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REVIEW ARTICLE

Iron and copper in male reproduction: a double-edged sword

Eva Tvrda · Rohan Peer · Suresh C. Sikka · Ashok Agarwal

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Abstract Iron and copper are essential trace nutrients playing important roles in general health and fertility. However, both elements are highly toxic when accumulating in large quantities. Their direct or indirect impact on the structure and function of male gonads and gametes is not completely understood yet. Excess or deficiency of either element may lead to defective spermatogenesis, reduced *libido*, and oxidative damage to the testicular tissue and spermatozoa, ultimately leading to fertility impairment. This review will detail the complex information currently available on the dual roles iron and copper play in male reproduction.

Keywords Iron · Copper · Male fertility · Spermatozoa · Oxidative stress · ROS

Introduction

Iron (Fe) and copper (Cu) are trace elements that constitute an essential ecophysiological component of cells and tissues present in the male reproductive system. In relatively small amounts, these micronutrients are essential cofactors of a broad array of bioactive molecules. However, their accumulation in large amounts may lead to metabolic disruptions which in turn could compromise male fertility. Thus, both elements may act as "double-edged swords" based on their ability to either maintain cellular homeostasis as micronutrients or to overturn this balance as catalysts responsible for serious structural and functional alterations. The aim of this review is to clarify the complex information available on both positive as well as negative effects these trace elements have on male reproduction.

Capsule Iron and copper play crucial roles in the physiology as well as pathology of male reproduction.

E. Tvrda · R. Peer · A. Agarwal (✉)
Center for Reproductive Medicine, Cleveland Clinic,
Cleveland, OH, USA
e-mail: agarwaa@ccf.org

E. Tvrda
Department of Animal Physiology, Slovak University of Agriculture,
Nitra, Slovakia

S. C. Sikka
Department of Urology, Tulane University School of Medicine,
New Orleans, LA, USA

Iron

Iron can be divided into two categories: heme and non-heme. Meat containing hemoglobin is the main source of heme iron. Heme in form of hemoproteins has diverse biological functions, including reversible binding of gases, enzyme catalysis and electron transport [1]. Non-heme iron, which is found in cereals, fruits, and green leafy vegetables rich in vitamin C [2], helps to stabilize optimal iron concentrations within the organism [3]. Heme iron is more readily absorbed than non-heme iron [2].

Anthropogenic sources of iron include steel industry, sewage and dust from mining [4]. Iron sulphate is furthermore used as a fertilizer and herbicide [5].

Iron is primarily found in hemoglobin of erythrocytes. Small amounts of Fe are present in muscle myoglobin, macrophages and blood plasma [6, 7].

Transferrin is the primary transport protein for Fe and represents an essential iron pool. When needed, Fe enters the cell via transferrin membrane receptor (TfR) mediated endocytosis. The micronutrient subsequently dissociates throughout the cytosol and is taken up by ferritin, the most effective Fe storage protein [8], which is able to bind up to 4,500 atoms of Fe in one molecule [9].

As a transition metal, iron can easily donate an electron during oxidation to its active ferric form (Fe^{3+}) or remain in a stable reduced ferrous state (Fe^{2+}), which is more commonly found in the cytoplasm [8, 10, 11].

Within the male reproductive system, Sertoli and Leydig cells are important sources of ferritin. The molecule acts as a readily available source of Fe for the developing spermatozoa, while providing an extra layer of protection to the testicular tissue [8, 9].

Both ferritin and transferrin are regulated by the iron regulatory proteins (IRP-1/IRP-2) found in the cytoplasm [6]. Cellular Fe absorption, concentration, and accumulation are regulated via hepcidin – a liver enzyme, together with ferroportin - an exporter protein, responsible for the excretion of iron from the cell [12] as shown in Fig. 1a.

Copper

Meat, shellfish, seeds, legumes, nuts, whole grains, potatoes, and chocolate are major sources of copper [13]. Common industrial sources of Cu include municipal garbage, agricultural chemicals, construction sites, electrical equipment and vehicles [14].

Cu has two oxidation states: cuprous (Cu^{+}) and cupric (Cu^{2+}). Cu^{2+} is fairly soluble whereas Cu^{+} solubility is in a submicromolar range [15]. In biological systems, copper is found mainly in the Cu^{2+} form since in the presence of oxygen or other electron acceptors Cu^{+} is readily oxidized to Cu^{2+}. Cu oxidation is reversible as Cu^{2+} can accept an electron from strong reductants such as ascorbate [16] and reduced glutathione [17].

Dietary copper is mainly bound to serum albumin and is carried to the liver. A small amount of Cu is excreted in bile whereas the rest binds to ceruloplasmin and is released into the bloostream [18]. An overview of Cu metabolism is shown in Fig. 1b.

Ceruloplasmin is the primary copper-binding protein; six Cu atoms bind to each molecule (Fig. 2). Within the testes, approximately 80 % of seminal ceruloplasmin is located in the Sertoli cells [19, 20].

The remaining Cu is bound to metallothioneins (MTs), storage proteins for both Cu and zinc [21]. MTs are known to detoxify a variety of heavy metals in the male reproductive system of mice, rats and humans [22, 23]. Two major MT isoforms and their corresponding mRNAs have been shown to be expressed primarily in Sertoli and spermatogenic cells to protect the germinal epithelium [24]. Furthermore, Sugihara et al. [25] detected an additional MT-like protein, tesmin, specifically expressed in spermatocytes as early as day 8 of postnatal development, coinciding with the entry of germ cells into meiosis. Therefore, it is currently used as an early marker of male germ line differentiation.

