EXAMINING THE ROLE OF IRON AND EXERCISE IN IMPROVING IRON STATUS AND PHYSICAL PERFORMANCE IN IRON-DEPLETED CHINESE WOMEN

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EXAMINING THE ROLE OF IRON AND EXERCISE IN IMPROVING IRON STATUS AND PHYSICAL PERFORMANCE IN IRON-DEPLETED CHINESE WOMEN

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Iron deficiency without anemia (IDNA) is widely prevalent in China, where conventional methods for improving iron status are not always feasible in low-resource areas. IDNA has known detrimental effects on physical performance. Conversely, intense exercise training increases the risk of IDNA. While iron supplementation and aerobic exercise have been examined individually, the impact of simultaneous iron supplementation and aerobic training remains unclear. The goal of this study was to examine the individual and combined effects of supplementation (ferrous sulfate or a traditional Chinese herbal treatment, *BaZhen KeLi*, BZ) and/or aerobic training on iron status and physical performance.

One hundred and nine IDNA Chinese women participated in an 8-week randomized trial with a 3x2 factorial design. Treatments included 42 mg elemental iron/day, 150 g BZ/day, or a placebo and aerobic training (5 sessions/week of 25 minutes cycling) or no training. Iron status and physical performance were assessed at weeks 0, 4, and 8. Physical performance was measured as oxygen consumption at maximum exertion (VO_2peak) and at the ventilatory threshold.

Training modified the serum ferritin response to iron supplementation. Both iron-supplemented groups improving in serum ferritin, but the iron-trained group had significantly lower serum ferritin than the iron-untrained group at week 8.

There were significant 2-way interactions between training and supplement for VO_2peak and ventilatory threshold. These variables were significantly higher in the iron-trained, iron-untrained, and placebo-trained groups compared to the placebo-untrained group, with no significant differences among these three groups for either variable.

BZ treatment did not significantly improve any measure of iron status compared to placebo. The BZ-trained group showed improvements in physical performance of equal magnitude to those of the iron- and placebo-trained groups. The BZ-untrained group showed no improvements in physical performance compared to the placebo-untrained group.

Collectively, this dissertation concludes that regular aerobic training reduces the apparent efficacy of iron supplements in improving iron status. Additionally, iron supplementation taken without training improves endurance performance in sedentary women, while iron supplementation with simultaneous training provides no added benefit. Finally, prescribing BZ may not be advisable for improving iron status or physical performance in IDNA women.

BIOGRAPHICAL SKETCH

Laura Marie Pompano was born and raised in Hanover, Virginia. She first became interested in nutrition and physical performance through her experiences as an athlete in high school and college. She received a B.S. in Biochemistry from Virginia Polytechnic Institute and State University, Blacksburg, VA in 2008 and a M.S. in Biological Chemistry from UCLA, Los Angeles, CA in 2010. In 2011, she entered the graduate program at Cornell University where she has worked on studies related to iron and physical performance in China, Rwanda, and the United States.

To my family.

For their love and encouragement through all of my adventures.

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Go Big Red.

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Chapter 1: Introduction

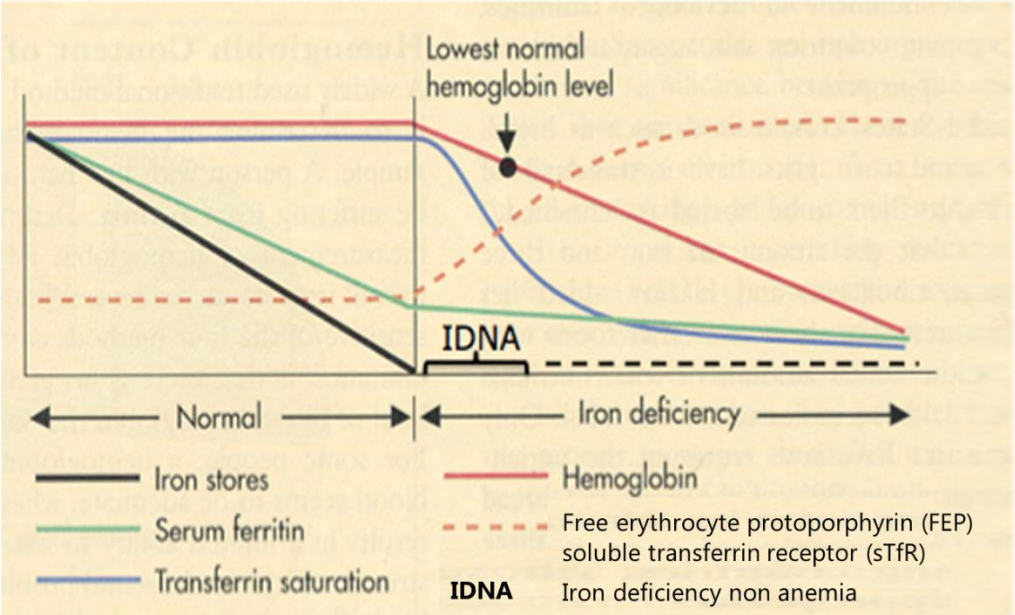
1.1 Iron Deficiency

Iron deficiency has persisted as the most common micronutrient deficiency in the world for years, affecting over 2 billion people in both developing and high-resource countries[1]. While severe iron deficiency results in roughly 800,000 deaths per year, according to the World Health Organization, there are also notable consequences of moderate iron deficiency (ID) on physical and cognitive ability that negatively impact daily life[1–5].

1.1.1 Biomarkers for Assessing Iron Status

There are many biomarkers available for assessing iron status, each representing different aspects of iron metabolism. Figure 1.1 shows the most commonly measured biomarkers for iron assessment and their relative concentrations across differing levels of iron status. The most widely used biomarkers for iron status assessment are: hemoglobin, serum ferritin, zinc protoporphyrin, mean cell volume, and soluble transferrin receptor[6]; however, transferrin saturation, mean corpuscular volume, erythropoietin, and others are also reported by some studies.

Figure 1.1: Iron Status Indicators (Modified from Guthrie and Picciano, 1995)



Hemoglobin (Hb), found in the red blood cells, binds oxygen and delivers it to tissues and cells. Red blood cells contain roughly 65-85% of the body's iron in the form of hemoglobin[7,8]. Accordingly, hemoglobin is the most commonly measured biomarker for iron status. Anemia is often used interchangeably with iron deficiency, but this use is erroneous because anemia can also occur as a result of other micronutrient deficiencies, pregnancy, chronic disease, and other conditions. According to a recent paper by Petry et al, only about 37-50% of the cases of anemia are due to iron-deficiency when defined using the current cut-offs for iron status indicators[9]. Therefore, while hemoglobin can be a good screening tool for assessing iron status and is more useful when severe iron deficiency is prevalent in the sample population, it is also necessary to measure other iron status biomarkers.

Besides hemoglobin, the most frequently reported iron status marker, and the marker promoted by the World Health Organization, is serum ferritin (sFer)[10]. Ferritin is an iron storage protein found in every cell, but that concentrates mainly in the liver. The ferritin level found in serum is proportional to the amount of ferritin stored in the liver (8 mg/L of stored iron for every 1 µg/L sFer) and is thus a good indicator for levels of stored iron[11,12]. As shown in **Figure 1.1**, ferritin levels decrease as iron stores diminish. Iron deficiency in adults is often defined as an sFer concentration less than 15 µg/L, a state that represents exhausted iron stores. Less severe iron deficiency, which reflects body iron depletion, represents lowered but not exhausted liver iron stores. Iron depletion varies in definition, but is typically defined as an sFer concentration between 20 and 30 µg/L[3,13,14]. Both clinical iron deficiency and iron depletion impact several important functions of daily life that will be discussed in later sections[8]. If sFer values indicate iron deficiency or depletion, but hemoglobin remains above the clinical cutoff that defines anemia, this state is called iron deficiency non-anemia (IDNA). If iron stores continue to worsen, hemoglobin levels can also decline below the clinical cutoff for anemia, resulting in iron deficiency anemia.

There are two major drawbacks of using sFer as a biomarker for iron status. First, sFer only represents the state of stored liver iron. Therefore, sFer is not a good indicator for processes such as erythropoiesis, functional iron such as myoglobin and Hb, or iron involved in cellular processes. Additionally, as an acute phase protein, sFer is highly affected by inflammation, increasing in concentration in the presence of inflammatory insults, obesity, or exercise[15]. This response is especially detrimental to

the assessment of iron stores in research environments where chronic illness or obesity are highly prevalent and can result in a measured prevalence of iron deficiency in a population that is falsely low. Depending on the purpose for which iron status was assessed (population surveys, clinical trials, etc.), there are several suggested ways for dealing with the association between inflammation and sFer values including: increasing the cutoff for ID, dropping those subjects with inflammation, or adjusting the sFer values using correction factors[16,17]. While these corrections can reduce the influence of inflammation on sFer concentrations, it is also helpful to measure other indicators of iron status to assure accurate iron status assessment.

In clinical research, soluble transferrin receptor (sTfR) is one of the more commonly measured biomarkers reported with sFer. Transferrin receptor is expressed on the membrane of cells and binds transferrin, the iron transport protein. Upon this binding, transferrin receptor facilitates the absorption of iron into the cell. sTfR expression increases with iron deficiency[11] because cells express more of the protein to attempt to import more iron. sTfR is also used to assess erythropoietic activity, as it increases in conditions of higher erythropoietic demand or ineffective erythropoiesis [17–19]. sTfR is directly related to iron deficiency, reflects the mass of red blood cell precursors, and is less affected by inflammation than sFer[20], collectively making it an excellent biomarker for iron status. However, there is currently no globally utilized standardized measure for sTfR. Each ELISA kit manufacturer uses its own scale, so quantification of sTfR is manufacturer-dependent and requires using reference intervals and deficiency cutoffs specific to the manufacturer. In order to standardize sTfR

between studies, the WHO released a reference reagent (material 07-202, made by NIBSC), which manufacturers can use to establish and standardize their reference values and deficiency definitions[21,22]. However, this reference standard is not yet frequently reported. More often, studies calculate and use manufacturer-specific correction values. This dissertation used the sTfR ELISA kit produced by RAMCO Laboratories to standardize all sTfR values from other labs. The recent Biomarkers Reflecting Inflammation and Nutritional Determinants of Anemia (BRINDA) project conducted by the US Center for Disease Control reported sTfR on the RAMCO scale (deficiency defined as sTfR > 8.3 mg/L), so this reference may become more standard in future reports[20].

A fourth measure of iron status is the estimation of total body iron. Total body Iron is an estimate that is calculated from the ratio of sTfR to sFer as shown below:

Total Body Iron (mg/kg) = −[log (sTfR/sFer ratio) − 2.8229]/0.1207

The total body iron measure was developed by Cook el al to provide a method to calculate absorption of iron during an intervention[23]. Cook described this value as a "quantitative estimate of body iron" that is a more sensitive tool for assessing effectiveness of intervention trials that can capture a wider range of iron status than either sFer or sTfR alone. Positive total body iron values indicate the presence of stored iron, whereas negative values indicate depletion of stores and iron deficiency in the tissue. The total body iron equation is best used in populations with little inflammation, and is therefore appropriate for this dissertation subject population, in which

inflammation was uncommon, as assessed by C-reactive protein (CRP) < 5mg/L and α-1-acid glycoprotein < 1mg/L (AGP).

1.2 Iron Absorption

Iron enters the body via the diet and is absorbed by the intestine. Iron excretion is constant at 1-2 mg/day and cannot be regulated by the body[7]. Therefore, the body's iron concentration is regulated at the point of absorption. The body's current iron status is a major regulator of the level of gut iron absorption. High iron stores in the body, indicated by high ferritin, result in lower absorption of iron in the intestine.

Figure 1.2: Regulation of iron absorption at the enterocyte[15]

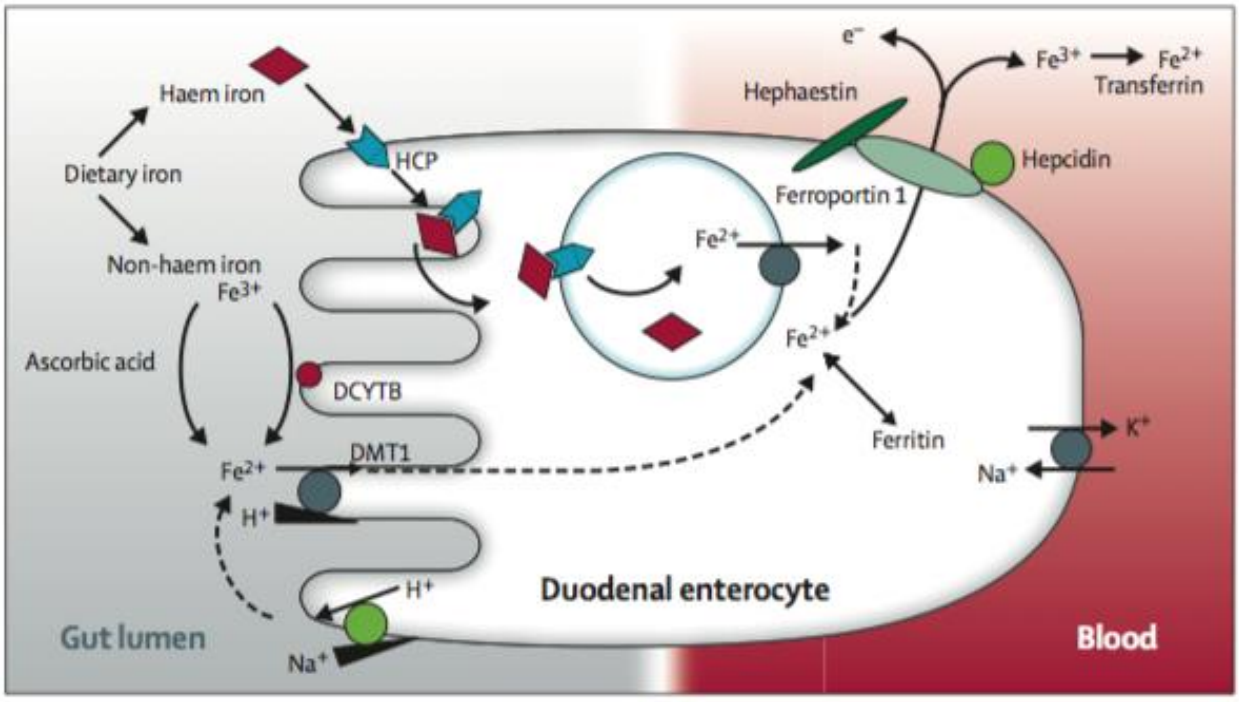


Figure 1.2 summarizes the iron absorption process. Ingested iron can be found in two forms: heme iron from animal products and inorganic non-heme iron from plant-

based foods. Heme iron is absorbed more efficiently than non-heme iron, is about 50% bioavailable, and is minimally impacted by body Fe stores, whereas non-heme iron is only 0.1 to 35% bioavailable, averaging towards the lower end of this range[24,25]. The two forms enter the enterocyte cells via different import proteins. Absorption of heme iron is not well understood as of this writing. Some studies suggest that it is endocytosed after binding the brush border of the enterocyte[26] or that it is taken up by the heme-responsive gene-1 protein[27]; however there does not appear to be enough evidence to conclusively determine the mechanism through which heme iron is absorbed. The main point of absorption for non-heme iron from the lumen into the enterocyte is through the apical membrane protein, divalent-metal transporter-1 (DMT1). Before inorganic iron can be imported into the enterocyte, it must be reduced to its ferrous (Fe^{+2}) form so it can be recognized by DMT1. One suggested reducing agent is the brush-border reductase, duodenal cytochrome B (DCYTB)[28]. Once inside the enterocyte, iron is either sequestered into the storage protein ferritin or exported for use via the export protein ferroportin in conjunction with the oxidizing protein ferroxidase hephaestin[15,26]. Unbound iron is highly reactive and can create harmful reactive oxygen species. Therefore, iron in the body is mainly found bound to various proteins. In the case of iron export to the bloodstream, it is bound to transferrin and transported throughout the body. If iron does not enter bloodstream, it is stored as the protein ferritin or hemosiderin. Ferritin is fairly ubiquitous throughout the body, but the primary location of iron storage is the liver.

1.3 Regulation of Iron Homeostasis

Iron homeostasis is regulated by the iron regulatory proteins, oxygen tension, and hepcidin at the cellular and systemic levels, respectively. The iron regulatory proteins (IRP1 and IRP2) sense cytosolic iron. In the iron deficient state, the IRPs bind to iron response elements in the mRNA that control multiple proteins involved in iron absorption and transport, promoting iron uptake and reducing iron storage and erythroid heme production[29]. More specifically, iron deficiency causes IRP1 to repress hypoxia-inducible factor 2α (HIF-2α) expression in the kidneys, which is responsible for several regulatory mechanisms controlling hypoxic response, erythropoiesis, and iron metabolism[30]. HIF-2α also stimulates iron absorption via downstream activation of proteins involved in absorption including DCYTB, DMT1, and ferroportin[31]. Furthermore, HIF activation also results in the expression of factors that ultimately reduce the use of pyruvate in the TCA cycle, limiting oxidative phosphorylation in ID conditions[32]. Under iron-replete conditions, the interactions between the IRP proteins and the various iron response elements do not occur, which results in the storage of iron as ferritin and increased erythropoiesis.

At the systemic level, the liver-derived hormone hepcidin controls iron homeostasis through its interactions with the only non-heme Fe cellular export protein, ferroportin[29]. Under iron-replete conditions, hepcidin binds ferroportin, resulting in its internalization and subsequent degradation and limits iron export to the plasma[33]. Thus, under normal conditions, hepcidin limits iron absorption, decreases recycling of iron from erythrocytes, and reduces iron stores. Hepcidin production is mediated by the iron

stores in the liver, transferrin saturation, erythropoietic demand, and systemic inflammation through several signaling pathways and through the acute phase response[33]. More specifically, hepcidin is primarily regulated by an increase in erythropoiesis[33]. Under normal conditions (the absence of thalassemias, etc.), erythropoiesis drives the release of the cytokine erythropoietin (EPO), which then down-regulates hepcidin production. Recent literature suggests that EPO may induce this inhibition via the hormone erythroferrone (ERFE), which suppresses hepcidin expression through a mechanism that is currently not well understood[29]. ERFE is expressed by both erythroblasts and skeletal muscle cells[29], and can thus respond to both erythropoietic demands from low-iron or hypoxia (erythroblasts) as well as increased demands induced by exercise (skeletal muscle), which will be discussed later. The relationship between erythropoiesis and hepcidin regulation remains somewhat unclear, though literature suggests that both EPO and iron status have interdependent roles in regulating hepcidin[29].

Additionally, erythroid cell differentiation involves transferrin receptor 2, a protein located in the developing precursor cell. Iron deficiency results in the destabilization and subsequent shedding of this receptor from the progenitor cell, which increases the sensitivity of the bone marrow to respond to EPO, thus increasing ERFE production and down-regulating hepcidin [29]. Collectively, these mechanisms suggest that iron deficiency influences several sites of erythropoietic control to reduce erythrocyte maturation while simultaneously increasing the amount of iron available for other, potentially more critical, functions.

1.4 Iron Status and Physical Performance

While impaired iron status is known to affect multiple aspects of physical performance, the metabolic mechanisms through which iron deficiency anemia and iron deficiency without anemia primarily produce these changes differ widely. This section will survey the literature examining first the effects of IDA on physical performance, then those of iron deficiency without anemia.

1.4.1 Iron Deficiency Anemia and Performance

Iron deficiency anemia negatively affects physical performance because the decreased Hb concentration in the body limits the amount of oxygen available for active muscles to use in aerobic respiration[34]. The observable outcome of this impairment is a lower maximal capacity to circulate and utilize oxygen, called VO_2max. Studies have demonstrated that IDA women who receive iron supplements and achieve replete iron levels show increases in VO_2max proportional to the measured changes in iron[35–37]. Lowered VO_2max from IDA is known to worsen physical performance in laboratory settings and free-living environments, lower total energy levels, and decrease daily physical activity[36,38,39].

Muscle myoglobin also impacts physical performance and may be affected by anemia or iron deficiency. Myoglobin is similar to hemoglobin in that it functions as an oxygen binding protein, transferring oxygen from the erythrocytes to the muscle tissue. Currently there is limited research investigating myoglobin's role in human physical performance in subjects with IDNA. However, several studies have suggested that

myoglobin concentration is negatively impacted by iron deficiency[40,41]. A review by Buratti et al summarizes findings from several studies that examined how myoglobin is affected by low oxygen conditions from high altitude or in subjects treated with low-dose erythropoietin[32]. The increased erythropoietic demand from high-altitude resulted in decreases in the three muscle myoglobin isoforms of 30-35%[42]. However, when subjects were chemically administered erythropoietin, which should theoretically stimulate erythropoiesis and inhibit hepcidin expression, there was no change in muscle iron content[43]. Buratti et al proposed that the discrepancy in muscle response is due to the iron demand of the stimulus, where high altitude induces a strong demand for iron and thus results in iron mobilization from muscle myoglobin, whereas in normal iron conditions, low-dose erythropoietin only induces a moderate iron demand, which can be met by other body iron stores in the body[32].

1.4.2 Iron Deficiency Without Anemia and Performance

While it is well established that IDA negatively affects maximal aerobic exercise, IDNA is also known to affect submaximal physical performance measures and lowers time spent in daily voluntary physical activity[3,13,44,45]. The literature involving IDNA subjects who were given iron in conjunction with any kind of physical training will be explored further in section 1.5.2.

Because iron deficiency without anemia does not, by definition, involve decreased levels of Hb, the metabolic explanation for the performance impairments seen in IDNA women are less related to oxygen delivery by Hb. For this reason, studies

involving only IDNA women do not typically show significant changes in VO_2max from iron supplementation alone[46–48]. Rather, observed effects are related to changes in aerobic respiration pathways such as the Citric Acid cycle, oxidative phosphorylation, and β-oxidation that rely heavily on iron-containing and iron-dependent proteins and enzymes (see section 1.5 below). While it is well established that IDNA negatively impacts physical performance by reducing oxidative capacity[34,49], it is not yet known exactly which enzymes in these processes are most affected by ID and which are associated with the observed impairments in submaximal exercise. It is also likely that these alterations in oxidative capacity compound the detrimental effect of restricted oxygen delivery that occurs in anemia, further impacting physical performance in this group. This relationship must be further clarified if we are to understand how iron deficiency exerts its effects on physical performance and activity.

1.5 Exercise Training and Physical Performance

This section will first look at the literature that has examined the role of aerobic exercise training in altering physical performance outcomes. It will then explore the role of iron in relation to these factors.

1.5.1 Physiological responses to aerobic training

1.5.1a Changes in bioenergetic pathways

Holloszy et al have shown that under normal, healthy conditions, exercise training induces metabolic changes in the bioenergetic pathways[50]. In his study, healthy rats underwent aerobic training 5 days a week for 1-6 weeks, depending on time of

sacrifice. Pre- and post-training muscle biopsies from both red and white tissue showed that the training resulted 70-140% increases in concentrations of various electron transport system enzymes and substrates in the trained rats. For example, cytochrome C increased 86% in trained rats compared to the sedentary group ($p < 0.001$). This study demonstrated that there is a strong relationship between the electron transport system, ATP production, and aerobic exercise training.

Following the findings detailed above, Holloszy et al. used the same aerobic training program to demonstrate the effects of aerobic training on Krebs cycle enzymes[51]. In this study, Holloszy observed a 34-101% increase in Kreb's cycle enzymes and substrates the gastrocnemius muscles of rats. The study concluded that while training affects oxidative phosphorylation more than the Kreb's cycle, there was a significant training effect on both metabolic pathways. Additionally, this study showed that because training-induced changes in oxidative phosphorylation were greater than in the Kreb's cycle, there must be some change in mitochondrial composition that is responsible for the increase in ATP production seen from aerobic training. This change is now known to be increased mitochondrial density and size, which allows for more oxidative phosphorylation and thus greater ATP production[51]. The changes observed in both the Kreb's cycle and oxidative phosphorylation enzymes are not due to increased specific activity of these enzymes. Rather, trained muscle has both larger and more numerous mitochondria than untrained muscle. Thus, in a trained muscle undergoing aerobic exercise, there are simply more of the necessary enzymes available to make ATP[52–54].

Aerobic exercise training also results in changes in fatty acid metabolism[55]. In a study by Molé et al, rats were exercised on a treadmill 5 days a week for 12 weeks. The results showed that trained rats had up to double the concentrations of palmityl CoA synthetase and palmityl CoA dehydrogenase – enzymes involved in muscle's ability to oxidize various fatty acids. Additionally, the trained group had higher levels of carnitine palmityl transferase, which catalyzes the reaction to bring fatty acids into the mitochondria to undergo oxidation.

In addition to mitochondrial enzyme changes in fatty acid metabolism, studies have shown that aerobic exercise training increases muscles' ability to metabolize triacylglycerols and lowers the amounts of catecholamine released [56–58]. The training-induced increases in the enzymatic capacity to utilize lipid fuel sources appears to be complemented by an increase in the number of intramyocellular triglycerides that are stored in the muscle fibers, particularly the Type 1 oxidative fibers, as evidenced by Type 1 fibers having three-folder greater intra-muscular triglyceride content compared to Type 2 fibers in muscle cross-sections prior to exercise[59]. This increase in triglycerides is specifically due to a larger number of lipid droplets in the muscle, as opposed to an increased volume of existing droplets. An increased number of lipid droplets allows for easier lipolysis for use due to the increased surface area of the additional droplets[59,60]. The increases in lipolytic enzymes, as well as the improvements in overall oxidative capacity from the increased size and number of mitochondria discussed earlier, collectively result in the trained muscle being better able to rely on intramyocellular triglycerides for fuel during exercise. This shift ultimately has a glycogen sparing effect

in the muscle, allowing the trained muscle to perform aerobic work longer before reaching an anaerobic state.

1.5.1b Changes in VO_2max

Aerobic exercise training increases VO_2max as a result of several training-induced adaptations. VO_2max is determined by cardiac output and the a-vO_2 difference, which are respectively defined as the amount of blood moved with each heartbeat and the amount of oxygen removed by the capillaries as it circulates through the body. Aerobic training positively impacts both of these factors. With long-term aerobic training, the left ventricular cavity of the heart increases in volume by around 25%[61,62]. The capillarization of the heart muscle itself is also increased, allowing more blood flow to the heart tissue, which decreases the energetic cost of breathing. Further complementing these cardiovascular changes, aerobic training induces a roughly 10-20% increase in plasma volume[63]. The expansion of plasma volume lowers blood viscosity, which enhances movement of blood through the capillaries and improves oxygen delivery to tissues[64]. Together, these changes allow the heart to move more blood per beat, increasing the stroke volume component of cardiac output in the VO_2max equation. Another component of VO_2max, a-vO_2 difference, or the amount of oxygen extracted into the muscles from the blood, also increases as a result of aerobic training[65]. This increase is accomplished partly by increased capillarization of the trained skeletal muscle, increased myoglobin concentrations in the muscle to accept oxygen, and the development of more aerobic (Type 1) muscle fibers, several of which are processes that are affected by iron bioavailability[32,66]. Together, these training

effects result in a higher overall VO_2max in aerobically trained subjects.

1.5.1c Changes in fatigue

Aerobic training also influences rate of fatigue, or the reduced ability of a muscle to generate force. One cause of fatigue can be the inability of nerves to sustain a muscle response. In untrained subjects, nerves are unable to maintain a high-frequency signal to the muscle, thereby lowering the force that the muscle produces[67,68]. The other aspect of fatigue is the onset of anaerobic metabolism within the muscle fibers themselves. This kind of fatigue results from a lack of aerobic energy substrates and subsequent lactic acid production, which will be discussed in the following sections[69].

During exercise, glucose is broken into two pyruvate molecules during glycolysis. The resulting pyruvates then enter the Kreb's cycle to produce ATP and high-energy molecules for later ATP production in oxidative phosphorylation. Both the Kreb's cycle and oxidative phosphorylation are highly aerobic and cannot proceed if available oxygen becomes insufficient – such as during strenuous exercise. In this scenario, the anaerobic process of glycolysis can still occur, producing two net ATPs and 2 pyruvate molecules. During sustained vigorous activity, muscle glycogen is broken down to provide glucose for glycolysis, which occurs rapidly to provide the muscles with ATP. However, this also leads to a large accumulation of pyruvate. This pyruvate is then shunted into the anaerobic process of fermentation, producing lactic acid as a product, which builds up in the muscle[69].

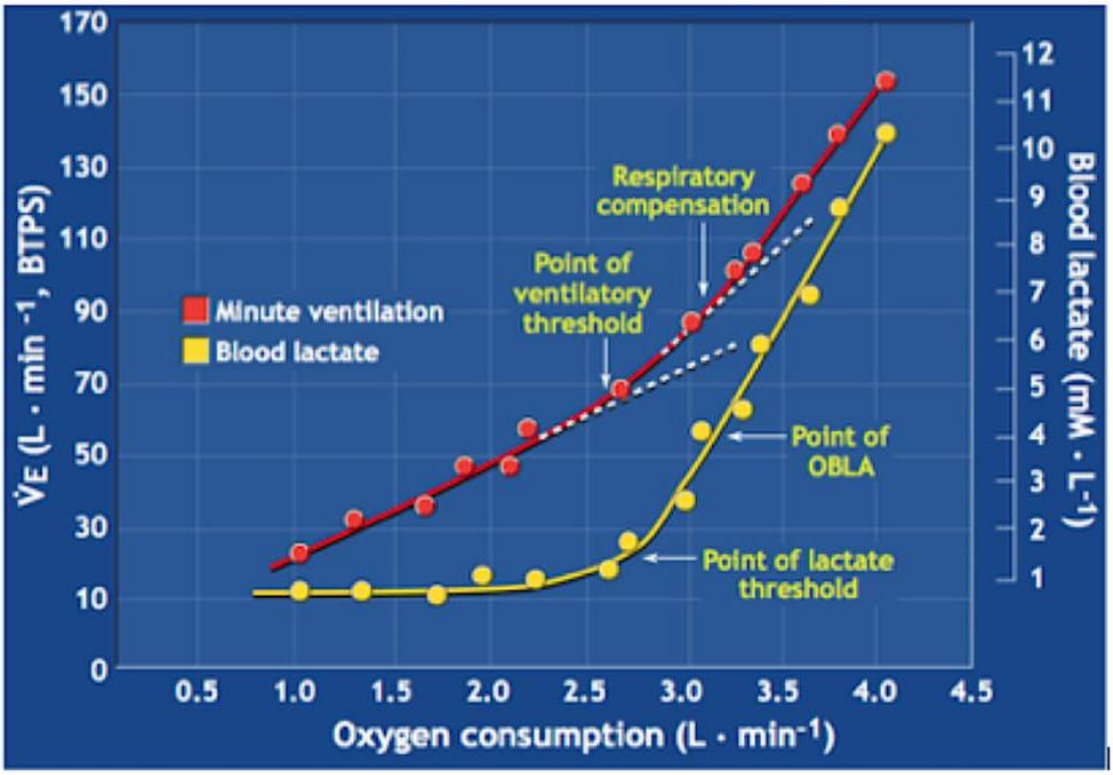
Muscular fatigue occurs as a result of both depleted substrates for energy

production and increased metabolite products that inhibit muscle function. During strenuous exercise, oxygen is depleted first, inhibiting aerobic respiration. Muscle glycogen and phosphocreatine stores are then depleted, inhibiting the rate of anaerobic respiration. At this point, the muscle has highly restricted access to energy producing substrates and cannot sustain the same level of activity. Simultaneously, the buildup of lactic acid may also influence muscle fatigue, though this research is controversial. It was initially believed that lactic acid buildup was the main cause of muscle fatigue[70], however recent literature suggests there may be multiple mechanisms involved in fatigue in addition to acidosis[71,72]. Regardless of the mechanism through which fatigue is induced, lactic acid concentration is an excellent proxy for muscle fatigue and the anaerobic threshold, or the point where anaerobic respiration begins during exercise.

During light to moderate activity, lactic acid builds up steadily but is cleared by the body at the same rate at which it is produced, with no net lactic acid accumulation. The flat portion of the bottom line in Figure 1.3 indicates this steady state. When exercise intensity increases, this balance shifts towards production and lactic acid concentration increases. The lactate threshold is the highest level of oxygen consumption (exercise intensity) that does not increase blood lactate by more than 1.0 mM from the resting level[69]. Blood lactate and the lactate threshold are technically easy to measure, but require constant sampling of blood, which can be problematic in practice. However, another measure, ventilatory threshold, corresponds well to the lactate threshold, is easily measured using expired air, and does not require blood sampling. The ventilatory threshold is the level of exercise where pulmonary ventilation

increases disproportionately to oxygen consumption (altered V_E/VO_2 ratio). Figure 1.3 clearly depicts that while the ventilatory threshold occurs at higher levels of minute ventilation than the lactate threshold, the inflection points for the lactate threshold and the ventilatory threshold occur at nearly the same volume of oxygen consumption. Thus, ventilatory threshold can be an excellent proxy for lactate threshold.

Figure 1.3: Lactate Threshold vs. Ventilatory Threshold (McArdle, Katch, and Katch 2010)



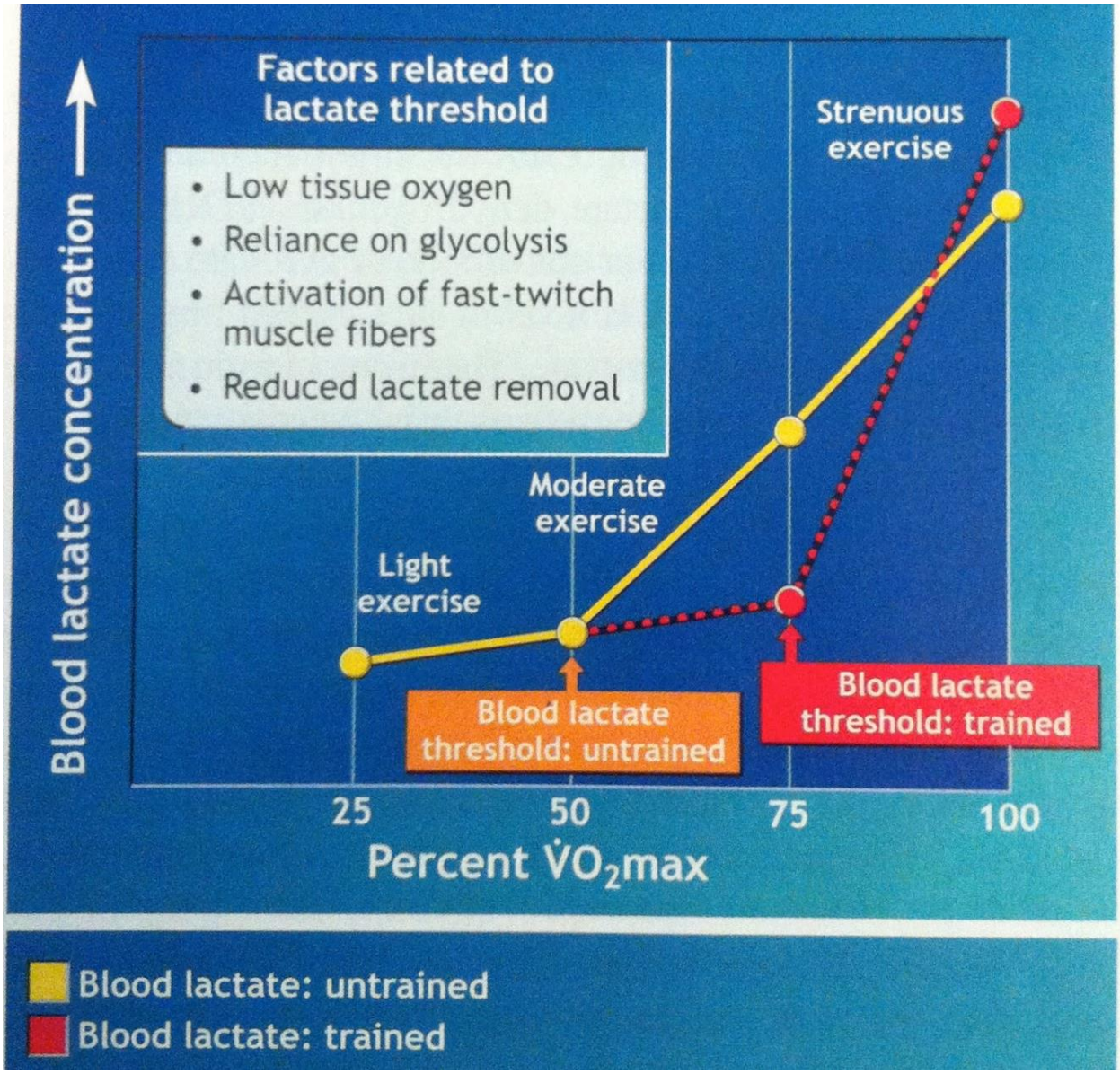
Abbreviations: VE – Pulmonary Respiration; BTSP - body temperature, ambient pressure, saturated (used for expressing a gas volume that has been expressed as if it were saturated with water vapor at body temperature (37°C) and at the ambient barometric pressure; used for measurements of lung volumes); OBLA - Onset of blood lactate accumulation

The onset of muscle fatigue is delayed by exercise training. Aerobic training has been shown to lower lactate levels in healthy subjects after exercise and delay the

occurrence of the lactate threshold[73]. This decrease is likely due to improved lactate clearance in trained subjects[74] as well as improved oxygen uptake and use[65]. Aerobic training results in the muscle having a higher proportion of Type 1 muscle fibers, which have a greater number of the transporters needed for lactate clearance than the Type 2 fibers. Figure 1.4 depicts the effects of training on lactate threshold.

As discussed in 1.5.1b, VO_2max increases proportionately with aerobic training. Therefore, as VO_2max increases, the lactate threshold can occur at a higher percent of maximum O_2 uptake.

Figure 1.4: Blood lactate concentration for trained and untrained subjects (McArdle, Katch, and Katch 2010)[69]



Abbreviations: VO_2max – maximal oxygen consumption

1.5.2 Iron status, aerobic training, and physical performance

As discussed in section 1.4, both IDA and IDNA have been shown to negatively impact physical performance. IDA is primarily affected by lowered Hb concentrations

restricting oxygen delivery to tissues[34,36], while both IDA and IDNA are affected by reduced oxidative capacity[34,49]. This section will explore the literature that examines changes in various physical performance outcomes in IDNA subjects who received iron supplementation as well as some kind of physical training. The literature on this topic examines all populations including trained athletes as well as untrained males and females. However, this dissertation involved only relatively sedentary females, therefore this section will focus on studies most relevant to our study population.

Zhu et al[45], gave a ferrous sulfate treatment or a placebo to IDNA women for 8 weeks and observed changes in their performance in a 15-km time trial. The study observed lower energy expenditure during the time trial in iron-supplemented subjects compared to controls. Additionally, during the time trial, iron-supplemented subjects worked at 5.1% lower VO_2 relative to VO_2max than controls. A different study by Brutsaert et al observed a group of IDNA women's maximal voluntary static muscle contractions during a dynamic knee extensor test before and after a 6-week iron supplementation (or placebo) period[75]. After supplementation, women in the placebo group showed no change in their ability to apply force during the leg-extension test, with force decreasing at the same rate over the 6-minute period before and after treatment. The iron group however, showed an attenuated rate of decrease in force – signifying an improved (slower) rate of muscle fatigue[75]. These studies and others[13,76] indicate that iron supplementation alone is enough to elicit changes in endurance performance.

The combined effect of iron supplementation and aerobic training on physical

performance in IDNA subjects has also been studied. More recently, a study by DellaValle et al randomized a group of 40 female IDNA collegiate rowers to receive either 100 mg ferrous sulfate per day or a visually identical placebo for 6 weeks during their training season[14]. The subjects performed a 4 km rowing time trial and a VO_2max test before and after 6 weeks of physical training. While both groups increased their fat-free mass and VO_2peak (maximum observed VO_2 during the test), the iron group showed significant improvements in sFer and larger improvements in energy expenditure and energetic efficiency than the control group. Additionally, the iron group had a slower lactate response during the first half of the time trial and at 5-minutes post-test.

Another study by Hinton et al, supplemented moderately active, IDNA women with either iron or a placebo for 6 weeks. All subjects also participated in 30 minutes of aerobic exercise training 5 days a week for the last 4 weeks of the study. Training ranged from a level of 75-85% of subjects' maximum heart rate and became progressively more challenging each week in order to induce a training effect. This study observed that in a 15 km time trial, the iron supplemented women decreased their time to complete the time trial more than the placebo group after identical training[3]. From this same study, Brownlie et al showed that VO_2max increased in all subjects after training, but the iron group showed a significantly greater improvement[77]. However, these findings were only seen in subjects with an elevated sTfR value (> 8.0 mg/L), indicating that the response was due more to a lack of available iron for cellular uptake and less related to iron stores. In another paper, Brownlie further showed that the iron

supplemented group worked at a significantly lower percent of their VO_2max during the time trial than placebo group after training – again after accounting for baseline sTfR status[45].

The role of exercise in affecting iron status has also been observed. Exercise training has been shown to negatively impact iron status[76,78]. Female soldiers who completed basic training displayed a 20% higher prevalence of iron deficiency than soldiers who had not yet completed basic training[78]. In a similar study, women were given either a placebo or iron supplementation before completing basic training. After training, sFer levels were significantly lower in the placebo group but not in the group that received iron.[78]

Another study by Magazanik et al. randomized 28 iron depleted women (mean sFer at baseline 24 µg/L) to receive either 160 mg of ferrous sulfate per day or an identical placebo[79]. The women then completed a 7-week intensive physical training program. VO_2max was assessed before, during, and after the treatment. At the midpoint of the study, the iron group had a 7.5% increase in VO_2max, compared to no change in the placebo group. While no significant difference was ultimately found in VO_2max after the 7-week training period, 66% of the placebo group had sFer values < 10 µg/L, whereas none of the iron group had such low values, suggesting that iron supplementation mitigated the negative impact of training on iron status.

