

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

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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.

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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 FeSO₄, 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 FeSO₄ (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.

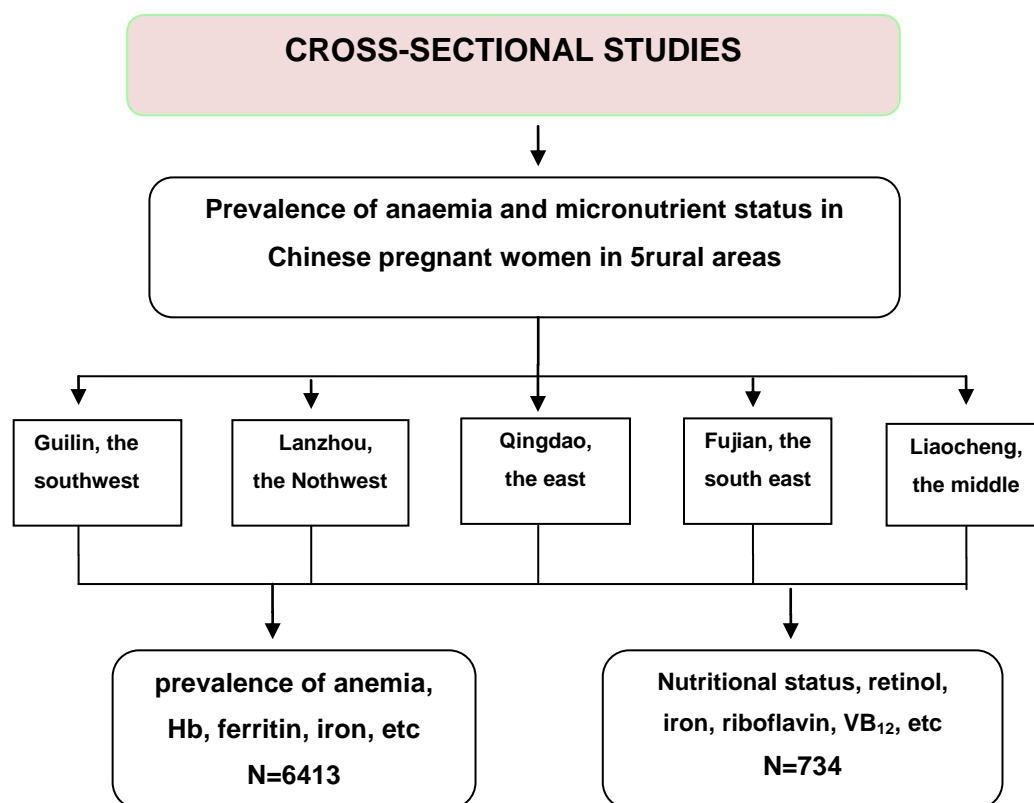


Figure 1.1 Profile of the cross sectional study.