Metabolic interactions between iron and copper

Close chemico-biological relations between the iron and copper metabolism have been recognized for many years [26]. Ceruloplasmin, a Cu-dependent ferroxidase, represents a fundamental bridge between Fe utilization and Cu status. It is associated with the oxidation of ferrous ion into ferric, enabling its transport with transferrin, which can carry only trivalent iron [27].

The presence of ceruloplasmin in semen was confirmed almost 50 years ago. Orlando et al. [19] suggest that it may be of testicular origin, serving as a marker of a proper seminiferous tubule function.

The ferroxidase properties of ceruloplasmin, and possibly ferritin, are negatively affected by low Cu levels in circulation. Subsequently, the Fe concentration decreases, possibly leading to an anemic state which can only be treated with Cu supplementation [28].

Iron, copper and cellular oxidative balance

Superoxide (O_2^{-}), hydrogen peroxide (H_2O_2), peroxyl (•ROO), and hydroxyl radical (•HO) are common types of reactive oxygen species (ROS) implicated in oxidative stress (OS) development [29]. Conversely, antioxidants including superoxide dismutase (SOD), catalase (CAT), vitamin E, selenium or glutathione (GSH), quench or neutralize ROS in order to decrease their ability to spread [30]. A proper balance between ROS and antioxidants is to be maintained in all cells. This equilibrium may shift towards the pro-oxidant state when ROS production is significantly increased or in case of antioxidant delpetion. The resulting state can subsequently lead to cellular damage or apoptosis [29, 30].

Iron and copper are important components of superoxide dismutase [31] and catalase [32] - two main antioxidant enzymes preventing fluctuations in ROS and protecting the cellular structure and function against oxidative damage. SOD spontaneously dismutates O_2^{-} to form oxygen (O_2) and H_2O_2, while CAT breaks H_2O_2 into O_2 and water (H_2O) [33]:

$$2\left(O_2^{-}\right) + 2H \rightarrow H_2O_2 + O_2\,(catalyzed\,by\,SOD)$$

$$H_2O_2 \rightarrow H_2O + 1/2O_2(catalyzed\,by\,CAT)$$

In a biological system, both Fe and Cu are essential for electron transport [15]. At the same time, their transition and redox characteristics when free or unbound, determine their reactivity with oxygen via the Haber–Weiss and/or Fenton reaction. ROS are overproduced as a consequence, being responsible for oxidative damage to biomolecules [34].

The Fenton and Haber-Weiss reaction are responsible for •HO generation driven by the reduction of iron or copper by superoxide in the presence of a catalyst,

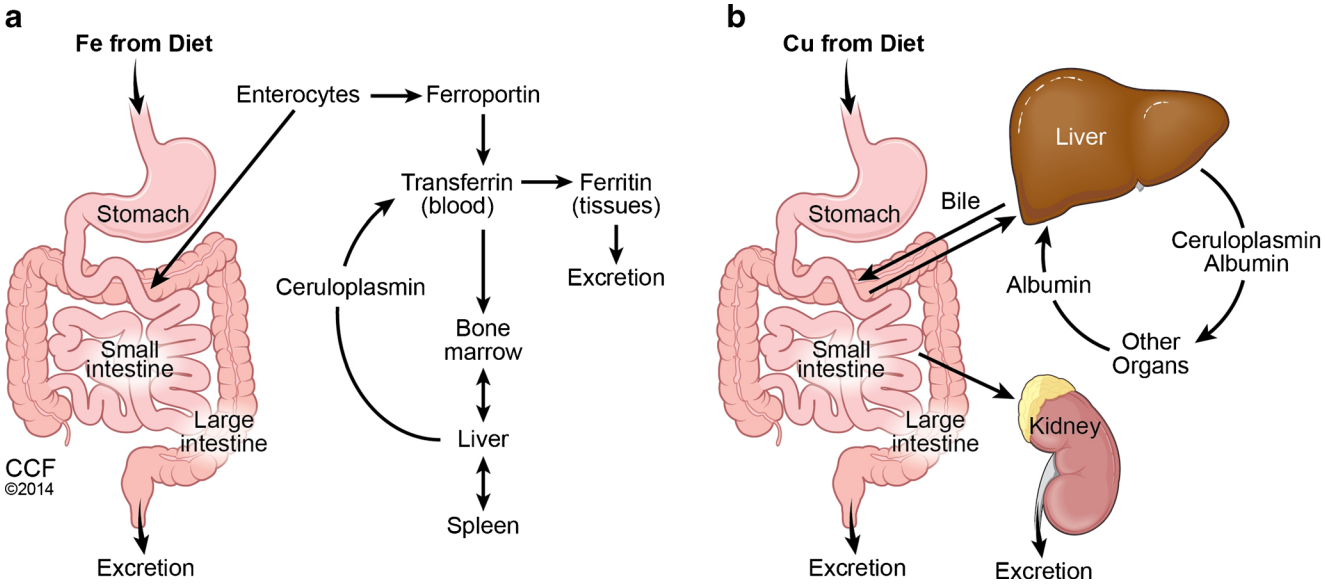

Fig. 1 A brief overview of iron **a** and copper **b** metabolism. 1–2 mg of Fe are ingested from the diet per day. The metal binds to ferroportin in the intestinal cells, and is subsequently transferred via transferrin. Iron in tissues is bound to ferritin, most commonly in the bone marrow (haemoglobin synthesis), liver and spleen (blood recycling and Fe storage). Iron is excreted either via defecation or blood loss.**b** 1.2–2 mg of dietary Cu are ingested per day. 25 % of this will be transported to the liver in the form of ceruloplasmin, which will serve as Cu pool. Ceruloplasmin and albumin transport Cu in organs. 25 % Cu will be excreted as urine while about 50 % Cu will be released as a methallothionein complex

which chelates to a specific ligand, thus enabling the redox cycling. Ordinarily, both processes are slow, but in the presence of free ferric or cupric ions, they occur aggressively, leading to chain reactions with a subsequent cellular damage [35, 36].