Finally, a study by Mielgo-Ayuso et al. randomized 22 national volleyball players to either a control group (no treatment) or an iron supplement group that received 325

mg of ferrous sulfate per day for 11 weeks while both groups performed the team's usual training routines[80]. After the treatment, the control group had significantly lower serum iron, sFer, and Hb than they had at baseline. The iron group displayed significant positive changes in the same iron measures, again suggesting that the iron treatment prevented a worsening of iron status. Additionally, the iron group displayed significantly greater improvements from baseline to week 8 in various strength scores than those observed in the placebo group. Collectively, these studies suggest that exercise training may result in worsened iron status, but that this effect can be attenuated by concurrent iron supplementation. Additionally, iron supplementation with simultaneous exercise training enhances strength, endurance, efficiency and other submaximal exercise measures without changes to VO_2max.

While numerous studies have compared the effects of iron supplementation versus placebo in trained women, to our knowledge, no study has used a design that has both iron and placebo as well was trained and untrained groups. Therefore, this dissertation used a 2x2 design to look at the effects of iron supplementation and aerobic exercise training in a manner that allowed for the examination of the independent and synergistic effects of iron supplementation and aerobic exercise training in a group of IDNA women.

1.6 Traditional Chinese Medicine Treatment for Iron Deficiency

Collectively, the data presented in sections 1.1 through 1.5 clearly demonstrate the impact that iron deficiency can have on physical performance and highlight the

importance of monitoring and addressing IDNA in a population. Many approaches are utilized to address iron deficiency in varying populations around the world, including dietary diversification, fortifying food products, and the use of dietary supplements. However, for some low-resource areas in rural China, these methods are less feasible due to issues of affordability and availability of supplements or fortified food products[81]. Therefore, it is critical to identify novel, sustainable, and affordable methods for addressing iron deficiency in these areas. One such possibility is using traditional Chinese medicinal herbal treatments. In many parts of China, traditional Chinese medicine is considered a "first line" of treatment that should be used before seeing a physician or using "Western" medicines[82]. While the majority of traditional Chinese herbal remedies have not been studied using rigorous scientific methodologies, a recent review of several Cochrane systematic reviews[83] concluded that many treatments had evidence of a positive effect or lacked sufficient evidence to determine an effect either way. This conclusion supports the idea that traditional Chinese herbal remedies do have the potential to be beneficial and should be studied for their efficacy in improving medical conditions or nutritional deficiencies like IDNA.

BaZhen KeLi (Chinese: 八珍颗粒, BZ) is a traditional Chinese medicinal treatment that is often consumed to address symptoms that would be considered by Western medicine to indicate iron deficiency or anemia, such as pallor and fatigue[84]. A Chinese medicine practitioner would assess iron deficiency as one of two types of *qi* deficiencies – generally meaning that the blood is lacking a vital "energy" called *qi*. BZ is an herbal mixture composed of 8 different plants, which when taken together, address

both of these two *qi* deficiencies. Table 1.1 lists the main ingredients of BZ and their functions in the body, according to Chinese medicinal theory. BZ is usually administered as a capsule containing various ratios of the eight components.

Table 1.1: BaZhen capsule ingredients

English Name	Latin Name	Chinese Name	g per capsule
White Atractylodes Rhizome	*Rhizoma Atractylodis Macrocephalae*	Bai Zhu, 白术	10
White Peony Root	*Radix Albus Paeoniae Lactiflorae*	Bai Shao, 白芍	10
Szechuan Lovage Root	*Rhizoma Ligustici Chuanxiong*	Chuan Xiong, 川芎	10
Chinese Angelica Root	*Radix Angelica Sinensis*	Dang Gui, 当归	10
Codonopsis Root	*Radix Codonopsitis Pilosulae*	Dang Shen, 党参	10
Poria (China Root)	*Scierotium Poriae Cocos*	Fu Ling, 茯苓	10
Honey Fried Licorice Root	*Radix Glycyrrhizae*	Zhi Gan Cao, 炙甘草	5
Chinese Foxglove Root	*Radix Rehmanniae*	Shi Di Huang 熟地黄	10

While BZ has long been used in traditional Chinese settings, recently its components have been examined in multiple research and clinical studies. In 2011 Wang et al[85] conducted a quality control study tailored to traditional Chinese herbs to identify potentially active substances in the medicinal formula as given in a traditional Chinese context. The paper faults previous studies of Chinese herbal treatments for testing only the identified “active” ingredients in herbs. Thus, Wang et al tested several formulations of the treatment including: a. the traditional mixture of components as typically prescribed, b. each plant individually, and c. a mixture of three of the previously

identified BZ molecular components[85] (ferulic acid, ligustilide, and senkyunolide A) to allow comparison of whole BZ to its various components. The group also tested two different manufacturers' BZ products, to obtain an idea of variance among on-the-market products. An absorption study was performed in the Caco-2 cell culture model for each of these seven BZ products or components. The absorbable components were then incubated with MCF-7 cells to determine and compare the gene expression profiles induced by each sample. The study found that in all of the repeated trials, the two brands of whole BZ treatments induced similar gene expression profiles - identifying nine differentially expressed genes from the whole treatments. None of the four individual herbal components or the active ingredient mixture clustered with the whole treatments' gene expression profiles and none showed the same set of differentially expressed genes. The Wang et al study concluded that traditional herbal medicines should be administered in their whole form, suggesting that *in vivo* reactions are likely not simply relying on one herb or one active molecule of an herb within the whole formula. The study recognized that the Caco-2/MCF-7 model used is not likely representative of the process that occurs in humans and thus called for more research involving BZ in its whole form in human studies to further explore BZ's effects on gene expression profiles in *vivo* settings.

A study by Li et al[86] examined the effects of BZ on physical fatigue in mice. A group of 32 mice were given either a placebo or varying concentrations of BZ (5, 10 and 20ml/ kg body weight) for 28 days. After supplementation, mice were subjected to a forced swimming test, after which blood lactate, blood urea nitrogen, and liver and

muscle glycogen were measured. Mice that received BZ extended their swimming time in a dose-dependent manner from 1.42 to 2.14 times that of the controls ($p<0.05$). Additionally, BZ mice showed lower levels of blood lactate and urea nitrogen, as well as higher levels of liver and muscle glycogen after the swimming test. This study suggests that BZ plays some role in physical performance, though more research is needed to clarify the mechanisms through which it does so.

Finally, several studies by Wang et al[87–89] examined the relationship between a polysaccharide in *Dang Gui* (Latin: *Angelica sinensis*, one of the herbs in BZ) and iron deficiency anemia. This series of papers showed that the *Angelica sinensis* polysaccharide (ASP) inhibits hepcidin expression. This mechanism potentially works through an inhibition of the STAT3/5 and SMAD4[88] pathways, inhibition of the JAK2 pathway[89], and increasing secretion of erythropoietin[87], which further inhibits hepcidin expression. Though this series of papers examines only one biological component of the BZ treatment, they suggest a biological mechanism through which BZ may directly influence iron homeostasis.

Collectively, these papers support the further examination of BZ in a more vigorous research setting for both its effects on iron status as well as physical performance outcome measures.

1.7 Research Aims

Previous research has justified the need for, but not allowed for the examination of the modifying effects of iron supplementation on improvements in performance from

aerobic training or regular exercise on the effectiveness of iron supplementation. This dissertation expands upon prior research that suggests that regular aerobic training can worsen iron status and that IDNA can impact physical performance and physical activity via a mechanism unrelated to Hb. Understanding these relationships could greatly enhance our understanding of iron metabolism and better inform the design of iron interventions in physically active populations. Therefore, the primary research objective of this dissertation is to examine the relationships between and potential modifying effects of regular aerobic training and/or changes in iron status on maximal and submaximal physical performance. This objective will be explored in the following aims:

1. To determine the modifying effects of exercise training on the effectiveness of iron supplementation in improving iron status assessed by sFer, sTfR, Hb, and body iron.
2. To examine the combined and independent effects of exercise training and changes in iron status on physical performance assessed by maximal and submaximal exercise tests.

The relationship between IDNA and physical activity and performance is directly related to larger public health concerns since decreased energetic efficiency and/or daily physical activity is associated with higher risks of obesity, diabetes and heart disease[90–93]. Given the high prevalence of ID in the global population, and the increasing rates of obesity, diabetes, and heart disease in both developed countries and those undergoing the nutrition transition, such as China, it is becoming increasingly more important to fully understand the functional consequences of ID in daily physical

activity and physical performance. The detrimental effects of iron deficiency on physical performance also have implications for economic productivity in developing countries. For example, agricultural workers rely heavily on their ability to perform physical tasks, such as picking crops. Iron deficiency has the potential to lower physical performance capacity of a large portion of the labor force, which would result in an overall lower economic productivity in areas with a high prevalence of iron deficiency[94,95]. Similarly, if physical activity negatively impacts iron status or the effectiveness of iron supplementation, those areas with a high prevalence of iron deficiency among manual laborers could be doubly affected. Finally, most iron interventions target women with IDA at the expense of a large population of IDNA women who could benefit greatly from iron interventions. This dissertation will promote the inclusion of IDNA in iron interventions by clarifying the negative impact of IDNA in active populations, such a rural laborers or athletes.

Additionally, it is imperative to identify new, sustainable and affordable methods for addressing IDNA in rural areas of China that cannot utilize the conventional methods of resolving iron deficiency. Given the popularity of traditional Chinese medicine in rural areas, as well as the promising literature that relates iron status and the Chinese herbal treatment BZ, it is possible that BZ may provide an alternative to iron supplementation. Therefore, this dissertation will also explore the third aim below:

3. To compare the effectiveness of using a traditional Chinese herbal treatment (BZ) to that of ferrous sulfate in improving iron status and/or physical performance of Chinese, IDNA women

Chapter 2: Methods

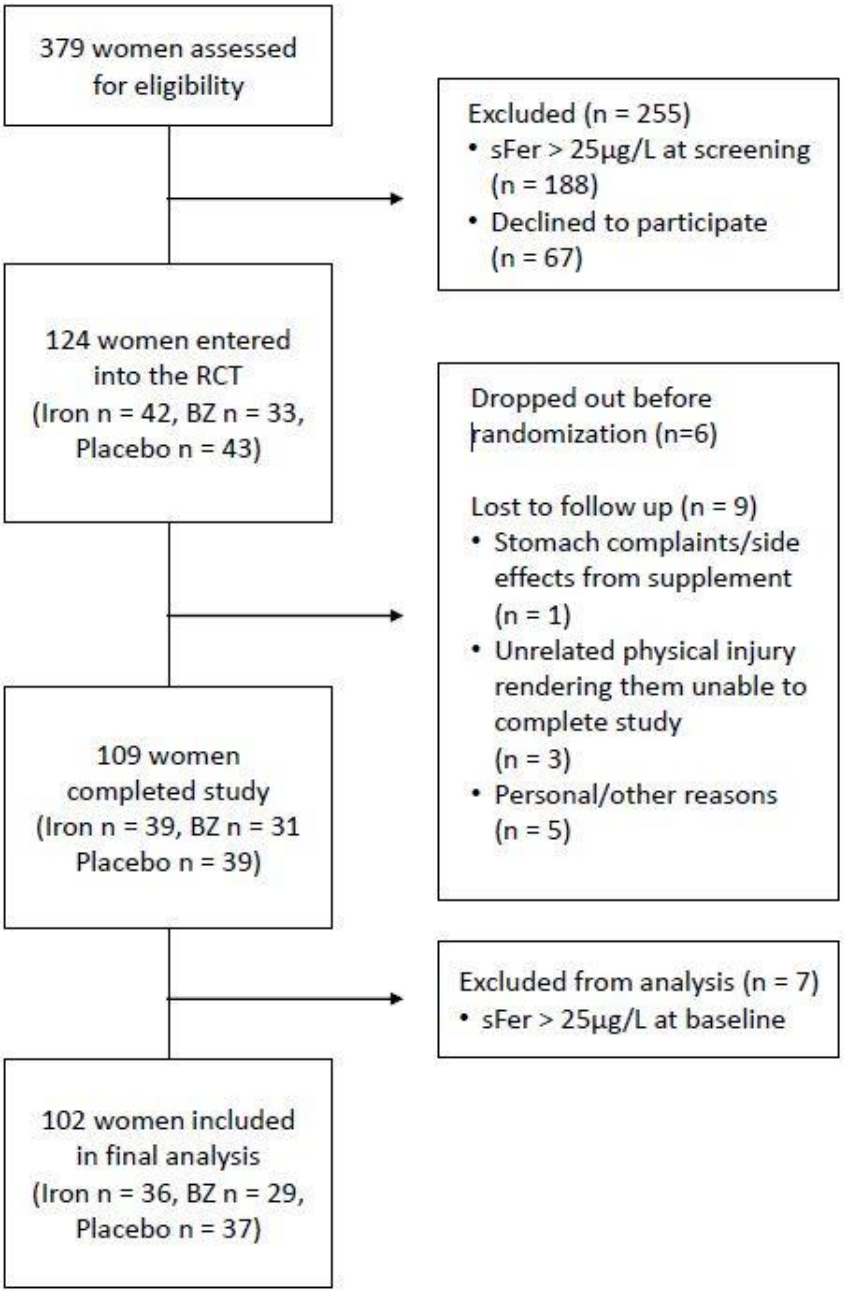
2.1 Kunming Randomized Control Trial

The objective of this study was to examine the individual and combined effects of an 8-week iron supplementation and aerobic exercise training program on biomarkers of iron status and the functional outcomes of physical performance (maximal and sub-maximal measures). A subset of participants was also included in the main trial to examine the efficacy of using a traditional Chinese herbal medicine as an alternative to Western iron supplementation. The methods for that subset will be presented in Section 2.2. The Consort diagram for the full study population is shown in Figure 2.1.

2.1.1 Study Design

The study for Aims 1 and 2 was a randomized, controlled, partially-blinded trial that took place at Kunming Medical University in Yunnan, China from February 2014 to July 2015. The 8-week intervention occurred in two waves, one from October to December of 2014 and the second from April to June 2015, referred to as waves 1 and 2, respectively. Both waves were identical in protocol. The study used a 2x2 factorial design with participants being randomly assigned to receive either 100 mg of ferrous sulfate twice daily or an identical placebo for a total of 42.2 mg of elemental iron per day. Both the iron and placebo supplements were prepared by the author in the research lab of Dr. Bing Su at the Kunming Zoology Institute, a part of the Chinese Academy of Sciences. Twenty iron and placebo capsules were randomly selected every four weeks throughout the study and analyzed for iron content.

Figure 2.1: Consort diagram for full study population



The iron content of the ferrous sulfate and placebo capsules was determined by ICP/MS to be 21.1 mg and 0.0 mg elemental iron per capsule, respectively.

Subjects were instructed to consume the capsules with their morning and evening meals, to do so with citrus juice to enhance absorption, and to avoid consuming capsules on an empty stomach. Thirty capsules were distributed to each subject in a bottle labeled with their unique subject ID every 2 weeks (weeks 0, 2, 4, and 6). Each subject received a log to record their daily capsule ingestion as well as information on medication use, illness, menstrual cycle status, any gastrointestinal complaints, physical activity, and musculoskeletal problems. Additionally, subjects were instructed to return their capsule bottles every two weeks with any remaining capsules, which were then counted to confirm the number of capsules reported as consumed on the capsule logs. Subjects were not told that the returned capsules would be counted to avoid their discarding any non-consumed capsules prior to returning their capsule bottles. Half of each capsule group was further randomized to receive either 8 weeks of aerobic exercise training or no training, resulting in a total of 4 groups in which supplement type was blinded but training group was not. The training protocol will be described in section 2.1.4.

In each wave, blood samples were taken at weeks 0, 4, and 8 as well as measurements of anthropometry, physical performance, dietary intake, and physical activity. Physical performance assessments will be described in section 2.1.5. Blood samples were assessed for 5 biomarkers: sFer, Hb, sTfR, alpha-1-acid glycoprotein (AGP), and C-reactive protein (CRP). Anthropometry included height, weight, mid-upper

arm circumference, and body fat as assessed by 4 caliper measurements of the bicep, triceps, suprailiac, and sub-scapular skinfold thicknesses (Lange calipers, Cambridge Scientific, Cambridge, MA). Dietary intake was assessed using a 4-day dietary record in which subjects were instructed to record all food and beverages they consumed, including water, during a specified Thursday to Sunday period. They were instructed to be as detailed as possible, giving volumes or weights when known and estimating "scoops" for the food served at the cafeterias on campus, which was served using a ladle. Subjects were asked not to alter their normal eating behaviors during the assessment.

2.1.2 Study Participants

This study was conducted in an iron depleted, non-anemic, and otherwise healthy group of female Chinese university students in Yunnan, China. Women aged 18 to 26 years were recruited locally for screening through in-class presentations and distributing flyers on campus. Inclusion criteria for the intervention trial were as follows:

a. Ages 18-26
b. Hb > 110g/L
c. No current pregnancy or pregnancy within the previous year
d. No recent infectious illness or fever
e. No current inflammation or chronic inflammatory diseases
f. No major musculoskeletal problems, medical conditions, or other injuries that would prevent them from completing the physical performance assessments and/or training
g. No history of eating disorders
h. Non-smoking
i. BMI between 17 and 25 kg/m^2
j. No consumption of medications that may affect dietary iron intake or absorption or that have anticoagulant properties.
k. No use of psychoactive drugs

Three hundred seventy-nine women were recruited in two waves (September-December 2014 and March-June 2015) from the student population at Kunming Medical University in Yunnan, China. Of these, 98 women were identified in the fall wave, and 281 were identified in the spring. In total, 191 were identified as iron-depleted without anemia, defined as Hb > 110 g/L and sFer < 25μg/L. Of the 191 women invited to participate, 124 agreed to enroll in the study. One hundred and nine women completed the full study (39 in wave 1, 70 in wave 2). In wave 1, three women dropped out citing the following reasons: left campus for family member's death, outside injury prevented continuation, and gastrointestinal discomfort from assigned capsule (n=1 for each). In wave 2, 6 women dropped out before randomization and another 6 women dropped out citing the following reasons: too time intensive (n=3), outside injury prevented continuation (n=2), left campus early after finishing final exams and could not complete week 8 testing (n=1). The women were distributed as shown in Table 2.1.

Table 2.1: Distribution of Subjects by Treatment Group, Final Count

	Iron	**Placebo**
Trained	20	18
Untrained	16	19

After examining the data, seven of the participants had a baseline sFer > 25 μg/L and were excluded from analysis for a final sample size of 102 women. The 102 women who were included in the final data analyses were randomly assigned to the following supplement groups: 36 iron, 37 placebo, and 29 BZ (as shown in Table 2.1). Chapters 3 and 4 describe analyses of only the iron and placebo groups (n = 73), while Chapter 5

examines the effects of BZ and includes all 102 subjects. The methods for the BZ analysis will be described in Section 2.2. Randomization occurred after screening, before the beginning of data collection at week 0.

Participants received compensation in the form of a tablet computer at the conclusion of the study. Additionally, to increase compliance, trained subjects were offered incentives in the form of small gifts when they completed a target number of training days (25, 32, and 40 days). Informed consent was obtained individually from the women participating in screening and again for the intervention phase of the study before the screening/baseline blood samples were taken. The Institutional Review Boards of Cornell University and Kunming Medical University approved the study protocols.

2.1.3 Blood Analyses

Whole blood samples were collected from participants in 4 mL Anti-Coagulant vacutainer tubes for analysis at screening and a batch analysis of weeks 0, 4, and 8 after the study was completed. Analyses were performed by two different labs, the First Affiliated Hospital of Kunming Medical University (FAH, Kunming, China) and the ShengSheng Logistics Life Science Supply Chain Solutions (SSLL, 上海生生物流有限公司, Shanghai, China), in waves 1 and 2, respectively.

In both waves, whole blood was refrigerated until transport (within 24 hours) to FAH. Hb was analyzed from whole blood at FAH in both waves using a Coulter LH 750 Hematology analyzer.

In wave 1, the remaining whole blood from each time point was centrifuged at FAH to separate the serum, which was divided into 500µL aliquots and stored at -80° C until being analyzed at FAH for sFer, sTfR, AGP, and CRP using a Siemens Advia 2400 automated analyzer. In wave 1, analyses were conducted within 48 hours of blood collection at each time point.

In wave 2, whole blood was centrifuged at Kunming Medical University to separate the serum, which was divided into 500 µL aliquots and stored at -80° C. After completing the week 8 blood draw, the samples from weeks 0, 4, and 8, as well as 35 samples from screening were shipped to Shanghai to be analyzed at SSLL. During shipping the temperature was maintained at -80° C, which was monitored hourly and adjusted as needed. At SSLL, serum was analyzed for sFer, sTfR, AGP, and CRP using a Siemens Advia 2400 automated analyzer.

The wave 2 screening (n=35) and all baseline samples (waves 1 and 2) were analyzed at both FAH and SSLL to allow for comparison between the labs. Studies have demonstrated the stability of ferritin, CRP, and TfR in both whole blood (for less than 96 hours)[96,97] or in plasma or serum frozen at -80° C (for up to 1 year)[98]. Therefore, the handling of the whole blood and subsequent storage before analyses in the present study are not likely to have had a large impact on the biomarker concentrations.

Additionally, 35 serum samples from each time point were analyzed by the author in the clinical laboratory of the Yunnan University for Traditional Chinese Medicine Affiliated Hospital. These samples were analyzed for sFer and sTfR using RAMCO laboratories Spectro Ferritin and Human Transferrin Receptor ELISA kits,

respectively. In order to match the scale used by Cook et al. to derive the Body Iron equation, all TfR values from FAH and SSLL were adjusted to the RAMCO Laboratories TfR scale using the following prediction equation, which was derived by plotting the TfR values obtained from the FAH and SSLL hospitals against the TfR values obtained from the RAMCO Laboratories ELISA kit run by the author (LMP) and calculating a regression line:

$$TfR_{Ramco} = (3.779*TfR_{lab}) + 0.400, R^2 = 0.93$$

There was a strong, significant correlation between the screening sFer values from the FAH and SSLL laboratories ($r = 0.86$, $p < 0.001$). Therefore, no correction factor was made between the FAH and SSLL values. However, the FAH hospital contacted the researchers (LMP) after the initial analyses to inform us that a different machine was used to run the Week 0 blood analyses than the machine that was used for the other time points and that because no standard was run on both machines, the wave 1, week 0 data was not valid. Therefore, for wave 1, the values used as baseline sFer are those that were obtained at the screening a week prior to the start of the study. Iron status was defined using Hb, sFer, and sTfR. Participants were classified as iron depleted if they had sFer < 25 µg/L and iron deficient if sFer < 15 µg/L. Using sTfR on the RAMCO Laboratories scale, iron deficiency was defined as a value > 8.3 mg/L[99]. The equation by Cook et al. for body iron described in Section 1.1 was used to calculate total body iron for each subject at each time point[23]. Using this measure, stores were considered depleted for body iron values < 0 mg Fe/kg body weight. AGP and CRP were used to assess inflammation. Inflammation cutoffs were established as CRP > 5

mg/L and AGP > 1 g/L[16].

2.1.4 Aerobic Training Protocol

The subjects assigned to training followed a training protocol based on that of Hinton et al. and adapted to an 8-week training period. Subjects trained for 8 weeks for 5 days each week on a cycle ergometer with adaptable workloads that was equipped to measure heart rate, revolutions per minute (RPM), and work output in Watts. Each session involved 25-minutes of cycling. The 25-minute session was divided into workloads that allowed subjects to obtain a target heart rate of 75-85% of their age-predicted maximum heart rate (HRmax). Time spent at the harder workload (85% HRmax) was increased each week to increase aerobic fitness of subjects as they progressed through training program. The training program is shown in Table 2.2.

Additionally, optional training days were offered on weekends to allow subjects to make-up any days they had missed during the week. The maximum number of training days was 40 days.

Table 2.2: Training Schedule by Week

Week	Minutes at 75% max HR	Minutes at 85% max HR
1	20	5
2	19	6
3	18	7
4	17	8
5	15	10
6	13	12
7	11	14
8	10	15

Abbreviations: max HR – maximum age-predicted heart rate (220 – age in years)

2.1.5 Assessing Physical Performance

Physical performance was measured at weeks 0, 4, and 8 using a graded exercise (VO_2max) performance test: The test was performed on a mechanically braked and calibrated cycle ergometer (Monark 884E). Oxygen consumption (VO_2) was determined while cycling at levels of effort ranging from rest to 100% of subjects' maximum capacity. Metabolic parameters were assessed using a portable metabolic measurement system (Cosmed K4B2, Cosmed, Rome, Italy), which analyzes heart rate, the breath-by-breath volume of respired air, and the concentrations of O_2 and CO_2 in the expired air. The subjects breathed room air through a two-way valve connected to

a facemask worn throughout the testing periods.

Calculated metabolic measures included oxygen consumption (VO_2 in mL/min), oxygen consumption per kilogram body weight (VO_2 mL/min/kg), pulmonary minute ventilation (VE in L/min), ventilatory threshold (VT, absolute in mL/min and VT as % of VO_2max), heart rate (HR beats/min), respiratory exchange ratio (RER = VCO_2/VO_2) and carbon dioxide production (VCO_2 in mL/min).

Subjects were asked not to perform any strenuous physical activities/exercise for two days before the exercise tests. Additionally, subjects were told not to consume food or caffeinated products for 3 hours prior to performance testing.

2.1.5a: VO_2max protocol

An exercise protocol adapted from that reported by Brownlie et al[77] was used to measure VO_2max. The test started when the subject's resting heart rate was <100 beats per minute. The test began with a 5-minute warm-up against only the empty weight basket (1 kg), cycling at 50 revolutions per minute (RPM). Workloads were then increased by 0.4 kg every 2 minutes, cycling at 50 RPM, until VO_2 did not increase more than 150 mL/min from the previous workload, signifying the subject was working at their VO_2max. If this condition could not be met (due to motivational or other factors), the test proceeded until the subject was unwilling or unable to continue. Subjects were considered to have reached VO_2max if the data from their test (collapsed into 60-second epochs) met two of the following conditions: a heart rate within 10 beats/min of their age-predicted maximum heart rate (220 - age in years), a blood lactate concentration > 8.0 mM, or a respiratory exchange ratio > 1.15. The highest measured

VO_2 achieved during this test was designated as VO_2peak. At baseline and week 8 only 69.9% and 60.3% of subjects, respectively, met the criteria for VO_2max. For subjects who failed to reach VO_2max, an estimated VO_2max (eVO_2max) was calculated by plotting heart rate against VO_2 and extrapolating to the VO_2 value for the age-predicted maximum heart rate. Ventilatory threshold (VT) was calculated by combining the ventilatory equivalencies, excess CO_2 production, and modified V-slope methods, as described by Gaskill et al[100]. Percent values at VT were calculated by taking the observed VO_2 value at VT and dividing by VO_2peak or eVO_2max. Blood lactate was measured immediately after testing via finger stick using a Lactate Plus portable blood lactate analyzer (Nova Biomedical, Waltham, MA, USA).

See **Appendix A** for data collection forms.

2.2 Traditional Chinese Medicine Study

2.2.1 Study Design

This study utilized the same protocol and subject population as the study described in Section 2.1, with the inclusion of 29 additional subjects who received the BZ treatment (13 trained and 16 untrained). It was a partially-blinded, randomized, controlled study. The objective of this study was to examine the effects of consuming a traditional Chinese herbal treatment, BaZhen KeLi, for 8 weeks on iron status and/or the physical performance in IDNA women (Aim 3). The BZ treatment was compared to both a placebo and to supplementation with ferrous sulfate. The BZ supplement was produced by the pharmacy of Yunnan University for Traditional Chinese Medicine. It was composed of the eight ingredients listed in Table 1.1. The study design was the

same as that described in Section 2.1.1 with the addition of a trained and untrained group that received BZ supplements in addition to the four treatment groups in the main study.

2.2.2 Study Participants

The recruitment process and final study participants were identical to those described in Section 2.1.2 for the Randomized Supplementation Trial. The final sample included in the data analyses for this study had 102 women, distributed among six groups as shown in Table 2.3.

Table 2.3: Participants in the BZ Study, By Treatment Group

	Iron	Placebo	BZ
Trained	20	19	13
Untrained	16	18	16

2.2.3 Outcomes

The blood analyses for the BZ study were identical to those described in Section 2.1.3, which included the samples from the additional 29 women in the BZ groups. Physical performance was assessed using the protocol described in section 2.1.5.

Chapter 3: Efficacy of iron supplementation may be misinterpreted using conventional measures of iron status in iron depleted, non-anemic women undergoing aerobic exercise training[1]

3.1 Abstract

*Background***:** Despite having known detrimental effects, iron deficiency remains the most common micronutrient deficiency in the world. Many interventions aiming to improve iron status involve physically active populations. Intense aerobic exercise training negatively affects iron status; however, the impact of regular moderate aerobic exercise on the effectiveness of iron supplementation remains unclear.

Objective: This study aimed to determine whether aerobic training modifies the assessment of the effectiveness of iron supplementation in improving conventional iron status measures.

Design: Seventy-two iron depleted, non-anemic Chinese women (serum ferritin (sFer) < 25 µg/L and hemoglobin >110 g/L) were included in an 8-week, partially-blinded, randomized control trial with a 2x2 factorial design including iron supplements (42mg elemental iron/day) or placebo and aerobic training (5 sessions/week of 25 minutes at 75-85% maximum heart rate) or no training. Linear mixed models were used to evaluate the relationship between supplement type, training, and changes in iron status over time, measured by sFer, hemoglobin, transferrin receptor, and estimated total body iron.

[1]This chapter contains the materials published in the *American Journal of Clinical Nutrition* under the same title. The chapter has not been modified from the printed edition except to match the Cornell University dissertation formatting requirements. The citation for this paper is shown below and a reprint is found in Appendix B.
Am J Clin Nutr. 2017 Dec;106(6):1529-1538. doi: 10.3945/ajcn.117.152777. Epub 2017 Nov 1.

Results: After treatment, both the iron-supplemented trained and untrained groups had significantly improved sFer, sTfR, and body iron values than either placebo group. Similarly, trained subjects had a significantly higher aerobic fitness measures than untrained subjects. Training modified the sFer response to supplementation (training by supplement interaction p = 0.07), with the iron trained group having significantly lower sFer than the iron untrained group at week 8 (31.8 ± 13.5 µg/L and 47.6 ± 15.7µg/L, respectively, p = 0.042), while there was no significant difference between the placebo trained and untrained groups (21.3 ± 12.2 µg/L and 20.3 ± 7.0 µg/L, respectively, p = 1.00).

Conclusions: Regular aerobic training reduces the apparent effectiveness of iron supplementation in improving sFer and calls into question whether conventional measures of iron status accurately reflect iron metabolism in physically active, non-anemic women.

3.2 Introduction

Iron deficiency anemia affects 20-34% of Chinese women of childbearing age[101,102]. This rate likely underestimates the prevalence of iron deficiency (ID) in the population because it does not account for ID without anemia [IDNA: hemoglobin >110g/L and serum ferritin < 15 µg/L] [103]. ID with and without anemia have negative consequences on physical performance and exercise capacity [3,104]. However, there is also evidence that aerobic training may have a negative influence on iron status that is potentially more concerning when considering iron interventions are frequently targeted

towards physically active populations [76,105,106].

Intense physical exercise lowers several measures of iron status including serum ferritin (sFer) and increases soluble transferrin receptor (sTfR), indicative of either compromised iron status [5,76,105,106] or redistribution of iron to be used in erythropoiesis and/or muscle formation [32,43,107,108]. Female soldiers who underwent a 9-week basic combat training showed declining iron status [78,105]. Providing an iron supplement [78] during basic combat training attenuated some of these changes, compared to placebo. While these findings occurred in highly intensive training programs, recreational physical activity can also negatively impact iron status. In a study by Hinton et al of IDNA women who underwent aerobic training, iron supplementation resulted in a modest increase in sFer (4.1µg/L), compared to those who received placebo[3]. Interestingly, the improvement in sFer in the iron supplemented, trained women in the Hinton et al study was less than half of that observed in a separate study of fit IDNA women who received similar iron supplementation but did not undergo training[109]. These findings suggest that improvements in sFer from iron supplementation may differ if women train during supplementation. This difference may be a result of an exercise-induced prioritization of erythropoietic demands and muscle growth over storing iron in the liver, as reflected by sFer concentration. While recent literature supports this idea of iron redirection away from the stores to support more functional uses of iron, most interventions aiming to improve iron status or anemia measure only Hb, sFer, and sometimes sTfR or estimated body iron, calculated from the ratio of sTfR to sFer, and do not measure other body iron pools. This oversight could potentially lead to erroneous

conclusions of the effectiveness of the intervention.

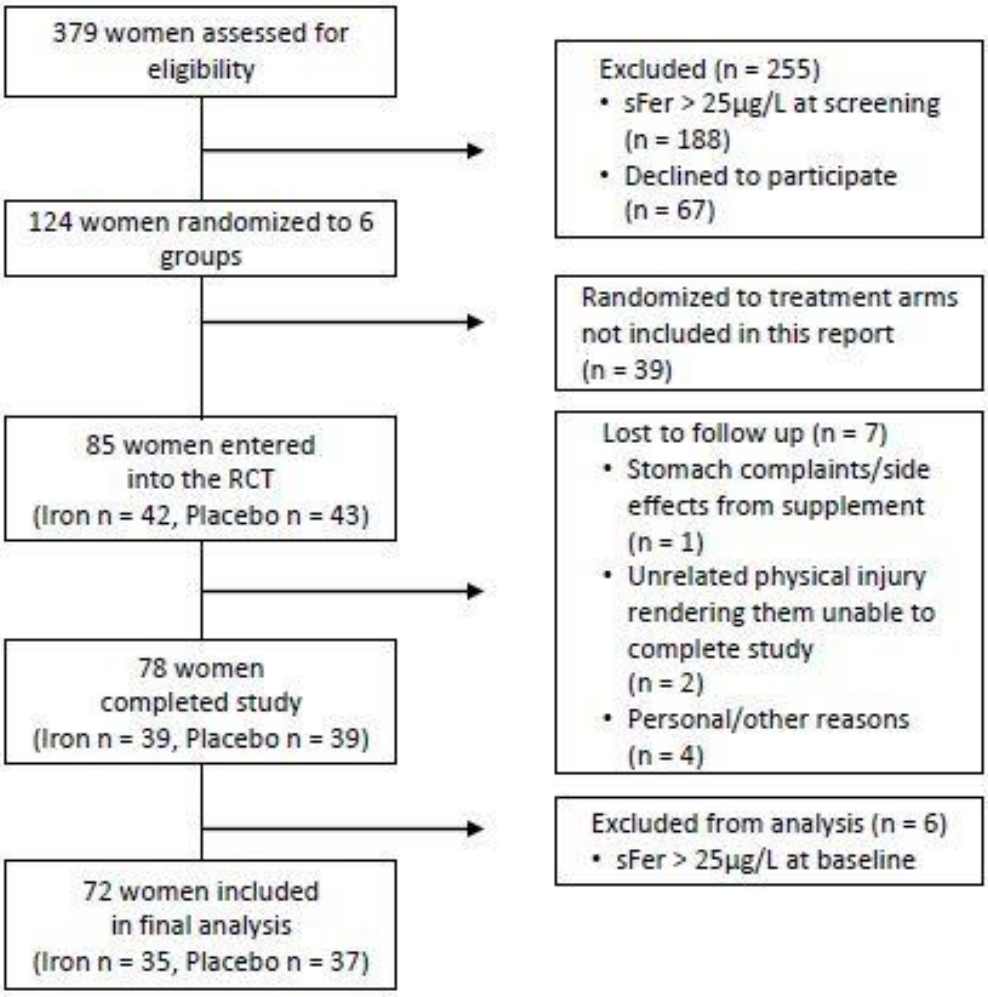
Further complicating the relationship between IDNA and exercise, research also suggests that women who are actively training perform better when given iron supplementation than those who train without supplementation [76,78], suggesting an intricate, but currently undefined, relationship between iron status, total body iron, exercise, and physical performance capacity. This relationship must be understood before we can properly design effective interventions to measure and target IDNA in physically active populations such as laborers, athletes, or non-athletes who want to benefit from improved fitness. While several studies have shown that training worsens traditional measures of iron status, to our knowledge no study has yet directly examined the interaction between training and iron supplementation to determine how training impacts the effectiveness of iron supplementation, as assessed by sFer and Hb concentrations. Therefore, the goal of this study is to determine whether 8 weeks of regular aerobic exercise on a cycle ergometer modifies the apparent effectiveness of concurrent iron supplementation in improving the traditional measures of iron status. We hypothesized that those women who received both iron and training would have smaller changes in sFer than women who did not train while taking iron.

3.3 Subjects and Methods

3.3.1 Subjects

The sampling scheme for this study is shown in **Figure 3.1.**

Figure 3.1: Consort Diagram for Chapter 3



[1] Abbreviations: RCT: randomized control trial; sFer: serum ferritin

Three hundred seventy-nine physically active, untrained 18- to 26-yr-old women were recruited in two waves (September-December 2014 and March-June 2015) from the student population at Kunming Medical University in Yunnan, China. Of these, 98 women were identified in the fall wave, and 281 were identified in the spring. In total, 191 were identified as iron-depleted without anemia, defined as Hb > 110 g/L and sFer

< 25 μg/L. Anemia was defined as <110 g/L to align with the Hb cutoff used by the First Affiliated Hospital of Kunming Medical University where the screening analyses were performed. The cutoff for iron depletion was set at 25 μg/L because prior research has shown that physical performance changes occur even in women who are iron depleted and not clinically iron deficient [8,10,110–112]. Screening, which included a medical history questionnaire, was conducted to identify and exclude women who met any of the following criteria: anemia (Hb < 110 g/L), current pregnancy or pregnancy within the previous year, current lactation or lactation within the previous year, recent infectious illness or fever, current inflammation or chronic inflammatory diseases, hemolytic anemia, chronic respiratory disease, musculoskeletal problems, history of eating disorders, smoking, BMI < 17 or > 25 kg/m^2, or recent consumption of iron supplements, vitamin supplements, or medications that may affect dietary iron intake or absorption or that had anticoagulant properties. Subjects also filled out a physical activity questionnaire that indicated they did not participate in regular exercise or organized sports, were interested in increasing their level of physical fitness, and were willing to comply with the full 8-week training program. Of the eligible women, 124 agreed to participate in the trial (45 fall, 79 spring). The recruitment process for this study was designed to recruit for two additional arms of the study. This paper focuses on the first four arms of the study, which included the iron and placebo supplemented groups. The other arms of the study included two groups who received a Chinese herbal supplement. Of the 124 women in the total study, 85 were randomized to the treatments relevant to this study.

Data from 6 women were excluded from statistical analyses because their sFer values at Week 0 increased above the screening cutoff of 25 µg/L, whereas their original screening values from two weeks prior indicated that they were iron depleted. Additionally, 9 women (from all six treatment arms) dropped out of the trial. The final sample size for this analysis was 72 women. Signed informed consent was obtained from each subject. The study was approved by the Cornell University Institutional Review Board and the Kunming Medical University Ethical Committee and registered under ClinicalTrials.gov #: NCT03002090.

3.3.2 Study Design

The experimental design of the study was a 2x2 randomized, double-blind, placebo-controlled intervention trial. Subjects were randomly assigned by the author (LMP) via a random number generator to treatment groups. Subjects received either 100 mg of ferrous sulfate or an identical placebo capsule twice daily for 8 weeks. Subjects and all investigators were blinded to supplement type until after the per-protocol analyses were completed. Sample size was based on consuming a minimum elemental iron concentration of 12 mg/capsule over 8 weeks. The final capsule concentration met this requirement. Previous work has demonstrated that iron status is improved after 4 weeks of iron supplementation at a similar dose of ferrous sulfate [3,113]. The capsules were prepared by the author with the use of gelatin capsules, ferrous sulfate, and dextrose filler (PCCA, Houston, TX). At weeks 0, 2, 4, 6, and 8 of each wave of the study, 20 capsules of each type were randomly selected from the batch and

stored in a sealed container in a cool, dry place until analyzed post-study by ICP/MS. After the study, the iron concentrations of the iron and placebo capsules were determined from this random sample to be 21.1 mg and 0.00 mg of elemental iron, respectively.

Subjects were instructed to consume the capsules with citrus juice during their morning and evening meals, and to avoid consuming the capsules on an empty stomach. Thirty capsules were distributed to each subject in a bottle labeled with their unique subject ID every 2 weeks (weeks 0, 2, 4, and 6). Each subject was instructed to complete a daily log to record capsule ingestion as well as information on medication use, illness, menstrual cycle status, any gastrointestinal complaints, physical activity, and musculoskeletal problems. Additionally, subjects were instructed to return their capsule bottles every two weeks with any remaining capsules, which were then counted as independent confirmation of the number of capsules reported as consumed on the daily logs.

One half of each supplement group was further randomly assigned to an exercise training program or no training to create four treatment groups: iron trained (FeTr), iron untrained (FeUn), placebo trained (PLTr), and placebo untrained (PLUn). The training protocol was adapted from a protocol previously published by Hinton et al that has produced measurable improvements in endurance and maximal oxygen consumption (VO_2max) [3]. Intensity of training increased each week according to each subject's heart rate. Training sessions were 25 minutes long and were divided into two target heart rates of 75% and 85% of age-estimated maximum, calculated as 220 (beats

per minute) minus age (years). The workload was adjusted as needed to maintain the target heart rate throughout the session. Minutes spent at 85% maximum heart rate increased each week to increase the difficultly of the training. The training protocol is shown in **Table 3.1**. Subjects trained Monday through Friday during each of the 8 weeks of the study. Optional training days were offered on weekends to allow subjects to make-up any days they had missed during the week. The maximum number of training days was 40 days.