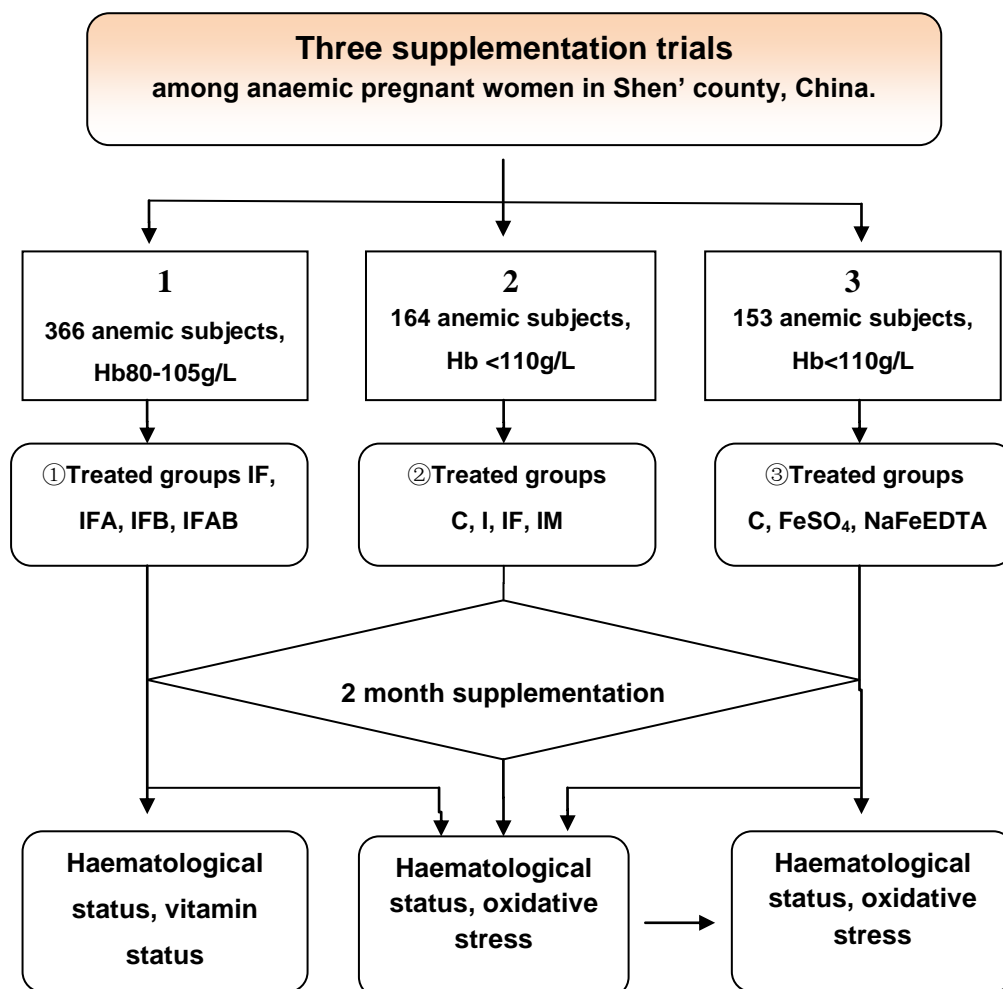


Figure 1.2 Profile of the randomized, double-blind controlled trials.

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.

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Chapter 2

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

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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 above 140 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 education in pregnant women

Items	total number	anemic number	prevalence (%)	<i>p</i>
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.

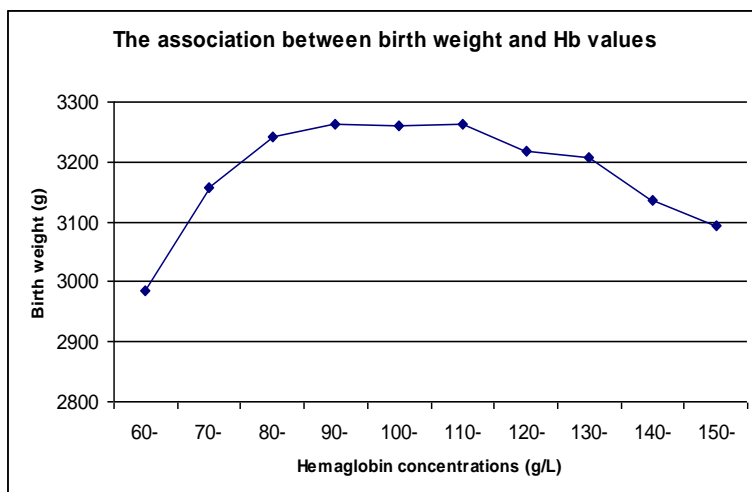


Figure 2.1 The association of average points of hemoglobin concentrations (Hb) with birth weight in 6413 pregnant women was shown on the curve. There was a range of higher levels of birth weights during 90 g/L to 140 g/L of hemoglobin concentrations. However, the lower birth weight on the curve was located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

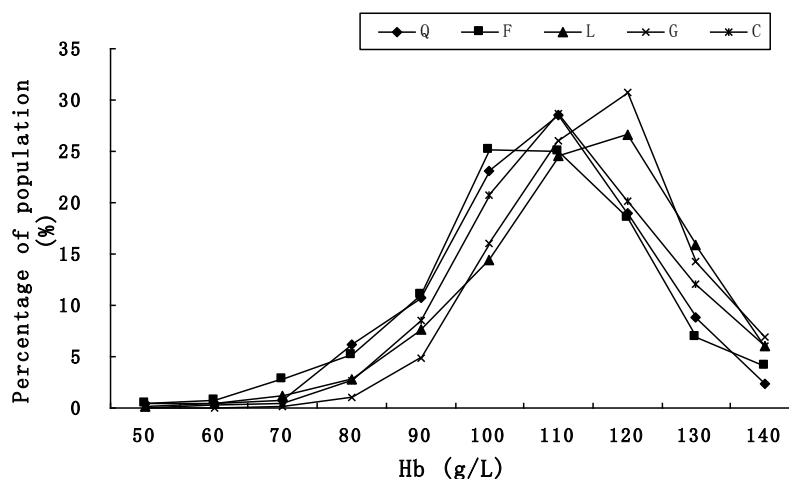


Figure 2.2 The figure shows the distribution of hemoglobin on five curves. The peaks of percentage of hemoglobin in Lanzhou (L) and Guilin (G) are at about 115 g/L, while other three peaks of hemoglobin in Qingdao (Q), Fuzhou (F) and Liaochen (C) are at about 95 g/L ~ 105 g/L. The difference among the 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 B₁₂ 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 B₁₂. 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 <80 g/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.

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Chapter 3

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

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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, B₁₂ and C, iron and zinc status parameters, and vitamin B₂ 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 B₂ 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 B₂ and B₁₂ 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 morbidity¹ and to low birth weight as well². 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 city³, 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 vitamins⁴.