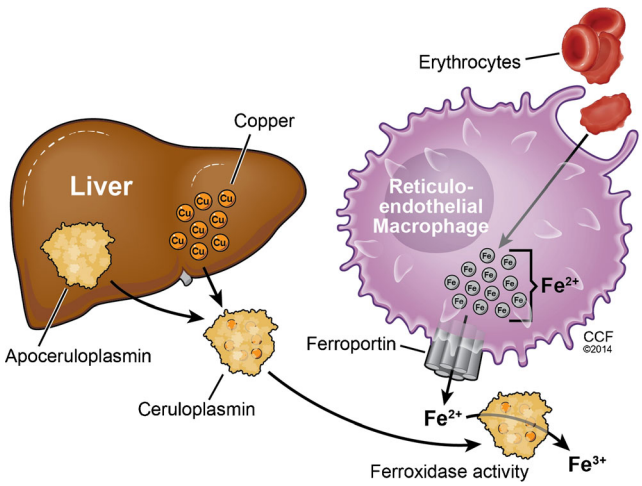

Fig. 2 Ceruloplamsin – evidence of the synergistic relationship between iron and copper Ceruloplasmin, an enzyme synthesized in the liver, exemplifies the interconnections between iron and copper. Structurally, it contains atoms of copper, which is why it is able to carry more than 95 % of the total copper in plasma. Ceruloplasmin exhibits a copper-dependent oxidase activity, which is directly associated with oxidation of ferrous iron into ferric iron. This assists in its transport in association with transferrin, as this can carry trivalent iron exclusively. Both Cu-transport as well as feroxidase activities are exhibited in the testes, primarily assisting in processes related to spermatogenesis

The Haber-Weiss reaction is the first step, involving a reduction of the ferric/cupric ion to ferrous/cuprous ion [36]:

$$Fe^{3+}/Cu^{2+}(ligand) + O_2^- \rightarrow Fe^{2+}/Cu^{+}(ligand) + O_2$$

The second step involves the actual Fenton reaction [35]:

$$Fe^{2+}/Cu^{+}(ligand) + H_2O_2 \rightarrow Fe^{3+}/Cu^{2+}(ligand) + OH^- + {}^{\cdot}HO$$

Net reaction [144]:

$$O_2^- + H_2O_2 \rightarrow OH^- + {}^{\cdot}HO + O_2$$

Simultaneously, transferrin and ferritin can also support the ROS generation [37, 38]. The heme found at the active site of Fe-containing proteins can be oxidized by hydrogen peroxide to release free iron, generate ferryl-heme as well as more ROS [39].

Metabolic roles of iron and copper in male reproduction

Iron

Iron is one of the most abundant mineral nutrients in the organism and plays a critical role in the synthesis of nucleic acids and proteins, electron transport, cellular respiration, proliferation and differentiation [11], all of which are intimately related to spermatogenesis and spermatozoa metabolism [8].

Three mammalian gene expressions are directly regulated by iron [11], two of which have an impact on male reproduction.

The protein kinase C-beta, a member of the protein kinase C family, has been localized in human semen and associated with flagellar motility [40]. Furthermore, Kalina et al. [41] detected the enzymatic family in distinct structures of human sperm (head, neck, and tail), thus proposing their involvement in various aspects of sperm physiology.

The type 5 isozyme of acid phosphatase is an Fe-containing molecule found in semen in large quantities and of prostatic origin. It is believed that its presence may be associated with the liquefaction process of semen [42]. Interestingly, increased amounts of this enzyme are connected to a variety of male diseases, especially prostate cancer, which led to its establishment as a clinically useful tumor marker [43].

Fe is involved in a variety of redox reactions catalyzed by cytochromes, with a subsequent energy production, drug and hormonal metabolism, propagation and activation of the defense systems via the nicotinamide adenine dinucleotide phosphate (NADP) oxidase [11]. The connection between iron and the Krebs cycle is further solidified by the mitochondrial aconitase enzyme [44]. Under ROS overproduction or in a state of iron deficiency, cellular respiration is inhibited by the nitrosylation of heme in mitochondrial enzymes aconitase and glyceraldehyde-3-phosphate dehydrogenase [45], leading to a depletion of adenosine triphosphate (ATP) and a subsequent loss of spermatozoa motility [46].

It is widely known that during spermatid development, mitochondria undergo dramatic events related to movement and shaping, including aggregation and fusion or elongation alongside the growing axoneme [47]. Disruptions of mitochondrial function typically affects male fertility - a phenotype that is easily screened for and characterized at the subcellular level. The mitoferrin gene product and other proteins involved in iron metabolism show enriched expression in the testes suggesting that mitochondrial iron metabolism plays a role in spermatogenesis [47, 48].

Metzendorf and Lind [48] established a *D. melanogaster mitoferrin* (*dmfrn*) mutant line and showed that the *dmfrn* mitochondrial iron importer is required for spermatid mitochondrial morphogenesis and thus for the development of mature and motile sperm. Moreover, this study showed that mitoferrin could play a direct role in mitochondrial dynamics, analogous with dual roles in both ATP synthesis and shaping of the inner mitochondrial membrane [48]. Further experiments in vertebrates and yeast demonstrated a role for the mitoferrin family members in Fe import from the cytosol into mitochondria [49, 50].

In addition, Nikolaev et al. [51] stated that Fe and nonhemic ferroproteins are involved in ejaculate thinning and viscosity, sperm pH, alongside with normal spermatogenesis.