Table 3.1: Daily training schedule, by week

Week	Minutes at 75% max heart rate	Minutes at 85% max heart rate
1	20	5
2	19	6
3	18	7
4	17	8
5	15	10
6	13	12
7	11	14
8	10	15

Training was performed on a stationary exercise bicycle (KangLe Exercise Products Company, Stationary Bicycle model B8.4E, Yunnan, China) equipped with digital output of work (watts) and cadence (rotations per minute). Subjects wore a T31

Polar heart rate monitor, which was read using a Polar A5 heart rate watch (Polar Electro Inc., Lake Success, NY). Trained research assistants recorded the watts, heart rate, and speed of each subject in a training log every 5 minutes throughout each session.

Daily physical activity, defined as minutes spent performing discretionary exercise, was assessed at weeks 0, 4, and 8 using the data from a daily log completed each day over the 56-day trial. Minutes of self-reported physical activity were totaled at each time point. Additionally, a physical activity frequency questionnaire was administered at Week 0 to assess similarity in habitual physical activity levels between groups. Participants were asked to maintain their normal pre-study activity patterns for the duration of the 8-week study period, regardless of whether they were in the trained or untrained groups.

Body composition and physical performance were measured before and after the study as well as at the 4-week midpoint. Dietary iron intake was assessed at Week 0 using a 4-day diet record that spanned from Thursday-Sunday. These records were analyzed for daily iron, inhibitors and enhancers of iron absorption, and macronutrient content using Nutrition Data System for Research Software (2016, University of Minnesota).

All subjects were compensated for study participation with a gift. Additionally, to increase compliance to the training program, trained subjects were offered incentives in the form of small gifts when they completed a target number of training days (25, 32, and 40 days). While all subjects received the same gift for participation in the study, the

smaller training-based incentives were only offered to the trained group.

3.3.3 Iron Status Measurements

Iron status was assessed at Weeks 0, 4, and 8. Whole blood was collected from the antecubital vein into 4 mL anti-coagulant vacutainers by a phlebotomist at the Kunming Medical University campus hospital. A small sample of whole blood was removed for Hb analysis. The remaining samples were stored at 4°C until centrifugation at 1600g for 10 minutes at room temperature within 24 hours. The serum was collected and separated into 0.5mL aliquots and frozen at -80°C until analysis. Blood was analyzed for sFer, sTfR, alpha-1-acid glycoprotein (AGP), and C-reactive protein (CRP) at the First Affiliated Hospital of Kunming Medical University in the first wave, and at the Shanghai Fenglin Clinical Laboratory in the second wave. Additionally, 75 samples from the second wave were run at both labs to allow for comparison between the labs. Hb was determined using a Coulter LH 750 Hematology analyzer (Beckman Coulter, Inc. Brea, CA). sFer, sTfR, AGP, and CRP were analyzed on a Siemens Advia 2400 automated analyzer (Siemens Healthcare, Erlangen, Germany). Estimated total body iron was derived from the ratio of sTfR to sFer using the equation reported by Cook et al [23] as:

$$\text{Total Body Iron} = -[\log(\text{sTfR}/\text{sFer}) - 2.8229] / 0.1207$$

This equation uses sTfR values from Ramco ELISA kits (Ramco Laboratories, Stafford, TX). Therefore, we converted the sTfR values derived from the Chinese laboratories to Ramco-adjusted sTfR values with the prediction equation below, which was derived from 35 random duplicate samples run on Ramco Laboratories sTfR ELISA kits.

$$\text{sTfR}_{\text{Ramco}} = (3.779*\text{sTfR}_{\text{lab}}) + 0.400,\ R^2 = 0.93$$

3.3.4 Physiological measurements

Height and weight were measured using previously described standard protocols [114]. Body composition was estimated from skinfold thicknesses measured with Lange calipers (Cambridge, MD) at the biceps, triceps, subscapular, and suprailiac sites. The Durnin and Womersley equation was used to calculate body density and percent body fat [115].

Physical performance was assessed using a VO_2max test at Weeks 0 and 8 on a mechanically braked and calibrated cycle ergometer (Monark 884E, Monark Exercise AB, Vansbro, Sweden). Oxygen consumption was determined while cycling at levels of effort ranging from rest to approximately 100% of subjects' maximum level of exertion. Metabolic parameters were assessed with a portable metabolic measurement system (Cosmed K_4B^2, Cosmed, Rome, Italy), which analyzes heart rate, the volume of respiratory air, and concentrations of oxygen and carbon dioxide in expired air. The subjects breathed room air through a two-way valve connected to a facemask worn during the testing sessions. Oxygen consumption per kilogram body weight (VO_2, mL/kg/min) was calculated from the portable metabolic unit's internal program.

Subjects were asked not to perform any strenuous physical activities/exercise for one day prior to the exercise tests, excluding daily training for the trained group. Trained subjects did not train on the day of their performance tests. Additionally, subjects were told not to consume food or caffeinated products for 3 hours prior to performance testing.

The VO_2max protocol was adapted from that used by Brownlie et al [77]. The test

started when the subject's heart rate was <100 beats per minute. The test began with a 5-minute warm-up with a workload of 1kg, cycling at 50 revolutions per minute (RPM). Workloads were then increased by 0.4kg every 2 minutes, cycling at 50 RPM, until VO_2 did not increase more than 150 mL/min from the previous workload, suggesting that the subject was working at their VO_2max. If this condition could not be observed, the test proceeded until the subject was unwilling or unable to continue. The subject was considered to have achieved VO_2max if 2 of the following conditions were met: subject reached: a respiratory exchange ratio > 1.10, a heart rate within 10 beats/min of their age-predicted maximum (220 – age), or blood lactate > 8.0mM. Blood lactate was assessed by finger stick using a Lactate Plus portable blood lactate analyzer (Nova Biomedical, Waltham, MA, USA) after completion of the final workload.

3.3.5 Compliance Analyses

Compliance to capsule consumption was assessed from 2 sources of documentation, pill counts from returned bottles and daily logs. The Pearson correlation coefficient between the capsules reported as missing in the daily logs and the capsules counted from the returned bottle was 0.39 (p=0.01), suggesting some discrepancies. In order to create a more reliable assessment of capsules consumed, two compliance variables were created: a conservative estimate and an average estimate. The conservative estimate was calculated by comparing the information from the capsule counts and the log for each two-week period and taking the higher number of missed capsules (reported or physically returned). The average estimate was created by taking the

average of the log and bottle counts. There were no group differences in number of capsules consumed for either the conservative or the average estimates.

3.3.6 Statistical Analyses

Sample size calculations were based on the Hinton et al[3] and Hinton and Sinclair[109] studies discussed previously, which suggest that a smaller change in sFer is observed when iron supplementation is given to women who are training. Sample size was determined to require 25 subjects in each of the four groups, which was expanded to 29 subjects per group after considering potential loss to follow-up in order to detect a 4.1µg/L change in sFer, or an effect size of approximately 0.8 standard deviations, after 8 weeks with 80% power and $\alpha = 0.05$[3]. Data were examined to verify normality of distribution using the Kolmogorov-Smirnov test, histograms, and qq-plots. Statistical analyses were performed on log-transformed variables for sFer and sTfR, which were found to have non-normal distributions. Measures of iron status at weeks 4 and 8 were analyzed using linear mixed models with fixed effects of supplement, training group, time, all 2- and 3-way interactions, and a random effect at the subject level. Baseline was included as a covariate for each measure of iron status. If the 3-way interaction was not significant in the full model, the 3-way interaction term was removed from the model and the model was rerun with all of the 2-way interaction terms. Any 2-way interaction terms that were not significant were also removed and a final model was run including only the significant 2-way interaction(s). Post-hoc pairwise comparisons were made with Bonferroni corrections made for multiple comparisons. Secondary analyses

were performed using linear models to test for relationships between variables. Residuals were examined for normality for all linear mixed models using the Kolmogorov-Smirnov test, histograms, and qq-plots. For a subgroup of those subjects who had a baseline sFer < 20 µg/L, the relative risk of resolving one's iron depletion (being iron depleted at baseline and iron replete at week 8) was calculated for all pairwise comparisons between groups. For all analyses, an α level of 0.05 was used to indicate statistical significance. For each result where a Bonferroni correction is indicated, the original *P* value was multiplied by the number of comparisons made and considered significant if: (*P* value times k comparisons) < 0.05. All statistical analyses were performed in SAS 9.4 (SAS Institute, Cary NC).

3.4 Results

3.4.1 Subject characteristics

There were 19 iron trained (FeTr), 16 iron untrained (FeUn), 18 placebo trained (PLTr), and 19 placebo untrained (PLUn) subjects included in the final analyses. Background characteristics including, age, anthropometry, and fitness level as assessed by VO_2max at baseline, blood biomarkers, and dietary intake at baseline are shown in **Table 3.2**.

Table 3.2: **Sample characteristics of total sample at Week 0 (n = 72)**[1]

Characteristic	Iron Trained	Iron Untrained	Placebo Trained	Placebo Untrained
Age (years)	20.6 ± 1.1	20.3 ± 0.9	20.7 ± 1.2	20.5 ± 1.7
Height (cm)	156.8± 5.7	156.9 ± 6.2	157.3 ± 4.7	157.4 ± 5.0
Weight (kg)	51.2 ± 6.8	51.4 ± 7.9	53.5 ± 6.1	49.7 ± 5.6
BMI (kg/m^2)	20.8 ± 2.8	21.0 ± 3.5	21.6 ± 2.1	20.0 ± 2.1
Body Fat (%)	25.2 ± 2.5	25.5 ± 3.2	26.0 ± 2.0	24.7 ± 2.0
sFer (µg/L)	14.0 ± 5.8	17.1 ± 4.2	14.4 ± 4.7	15.2 ± 5.7
Hb (g/L)	135 ± 10.3	141 ± 5.2	132 ± 12.8	133 ± 9.7
sTfR (mg/L)	6.8 ± 3.2	5.3 ± 0.8	5.8 ± 1.5	6.1 ± 1.7
Body Iron (mg/kg)	1.1 ± 2.6	2.7 ± 0.9	1.8 ± 1.8	1.7 ± 1.9
VO_2max (mL/min/kg)	42.7 ± 4.8	42.4 ± 5.6	41.4 ± 5.3	42.8 ± 5.0
Physical activity (min)[2]	59.8 ± 91.1	56.1 ± 60.5	52.9 ± 71.7	49.0 ± 65.9
AGP (g/L)	0.61 ± 0.08	0.61 ± 0.09	0.64 ± 0.10	0.59 ± 0.09
CRP (mg/L)[3]	0.04 (0.00, 0.01)	0.22 (0.00, 0.01)	0.20 (0.00, 0.30)	0.10 (0.00, 0.21)
Dietary Intake, daily				
Calories, kcal	1536 ± 278	1573 ± 333	1609 ± 324	1582 ± 318
Fat, g	51.5 ± 12.6	57.2 ± 15.0	56.6 ± 13.3	54.8 ± 16.9
Protein, g	59.5 ± 20.3	60.2 ± 18.4	59.5 ± 17.5	58.2 ± 20.0
Carbohydrate, g	213.5 ± 43.5	204.3 ± 49.3	215.2 ± 47.2	216.0 ± 51.6
Iron, mg	11.7 ± 4.5	13.0 ± 5.8	11.5 ± 3.6	12.3 ± 3.7
Calcium, mg	323.4 ± 116.4	340.0 ± 145.5	322.5 ± 158.4	388.8 ± 190.2
Ascorbic Acid, mg	79.5 ± 40.8	60.2 ± 37.5	63.1 ± 41.8	67.0 ± 37.9
Phytic Acid, mg	534.0 ± 196.9	597.8 ± 251.5	519.1 ± 246.9	598.3 ± 252.5
n	19	16	18	19

[1] Values are mean ± standard deviations
[2] Minutes of physical activity per week, defined as discretionary exercise, based on a self-reported questionnaire
[3] Value for CRP is given as mean with 1st and 3rd quartiles, due to skewed distribution
Abbreviations: Stdev: standard deviation; sFer: serum ferritin; Hb: hemoglobin; sTfR: soluble transferrin receptor; VO_2max: maximal oxygen consumption; AGP: α-1-acid glycoprotein; CRP: c-reactive protein

At baseline, all 72 subjects were iron depleted but not anemic. Of these 72 women, 39 women (54.2%) were clinically iron deficient defined as sFer less than 15.0µg/L, 5 women (6.9%) had sTfR values greater than 8.3mg/L, and 12 women (16.7%) had body iron less than 0 mg/kg. Consistent with spending slightly less than one hour of discretionary physical exercise per week, VO_2max levels indicated subjects were at a moderate to average fitness level, with no differences between the groups. There were no statistically significant differences in body weight or composition between the four groups before or after the study, nor were there significant changes during the 8-week study period (data not shown). Likewise, there were no differences in reported symptomology between the four treatment groups at any time point. Additionally, there were no differences between groups for daily iron, calcium, ascorbic acid, phytic acid, fat, carbohydrate, protein, or total caloric intake (Table 3.2). Of the 72 subjects, 13 women (18.1%) had a daily iron intake under the current U.S. estimated average requirement of 8.1 mg/day [116]. There were no significant differences in inflammation, measured as CRP and AGP, between the four groups at any time point in the study, nor were there significant changes between time points during the 8-week study period (data not shown). During the 8-week study, there were no participants who indicated inflammation based on AGP > 1.0 g/L. Only one subject was determined to have inflammation based on CRP > 5.0 mg/L, which occurred at week 4. All models were run including this subject and again excluding this subject, but no differences were found in the results of these analyses, so the subject was included for all analyses reported here.

3.4.2 Compliance to treatments

The iron and placebo groups returned 81% and 80% of their capsule bottles, respectively, and completed 96% and 92% of their daily capsule logs, respectively. There were no significant differences between the number of bottles returned or the percent of daily logs completed by the combined iron and combined placebo groups (t-test, $p = 0.87$) or in the four individual treatment groups (one-way ANOVA, $p = 0.31$). Estimates of the amount of iron consumed by the two iron groups are shown in **Table 3.3**.

Table 3.3: Total capsules and iron consumed by iron supplemented groups over 8 weeks[1, 2]

	Iron Trained		Iron Untrained	
	Conservative Estimate	**Average Estimate**	**Conservative Estimate**	**Average Estimate**
Capsules consumed[3]	95 ± 13	98 ± 11	97 ± 10	101 ± 7
Iron Consumed (g/8 weeks)	2.0 ± 0.3	2.1 ± 0.2	2.1 ± 0.2	2.1 ± 0.1
n	19	19	16	16

[1]Values are means ± standard deviations

[2] No group differences were observed for number of capsules consumed or total iron consumed for either estimate using one-way ANOVA with a $p < 0.05$

[3] Maximum possible capsules = 112

Details of the training sessions attended by each group are shown in **Table 3.4**. The average number of training sessions attended by subjects in the FeTr and PLTr

groups was not statistically significantly different (t-test, p = 0.76). The FeTr and PLTr groups achieved an average of 90.5% ± 7.8% and 91.9% ± 5.6%, respectively, of the target heart rate over the 8 weeks of training, which was not significantly different (t-test, p = 0.52). In the combined training groups, 80% of target heart rate was achieved on 91% of all days trained. Additionally, minutes spent in self-reported physical activity outside of the assigned training groups did not differ between groups at any time point. At baseline and week 8, 88% and 92% of subjects reached VO_2max. There was no difference in the percent of each of the groups that reached VO_2max at baseline or Week 8 (Chi-Square p=0.92 and p=0.13, respectively), suggesting there was equal compliance to the testing protocol between groups.

Table 3.4: Training sessions attended, by group[1]

Group	N	Days Trained[2]	Range[3]	% Attending ≥ 30 days	Percent target HR[2]
Iron Trained	19	33.2 ± 5.7	21-40	73.7 (n=14)	90.5 ± 7.8
Placebo Trained	18	32.7± 3.7	28-40	77.8 (n=14)	91.9 ± 5.6

[1] No significant differences were observed between training groups for any variable using a t-test, p > 0.05.

[2] Values are means ± standard deviation.

[3] Maximum possible number of training sessions was 40 sessions.

Abbreviations: HR: heart rate

3.4.3 Response to Iron Treatment

Table 3.5 shows the week 8 values for each iron biomarker as well as VO_2max after 8 weeks of treatment. Unadjusted means and standard deviations for each group, by time point can be found in Supplemental Table 1 in the online supporting materials. No significant 3-way interaction effects (supplement type by training group by time) were observed for any of the outcome measures in the linear mixed models (data not shown), nor were there significant interaction effects between time and training in any model. Significant training by supplement type interactions were observed for sFer and Hb, which will be discussed separately.

There was a significant interaction effect between time and supplement type for sTfR (interaction $p = 0.039$). At week 8 the iron group had a significantly lower sTfR concentration than the placebo group (4.9 ± 0.9 mg/L and 5.9 ± 1.8 mg/L, respectively, $p < 0.001$). No significant two-way interaction effects were observed for Body Iron. However, the iron group had significantly higher body iron than the placebo group (5.7 ± 1.9 mg/kg and 2.8 ± 2.5 mg/kg, respectively, $p < 0.001$).

Table 3.5: Effects of training and supplement type[1]

Group	n	sFer (µg/L)	Hb (g/L)	sTfR (mg/L)	Body Iron (mg/kg)	VO_2max (L/min/kg)
FeTr	19	31.8 ± 13.5	139.4 ± 8.0	5.3 ± 1.0	4.7 ± 1.9	43.3 ± 4.1
FeUn	16	47.6 ± 15.7	143.3 ± 6.7	4.4 ± 0.7	6.9 ± 1.3	40.8 ± 4.3
PLTr	18	21.3 ± 12.2	137.3 ± 9.4	5.9 ± 1.3	2.6 ± 2.8	42.4 ± 3.5
PLUn	19	20.3 ± 7.0	135.3 ± 10.3	5.9 ± 2.3	2.9 ± 2.2	38.8 ± 4.5
Supplement p value[2]		<0.001	0.003	<0.001	<0.001	0.28
Training p value[2]		0.034	0.66	0.018	0.023	<0.001
Interaction p value[2]		0.072	0.030	0.86	0.17	0.50
Supplement Effect[3]						
FeTr - PLTr *P* value		<0.001	1.00			
FeUn - PLUn *P* value		<0.001	0.003			
Training Effect[3]						
FeTr - FeUn *P* value		0.042	0.43			
PLTr - PLUn *P* value		1.00	1.00			

[1] Values are unadjusted means ± standard deviation, at week 8

[2] Results of linear mixed models, adjusted for baseline. Interaction p value represents the supplement type by training group interaction.

[3] P values of post-hoc pairwise comparisons with Bonferroni corrections, reported for measures where a significant training-by-supplement group interaction was observed.

Abbreviations: sFer: serum ferritin; Hb: hemoglobin; sTfR: soluble transferrin receptor; VO_2max: maximal oxygen capacity; FeTr: iron trained; FeUn: iron untrained; PLTr: placebo trained; PLUn: placebo untrained

3.4.4 Response of VO2max to Aerobic Training

There was no interaction observed between training group and supplement type for VO_2max; however, a significant training effect was observed. The trained group had a significantly higher VO_2max after treatment than the untrained group (42.8 ± 3.8 mL/min/kg and 39.7 ± 4.5 mL/min/kg, respectively, linear mixed model $p < 0.001$). Within the training group, the number of training sessions attended (range: 21-40 sessions) was not significantly associated with the 8-week change in VO_2max (linear regression, $r^2 = 0.01$, $p = 0.42$).

3.4.5 Interaction of Supplement and Training

Significant interaction effects between supplement type and training group were observed for sFer and Hb ($p = 0.072$ and 0.030, respectively, Table 3.5). The study was not powered at the 0.05 level to detect a clinically relevant 2-way interaction between training and supplement type, such as that observed for sFer. Additionally, the calculated sample size of 25 women per group was not achieved due to research conditions in the field. Therefore, the P value for the interaction ($p = 0.07$) was treated as being significant even though it did not reach the pre-specified 0.05 level. A reduced model was run including only the supplement-by-training group interaction term, where it retained its significance ($p = 0.07$). Post-hoc pairwise comparisons between supplement and training groups were adjusted for multiple comparisons using a Bonferroni correction (Table 3.5).

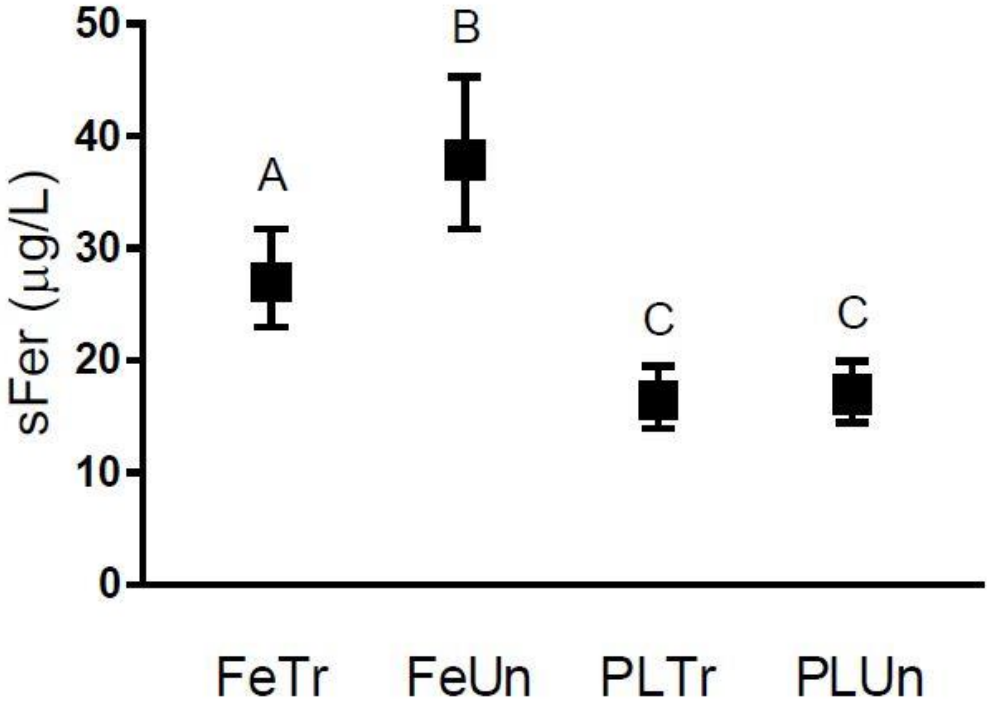
In examining the training-by-supplement interaction for sFer, shown in **Figure 2**,

both the iron trained and iron untrained groups showed significantly higher sFer values than either of the placebo groups. However, sFer in the subjects who received training in addition to iron supplementation was significantly lower than that of those subjects who did not train. For the training-by-supplement interaction for Hb, the FeUn group had a significantly higher Hb after treatment than the PLUn group. There were no other significant post-hoc comparisons for the training-by-supplement interaction for Hb.

Academic semester of testing was included as a covariate in all linear mixed models but was not statistically significant. The interaction between training and supplementation for the sFer model was maintained when baseline age, height, weight, and VO_2max were included as covariates in the models predicting sFer. None of these covariates were significant at $p < 0.05$. In order to determine whether the changes observed in sFer were independent of Hb, a model was run including Hb as a covariate. The significance of the training-by-supplement interaction and all trends in the post-hoc comparisons were maintained when Hb was included as a covariate (interaction $p = 0.09$), while Hb was not significant in the model ($p = 0.81$).

To examine the biological plausibility of the main findings, several secondary analyses were conducted. No relationship was found between the number of days trained and change in sFer in either of the supplement groups. Additionally, there was no significant association between the number of capsules consumed and the change in sFer in either supplement group, even when sFer status at baseline was included as a covariate. The same result was seen using the more conservative compliance measure for the number of capsules consumed. However, when only those subjects who

Figure 3.2: LS means for the training by supplement type interaction for serum ferritin [1, 2, 3]



[1] Values are post-hoc pairwise comparisons between supplement and training groups with 95% Confidence interval bands for the reduced model that did not include time point. The P value for the supplement by training group interaction was p = 0.07.

[2] N per group: 19 FeTr, 16 FeUn, 18 PLTr, and 19 PLUn subjects

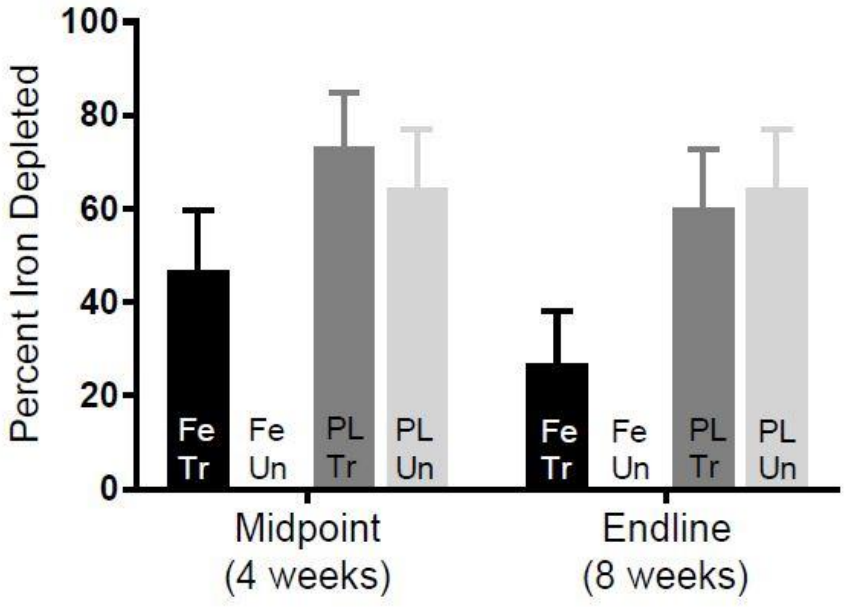
[3] A, B, and C indicate that post-hoc pairwise comparisons between supplement and training groups had significant differences from all groups with differing letters. Results are from linear mixed models controlling for baseline, where Bonferroni corrections for multiple comparisons have been applied to the post-hoc pairwise comparisons between groups.

Abbreviations: sFer: serum ferritin; FeTr: iron trained; FeUn: iron untrained; PLTr: placebo trained; PLUn: placebo untrained

returned 100% of their capsule bottles and daily logs were examined (n=25), there was a positive relationship between the number of capsules consumed and the change in sFer for the iron group (r^2=0.39, p=0.04) but no relationship in the placebo group (data not shown). Subjects who had the highest sTfR concentrations at Week 0 (i.e. the lowest tissue iron levels) displayed the largest decreases in sTfR over the 8-week study period when given iron supplements, but no change was observed in those given the placebo (supplement by week 0 sTfR concentration interaction, linear model, p <0.001). This same trend was observed for body iron, with those subjects starting with the lowest body iron showing the largest improvements in body iron over the 8-week study (supplement-by-week 0 body iron concentration interaction p = 0.03).

In a subgroup analysis of subjects with more severe iron depletion (sFer < 20 µg/L) at baseline, 100% of the FeUn group repleted their iron stores by 4 weeks, and this resolution persisted until 8 weeks. Conversely, after 4 weeks only 53.5% ± 12.9% of the FeTr group had resolved their iron depletion and only 73.3% ± 11.4% by week 8. **Figure 3.3** shows the prevalence of iron depletion within each group at each time point. Relative risk (RR) calculations were performed to determine pairwise differences in percent resolution between the four treatment groups.

Figure 3.3: Percent of women with iron depletion (sFer < 20μg/L) by treatment group at 4 and 8 weeks [1, 2]



[1] All subjects were iron depleted (sFer < 20 μg/L) at baseline. N per group: 19 FeTr, 16 FeUn, 18 PLTr, and 19 PLUn subjects

[2] Values are percent, bars are standard error

[3] No iron-untrained subjects were depleted at Week 4 or 8; i.e. there was total resolution of iron depletion in this group

Abbreviations: FeTr: iron trained; FeUn: iron untrained; PLTr: placebo trained; PLUn: placebo untrained

At the end of the 8-week study period, the FeTr group was no more likely to be iron replete than the PLTr or PLUn group (FeTr/PLTr: RR=1.83, 95% CI: 0.92, 3.66, p = 0.51; FeTr/PLUn: RR=2.05, 95% CI 0.95, 4.42, p = 0.39); however, the FeUn group was 2.5 times more likely to be replete than the PLTr group and 2.8 times more likely to be replete than the PLUn group (RR=2.50, 95% CI: 1.35, 4.65, p<0.05, and RR=2.80, 95% CI: 1.39, 5.65, p<0.05, respectively).

3.5 Discussion

Improvements in iron status measures in both of the iron-supplemented groups demonstrate that the supplementation regimen was adequate to elicit changes in iron status. After 8 weeks of treatment, the iron-supplemented group had significantly higher sFer and body iron and significantly lower (indicating better iron status) sTfR concentrations than the placebo group. Previous studies have shown that 100 mg/day of ferrous sulfate is sufficient to cause changes in iron status markers of similar magnitudes as those observed in this study [3]. Similarly, post-training improvements in VO_2max in the trained groups confirm that the training program was sufficient to induce physiological adaptations. The high compliance to the training program (89% of subjects attending > 28 days) likely explains this effect, though the narrow range in the number of sessions attended likely limits our ability to find a dose-response effect of training on VO_2max.

Aerobic training lowered the effectiveness of iron supplementation in improving traditional measures of iron status, as evidenced by the significant training-by-supplement interaction in linear mixed models of sFer effects. While both iron supplemented groups showed significant improvements in sFer, the improvements of the trained subjects were significantly smaller than those of the untrained group. One interpretation of this finding is that subjects who trained while taking iron supplements did not benefit from the supplements as much as those who did not train.

Additionally, by week 8, 100% of the FeUn group had resolved their iron depletion, as defined by sFer < 20 µg/L, compared to only 73% of the FeTr group.

These findings are consistent with those of McClung and colleagues, who found that participation in basic combat training decreased iron status in female soldiers [78,117].

While the modifying effect of aerobic training on iron status is demonstrated by the results of this study, the physiological mechanism explaining this effect remains unclear. Possible explanations include: reduced absorption of iron due to exercise-induced inflammation, increased demand for iron in the oxidative pathways, or increased production of total body Hb or myoglobin, which could be supported by redirecting iron from iron stores toward erythropoietic processes [32,63,118,119].

There is currently conflicting evidence about whether long-term, moderate exercise results in a chronic inflammatory state that could affect iron status. Both acute and chronic inflammation result in increased concentrations of hepcidin, the primary regulator of iron absorption in the body. While studies have demonstrated a relationship between increased hepcidin expression and lower iron status after acute exercise [120,121], few studies have shown a long-term impact [121,122]. Outside of the role of hepcidin, several studies have shown that inflammation induced by exercise [123] or chemical injection [124,125] results in decreased expression of proteins required for iron absorption into the enterocyte. While this study did not detect inflammation using CRP or AGP, inflammation may still have been present in this population. This study did not measure hepcidin, its mediator IL-6, or proteins involved in iron absorption. Therefore, we cannot determine whether inflammation or absorption differed between the FeTr and FeUn groups.

There is evidence that exercise-induced increases in erythropoiesis draw iron

from the other iron stores in the body to meet the increased iron demand [32,126], which could explain the smaller increase in sFer observed in the FeTr group compared to the FeUn group. However, the details of muscle iron homeostasis in conditions of high erythropoietic demand, such as long-term aerobic training, remain unclear. Some studies have shown the increased iron demand for erythropoiesis draws iron from myoglobin [32], whereas others suggest it comes from the liver [107,126]. It is possible that the iron entering the body in the FeTr group was used directly for erythropoiesis or muscle growth. In this study, the FeUn group showed significantly higher Hb after iron supplementation than the PLUn group; however, the FeTr group did not have significantly higher Hb than the other groups. This finding may support the idea that training increased the erythropoietic demand for iron, and the supplemented iron was used directly for this process rather than being stored as ferritin. However, if training were increasing the demand for iron for erythropoiesis, it would therefore be expected that the PLTr group would show decreases in their serum ferritin, which was not observed. It is possible that iron contained in muscle myoglobin was diverted for use in erythropoiesis in the PLTr group; however, this study was unable to assess myoglobin and thus cannot determine whether this was the case.

Finally, it is possible that there was an increased iron demand in skeletal muscle mitochondrial bioenergetics pathways, as many enzymes and cofactors in energy production are iron dependent. It has been shown that ID impairs mitochondrial function[32] and that aerobic exercise training in combination with iron supplementation improves mitochondrial function and density[127–129]. Therefore, it is also feasible that the

iron in the FeTr group was used to synthesize the enzymes needed for increased energy production or skeletal muscle growth known to occur with training[32,130].

One limitation of this study was its short duration, which may have been insufficient to observe any long-term effects. Additionally, blood and exercise data were not collected for women who dropped out, thus limiting our ability to perform a true intention-to-treat analysis. The subjects who dropped out or were excluded from analyses due to discrepancies in their sFer values at baseline may have been systematically different than those who were retained; however, there were no baseline differences between those subjects who dropped out or were excluded and those who were retained. The study may have had increased type I error due to the assessment of multiple biomarker variables. Additionally, the training group was not blinded, potentially introducing bias. Finally, this study was unable to measure other, potentially more appropriate measures of iron status such as those involved in inflammation, muscle growth, or erythropoiesis. Further investigation requires more invasive or more expensive analysis techniques, such as metabolomics, that would provide a more comprehensive view of iron metabolism. However, our results support the idea that sFer and Hb may not be the best biomarkers for iron status in non-anemic, physically active populations.

3.6 Conclusion

We have demonstrated that regular, aerobic training diminishes the effectiveness of iron supplementation on improving sFer in iron-depleted, non-anemic women, compared to untrained women. Iron supplementation was still able to increase sFer in

trained women, but at a slower rate. This finding could have implications for interventions aiming to improve iron status in physically active populations, such as women who train casually to improve fitness in developed countries or in developing countries where heavy manual labor is necessary for economic livelihoods. Typically, iron supplementation interventions can run for 4-6 weeks at the dose used in this study[3,14]. This dose can be sufficient in that time frame, as evidenced by the 100% resolution of iron depletion in the iron untrained group. However, our study suggests that longer interventions may be necessary to improve iron stores of active women. Further research should explore whether a larger dose of iron can counteract the modifying effect that daily exercise has on improvements in sFer and whether this effect has any long-term impacts. Additionally, further research should determine an optimal iron intervention dose and duration for active populations and develop other, more appropriate biomarkers that are more indicative of iron involved in muscle metabolism and erythropoietic processes.

3.7 Chapter Acknowledgements

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Chapter 4: Aerobic training and elevating iron status equally improve submaximal performance

4.1 Abstract

Introduction/Purpose: Iron deficiency persists as the most common micronutrient deficiency globally, despite having known detrimental effects on physical performance. While iron supplementation and aerobic exercise have been examined individually and are both known to improve physical performance, the impact of simultaneous iron supplementation and aerobic training remains unclear. The goal of this study was to examine the individual and combined effects of iron supplementation and aerobic training on improving maximal and submaximal physical performance measures.

Methods: Seventy-three iron depleted, non-anemic women (serum ferritin < 25 µg/L and hemoglobin > 110 g/L) participated in a partially-blinded, 8-week randomized placebo-controlled trial with a 2x2 factorial design including aerobic exercise training (5 days/week of 25 minutes at 75-85% maximum heart rate) or no training and iron supplementation (42 mg elemental iron/day) or placebo. Linear models were used to examine relationships between training, supplement, and changes in exercise performance, measured as oxygen consumption (VO_2) at maximal exertion (estimated VO_2max and VO_2peak) and at the ventilatory threshold (absolute VO_2 and percent of estimated maximum VO_2).

Results: There were significant 2-way interactions between training and supplement for VO_2peak, VO_2 at the ventilatory threshold, and the percent of estimated VO_2max at which the threshold occurred. These variables were significantly higher in the iron-

trained, iron-untrained, and placebo-trained groups compared to the placebo-untrained group. However, there were no significant differences between these three groups themselves for any performance variable.

Conclusions: Iron supplementation given to sedentary women resulted in increases in submaximal physical performance of the same magnitude as those produced by aerobic training. Furthermore, there was no an additive effect of supplemental iron to aerobic training above the effect of either treatment alone.

4.2 Introduction

In China, 20-34% of women of childbearing age suffer from iron deficiency anemia. This rate does not account for the prevalence of iron deficiency without anemia [IDNA, defined as Hemoglobin (Hb) > 110 g/L and serum ferritin (sFer) < 15 μg/L], which can be as high as double the rate of anemia in some populations[131]. The widespread prevalence of IDNA has consequences beyond basic nutritional deficiencies, as iron deficiency with and without anemia are also known to impair both endurance performance and maximal exercise capacity[132–134]. These functional consequences have implications for a wide range of women including athletes and those trying to increase physical performance for health reasons in high-income countries as well as manual laborers in developing areas. There is evidence that improving iron status can have positive impacts on physical performance in active women[3,78]; however, the relationship between iron status and physical performance when iron status and fitness level are changing simultaneously remains unclear.

It is well-established that the worsened physical performance (measured as maximal oxygen consumption, VO_2max) observed in IDA is a result of impaired O_2 delivery to tissues[36,49]. However, IDNA is also known to negatively impact physical performance outside of the role Hb plays in oxygen delivery. In iron deficient animal models where Hb was replete, worsened endurance capacity was determined to be a result of impaired muscle oxidative capacity from reduced mitochondrial enzyme productivity, not impaired oxygen delivery[34,49]. This effect of IDNA has also been observed at the functional level in human studies of physical performance that have demonstrated impaired aerobic capacity, endurance, and energetic efficiency in IDNA women[3,45,104]. When a group of IDNA women were given 100 mg/day of ferrous sulfate or a placebo and aerobically trained 5 days/week for six weeks, those women who were given iron adapted better to training than those given a placebo as demonstrated in performance tests, with a shorter time-to-completion, lower respiratory exchange ratios, and increased work rates during a 15 km time-trial on a cycle ergometer[3]. Untreated IDNA also impairs the typical adaptations to aerobic exercise training, including reducing endurance capacity[104] and lower VO_2max[77] values after training compared to iron replete women.

While these studies all provide evidence of the positive effects that iron supplementation can have on the adaptive response to aerobic training, there is currently little evidence of the effect that iron supplementation has on the physical performance of women who are not physically active. To our knowledge, no study has yet examined whether changing iron status from iron supplementation modifies the

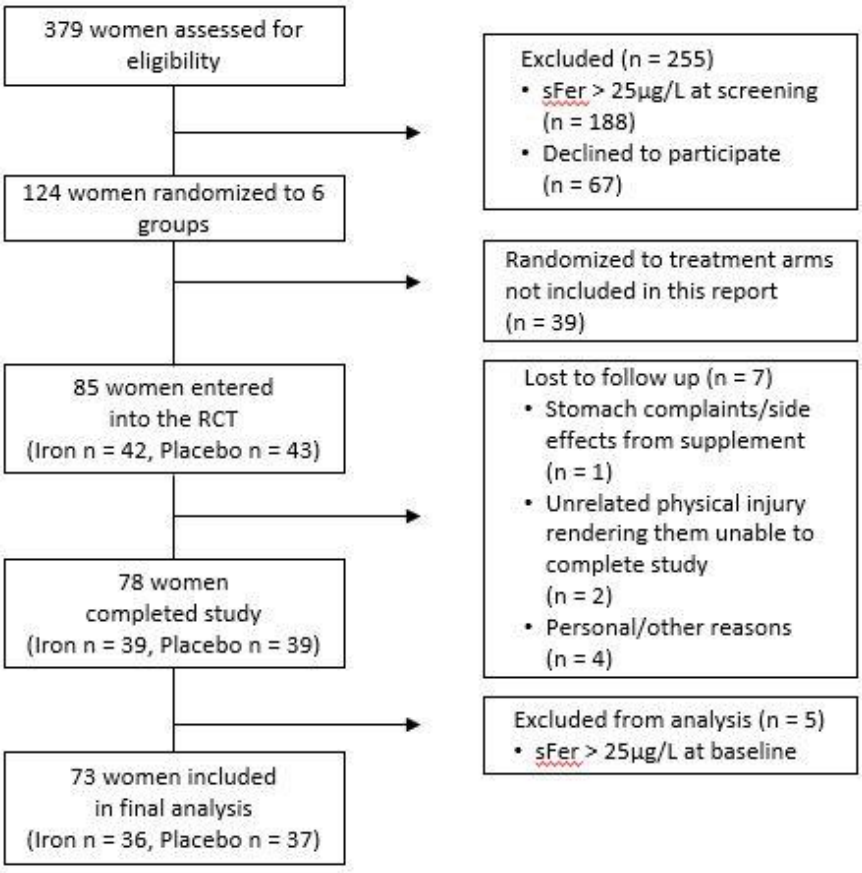
adaptations in fitness level that occur from concurrent regular aerobic training. Therefore, the goals of this study were first to determine whether 8 weeks of iron supplementation modifies the response to aerobic exercise training in IDNA women and second to examine how supplemental iron impacts the physical performance of sedentary women who do not train, thus comparing the individual and combined effects of iron supplementation and aerobic training on physical performance. We hypothesized that women who receive iron while training will show the greatest improvements in physical performance outcomes compared to women who receive either treatment alone. Additionally, we expect that women who receive either training or iron alone will show improvements in physical performance measures compared with those who received no training and the placebo treatment.

4.3 Subjects and Methods

4.3.1 Subjects

This study took place in two sessions (fall semester: September – December 2014 and spring semester: March – June 2015). Ninety-eight women participated in screening during the fall session, and another 281 were screened in the spring session (**Figure 4.1)**.