Anemia during pregnancy has been attributed not only to increased iron requirements during the second and the third trimester of gestation⁵, but also to micronutrient deficiency. Deficiencies of iron and vitamin A were among the major contributory factors⁶. 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 indices⁹. 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 °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 B₁₂ 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 radioimmunoassay¹⁰, 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 status¹². 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 B₁₂ were measured by radioimmunoassay method (Diagnostic Products Corporation DPC, USA)¹³. Serum concentrations 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 Group¹⁴, 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 < 30 µg/dL,

ascorbic acid <400 µg/dL, riboflavin/creatinine <80 µg/g in urine, folic acid <3.0 ng/mL and vitamin B₁₂<200pg/mL¹⁵.

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 B₂ 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 B₂ in urine (-21%) in the anemia group were significantly lower. There were no significant differences of serum copper levels, vitamin B₁₂ and folate.

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

Items	Hb<110g/L		Hb≥110g/L		Difference (%)	<i>p</i>
	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		<i>p</i>
		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 B₂/creatinine in urine. Moreover, the curves of Hb<110g/L and Hb≥110g/L in ferritin, serum iron, vitamin C and vitamin B₂/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.

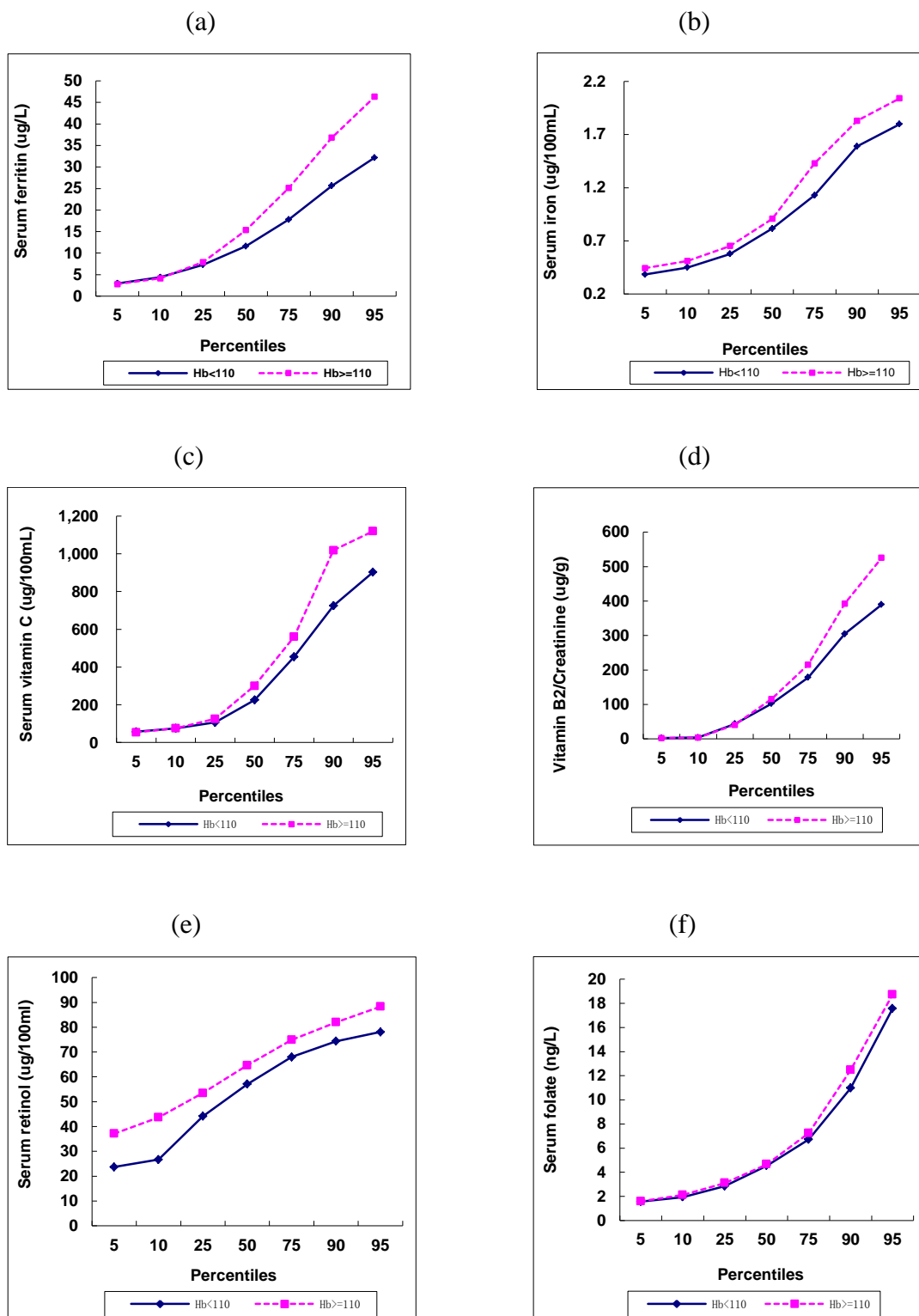


Figure 3.1 The distributions of concentrations of ferritin (a), serum iron (b), serum vitamin C (c), vitamin B₂ in urine (d), serum retinol (e) and serum folate (f) according to presence or absence of anemia are presented both in Hb < 110 and Hb ≥ 110.