The importance of iron in male fertility has been shown in a variety of *in vivo* and *in vitro* studies. According to Kanwal et al. [52], it was only iron from all the bulk elements evaluated in the seminal plasma of Niki-Rawi bulls, which was significantly and positively correlated with sperm motility. Tvrda et al. [53] showed that iron quantified in bovine seminal plasma was positively associated with sperm motility characteristics.

An *in vitro* study showed that iron (≤250 μM/L $FeSO_4.7H_2O$) sustained spermatozoa motility and energy metabolism, a key factor supporting spermatozoa function. Inversely, the sperm motility increased with low *in vitro* concentrations of iron (≤62.50 μM/L $FeSO_4.7H_2O$) [54].

Copper

Similarly to iron, the ability to participate in one-electron reactions has allowed Cu to gain a strong foothold in the redox reactions of living matter [15].

A direct or indirect participation of this element in a variety of oxidation and reduction reactions is closely related to its function as a metal cofactor for numerous enzymes e.g., diamine oxidase, copper/zinc (Cu/Zn) SOD, cytochrome c oxidase and tyrosinase [55].

As a terminal oxidase, the cytochrome c oxidase (Cyc) is located in the inner mitochondrial membrane. A two-atom copper center (CuA) accepts electrons from cytochrome c (Cyt c) and transfers them via a low-spin heme group (heme a) to a heme a3–CuB binuclear center where the four-electron reduction of oxygen to water occurs [56]. This electron carrier thus acts as the terminal complex of the electron transport chain, making the enzyme an integral component of oxygen consumption and energy production. Cyc also plays a role in oxidative phosphorylation as a transporter of protons across the mitochondrial membrane (Fig. 3) [56, 57].

Sperm motility is dependent on aerobic energy metabolism, of which the apparent rate-limiting step of the mitochondrial respiratory chain is catalyzed by Cyc. Hüttermann et al. [58] reported the occurrence of a Cyc subunit VIb displaying a testes and spermatozoa-specific isoform in humans, bulls, rats, and mice (Cyc VIb-2), facilitating aerobic metabolism when needed [59].

Diamine oxidase (DAO) is a different Cu-dependent enzyme that degrades polyamines and is present at very high levels in the human seminal plasma. The enzyme is assumed to originate from the prostate and upper genital tract (testis and/or epididymis). As shown by Le Calve et al. [60], DAO activity was strongly correlated with a variety of prostatic markers, thus it may be related to significant changes in the spermatozoa metabolism [61].

A number of human and animal studies have demonstrated that Cu has direct and positive effects on semen quality parameters. Cu levels in the seminal plasma were lower in