Figure 4.1: Consort Diagram for Chapter 4[1]



[1] Abbreviations: RCT: randomized control trial; sFer: serum ferritin

Of these women, 191 were identified as iron-depleted and non-anemic, defined as sFer < 25 µg/L and Hb > 110 g/L. A cutoff of 25 µg/L was used for iron depletion because prior literature suggests that physical performance and iron stores change can occur in iron-supplemented women who are not clinically iron deficient but still have depleted iron stores[8,10,112]. Anemia was defined as Hb > 110 g/L to align with the standards of care used by the First Affiliated Hospital of Kunming Medical University, where the screening blood analyses were conducted. Subjects were screened to

identify and exclude those women who met any of the following criteria: history of eating disorders, smoking, BMI < 17 or > 25 kg/m^2, anemia (Hb < 110 g/L), hemolytic anemia, current pregnancy or pregnancy within the previous year, current lactation or lactation within the previous year, recent infectious illness or fever, current inflammation or chronic inflammatory diseases, chronic respiratory disease, musculoskeletal problems, or recent consumption of iron supplements, vitamin supplements, or medications that may affect dietary iron intake or absorption or that had anticoagulant properties. Additionally, subjects completed a physical activity questionnaire that indicated they were not participating in any organized sports or regular exercise activities, had an interest in increasing their current fitness level, and would be willing to comply with the full 8-week training regimen if randomized to a training group. Out of 191 eligible women, 124 agreed to participate in the study (45 fall, 79 spring). Recruitment for this study included selecting subjects for two arms that received a Chinese herbal supplement and are not included in this analysis (see Chapter 5), which includes only the iron and placebo supplemented groups. Out of the 124 study participants, 85 were randomized to the four arms relevant to this study.

During the study, 9 women (from all four treatment arms) dropped out of the trial for personal and health reasons. Another 5 women were excluded from statistical analyses because they were identified as having baseline sFer > 25 µg/L in final blood analyses, which were conducted in a single batch consisting of weeks 0 and 8 together after the study's completion. Therefore, despite having been identified as iron depleted (sFer values 23.5 - 24.9 µg/L) at the initial screening, these subjects were excluded

from the final analyses. The final sample size for this analysis was 73 women. Signed informed consent was obtained from each subject. The study was approved by the Cornell University Institutional Review Board and the Kunming Medical University Ethical Committee and registered under ClinicalTrials.gov #: NCT03002090.

4.3.2 Study Design

This study used a 2x2, double-blind, randomized, placebo-controlled intervention design. Subjects were randomly assigned by the author (LMP) to treatment groups via a random number generator. Subjects were randomized to receive training or no training and iron or placebo capsules, creating a total of four groups: iron trained (IT), iron untrained (IU), placebo trained (PT), and placebo untrained (PU). A training protocol published by Hinton et al that has been shown to produce measurable changes in performance measures was adapted to meet an 8-week training schedule[3]. The adapted training protocol is shown in **Table 3.1**.

Subjects were asked to train 5 days per week for 8 weeks, with the maximum number of training days being 40. Each training session lasted 25 minutes, which was divided into time spent pedaling at 60 revolutions per minute (RPM) at 75% or 85% of subjects' age-predicted maximum heart rate (220 beats per minute – years of age). The first two minutes of the 25-minute session were considered a warm-up period and were conducted at the lowest workload on the training ergometer. After the first two minutes, the workload (in watts) was adjusted to ensure subjects were meeting their target heart rate throughout the session. Each week the time spent at 85% of maximum heart rate

was increased in order to continually challenge the subjects as their fitness level improved.

Training was performed on a stationary exercise bicycle (KangLe Exercise Products, Stationary Bicycle model B8.4E, Yunnan, China) equipped with digital output of work (watts) and cadence (RPM). Heart rate was read from a Polar A5 heart rate watch (Polar Electro Inc., Lake Success, NY) synced to a T31 Polar heart rate monitor worn around the subject's chest. Trained research assistants recorded the watts, heart rate, and speed of each subject in a training log every 5 minutes throughout each session.

Additionally, according to their randomization group, all subjects received either 100 mg of ferrous sulfate or an identical placebo capsule, twice daily throughout the 8-week study period (200 mg total/day). Investigators and subjects were both blinded to capsule type until after the intent-to-treat data analyses were conducted. All capsules were prepared by the author (LMP) using dextrose filler, ferrous sulfate, and gelatin capsules (PCCA, Houston, TX). During the study, a random selection of 20 capsules was taken from each supplement and placebo batch and stored in a sealed container in a cool, dry location until post-study analysis via ICP/MS. Sample size calculations were based on the assumption that subjects would consume a minimum of 12 mg of elemental iron per capsule over the 8 weeks. The iron concentrations of the placebo and iron capsules were determined to be 0.00 mg and 21.1 mg ± 1.1 mg elemental iron per capsule, respectively, thus meeting that requirement. Previous studies have shown that iron status can be increased after a 4-week course of a similar iron dose[3,45].

Subjects were instructed to consume the capsules with citrus juice during their morning and evening meals in order to avoid gastrointestinal side effects and maximize absorption. Every 2 weeks, beginning at week 0, 30 capsules were distributed to each subject in a bottle labeled with their individual subject ID number. Subjects were asked to record their morning and evening capsule ingestion, daily medication use, menstrual cycle status, illnesses, injuries, and physical activity on a daily log that was distributed with the capsule bottles every 2 weeks. Additionally, subjects returned their old capsule bottles along with any remaining capsules every two weeks. Returned capsules were counted as an independent confirmation of the values reported by the subjects on the daily logs.

Subjects were instructed to record the minutes and type(s) of physical activity they performed each day, excluding that of the training program, on their daily logs throughout the study period. Minutes of self-reported physical activity were tallied over the 8-week study period and METs were calculated from the 2011 Compendium of Physical Activities[135]. Furthermore, participants completed a physical activity frequency questionnaire prior to the study to assess habitual physical activity levels among groups. During the study, subjects were instructed to maintain their regular activity patterns throughout the 8-week study duration, regardless of whether they were participating in the training program.

Dietary assessments were conducted at baseline using a 4-day diet record from Thursday to Sunday before the study began. Records were analyzed for daily intake of: iron, inhibitors and enhancers of iron absorption, and macronutrient content using

Nutrition Data System for Research Software (2016, University of Minnesota). Additionally, percent body fat was estimated, and mid-upper arm circumference was measured before and after the study.

All subjects received a gift as compensation for their participation in the study. Trained subjects were offered additional incentives to increase compliance to the training program. These incentives were small gifts when they completed 25, 32, and 40 training sessions. While all subjects received the same gift for participation in the full study, only trained subjects were eligible for the smaller incentive gifts.

4.3.3 Physiological measurements

Weight and height were measured using standard protocols that have been described previously[114]. Body composition was estimated from skinfold thicknesses measured in triplicate with Lange calipers (Cambridge, MD) at the triceps, biceps, subscapular, and suprailiac sites. The Durnin and Womersley equation was used to calculate body density and percent body fat[115].

A graded exercise test was performed at baseline and at week 8 using a mechanically braked and calibrated cycle ergometer (Monark 884E, Monark Exercise AB, Vansbro, Sweden). Oxygen consumption was measured during the test, which varied in difficulty from rest to approximately 100% of each subject's maximal exertion level. A portable metabolic measurement system (Cosmed K_4B^2, Cosmed, Rome, Italy) in which subjects breathed room air via a two-way valve on a facemask was used to assess metabolic parameters including: volume of respired air (VE), concentrations of

oxygen and carbon dioxide in expired air, and heart rate (HR).

Subjects were instructed not to participate in any strenuous physical activity within 24 hours of their exercise test. Subjects in the training group attended the training session on the day before their test but did not train on the day of the VO_2max test itself. Additionally, subjects were also instructed not to consume any foods or caffeinated products within 3 hours of their scheduled test. None of the subjects reported smoking during the study.

The testing protocol was adapted from a similar test used by Brownlie et al[77]. Testing began once the subject's heart rate was below 100 beats per minute. After a 5-minute warm-up where subjects pedaled at 50 RPM against a 1 kg workload, workloads were increased by 0.4 kg every 2 minutes until VO_2 increased no more than 150 mL/min from the prior workload. Because attaining this level is quite challenging for most subjects, it was expected that many women would be unable to reach a plateau in VO_2. Therefore, if no plateau was reached, the test proceeded until the subject could not continue or voluntarily ceased pedaling. Subjects were considered to have reached VO_2max if they achieved any two of the following conditions: a heart rate within 10 beats/min of their age-predicted maximum heart rate (220 - age), a blood lactate concentration > 8.0 mM, or a respiratory exchange ratio > 1.15. The highest measured VO_2 achieved during this test was designated as VO_2peak. At baseline and week 8 only 69.9% and 60.3% of subjects, respectively, met the criteria for VO_2max during exercise testing. Therefore, for subjects who failed to meet VO_2max criteria, an estimated VO_2max (eVO_2max) was calculated by plotting heart rate against VO_2 and extrapolating

to the VO_2 value for the age-predicted maximum heart rate (220 - age). The variable eVO_2max thus reflects the measured VO_2max for those subjects who met the VO_2max criteria and the estimated value for those subjects who did not meet the criteria.

Ventilatory threshold (VT) was calculated by combining three methods (ventilatory equivalencies, excess CO_2 production, and modified V-slope), as described by Gaskill et al[100]. This combined method has been shown to be appropriate for women of the fitness level and general healthy state as those in this study and improves the accuracy of the VT calculation compared to any one method alone[100]. Percent of VO_2peak or eVO_2max at VT was calculated by taking the observed VO_2 value at VT and dividing by the peak or estimated VO_2max value, respectively.

Blood lactate was measured immediately after completion of the last grade of the performance test via finger stick using a Lactate Plus portable blood lactate analyzer (Nova Biomedical, Waltham, MA, USA).

4.3.4 Iron status measurements

Iron status was measured before and after the 8-week study period from whole blood that was collected from the antecubital vein into 4 mL serum collection vacutainers by a licensed phlebotomist at Kunming Medical University's student hospital. Immediately after the blood draw, a small sample of whole blood was analyzed for Hb. The remaining samples were stored at 4°C for no longer than 24 hours, when then were centrifuged at 1600 g for 10 minutes at room temperature. After centrifugation, serum was separated into 0.5 mL aliquots and frozen at -80° C for later

analyses. Samples from weeks 0 and 8 were analyzed for sFer, sTfR, alpha-1-acid glycoprotein (AGP), and C-reactive protein (CRP) at the First Affiliated Hospital of Kunming Medical University and at the Shanghai Fenglin Clinical Laboratory in the fall and spring sessions, respectively. To allow for comparisons between the two labs, 75 samples from the second wave were run at both labs and correlations between measures were examined using Pearson correlations (adjusted $R^2 = 0.86$) and Bland-Altman plots. sFer, sTfR, AGP, and CRP were all analyzed on a Siemens Advia 2400 automated analyzer (Siemens Healthcare, Erlangen, Germany). Hb was determined using a Coulter LH 750 Hematology analyzer (Beckman Coulter, Inc. Brea, CA). Total body iron was calculated using the ratio of sTfR to sFer and the equation reported by Cook et al[23]:

$$\text{Total Body Iron (mg/kg)} = - [\log(\text{sTfR/sFer}) - 2.8229] / 0.1207$$

Cook's equation uses sTfR values from Ramco ELISA kits (Ramco Laboratories, Stafford, TX). To match these values, 35 random duplicate samples of serum analyzed at the Chinese laboratories were also run on Ramco Laboratories sTfR ELISA kits to determine the following conversion equation:

$$\text{sTfR}_{\text{Ramco}} \text{ (mg/L)} = (3.779 \times \text{sTfR}_{\text{lab}}) + 0.400, R^2 = 0.93$$

4.3.5 Compliance Analyses

Compliance to training was assessed via a training score, calculated as the number of days trained multiplied by the total percent of the target heart rate achieved each day throughout training. This score should therefore reflect both the quantity and

the intensity of the training that the subject received. The highest possible training score was 40, which reflects both perfect attendance and 100% attainment of target heart rate on all training days. Additionally, percent of target RPM was used to reflect the subjects' compliance to the instructions to maintain a constant speed of 60 RPM throughout the training program.

Compliance to capsule ingestion was assessed in two ways, from the daily logs filled out by the participants and the from actual count of capsule remaining in the returned bottles every two weeks. The Pearson correlation coefficient between the capsules returned in the capsule bottles and those reported as missing on the daily longs was 0.39 ($p = 0.01$), which indicates there were discrepancies between the two measurements. Therefore, in order to make a more reliable estimate of capsule consumption, two compliance variables were calculated: an average estimate and a conservative estimate. The average estimate was calculated by taking the average of the number of capsules returned and the number of capsules reported as taken in the logs. The conservative estimate was created by comparing the reported consumption to the returned capsule counts and taking the higher number of missed capsules (reported or returned physically) as the number missed. No significant group differences were found in the number of capsules taken using either method. Additionally, both methods were used in the compliance analyses and produced the same results. Therefore, only the conservative estimate is reported.

4.3.6 Statistical Analyses

Sample size calculations were based on two studies that suggest that iron supplementation benefits unfit women participating in an aerobic training program similar to the program used in this study[3,109]. Sample size was determined to require 25 subjects in each of the four groups, which was expanded to 29 subjects per group after anticipated attrition, to detect a 4.2 μg/L difference in sFer between groups, or 0.8 standard deviations. Change in sFer that corresponded to biologically meaningful changes in physical performance with 80% power and $\alpha = 0.05$. Log transformations were made for variables found to have non-normal distributions (sFer and sTfR). Group differences at baseline were tested using one-way ANOVA. Physical performance measures at week 8 were analyzed using linear models testing for the main effects of supplement and training group as well as the training group by supplement interaction[136]. Baseline values were included as a covariate for each measure of physical performance. Semester (fall or spring session) of testing was included in all models. For all analyses, statistical significance was defined as $p < 0.05$ for single variables or $p < 0.1$ for interaction terms. Post-hoc pairwise comparisons were made with Tukey corrections for multiple comparisons. Secondary analyses were performed using linear models to test for relationships between variables with a significance level defined as $p < 0.05$. All statistical analyses were performed using SAS 9.4 (SAS Institute, Cary NC).

4.4 Results

4.4.1 Subject characteristics

There were 20 iron trained, 16 iron untrained, 19 placebo trained, and 18 placebo untrained subjects included in the final analyses. There were no statistically significant differences between groups for any background measure on the basis of one-way ANOVA **(Table 4.1)**. All subjects began the study iron depleted, but not anemic. At baseline, 39 of the 73 subjects (53.4%) were clinically iron deficient, defined as sFer < 15.0 µg/L, 12 women (16.4%) had body iron < 0 mg/kg, and 5 women (6.8%) had sTfR values > 8.3 mg/L.

There were no significant group differences in body composition or weight at baseline, nor were there significant changes in weight or body composition during the 8-week study in any group (data not shown). Daily dietary intake of iron, ascorbic acid, calcium, phytic acid, carbohydrates, fat, protein, or total caloric intake did not vary across groups at baseline. Additionally, there were no differences in reported symptomology across the four groups during the study. Inflammation, measured as CRP and AGP, showed no significant group differences at baseline (Table 4.1) or after the 8-week study period (data not shown). During the study period, no participant demonstrated clinical inflammation based on AGP or CRP (AGP > 1.0 g/L or CRP > 5.0 mg/L).

Table 4.1: Chapter 4 baseline characteristics, by intervention group[1, 2]

Characteristic	Iron Trained	Iron Untrained	Placebo Trained	Placebo Untrained
Anthropometry				
Age (yr)	20.5 ± 1.0	20.2 ± 0.8	20.6 ± 1.3	20.6 ± 1.8
Height (cm)	157.1 ± 5.7	156.9 ± 6.2	157.3 ± 4.7	157.5 ± 5.2
Weight (kg)	52.2 ± 7.3	51.6 ± 7.9	53.2 ± 6.1	51.0 ± 5.5
BMI (kg/m^2)	21.2 ± 2.9	21.0 ± 3.5	21.5 ± 2.1	20.6 ± 2.1
Body Fat (%)	29.3 ± 3.4	28.3 ± 5.1	29.1 ± 4.2	28.8 ± 2.3
Blood Biomarkers				
Hb (g/L)	135.4 ± 8.8	140.7 ± 5.2	137.2 ± 10.1	135.3 ± 6.5
sFer (µg/L)	14.6 ± 6.1	17.1 ± 4.2	14.2 ± 4.6	15.5 ± 5.8
sTfR (mg/L)	6.7 ± 3.1	5.3 ± 0.8	5.9 ± 1.5	6.0 ± 1.7
Body Iron (mg/kg)	1.3 ± 2.6	2.7 ± 0.9	1.7 ± 1.8	1.8 ± 1.9
AGP	0.62 ± 0.09	0.61 ± 0.09	0.64 ± 0.09	0.58 ± 0.09
CRP	0.06 ± 0.12	0.22 ± 0.77	0.21 ± 0.35	0.08 ± 0.13
Performance Measures				
Physical activity (min)[3]	59.8 ± 91.1	56.1 ± 60.5	52.9 ± 71.7	49.0s ± 65.9
VO_2peak (mL/min/kg)	36.1 ± 4.4	37.3 ± 3.9	36.0 ± 4.2	36.0 ± 3.7
eVO_2max (mL/min/kg)	40.4 ± 6.0	40.4 ±4.3	40.0 ± 4.5	40.2 ± 3.8
Peak HR (beats/min)	185.5 ± 10.0	190.5 ± 8.5	186.9 ± 9.5	185.6 ± 13.1
Post-test lactate (mmol)	10.4 ± 2.2	11.7 ± 2.0	11.9 ± 3.4	11.5 ± 2.1
VO_2 at VT (mL/min/kg)	27.2 ± 4.2	28.8 ± 3.3	28.2 ± 2.9	29.4 ± 3.2
Percent eVO_2max at VT (%)	77.0 ± 8.3	80.0 ± 5.8	81.1 ± 6.1	84.9 ± 4.1
Daily Dietary Intake				
Calories, kcal	1543 ± 272	1573 ± 333	1602 ± 315	1587 ± 327
Fat, g	52 ± 12	57 ± 15	56± 13	55 ± 17
Protein, g	59 ± 20	60 ± 18	59 ± 17	59 ± 21
Carbohydrate, g	215 ± 43	204 ± 49	216 ± 46	216 ± 53
Iron, mg	12 ± 4	13 ± 6	12 ± 4	12 ± 4
Calcium, mg	325 ± 114	340 ± 146	321 ± 154	394 ± 195
Ascorbic Acid, mg	94 ± 57	60 ± 38	65 ±41	83 ± 80
Phytic Acid, mg	544 ± 184	662 ± 340	533 ± 247	577 ± 252
n	20	16	19	18

[1] Values are means ± standard deviations

[2] There were no significant differences between the four treatment groups at baseline for any anthropometric, blood biomarker, physical performance measure, or dietary intake measure, on the basis of one-way ANOVA with an alpha of 0.05.

[3] Minutes of discretionary physical activity per week, above walking to and from classes, based on a self-reported questionnaire

Abbreviations: VO_2peak: highest measured oxygen consumption, eVO_2max: calculated maximal oxygen consumption; VT: ventilatory threshold; VCO_2: volume of exhaled carbon dioxide; VE: minute ventilation; HR: heart rate; RER: respiratory exchange ratio; OP: oxygen pulse

Physical performance measures indicated that the women began the study at an average fitness level, with an average VO_2peak of 36.8 ± 4.5 $mL \cdot min^{-1} \cdot kg^{-1}$ and an average VO_2 at VT of 28.3 ± 3.5 $mL \cdot min^{-1} \cdot kg^{-1}$ (80.7 ± 6.8% and 66.7 ± 8.3% of VO_2peak and eVO_2max, respectively**)**. Additionally, the four treatment groups did not differ in minutes of self-reported physical activity or metabolic equivalents (METs) expended per week during the 8-week study period, not including the exercise performed as part of the study's training regimen for the trained subjects (data not shown, one-way ANOVA, $p > 0.05$).

4.4.2 Compliance to Treatments

The IT and PT groups attended the same number of training days, reached the same percent target heart rate, and achieved the same training score during the 8-week period (32.9 ± 5.7 days, 90.5 ± 7.8%, and 30.3 ± 5.6 score; and 32.7 ± 3.7 days, 91.9 ± 5.6%, and 30.6 ± 3.4 score, t-tests, $p = 0.92$, $p = 0.52$, and $p = 0.83$, respectively).

There were no differences in the percent of capsule bottles returned by each of the four groups or by combined supplement types (one-way ANOVA $p = 0.38$ and t-test $p = 0.81$, respectively, **Table 4.2**). Additionally, there were no differences in the percent of daily logs completed by group or by supplement type (one-way ANOVA $p= 0.51$ and t-test $p = 0.20$). The amount of iron consumed over the 8-week study period was the same in the iron-trained and iron-untrained groups (2.0 ± 0.3g and 2.1 ± 0.2g, respectively, one-way ANOVA, $p=0.76$).

Table 4.2: Capsule compliance between supplement groups[1, 2]

	Iron	Placebo
Percent capsule bottles returned (%)	82 ± 3	80 ± 3
Percent daily logs completed (%)	96 ± 7	92 ± 20
Capsules consumed (# capsules)	96 ± 12	99 ± 12
Total iron consumed from supplement (g/8 weeks)	2.0 ± 0.3	0
n	36	37

[1] Values are means ± standard deviations

[2] There were no differences between supplement groups for any measure on the basis of a t-test with an alpha of 0.05

4.4.3 Treatment Effects on Iron Status

There was a significant interaction between training group and supplement type for sFer (**Table 4.3**, interaction p = 0.011). Post-hoc pairwise comparisons indicated that the IT and IU groups both had statistically significantly higher sFer concentrations than the PT or PU groups. Furthermore, the IT group had a significantly lower sFer concentration at week 8 than the IU group (p = 0.012). No interaction was observed for Hb (interaction p = 0.67), but a significant supplement effect was observed with the iron-supplemented group having a higher Hb at week 8 than the placebo (141 g/L and 137 g/L, respectively, p = 0.025).

Table 4.3: Iron status at 8-weeks, by treatment group[1]

Group	n	sFer (µg/L)	Hb (g/L)
Iron Trained	19	33.2 ± 2.8	139.7 ± 1.8
Iron Untrained	16	46.9 ± 3.2	141.9 ± 2.1
Placebo Trained	19	21.5 ± 2.9	136.2 ± 1.8
Placebo Untrained	18	21.8 ± 3.0	136.7 ± 1.9
Effect of Supplement, p value[2, 3]		<0.001	0.025
Effect of Training, p value		0.06	0.481
Interaction, p value		0.011	0.67
Post-hoc Group Comparisons[4]			
IT - IU *p* value		0.012	1.00
IT - PT *p* value		0.027	1.00
IT - PU *p* value		0.036	1.00
IU - PT *p* value		<0.001	0.26
IU - PU *p* value		<0.001	0.45
PT - PU *p* value		1.00	1.00

1 Values are unadjusted means ± standard errors, at week 8

2 Details of main effect on Hb: Iron group 140.8 ± 1.4 g/L, placebo group 136.5 ± 1.3 g/L (mean ± standard error)

3 Results of a linear model, comparing values at 8 weeks for sFer or Hb after adjusting for baseline value and semester; Interaction p value represents the supplement type by training group interaction

4 P values are from post-hoc comparisons between groups with Tukey corrections for multiple comparisons

Abbreviations: sFer: serum ferritin; Hb: hemoglobin

4.4.4 Treatment Effects on Maximal Exercise Performance

There was a significant interaction effect between training group and supplement type for VO_2peak at 8 weeks (interaction p = 0.035, **Table 4.4**). Post-hoc pairwise

comparisons showed that the IT and PT groups had a significantly higher VO_2peak at 8 weeks than the PU ($p = 0.007$ and 0.002, respectively). Additionally, VO_2peak in the IT group showed a trend of being higher than that of the PU group, though this trend did not reach the $p < 0.05$ significance level ($p = 0.060$).

Despite the interaction between training group and supplement type observed for VO_2peak, there was no interaction observed for eVO_2max at week 8. However, there was a significant training effect for eVO_2max ($p < 0.001$). After 8 weeks of treatment, the trained group had an eVO_2max of 43.7 ± 3.1 $mL \cdot min^{-1} \cdot kg^{-1}$ while the placebo group was significantly lower at 40.3 ± 3.0 $mL \cdot min^{-1} \cdot kg^{-1}$. Additionally, there was a supplement effect observed for the percent of estimated VO_2max attained at VO_2peak. After 8 weeks, the iron group achieved 96.6 ± 7.8% of eVO_2max at peak while the placebo group achieved 92.2 ± 7.9% of eVO_2max at peak ($p=0.021$).

Table 4.4: Physical performance measures at week 8 by intervention group

Group	n	VO_2 peak (mL/min/kg)	eVO_2max (mL/min/kg)	Percent eVO_2max at Peak	Post-test lactate (mMol)	VO_2 at VT (mL/min/kg)	VT as % VO_2peak	VT as % eVO_2max
IT[1]	20	37.7 ± 0.7	43.4 ± 0.6	94.5 ± 1.8	10.0 ± 0.8	34.0 ± 0.7	88.6 ± 1.5	76.3 ± 1.7
IU[1]	16	37.0 ± 0.8	40.8 ± 0.7	98.7 ± 2.0	10.2 ± 0.8	31.8 ± 0.8	86.2 ± 1.6	76.6 ± 1.9
PT[1]	18	38.1 ± 0.7	44.0 ± 0.7	92.9 ± 1.8	10.4 ± 0.7	32.4 ± 0.7	85.0 ± 1.5	76.2 ± 1.7
PU[1]	19	34.2 ± 0.7	39.9 ± 0.7	91.5 ± 1.9	10.2 ± 0.7	27.3 ± 0.7	82.2 ± 1.6	69.6 ± 1.8
Supp P[2]		0.12	0.84	0.021	0.77	<0.001	0.018	0.047
Train P[2]		0.003	<0.001	0.47	0.97	<0.001	0.10	0.078
Int. P[3]		0.035	0.30	0.14	0.80	0.048	0.91	0.059
Post-hoc pairwise comparisons for significant interactions[4]								
IT-IU		0.92				0.14		1.00
IT-PT		0.97				0.34		1.00
IT-PU		0.007				<0.001		0.037
IU-PT		0.71				0.94		1.00
IU-PU		0.060				<0.001		0.043
PT-PU		0.002				<0.001		0.050
LS Means for training effects[5]								
Trained			43.7 ± 0.5*	93.7 ± 1.3			86.8 ± 1.1	
Untrained			40.3 ± 0.5	95.1 ± 1.4			84.2 ± 1.1	
LS Means for supplement effects[5]								
Iron			42.1 ± 0.5	96.6 ± 1.3*			87.4 ± 1.1*	
Placebo			41.9 ± 0.5	92.2 ± 1.3			83.6 ± 1.1	

[1]Results of linear models adjusted for baseline and semester, values are mean ± standard error, significance defined as $p < 0.05$ for single variables and $p < 0.1$ for interaction terms

[2] P values for main effects of supplement type (Supp P) and training group (Train P)

[3] P value for the training group by supplement type interaction

[4] P values for post-hoc pairwise comparisons for interaction terms, adjusted for multiple comparisons with a Tukey correction

[5] LS means ± standard error of post-hoc comparisons for main effects of training and supplement.

* Indicates significant difference of main effect at a level of $p < 0.05$

Abbreviations: eVO_2max: estimated maximal oxygen consumption; IT: iron trained group; IU: iron untrained group; PT: placebo trained group; PU: placebo untrained group

4.4.5 Treatment Effects on Submaximal Exercise Performance

At week 8, there were significant interaction effects between training group and supplement type for both VO_2 at VT and the percent of eVO_2max at which VT occurred **(Table 4.4).** For each of these variables, the IT, IU, and PT groups all attained a significantly higher value or reached VT at a higher percent of estimated maximum than the PU group. Notably, while the IT, IU, and PT groups all showed significantly improved performance for both absolute and percent eVO_2max, there were no statistically significant differences between the IT, IU, and PT groups themselves.

Main effects of training and supplement were examined for percent of VO_2peak at VT, for which there was no significant interaction term. A significant supplement effect was observed for percent of VO_2peak at VT, where the iron group reached VT at 87.4 ± 6.6% of VO_2peak, while the placebo group reached 83.6 ± 6.7% at VT (one-way ANOVA p = <0.001).

Academic semester of testing was included as a covariate in all linear regression models for submaximal and maximal exercise performance but was not statistically significant. For all variables where an interaction between training and supplement was observed, the interaction was maintained when baseline age and 8-week change in weight or fat free mass were included in the models. Age and change in weight or fat free mass were not significant in any model. Finally, including 8-week change in Hb as a covariate did not change the significance of the

interactions between training and supplement for any of the performance measures at 8 weeks. Change in Hb was not significant in any model except for the percent of estimated VO_2max reached at VO_2peak (change in Hb p = 0.017); however, the significant effect of Hb in this model did not impact the significance or size of the supplementation effect (p = 0.019).

4.4.6 Secondary analyses

Several secondary analyses were conducted in order to examine the biological plausibility of the main findings. There was no relationship between the number of training days attended or the training score achieved and any of the performance outcomes in the trained subjects. In the iron supplemented group there was a significant, positive relationship between the number of capsules consumed and eVO_2max, controlling for baseline (data not shown, linear regression, p =0.039). There were no significant relationships between changes in sFer or Hb and changes in performance measures (data not shown).

4.5 Discussion

To our knowledge, this is the first study to examine the combined effects of simultaneous aerobic exercise training and supplemental iron on physical performance. The objectives were to analyze the independent effects of improved fitness and improved iron status on maximal and submaximal exercise performance

measures as well as to determine whether consumption of supplemental iron modifies the known physiological adaptations to aerobic training. A recent meta-analysis conducted by Pasricha et al examined 22 existing studies that gave iron or placebo supplements to participants, with some studies having all subjects undergoing training and others having no training program. While the Pasricha review provides an important analysis of the impacts of iron status on aerobic fitness in trained or untrained subjects, none of the included studies had trained and untrained groups together in the same study. Therefore, it lacked the ability to directly examine the interaction between training and supplementation that the current study provides. Nevertheless, the Pasricha et al review found that in trained women, VO_2max improved more in IDNA women who were given oral iron supplements compared to those who trained without additional iron[134]. The results of the present study partially agree with the Pasricha et al findings in suggesting that both iron supplementation and aerobic training individually improved VO_2peak; however, the present study did not detect an additive effect of supplemental iron and concomitant aerobic training for VO_2peak, nor did this study observe a supplement effect (main or interaction) for eVO_2max. However, the Pasricha meta-analysis used VO_2max and VO_2peak data interchangeably in their maximal performance analyses, and no secondary analyses were done comparing peak to max after iron supplementation. This study examined both estimated VO_2max and VO_2peak separately and found different results for these two variables. Therefore, it is unclear whether results of the present study directly

contradict the results of the Pasricha et al analysis.

The Pasricha et al meta-analysis also found that during submaximal exercise, iron supplementation in trained women resulted in a lower percent of VO_2max being reached while exercising at a given submaximal workload, compared to those who received a placebo. Similarly, the present study found that iron supplementation had a significant impact on VO_2 at VT as well as the percent of estimated maximum at which VT was reached, both of which reflect endurance capacity at differing exercise intensities. However, the present study was able to further expand upon previous findings by examining the interaction between simultaneous iron supplementation and aerobic training. The results of the present study indicate that training, iron supplementation, and their combination all result in statistically significant improvements in several measures of submaximal exercise performance, and that the effects of iron supplementation are of the same magnitude as those produced from aerobic training, with or without iron supplementation. In considering the biological mechanism through which iron supplementation could improve physical performance to the same degree as aerobic training, the impacts of both treatments on physical performance need to be examined.

It is widely accepted that aerobic training results in a combination of muscular, cardiovascular, biochemical, and respiratory adaptations that increase aerobic exercise performance. Collectively, these training-induced adaptations, which have been reviewed extensively[137,138], improve both maximal and submaximal exercise

performance. These adaptations include: increasing plasma volume, myoglobin stores, and capillarization of the heart and skeletal muscle, shifting of fast glycolytic to fast-oxidative glycolytic muscle fibers, increasing the number of slow oxidative muscle fibers, increasing stroke volume, and increasing the protein content and activity of enzymes involved in aerobic respiration [50,69,137,139,140]. Training-induced adaptations allow for increased energy production and more efficient cellular respiration, allowing trained subjects to sustain exercise at a higher percentage of maximal aerobic capacity without crossing the anaerobic threshold[69]. In accordance with these adaptations, this study found that subjects who trained, regardless of supplementation group, had significant improvements in several measures of maximal and submaximal exercise performance. Consistent with the literature, these improvements suggest that the trained subjects had both improved maximal oxygen consumption and reached their anaerobic threshold later than the untrained subjects.

Similarly, iron supplementation also resulted in improvements in both maximal and submaximal exercise performance. Significant supplement effects were observed for VO_2peak, the percent of eVO_2max achieved at VO_2peak, VO_2 at VT, and the percent of both VO_2peak and eVO_2max at which VT occurred. Interestingly, while a significant iron effect was observed for percent of eVO_2max achieved at VO_2peak, no change in eVO_2max itself was observed between the iron and placebo groups. The Pasricha et al meta-analysis did not differentiate between VO_2max and VO_2peak data in their analysis of maximal performance. However, by analyzing

eVO_2max and VO_2peak separately, this study was able to expand upon the conclusion of the Pasricha et al analysis by suggesting that while IDNA women who received supplemental iron were able to attain a higher percentage of their existing maximal capacity, estimated maximum oxygen consumption itself was not improved by supplemental iron.

There are several ways in which supplemental iron could have led to the observed increases in peak performance and performance at VT in the IU group. ID interferes with several signaling pathways required for muscle growth as well as shifting for mitochondria towards a heavier reliance on glycolytic activity, triggering mitophagy, negatively impacting the cristae and other mitochondrial structures, and decreasing mitochondrial ability to properly use fats as a fuel source[32,141,142]. Several enzymes in the TCA cycle and the electron transport chain also require iron to function, further impacting fuel oxidization[40]. Furthermore, iron is required to synthesize myoglobin, the skeletal muscle protein responsible for storing and providing oxygen to the mitochondria for respiration[42]. Providing supplemental iron could potentially help reverse these effects. At the functional level, providing supplemental iron to IDNA women could result in increased oxidative capacity and energy production, more efficient aerobic respiration, and increased muscle myoglobin. These metabolic changes would then result in VT occurring at a higher percentage of maximum values, as seen in the IU women in this study.

Furthermore, as this study was conducted in non-anemic women and Hb did

not change greatly in any group, the changes resulting from iron supplementation were potentially more relevant to skeletal muscle and mitochondrial function, rather than oxygen circulation. These systems are responsible more for aerobic endurance than maximal performance, which could explain why the IU women reached a higher VO_2peak and percent of estimated VO_2max at peak but did not improve in estimated maximal oxygen consumption capacity itself. Therefore, while operating through different mechanisms, the increased muscle growth, mitochondrial productivity and efficiency, and increased myoglobin stores resulting from increased iron status could produce the same effects as the increased mitochondrial size and density associated with aerobic training.

While the ergogenic effects of both iron supplementation and aerobic training on submaximal exercise are clearly demonstrated by the results of this study and corroborated by previous literature, it remains unclear as to why there was no additive benefit of iron supplementation and training together. The beneficial effect of providing iron supplementation to trained women has been reported in the literature[3,77,78]. Previous studies did not include trained and untrained subjects together and were therefore unable to examine the training and iron effects individually. However, these studies observed increases in physical performance with iron supplementation in the form of decreased times and better performance when completing various time-trials [3,14,78,104] or an attenuation of the rate of fatigue in dynamic knee extensions. Interestingly, while the results of the current study do not

support that iron supplementation during training provides additional benefit over iron supplementation or aerobic training alone, they also do not directly contradict the results of the existing literature.

The referenced studies utilized different exercise tests than the one performed in this study. Specifically, the study by Brutsaert et al used a knee extension test, which is a more power-based exercise rather than an aerobic test. The difference in the primary energy system used to perform a knee extension test compared to that used in the VO_2max test (glycolytic versus aerobic) from this protocol makes comparisons between the studies difficult. Similarly, the other studies that found a beneficial effect of iron above that of training all utilized endurance-based time trials. The exercise test conducted in the present study was a VO_2max test, which is a test that increases in difficultly progressively until exhaustion. It is likely that the VO_2max test used by this study was unable to capture the same aerobic steady-state produced by the time-trials used by other studies and was therefore unable to detect an additive effect of iron and exercise on aerobic energy production. A study by Perkkiö et al found that when iron deficient rats underwent aerobic training, their endurance capacity increased six-fold, while no change in performance was detected from a higher-intensity performance test in this same group[143], suggesting that a steady-state endurance test may have been more appropriate to detect a beneficial effect of supplemental iron while training.

Alternatively, it is possible that no additive effect of supplemental iron was

seen in the IT group because the additional iron provided was sufficient to begin suppressing the regulatory mechanisms meant to conserve iron in the IDNA state but was not sufficient to both reverse the negative impacts of iron deficiency while also meeting the increased physiological demands of training. While hypoxia is known to trigger HIF2α-mediated increases in erythropoiesis, in conditions where iron availability is low, the body also limits erythropoietin production through several signaling pathways including the IRP1 response, ultimately reducing the amount of iron used in erythropoiesis[29,144,145], which could potentially impact the expected response to aerobic training. It is possible that the PT group in this study may have been operating under these suppressed conditions, which may have limited the training-induced improvements in performance that would be expected from an iron replete group undergoing the same training. This idea is supported by the observation that the IU group showed improvements in performance of equal magnitude as those in the PT group. It is possible that the benefits from training in the IDNA state, in which erythropoiesis and other metabolic processes do not function at their full capacity, are reduced to the point that simply improving iron status can elicit the same magnitude of changes in performance.

Furthermore, the iron provided to the IT group may have been sufficient to reverse the restrictions on these pathways, thus increasing the erythropoietic demand from training, which could translate to improvements in maximal or submaximal performance from improved oxygen carrying-capacity. However, it is

possible that the amount of supplemental iron given to the IT group was insufficient to meet this increased erythropoietic demand while also fully meeting the needs of the skeletal muscle, mitochondria, and other systems impaired by the ID state. One potential explanation for an iron insufficiency despite supplementation would be decreased iron absorption due to an exercise-induced inflammatory response. The subsequent prioritization of available iron for erythropoiesis could come at the cost of negating the expected improvements in performance that result from increasing iron status discussed above. Such prioritization of erythropoiesis at the expense of other iron-dependent processes has been reported previously[42]. In this case, the expected effects of training or iron repletion would be less than those observed from either treatment alone, and, as seen in this study, could result in an improvement of equal magnitude to either treatment alone. However, because this study was not able to take muscle biopsies or measure other iron biomarkers, it is not possible to conclude what metabolic changes occurred with aerobic training, iron supplementation, or their combination.

One limitation of this study is that it was unable to measure other iron homeostasis indicators such as myoglobin, hepcidin, or proteins involved in erythropoiesis or iron absorption, which could provide insight as to how the iron was being utilized by the IT group compared to the other groups and whether iron was being redistributed. Further research should investigate whether providing iron supplementation for a longer duration would eventually elicit a larger response from

the IT group. Additionally, the exercise tests used in the literature vary widely, making it difficult to compare the results of this study to those that have been reported in similar studies. This study was conducted in non-anemic women who were not regularly participating in intense aerobic training. Therefore, the results of this study cannot be extrapolated to IDA or other anemic women, or women who are more fit or professionally trained athletes.

4.6 Conclusion

We have demonstrated that iron supplementation in sedentary IDNA women produces improvements in submaximal exercise performance of equal magnitude to those created by aerobic exercise training, with or without iron supplementation. Additionally, providing iron supplements to women during training did not have an additive benefit above that of either training or iron supplementation alone. The improvements in submaximal performance of the IU group support the idea that supplemental iron is used by sedentary women to increase mitochondrial productivity. Further research should be conducted to understand whether the added stress of aerobic training redirects the iron supplied by a dietary supplement away from mitochondrial bioenergetics pathways, how it is subsequently being used, and whether it affects bioavailability of supplemental iron.

Chapter 5 Assessing the feasibility of using a traditional Chinese herbal supplement, BaZhen KeLi, to address iron deficiency in Chinese women

5.1 Abstract

Background: Iron deficiency can be treated with elemental iron supplementation; however, in some areas of China, supplements are not commonly used. *BaZhen KeLi (BZ)*, a combination of 8 Chinese herbs, is often prescribed to treat symptoms that align with the Western definitions of iron deficiency. Despite the frequency of its use, there is currently no evidence in humans that BZ improves iron status or its impacts on physical performance. *Objectives*: This study aimed to compare the efficacy of using BZ to that of an iron supplement or placebo in improving iron status and physical performance. *Design*: 102 iron-depleted, non-anemic Chinese women (serum ferritin < 25 µg/L, hemoglobin > 110 g/L) were included in an 8-week, partially-blind randomized control trial with a 3x2 factorial design. Treatments included 42 mg elemental iron/day, 150 g BZ/day, or a placebo and aerobic training (5 sessions/week of 25 minutes cycling) or no training. Linear models were used to evaluate the relationships between supplement type, training group, and changes in iron status or physical performance. *Results*: Eight weeks of BZ treatment did not significantly improve any measure of iron status compared to placebo. The BZ-trained group showed improvements in physical performance of equal magnitude to those of the iron- and placebo-trained groups. The BZ-untrained group showed no

improvements in physical performance compared to the placebo-untrained group. *Conclusions*: BZ treatment does not improve any measure of iron status in iron-depleted women, nor does it have an ergogenic effect on physical performance when given alone or in combination with aerobic training.

5.2 Introduction:

Despite improvements in recent years, iron deficiency with and without anemia remains a large public health concern in China. Among women of childbearing age, between 20-34% are anemic (hemoglobin (Hb) < 110 g/L)[81], a rate that likely underestimates the prevalence of iron deficiency in the population because it does not include those women who are iron deficient but not anemic[103]. Beyond the nutritional impacts of iron deficiency itself, iron deficiency without anemia [IDNA: Hb > 110 g/L and serum ferritin < 15 μg/L] also results in decreased cognitive performance, impaired aerobic exercise capacity, and a reduced amount of time spent performing voluntary physical activity[5]. Collectively, IDNA and anemia not only result in a decreased productivity and quality of life at the individual level, but also impact the economic productivity of China as a whole, reducing the Chinese gross domestic product by between 2-3%[81].