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 B₂ 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 evaluation¹⁶, 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 B₁₂, folate, vitamin A and even zinc contribute either singly or in combination to maternal anemia¹⁷. 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 B₁₂. However, iron deficiency rarely occurs in isolation and is often accompanied by other micronutrient deficiencies¹⁸. Makola¹⁹ 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 foods²⁰. 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 supplementation²¹. 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 absorption²². 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 interaction²³. In this study, there were 11.2% and 13.9 % of subnormal vitamin B₁₂ (<200 pg/mL) in anemic and non anemic pregnant women respectively, which may have confounded the enhancing effect of ascorbic acid on iron status²⁴. Vitamin B₂ in urine, estimated by a ratio of vitamin B₂ 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 hemoglobin²⁷. 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 countries²⁸. 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 pregnancy²⁹.

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%, respectively³⁰. 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 education⁴. 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.

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Chapter 4

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

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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) ≤ 105 g/L] were randomly assigned to four groups, all receiving daily 60 mg iron and 400 μ g 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 μ g 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 < 110 g/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 worthwhile 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 ≤ 105 g/L in the study; therefore, 178 pregnant women did not meet the inclusion criteria, and a further 89 women with Hb ≤ 105 g/L refused to take any “drugs” during pregnancy. Finally, 366 women remained [Hb: >80 and ≤ 105 g/L, no dietary supplements during 2 months, no abnormal pregnancy response and receiving daily folic acid (400 μ g)]. 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 μ g 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 questionnaire which 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 \leq 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.

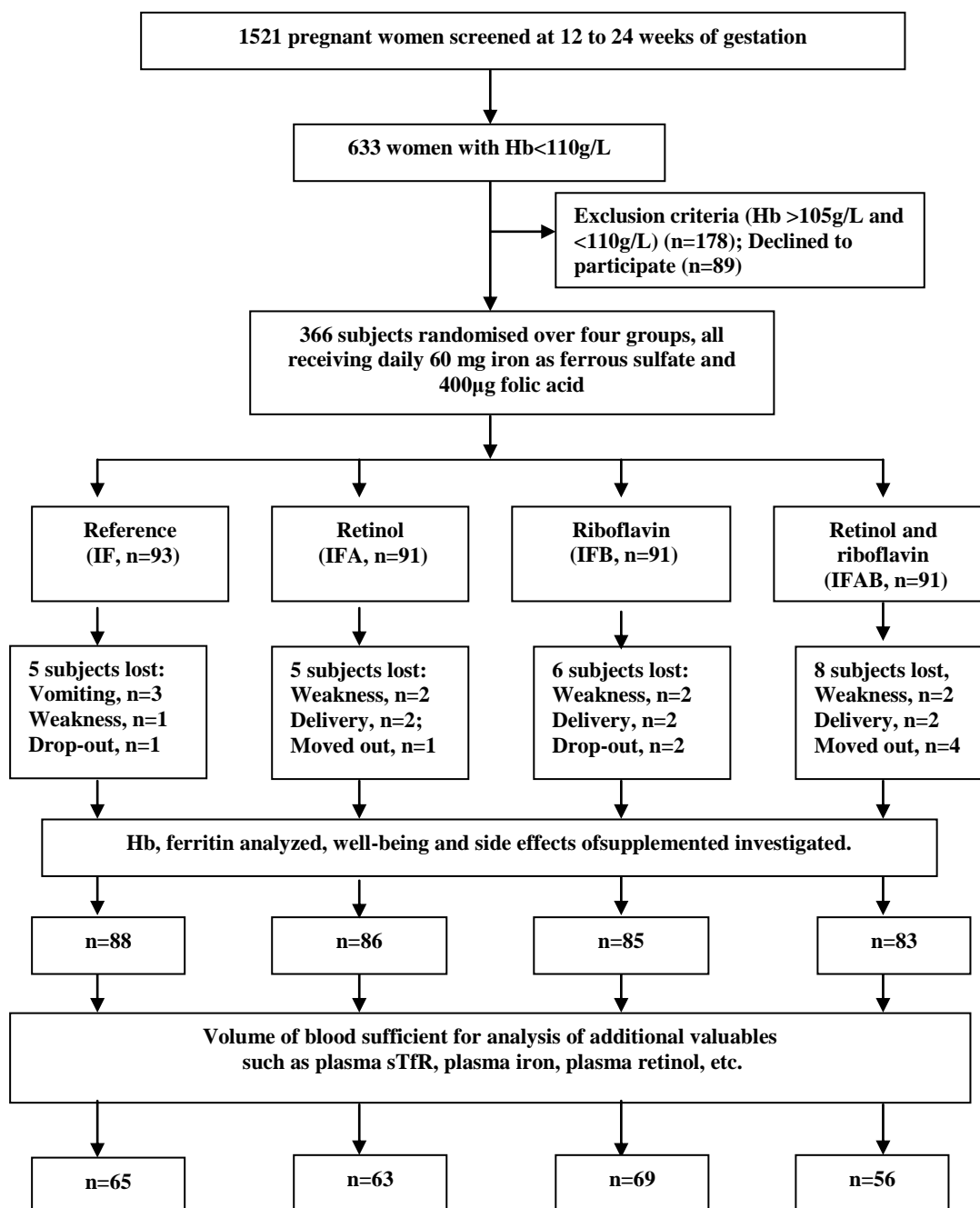


Figure 4.1 The trial profile.