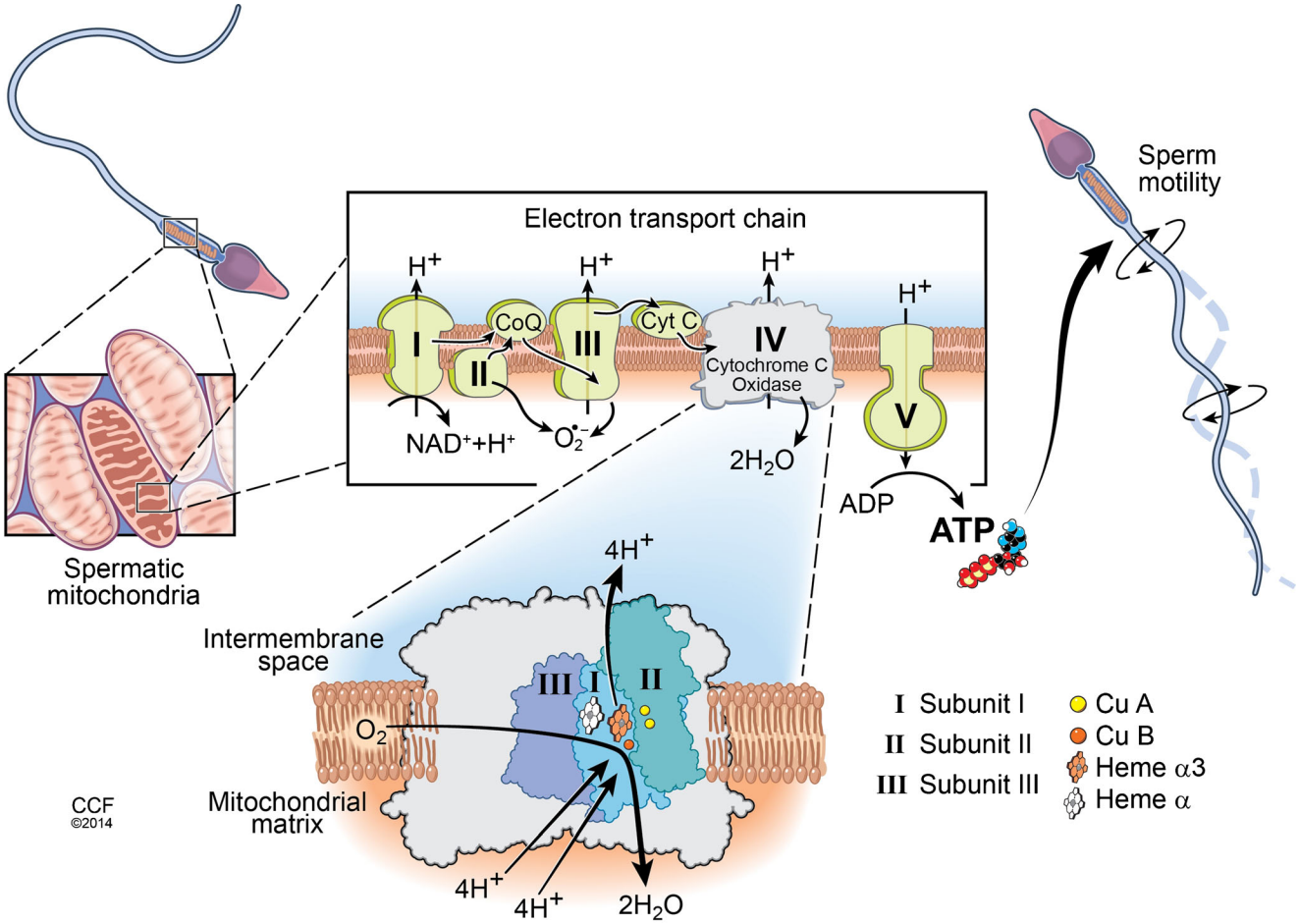

Fig. 3 Cytochrome c oxidase This enzyme is located in the inner mitochondrial membrane, facilitating the oxidation of cytochrome c^{2+} to cytochrome c^{3+} as well as the reduction of oxygen to water. Therefore, it plays an integral role in oxidative phosphorylation and energy metabolism. A specific isomer is present in the testes, allowing maximal aerobic metabolism with a subsequent production of ATP needed for spermatozoa motility

azoospermic males compared to healthy controls in a study by Saleh et al. [62]. Furthermore, Akinloye et al. [63] showed that Cu had significant positive effects on semen volume. Abdul-Rasheed [64] noted a significant decrease of Cu levels in the seminal plasma of azoospermic patients, leading to a concomitant decrease in the SOD activity and a higher risk of oxidative stress. Wong et al. [65] demonstrated a weak but significant positive correlation between blood Cu and sperm motility. Furthermore, Machal et al. [66] reported a positive correlation between the Cu concentration in bovine blood plasma, sperm count as well as progressive motility.

In vitro addition of low concentrations of $CuSO_4$ to a semen extender improved the total antioxidant capacity of the ejaculate of water buffaloes. The *in vitro* presence of copper improved the antioxidant capacity of the semen samples [67]. Furthermore, it has been shown that Cu modulated the Cu/Zn SOD activity, and the addition of Cu to a testicular cell culture increased the enzymatic activity of Cu/Zn SOD [68].

Iron, copper and seminal oxidative balance

As illustrated earlier, CAT contains heme and thus is an Fe-dependent enzyme [32], whereas copper is a major contributor to the functionality of Cu/Zn-SOD [31], both of which are commonly found in the ejaculate, and are produced by the testis, epididymis, accessory reproductive organs, as well as spermatozoa [69].

Diverse studies have reported on the role both enzymes play as antioxidants in reproductive biology. SOD protects spermatozoa against spontaneous O_2 toxicity and lipid peroxidation (LPO) [70, 71]. SOD and CAT also remove superoxide radicals generated by NADPH-oxidase in neutrophils and may play important roles in protecting spermatozoa during genitourinary inflammation [72].

SOD and CAT activities were positively associated with semen quality parameters in mammals including rapid progressive motility, nonprogressive motility, viability and spermatozoa concentration [71, 73, 74]. Inversely, decreased activities of both enzymes were observed in infertile men

[75–77]. Furthermore, *in vitro* supplementation of both enzymes has led to a significant improvement of human, dog and stallion semen [78–80].

Tvrda et al. [81] showed that physiological levels of iron and copper exhibited a positive relationship with a variety of antioxidant markers of bovine seminal plasma and spermatozoa, followed by a negative association with LPO.

Lastly, Cu/Zn SOD deficient mice usually develop smaller testes [82], and their spermatozoa have reduced motility and average path velocity. Moreover, it has recently been demonstrated that Cu/Zn SOD deficient mice have a reduced capability to penetrate the zona pellucida during *in vitro* fertilization [83].

Male reproductive complications associated with iron and copper deficiencies

Iron

Anemia (a decrease in the number of erythrocytes or hemoglobin leading to a lower capacity to transport oxygen) is the most common pathology resulting from a direct Fe deficiency. Furthermore, the disease may be triggered indirectly by low Cu levels with a subsequent negative impact on ceruloplasmin and its ferroxidase properties, followed by low Cu and Fe levels to be released throughout the organism [84].

Anemia induces a significant hypoxic environment in the testes. Spermatogenesis occurs under a high proliferation rate, demanding considerable oxygen consumption. However, under anemic conditions the testicular PO_2 is relatively low, oxygen diffusion is slow and the testicle has little capacity to increase total blood flow [85]. Therefore, males presenting with iron or copper deficiency-related anemia may present with poor semen parameters [86]. Common anemia may be manageable with supplementation or intravenous therapy, significantly enhancing the hormonal as well as semen profile of the patients [87].

Infertility is a known complication in males with sickle cell disease, which is usually accompanied by low levels of ferritin and a general iron deficiency [88–90]. This has been attributed to a relative primary gonadal failure and a delayed or impaired sexual development [90, 91]. Men with sickle cell disease generally have a smaller ejaculate volume, poorer sperm motility, reduced sperm density, and fewer spermatozoa with normal morphology [89]. Most importantly, such patients are often diagnosed with primary testicular failure, characterized by low levels of testosterone [91–95], aggravated by impotence secondary to earlier priapism [92]. Interestingly hydroxyurea, a drug used to manage sickle cell anemia, is detrimental to male reproduction. Reported side effects include testicular atrophy, low sperm count, poor spermatozoa motility and morphology, as well as germ cell DNA damage [96, 97].