While IDNA and its negative consequences can be treated with iron supplements or multivitamins and minerals, the use of Western multivitamins or iron supplements is not common among Chinese populations – especially those in the rural areas that are more at risk for IDNA[81]. Instead, many people rely on traditional Chinese

medicine (TCM) to diagnose and treat the symptoms of IDNA and anemia. In 2015, between 19 and 25 percent of mainland Chinese adults reported using TCM[146]. Furthermore, the popularity of these treatments is growing in the US, with 38.3% of American adults reporting use of at least one type of complementary health product or treatment in 2016[147], and some reports showing usage rates as high 67% in US women of reproductive age[148]. Despite its popularity, TCM herbal treatments are not regulated by the US government and few rigorous scientific studies are available validating the efficacy or the nutritional composition of popular herbal treatments in their intended populations. Furthermore, little research has been done examining herbal supplements in their whole form, as they are consumed in TCM - compared to the examination of a single active chemical ingredient.

In Western medicine, iron deficiency is defined by serum ferritin (sFer) and hemoglobin (Hb) concentrations in the blood. However, within TCM, IDNA and anemia are generally diagnosed as a “blood deficiency”, a “qi deficiency”, or a combination of the two[149] and are usually treated with traditional herbal remedies. One common herbal treatment for such “deficiencies” is the traditional Chinese herbal treatment called *BaZhen KeLi* (BZ) that is comprised of 8 Chinese medicinal plants[150]. Though it is widely administered to treat a range of symptoms including anemia, fatigue, pale complexion, dizziness, and others, few studies have examined the efficacy of BZ in directly improving Hb or iron status.

In a study using 80 anemic female mice, oral administration of BZ for 10 days

resulted in an increase in red blood cells, Hb, and hematocrit and also stimulated transcription of the erythropoietin mRNA in the kidney and liver, which stimulates erythropoiesis[149]. Other studies have examined a polysaccharide isolated from the traditional herb *Radix Angelica Sinensis*, a key ingredient in BZ, for its use in improving iron status in rats. These studies have shown that in a group of 36 Sprague–Dawley rats, this polysaccharide down-regulates hepcidin expression and stimulates erythropoietin secretion , which could be beneficial to iron status[87–89,151]. Additionally, two studies have shown that administering this herb to exercising rodents improved their exercise performance by protecting against fatigue in mice[152] and improved exercise-induced anemia by increasing red blood cell count and Hb concentration in rats[153].

While these studies demonstrate the potential of using BZ to treat iron deficiency with or without anemia or their associated detrimental effects on physical performance, to our knowledge, no study has ever tested the efficacy of BZ or its potential active ingredients in treating these conditions in humans. Therefore, the goal of this study was to compare the efficacy of BZ supplementation with that of ferrous sulfate supplements or a placebo in improving iron status in IDNA women. We hypothesize that BZ will increase markers of iron status compared to placebo, but to a lesser extent than the ferrous sulfate supplement. Additionally, we hypothesize that those subjects taking BZ will demonstrate a longer time necessary to reach the point in exercise where ventilation increases disproportionately to the amount of

oxygen consume (ventilatory threshold, VT) and a decreased total blood lactate levels during submaximal exercise than placebo subjects, but to a lesser extent than subjects taking ferrous sulfate supplements. Though previous studies have not tested the impacts of BZ or its components on improving maximal physical performance, they consistently showed an increase in Hb and inhibition of hepcidin. These changes are known to improve maximal performance, as they increase oxygen delivery to exercising tissues[34,90]. Therefore, we also hypothesize that BZ treatment will increase subjects' highest achieved oxygen consumption during testing (VO_2peak) compared to placebo, but to a lesser extent than in those who receive iron supplementation.

5.3 Methods

5.3.1 Subjects

The Consort diagram for this study is shown in **Figure 2.1**. There were 379 untrained but moderately active women recruited from September to December 2014 and from March to June 2015. The women were students between the ages of 18 and 26 years at Kunming Medical University. Of the recruited women, 124 were identified as IDNA (sFer < 25μg/L and Hb > 110 g/L). The cutoff for IDNA of sFer < 25 μg/L was selected because literature supports that physical performance can improve in women of this sFer range, despite not being clinically iron deficient [8,112]. A Hb cutoff of 110 g/L, which is lower than the 120 g/L cutoff used in Western medicine,

was used to define anemia in order to align with the cutoff used at the First Affiliated Hospital of Kunming Medical University, who performed the screening analyses.

Screening was conducted to identify and exclude women who met any of the following exclusion criteria: recent infectious illness or fever, current pregnancy or lactation or pregnancy/lactation within the previous year, hemolytic anemia, current inflammation or chronic inflammatory diseases, musculoskeletal problems, chronic respiratory disease, history of eating disorders, smoking, or regular/recent consumption of iron supplements, multivitamins, or medications that had anticoagulant properties or that could affect iron intake or absorption. Subjects completed a questionnaire that indicated they were not regularly participating in exercise or sports teams and were willing to comply with the full 8-week aerobic training schedule if randomized to a training group. In total, 124 women agreed to participate in the trial over the two semesters (45 fall, 79 spring). Of these, six women who had initially agreed to participate did not respond to further communications, leaving 118 women in the study who were randomized to the six groups.

During the study 9 women dropped out of the trial for personal and health reasons (3 iron, 4 placebo, and 2 BZ). Additionally, the data from 7 women were excluded from the statistical analysis because it was found that their sFer concentrations at study week 0 were above the cutoff of 25 µg/L, despite having screening values from two weeks prior indicated that they were iron depleted (23.5 - 24.9µg/L). The final sample size for this analysis was 102 women. Signed informed

consent was obtained from each subject. The study was approved by the Cornell University Institutional Review Board and the Kunming Medical University Ethical Committee and registered under ClinicalTrials.gov #: NCT03002090.

5.3.2 Study Design

The study used a 3x2 randomized, partially-blinded, placebo-controlled intervention design. A random number generator was used by the author (LMP) to randomly assign participants to the six treatment groups. Subjects received one of the following three supplements: 100 mg of ferrous sulfate, an identical placebo capsule, or a BZ capsule containing the ingredients shown in **Table 1.1**. Capsules were consumed twice a day, one in the morning and one in the evening with meals (2 capsules per day total). The researchers and participants were both blinded to the identity of the iron and placebo capsules. The BZ capsules were prepared locally by Dr. YuXu He at Yunnan University for Traditional Chinese Medicine and were visually distinct from the iron and placebo capsules. Therefore, while the participants were not told which group they were in, the BZ group was aware that their capsules were different. Blinding was maintained until after the initial per-protocol analyses were completed. The sample size was determined under the assumption of subjects consuming at least 12 mg elemental iron per capsule over the 8-week study period. The final iron capsule concentration met this requirement. Previous studies have shown that a 4-week treatment with this dose of ferrous sulfate is sufficient to

improve iron status as measured by the indicators used by the present study[3,113]. The iron and placebo capsules were prepared by one of the authors (LMP) using ferrous sulfate, dextrose filler, and gelatin capsules (PCCA, Houston, TX). Every two weeks during the study, 20 capsules of each type were randomly selected from the full batch and stored in a cool, dry place until their analysis by ICP/MS after the study was completed. A random sample of 20 of each of the iron, placebo, and BZ capsules were analyzed for iron content and found to have 21.1 mg, 0.00 mg, and 0.49 mg elemental iron per capsule, respectively.

Subjects were asked to consume the capsules along with their morning and evening meals and encouraged to consume them along with citrus juice to increase iron absorption. Every two weeks, 30 capsules were delivered to each subject in a bottle labeled with their subject ID. Subjects were also given a daily log on which they recorded capsule ingestion, menstrual cycle status, any gastrointestinal or other health complaints, medication use, and voluntary physical activity. On weeks 2, 4, 6, and 8, subjects were asked to return their capsule bottles along with any unconsumed capsules, which were counted as an independent confirmation of the capsule counts reported in the daily log.

Half of each of the three supplement groups were randomly assigned to an 8-week aerobic exercise training program or no training, creating a total of six treatment groups: iron trained (FeTr), iron untrained (FeUn), placebo trained (PLTr), placebo untrained (PLUn), BZ trained (BzTr), and BZ untrained (BzUn). The training protocol

was adapted from a training program published by Hinton et al, which resulted in significant improvements in both maximal and submaximal exercise performance[3]. The training protocol is shown in **Table 2.2**. Each 25-minute session was divided into time spent at 75% and 85% of subjects' age-predicted maximum heart rate (220 beats per minute minus years of age). Each week the time spent at 85% maximum heart rate was increased to account for increasing fitness as the training program progressed. Training occurred every Monday through Friday in the 8 weeks of the study, with additional make-up days being offered on weekends if subjects missed a session during the week. The maximum number of training days was 40 sessions.

Training was conducted using stationary exercise bicycles (KangLe Exercise Products Company, Stationary Bicycle model B8.4E, Yunnan, China) that provided a digital output of cadence (rotations per minute) and work (watts). Heart rate was monitored using T31 Polar heart rate monitors and Polar A5 heart rate watches (Polar Electro Inc., Lake Success, NY). Research assistants recorded heart rate, speed, and watts of each subject in training logs every 5 minutes throughout each training session.

Minutes of self-reported voluntary/discretionary physical activity were measured using the daily logs collected at weeks 0, 4, and 8. Additionally, at baseline, habitual physical activity levels were assessed via a physical activity frequency questionnaire to compare similarity between groups. Participants were requested to maintain their typical physical activity patterns throughout the 8-week

study period, regardless of whether the group they were randomized to was trained or untrained.

Maximal and submaximal physical performance, as well as body composition, were measured at study weeks 0, 4, and 8. Macronutrient content, dietary iron, and iron enhancers and inhibitors were assessed at week 0 using a 4-day diet record. Dietary analyses were conducted using Nutrition Data System for Research Software (2016, University of Minnesota).

Participants were compensated for completing the study with a gift. Additionally, subjects randomized to the training groups were offered incentives to improve compliance to the training program. Small gifts were provided when subjects reached 25, 32, and 40 days of training. The incentives were only offered to the trained subjects, while all subjects received the same larger gift for their participation in the study.

5.3.3 Iron Status Measurements

At study weeks 0, 4, and 8, blood was drawn by a licensed phlebotomist from the antecubital vein into 4 mL Anti-Coagulant vacutainers at the Kunming Medical University campus hospital. A small sample of whole blood was immediately analyzed for Hb concentration using a Coulter LH 750 Hematology analyzer (Beckman Coulter, Inc. Brea, CA). The remaining blood was stored for no longer than 24 hours at 4°C until it was centrifuged at 1600 g for 10 minutes at room

temperature. Serum was separated into 0.5 mL aliquots and frozen at -80°C until being analyzed for sFer, sTfR, alpha-1-acid glycoprotein (AGP), and C-reactive protein (CRP) using a Siemens Advia 2400 automated analyzer (Siemens Healthcare, Erlangen, Germany). Blood analyses were conducted at the First Affiliated Hospital of Kunming Medical University and the Shanghai Fenglin Clinical Laboratory in the first and second semesters, respectively. To allow for comparison between the labs, 75 samples from the second semester were run at both labs. Estimated total body iron was calculated using the ratio reported by Cook et al[23] as:

$$\text{Total Body Iron (mg/kg)} = -[\log(\text{sTfR}/\text{sFer}) - 2.8229] / 0.1207$$

The total body iron equation uses the sTfR values from an ELISA kit produced by Ramco Laboratories (Stafford, TX). To align with this scale, sTfR values from this study were converted to the Ramco scale using the following prediction equation derived from 34 random duplicate samples run on Ramco Laboratories sTfR ELISA kits:

$$\text{sTfRRamco} = (3.779 \times \text{sTfRlab}) + 0.400,\ R^2 = 0.93$$

5.3.4 Physiological measurements

Height, weight, and body composition were measured using previously described standard methods[114,115]. Percent body fat was estimated using the Durnin and Womersley equation with skinfold thicknesses from the bicep, triceps, subscapular, and suprailiac sites measured with Lange calipers (Cambridge, MD).

A maximal exercise test (VO_2max test) was used to assess physical performance at study weeks 0 and 8. Tests were conducted on a mechanically braked and calibrated cycle ergometer (Monark 884E, Monark Exercise AB, Vansbro, Sweden). The test required subjects to cycle at efforts ranging from rest to approximately 100% of their maximum exertion level. During the test, a portable metabolic measurement system (Cosmed K_4B^2, Cosmed Rome, Italy) was used to measure volume of respired air, heart rate, and concentrations of O_2 and CO_2 in expired air. Oxygen consumption was standardized to body weight (VO_2, mL/kg/min) to allow for comparison between subjects. For the 24 hours prior to performing the VO_2max test, subjects were asked not to engage in strenuous physical activity. Subjects in the three trained groups did not participate in training sessions on the day of their VO_2max tests. Additionally, subjects were instructed to avoid food and caffeinated beverages three hours before testing.

The VO_2max testing protocol was adapted from previously published methods[77]. After allowing the subject's resting heart rate to fall below 100 beats per minute, the test began with a 5-minute warm up pedaling against 1 kg at a cadence of 50 revolutions per minute (RPM). After the warm up period, workload was increased every two minutes by 0.4 kg while subjects maintained the 50 rpm cadence. This process continued until VO_2 did not increase by more than 150 mL/min after the addition of weight – indicating subjects had reached their VO_2max. However, as the subjects in this study were unaccustomed to such rigorous exercise

levels, it was expected that many would be unable to attain this plateau in VO_2. Therefore, the test continued either until the plateau was reached or subjects ceased pedaling or were unable to continue. Subjects were considered to have achieved VO_2peak (highest observed VO_2) if any 2 of the following conditions were met: a heart rate within 10 beats/min of the predicted maximum heart rate for their age (220 – age in years), a respiratory exchange ratio (ratio of expired CO_2 to O_2) greater than 1.15, or a blood lactate concentration greater than 8.0 mM. Blood lactate was assessed immediately upon completion of the VO_2max test via finger stick using a Lactate Plus portable blood lactate analyzer (Nova Biomedical, Waltham, MA, USA).

5.3.5 Compliance Analyses

Compliance to training was assessing using a training score that was calculated as the total percent of the target heart rate achieved each day multiplied by the number of days the subject trained. Additionally, the percent of the target training speed (60 rpm) each subject maintained was used as a separate indicator to estimate subjects' compliance to the training instructions.

Compliance to the supplementation groups was assessed by comparing the daily logs filled out by the subjects to the physical capsule counts obtained from the returned bottles. A combined estimate of capsules consumed was calculated by comparing the self-reported consumption to the returned capsule counts and taking the larger number of missed capsules (reported or returned physically) as the number

of capsules skipped.

5.3.6 Statistical Analyses

Sample size was determined to require 25 subjects per group to detect a 0.8 standard deviation change (4.2 μg/L) in sFer that corresponded to biologically meaningful improvements in physical performance at an $\alpha = 0.05$ with 80% power[3,109]. Variables were examined for normality of distribution using Kolmogorov-Smirnov test, histograms, and qq-plots. For sFer and sTfR, statistical analyses were run using log transformations because these variables were found to have non-normal distributions. Differences between groups at baseline were tested using one-way ANOVA. Certain measures of iron status and physical performance at week 8 were analyzed using linear models[136]. For all models, baseline values and semester of testing were included as covariates. Baseline age was also included in all models because it was found to be significantly different between groups at baseline. For all analyses, statistical significance was defined as $p < 0.05$ for single variables and $p < 0.1$ for interaction terms. For significant main and interaction effects, post-hoc pairwise comparisons of the week 8 values were made with a Tukey correction for multiple comparisons. Secondary analyses were conducted with linear models using a significance level of $p < 0.05$. Statistical analyses were conducted using SAS 9.4 (SAS Institute, Cary, NC).

5.4 Results

5.4.1 Subject characteristics

Despite randomization, the BZTr group was significantly younger than the FeTr, PLTr, and PLUn groups at baseline (**Table 5.1**). There were no other differences observed in any background measure at baseline on the basis of one-way ANOVA. At baseline (week 0), 52 of the 102 subjects (51.0%) were clinically iron deficient, defined as sFer < 15.0 µg/L, 18 women (17.7%) had body iron < 0 mg/kg, 9 women (8.8%) had sTfR values > 8.3 mg/L, and 3 women had Hb < 120 g/L (2.9%).

No change in weight or BMI was observed for any group from baseline to week 8; however, those women who received BZ had a significantly lower percent body fat at week 8 than those who received either iron or placebo, after adjusting for baseline body fat (28.4 ± 1.5, 29.5 ± 1.2, and 29.4 ± 1.3, respectively, linear model $p = 0.007$). At baseline, there were no differences in any dietary measure including daily intake of: calories (kcals), protein, carbohydrates, fat, iron, ascorbic acid, calcium, or phytic acid. There were also no differences in inflammation at baseline or during or after the 8-week study on the basis of α-1-acid glycoprotein (AGP) or C-reactive protein (CRP). There were no subjects at week 0 or 8 with elevated AGP or CRP (AGP > 1 mg/L, CRP > 5 mg/L). Additionally, there were no differences between groups in reported symptomology during the 8-week study on the basis of symptoms reported in the daily log.

Table 5.1: Baseline characteristics by group

Characteristic	Iron Trained	Iron Untrained	Placebo Trained	Placebo Untrained	BZ Trained	BZ Untrained
Age (yr)	20.5 ± 1.0	20.2 ± 0.8	20.6 ± 1.3	20.6 ± 1.8	19.2 ± 1.0*	20.3 ± 1.3
Height (cm)	157.1 ± 5.7	156.9 ± 6.2	157.3 ± 4.7	157.5 ± 5.2	156.3 ± 3.9	159.5 ± 5.8
Weight (kg)	52.2 ± 7.3	51.6 ± 7.9	53.2 ± 6.1	51.0 ± 5.5	48.6 ± 4.6	50.0 ± 7.1
BMI (kg/m^2)	21.2 ± 2.9	21.0 ± 3.5	21.5 ± 2.1	20.6 ± 2.1	19.9 ± 1.7	19.6 ± 2.1
Body Fat (%)	29.3 ± 3.4	28.3 ± 5.1	29.1 ± 4.2	28.8 ± 2.3	28.4 ± 3.5	28.4 ± 3.3
Hb (g/L)	135.4 ± 8.8	140.7 ± 5.2	136.0 ± 10.5	135.3 ± 6.5	131.8 ± 8.9	133.9 ± 10.0
sFer (µg/L)	14.6 ± 6.1	17.1 ± 4.2	14.2 ± 4.6	15.5 ± 5.8	14.9 ± 5.8	17.2 ± 5.8
sTfR (mg/L)	6.7 ± 3.1	5.3 ± 0.8	5.9 ± 1.5	6.0 ± 1.7	7.0 ± 2.1	5.7 ± 1.5
Body Iron (mg/kg)	1.3 ± 2.6	2.7 ± 0.9	1.7 ± 1.8	1.8 ± 1.9	1.2 ± 2.0	2.4 ± 1.9
AGP (mg/L)	0.62 ± 0.09	0.61 ± 0.09	0.64 ± 0.09	0.58 ± 0.09	0.61 ± 0.08	0.58 ± 0.09
CRP (mg/L)	0.06 ± 0.12	0.22 ± 0.77	0.21 ± 0.35	0.08 ± 0.13	0.02 ± 0.04	0.03 ± 0.05
Daily Dietary Intake[1]						
Calories (kcal)	1543 ± 272	1573 ± 333	1602 ± 315	1587 ± 327	1482 ± 315	1609 ± 781
Fat (g)	51.7 ± 12.3	57.2 ± 15.0	56.0 ± 13.1	55.3 ± 17.2	49.8 ± 15.6	51.9 ± 26.7
Protein (g)	59.1 ± 19.9	60.2 ± 18.4	59.0 ± 17.1	58.7 ± 20.5	61.8 ± 25.5	61.4 ± 32.4
Carbohydrate (g)	215 ± 43	204 ± 49	216 ± 46	216 ± 53	198 ± 27	224 ± 128
Iron (mg)	11.7 ± 4.3	13.0 ± 5.8	11.6 3.5±	12.3 ± 3.8	13.6 ± 4.7	12.3 ± 7.6
Calcium (mg)	325 ± 114	340 ± 146	321 ± 154	394 ± 195	363 ± 133	319 ± 217
Ascorbic Acid (mg)	94.1 ± 57.3	60.2 ± 37.5	64.5 ±41.0	82.6 ± 79.5	90.1 ± 45.3	80.7 ± 66.1
Phytic Acid (mg)	544 ± 184	662 ± 340	533 ± 247	577 ± 252	444 ± 162	545 ± 397
Physical Performance Measures						
VO_2peak (mL/min/kg)	35.3 ± 4.5	36.0 ± 3.5	35.0 ± 4.0	34.6 ± 3.3	37.8 ± 3.4	35.5 ± 7.4
VO_2 at VT (mL/min/kg)	27.2 ± 4.2	28.8 ±3.3	28.2 ± 2.9	29.4 ± 3.2	32.4 ± 3.1	29.7 ± 5.7
Post-test lactate (mmol)	10.4 ± 2.2	11.7 ± 2.0	11.9 ± 3.4	11.5 ± 2.1	10.4 ± 2.3	10.8 ± 2.0
n	20	16	19	18	13	16

***** Indicates significant group difference at week 0 on the basis of one-way ANOVA at $p < 0.05$

[1] Dietary intake data excludes nutrients from iron or BZ supplements

Abbreviations: BZ – *BaZhen KeLi*; Hb – hemoglobin; sFer – serum ferritin; sTfR – soluble transferrin receptor; AGP – α-1-acid glycoprotein; CRP – C-reactive protein; VO_2peak – maximum observed oxygen consumption; VO_2 at VT – volume of oxygen consumed at the ventilatory threshold, or the point where the rate of respiration increases disproportionately to the amount of oxygen consumed

Participants began the study at an average level of fitness, based on a mean VO_2peak of 35.6 ± 4.6 mL/min/kg and a mean VO_2 at VT of 29.1 ± 4.0 mL/min/kg. The number of self-reported minutes spent doing physical activity did not differ between the groups, nor did the kcals expended per week over the 8-week period (data not shown, one-way ANOVA, $p > 0.05$). Across the 8-week study period, the average number of kcals expended per week in voluntary physical activity outside of the study's training program was 271 kcal ± 377 kcal.

5.4.2 Compliance to Treatments

The three trained groups all attended approximately 32 training days, during which they achieved the same percent of target heart rate and percent of target pedaling speed (**Table 5.2**).

There were no significant differences in the number of capsule bottles returned from each of the three supplement groups or the percent of the daily logs completed by each of the groups (**Table 5.3**). The amount of iron consumed from the capsules by the iron and BZ groups over the 8-week study period is shown in **Table 5.3**. The iron group consumed approximately 5 times the daily amount of iron required to prevent deficiency in 50% of the population (estimated average requirement, EAR) while the BZ group consumed only 12.3% of the EAR each day.

Table 5.2: Compliance to Training, by training group[1]

	Iron Trained	Placebo Trained	BZ Trained	P Value[2]
Days trained (out of 40)	32.9 ± 5.6	32.7 ± 3.7	32.4 ± 5.1	0.96
Range (out of 40)	21-40	28-40	23-40	
Training Score (out of 40)	30.3 ± 5.6	30.6 ± 3.4	29.7 ± 5.5	0.89
% Target HR	90.5 ± 7.8	91.9 ± 5.6	90.8 ± 3.8	0.76
% Target RPM	99.0 ± 5.3	100 ± 0.0	98.3 ± 5.7	0.41
n	18	18	13	

[1]Values are mean ± standard deviation

[2] P values are from one-way ANOVA

Abbreviations: BZ – *BaZhen KeLi*; HR – heart rate; RPM – rotations per minute

Table 5.3: Capsule Compliance[1]

	Iron	Placebo	BZ	P value[2]
Percent capsule bottles returned	81.9 ± 25.1	80.4 ± 28.3	87.9 ± 15.8	0.36
Percent daily logs completed	96.1 ± 7.1	91.6 ± 19.5	90.0 ± 17.1	0.63
Capsules consumed (#)	95.9 ± 11.9	99.3 ± 12.1	96.7 ± 11.1	0.88
Iron consumed (g/8 weeks)	2.02 ± .25	0.0 ± 0.0	0.05 ± 0.01	<0.001
n	36	37	29	

[1]Values are means ± standard deviation

[2] P values are from one-way ANOVA

Abbreviations: BZ – *BaZhen KeLi*

5.4.3 Treatment Effects on Iron Status

At week 8, there were no significant interactions between training group and supplement type for any measure of apparent iron status, controlling for baseline values. There were also no significant main effects of training for any iron status measure; however, there were significant main effects of supplement type for sFer, Hb, sTfR, and body iron (**Table 5.4**). At week 8, the iron-supplemented group had significantly higher sFer and body iron, and significantly lower sTfR (indicating better iron status), than either the placebo or BZ supplemented groups. There were no significant differences between the BZ group and the placebo group for any of the three iron measures.

5.4.4 Treatment Effects on Physical Performance

There were significant interaction effects between training group and supplement type for both VO_2peak and VO_2 at VT (**Table 5.5**). Post-hoc pairwise comparisons for the significant interaction effects, with corrections for multiple comparisons, are shown in Table 5.5. There were no significant interactions or main effects observed for post-test blood lactate concentration.

Table 5.4: BZ Study Iron Biomarkers at Week 8

Group	n	sFer (µg/L)	Hb (g/L)	sTfR (mg/L)	Body Iron (mg/kg)
Iron[1]	36	39.6 ± 16.5	141.1 ± 7.5	4.9 ± 0.9	5.8 ± 2.0
Placebo[1]	37	20.8 ± 9.8	136.3 ± 9.8	5.9 ± 1.9	2.8 ± 2.5
BZ[1]	29	18.5 ± 10.2	132.6 ± 8.6	5.9 ± 1.4	2.3 ± 2.4
Interaction p value[2]		0.43	0.61	1.00	0.55
Training p value[2]		0.07	0.78	0.11	0.053
Supplement p value[2]		<0.001	0.050	<0.001	<0.001
Post-hoc Group Comparisons for Supplement Effect					
Fe-PL *P* value		<0.001	0.057	<0.001	<0.001
Fe-BZ *P* value		<0.001	0.17	<0.001	<0.001
PL-BZ *P* value		0.90	0.99	0.68	0.80

1 Values are unadjusted means ± standard errors, at week 8

2 Results of a linear model, adjusted for baseline, comparing values at 8 weeks in each variable. Interaction p value represents the supplement type by training group interaction.

3 P values are from post-hoc comparisons between groups with Tukey corrections for multiple comparisons

Abbreviations: sFer: serum ferritin; Hb: hemoglobin, BZ: *BaZhen KeLi*

Table 5.5: BZ Study Physical Performance Measures at Week 8[1]

Group	n	VO_2peak (mL/min/kg)	VO_2 at VT (mL/min/kg)	Post-VO_2 Test Lactate (mmol)
Iron Trained	20	37.8 ± 5.1	33.2 ± 5.0	9.8 ± 3.3
Iron Untrained	16	37.6 ± 4.4	32.2 ± 2.6	10.3 ± 3.9
Placebo Trained	19	37.8 ± 3.0	32.2 ± 3.9	10.6 ± 2.2
Placebo Untrained	18	33.9 ± 4.2	28.2 ± 3.9	10.1 ± 2.4
BZ Trained	13	35.3 ± 3.6	33.1 ± 3.6	9.4 ± 2.3
BZ Untrained	16	33.6 ± 4.9	29.3 ± 3.8	9.8 ± 2.0
Interact. p value[2]		0.047	0.093	0.95
Training p value[3]		0.016	<0.001	0.92
Supp. p value[3]		0.007	<0.001	0.66

Post hoc pairwise comparisons for significant interaction terms[4]

Is there a training effect in all trained groups? (Trained groups vs. true placebo)		
FeTr vs. PLUn	0.019	<0.001
PLTr vs. PLUn	0.006	<0.001
BZTr vs. PLUn	1.00	0.044
Does BZ impact training effect? (BZ trained vs. other trained groups)		
BZTr vs. FeTr	0.17	0.27
BZTr vs. PLTr	0.07	0.92
Does BZ have ergogenic effect alone or in addition to training?		
BZTr vs. BZUn	1.00	0.46
BZUn vs. PLUn	1.00	0.77

[1] Results of linear models adjusted for baseline and semester, values are LS means ± standard errors, significance defined as $p < 0.05$ for single variables and $p < 0.1$ for interaction terms

[2] P value for the interaction between training group and supplement type

[3] P values for main effects of training group and supplement type (Supp. P)

[4] P values for post-hoc pairwise comparisons, corrected for multiple comparisons with a Tukey correction

Abbreviations: VO_2peak: highest observed oxygen consumption during VO_2max test; VO_2 at VT: oxygen consumption at ventilatory threshold; post- VO_2 test lactate: blood lactate level at completion of VO_2max test; BZ: *BaZhen KeLi*; Interact: interaction; Supp: supplement; FeTr: iron trained group; FeUn: iron untrained group; PLTr: placebo trained group; PLUn: placebo untrained group; BZTr: *BaZhen KeLi* trained group; BZUn: *BaZhen KeLi* untrained group

Academic semester of testing and baseline age were included as covariates in all linear regression models for both blood biomarkers and physical performance measures but were not statistically significant in any model. For variables where an interaction was observed between supplement type and training group, the interaction was maintained when change in Hb and baseline body fat were included in the models. Hb and body fat were not significant in any model. Additionally, including the number of capsules consumed as a covariate did not alter the relationships observed for any blood biomarker or physical performance measure.

5.4.5 Secondary analyses

Secondary analyses were conducted to examine the biological plausibility of the primary results. First, the relationship between the amount of iron consumed and the resulting change in apparent iron status and physical performance measures was examined in subjects who consumed either BZ or iron supplements. Significant relationships were observed between the amount of iron consumed and all measures of iron status at week 8, adjusting for baseline values (linear regression, sFer estimate 1.7 ± 0.2 µg/L, $p < 0.001$; Hb estimate 3.7 ± 1.0 g/L, $p < 0.001$; sTfR estimate -0.47 ± 0.12 mg/L, $p < 0.001$; body iron estimate 1.7 ± 0.2, $p < 0.001$). However, there were no significant relationships observed between the amount of iron consumed and any physical performance measure at week 8, adjusting for baseline (linear regression, $p = 0.10$, $p = 0.16$, and $p = 0.41$ for VO_2peak, VO_2 at VT,

and post-test blood lactate, respectively). Additionally, among all participants, there was a significant positive relationship between the 8-week change in sFer and 8-week VO_2peak, adjusting for baseline (estimate and SEM: 0.05 ± 0.02, p = 0.038). The same trend was seen for the relationship between 8-week change in sFer and VO_2 at VT (estimate and SEM: 0.05 ± 0.03, p = 0.070). No significant relationships were observed between the baseline to 8-week change in Hb, sTfR, or body iron and any performance measure.

To examine the relationship between supplement and iron status markers in those women who should benefit most, we examined the effect of BZ supplementation in a subgroup of subjects with the most severe iron deficiency at baseline. Separate subgroup analyses were run in those women who began the study with the lowest Hb concentrations at week 0 (women below the median value, Hb < 136.0 g/L, n = 46) and those with clinical iron deficiency at week 0 (sFer < 15.0 µg/L, n = 52). For both of these subgroups, when compared to the full study population, there were no differences found in the significance levels of the main or interaction effects for supplement or training group in any of the tested outcome measures. Additionally, there were no differences in the direction of the relationships between the adjusted means for any main or interaction effect (data not shown).

Functional anemia occurs when Hb is not below the clinical cutoff but still improves with iron supplementation. In this study, women who were functionally anemic could potentially respond to supplementation differently than those who were

not by directing the supplemental iron toward Hb production rather than towards improving iron stores or tissue iron (represented by sFer and sTfR, respectively). This reprioritization of iron could then alter the subjects' response to training and impact the physical performance measures. To assess for the implications of functional anemia, a subgroup analysis was conducted in those women who did not show functional anemia – defined as women whose 8-week change in Hb was of a magnitude that was less than one standard deviation of that observed in the placebo untrained group, which should represent the natural variation in Hb (change in Hb < 9.2 g/L, n = 76). There were no differences in any of the measured biomarkers of iron status found in the significance levels of the main or interaction effects for supplement or training group for this group compared to the full study population (data not shown). For the physical performance measures, no differences in the results for maximal performance were observed between the full sample and those who were not functionally anemic. For submaximal performance, the interaction effect was no longer significant; however, there was a significant supplement effect ($p = 0.002$). Post-hoc pairwise comparisons showed that while the iron group performed significantly better than the placebo group, there was no effect for BZ on submaximal performance compared to placebo. The same results were found in those subjects who met the criteria for functional anemia (n=26), with no differences in significance level for any iron biomarker between the functionally anemic subgroup and the full sample (data not shown).

Within the trained subjects, there was a significant relationship between the number of days trained and the post-test blood lactate concentration (linear regression, estimate -0.17, $p = 0.027$). However, in this model there was not a significant interaction effect between the number of days trained and supplement group, nor was there a significant main effect of supplement group. The same relationships were seen for the percent of target heart rate achieved during training and post-test blood lactate concentration (data not shown).

5.5 Discussion

To our knowledge, this is the first study to examine the efficacy of BZ in improving iron status (as assessed by sFer, sTfR, and Hb) or its associated impacts on physical performance in humans. The objectives were to analyze the efficacy of BZ supplements in improving several measures of iron status compared to conventional iron supplements or a placebo control as well as to examine whether BZ is effective at resolving the impaired physical performance known to occur in IDNA women.

While there are no studies examining the impact that BZ has on iron status in humans, several studies in rodents have shown that BZ[149] or a polysaccharide derived from *Angelica Sinensis* (one of its component herbs)[87–89,151,153] at a dose of 1g/kg body weight can increase hemoglobin concentration and reduce hepcidin expression, which help improve iron status. The results of this study contrast these previous findings, suggesting that BZ has no effect on any observed measure of iron

status. Iron supplementation significantly improved iron status as measured by sFer, sTfR, and body iron after 8 weeks of supplementation compared to both the placebo and the BZ groups. However, no significant differences were observed between the BZ and placebo groups for any iron biomarker, suggesting that BZ had no effect on either iron storage or cellular iron. A similar trend was observed for Hb, with no significant difference between the BZ and placebo groups.

These findings directly contrast those of previously published studies that found that BZ or its components improved hemoglobin counts in iron deficient anemic rats[151]. The present study was conducted in non-anemic women, and therefore may have missed any effects of BZ treatment that occur only in the most severely iron deficient anemic women. However, the study was designed to measure iron deficiency at varying severities, using sFer and sTfR – biomarkers that are more sensitive to less severe stages of iron deficiency. If BZ were improving iron status, these more sensitive biomarkers would be the first to respond. However, while these markers all showed significant improvements from iron supplementation, there was no change whatsoever in any of these more sensitive biomarkers in the BZ group, suggesting that BZ treatment is not effective at improving apparent iron status.

Additionally, when examining a subset of subjects who had Hb concentrations below the median value at baseline (Hb < 136.0 g/L, n =46) or a separate subset who were clinically iron deficient (sFer < 15 µg/L, n = 52), there was still no relationship between BZ treatment and improvement in Hb or any other iron status marker

measured in this study. This finding is consistent with the analysis of the BZ iron content, which showed that each capsule contained only 0.49 mg of iron. The intervention provided two capsules per day, delivering about 1 mg of iron to the subjects each day, an amount equivalent to only 12.5% of the iron estimated to meet the daily needs of 50% of adult women[116]. Collectively, these findings partially agree with the hypothesis in suggesting that BZ treatment is not as effective as iron supplementation in improving apparent iron status. However, contrary to the hypothesis, this study also found that treatment with BZ is no more effective than a placebo in improving apparent iron status in IDNA women.

This study also aimed to determine whether BZ treatment is effective in improving physical performance or enhancing the impact of aerobic training. Several studies have found that various components of BZ (the previously mentioned *Angelica Sinensis* polysaccharide or a mixture of 4 of the 8 herbs in BZ called "*Si Wu Tang*") have been shown to improve aerobic exercise performance in both iron replete and anemic rodents[86,153]. In a study by Yeh et al[152], iron-replete mice given *Angelica Sinensis* and/or 6-weeks of aerobic training showed increased endurance during a swim test, decreased blood lactate after the test, and increased post-exercise liver and muscle glycogen concentration. The Yeh et al study also observed a potential ergogenic effect of the herb when taken during training beyond that observed when training with placebo[152]. However, the present study found that 8 weeks of aerobic training with simultaneous treatment with BZ had no significant

impact on maximal or submaximal performance or post-test lactate concentration in women. The FeTr, PLTr, and BZTr groups all showed statistically significant improvements in submaximal performance that are consistent with the improvements in physical performance observed in others studies that have used a similar training protocol[3]. As there were no significant differences among the three trained groups for maximal or submaximal exercise performance, it is likely that the improvements in performance in the BZTr group were due solely to the training program and not to any beneficial effect of BZ itself.

Furthermore, the results of this study do not support that BZ treatment without training has any impact on maximal or submaximal physical performance or blood lactate concentration. While the FeUn group showed a significant improvement in submaximal exercise performance compared to the PLUn group, no effect of BZ was observed for maximal or submaximal exercise outside of the effect of training.

Finally, there were no significant differences in physical performance measures between the BZTr group and the BZUn group, which somewhat contradicts the finding that the BZTr group had significant improvements in physical performance compared to the PLUn group. There are two possibilities that may explain this finding. First, it is possible that the BZ treatment in the BZUn group did have a small ergogenic effect, increasing the performance of the BZUn group slightly above that seen at baseline, but not by enough to be significantly different from the PLUn group at week 8. Alternatively, it is possible that because the BZTr group had the smallest

sample size (n=13), there was not enough statistical power to observe the same level of significance seen in the relationships between the PLTr-PLUn groups and the FeTr-FeUn groups. For both maximal and submaximal exercise performance measures, the BZTr group showed better performance measures than the BZUn group, suggesting that this sample size may have been a limitation for this comparison.

There are several biological mechanisms that could explain why the results of this study contrast the previously published work. First, all of the previously reported studies have been conducted in mice or rats, which may limit the generalizability of their findings to humans. Additionally, this study gave BZ as it is generally prescribed by TCM practitioners – as a combination of 8 herbs consumed in a single capsule twice a day. The Tian et al study used a *BaZhen* decoction (liquid distilled from the BZ herbs), while all of the other cited studies used only components of BZ such as the four-herb combination, *Si Wu Tang*, or a single polysaccharide isolated from one of the BZ herbs. It is possible that one or more ingredients in the full herbal recipe may be blocking the effect seen from using the single polysaccharide or a smaller combination of herbs.

It is also possible that the dosage of BZ per kg body weight used in this study differed from that used in previously published rodent studies. Because most prior studies use do not use the full BZ recipe, it is difficult to directly compare the dose per kg body weight; however, in the Tian et al study, between three and fifteen g of each

of the 8 herbs in BZ were put into 1L of distilled water to make the decoction. This study used five to ten g of each of the 8 herbs per pill, for a total of ten to twenty g of each herb consumed per day. Therefore, while the absolute concentration of the herbs in each dose is similar, the large difference in body weight between rats and humans could potentially decrease the potency of the BZ treatment in humans. However, because this study did not provide different dosages of BZ to the participants, we are unable to assess how a stronger dose or greater number of capsules per day could have altered the findings.

One limitation of this study is that we were not able to fully blind the subjects to the capsules. Due to constraints at the TCM hospital where the BZ capsules were prepared, the BZ capsule shell was visually distinct from the iron and placebo capsule shells. Therefore, while the subjects in the BZ groups did not know what treatment group they were in, they were able to observe that their capsules were visually different than the other subjects' capsules. However, the compliance analyses show that there were no significant differences in compliance to capsule consumption across groups.

Additionally, the researchers were not blinded to the BZ group, which could potentially lead to bias in the physical performance data collection. To assess for potential measurement bias in the BZ group, subjects' maximum heart rate was compared across week 0 and after 8 weeks of treatment, adjusting for baseline. There were no significant differences in maximum heart rate at baseline or week 8

(linear models p = 0.41 and p = 0.08). Because the p value for this secondary analysis was close to the significance cutoff of $p < 0.05$, post-hoc pairwise comparisons with a Tukey correction for multiple comparisons were examined to compare each BZ group with its placebo group counterpart. There were no significant differences between the BZTr and PLTr groups (mean and standard error 186.8 ± 2.0 and 188.1 ± 1.5, respectively, p = 1.00), nor were there differences between the BZUn and PLUn groups (mean and standard error 190.6 ± 1.8 and 187.6 ± 1.6, p = 0.82). Additionally, the percent of subjects achieving a heart rate within 10 beats per minute of their age predicted maximum heart rate (220-age) did not differ between groups at week 8 (Chi-square p = 0.22). There were also no significant changes in maximum heart rate across any treatment group from baseline to week 8. Maximum heart rate is predominantly determined by age and would not be expected to change from either aerobic training or iron supplementation[154]. Therefore, if there were differences in the maximum heart rate achieved during testing, it could be a result of differential treatment on behalf of the researchers. However, as no differences were observed between the supplement groups, there is no indication that such measurement bias occurred.

Furthermore, due to limitations in the field, this study was unable to recruit the target number of women per group and may be limited in its statistical power to detect statistically significant differences between the six treatment groups. Despite the small sample size in the BZ groups, the primary outcomes of interest all showed

low variances of similar or smaller magnitude to those of the iron and placebo groups, which suggests that the BZ groups did not have outliers that influenced the mean values in a major way. Finally, this study was conducted in sedentary IDNA women. Therefore, the results of this study may not be as relevant to severely anemic women, or women who are more fit or professionally trained athletes.

