese Food Safety Committee, Disease Prevention and Control Committee, and Nutritional Standard Committee. A member of Chinese Degree Committee of the State Council in Public Health and Preventive Medicine.

Prof. Ma' research field is nutritional epidemiology, such as children and overweight, anemia pregnancy and micronutrient supplementation, nutrition and health education in elderly people, nutritional deficiency, antioxidants or vitamins and DNA damage and repair, magnesium and vitamin E and diabetes, diabetes and tuberculosis, etc. Moreover, more than 100 papers were published in Chinese and English journals.

Prof. Ma was graduated from the Xinjiang Medical University in July 1983 for bachelor degree of preventive medicine, and got a master degree of human nutrition in May 1988. He co-worked with Dr. Collins in the Rowett Research Institute, Scotland in 1992-1993 on nutrition and DNA damage and repair, and modifying the method of single gel cell electrophoreses better, which were published in the journals of cancer Res (1996), and Mutatio Res (1995) respectively. He had several visits to Division of Human Nutrition, Wageningen University, Netherlands for the research collaboration during 2001-2012. In 2001-2002, prof. Ma had stayed in USDA Nutrition Research Center on Aging, Tufts University, Boston USA for 8 months for the collaborative research on antioxidant nutrients and aging.

Since 2000, Aiguo Ma has got and finished several projects supported by China Nature Science Fund, local government funds, Nestle Foundation, Danone Center Fund in China, World Diabetes Foundation (WDF), etc. The study of the projects supported by Nestle Fondation and partly by Danone Center Fund in China was presented in this thesis.

Publications in English

1. Sun Y, Ma A, Li Y, Han X, Wang Q, Liang H.Vitamin E supplementation protects erythrocyte membranes from oxidative stress in healthy Chinese middle-aged and elderly people.Nutr Res. 2012;32(5):328-34.
2. Ma AG, Ge S, Zhang M, Shi XX, Schouten EG, Kok FJ, Sun YY, Han XX.Antioxidant micronutrients improve intrinsic and UV-induced apoptosis of human lymphocytes particularly in elderly people.J Nutr Health Aging. 2011;15(10):912-7.
3. Wang Q, Han X, Ma A, Wang Y, Bygbjerg IC, Li G, Sun Y, Zhou X.Screening and intervention of diabetes mellitus in patients with pulmonary tuberculosis in poverty zones in China: Rationale and study design. Diabetes Res Clin Pract. 2011 Dec 5. [Epub ahead of print]
4. Han XX, Sun YY, Ma AG, Yang F, Zhang FZ, Jiang DC, Li Y.Moderate NaFeEDTA and ferrous sulfate supplementation can improve both hematologic status and oxidative stress in anemic pregnant women. Asia Pac J Clin Nutr. 2011;20(4):514-20.
5. Han XX, Zhang M, Ma AG, Ge S, Shi XX, Sun YY, Wang QZ, Liang H.Antioxidant micronutrient supplementation increases erythrocyte membrane fluidity in adults from a rural Chinese community. Br J Nutr.2011;106(11):1676-82.
6. Ma AG, Schouten EG, Ye Sun YY, Yang F, Han XX, Zhang FZ, Jiang DC, Kok FJ. Supplementation of iron alone and combined with vitamins improves haematological status, erythrocyte membrane fluidity and oxidative stress in anaemic pregnant women. Br J Nutr. 2010; 104:1655-61.
7. Bao W, Ma A, Mao L, Lai J, Xiao M, Sun G, Ouyang Y, Wu S, Yang W, Wang N, Zhao Y, Fu J, Liu L. Diet and lifestyle interventions in postpartum women in China: study design and rationale of a multicenter randomized controlled trial.BMC Public Health. 2010; 27;10:103.
8. Wang Q, Sun Y, Ma A*, Li Y, Han X, Liang H. Effects of vitamin e on plasma lipid status and oxidative stress in chinese women with metabolic syndrome. Int J Vitam Nutr Res. 2010;80:178-87.
9. Sun YY, Ma AG*, Yang F, Zhang FZ, Luo YB, Jiang DC, Han XX, Liang H. A combination of iron and retinol supplementation benefits iron status, IL-2 level and

lymphocyte proliferation in anemic pregnant women. Asia Pac J Clin Nutr. 2010;19:513-9.

10.Dou M, Ma AG*, Wang QZ, Liang H, Li Y, Yi XM, Zhang SC. Supplementation with magnesium and vitamin E were more effective than magnesium alone to decrease plasma lipids and blood viscosity in diabetic rats.Nutr Res. 2009;29:519-24.

11.Li Y, Ma A*, Sun Y, Liang H, Wang Q, Yi X, Han X. .Magnesium status and dietary intake of mid-old people in a rural area of China. Magnes Res. 2009;22:66-71.

12. Ma AG, Schouten E, Wang Y, Xu RX, Zheng MC, Li Y, Sun YY, Wang QZ. . Anemia prevalence among pregnant women and birth weight in five areas in China. Med Princ Pract. 2009;18:368-72.