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 \pm SD or means \pm 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 <12 μ g/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 treatment¹

Items	IF ²		IFA ²		IFB ²		IFAB ²	
Characteristics	n ³		n ³		n ³		n ³	
Age, <i>y</i>	88	28.1 ± 3.6	86	28.2 ± 3.6	85	28.0 ± 3.7	83	28.1 ± 3.5
Gestational age, <i>wk</i>	88	20.9 ± 5.6	86	21.1 ± 5.6	85	20.7 ± 5.4	83	20.7 ± 5.5
Parity, <i>n</i>	88	1.3 ± 0.5	86	1.3 ± 0.5	85	1.3 ± 0.4	83	1.3 ± 0.4
Indicators								
Hemoglobin, <i>g/L</i>	88	96.6 ± 7.3	86	96.8 ± 6.4	85	96.9 ± 7.1	83	97.0 ± 7.2
Plasma Ferritin ³ , <i>µg/L</i>	88	14.9 ± 8.4	86	15.0 ± 6.6	85	14.9 ± 6.1	83	14.9 ± 7.0
Plasma sTfR, <i>mg/L</i>	65	11.9 ± 4.6	63	11.9 ± 4.9	69	12.0 ± 4.3	56	11.9 ± 4.1
Plasma iron, <i>µmol/L</i>	65	12.9 ± 1.7	63	12.9 ± 1.9	69	13.0 ± 1.9	56	12.9 ± 2.0
EP, <i>µmol/mol heme</i>	65	80.4 ± 83.1	63	82.2 ± 97.7	69	80.4 ± 74.2	56	83.5 ± 63.4
Total iron binding capacity, <i>µmol/L</i>	65	67.4 ± 10.5	63	66.9 ± 10.2	69	67.8 ± 9.4	56	65.9 ± 11.2
Plasma retinol, <i>µmol/L</i>	65	1.25 ± 1.11	63	1.20 ± 1.11	69	1.19 ± 0.96	56	1.24 ± 0.95
Plasma Folic acid, <i>nmol/L</i>	65	8.38 ± 3.62	63	8.61 ± 4.30	69	7.93 ± 3.40	56	8.38 ± 3.62
Urine Riboflavin, <i>mmol/mol</i>	65	0.22 ± 0.17	63	0.23 ± 0.22	69	0.22 ± 0.20	56	0.21 ± 0.19

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

Table 4.2 Changes in hematologic indicators in anemic pregnant women during intervention with retinol and riboflavin¹.

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 retinol and riboflavin¹.

Indicators	Group ²	n ²	Baseline	Change from baseline	Difference from IF	P-value for difference
Plasma retinol, $\mu\text{mol/L}$						
	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 Intervention with 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)*

¹Moderate anemia: hemoglobin 80-105g/L; all anemia: Hb<110g/L; Iron deficiency anemia: Hb<110g/L and plasma ferritin<12µg/L; ²see Figure 4.1 for groups and sample size; ³PR, 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

¹Retinol treatment including IFA and IFAB groups, n=169 (125 in sTfR);

²Riboflavin 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)

¹Well 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; gastrointestinal side effects: including nausea, abdominal discomfort, vomiting, etc. ²See Figure 4.1 for groups and sample size. ³Proportions of well being or side-effects in four groups. ⁴PR, 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°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 worthwhile for pregnant women in rural China.

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Chapter 5

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

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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 with 15.8, 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 ($80\text{g/L} < \text{Hb} < 110\text{ g/L}$), 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 $400\mu\text{g}$ folic acid, IM ($n=41$) with 60 mg iron, $400\mu\text{g}$ 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% ($\alpha=0.05$) significance level and a power of 0.80 ($\beta=0.20$), 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, 2×10^{-6} mol/L) was added before being incubated in a water bath at 25°C 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:

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

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 \pm 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**).