5.6 Conclusion

We did not find any significant impact of BZ on any measure of iron status in IDNA women. Additionally, we did not find that BZ has an ergogenic effect on maximal or submaximal physical performance or post-exercise blood lactate concentration. Considering the prevalence of the use of TCM, and the popularity of BZ and its constituent herbs for treatment of fatigue, anemia, pallor, and other symptoms associated with iron deficiency, it is important that TCM and Western medicine practitioners be made aware that this herbal treatment does not appear to have any benefit for iron-depleted women and should not be prescribed for treatment of iron deficiency without anemia, especially at the expense of prescribing conventional therapeutic iron supplements. Further research should examine whether other preparations of the *BaZhen* herbs together or *Angelica Sinensis* alone have an ergogenic effect on submaximal physical performance.

Chapter 6: General Discussion

6.1: Interplay of exercise and iron homeostasis – gaps in the literature

The collective aim of Chapters 3 and 4 was to understand how iron homeostasis and aerobic training mutually influence each other in IDNA women. Chapter 3 concluded that when women train while taking iron supplements, the improvements in sFer in the trained group are significantly less than those observed in the women who received iron but did not train, suggesting that training reduces the apparent efficacy of supplementation. Chapter 4 then explored the ergogenic effects of iron supplementation on maximal and submaximal physical performance. The data presented in that chapter suggest that while supplemental iron improves submaximal physical performance as much as training without supplemental iron, there is no additive benefit of supplemental iron above that of training alone. Collectively, these findings suggest that when iron status and aerobic fitness change simultaneously, there is a prioritization of available iron to support key processes such as erythropoiesis, myoglobin production, or the bioenergetics pathways[34,49,66,155–157]. It is also possible that the iron was either not being absorbed perhaps due to exercise-induced inflammation[32], or that the exercise was increasing iron losses[8]. This dissertation was unable to determine exactly how iron was being utilized by women in various treatment groups, a shortcoming that will be discussed in the following sections. However, comparing the changes in the measured iron biomarkers in each treatment group could provide insight into what metabolic pathways were

differentially affected by training and iron supplementation.

Unfortunately, biomarkers for assessment of iron homeostasis remain an imperfect tool. Section 6.2 will address some of the shortcomings of the conventional measures of iron status. It will also explore several potential methods for assessing iron homeostasis that could help provide a more comprehensive understanding of each of the body iron pools than that which is created with the current set of commonly used biomarkers. With the caveat that future research should account for the current limitations in iron status biomarkers, Section 6.3 will then examine how to address iron deficiency in active populations. This dissertation demonstrates the impact that regular aerobic activity can have on iron status. However, current recommendations for daily iron intake do not account for physical activity level. Section 6.4 will then broaden in scope to consider how to address the issue of iron deficiency in China, both in its current context where many women are predominantly sedentary[158] as well as if Chinese women should begin to be more active, as recommended by the new physical activity guidelines produced by the Chinese government[159]. Section 6.5 further expands this topic by examining the potential for using traditional Chinese medicine to address iron deficiency in China, a topic for which there is currently underwhelming scientific evidence. Finally, Sections 6.6 and 6.7 will present directions for future research and summarize the overall findings and concepts reported in this dissertation.

6.2 Biomarkers for the assessment of iron status

One of the consistent problems revealed by the results reported in Chapters 3 and 4 is the inability to decipher how the supplemental iron was being differentially utilized by the iron-trained group compared to the iron-untrained group.

This dissertation was able to examine how the combination of iron supplementation and aerobic training impacts the outcomes of iron status and physical performance, but only at a somewhat superficial level. Iron homeostasis is an intricate and complicated balance of many hormones, cytokines, proteins, and other factors. This dissertation used three of the most commonly measured iron status biomarkers (sFer, sTfR, and Hb) as well as two measures of inflammation to try and create a more comprehensive picture of what was happening at the metabolic level. However, as indicated in the previous chapters, it is clear that this set of biomarkers was insufficient for deducing the exact changes that occurred in this study across the various iron pools.

As mentioned in the discussions of Chapters 3 and 4, there are several additional biomarkers that could have provided a more thorough explanation for the results observed in this study. Of particular importance are those that represent erythropoietic activity such as erythropoietin and erythroferrone as well as hepcidin, which regulates iron on the systemic level via changes in absorption[29]. With these markers, we would have been better able to determine whether there was an exercise-induced up-regulation of erythropoiesis in the iron-trained group. Increases

in erythropoiesis and Hb production are known adaptations to aerobic training[137], though it is less clear how IDNA alters this response. Because there were still significant improvements in submaximal performance in the placebo-trained group, it is clear that IDNA women still benefit from aerobic training, despite their iron deficiency. Similarly, the iron-untrained women showed improvements in submaximal physical performance from iron supplementation without any training. As explained in Chapter 4, these improvements in performance from training and iron supplementation are likely due to somewhat different metabolic mechanisms and are not likely due to changes in oxygen delivery from changes in Hb concentration. However, without markers of erythropoiesis and systemic iron homeostasis, it is not possible to determine whether these mechanisms were primarily due to changes in erythropoiesis, bioenergetics, myoglobin, or some combination of these or other factors for each treatment group.

Another possible consequence of aerobic training that could have partially contributed to the observed changes in iron status indicators is exercise-induced inflammation that reduces iron absorption. As discussed in Chapter 3, the literature on whether long-term, moderate exercise results in a chronic inflammatory state shows conflicting results. In both acute and chronic inflammation, hepcidin levels in the body are increased, resulting in the internalization of ferroportin, and ultimately reducing overall iron levels in the body. While many studies have demonstrated a clear relationship between increased hepcidin expression and lower iron status after

acute bouts of exercise[120,160], few studies have shown a long-term impact of exercise-induced inflammation (increased hepcidin) on apparent iron status[122,160,161]. Dominguez et al found that while there was a direct relationship between intense aerobic exercise and hepcidin levels, this relationship was transient and would not support a chronic elevation of hepcidin from long-term exercise training. Additionally, in comparing hepcidin and sFer levels of athletes after a basketball training season, Zieman et al found that while there was increased hepcidin after the training season compared to before the competitive season, there was no relationship between hepcidin and sFer. Due to the conflicting evidence about the influence of long-term exercise on hepcidin-mediated changes in sFer, it remains unclear whether the lower sFer levels in the iron-trained group were due to decreased iron absorption resulting from increased hepcidin. Directly measuring hepcidin in future iron interventions with an exercise component could provide insight into this relationship.

Exercise-induced inflammation may also influence iron absorption beyond the role of hepcidin-mediated internalization of ferroportin. Several rodent studies have shown that inflammation induced by exercise (5-week treadmill training with progressive loading)[123] or by chemical injection[124,125] results in decreased expression of DMT1 and Cytochrome B, two proteins required for absorption of iron into the enterocyte. These mechanisms were found to be unrelated to the role of hepcidin in lowering iron export from the enterocyte, suggesting that exercise may impact iron absorption in more than one way. This dissertation was not able to measure

biomarkers of inflammation and iron absorption such as hepcidin, IL-6 (the primary mediator of inflammation mediated changes in hepcidin), DMT-1, and duodenal Cytochrome B. These markers could have provided clearer insight into whether the training regimen was inducing inflammation or increasing hepcidin levels either acutely or over the full 8-week study duration. Additionally, with these measures we could have examined the association between hepcidin levels and sFer concentration. While no inflammation was detected in any group of this study using CRP or AGP, it is possible that the training was inducing inflammation not captured by these two biomarkers.

Furthermore, all of the above arguments for using various biomarkers are conditional on the assumption that those biomarkers will accurately reflect total body iron, stored iron, and functional iron in the tissues. However, several of the most commonly measured iron status biomarkers can be affected by inflammation, misrepresenting the apparent iron status of the affected individual(s). If the trained subjects were experiencing an inflammatory response, it could result in misinterpreting the elevated sFer levels. The iron-trained group already displayed significantly smaller improvements in sFer than the iron-untrained group. If inflammation were elevating their sFer levels, this could mean that the severity of iron depletion remaining in the iron-trained group was greater than what was concluded in Chapter 3. This would indicate that regular aerobic physical activity actually has far larger negative implications for both iron status and its accurate assessment than

concluded by this study. However, without a more comprehensive set of inflammatory and iron status biomarkers, it is not possible to determine whether this type of exercise-induced inflammation occurred and the extent to which it may have influenced the results.

The inability of this study to distinguish the metabolic mechanisms explaining the changes observed in performance and iron status is not a flaw of its design, especially given the particular constraints presented by conducting research in the Chinese academic setting (restrictions on transporting blood, where blood draws could occur, etc.). Indeed, this set of biomarkers is recommended by the WHO and many iron supplementation studies measure only sFer, Hb, and sTfR[6]. One major reason for this potential "oversight" may be the prohibitory expense of measuring multiple biomarkers or the difficult logistics of measuring more than a few biomarkers in large, international studies. However, as technology progresses and biomarkers like erythroferrone, erythropoietin, and hepcidin can be easily assessed on automated systems rather than via individual ELISA kits, the frequency of their measurement may increase.

Furthermore, technologies like metabolomics may vastly improve our ability to understand what is happening on the metabolic level in a much more comprehensive way than previously possible. Untargeted metabolomics would provide insight not only about the iron and inflammation biomarkers mentioned above, but also on other markers of bioenergetics, inflammation, and general iron homeostasis that may not

normally be considered. Metabolomics has already been used to study both iron bioenergetics[162,163] and exercise physiology[164–166]; however, as of this writing, no study has yet been published that utilizes this tool to examine how changing iron status and fitness level mutually influence each other at the metabolic level.

Using this technology would allow researchers to examine in far greater detail the biological mechanisms underlying the changes in physical performance that occur from changes in iron status and aerobic training. Additionally, it could potentially even help inform the development of new biomarkers that may be more representative of processes of interest. For example, because they are not frequently reported in the literature that examines iron status in physically active women, it is not clear whether erythropoietin or erythroferrone would be more indicative of what is happening at the cellular and systemic levels in response to simultaneous iron supplementation and aerobic training. By examining each of the known biomarkers in context of the other factors involved in erythropoiesis, iron metabolism, energy production, and inflammation, it may be possible to create a recommendation for a standard set of biomarkers that better represents iron homeostasis in active populations.

6.3 Iron nutrition in physically active populations

Assuming that the biomarkers measured can accurately reflect iron status in an intervention, clinical setting, or population study, the problem of how to address iron deficiency in physically active populations still remains. Collectively, the results of

this dissertation support the idea that iron nutrition in physically active women differs from that of sedentary women. These differences should be accounted for when planning future iron interventions in populations that are, or plan to become, physically active. The findings in Chapter 3 support those of the previous literature, showing that regular aerobic training has an apparent detrimental effect on iron status. This dissertation further advanced the published literature in showing that providing a dose of iron that falls in the upper range of a conventional intervention dose did not improve conventional measures of iron stores as expected in trained women. This response is problematic for several reasons.

Assuming that the biomarkers measured accurately reflected iron status, the dose of iron provided, 42.2 mg of elemental iron per day, was not enough to fully resolve the iron-trained group's iron depletion as measured by sFer within an 8-week time frame. Conventional iron supplementation is often administered to iron deficient women in similar or smaller doses than those used here[3,78,109]. Even in the most severe cases of iron deficiency, where iron deficiency anemia prevalence is quite high (>40%), the World Health Organization recommends providing 60 mg elemental iron/day iron for a duration of 3 months[10]. This study gave 70% of that amount for an 8-week duration and was not able to fully resolve the iron depletion of the women who trained while taking iron supplements. Though it is difficult to directly compare the WHO recommendation to the dose used in this study due to the differences in duration and iron concentration as well as the severity of iron depletion or deficiency,

the results of this study may suggest that the WHO recommended iron supplementation regimen might not be sufficient to resolve iron depletion, iron deficiency, or iron deficiency anemia in active populations.

Further research should investigate whether active women who receive either the WHO recommendation of 60 mg/day (via various iron compounds including ferrous fumarate, ferrous sulfate, etc.) or the 200 mg ferrous sulfate (42.2 mg elemental iron) used in this study are able to fully resolve their iron depletion if supplemented for a longer duration. However, establishing an optimal dose of iron for iron-depleted, physically active women may be problematic because of the potential negative side effects of iron supplementation, including gastric distress. The WHO states that: “Adherence frequently diminishes due to intolerance when more than one iron tablet of 60 mg is required.” Interestingly, as defined by the U.S. Institute of Medicine, the current tolerable upper limit of iron intake for nominally health adults is 45 mg of elemental iron per day, based on avoiding the adverse effect of gastrointestinal distress[116]. However, this study provided 42.2 mg of elemental iron as ferrous sulfate per day to subjects and had only one participant, in the iron untrained group, report any gastrointestinal discomfort presumably attributable to supplementation (though another subject complained she was having GI distress as a result of wearing the Actigraph accelerometer belt). Based on this study, it may be possible that IDNA women, especially those who are physically active, can tolerate a higher dosage of iron than iron replete and/or sedentary women. Therefore, it is

important to determine a dosage regimen that provides enough elemental iron to resolve the physiological iron deficiency while still being tolerated by the individual.

Additionally, it could be beneficial for the WHO to consider revising its guidelines for practitioners to indicate that the current recommendation may not be adequate for iron depleted, active populations such as manual laborers, athletes, people who regularly perform vigorous physical activity for general fitness purposes, or sedentary people who begin a regular physical activity regimen.

This research may also have implications for iron replete individuals who are currently, or are planning to become, highly active. Previous literature shows that intense regular, physical activity significantly increases the risk of iron deficiency in women and that this detrimental effect of exercise may be prevented by supplemental iron intake[78,117,167,168]. However, the US Institute of Medicine's Estimated Average Requirement (EAR) for iron, or the average amount of daily iron consumption required to meet physiological demands, fails to account for an individual's physical activity level. The current recommendation only accounts for age, gender, and pregnancy or lactation status of the individual as well as an assumed bioavailability of iron of 18% in the typical omnivorous American diet[116]. Therefore, the current recommendation that menstruating women between the 19 and 50 years of age consume 8.1 mg of dietary iron per day (which equates to 1460 µg absorbed iron per day) may be insufficient for highly active populations. Because these women are at higher risk for iron deficiency due to their activity, they may

require a higher daily intake to maintain a healthy iron status as reflected by the conventional measures of sFer and sTfR. The EAR value is calculated using a factorial method encompassing basal iron loses, menstrual loses, and absorption. Both iron loses and absorption are potentially impacted by physical activity[8,123,169], suggesting that the iron requirement should be adjusted for women who are more physically active. Further research should investigate whether the average daily physiological iron requirement is higher in regularly active women.

Creating an iron intake recommendation for “active” women may be further complicated by the definition used for activity. At the time of this writing, there was very little published literature examining how the known effects of regular exercise on iron status are altered when exercise is more or less intense, or continued for a longer duration, than that reported in this dissertation or the published literature (5 days per week, 25-30 minutes per day, cycling at 75-85% of maximum age-predicted maximum heart rate)[3]. Therefore, it is currently unclear how physical activity impacts iron homeostasis when only performed a few days a week or at a low percentage of age-predicted maximum heart rate in untrained women. Additionally, it is unclear whether a similar response would be observed in highly trained women who underwent longer or more intense exercise sessions.

Similarly, there is very little published literature concerning iron status and the duration of exercise training, both in terms of the length of exercise performed each day as well as the duration of the training program undertaken. A study by Comassi

et al examined the effect of the duration of a single bout of intense exercise (Ironman or Half Ironman triathlons) in elite male athletes on inflammation and iron homeostasis[170]. The Comassi study found that the inflammatory response induced by the exercise was dependent on the duration of the activity. They also reported lower apparent iron status in those with increased duration of activity. This finding suggests that duration of single-bouts of exercise does play a role in iron homeostasis; however, the Comassi report does not indicate whether these effects are sustained with long-term training programs. Additionally, it only included elite male athletes, which make the results difficult to extrapolate to less fit individuals or to females.

Future research should investigate the impacts of exercise regimens of varying duration and intensity on several measures of iron status to more fully understand when and how iron homeostasis is impacted by physical activity. Additionally, these studies should be conducted in both untrained and trained populations to understand how initial fitness level impacts this relationship. However, as discussed in Section 6.2, markers of iron status may misrepresent iron homeostasis in active populations. Therefore, research investigating duration and intensity of exercise should also account for acute and chronic changes in inflammation, myoglobin, and erythropoiesis to create a more comprehensive understanding of how these factors affect iron homeostasis. This research should be conducted in populations of varying fitness levels. Many reported findings on iron status and exercise used elite athletes or military recruits[117,170,171], while there is very

little reported evidence for these relationships in recreationally active or sedentary individuals. Furthermore, there is little literature published that investigates this relationship in IDNA males. Except for menstruation, there is no physiological evidence that iron homeostasis in males differs from that of females. In order to create iron intake recommendations that account for physical activity and are applicable to the general population (male or female, US or Chinese), it must first be understood how intensity and duration of exercise, gender, and current fitness level influence the relationship between aerobic training and iron homeostasis.

6.4: TCM to Address Iron Deficiency in China

This dissertation focuses on the relationship between changing iron status, fitness level, and physical performance outcomes. The previous sections have highlighted the need to develop recommendations for iron supplementation regimens in active, iron deficient women as well as for preventing iron deficiency in healthy women who are highly active. However, it is also important to understand these relationships in the context of China and its cultural views on both exercise and Western medicine.

According to the 2009 China Health and Nutrition Survey, iron deficiency without anemia affects 28.4% of Chinese women between 18 and 50 years of age[172]. This number does not include the additional 10.7% of women in this age group who have iron deficiency anemia[173], bringing the collective number of Chinese women of

childbearing age affected by some level of iron deficiency to nearly 40%. This prevalence rate matches that found in both the study reported in this dissertation as well as an unpublished pilot study that we conducted in Shanghai in 2012, though the samples used in the Shanghai and dissertation studies were not necessarily representative of the greater Chinese population. According to the World Bank, in 2016 there were over 321 million women in this age range. Therefore, resolving iron deficiency in this population could potentially impact the health of 128 million Chinese women.

Improving iron status in China is not as simple as recommending that all affected women consume conventional “Western” iron supplements. The use of dietary supplements to deliver elemental iron or combined as multivitamins plus minerals is low in China[174]. Recent literature suggests that 9.2% of women use some form of commercially produced dietary supplement, but these are not specific to iron[175,176]. A recent study in Chinese children found that only 3.9% of children under 3 years of age were consuming commercial iron supplements, which suggests that iron supplementation use is particularly low in China.

While the use of commercial dietary supplements and multivitamins and minerals is low in China, the use of traditional Chinese herbal medicines is much higher[175]. These medicines should be prescribed by a Chinese medicine doctor, but many formulations are available over-the-counter. Both prescription and over-the-counter forms of Chinese medicine are commonly used by Chinese adults as they

are considered a more traditional and preferred form of treatment that should be used prior to using Western medicines[177]. It is this preference for using traditional herbal treatments over commercial iron supplementation that necessitates the study of the efficacy of these treatments in improving iron status using rigorous scientific methods. However, there is currently a paucity of sound scientific evidence on the effects of most traditional Chinese herbal medicines[83,178], making it difficult to create evidence-based recommendations on using them to treat conditions such as iron deficiency. To highlight this point, despite the frequency of its use in both China and the US, to our knowledge, the research reported in this dissertation is the first study in which *BaZhen KeLi* has been tested in humans for its impacts on either iron status or physical performance.

While this study does not support the use of BZ to treat iron deficiency or its consequences on physical performance, it is possible that other formulations of its components, such as the four-herb combination called *Si Wu Tang*, could still improve iron status[86,149]. It is also possible that other traditional herbal treatments may impact iron status but have not yet been tested. A 2009 analysis of Cochrane reviews concerning varying kinds of Traditional Chinese Medicinal therapies concluded that of the 70 Cochrane systematic reviews examined, 27 reviews suggested some kind of benefit from the treatments while another 41 reviews concluded there was not sufficient, high-quality evidence to determine the efficacy of the reviewed treatments[179]. This finding supports a recommendation that more

research is needed to evaluate the efficacy of using Chinese herbal medicines to treat IDNA and/or anemia in humans.

Additionally, there are several other methods that could be used alone or in combination with TCM to address iron deficiency in China. A 2008 review by the Chinese Center for Disease Control evaluated the cost-effectiveness of using supplements, dietary diversification, food fortification, and biofortification to resolve iron deficiency in China[81]. In 2003, China implemented fortification programs for soy sauce and wheat flour. These programs have been successful in the populations that have access to or can afford to regularly purchase and consume these products; however, the review concluded that no further fortification programs are needed. The publication also concluded that while all of the intervention approaches examined have some degree of effectiveness, the most feasible and promising methods at the national level are promoting the use of biofortification and dietary diversification. However, they recognized the cost limitations for suggesting dietary diversification for low-resource subgroups and therefore stressed the importance of biofortification. The 2009 review by Ma and colleagues, which as of this writing was the most recent English-language review addressing this issue in China, made no mention of using traditional Chinese medicine to address iron deficiency in the Chinese population. It is unclear whether this oversight is because there is currently insufficient scientific evidence to make recommendations or because it is not considered a realistic method for addressing this issue at the population level. If more evidence on the

efficacy of TCM treatments in improving iron status is reported in peer-reviewed scientific publications, evaluating the combination of using traditional Chinese medicines with methods such as dietary diversification or the cultivation and consumption of biofortified crops may become a feasible strategy for addressing iron deficiency in the Chinese population.

6.5: Physical activity, obesity, and iron status in Chinese women

The role of physical activity in the Chinese culture is somewhat different than it is in the United States. Voluntary, recreational physical activity is less common in China than it is in the U.S., particularly among women[175]. A 2014 review of physical activity in China found that “active leisure” activities do not contribute much to the physical activity of Chinese adults[158]. Rather, the majority of physical activity performed by Chinese adults was occupational in nature. Additionally, the amount of total daily recreational physical activity performed by the average Chinese woman has decreased somewhat over the last two decades[158]. This decline in physical activity, along with a shift from a traditional to “Western” style diet, is cited as one of the primary reasons underlying the increasing rates of obesity in the last three decades[180,181].

The prevalence of overweight and obesity (BMI > 25 kg/m^2) in China has increased at an alarming rate since the early 1990’s, affecting 29.2% of Chinese adults in 2009[181]. To prevent the chronic diseases that are associated with overweight and obesity, the Chinese Ministry of Health is developing a national

framework that includes a physical activity recommendation[180]. In 2009, the Chinese government created national fitness recommendations to help increase daily voluntary physical activity in China and in 2010 they released a set of physical activity guidelines for Chinese adults[180]. Understanding the implications that increasing general physical activity levels may have for iron status in China is somewhat difficult, as it is unclear how effective these new guidelines and programs will be and whether the levels of exertion achieved will be high enough to impact, or be impacted by, iron nutrition.

In 2011, the average Chinese female performed less than three MET-hours of physical activity per week[158]. MET-hours reflect the intensity of an exercise and its duration, so 3 MET hours per week is the equivalent of doing an activity that requires 3 times the amount of energy expended at rest for one hour. Examples of this intensity of activity include walking at 3 miles per hour or using a stationary bicycle with very light effort[135]. Accordingly, it may be that the average Chinese woman is not currently physically active enough to be at the higher risk of iron deficiency associated with regular aerobic activity, such as that which was reported in the trained women in this dissertation. However, this means that the current 40% prevalence of iron deficiency in Chinese women[173] occurred despite the population being largely sedentary. The trained women in this study were biking at a speed classified as vigorous physical activity (6.5 METs) by the 2011 Compendium of Physical Activities[135]. This equates to 13.5 MET-hours per week, or about 4.5 times

the amount of an average Chinese woman. Based on the results reported in Chapter 3, it is clear that this amount of aerobic training (25 minutes per day at 75-85% of maximum heart rate, for an average of 4 days per week) was sufficient to impact conventional indicators of iron status and stored liver iron. This amount of exercise would not be difficult to achieve over the course of a week. For example, a woman who walks for 1 hour at 3 miles per hour 5 days per week would achieve roughly the same number of MET-hours per week as the women in the trained group in this dissertation. Given the relationship between regular exercise and decreased apparent iron status as assessed by sFer, sTfR, and Hb[76,117], the prevalence of iron deficiency in China could potentially be exacerbated further if there is a significant increase in the frequency of leisure-time exercise in the female Chinese population because iron requirements would increase with increasing activity levels (though as mentioned in Section 6.3, it remains unclear how varying duration and intensity of exercise impact this relationship).

It is important that as the Chinese government and research institutions continue to promote physical activity as a means to control obesity and metabolic syndrome in the Chinese population, that they also carefully monitor women's iron status. Obesity often has comorbid iron deficiency and anemia, though paradoxically, about one-third of patients with metabolic syndrome have elevated ferritin levels[182]. While the relationship between obesity, inflammation, hepcidin, and iron homeostasis is still being studied, it is becoming clearer that obesity and metabolic syndrome can

negatively impact iron status through inflammation and women with these conditions should be monitored closely to prevent iron deficiency[183]. Further complicating this relationship is that women who are iron deficient or anemic are known to be less active in free-living physical activity[44]. Therefore, it is unclear how widely the physical activity recommendations will be adopted in populations where iron deficiency and anemia are more widely prevalent.

Based on the collective findings in this dissertation, several general recommendations can be made for improving and monitoring iron status in China. Chinese health care practitioners should be trained to order tests for sFer and, if monetarily feasible as technology progresses, sTfR in addition to Hb to identify iron deficiency in its early stages before it progresses to anemia. Additionally, medical practitioners and health educators should encourage the use of proven efficacious interventions and treatment regimens that use commercial iron supplements, dietary diversity, or iron-fortified food products to improve iron status or prevent decrements in iron status as women increase their daily physical activity levels. Traditional herbal medicine practitioners should be advised that the formulation of BZKL used in this dissertation is not known to be effective for treating iron deficiency and should be avoided until more conclusive evidence is available for its efficacy in improving iron status or physical performance. Finally, as public health researchers develop interventions aiming to reduce obesity and metabolic syndrome, they should consider the implications that changing physical activity patterns may have on the iron status

of their target populations, as well as how iron deficiency may limit women from adopting physical performance recommendations, and account for this effect in their study designs. Similarly, when designing interventions aiming to improve iron status in Chinese women, researchers should be aware of how active their participants are, as they may need to adjust their intervention dose as discussed in Section 6.3.

6.6: Directions for future research

This dissertation has helped clarify several aspects of how improved iron status and aerobic training interact to affect physical performance and measures of iron status in Chinese women. It has also raised several new questions that can help guide the future of this field of study.

First, this study was conducted in a group of IDNA, but otherwise healthy, sedentary females, which allowed for the examination of the research questions proposed in Aims 1 through 3 of this dissertation. However, the results here may be limited in their generalizability to other populations. Future research could examine a similar research question in other populations to understand how factors such as anemia, level of fitness or regular physical activity at baseline, as well as baseline weight/body composition or gender alter the results reported in this dissertation. Examining the relationship between aerobic training and changing iron status would be particularly interesting, as the added physiological stressor of anemia would likely influence the metabolism of supplemental iron. Understanding how iron is differentially distributed in the body to support various metabolic adaptations in iron

deficient anemic individuals undergoing aerobic training, compared to IDNA individuals undergoing the same training would help clarify the basic mechanism of iron metabolism across varying levels of iron status.

Future research should also address the question of how iron deficiency impacts the typical adaptations to aerobic training using methodologies that allow for a more detailed examination of energy metabolism and muscle physiology. Of foremost interest is elucidating the metabolic changes underlying the observed changes in iron status markers and physical performance. As discussed in sections 6.2 and 6.3, there are several biomarkers that, if measured, could help understand these changes. Future research should be conducted that reproduces the study reported here with several added measurements. Specifically, future studies should measure a larger number of iron biomarkers including erythropoietin and erythroferrone to understand how erythropoiesis is influenced and hepcidin and IL-6 to understand how general iron homeostasis and inflammation are altered. Additionally, if possible, studies should also try and assess DMT1 and Cytochrome B, perhaps via mRNA expression, to clarify whether exercise-induced inflammation is altering iron absorption in the trained women.

Furthermore, future research should examine how aerobic training in the iron deficient state impacts muscle metabolism and growth. To address this question, it would be particularly promising to measure muscle metabolites and myoglobin during exercise before and after the 8-week treatment period used in this dissertation. This

could be achieved by taking muscle biopsies from the quadriceps muscle (a muscle that is trained heavily in cycling) of the women before and after the 8-week study period both at rest and after an exercise test that would achieve both an aerobic steady state (to observe endurance capacity) as well as maximal exertion (to allow for examination of both VO_2max and VO_2peak). Muscle biopsies would also allow us to examine changes in muscle fiber type and intramuscular fuel stores, which would help clarify how iron deficiency impacts the typical adaptations to aerobic training. Combining the expanded biomarker collection and muscle biopsies with untargeted metabolomics would also further improve the ability of such a study to make comprehensive conclusions about the changes in iron homeostasis, muscle metabolism before and after exercise, and how these factors interact in response to the treatments used in the current research.

Additionally, it is likely that other biomarkers may be more reflective of general iron homeostasis than only examining sFer, Hb, and sTfR. As mentioned previously, the use of global metabolomics may help inform the design of a better panel of iron biomarkers or even potentially new metabolites altogether for use in future iron studies.

6.7: Conclusions

To summarize, this 3x2 factorial intervention study helped elucidate the relationship between simultaneous improvements in iron status and fitness level and how they impact physical performance in Chinese university women. Chapter 3

demonstrated that iron deficient, non-anemic women who undergo regular aerobic training while taking iron supplements do not improve their estimated iron stores as much as women who remain sedentary during supplementation. However, while it is clear that iron stores in trained women do not increase as expected, it is unclear how the supplemental iron is being used by these women (for example, whether it is being shuttled to myoglobin, erythropoiesis, or other unmeasured pathways or storage sites) and whether any exercise-induced inflammation is occurring that alters iron absorption.

Chapter 4 explored the influence that supplemental iron has on physical performance and/or the physiological adaptations to aerobic training. Iron supplementation improved endurance performance at both maximal and submaximal exercise intensities in iron-deficient, non-anemic women. The results suggested that iron supplementation alone increased submaximal performance to the same extent as training without supplementation. Interestingly, this study also found that taking supplemental iron during aerobic training had no additive benefit on physical performance compared to either treatment alone. This finding contradicts previous studies, which showed an ergogenic effect of supplemental iron above that of training alone[3].

Furthermore, though not discussed in detail in Ch. 4, this is the first study to separately examine VO_2max and VO_2peak as markers of different physiological states – maximal capacity for oxygen consumption and endurance at near-maximal

intensities, respectively. The results of this dissertation agree with previous studies that suggest that iron deficiency without anemia should not impact VO_2max[5], while also expanding upon the findings of the Pasricha et al review[134] that concluded that iron supplementation does improve VO_2max, which was measured as a combined variable of VO_2max and VO_2peak together.

Finally, Chapter 5 examined the efficacy of using a traditional Chinese herbal medicine, *BaZhen KeLi*, on improving iron status and physical performance in iron-deficient, non-anemic women. To our knowledge, this was the first time that the efficacy of this treatment has been tested in humans, despite its frequent use in both China and the United States. This study found that *BaZhen KeLi* had no impact whatsoever on any measure of iron status measured and does not improve maximal or submaximal physical performance. Additionally, this treatment did not have any ergogenic effect when given during aerobic training. Based on these findings, Traditional Chinese Medicine practitioners should be advised not to prescribe this treatment for symptoms of iron deficiency or iron deficiency anemia, especially in lieu of conventional iron supplements where feasible. Additionally, healthcare practitioners in both China and the US should be aware of iron deficient and anemic patients' self-care practices and caution against using this treatment as a home remedy to improve iron status in lieu of standard iron supplementation. Based on the literature, it is possible that one or more of the components in *BaZhen KeLi* may affect iron status or physical performance, but further research is needed to clarify

whether different formulations or dosages of BZKL, or even single active ingredients, are effective in increasing iron status or physical performance in women with varying degrees of iron deficiency or IDA.

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Appendix A: Data Collection Forms

A1: Screening Documents (Chinese)
A1.1: Screening informed consent

知情同意书 1——受试对象筛选过程

我们邀请您参与一项科学研究的筛选过程。表格的设计是为了给您提这项研究有关信息。我们将为您描述这项研究并对您的任何疑问做出解答。

项目名称：铁在有氧训练和机体性能之间关系的功能和代谢作用。本研究的负责人是美国康奈尔大学的 Laura Pompano，她的导师是康纳尔大学的 Jere Haas 博士和昆明医科大学的蔡乐博士。

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研究目的：
本次调查的目的是决定您是否有资格参与该项研究。这是一项为期 8 周的研究，检查铁缺乏如何影响其他因素以及如何被运动影响。本次研究有两组受试者：铁缺乏非贫血妇女和健康妇女。我们将基于筛选试验时您体内铁的含量将您分到相应的小组。这

次研究的主要目的是阐明机体铁缺乏状态，有氧运动和机体性能之间的关系。

研究方法：
我们希望您能认真审阅参与本次研究所需的一系列标准。如果您符合这些标准，我们希望您能允许护士使用无菌针管在手臂血管处抽取 4ml 的血液。这些血液将会用于实验室内测量您体内的铁含量。我们还需要通过一个简单的健康问卷和测量您的身高体重，以便了解您的健康状况。抽血时间不超过 15 分钟。

风险和不适：
从手臂处抽血的并发症包括轻微出血,擦伤或感染。为防止意外,将由一名有经验的护士进行抽血。如果您确实在抽血后出现出血,擦伤或感染等情况,医生/护士将免费为您进行治疗。

好处/利益：
参加本次筛选试验的一个优点是，在筛选试验完成后您将了解自己体内铁状态。

参与成本：
参加本次筛选试验完全属于自愿、免费的。

参与回报：
为感谢您的参与，完成筛查的受试者将收到我们提供的小礼物一份。

血液样本的处理：
在血红蛋白和血清铁蛋白筛选完成后，您的血液样本将会进行卫生处理，不会有样本会保留用于其它血液分析。

测试结果异常：
如果我们在抽血或后续分析中得到任何异常结果，我们都会在 10 天内通知您这些结果并建议您联系您的私人医生进行随访。请注意，作为研究者，我们并未经过进行诊断或治疗的训练。

如果您在本研究中受伤
在面对任何研究相关活动所导致的伤害时，我们将提供包括急救、应急处理、所需随访护理等治疗措施。这些治疗费用将通过普通方式由您或您的保险公司支付。如果您认为您受到了研究相关的损伤，请联系芮溧老师，研究顾问电话为 87165922921。

隐私和保密
所获得的数据将被安全储存并以匿名形式（没有表示能将您和您的信息联系在一起）报道。只有负责调查或伦理委员会的成员才可以访问严格保密的原始数据。如果您符

合资格，我们将邀请您参加本次研究。

自愿参与原则

作为参与者，你有权在任何时候推出筛选试验，并且不需要说明原因或承担负面后果。退出时无需退回之前所获得的补偿，也对您和这次试验所在学校间的关系无任何影响。

研究者、医生或赞助的退出

研究者、医生或赞助商在任何时候都可能停止研究或将您从研究中剔除。他们应该是从您的最佳利益判断并做出决定。如果您在相关研究中受伤，如果您需要额外的或不同的药物/治疗，或者如果您不遵守研究计划。他们可以因为各种行政或医疗原因，未经您同意而将您从研究中剔除。

若在筛选过程中发现年龄在 18 周岁以下或者有严重贫血（血红蛋白<90g/L）的女性将不能参与本次研究。所有的潜在参与者将由昆明医科大学公共卫生学院的芮溧博士跟进。

Subject Screening ID #: _____

如果您有以下情况时，请务必说明：
1、能阻止您在固定自行车上锻炼的身心障碍
2、去年一年内处于怀孕或哺乳期
3、吸烟
4、服用精神药物
5、最近又发烧、肺部或胃肠道疾病
6、已知有炎症或慢性炎症疾病
7、溶血性贫血
8、肌肉、骨骼问题
9、目前或过去饮食失调

（在上述的条件里，如果你能满足其中一项，请选择有） 有___ 没有____

问题：
1、近期，你有服用任何药物吗？ 是___ 不是____
如果是，请说明：

2、近期，你有服用维生素，矿物质，或营养补充剂吗？ 是___ 不是____
如果是，请说明：

3. 如果你被录取，你愿意遵守全部 8 周口服维生素或者中药吗？ 是___ 不是____

4. 如果你被录取，你愿意遵守全部 8 周的体能训练计划吗？ 是___ 不是____

5. 如果参与研究，你愿意遵守全部 8 周不放假吗?（包括国庆节） 是___ 不是____

如果您有任何的疑问

进行此项主要人员为美国康奈尔大学的研究生劳拉。如果您有任何疑问，请在现场进行咨询。如果你以后有问题，你可以联系公共卫生学院讲师芮溧，电话13888531601。如果您有任何问题或关心关于作为研究的受试者您的权利，你可以联系昆明医科大学或康奈尔大学或访问他们的网站的机构审查委员会（IRB）来了解参与人权利。

同意声明

我已阅读以上材料，并以了解相关信息。我同意参加本次筛选研究这一阶段。

姓名：_____ 日期：_____

本研究的负责人：_____ 日期：_____

本知情同意书将被研究保留至研究结束后至少 5 年。

A1.2 Screening Physical Activity Questionnaire

国际身体活动问卷 **Screening ID #:_____**

我们对找寻人们在他们日常生活中多种的身体活动有兴趣，这个问卷会问你在最近 7 天花在身体活动的时间，请回答每一个问题，甚至如果你想自己是一个没有活动的人，请想一想你在工作时的活动、像是你在家里或园艺的部份、从一个地方到一个地方及在你空闲的时间运动或娱乐。

1. 想一想在最近 7 天里你做过所有**强而有力**的活动，**强而有力**的身体活动是指以费力的身体负荷且让你呼吸较正常更为急促的活动，仅回想你所做过每次至少 10 分钟的那些身体活动。

   最近 **7 天**里，你花多少天做强而有力的身体活动, 像是提重物, 苦力, 有氧运动或快骑脚踏车？

   a. _____ 每周**几天**
   b. _____ 没有强而有力的身体活动 → 跳到问题 2

   在参与强有力身体活动的那些天，你通常花多少时间做强而有力的身体活动？

   a. _____ 每天**几分钟**
   b. _____ 不知道/不确定

   你是否经常参与类似于足球比赛一类的剧烈运动?

   c. _____ 参加
   d. _____ 不参加

2. 想一想最近 7 天你做过所有**适度**的活动，**适度**的活动是指以适度的身体负荷并且让你呼吸比正常费力一些的活动。

   最近 7 天里，你花多少时间做适度的身体活动，像是提轻的物品、正常的速度骑脚踏车或网球双打？不包含走路。

   a. _____ 每周**几天**
   b. _____ 没有适度的身体活动 → 跳到问题 3

   在参与适度身体活动的那些天，通常你花多少时间做适度的身体活动？

   a. _____ 每天**几分钟**
   b. _____ 不知道/不确定

3. 想一想最近 7 天你花多少时间在**走路**，包含工作、在家、从某地到某地、娱乐、游戏或休闲时的走路。 最近 7 天里，你花多少天走每次至少 10 分钟的路？

   a. _____ 每周**几天**
   b. 沒有走路 → 跳到问题 4

   在走路的那些天，你通常花多少时间在走路？

   a. _____ 每天**几分钟**

b. _____ 不知道/不确定

4. 最後的问题是在最近连续 7 个非假日时间（扣除周六与周日）你花多少时间在坐着，包含花在工作、家里、做作业及休闲时的坐着，这或许可以包含花在书桌、拜访朋友、读书或看电视的躺着或坐着。
   a. _____ 每天**几分钟**
   b. _____ 不知道/不确定

問卷最後感謝你的參與!

A1.3 Screening Health History Questionnaire

健康历史调查:

请您如实回答下面的问题，您的所有信息将会保密。

1. 姓名: _____
2. 年龄: _____
3. 专业: _____
4. 民族：_____
5. 最后的月经期：_____
6. 你每个月抽几支烟？ □ 0 □ 1-5 □ 6-10 □ 10+
7. 您是否有如下一种或多种情况： □ 是 □ 不是
   a. 近期得感染性疾病
   b. 近期发热
   c. 炎症或慢性炎性疾病
   d. 溶血性贫血
   e. 慢性呼吸系统疾病
   f. 肌肉骨骼系统问题
   g. 进食障碍的历史
8. 您是否有别的问题（例如：发烧、头疼、胃部不适、疲劳等等）？如果有，请具体说明：
9. 您是否:
   a. 怀孕 □ 是 □ 不是
   b. 处于哺乳期 □ 是 □ 不是
   c. 服用精神类药物 □ 是 □ 不是
   d. 服用维生素 □ 是 □ 不是
   e. 如果服用精神类药物或者维生素，请具体说明：
10. 您是否会骑自行车? □ 是 □ 否
11. 您现在能否骑自行车？（是否有身体不适） □ 能 □ 不能

这个页是研究者完成的。 你不要填空。

Screening Data:

1. Screening ID:
2. Height: _____ cm
3. Weight: _____ kg
4. Hb: _____ g/dL
5. Body fat:

Site	1st Measurement (mm)	2nd Measurement (mm)	Average (mm)
Bicep			
Tricep			
Subscapular			
Suprailiac			
MUAC			

6. Gift Received?
7. Subject signature of gift received: _____

Lab Test Results:

1. Hb: _____
2. sFer: _____

A2: Screening Documents (English)
A2.1 Screening informed consent
Screening Session (FORM 1A)

We are asking you to participate in a screening process for a research study. This form is designed to give you information about this study. We will describe this study to you and answer any of your questions.