13. Ma AG, Schouten EG, Wang Y, Xu RX, Zheng MC, Li Y, Wang Q, Sun Y. Micronutrient status in anemic and non-anemic Chinese women in the third trimester of pregnancy. Asia Pac J Clin Nutr. 2009;18(1):41-7.

14. Ma AG, Schouten EG, Zhang FZ, Kok FJ, Yang F, Jiang DC, Sun YY, Han XX. Retinol and riboflavin supplementation decreases the prevalence of anemia in Chinese pregnant women taking iron and folic Acid supplements. J Nutr. 2008;138:1946-50.

15. Liang H, He J, Ma AG, Zhang PH, Bi SL, Shi DY. Effect of ethanol extract of alga Laurencia supplementation on DNA oxidation and alkylation damage in mice.Asia Pac J Clin Nutr. 2007;16 Suppl 1:164-8.

16. Ma AG, Chen XC, Xu RX, Zheng MC, Wang Y, Li JS. Comparison of serum levels of iron, zinc and copper in anaemic and non-anaemic pregnant women in China. Asia Pac J Clin Nutr. 2004;13(4):348-52.

17. Ma AG, Chen XC, Wang Y, Xu RX, Zheng MC, Li JS. The multiple vitamin status of Chinese pregnant women with anemia and nonanemia in the last trimester. J Nutr Sci Vitaminol (Tokyo). 2004;50(2):87-92.

18. Kim Y, Ma AG, Kitta K, Fitch SN, Ikeda T, Ihara Y, Simon AR, Evans T, Suzuki YJ.Anthracycline-induced suppression of GATA-4 transcription factor: implication in the regulation of cardiac myocyte apoptosis. Mol Pharmacol. 2003；63（2）：368-77

19. Ma A, Chen X, Zheng M, Wang Y, Xu R, Li J. Iron status and dietary intake of Chinese pregnant women with anaemia in the third trimester. Asia Pac J Clin Nutr. 2002;11 (3):171-5.

20. Duthie SJ, Ma A, Ross MA, Collins AR.Antioxidant supplementation decreases oxidative DNA damage in human lymphocytes.Cancer Res. 1996；56（6）：1291-1295

21. Collins AR, Ma AG, Duthie SJ. The kinetics of repair of oxidative DNA damage (strand breaks and oxidised pyrimidines) in human cells. Mutat Res. 1995;336(1):69-77

OVERVIEW OF COMPLETED TRAINING ACTIVITIES

Discipline specific activities

Training at USDA Human Nutrition Research Center on Aging, Tufts University, 2001-2002
13th International Carotenoid Symposium, Honolulu, Hawaii, USA, 2002
18th International Congress of Nutrition of the IUNS, Nutrition Safari, South Africa, 2005
Visit to International Institute of Public Health, Copenhagen University, Denmark, 2008
40th International Lung Health Conference, Cancun, Mexico, 2009
Visit to USDA Human Nutrition Research Center on Aging, Boston, USA, 2011
DM-TB stakeholder meeting, Beijing, China, 2011
Nestlé Research Symposium China, Beijing, China, 2011
Experimental Biology 2011 Annual Meeting, Washington DC, USA, 2011
Korea-China International Phytonutrient Symposium, Seoul, Korea, 2011
XI Asian Congress of Nutrition, Singapore, 2011
43th International Lung Health Conference, Kuala Lumpur, Malaysia, 2012

General courses

Exemption of general courses because of working experience

Optionals

Preparation PhD research proposal
Assistant in VLAG Nutritional and Lifestyle Epidemiology courses, Beijing (2001) and Nanjing (2004), China

List of abbreviations

CI	confidence intervals
CRH	corticotropin-releasing hormone
DMT-1	divalent metal transporter 1
DPH	1,6-diphenyl-1,3,5-hexatriene
EGRAC	erythrocyte glutathione reductase activity coefficient
EP	erythrocyte protoporphyrin
$FeSO_4$	ferrous sulfate
GSH-px	glutathione peroxidase
Hb	hemoglobin
HPLC	high-performance liquid chromatography
ID	iron deficiency
IDA	anemia
MDA	malondialdehyde
NaFeEDTA	sodium iron ethylenediaminetetraacetate
PR	prevalence ratios
PRF	prolonged release formulation
RBC	red blood cell
RDA	recommended daily allowance
ROS	reactive oxygen species
RR	relative risk
SOD	superoxide dismutase
sTfR	soluble transferrin receptor
TIBC	total iron-binding capacity
TRF	transferrin
VAD	vitamin A deficiency
ρ	polarisation
η	microviscosity

This PhD program and the printing of this thesis was funded by the Division of Human Nutrition, Wageningen University, The Netherlands.

Cover design: Chen Minjian and Ma Yan

Lay out: Ma Aiguo and Zheng Ying

Printed : Propress, Wageningen, The Netherlands.