Beta thalassemia, a disease caused by a reduced production of beta chains of hemoglobin leading to iron deficiency, has been shown to be highly associated with male infertility, sexual dysfunction, lack of effective pubertal growth, and inadequate sexual development [98, 99]. However hormonal complications are common in β-thalassemia patients due to iron overload from blood transfusions, administration of chelating agents, or splenectomy. Iron overload can furthermore be caused by the body's demand for red blood cells in the absence of hemoglobin [100, 101]. Hypogonadism and abnormal spermatogenesis have been identified as primary causes of sexual dysfunction seen in β-thalassemia patients [100, 102]. The hypogonadism-like symptoms are related to either anemia [103] or iron accumulation in the pituitary gland [104]. Treating infertile male patients with β-thalassemia with growth hormone and gonadotropins improved semen parameters [105]. Furthermore, cryopreservation is recommended to thalassaemia patients prior to a full manifestation of the toxic effects of the disease [106].

Copper

Cu deficiency can result in higher levels of ROS, Fe toxicity, and downregulation of enzymatic activity. All of these have been shown to negatively impact sperm parameters and thus male fertility [83] (Fig. 4b).

A large study on experimentally induced Cu deficiency was performed on rams. Cu deficient animals produced ejaculates of lower volume, lower sperm concentration, poorer sperm motility and morphology. Histologically, the seminiferous tubules of the deficient animals were less developed and less active, mainly due to the inactivity of the Sertoli cells. However, after the copper deficiency was reversed, the above parameters reverted to normal [107, 108].

Decreased sperm count and motility have also been reported in Cu deficient rats [109]. Histomorphological examination of Cu-deficient goat testes showed an inactive germinal epithelium with signs of mild testicular degeneration [110]. Furthermore, poor semen quality in male mice with copper deficiencies led to a decreased ability of *in vivo* oocyte fertilization [82].

As seen in Cu-deficient murine embryos, low levels of ceruloplasmin result in Fe toxicity, whereas low levels of cytochrome c oxidase and catalase may lead to high levels of ROS [111]. The structure of metallothionein [112] and gluthathione [113], both ROS scavengers, may be altered by a lack of Cu with a subsequent loss of their functionality. This unregulated OS leads to DNA damage in the cell, which has been detected in Cu-deficient animals [114]. Furthermore, a study of testes-specific Cyc knockout mice in which the final electron transfer step is eliminated revealed reduced ATP

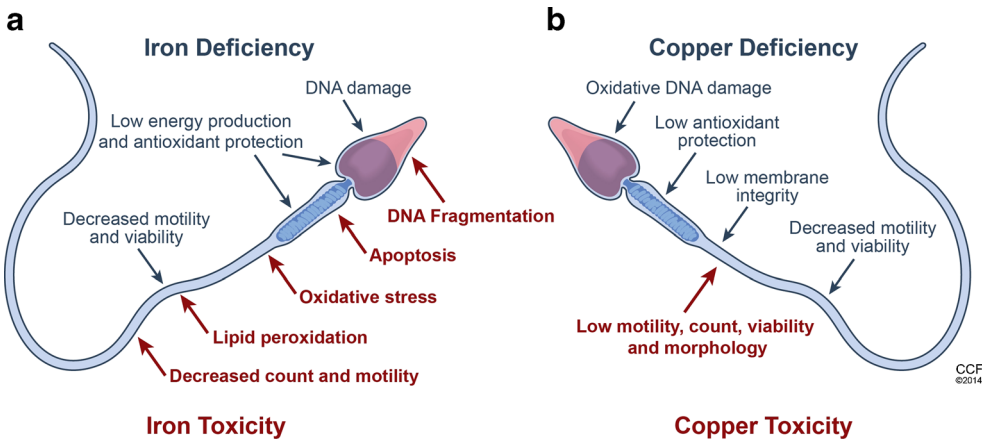

Fig. 4 The effects of iron **a** and copper **b** on the spermatozoon. Based on the currently available data, it may be concluded that deficiencies of both iron and copper may lead to reduced spermatozoa vitality, DNA damage, and a high risk of oxidative damage. Inversely, overload of one or both trace elements may cause disturbances to spermatogenesis as well as to crucial sperm cell structures accompanied by oxidative stress and cell death

concentrations, spermatozoa motility and overall fertility [115].

Male reproductive toxicity associated with iron and copper overload

Iron

If due to genetic, lifestyle, and environmental factors transferrin is unable to effectively regulate the amount of iron in the body, this will accumulate to toxic levels, with a negative impact on spermatozoa production [116] (Fig. 4a).

Wise et al. [8] showed that Fe and ferritin levels were negatively correlated with testicular weight in boars. Furthermore, boars with high Fe levels produced fewer spermatozoa. As the testicular Fe concentration increased, the daily spermatozoa production (DSP) declined. The study concluded that high levels of ferritin are associated with hypogonadism.

Excessive dietary doses of Fe resulted in testicular atrophy, morphological changes in the testes, impaired spermatogenesis, epididymal lesions and impaired reproductive performance [117–119].

In vitro experimental administration of $FeSO_4.7H_2O$ at doses≥125 μM/L significantly inhibited a variety of motion parameters in bulls [54].

As an autosomal recessive disorder, hereditary hemochromatosis disturbs the regular iron metabolism, leading to high levels of free iron in the organism. The HFE (High Iron Fe) gene controls the expression of hepcidin, which equally to transferrin, controls the circulation of iron in the body [120]. Fe toxicity develops as a consequence of C282Y and/or H63D mutations in the HFE gene (Fig. 5). Fe accumulation ultimately reaches a critical point when transferrin is unable to effectively manage the large Fe amounts in the organism [121].

Iron toxicity in association with hypogonadism results in atrophied testes with morphological changes and lesions in the seminiferous tubules, epididymes and Sertoli cells [122]. Sperm DNA damage was also observed, which has a high risk of inheritance to offspring [123].