Project Title: Defining the Functional and Metabolic Role of Iron in the Relationship Between Aerobic Training and Physical Performance

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Tel: 86-871-5922921 (office)

What the study is about
The purpose of this research is to determine if you qualify to be included in this study. The study will be an 8-week study examining how iron deficiency affects and is affected by exercise. The study will have two groups of participants: iron deficient non-anemic and healthy women. You will be assigned to a group based on your body iron status at screening. The primary aim of the study is to clarify the relationship between body iron status, aerobic exercise training, and physical performance

What we will ask you to do
We will ask you to review a list of criteria for participation. If you meet these criteria, we will ask you to allow a nurse to draw 4mL of blood using a sterile needle from a blood vessel in your arm. The blood will be taken to the laboratory and it will be used to measure the amount of iron in your body. We will also ask simple questions about your general health via a general health questionnaire and record your weight and height. The blood draw should take no more than 15 minutes.

Risks and discomforts
The disadvantages/ risks of having blood drawn from the arm are slight bleeding, bruising or infection. To prevent these risks, blood will be drawn by an experienced nurse. If you do have any bleeding, bruising or infection after the blood draw, you will be cared for by the nurse and/or doctor at no cost to you.

Benefits
An advantage of participating in the screening session is that you will learn your iron status after the screening exercise has been completed.

Cost of participating
Your participation in the screening session is entirely voluntary and free of charge.

Payment for participation
You will receive a small gift for your participation.

Use of Tissue Samples/DNA for Future Studies
After screening for hemoglobin and serum ferritin, your blood sample will be disposed of in a sanitary way. No samples will be retained for future analysis.

Abnormal Test Results
In the event that we get back any abnormal results from the blood draw or subsequent analyses, we will inform you about these results within 10 days and recommend you contact your private medical provider for follow-up. Please be advised that as researchers, we not trained to diagnose or treat medical conditions.

If you are injured by this research
In the event that any research-related activities result in an injury, treatment will be made available including first aid, emergency treatment, and follow-up care as needed. Cost for such care will be billed in the ordinary manner to you or your insurance company. No reimbursement, compensation, or free medical care is offered by Cornell University. If you think that you have suffered a research-related injury, contact Dr. Le Cai, the research advisor immediately at 87165922911.

Privacy/Confidentiality
The obtained data will be stored safely and reported in an anonymous form with no identifiers that can associate you with your data. Only the responsible investigators and/or the members of the ethical committee have access to the original data under strict confidentiality. If you qualify, you will be invited to join the study.

Taking part is voluntary
As a participant, you have the right to withdraw from the screening session at any time without needing to specify any reasons or facing negative consequences. Withdrawing will have no effect on the compensation earned before withdrawing or on your relationship with the universities involved with the research.

Withdrawal by investigator, physician, or sponsor
The investigators, physicians or sponsors may stop the study or take you out of the study at any time should they judge that it is in your best interest to do so, if you experience a study-related injury, if you need additional or different medication/treatment, or if you do not comply with the study plan. They may remove you from the study for various other administrative and medical reasons. They can do this without your consent.

Women under the age of 18 or found to have severe anemia (Hb<90g/L) in the screening process will not be invited to participate in the study. All potential participants will be examined by a project physician, Li Rui, M.D. from the public health department at KMU.

Please indicate if you have any of the following conditions:

1. physical/mental disability that prevents you from exercising on a stationary bicycle
2. pregnancy or lactating within the last year
3. smoke cigarettes
4. taking psychoactive drugs
5. current/recent fever, respiratory, or gastrointestinal illness
6. known inflammatory or chronic inflammatory disease
7. hemolytic anemia
8. musculoskeletal problems
9. current or past eating disorder
10. other preexisting medical condition

Yes _____ No _____
(If you have one or more of the 10 conditions listed above, please selected YES)

Are you taking any kind of medication? Yes___ No____
If Yes, please explain:

Are you taking any kind of vitamin, mineral, or nutritional supplement? Yes___ No____
If Yes, please explain:

You must complete a physical activity questionnaire that indicates you currently do not participate in regular exercise or organized sports, are interested in increasing their level of physical fitness, and willing to comply with the full 8-week training program.

If you have questions

The main researcher conducting this study is Laura Pompano, a graduate student at Cornell University in the U.S.A. Please ask any questions you have now. If you have questions later, you may contact Laura Pompano at lmp262@cornell.edu or (I will insert my Chinese phone number here once I acquire it in China). If you have any questions or concerns regarding your rights as a subject in this study, you may contact the Institutional Review Board (IRB) for Human Participants at KMU or at Cornell or access their website.

KMU:
*I will insert this information as soon as Dr. Cai provides it to me. *

Cornell:
+1-607-255-5138
http://www.irb.cornell.edu.

You may also report your concerns or complaints anonymously through Ethicspoint online at www.hotline.cornell.edu or by calling toll free at +1-866-293-3077. Ethicspoint is an independent organization that serves as a liaison between the University and the person bringing the complaint so that anonymity can be ensured.

You will be given a copy of this form to keep for your records.

Statement of Consent
I have read the above information, and have received answers to any questions I asked. I consent to take part in this phase of the study screening.

Your Signature_____ Date_____

Your Name (printed)_____

Signature of person obtaining consent_____ Date_____

Printed name of person obtaining consent_____

This consent form will be kept by the researcher for at least five years beyond the end of the study.

A2.2 Screening Physical Activity Questionnaire

INTERNATIONAL PHYSICAL ACTIVITY QUESTIONNAIRE

We are interested in finding out about the kinds of physical activities that people do as part of their everyday lives. The questions will ask you about the time you spent being physically active in the **last 7 days**. Please answer each question even if you do not consider yourself to be an active person. Please think about the activities you do at work or school, as part of your house and yard work, to get from place to place, and in your spare time for recreation, exercise or sport.

1. Think about all the vigorous activities that you did in the last 7 days. Vigorous physical activities refer to activities that take hard physical effort and make you breathe much harder than normal. Think only about those physical activities that you did for at least 10 minutes at a time.

   During the **last 7 days**, on how many days did you do **vigorous** physical activities like heavy lifting, running, aerobics, or fast bicycling?
   a. _____ days
   b. No vigorous physical activities → ***Skip to question 2***

   How much time did you usually spend doing vigorous physical activities in a typical day?
   a. _____ Minutes per day
   b. _____ Don't know/Not sure

   Do you participate in organized sports or regular vigorous physical activity? (Ex: running program, organized sports leagues, etc)
   a. _____ Yes
   b. _____ No

2. Think about all the **moderate** activities that you did in the **last 7 days**. **Moderate** activities refer to activities that take moderate physical effort and make you breathe somewhat harder than normal. Think only about those physical activities that you did for at least 10 minutes at a time.

   During the **last 7 days**, on how many days did you do **moderate** physical activities like carrying light loads, bicycling at a regular pace, or badmitton? Do not include walking
   a. _____ days
   b. No moderate physical activities →***Skip to question 3***

   How much time did you usually spend doing moderate physical in a typical day?
   a. _____ Minutes per day
   b. _____ Don't know/Not sure

3. Think about the time you spent **walking** in the **last 7 days**. This includes at work and at home, walking to travel from place to place, and any other walking that you might do solely

for recreation, sport, exercise, or leisure. During the **last 7 days**, on how many days did you **walk** for at least 10 minutes at a time?

a. **_____** days
b. No walking → ***Skip to question 4***

How much time did you usually spend **walking** in a typical day?

a. **_____ minutes per day**
b. _____ Don't know/Not sure

4. The last question is about the time you spent **sitting** on weekdays during the **last 7 days**. Include time spent at work, at home, while doing course work and during leisure time. This may include time spent sitting at a desk, visiting friends, reading, or sitting or lying down to watch television.
   During the last 7 days, how much time did you spend sitting on a week day?
   a. **_____ minutes per day**
   b. _____ Don't know/Not sure

This is the end of the questionnaire, thank you for participating.

A2.3 Screening Health History Questionnaire

Health History Form:

Please answer this form honestly and to the best of your ability. This information will be kept private and will only be used for this study by the researchers.

1. Name:

2. Age:

3. Major:

4. Chinese ethnicity/minority group: _____

5. Height _____ m

6. Weight _____ kg

7. Date of Last Period _____

8. How many cigarettes do you smoke per month?
   □ 0 □ 1-5 □ 6-10 □ 10+

9. Do you have **one or more** of the following health conditions: □ Yes □ No
   a. recent infectious illness
   b. recent fever
   c. current inflammation or chronic inflammatory disease
   d. hemolytic anemia
   e. chronic respiratory disease
   f. musculoskeletal problems
   g. history of eating disorder

10. Do you have any other present health complaints or problems (ex: fever, headache, stomach problems, tired, etc.)? If yes, please describe.

11. Are you:
    a. Pregnant □ Yes □ No
    b. Lactating □ Yes □ No
    c. Taking any psychoactive medications? □ Yes □ No
    d. Taking any vitamins? □ Yes □ No
    e. If yes to C or D: Please list which medications / vitamins you are taking:

12. Do you know how to ride a bicycle? □ Yes □ No

Are you currently physically able to ride a bicycle? □ Yes □ No

A3: Main Study Informed Consent (English and Chinese)
A3.1 Chinese

知情同意书 2——正式研究

我们邀请您参与一项科学研究的筛选过程。以下是这项研究的有关信息，如果有任何疑问，我们将为您做出解答。

项目名称：铁在有氧训练和机体性能之间关系的功能和代谢作用。本研究的负责人是美国康奈尔大学的博士研究生 Laura Pompano，她的导师是康纳尔大学的 Jere Haas 博士和昆明医科大学的蔡乐博士。

研究目的：这是一项为期 8 周的研究，检查铁缺乏如何影响其他因素以及如何被运动因素影响。本次研究有两组受试者：铁缺乏非贫血妇女和健康妇女。我们将根据筛选试验中您体内铁的含量值将您分到相应的小组。本研究旨在阐明机体铁缺乏状态，有氧运动和机体功能之间的关系。

研究步骤

第 1 部分：补充剂：

如果您愿意参加该研究，您将被随机分配到三个组（硫酸亚铁补充剂组、八珍颗粒补充剂组、安慰剂组）中的其中一组，请您连续 8 周口服硫酸亚铁补充剂（每天两次，每次 50mg），或八珍颗粒补充剂（每天两次每次，600mg），或相似的安慰剂（每日 2 次）。为了保证研究的完整性，我们要求您不能与你朋友或同学交换补充剂。我们要求您一天服药两次，并每周向我们提供您的用药频率记录。为减少胃肠道不适的风险，我们建议您在用餐时服用补充剂。为了提高营养物质的吸收，我们建议你服用补充剂的同时服用富含维生素 C 的食物，例如柚子、橙子或荔枝等。

第 2 部分：运动试验

本次研究将在第 0 周、4 周和 8 周各进行一次运动试验，即整个研究过程共进行 3 次运动试验，该运动试验用来评估你的身体状况。

每次试验包括两项运动测试。第一项测试是用来测量在运动过程中最大吸收和利用氧的能力。在测试过程中，我们将采用无菌一次性采血针对手指进行针刺，并收集一滴血以便观察血乳酸浓度。该检查在第一项测试中将进行多次。第二项测试的目的是检测机体效率。该测试将根据第一项测试结果确定在三个工作负荷下进行。在第二和第三的负荷后，会抽取 4ml 的臂肘静脉血。

每次进行的两项运动测试要间隔两天，每项测试要求在固定脚踏车上踩踏约 15-30 分钟，测试期间要求戴面罩用于呼吸和心率的监测。

第 3 部分：抽血

所有的静脉抽血将由有资格的执业医师执行。抽血将在研究中以下时间点进行。

a. 在第 0 周，4 周和 8 周：在非运动、静息状态时收集 4ml 臂肘前静脉血。一部分血用于评估血红蛋白。将剩余的血液向下旋转分离出血清，并收集，冷冻，存储用于以后的分析。

b.在第 0 周，4 周，和 8 周：如本文第 2 部分描述的那样，将于第二个运动试验各阶段后抽取 4ml 血液。将血液旋转到血清分离，并收集，冷冻，存储用于以后的分析。

c.在第 0 周，4 周和 8 周：在第一个运动试验的每次负荷结束后对手指戳刺。需要对每个手指进行采血。取决于测试期间你的实际情况，将收集 2-5 滴血。对这部分不进行存储。

第 4 部分：体力活动评估

在研究开始前，我们会要求你完成一个体力活动调查问卷，以便总结您的体力活动模式。在第 0 周，4 周和 8 周时，要求在腰带上佩戴小型加速度计装置以便测量每天的体力活动。应佩戴连续 5 天（一般选择某一周的星期三至星期天），并且每天都要求全天佩戴。你可以将其戴在上衣或裤子上，这取决于你的个人喜好。当您睡觉、游泳、洗澡或做任何需要在水中进行的活动时，应脱去装置。在下雨时您可以佩戴装置，但在这种情况下，我们要求装置放在衣服里面。

第 5 部分：膳食评价

在研究过程中的第 0 周，4 周和 8 周，需要完成一个为期 4 天的膳食评价。这种评估需要记录连续 4 天的饮食情况。要求这期间保持正常的饮食习惯以便提供准确的能够代表平时的饮食情况的信息。

第 6 部分：有氧锻炼

随机选取一些参与者进行有氧运动训练。如果选择了这个训练，要求在佩戴心率监视器的情况下进行骑自行车的训练，总共训练 8 周，每周 5 天，每天 30 分钟。训练将在昆明医科大学护理学院进行。工作人员将监视训练进程。训练将由负荷为 75%的最大心率开始（大约每分钟 150 次），并在 8 周的训练时间内进展到 85%的最大心率（约每分钟 170 次）。

第 7 部分：允许储存的血液样本用于未来的分析

采集的血液样本（如第三部分描述）将用于分析铁含量、炎症和代谢物的浓度。研究开始后，对您采集的所有标本及其他登记表，都使用代码，避免您的相关信息被泄露。

小结（第 1-6 部分）：

持续 8 周给予补充剂。在 3 个实验时间点（0 周，4 周和 8 周），评价运动试验 2 天/每个运动测试项目，共计 6 个运动试验，每个持续 15-30 分钟。第一个运动试验通过戳手指测量血乳酸浓度。第二运动试验期间，分三次采集静脉血（休息时，负荷 1 后，测试结束后），并存储用于以后的营养分析。体力活动评估将需要在三个不同的

场合连续 5 天佩戴加速度装置器加速度计装置，总共有 15 天需佩戴装置。最后，如果参加运动锻炼，锻炼时间为每周 5 天每天 30 分钟并持续 8 周。

风险和不适

第 1 部分：有些人可能在服用补充剂后出现胃肠不适（腹痛，腹泻，便秘）的情况。为了尽量减少这种风险，建议你服用补充剂的同时服用维生素 C 片，以便助于提高补充剂的吸收。

第 2 部分：有些人可能会在运动测试后感到疲倦、疼痛甚至晕厥。测试期间，将监测心率和身体状况，以便将昏厥发生的风险最小化。如果研究人员认为你处于昏迷高风险期，我们将停止测试并采取适当的预防措施来降低这种风险。试验完成后将提供水和零食的，以促进全面恢复。

第 3 部分：从胳膊处抽血的缺点/风险，如轻微出血、擦伤或感染。为了避免这些风险，由执业医师抽血。**如果您确实在抽血后出现出血，擦伤或感染等情况，医生/护士将免费为您进行治疗。**

第 4-5 部分：没有已知的与佩戴加速度计装置或完成相关的膳食评价有关的风险。

第 6 部分：由于运动试验的风险与日常训练的风险相同，故请参阅第 2 部分风险。

第 7 部分：没有已知的风险是与血液或血清存储相关的。您的个人信息将不会被储存。样品将被保存只有一个 ID 代码，并不能追溯到您。只有主要研究人员有机会接触这些样本。

优点

第 1 部分：如果选择接受补铁剂或八珍颗粒，在 8 周研究期间您的体内贮存铁量可能得到改善。

第 2 部分：在运动试验完成后了解您的身体状态。

第 3 部分：在完成血液分析后能了解您的铁含量和炎症状态。

第 4 部分：在日常的基础上了解如何有效的锻炼。

第 5 部分：没有已知的对参与者的利益。

第 6 部分：如果选择接受训练，你的身体水平可能在 8 个星期的训练期得到提升。

第 7 部分：没有已知的对参与者的利益。

参与回报

在您完成研究要求的所有内容（包括 8 周的补充，6 次运动试验，3 次体育活动评估以及如果分配到训练组 3 次评估饮食和有氧运动训练），为感谢您的参与将送上一份价值 300 人民币的礼物。在研究最后，如果您的血铁水平仍然未达到正常水平，我们可以为您提供一份研究过程中关于您各种检查结果的报告，以利于您的下一步治疗，但我们不能再为您提供任何的补充剂。

如果您在本研究中受伤

在面对任何研究相关活动所导致的伤害时，我们将提供包括急救、应急处理、所需随访护理等治疗措施。这些治疗费用将通过普通方式由您或您的保险公司支付。如果您认为您受到了研究相关的损伤，请联系芮溧老师，研究顾问电话为 0871-65922921。

隐私和保密

所获得的数据将被安全储存并以匿名形式（没有表示能将您和您的信息联系在一起）报道。只有负责调查或伦理委员会的成员才可以访问严格保密的原始数据。如果您符合资格，我们将邀请您参加本次研究。

自愿参与原则

作为参与者，你有权在任何时候退出筛选试验，并且不需要说明原因或承担负面后果。退出时无需退回之前所获得的补偿，也对您和这次试验所在学校间的关系无任何影响。

研究者、医生或赞助的剔出

研究者、医生或赞助商在任何时候都可能停止研究或将您从研究中剔除。他们应该是从您的最佳利益判断并做出决定。如果您在相关研究中受伤，如果您需要额外的或不同的药物/治疗，或者如果您不遵守研究计划。他们可以因为各种行政或医疗原因，未经您同意而将您从研究中剔除。

如果您有任何的疑问

你会得到本知情同意书的复印件。进行此项研究的主要人员为美国康奈尔大学的研究生劳拉。如果您有任何疑问，请在现场进行咨询。如果你以后有问题，你可以联系公共卫生学院讲师芮溧，电话 13888531601。如果您有任何问题或关心关于作为研究的受试者您的权利，你可以联系昆明医科大学或康奈尔大学或访问他们的网站的机构审查委员会（IRB）来了解参与人权利。

您将获得这种形式的副本保存在您的记录。

知情同意书 2

请签字这项研究中，你同意参加的每个部分：

第 1 部分 – 补充：_____

第 2 部分 – 运动测试：_____

第 3 部分 – 抽血：_____

第 4 部分 – 身体活动评估：_____

第 5 部分 – 膳食评估：_____

第 6 部分 – 运动训练（如随机分配）：_____

第 7 部分 – 多余的血清对未来营养分析存储：_____

同意声明

我已阅读以上材料，并以了解相关信息。我同意参加本项目的正式研究。

姓名：_____ 日期：_____

_____

本研究的负责人：_____ 日期：_____

本知情同意书将被研究保留至研究结束后至少 5 年。

A3.2 English

FORM 1B

We are asking you to participate in a research study. This form is designed to give you information about this study. We will describe this study to you and answer any of your questions.

Project Title: Defining the Functional and Metabolic Role of Iron in the Relationship Between Aerobic Training and Physical Performance

Principal Investigator: Laura Pompano
Division of Nutritional Sciences
1168 West Chunrong Street, Yuhua Avenue, Chenggong New District
Kunming, Yunnan Province, China 650500
Email: lmp262@cornell.edu
Telephone: (Insert Chinese phone number once acquired)

Cornell Faculty Advisor Dr. Jere Haas
Division of Nutritional Sciences
220 Savage Hall, Cornell University, Ithaca, NY 14853 USA
Phone: (607) 255-2665
Fax: (607) 255-1033
Email: jdh12@cornell.edu

Chinese Faculty Advisor Dr. Le Cai
Department of Nutrition and Food Sciences
School of Public Health, Kunming Medical University
1168 West Chunrong Street, Yuhua Avenue, Chenggong New District
Kunming, Yunnan Province, China 650500
Email: caile002@hotmail.com
Tel: 86-871-5922921 (office)

What the study is about

The study will be an 8-week study examining how iron deficiency affects and is affected by exercise. The study will have two groups of participants: iron deficient non-anemic and healthy women. You would be assigned to a group based on your body iron status at screening. The primary aim of the study is to clarify the relationship between body iron status, aerobic exercise training, and physical performance

What we will ask you to do

Part 1: Supplementation:

We will ask you to consume either a ferrous sulfate supplement (50mg, twice daily), a Ba Zhen Ke Li supplement (600mg, twice daily), or an identical placebo (twice daily) for 8 weeks. You will be randomly assigned to one of these three groups. To maintain the integrity of the research, we ask that you do not trade supplements with your friends or peers. You will be asked to take your given supplement twice daily, and provide records of how often you took the supplement each week. To minimize risk of gastrointestinal discomfort, we advise that you take your supplements with food. To improve absorption of the nutrients, we advise that you consume the supplements with foods containing vitamin C, such as pumelo, oranges, or lychee.

Part 2: Exercise testing

We will assess your physical performance via two exercise tests that will occur at weeks 0, 4, and 8 during the study. Each session, the tests will occur over two days. Each test requires you to pedal on a stationary cycle ergometer for approximately 15-30 minutes. During the tests you will wear a facemask and your heart rate (HR) will be monitored.

The first test is designed to measure your maximum ability to uptake and use oxygen during exercise. During the test, we will monitor your blood lactate concentration by collecting a small drop of blood from a finger-stick using sterile, disposable lancets. This measurement will occur several times during the first test.

The second test is designed to see how efficiently your body is. The test will be conducted at three workloads, determined by the results of your first test. After the 2nd and 3rd workloads, a licensed phlebotomist will collect 4mL of blood from the antecubital vein in your arm.

Part 3: Blood Draws

All venous blood draws will be performed by a licensed phlebotomist. Blood will be collected at the following points during the study.

a. At weeks 0, 4, and 8: 4mL of blood will be collected from the antecubital vein in your arm while you are in a non-exercised, resting state. A portion of this blood will be used to assess hemoglobin. The remainder will be spun down to separate the serum, which will be collected, frozen, and stored for later analyses.

b. At weeks 0, 4, and 8: 4mL of blood will be collected after each phase of the 2nd exercise test, as described in Part 2 of this document. This blood will be will be spun down to separate the serum, which will be collected, frozen, and stored for later analyses.

c. At weeks 0, 4, and 8: several finger sticks will be performed during the first exercise test, one at the end of each workload. One drop of blood is needed per finger stick. You will receive between 2-5 sticks, depending on your performance during the test. No blood will be

stored from this section.

Part 4: Physical Activity Assessment
At the beginning of the study, we will ask you to complete a Physical Activity Questionnaire that summarizes your typical physical activity patterns.

On three occasions, we will ask you to wear a small accelerometer device on an elastic belt around the waist to measure daily physical activity. The device should be worn all day, every day for 5 consecutive days including two weekend days. You can wear it under or above the clothing, depending on your personal preference. You should remove the device when you go sleep, swim, take a shower, or do any activity that will submerge the device in water. You can wear the device if it is raining, but in this situation we request you wear the device under your clothing. This assessment will occur once at week 0 and once during weeks 1-8 of the study.

Part 5: Dietary Assessment
At three occasions during the study, you will be asked to complete a 4-day dietary assessment. This assessment will require you to record the foods and beverages that you consume for a consecutive 4-day period. We request that you maintain your normal dietary habits during this period to provide accurate representation of your usual diet.

Part 6: Aerobic training
Some participants will be randomly selected to participate in aerobic exercise training. If you are selected for this training, we will ask you to complete 30 minutes of exercise on a training bicycle while wearing a heart rate monitor. Training will occur in the public health building of KMU located at:

Department of Nutrition and Food Sciences
School of Public Health, Kunming Medical University
1168 West Chunrong Street, Yuhua Avenue, Chenggong New District
Kunming, Yunnan Province, China 650500

Training will be monitored by our researchers. You will be trained for 8 weeks, 5 days per week for 30 minutes each day. The training will begin at a workload equal to 75% of your maximum heart rate (approximately 150 beats per minute), and progress in difficulty over the 8-week training period up to 85% of your maximum heart rate (approximately 170 beats per minute).

Part 7: Permission to store blood samples for future analyses
The blood samples collected (as described in Part 3) will be analyzed for iron content, inflammation, and metabolite concentrations. If you consent, all remaining blood or serum samples will be stored at negative 80®C for later nutrient, metabolite, proteome, or genetic

analyses in future studies. Stored samples will be de-identified and will have no personal information associated with them that could link them to you.

Summary of Requirements (Parts 1-6):
Supplementation will last for 8 weeks. Assessment of exercise testing will take 2 days per session and involve three separate sessions (weeks 0,4, and 8), for a total of 6 exercise tests each lasting 15-30 minutes. Finger sticks will be performed during the first exercise test to measure blood lactate concentration. Venous blood will be collected three times during the second exercise test (at rest, after workload 1, after end of test) and stored for use in later nutritional analyses. Physical activity assessment will require wearing an accelerometer device for 5 consecutive days on two separate occasions during the study, for a total of 10 days wearing the device. Finally, if selected for training, training will last 8 weeks and take place 5 days a week for 30 minutes each day.

Risks and discomforts
Part 1: Some subjects may experience gastrointestinal discomfort (cramping, diarrhea, constipation) from the supplementation. To minimize this risk, you are advised to consume your tablets with meals and vitamin C, which helps improve absorption of the supplement.

Part 2: Some subjects may feel tired, sore, or faint after the exercise tests. During the test, we will monitor your heart rate and physical condition to minimize risk of fainting. If the researchers consider you at high risk for fainting, we will stop the test and take appropriate precautions to minimize this risk. We will provide water and snacks after completion of the test to promote full recovery from exercise.

Part 3: The disadvantages/ risks of having blood drawn from the arm are slight bleeding, bruising or infection. To prevent these risks, blood will be drawn by a licensed phlebotomist. If you do have any bleeding, bruising or infection after the blood draw, you will be cared for by the nurse and/or doctor at no cost to you.

Part 4-5: No known risks are associated with wearing the accelerometer device or completing the dietary assessment.

Part 6: Please see risks for Part 2, as the risks for exercise testing are the same as the risks of daily training.

Part 7: No known risks are associated with storage of blood or serum. Your personal information will not be stored. The samples will be stored only with an ID code, which is not traceable to you. Only the primary researchers will have access to these samples.

Benefits
Part 1: If selected to receive iron supplementation or Ba Zhen Ke Li supplementation, your body iron stores may improve over the 8 week study.

Part 2: You will learn your fitness level after completion of the exercise testing.

Part 3: You will learn your iron and inflammation status after completion of the blood analyses.

Part 4: You will learn how physically active you are on a daily basis.

Part 5: No known benefit to participant.

Part 6: If selected to receive exercise training, your fitness level may improve during the 8-week training period.

Part 7: No known benefit to participant.

Payment for participation
Upon completion of the study (including the 8 week supplementation, six exercise tests, two physical activity assessments, three dietary assessments, and aerobic exercise training if assigned to a training group) you will receive a gift for your participation valued at $50 USD, or 300 元.

Privacy/Confidentiality
The obtained data will be stored safely and reported in an anonymous form with no identifiers that can associate you with your data. Only the responsible investigators will have access to the original data under strict confidentiality.

Taking part is voluntary
As a participant, you have the right to withdraw from the study at any time without needing to specify any reasons or facing negative consequences. Withdrawing will have no effect on the compensation earned before withdrawing or on your relationship with the universities involved with the research.

Withdrawal by investigator, physician, or sponsor
The investigators or KMU physicians may stop the study or take you out of the study at any time should they judge that it is in your best interest to do so, if you experience a study-related injury, if you need additional or different medication/treatment, or if you do not comply with the study plan. They may remove you from the study for various other

administrative and medical reasons. They can do this without your consent.

If you have questions

The main researcher conducting this study is Laura Pompano, a graduate student at Cornell University. Please ask any questions you have now. If you have questions later, you may contact Laura Pompano at lmp262@cornell.edu (I will insert my Chinese phone number here once I acquire it in China). If you have any questions or concerns regarding your rights as a subject in this study, you may contact the KMU or Cornell Institutional Review Board (IRB) for Human Participants at:

KMU:
*I will insert this information as soon as Dr. Cai provides it to me. *

Cornell:
+1-607-255-5138
http://www.irb.cornell.edu.

You may also report your concerns or complaints anonymously through Ethicspoint online at www.hotline.cornell.edu or by calling toll free at 1-866-293-3077. Ethicspoint is an independent organization that serves as a liaison between the University and the person bringing the complaint so that anonymity can be ensured.

You will be given a copy of this form to keep for your records.

Statement of Consent
I have read the above information, and have received answers to any questions I asked. I consent to take part in the study.

Please initial for each part of the study in which you agree to participate:

Part 1 – Supplementation: _____

Part 2 - Exercise testing: _____

Part 3 – Blood draws: _____

Part 4 – Physical Activity Assessment: _____

Part 5 – Dietary Assessment: _____

Part 6 – Daily exercise training (if randomly assigned): _____

Part 7 - Storage of excess blood serum for future nutritional analyses: _____

Your Signature_____ Date_____

Your Name (printed)_____

Signature of person obtaining consent_____ Date_____

Printed name of person obtaining consent_____

This consent form will be kept by the researcher for at least five years beyond the end of the study

A4: VO_2max testing form

Test Title: VO_2max

Subject Name:_____ Date:_____

Height:_____ (cm) Weight:_____ (kg) Age: ___

Max HR _____ MaxHR +/- 10 BPM: _____

Temperature:_____ Barometric Pressure:_____ Rel. Humidity:_____ %

Test Start Time:_____

Mask Size: Pet XS Sm

Seat Height: _____

Time (min)	Heart Rate	RPM	Work Load (kg)	WL#	Legs have feeling?	Breathing faster?
0:00 (start)		**Start Test**	**Start Test**	Start	yes/no	yes/no
1:00			1 kg	Warm-up		
2:00			1 kg	Warm-up		
3:00			1 kg	Warm-up		
4:00			1 kg	Warm-up		
5:00			kg	**WL1**		
6:00			kg	**WL1**		
7:00			kg	**WL2**		
8:00			kg	**WL2**		
9:00			kg	**WL3**		
10:00			kg	**WL3**		
11:00			kg	**WL4**		
12:00			kg	**WL4**		
13:00			kg	**WL5**		
14:00			kg	**WL5**		
15:00			kg	**WL6**		
16:00			kg	**WL6**		
17:00			kg	**WL7**		
18:00			kg	**WL7**		

Post-Test Blood Lactate Level: _____ mmol

A5: Daily Log (English and Chinese)
A5.1 Chinese

口服补充剂日志

受试者 # _____ 周 _____

1 请每天准确记录你是否吃丸剂

	周一	周二	周三	周四	周五	周六	周日
早上	☐ 是 ☐ 否	☐ 是 ☐ 否	☐ 是 ☐ 否	☐ 是 ☐ 否	☐ 是 ☐ 否	☐ 是 ☐ 否	☐ 是 ☐ 否
晚上	☐ 是 ☐ 否	☐ 是 ☐ 否	☐ 是 ☐ 否	☐ 是 ☐ 否	☐ 是 ☐ 否	☐ 是 ☐ 否	☐ 是 ☐ 否

2. 下面每个问题，请你表示是或否

	周一	周二	周三	周四	周五	周六	周日
你在来月经吗？	☐ 是 ☐ 否	☐ 是 ☐ 否	☐ 是 ☐ 否	☐ 是 ☐ 否	☐ 是 ☐ 否	☐ 是 ☐ 否	☐ 是 ☐ 否
你是否出现胃肠问题？	☐ 是 ☐ 否	☐ 是 ☐ 否	☐ 是 ☐ 否	☐ 是 ☐ 否	☐ 是 ☐ 否	☐ 是 ☐ 否	☐ 是 ☐ 否
你是否患其他疾病或受外伤？如果是，请简要描述。	☐ 是 ☐ 否 问题：	☐ 是 ☐ 否 问题：	☐ 是 ☐ 否 问题：	☐ 是 ☐ 否 问题：	☐ 是 ☐ 否 问题：	☐ 是 ☐ 否 问题：	☐ 是 ☐ 否 问题：
你是否吃了别的药？如果是，请简要描述。	☐ 是 ☐ 否 药：	☐ 是 ☐ 否 药：	☐ 是 ☐ 否 药：	☐ 是 ☐ 否 药：	☐ 是 ☐ 否 药：	☐ 是 ☐ 否 药：	☐ 是 ☐ 否 药：
你是否参与体力活动？如果是，请简要描述。	☐ 是 ☐ 否 活动：	☐ 是 ☐ 否 活动：	☐ 是 ☐ 否 活动：	☐ 是 ☐ 否 活动：	☐ 是 ☐ 否 活动：	☐ 是 ☐ 否 活动：	☐ 是 ☐ 否 活动：

	多久？	多久？	多久？	多久？	多久？	多久？	多久？

A5.2 English

Capsule ingestion log

Subject # _____ Week # _____

For each day, please indicate whether you took your pill in the morning and evening.

	Monday	**Tuesday**	**Wednesday**	**Thursday**	**Friday**	**Saturday**	**Sunday**
Morning	☐ Yes ☐ No	☐ Yes ☐ No	☐ Yes ☐ No	☐ Yes ☐ No	☐ Yes ☐ No	☐ Yes ☐ No	☐ Yes ☐ No
Evening	☐ Yes ☐ No	☐ Yes ☐ No	☐ Yes ☐ No	☐ Yes ☐ No	☐ Yes ☐ No	☐ Yes ☐ No	☐ Yes ☐ No

For each question below, please indicate yes or no.

	Monday	**Tuesday**	**Wednesday**	**Thursday**	**Friday**	**Saturday**	**Sunday**
Are you menstruating?	☐ Yes ☐ No	☐ Yes ☐ No	☐ Yes ☐ No	☐ Yes ☐ No	☐ Yes ☐ No	☐ Yes ☐ No	☐ Yes ☐ No
Did you have GI complaints?	☐ Yes ☐ No	☐ Yes ☐ No	☐ Yes ☐ No	☐ Yes ☐ No	☐ Yes ☐ No	☐ Yes ☐ No	☐ Yes ☐ No
Did you have other illness or injury? If yes, please describe.	☐ Yes ☐ No	☐ Yes ☐ No	☐ Yes ☐ No	☐ Yes ☐ No	☐ Yes ☐ No	☐ Yes ☐ No	☐ Yes ☐ No
Did you consume other medications? If yes, please describe.	☐ Yes ☐ No	☐ Yes ☐ No	☐ Yes ☐ No	☐ Yes ☐ No	☐ Yes ☐ No	☐ Yes ☐ No	☐ Yes ☐ No
Did you engage in physical activities? If yes, please describe.	☐ Yes ☐ No	☐ Yes ☐ No	☐ Yes ☐ No	☐ Yes ☐ No	☐ Yes ☐ No	☐ Yes ☐ No	☐ Yes ☐ No

A6: Four-day Diet Record (English and Chinese)

Instructions for Completing the 4-Day Food Record

It is extremely important that you take this part of the study seriously. We need for you to be as complete and specific as possible.

1. Please write down everything you eat or drink as soon as possible after you consume it for the next four days.
2. Include anything you "eat", including hard candy, gum, etc.
3. Please fill out each column for each food item
    a. Type of Food—We need to know what types of foods you eat so that we can determine the nutrients in your overall diet.
    b. Time – We need this to see if timing of eating makes a difference in the things we are measuring
    c. Place –We need to know where you ate the food so that we can use cafeteria recipes to figure out what all of the ingredients are.
    d. Amount – We need this to be able to enter your food intake correctly into the computer.

Here are some tips for estimating the amount of food you eat.

- Try to use amounts such as grams, etc. if possible, but just describe the volume if you are not sure.
- If the item is a standard size at a restaurant, you can just say exactly what the item was and what size (small/medium/large, 10cm/20cm, single/double, etc.) – remember to say WHERE you ate.
- If you make the food yourself, tell us how many pieces/slices of EACH ingredient in stir fry, how much of EACH sauce or topping, etc.
- For drinks, tell us whether it was regular or diet, sweet or unsweetened, and the SIZE (small/medium/large, 500mL/1L), and anything you added (i.e. 珍珠, sugar, etc.)
- If the item is one "pack" or "package", please tell us the size of the package – should be on the package itself
- For any foods that you can, especially rice/馒头/面包, etc., please tell us the BRAND NAME and PRODUCT NAME of the food you ate.

指导完成为期 4 天的饮食记录

您采取这部分研究的严重是极其重要的。我们需要你尽可能完整和明确化。

1. 请写下所有你吃或尽快与您使用它在未来四年天后饮用。
2. 包括任何你“吃”，包括硬糖，口香糖等。
3. 请填写各栏每一个食品项目
    a. 类型食品，我们需要知道你吃什么类型的食物，这样我们就可以在你的整体饮食决定的营养素。
    b. 时间 – 我们需要这个，看看吃的时机使得我们所测量的东西有差别
    c. 地方 – 我们需要知道你在那里吃饭，这样我们就可以使用食堂菜谱找出所有的成分都是。
    d. 量 – 我们需要这是能够正确输入您的食物摄入到计算机。

下面是估算的食物你吃的量的一些技巧。

- 尽量使用如克等，如果可能的数量，而只是描述了音量，如果你不能确定。
- 如果项目是在一家餐厅标准尺寸，你可以说正是这个项目是什么尺寸（小/中/大，10 厘米/ 20 厘米，单/双，等） – 记得说你吃了。
- 如果你自己做的食物，告诉我们有多少个/每种成分的翻炒，多少每个酱或摘心片等。
- 适用于饮料，告诉我们，无论是规律或饮食，甜或不甜的，和你增加的大小（小/中/大，500 毫升/公升），和任何东西（即珍珠，糖等）
- 如果该项目是一“包”或“包”，请告诉我们包的大小 – 应该是在包装本身
- 对于任何食物，你可以，特别是大米/馒头/面包等，请告诉我们你吃的食物的品牌名称和产品名称。

ID # _____ 第一天

食物的种类	你在哪儿吃？（家庭，食堂，餐厅等）	什么时候吃？	你吃了多少这种菜？

ID # _____ 第二天

食物的种类	你在哪儿吃？（家庭，食堂，餐厅等）	什么时候吃？	你吃了多少这种菜？

ID # _____ 第三天

食物的种类	你在哪儿吃？（家庭，食堂，餐厅等）	什么时候吃？	你吃了多少这种菜？

ID # _____ 第四天

食物的种类	你在哪儿吃？（家庭，食堂，餐厅等）	什么时候吃？	你吃了多少这种菜？

Appendix B: AJCN Publication Reprint

The following 10 pages contain the publication that resulted from the work reported in Chapter 3. The citation for this publication is:

Pompano, LM and Haas JD. Efficacy of iron supplementation may be misinterpreted using conventional measures of iron status in iron-depleted, nonanemic women undergoing aerobic exercise training. Am J Clin Nutr. 2017 Dec;106(6):1529-1538. doi: 10.3945/ajcn.117.152777. Epub 2017 Nov 1.

AJCN. First published ahead of print November 1, 2017 as doi: 10.3945/ajcn.117.152777.

Efficacy of iron supplementation may be misinterpreted using conventional measures of iron status in iron-depleted, nonanemic women undergoing aerobic exercise training

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Division of Nutritional Sciences, Cornell University, Ithaca, NY

ABSTRACT

Background: Despite its known detrimental effects, iron deficiency remains the most common micronutrient deficiency in the world. Many interventions that aim to improve iron status involve physically active populations. Intense aerobic exercise training negatively affects iron status; however, the impact of regular moderate aerobic exercise on the effectiveness of iron supplementation remains unclear.

Objective: This study aimed to determine whether aerobic training modifies the assessment of the effectiveness of iron supplementation in improving conventional iron status measures.

Design: Seventy-two iron-depleted, nonanemic Chinese women [serum ferritin (sFer) <25 μg/L and hemoglobin >110 g/L] were included in an 8-wk, partially blinded, randomized controlled trial with a 2 × 2 factorial design including iron supplements (42 mg elemental Fe/d) or placebo and aerobic training (five 25-min sessions/wk at 75–85% of maximum heart rate) or no training. Linear mixed models were used to evaluate the relation between supplement type, training, and changes in iron status over time, measured by sFer, hemoglobin, soluble transferrin receptor (sTfR), and estimated total body iron.

Results: After treatment, both the iron-supplemented trained and untrained groups showed significantly improved sFer, sTfR, and body iron values compared with either of the placebo groups. Similarly, trained participants had significantly higher aerobic fitness measures than untrained participants. Training modified the sFer response to supplementation (training by supplement interaction, $P = 0.07$), with the iron-supplemented trained group having significantly lower sFer than the iron-supplemented untrained group at week 8 (mean ± SD: 31.8 ± 13.5 and 47.6 ± 15.7 μg/L, respectively; $P = 0.042$), whereas there was no significant difference between the placebo trained and untrained groups (21.3 ± 12.2 and 20.3 ± 7.0 μg/L, respectively; $P = 1.00$).

Conclusions: Regular aerobic training reduces the apparent effectiveness of iron supplementation in improving sFer and calls into question whether conventional measures of iron status accurately reflect iron metabolism in physically active, nonanemic women. This trial was registered at clinicaltrials.gov as NCT03002090. *Am J Clin Nutr* doi: https://doi.org/10.3945/ajcn.117.152777.

Keywords: iron deficiency, supplementation, aerobic training, serum ferritin, body iron

INTRODUCTION

Iron deficiency anemia affects 20–34% of Chinese women of childbearing age (1, 2). This rate likely underestimates the prevalence of iron deficiency (ID) in the population because it does not account for ID without anemia [IDNA; hemoglobin >110 g/L and serum ferritin (sFer) <15 μg/L] (3). ID with anemia and IDNA have negative consequences on physical performance and exercise capacity (4, 5). However, there is also evidence that aerobic training may have a negative influence on iron status, which is potentially more concerning when considering that iron interventions are frequently targeted toward physically active populations (6–8).