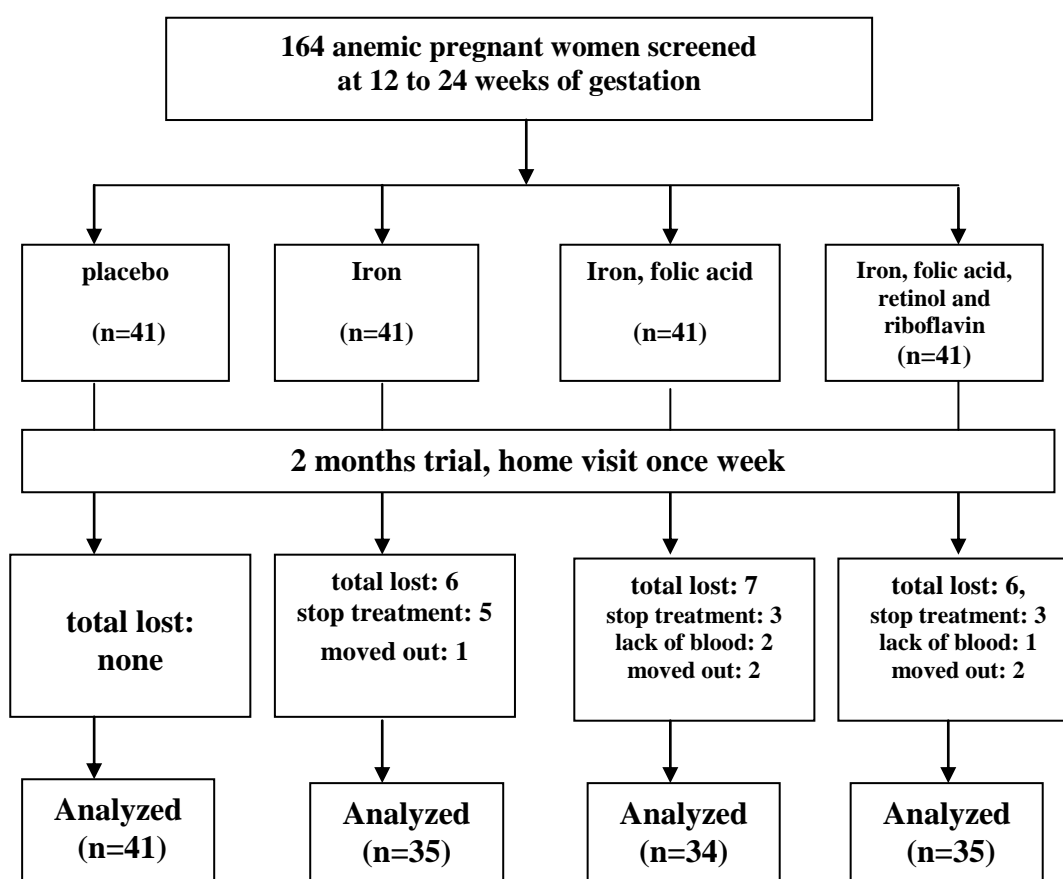


Figure 5.1 The trial profile. The trial enrollment was conducted from 2004 to 2006. Of a total of 366 women were eligible, whom we took a random sample of 164, and they were allocated to the intervention groups by order of randomization. In the intervention study, complete data were available on 145, 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 $\mu\text{mol/L}$, 5.5 $\mu\text{mol/L}$ and 9.7 $\mu\text{mol/L}$ for plasma iron (all p values <0.001), and 2.8 $\mu\text{g/L}$, 3.6 $\mu\text{g/L}$ and 11.0 $\mu\text{g/L}$ for ferritin (all p values <0.001). The increases of plasma retinol and urine riboflavin in IM were 26.75 $\mu\text{g/dL}$ ($p < 0.001$) and 61.59 mg/g creatinine ($p < 0.001$), respectively. For plasma folic acid the increase was 4.06 $\mu\text{g/L}$ in the IF group ($P < 0.001$) and 4.65 $\mu\text{g/L}$ 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).