Males with hemochromatosis tend to have symptoms related to impotence and lack of sexual desire due to Fe toxicity in the pituitary gland. This reduces the regular flow of gonadotropins leading to decreased testosterone levels [124].

Copper

Genetic preconditions, metabolic diseases and environmental pollution are the main factors leading to Cu overload [34], with a direct or indirect impact on male fertility (Fig. 4b). Negative correlations between Cu quantified in the seminal plasma and spermatozoa quality parameters such as motility, viability and morphology have been shown in several human studies [125, 126].

In vivo copper administration by gavage in rats resulted in increased testicular apoptosis and structural abnormalities such as atrophic and sclerotic tubules accompanied by a reduction in spermatogonial and Sertoli cells [127].

Knazicka et al. [128] indicated that at high doses (>19.09 μg/mL) Cu^{2+} negatively affected spermatozoa motility and mitochondrial activity in bulls. Roychoudhury et al. [129] found that $CuSO_4$ at doses>3.70 μg/mL had a negative effect on rabbit spermatozoa motility, morphology and membrane integrity. Similar results were observed by Rebrelo et al. [130] in human spermatozoa at Cu^{2+} concentrations of 100 μg/mL.

Wilson's disease, a different autosomal recessive disorder related to copper accumulation in the liver, brain, testes and eyes, has been shown to result in hypogonadism, impotence, and defective spermatogenesis [131].

Iron, copper and seminal oxidative stress

ROS are active participants in sperm capacitation, hyperactivation, and sperm-oocyte fusion. However, because spermatozoa

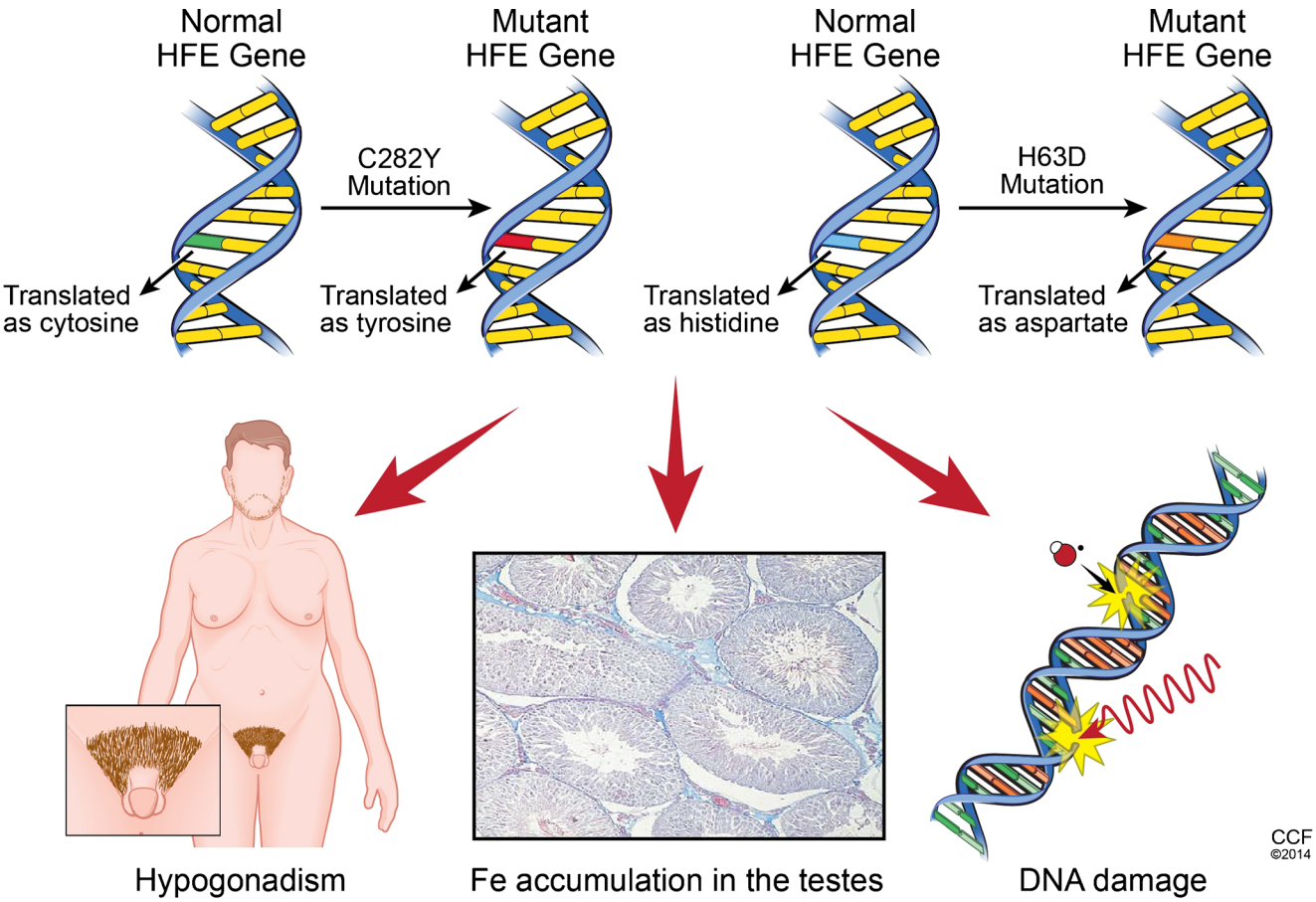

Fig. 5 The genetics of hereditary hemochromatosis Hereditary hemochromatosis is an autosomal recessive disorder that disturbs regular iron metabolism and subsequently leads to high levels of free iron in the organism. Iron toxicity develops as a consequence of C282Y and H63D mutation variants in the HFE gene. This disease leads to atrophied testes with pathological alterations in their structure, endocrine imbalance and a high risk of DNA damage, transmittable to offspring

lack cytoplasm – an important component containing antioxidants to counteract the damaging effect of ROS. Also, particularly large amounts of polyunsaturated fatty acids in the spermatozoa membrane can be easily degraded by ROS-derived lipid peroxidation [10, 132].