Intense physical exercise lowers several measures of iron status, including sFer, and increases soluble transferrin receptor (sTfR), indicative of either compromised iron status (6–9) or redistribution of iron to be used in erythropoiesis or muscle formation (10–13). Female soldiers who underwent 9 wk of basic combat training showed declining iron status (7, 14). The provision of an iron supplement (14) during basic combat training attenuated some of these changes compared with placebo. Although these findings occurred in highly intensive training programs, recreational physical activity can also negatively affect iron status. In a study by Hinton et al. (4) in women with IDNA who underwent aerobic training, iron supplementation

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Supplemental Table 1 is available from the "Online Supporting Material" link in the online posting of the article and from the same link in the online table of contents at http://ajcn.nutrition.org.

Address correspondence to LMP (e-mail: lmp262@cornell.edu).

Abbreviations used: AGP, α1-acid glycoprotein; CRP, C-reactive protein; FeTr, iron trained; FeUn, iron untrained; ID, iron deficiency; IDNA, iron deficiency without anemia; PLTr, placebo trained; PLUn, placebo untrained; sFer, serum ferritin; sTfR, soluble transferrin receptor; $\dot{V}O_2$max, maximal oxygen consumption.

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resulted in a modest increase in sFer (4.1 μg/L) compared with those who received placebo. Interestingly, the improvement in sFer in the iron-supplemented trained women in the Hinton and Sinclair study (15) was less than half of that observed in a separate study in fit IDNA women who received similar iron supplementation but who did not undergo training. These findings suggest that improvements in sFer from iron supplementation may differ if women train during supplementation. This difference may be a result of an exercise-induced prioritization of erythropoietic demands and muscle growth over storing iron in the liver, as reflected by sFer concentration. Although the current literature supports this idea of iron redirection away from the stores to support more functional uses of iron, most interventions that aim to improve iron status or anemia measure only hemoglobin, sFer, and sometimes sTfR or estimated body iron, calculated from the ratio of sTfR to sFer, and do not measure other body iron pools. This oversight could potentially lead to erroneous conclusions of the effectiveness of the intervention.

Further complicating the relation between IDNA and exercise, research also suggests that women who are actively training perform better when given iron supplementation than do those who train without supplementation (6, 14), which suggests an intricate, but currently undefined, relation between iron status, total body iron, exercise, and physical performance capacity. This relation must be understood before we can properly design effective interventions to measure and target IDNA in physically active populations, such as laborers, athletes, or nonathletes who want to benefit from improved fitness. Although several studies have shown that training worsens traditional measures of iron status, to our knowledge no study has directly examined the interaction between training and iron supplementation to determine how training affects the effectiveness of iron supplementation, as assessed by sFer and hemoglobin concentrations. Therefore, the goal of this study was to determine whether 8 wk of regular aerobic exercise on a cycle ergometer modifies the apparent effectiveness of concurrent iron supplementation in improving the traditional measures of iron status. We hypothesized that those women who received both iron and training would have smaller changes in sFer than women who did not train while taking iron.

METHODS

Participants

The sampling scheme for this study is shown in **Figure 1**. A total of 379 physically active, untrained 18- to 26-y-old women were recruited in 2 waves (September–December 2014 and March–June 2015) from the student population at Kunming Medical University in Yunnan, China. Of these, 98 women were identified in the fall wave and 281 were identified in the spring

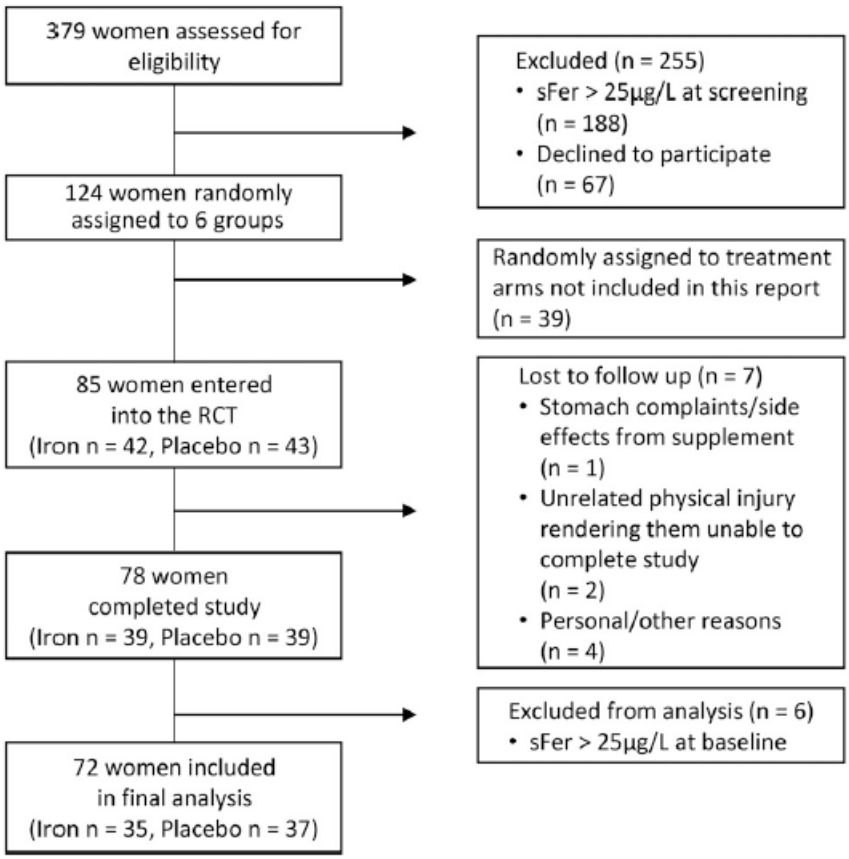

FIGURE 1 Consolidated Standards of Reporting Trials diagram. RCT, randomized controlled trial; sFer, serum ferritin.

wave. In total, 191 were identified as iron depleted without anemia, defined as hemoglobin >110 g/L and sFer <25 μg/L. Anemia was defined as <110 g/L to align with the hemoglobin cutoff used by the First Affiliated Hospital of Kunming Medical University where the screening analyses were performed. The cutoff for iron depletion was set at 25 μg/L, because previous research has shown that physical performance changes occur even in women who are iron depleted and not clinically iron deficient (16–20). Screening, which included a medical history questionnaire, was conducted to identify and exclude women who met any of the following criteria: anemia (hemoglobin <110 g/L), current pregnancy or pregnancy within the previous year, current lactation or lactation within the previous year, recent infectious illness or fever, current inflammation or chronic inflammatory diseases, hemolytic anemia, chronic respiratory disease, musculoskeletal problems, history of eating disorders, smoking, BMI (in kg/m^2) <17 or >25, or recent consumption of iron supplements, vitamin supplements, or medications that may affect dietary iron intake or absorption or that had anticoagulant properties. Participants also filled out a physical activity questionnaire that indicated they did not participate in regular exercise or organized sports, were interested in increasing their level of physical fitness, and were willing to comply with the full 8-wk training program. Of the eligible women, 124 agreed to participate in the trial (45 in the fall wave and 79 in the spring wave). The recruitment process for this study was designed to recruit for 2 additional arms of the study. This article focuses on the first 4 arms of the study, which included the iron- and placebo-supplemented groups. The other arms of the study included 2 groups who received a Chinese herbal supplement. Of the 124 women in the total study, 85 were randomly assigned to receive the treatments relevant to this study.

Data from 6 women were excluded from statistical analyses because their sFer values at week 0 increased above the screening cutoff of 25 μg/L, whereas their original screening values from the previous 2 wk indicated that they were iron depleted. In addition, 7 women dropped out of the trial. The final sample size for this analysis was 72 women. Signed informed consent was obtained from each participant. The study was approved by the Cornell University Institutional Review Board and the Kunming Medical University Ethical Committee and registered under clinicaltrials.gov as NCT03002090.

Study design

The experimental design of the study was a 2 × 2 randomized, double-blind, placebo-controlled intervention trial. Participants were randomly assigned by one of the authors (LMP) via a random-number generator to treatment groups. Participants received either 100 mg ferrous sulfate or an identical placebo capsule 2 times/d for 8 wk. Participants and all of the investigators were blinded to supplement type until after the per-protocol analyses were completed. Sample size was based on consumption of a minimum elemental iron concentration of 12 mg/capsule over 8 wk. The final capsule concentration met this requirement. Previous work has shown that iron status improves after 4 wk of iron supplementation at a similar dose of ferrous sulfate (4, 21). The capsules were prepared by LMP with the use of gelatin capsules, ferrous sulfate, and dextrose filler (Professional Compounding Centers of America). At weeks 0, 2, 4, 6, and 8 of each wave of the study, 20 capsules of each type were randomly selected from the batch and stored in a sealed container in a cool, dry place until analyzed poststudy by inductively coupled plasma mass spectrometry. After the study, iron concentrations of the iron and placebo capsules were determined from this random sample to be 21.1 and 0.00 mg elemental Fe, respectively.

Participants were instructed to consume the capsules with citrus juice during their morning and evening meals and to avoid consuming the capsules on an empty stomach. Thirty capsules were distributed to each participant in a bottle labeled with their unique subject identification number every 2 wk (weeks 0, 2, 4, and 6). Each participant was instructed to complete a daily log to record capsule ingestion as well as information on medication use, illness, menstrual cycle status, any gastrointestinal complaints, physical activity, and musculoskeletal problems. In addition, they were instructed to return their capsule bottles every 2 wk with any remaining capsules, which were then counted as an independent confirmation of the number of capsules reported as consumed on the daily logs.

One-half of each supplement group were further randomly assigned to an exercise training program or to no training to create 4 treatment groups: iron trained (FeTr), iron untrained (FeUn), placebo trained (PLTr), and placebo untrained (PLUn). The training protocol was adapted from a protocol previously published by Hinton et al. (4) that has produced measurable improvements in endurance and maximal oxygen consumption ($\dot{V}O_2$max). The intensity of training increased each week according to each participant's heart rate. Training sessions lasted 25 min and were divided into 2 target heart rates of 75% and 85% of age-estimated maximum, calculated as 220 (beats per minute) minus age (years). The workload was adjusted as needed to maintain the target heart rate throughout the session. Minutes spent at 85% of maximum heart rate increased each week to increase the difficulty of the training. The training protocol is shown in **Table 1**. Participants trained Monday through Friday during each of the 8 wk of the study. Optional training days were offered on weekends to allow participants to make-up any days they had missed during the week. The maximum number of training days was 40.

Training was performed on a stationary exercise bicycle (model B8.4E; KangLe Exercise Products Company) equipped with digital output of work (watts) and cadence (rotations per minute). Participants wore a T31 Polar heart rate monitor, which was read by using a Polar A5 heart rate watch (Polar Electro Inc.).

TABLE 1
Daily training schedule, by week

Week	75% of maximum heart rate, min	85% of maximum heart rate, min
1	20	5
2	19	6
3	18	7
4	17	8
5	15	10
6	13	12
7	11	14
8	10	15

Trained research assistants recorded the watts, heart rate, and speed of each participant in a training log every 5 min throughout each session.

Daily physical activity, defined as minutes spent performing discretionary exercise, was assessed at weeks 0, 4, and 8 by using the data from a daily log completed each day over the 56-d trial. Minutes of self-reported physical activity were totaled at each time point. In addition, a physical activity frequency questionnaire was administered at week 0 to assess similarity in habitual physical activity levels between groups. Participants were asked to maintain their normal prestudy activity patterns for the duration of the 8-wk study period, regardless of whether they were in the trained or untrained groups.

Body composition and physical performance were measured before and after the study as well as at the 4-wk midpoint. Dietary iron intake was assessed at week 0 by using a 4-d diet record that spanned from Thursday to Sunday. These records were analyzed for daily iron, inhibitors and enhancers of iron absorption, and macronutrient content by using Nutrition Data System for Research Software (2016; University of Minnesota).

All of the participants were compensated for study participation with a gift. In addition, to increase compliance to the training program, trained participants were offered incentives in the form of small gifts when they completed a target number of training days (25, 32, and 40 d). Although all of the participants received the same gift for participation in the study, the smaller training-based incentives were only offered to the trained group.

Iron status measurements

Iron status was assessed at weeks 0, 4, and 8. Whole blood was collected from the antecubital vein into 4-mL EDTA-coated tubes by a phlebotomist at the Kunming Medical University campus hospital. A small sample of whole blood was removed for hemoglobin analysis. The remaining samples were stored at 4°C until centrifugation at 1600 × *g* for 10 min at room temperature (23°C) within 24 h. The serum was collected and separated into 0.5-mL aliquots and frozen at −80°C until analysis. Blood was analyzed for sFer, sTfR, α1-acid glycoprotein (AGP), and C-reactive protein (CRP) at the First Affiliated Hospital of Kunming Medical University in the first wave and at the Shanghai Fenglin Clinical Laboratory in the second wave. In addition, 75 samples from the second wave were analyzed at both laboratories to allow for comparison between the laboratories. Hemoglobin was determined by using a Coulter LH 750 Hematology analyzer (Beckman Coulter Inc.). sFer, sTfR, AGP, and CRP were analyzed by using a Siemens Advia 2400 automated analyzer (Siemens Healthcare). Estimated total body iron was derived from the ratio of sTfR to sFer by using the equation reported by Cook et al. (22) as follows:

$$\text{Total body iron} = -[\log(\text{sTfR}/\text{sFer}) - 2.8229]/0.1207 \quad (1)$$

This equation uses sTfR values from Ramco ELISA kits (Ramco Laboratories). Therefore, we converted the sTfR values derived from the Chinese laboratories to Ramco-adjusted sTfR values with the prediction equation below, which was derived from 35 random duplicate samples analyzed by using Ramco Laboratories sTfR ELISA kits.

$$\text{sTfR Ramco} = (3.779 \times \text{sTfR}_{\text{lab}}) + 0.400,\ R^2 = 0.93 \quad (2)$$

Physiologic measurements

Height and weight were measured with the use of previously described standard protocols (23). Body composition was estimated from skinfold thicknesses measured with Lange calipers at the biceps, triceps, subscapular, and suprailiac sites. The Durnin and Womersley (24) equation was used to calculate body density and percentage of body fat.

Physical performance was assessed by using a $\dot{V}O_2$max test at weeks 0 and 8 on a mechanically braked and calibrated cycle ergometer (Monark 884E; Monark Exercise AB). Oxygen consumption was determined while cycling at levels of effort ranging from rest to ∼100% of participants' maximum level of exertion. Metabolic variables were assessed with a portable metabolic measurement system (Cosmed K_4B^2; Cosmed), which analyzes heart rate, the volume of respiratory air, and concentrations of oxygen and carbon dioxide in expired air. The participants breathed room air through a 2-way valve connected to a facemask worn during the testing sessions. Oxygen consumption per kilogram of body weight ($\dot{V}O_2$, milliters per kilogram per minute) was calculated from the portable metabolic unit's internal program.

Participants were asked not to perform any strenuous physical activities or exercise for 1 d before the exercise tests, excluding daily training for the trained group. Trained participants did not train on the day of their performance tests. In addition, participants were told not to consume food or caffeinated products for 3 h before performance testing.

The $\dot{V}O_2$max protocol was adapted from that used by Brownlie et al. (25). The test started when the participant's heart rate was <100 beats/min. The test began with a 5-min warm-up with a workload of 1 kg, cycling at 50 × *g*. Workloads were then increased by 0.4 kg every 2 min, cycling at 50 × *g*, until $\dot{V}O_2$ did not increase by >150 mL/min from the previous workload, suggesting that the participant was working at her $\dot{V}O_2$max. If this condition could not be observed, the test proceeded until the participant was unwilling or unable to continue. The participant was considered to have achieved $\dot{V}O_2$max if 2 of the following conditions were met: reaching a respiratory exchange ratio >1.10, a heart rate within 10 beats/min of their age-predicted maximum (220 – age), or blood lactate >8.0 mM. Blood lactate was assessed by finger stick with the use of a Lactate Plus portable blood lactate analyzer (Nova Biomedical) after completion of the final workload.

Compliance analyses

Compliance to capsule consumption was assessed from 2 sources of documentation: capsule counts from returned bottles and daily logs. The Pearson correlation coefficient between the capsules reported as missing in the daily logs and the capsules counted from the returned bottle was 0.39 ($P = 0.01$), suggesting some discrepancies. To create a more reliable assessment of

capsules consumed, 2 compliance variables were created: a conservative estimate and an average estimate. The conservative estimate was calculated by comparing the information from the capsule counts and the log for each 2-wk period and taking the higher number of missed capsules (reported or physically returned). The average estimate was created by taking the average of the log and bottle counts. There were no group differences in the number of capsules consumed for either the conservative or the average estimates.

Statistical analyses

Sample size calculations were based on the Hinton et al. (4) and Hinton and Sinclair (15) studies discussed previously, which suggested that a smaller change in sFer is observed when iron supplementation is given to women who are training. Sample size was determined to require 25 participants in each of the 4 groups, which was expanded to 29 participants/group after considering potential loss to follow-up to detect a 4.1-μg/L change in sFer or an effect size of ~0.8 SDs after 8 wk with 80% power and $\alpha = 0.05$ (4). Data were examined to verify normality of distribution by using the Kolmogorov-Smirnov test, histograms, and qq-plots. Statistical analyses were performed on log-transformed variables for sFer and sTfR, which were found to have non-normal distributions. Measures of iron status at weeks 4 and 8 were analyzed by using linear mixed models with fixed effects of supplement, training group, time, all 2- and 3-way interactions, and a random effect at the participant level. Baseline was included as a covariate for each measure of iron status. If the 3-way interaction was not significant in the full model, the 3-way interaction term was removed from the model and the model was reanalyzed with all of the 2-way interaction terms. Any 2-way interaction terms that were not significant were also removed and a final model was analyzed that included only the significant 2-way interaction or interactions. Post hoc pairwise comparisons were made, with Bonferroni corrections for multiple comparisons. Secondary analyses were performed by using linear models to test for relations between variables. Residuals were examined for normality for all linear mixed models by using the Kolmogorov-Smirnov test, histograms, and qq-plots. For a subgroup of those participants who had a baseline sFer <20 μg/L, the RR of resolving one's iron depletion (being iron depleted at baseline and iron replete at week 8) was calculated for all pairwise comparisons between groups. For all of the analyses, an α level of 0.05 was used to indicate significance. For each result for which a Bonferroni correction was indicated, the original P value was multiplied by the number of comparisons made and considered significant if ($P \times k$ comparisons) < 0.05. All of the statistical analyses were performed in SAS 9.4 (SAS Institute).

RESULTS

Participant characteristics

There were 19 FeTr, 16 FeUn, 18 PLTr, and 19 PLUn participants included in the final analyses. Background characteristics, including age, anthropometric measures, and fitness level as assessed by $\dot{V}O_2$max at baseline, blood biomarkers, and dietary intake at baseline, are shown in **Table 2**. At baseline, all

TABLE 2
Characteristics of total sample at week 0[1]

Characteristics	Iron trained (n = 19)	Iron untrained (n = 16)	Placebo trained (n = 18)	Placebo untrained (n = 19)
Age, y	20.6 ± 1.1	20.3 ± 0.9	20.7 ± 1.2	20.5 ± 1.7
Height, cm	156.8 ± 5.7	156.9 ± 6.2	157.3 ± 4.7	157.4 ± 5.0
Weight, kg	51.2 ± 6.8	51.4 ± 7.9	53.5 ± 6.1	49.7 ± 5.6
BMI, kg/m^2	20.8 ± 2.8	21.0 ± 3.5	21.6 ± 2.1	20.0 ± 2.1
Body fat, %	25.2 ± 2.5	25.5 ± 3.2	26.0 ± 2.0	24.7 ± 2.0
sFer, μg/L	14.0 ± 5.8	17.1 ± 4.2	14.4 ± 4.7	15.2 ± 5.7
Hemoglobin, g/L	135 ± 10.3	141 ± 5.2	132 ± 12.8	133 ± 9.7
sTfR, mg/L	6.8 ± 3.2	5.3 ± 0.8	5.8 ± 1.5	6.1 ± 1.7
Body iron, mg/kg	1.1 ± 2.6	2.7 ± 0.9	1.8 ± 1.8	1.7 ± 1.9
$\dot{V}O_2$max, mL · min^{-1} · kg^{-1}	42.7 ± 4.8	42.4 ± 5.6	41.4 ± 5.3	42.8 ± 5.0
Physical activity,[2] min	59.8 ± 91.1	56.1 ± 60.5	52.9 ± 71.7	49.0 ± 65.9
AGP, g/L	0.61 ± 0.08	0.61 ± 0.09	0.64 ± 0.10	0.59 ± 0.09
CRP,[3] mg/L	0.04 (0.00, 0.01)	0.22 (0.00, 0.01)	0.20 (0.00, 0.30)	0.10 (0.00, 0.21)
Daily dietary intake				
Energy, kcal	1536 ± 278	1573 ± 333	1609 ± 324	1582 ± 318
Fat, g	51.5 ± 12.6	57.2 ± 15.0	56.6 ± 13.3	54.8 ± 16.9
Protein, g	59.5 ± 20.3	60.2 ± 18.4	59.5 ± 17.5	58.2 ± 20.0
Carbohydrate, g	213.5 ± 43.5	204.3 ± 49.3	215.2 ± 47.2	216.0 ± 51.6
Iron, mg	11.7 ± 4.5	13.0 ± 5.8	11.5 ± 3.6	12.3 ± 3.7
Calcium, mg	323.4 ± 116.4	340.0 ± 145.5	322.5 ± 158.4	388.8 ± 190.2
Ascorbic acid, mg	79.5 ± 40.8	60.2 ± 37.5	63.1 ± 41.8	67.0 ± 37.9
Phytic acid, mg	534.0 ± 196.9	597.8 ± 251.5	519.1 ± 246.9	598.3 ± 252.5

[1] Values are means ± SDs unless otherwise indicated; n = 72. AGP, α1-acid glycoprotein; CRP, C-reactive protein; sFer, serum ferritin; sTfR, soluble transferrin receptor; $\dot{V}O_2$max, maximal oxygen consumption.
[2] Minutes of physical activity per week, defined as discretionary exercise on the basis of a self-reported questionnaire.
[3] Values are means with first and third quartiles in parentheses, due to skewed distribution.

TABLE 3
Total capsules and iron consumed by iron-supplemented groups over 8 wk[1]

	Iron trained (n = 19)		Iron untrained (n = 16)	
	Conservative estimate	Average estimate	Conservative estimate	Average estimate
Capsules consumed,[2] n	95 ± 13	98 ± 11	97 ± 10	101 ± 7
Iron consumed, g/8 wk	2.0 ± 0.3	2.1 ± 0.2	2.1 ± 0.2	2.1 ± 0.1

[1] Values are means ± SDs. No group differences were observed for number of capsules consumed or total iron consumed for either estimate by using 1-factor ANOVA with $P < 0.05$.
[2] Maximum possible capsules = 112.

72 participants were iron depleted but not anemic. Of these 72 women, 39 (54.2%) were clinically iron deficient defined as sFer <15.0 μg/L, 5 women (6.9%) had sTfR values >8.3 mg/L, and 12 women (16.7%) had body iron <0 mg/kg. Consistent with discretionary physical exercise of slightly <1 h/wk, $\dot{V}O_2$max levels indicated that participants had a moderate to average fitness level, with no differences between the groups. There were no significant differences in body weight or composition between the 4 groups before or after the study, nor were there significant changes during the 8-wk study period (data not shown). Likewise, there were no differences in reported symptomology between the 4 treatment groups at any time point. In addition, there were no differences between groups for daily iron, calcium, ascorbic acid, phytic acid, fat, carbohydrate, protein, or total caloric intake (Table 2). Of the 72 participants, 13 women (18.1%) had a daily iron intake under the current US Estimated Average Requirement of 8.1 mg/d (26). There were no significant differences in inflammation, measured as CRP and AGP, between the 4 groups at any time point in the study, nor were there significant changes between time points during the 8-wk study period (data not shown). During the 8-wk study, there were no participants who indicated inflammation on the basis of AGP >1.0 g/L. Only 1 participant was determined to have inflammation on the basis of CRP >5.0 mg/L, which occurred at week 4. All of the models were analyzed including this participant and again excluding this participant, but no differences were found in the results of these analyses, so the participant was included for all analyses reported here.

Compliance to treatments

The iron and placebo groups returned 81% and 80% of their capsule bottles, respectively, and completed 96% and 92% of their daily capsule logs, respectively. There were no significant differences between the number of bottles returned or the percentage of daily logs completed by the combined iron and combined placebo groups (t test, $P = 0.87$) or in the 4 individual treatment groups (1-factor ANOVA, $P = 0.31$). Estimates of the amount of iron consumed by the 2 iron groups are shown in **Table 3**.

Details of the training sessions attended by each group are shown in **Table 4**. The average number of training sessions attended by participants in the FeTr and PLTr groups was not significantly different (t test, $P = 0.76$). The FeTr and PLTr groups achieved mean ± SD percentages of 90.5% ± 7.8% and 91.9% ± 5.6%, respectively, of the target heart rate over the 8 wk of training, which was not significantly different (t test, $P = 0.52$). In the combined training groups, 80% of the target heart rate was achieved on 91% of all days trained. In addition, minutes spent in self-reported physical activity outside of the assigned training groups did not differ between groups at any time point. At baseline and week 8, 88% and 92% of participants, respectively, reached $\dot{V}O_2$max. There was no difference in the percentage of each of the groups who reached $\dot{V}O_2$max at baseline or week 8 (chi-square test: $P = 0.92$ and $P = 0.13$, respectively), suggesting there was equal compliance to the testing protocol between groups.

Response to iron treatment

Table 5 shows the week 8 values for each iron biomarker as well as $\dot{V}O_2$max after 8 wk of treatment. Unadjusted means and SDs for each group, by time point, are shown in **Supplemental Table 1**. No significant 3-way interaction effects (supplement type by training group by time) were observed for any of the outcome measures in the linear mixed models (data not shown), nor were there significant interaction effects between time and training in any model. Significant training by supplement type interactions were observed for sFer and hemoglobin, which will be discussed separately.

There was a significant interaction effect between time and supplement type for sTfR (P-interaction = 0.039). At week 8, the iron group had a significantly lower sTfR concentration than did the placebo group (4.9 ± 0.9 and 5.9 ± 1.8 mg/L, respectively; $P < 0.001$). No significant 2-way interaction effects were observed for body iron. However, the iron group had significantly higher body iron than the placebo group (5.7 ± 1.9 and 2.8 ± 2.5 mg/kg, respectively; $P < 0.001$).

Response of $\dot{V}O_2$max to aerobic training

There was no interaction observed between training group and supplement type for $\dot{V}O_2$max; however, a significant training

TABLE 4
Training sessions attended, by group[1]

Group	n	Days trained	Range[2]	Attended ≥30 d, % (n)	Percentage of target heart rate
Iron trained	19	33.2 ± 5.7[3]	21–40	73.7 (14)	90.5 ± 7.8
Placebo trained	18	32.7 ± 3.7	28–40	77.8 (14)	91.9 ± 5.6

[1] No significant differences were observed between training groups for any variable by using a t test, $P > 0.05$.
[2] The maximum possible number of training sessions was 40 sessions.
[3] Mean ± SD (all such values).

TABLE 5
Effects of training and supplement type[1]

Group	sFer, μg/L	Hemoglobin, g/L	sTfR, mg/L	Body iron, mg/kg	$\dot{V}O_2$max, L · min^{-1} · kg^{-1}
FeTr (n = 19)	31.8 ± 13.5	139.4 ± 8.0	5.3 ± 1.0	4.7 ± 1.9	43.3 ± 4.1
FeUn (n = 16)	47.6 ± 15.7	143.3 ± 6.7	4.4 ± 0.7	6.9 ± 1.3	40.8 ± 4.3
PLTr (n = 18)	21.3 ± 12.2	137.3 ± 9.4	5.9 ± 1.3	2.6 ± 2.8	42.4 ± 3.5
PLUn (n = 19)	20.3 ± 7.0	135.3 ± 10.3	5.9 ± 2.3	2.9 ± 2.2	38.8 ± 4.5
P[2]					
Supplement	<0.001	0.003	<0.001	<0.001	0.28
Training	0.034	0.66	0.018	0.023	<0.001
Interaction	0.072	0.030	0.86	0.17	0.50
Supplement effect,[3] P					
FeTr compared with PLTr	<0.001	1.00			
FeUn compared with PLUn	<0.001	0.003			
Training effect,[3] P					
FeTr compared with FeUn	0.042	0.43			
PLTr compared with PLUn	1.00	1.00			

[1] Values are unadjusted means ± SDs at week 8. FeTr, iron trained; FeUn, iron untrained; PLTr, placebo trained; PLUn, placebo untrained; sFer, serum ferritin; sTfR, soluble transferrin receptor; $\dot{V}O_2$max, maximal oxygen consumption.
[2] Results of linear mixed models, adjusted for baseline. Interaction P values represent the interaction of supplement type by training group.
[3] P values derived by post hoc pairwise comparisons with Bonferroni corrections, reported for measures in which a significant training-by-supplement-group interaction was observed.

effect was observed. The trained group had a significantly higher $\dot{V}O_2$max after treatment than did the untrained group (42.8 ± 3.8 and 39.7 ± 4.5 mL · min^{-1} · kg^{-1}, respectively; linear mixed model $P < 0.001$). Within the training group, the number of training sessions attended (range: 21–40 sessions) was not significantly associated with the 8-wk change in $\dot{V}O_2$max (linear regression: $r^2 = 0.01$, $P = 0.42$).

Interaction of supplement and training

Significant interaction effects between supplement type and training group were observed for sFer and hemoglobin ($P = 0.072$ and 0.030, respectively; Table 5). The study was not powered at the 0.05 level to detect a clinically relevant 2-way interaction between training and supplement type, such as that observed for sFer. In addition, the calculated sample size of 25 women/group was not achieved due to research conditions in the field. Therefore, the P value for the interaction (P = 0.07) was treated as being significant, even though it did not reach the prespecified 0.05 level. A reduced model was analyzed that included only the supplement-by-training group interaction term, where it retained its significance (P = 0.07). Post hoc pairwise comparisons between supplement and training groups were adjusted for multiple comparisons with the use of a Bonferroni correction (Table 5).

In examining the training-by-supplement interaction for sFer, shown in **Figure 2**, both the FeTr and FeUn groups showed significantly higher sFer values than either of the placebo groups. However, sFer in the participants who received training in addition to iron supplementation was significantly lower than that in those participants who did not train. For the training-by-supplement interaction for hemoglobin, the FeUn group had significantly higher hemoglobin concentration after treatment than the PLUn group. There were no other significant post hoc comparisons for the training-by-supplement interaction for hemoglobin.

Academic semester of testing was included as a covariate in all linear mixed models but was not significant. The interaction between training and supplementation for the sFer model was maintained when baseline age, height, weight, and $\dot{V}O_2$max were included as covariates in the models predicting sFer. None of these covariates were significant at $P < 0.05$. To determine whether the changes observed in sFer were independent of hemoglobin, a model was analyzed including hemoglobin as a covariate. The significance of the training-by-supplement interaction and all trends in the post hoc comparisons were maintained when hemoglobin was included as a covariate

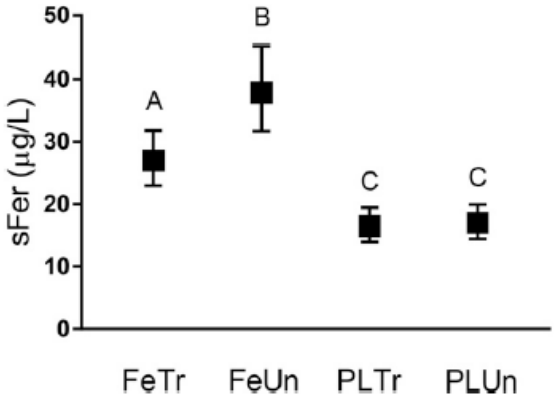

FIGURE 2 Least-square means for the training-by-supplement-type interaction for serum ferritin. Values are post hoc pairwise comparisons between supplement and training groups, with 95% CI bands for the reduced model that did not include time point. n = 19, 16, 18, and 19 for FeTr, FeUn, PLTr, and PLUn groups, respectively. $P = 0.07$ for the supplement-by-training-group interaction. Uppercase letters indicate significant differences between groups with differing letters. Results are from linear mixed models controlling for baseline, in which Bonferroni corrections for multiple comparisons were applied to the post hoc pairwise comparisons between groups. FeTr, iron trained; FeUn, iron untrained; PLTr, placebo trained; PLUn, placebo untrained; sFer, serum ferritin.

(P-interaction = 0.09), whereas hemoglobin was not significant in the model (P = 0.81).

To examine the biological plausibility of the main findings, several secondary analyses were conducted. No relation was found between the number of days trained and change in sFer in either of the supplement groups. In addition, there was no significant association between the number of capsules consumed and the change in sFer in either of the supplement groups, even when sFer status at baseline was included as a covariate. The same result was seen with the use of the more conservative compliance measure for the number of capsules consumed. However, when only those participants who returned 100% of their capsule bottles and daily logs were examined (n = 25), there was a positive relation between the number of capsules consumed and the change in sFer for the iron group (r^2 = 0.39, P = 0.04) but no relation in the placebo group (data not shown).

Participants who had the highest sTfR concentrations at week 0 (i.e., the lowest tissue iron concentrations) showed the largest decreases in sTfR over the 8-wk study period when given iron supplements, but no change was observed in those given the placebo (supplement by week 0 sTfR concentration interaction, linear model $P < 0.001$). This same trend was observed for body iron, with those participants starting with the lowest body iron showing the largest improvements in body iron over the 8-wk study (supplement-by-week 0 body iron concentration interaction, P = 0.03).

In a subgroup analysis of participants with more severe iron depletion (sFer <20 μg/L) at baseline, 100% of the FeUn group repleted their iron stores by 4 wk, and this resolution persisted until 8 wk. Conversely, after 4 wk, only 53.5% ± 12.9% of the FeTr group had resolved their iron depletion and only 73.3% ± 11.4% had resolved their iron depletion by week 8. **Figure 3** shows the prevalence of iron depletion within each group at each time point. RR calculations were performed to determine pairwise differences in percentage resolution between the 4 treatment groups. At the end of the 8-wk study period, the FeTr group was no more likely to be iron replete than the PLTr or PLUn group (FeTr/PLTr: RR = 1.83; 95% CI: 0.92, 3.66; P = 0.51; FeTr/PLUn: RR = 2.05; 95% CI: 0.95, 4.42; P = 0.39); however, the FeUn group was 2.5 times more likely to be replete than the PLTr group and 2.8 times more likely to be replete than the PLUn group [RR: 2.50 (95% CI: 1.35, 4.65; $P < 0.05$) and 2.80 (95% CI: 1.39, 5.65; $P < 0.05$), respectively].

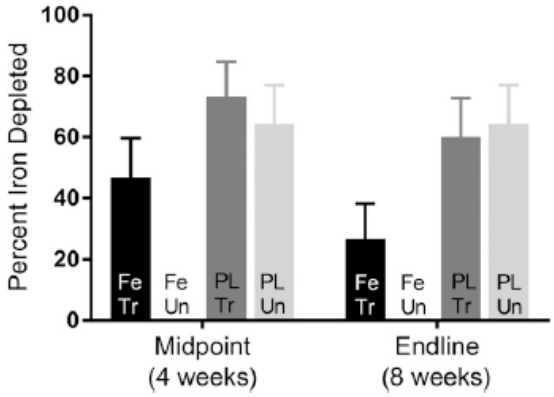

FIGURE 3 Women with iron depletion (sFer <20 μg/L) by treatment group at 4 and 8 wk. All of the participants were iron depleted (sFer <20 μg/L) at baseline. n = 19, 16, 18, and 19 for FeTr, FeUn, PLTr, and PLUn groups, respectively. Values are percentages ± SEs. No FeUn participants were depleted at week 4 or 8 (i.e., there was total resolution of iron depletion in this group). FeTr, iron trained; FeUn, iron untrained; PLTr, placebo trained; PLUn, placebo untrained; sFer, serum ferritin.

DISCUSSION

Improvements in iron status measures in both of the iron-supplemented groups show that the supplementation regimen was adequate to elicit changes in iron status. After 8 wk of treatment, the iron-supplemented groups had significantly higher sFer and body iron and significantly lower (indicating better iron status) sTfR concentrations than the placebo groups. Previous studies have shown that 100 mg ferrous sulfate/d is sufficient to cause changes in iron status markers of similar magnitudes as those observed in this study (4). Similarly, post-training improvements in $\dot{V}O_2$max in the trained groups confirm that the training program was sufficient to induce physiologic adaptations. The high compliance to the training program (89% of participants attended >28 d) likely explains this effect, although the narrow range in the number of sessions attended likely limits our ability to find a dose-response effect of training on $\dot{V}O_2$max.

Aerobic training lowered the effectiveness of iron supplementation in improving traditional measures of iron status, as evidenced by the significant training-by-supplement interaction in linear mixed models of sFer effects. Although both iron-supplemented groups showed significant improvements in sFer, the improvements in the trained participants were significantly smaller than those in the untrained group. One interpretation of this finding is that participants who trained while taking iron supplements did not benefit from the supplements as much as those who did not train.

In addition, by week 8, 100% of the FeUn group had resolved their iron depletion, as defined by sFer <20 μg/L, compared with only 73% of the FeTr group. These findings are consistent with those of McClung et al. (14), who found that participation in basic combat training decreased iron status in female soldiers (27).

Although the modifying effect of aerobic training on iron status is shown by the results of this study, the physiologic mechanism explaining this effect remains unclear. Possible explanations include reduced absorption of iron due to exercise-induced inflammation, increased demand for iron in the oxidative pathways, or increased production of total body hemoglobin or myoglobin, which could be supported by redirecting iron from iron stores toward erythropoietic processes (10, 28–30).

There is currently conflicting evidence about whether long-term moderate exercise results in a chronic inflammatory state that could affect iron status. Both acute and chronic inflammation results in increased concentrations of hepcidin, the primary regulator of iron absorption in the body. Although studies have shown a relation between increased hepcidin expression and lower iron status after acute exercise (31, 32), few studies have shown a long-term impact (32, 33). Outside of the role of hepcidin, several studies have shown that inflammation induced by exercise (34) or chemical injection (35, 36) results in decreased expression of proteins required for iron absorption into the enterocyte. Although this study did not detect inflammation with the use of CRP or AGP, inflammation may still have been

present in this population. This study did not measure hepcidin, its mediator IL-6, or proteins involved in iron absorption. Therefore, we cannot determine whether inflammation or absorption differed between the FeTr and FeUn groups.

There is evidence that exercise-induced increases in erythropoiesis draw iron from the other iron stores in the body to meet the increased iron demand (10, 37), which could explain the smaller increase in sFer observed in the FeTr group than in the FeUn group. However, the details of muscle iron homeostasis in conditions of high erythropoietic demand, such as long-term aerobic training, remain unclear. Some studies have shown that the increased iron demand for erythropoiesis draws iron from myoglobin (10), whereas others suggest that it comes from the liver (11, 37). It is possible that the iron entering the body in the FeTr group was used directly for erythropoiesis or muscle growth. In this study, the FeUn group showed significantly higher hemoglobin after iron supplementation than the PLUn group; however, the FeTr group did not have significantly higher hemoglobin than the other groups. This finding may support the idea that training increased the erythropoietic demand for iron and that the supplemented iron was used directly for this process rather than being stored as ferritin. However, if training were increasing the demand for iron for erythropoiesis, it would therefore be expected that the PLTr group would show decreases in their serum ferritin, which was not observed. It is possible that iron contained in muscle myoglobin was diverted for use in erythropoiesis in the PLTr group; however, this study was unable to assess myoglobin and thus cannot determine whether this was the case.

Finally, it is possible that there was an increased iron demand in skeletal muscle mitochondrial bioenergetics pathways, because many enzymes and cofactors in energy production are iron dependent. It has been shown that ID impairs mitochondrial function (10) and that aerobic exercise training in combination with iron supplementation improves mitochondrial function and density (38–40). Therefore, it is also feasible that the iron in the FeTr group was used to synthesize the enzymes needed for increased energy production or skeletal muscle growth known to occur with training (10, 41).

One limitation of this study is its short duration, which may have been insufficient to observe any long-term effects. In addition, blood and exercise data were not collected for women who dropped out, thus limiting our ability to perform a true intention-to-treat analysis. The participants who dropped out or were excluded from analyses due to discrepancies in their sFer values at baseline may have been systematically different from those who were retained; however, there were no baseline differences between those participants who dropped out or were excluded and those who were retained. The study may have had increased type I error due to the assessment of multiple biomarker variables. In addition, the training group was not blinded, potentially introducing bias. Finally, this study was unable to measure other, potentially more appropriate measures of iron status, such as those involved in inflammation, muscle growth, or erythropoiesis. Further investigation requires more invasive or more expensive analysis techniques, such as metabolomics, that would provide a more comprehensive view of iron metabolism. However, our results support the idea that sFer and hemoglobin may not be the best biomarkers for iron status in nonanemic, physically active populations.

In conclusion, we have shown that regular aerobic training diminishes the effectiveness of iron supplementation on improving sFer in iron-depleted, nonanemic women compared with untrained women. Iron supplementation was still able to increase sFer in trained women, but at a slower rate. This finding could have implications for interventions aiming to improve iron status in physically active populations, such as in women who train casually to improve fitness in developed countries or in developing countries where heavy manual labor is necessary for economic livelihoods. Typically, iron supplementation interventions can have durations of 4–6 wk at the dose used in this study (4, 42). This dose can be sufficient in that time frame, as evidenced by the 100% resolution of iron depletion in the FeUn group. However, our study suggests that longer interventions may be necessary to improve iron stores in active women. Further research should explore whether a larger dose of iron can counteract the modifying effect that daily exercise has on improvements in sFer and whether this effect has any long-term impacts. In addition, further research should determine an optimal iron intervention dose and duration for active populations and develop other, more appropriate biomarkers that are more indicative of iron involved in muscle metabolism and erythropoietic processes.

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The authors' responsibilities were as follows—LMP: conducted the research, analyzed the data, and had primary responsibility for final content; and both authors: designed the research, wrote the manuscript, and read and approved the final manuscript. Neither of the authors reported a conflict of interest related to the study.

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