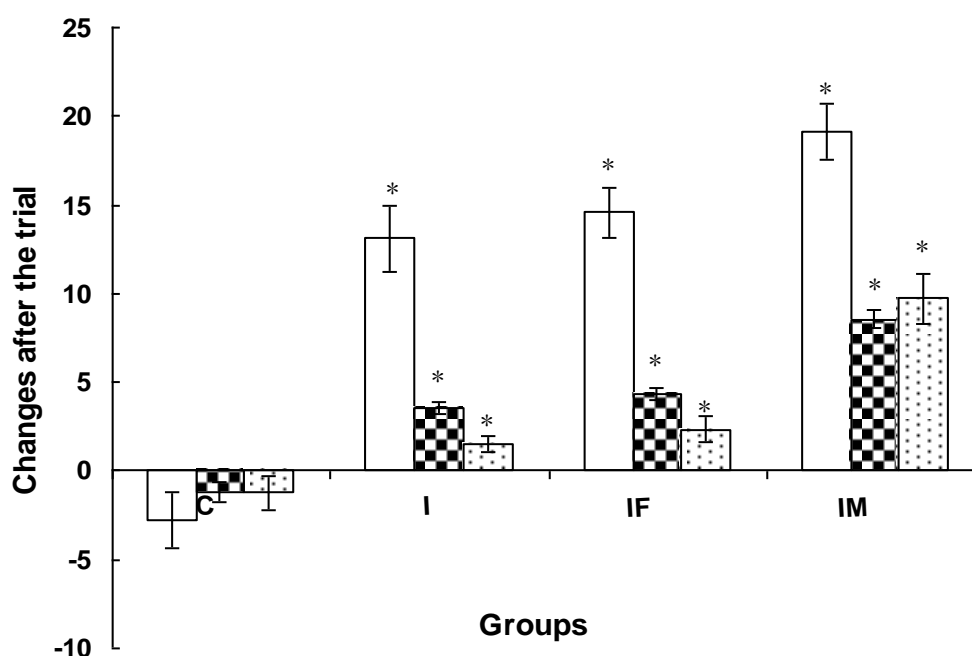


Fig. 5.2 A comparison of hematologic status between before and after the trial. The blank bars represent the mean and standard deviation of hemoglobin concentration (Hb), the square bars for plasma iron concentration and the dot bars for ferritin with significant changes between groups; group C as placebo, 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 2.56 $\mu\text{mol/L}$. Moreover, the increase of GSH-Px activity and decrease of MDA level were 50.2 IU/ml and 2.01 $\mu\text{mol/L}$ 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<0.05; 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<0.05; 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 study, 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 favorable 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

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There are no conflicts of interest.

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Chapter 6

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

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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 $80 \leq \text{Hb} < 110 \text{g/L}$, 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 $\mu\text{mol/L}$ and 7.19 $\mu\text{mol/L}$ for plasma iron (both p values < 0.001), 2.63 $\mu\text{g/L}$ and 8.99 $\mu\text{g/L}$ for ferritin (both p values < 0.05) in I and IE groups, respectively, compared with the control group. Glutathione peroxidase (GSH-Px) activities increased by 32.6 IU/ml and 75.3 IU/ml, and malondialdehyde (MDA) levels decreased by 0.70 $\mu\text{mol/L}$ and 1.12 $\mu\text{mol/L}$ 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 $\mu\text{mol/L}$, 6.36 $\mu\text{g/L}$ 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 world¹. Prevalence of anemia is still high in rural areas in China². 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 avoided⁵.

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 world⁸⁻¹⁰, 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 content¹¹. 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 delivery¹². 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 pregnancy¹³.

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 $80 \leq \text{Hb} < 110$ 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 debinded 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 radioimmunoassay¹⁴. 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, respectively¹⁵. 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 blood¹⁶. 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**).

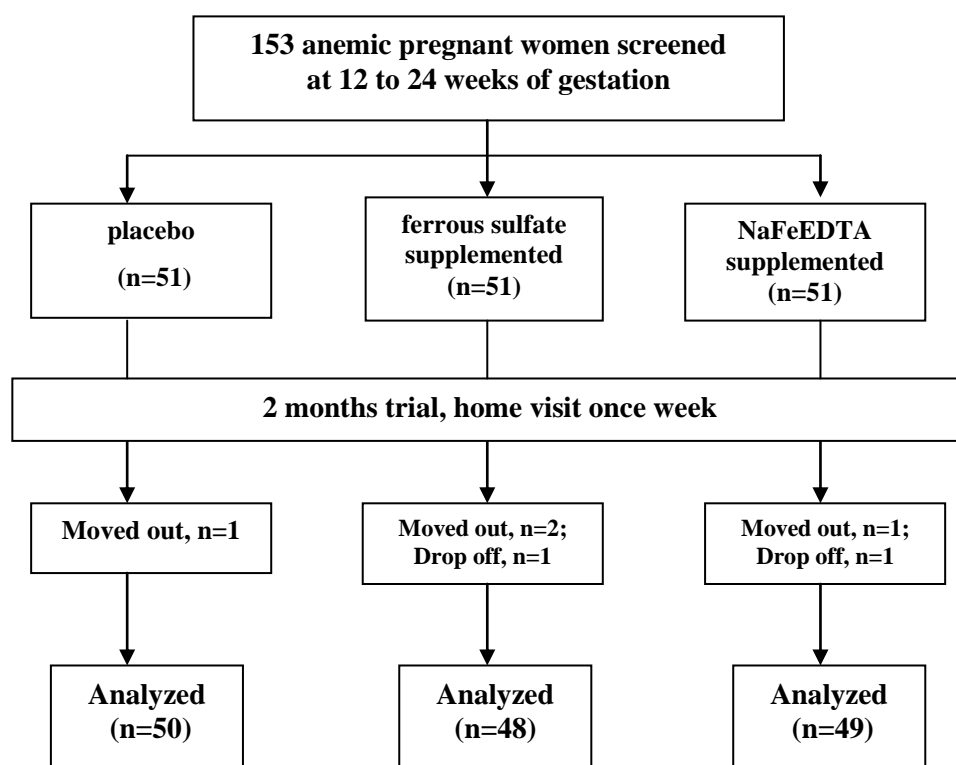


Figure 6.1 The trial profile. After ascertainment of eligibility, 153 consenting pregnant women were enrolled in the study. After 2 month trial, complete data were available on 147. There were no substantial differences between the groups in any of the baseline characteristics.

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 $\mu\text{mol/L}$ in plasma iron and -1.59 $\mu\text{g/L}$ 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 $\mu\text{mol/L}$ for plasma iron (both p values <0.001), 2.6 and 9.0 $\mu\text{g/L}$ 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 $\mu\text{mol/L}$ and 6.37 $\mu\text{g/L}$, 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 $\mu\text{mol/L}$ in the two treated 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	
<i>n</i>	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

¹Indicators 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. ²group C as placebo, group I supplemented with 60 mg iron as ferrous sulfate; group IE with 60 mg iron as NaFeEDTA; ³p-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

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

¹Indicators and abbreviations: MDA, malondialdehyde; SOD, superoxide dismutase; GSH-Px, glutathione peroxidase. ²group C as placebo, group I supplemented with 60 mg iron as ferrous sulfate; group IE with 60 mg iron as NaFeEDTA; ³Changes: value at end of trial subtracted from baseline value within group;

⁴Differences: changes in the group I (or IE)- changes in the control group, respectively; ⁵Differences: 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 pregnancy¹⁷. However, pathologic pregnancies including gestational diabetes mellitus (GDM), obesity and hypertensive disorders are associated with a heightened level of oxidative stress¹⁸⁻²⁰. 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 damage²¹. Untreated iron deficiency is deleterious, while iron overload could promote the generation of free radicals and result in cellular damage²². It is, therefore, important to maintain optimal iron intake²³. Low doses of oral ferrous iron (36mg/d) did not unfavorably change the physiological pattern of parameters of oxidation²⁴. Daily supplementation of 100mg iron as ferrous sulfate during the second half of pregnancy was recommended to address the corresponding iron requirements²⁵, and risks associated with oxidative stress were not observed in women supplemented with 120mg iron once or twice per week²⁶. Suggested guidelines are 80-100mg ferrous iron daily, for which there are no documented side effects⁵. 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 volumes²⁷.

Pregnant women have an increased demand for iron to expand about 30% of their erythrocyte mass and generate the iron supply to the growing fetus²⁸, which results in a decrease of hematocrit of about 3% to 5% between gestational weeks 20 and 30²⁹. Therefore, 80-100 mg ferrous iron/day for women with iron deficiency (ID) and iron deficiency anemia (IDA) should be recommended³⁰.

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 iron³³. Iron absorption from NaFeEDTA might be two to three times higher than from electrolytic iron³⁴. NaFeEDTA increased both Hb concentration and serum ferritin concentration substantially in iron deficient populations³⁵. 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/d³⁶.

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.

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AUTHORS DISCLOSURES

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

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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 B₂ in urine and B₁₂ 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 ≤ 105 g/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 $80\text{g/L} < \text{Hb} \leq 105\text{g/L}$, 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 < 110 g/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 $80 \leq \text{Hb} < 110$ g/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, $n = 2 \left(\frac{(t_{2\alpha} + t_{2\beta}) * SE}{\delta} \right)^2$, ($t_{2\alpha} = 1.645$, $t_{2\beta} = 1.282$). 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 ($\bar{\delta}$) 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°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 ($>12\text{h}$) 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 anemia prevalence increased from the first (29.6%) to the second (33.0%) and third (56.2%) trimesters in South-East China, (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 B₂ 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 B₁₂, 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, B₁₂, 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 on a 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, such 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.

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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 B₁₂ 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

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deteriorated in 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 ($Hb < 110\text{g/L}$) 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 ernstiger volksgezondheidsprobleem bij zwangere vrouwen 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 micronutriënten om het effect hiervan op anemie en micronutriëntenstatus vast te stellen en om mogelijke bijwerkingen te evalueren.

De prevalentie van ijzergebreksanemie en micronutrientdeficiënties 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 deelnemers 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 ($< 700\mu\text{g/L}$) en ijzerdepletie (ferritine $< 12\mu\text{g/L}$) waren bij anemische vrouwen met 39,7% en 52,6% significant hoger dan bij niet-anemische vrouwen met respectievelijk 23,9% en

Summary

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 membraanfluiditeit 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 parameters, en significante stijgingen in glutathion peroxidase (GSH-px) activiteit en daling van malondialdehyde (MDA). Bovendien daalden fluorescentie polarisatie (ρ) en microviscositeit (η), indicatoren van membraanfluiditeit, 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 membraanfluiditeit 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.

Summary

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 $<700\mu\text{g/L}$ ）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 B₂ 和维生素 B₁₂ 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 $<105\text{g/L}$ ）孕妇，采用铁+叶酸（IF）、铁+叶酸+维生素 A（IFA）、铁+叶酸+维生素 B₂（IFB）和铁+叶酸+维生素 A+维生素 B₂（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 B₂ 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 B₂ 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（FeSO₄），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。

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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 province. 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 epidemiology, 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 established 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.

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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.

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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

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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 Foundation and partly by Danone Center Fund in China was presented in this thesis.

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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

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