Generally, Fe overload increases OS in the testes and epididymis causing a depletion of lipid-soluble antioxidants such as alpha-tocopherol, ubiquinol-9, and ubiquinol-10, accompanied by damage to the lipids, proteins and DNA, impaired spermtaogenesis and a subsequent infertility [118, 133, 134]. Furthermore, Fe intoxication to epididymal and testicular cells *in vivo* as well as *in vitro* may cause oxidative damage in rat sperm DNA [135].

Male patients with diseases related to iron overload present with substantial oxidative damage to the spermatozoa, worsened by reduced antioxidant concentrations. The lack of vitamin E and C is commonly observed in such patients [136]. Iron overload and a subsequent free radical formation is ultimately detrimental to the testicular structure [134].

In vivo animal studies showed impaired sperm maturation and iron-dependent OS in rat. The amount of dietary iron was positively correlated with the extent of its deposition. Furthermore, the antioxidant concentration was lower in the testes of rats that had excess iron in their diet. High LPO occurred as the antioxidants were overpowered by Fe overload [134, 135]. Moreover, a collection of studies have concluded that Fe deposition and accumulation can be detrimental to sperm DNA. Oxidative damage to sperm DNA generated by iron were dose dependent, leading to apoptosis and a shorter sperm life span [106, 135].

Finally, lipid peroxidation as a consequence of the Fe-catalyzed Fenton reaction was previously observed in human [137], mouse [138], goat [139], bovine [140] and equine spermatozoa [141].

Similarly, copper toxicity also leads to ROS production followed by protein and lipid oxidation [142], which are negatively correlated with sperm motility and viability [81]. Once taken up into a cell, excess Cu is reduced to cuprous ions that readily bind with sulfhydryl groups [143] and ultimately inhibit ATP production by interfering with the electron transport [144]. Furthermore, Cu accumulates in the sperm mitochondria [145], decreasing the mitochondrial membrane

potential while causing ROS to form and leading to oxidative damage [146].

Strategies to minimize the negative impact of iron and copper on male fertility

Chelation therapy

Chelators are chemical agents of different chemical properties and biological effects, used in the treatment of metal poisoning. The chelate effect is based on the enhanced affinity of chelating ligands for a metal ion. Metal chelators are currently widely used to chelate iron and copper overload in diverse patients. Several studies have shown that chelation prevents the deleterious effects of metal accumulation in the organism [147–150], including male reproductive system, suggesting its potential for the protection against disorders during metal overload.

Deferoxamine is a common iron chelating agent [151] decreasing free Fe levels by inhibiting ferritin mRNA translation. Deferiprone is a different agent chelating free iron to prevent the propagation of Fe-dependent OS. It has been suggested that Fe chelation therapies could be improved by taking into consideration the involvement of a higher rate of ferritin production or depletion [152]. Chelation reversing iron overload may furthermore ameliorate the manifestations of hereditary hemochromatosis and help male patients with related reproductive complications [153].

On the other hand, deferoxamine has been shown to negatively impact the functionality of gonadotropic hormones. As such, it has antiproliferative effects during spermatogenesis and frequently causes oligospermia or asthenozoospermia [154].

Cu toxicity has been treated with other chelators such as d-penicillamine and trientine [155], which have been shown to vastly reduce the symptoms associated with diseases related to Cu accumulation. Zinc supplementation has also been used as it allows metallothionein to be absorbed into sites of Cu accumulation (e.g., small intestine), which easily blocks the movement of copper into the bloodstream [156]. Bathocuproine disulfonic acid is a chelator binding to free Cu and prevents further damage resulting from Cu accumulation.

Antioxidants

As oxidative stress from iron and copper overload is parcially responsible for male reproductive dysfunction, a potential strategy may be antioxidant therapy.

N-acetyl cysteine (NAC) has been generally used as an antioxidant in metal poisoning [157]. Several studies suggest the beneficial effect of NAC in oxidative stress by decreasing ROS production in health complications resulting from metal toxicity [158, 159].

The interaction between metal toxicity and vitamin E is well characterized. Vitamin E is an essential lipid-soluble antioxidant [30], thus represents the first line of defense against the peroxidation of polyunsaturated fatty acids (PUFAs) in the cellular and sub-cellular membrane phospholipids [160]. It is a major chain-breaking antioxidant in membranes directly neutralizing O_2^-, H_2O_2 and •HO [30]. Several studies have shown that vitamin E can prevent the majority of iron-mediated damage both in *in vitro* cultures [160], as well as in iron-loaded animals [161].

The a chain-breaking antioxidant action of manganese (Mn^{2+}) has been studied and confirmed on various peroxidizing systems [162]. It is an essential component of several enzymes, some of which (SOD, pseudocatalase) are involved in redox processes [163]. It has been shown that Mn^{2+} supplementation to human and bovine spermatozoa pretreated with ferrous ascorbate (FeAA) is favourable to preserve *in vitro* spermatozoa motility, viabiliy as well as a proper protection of the glutathione cycle [164].

High concentrations of Zn have previously been shown to provide cell protection from oxidative insult and subsequent apoptosis [165] as a result of copper-induced toxicity, as the tumor-suppressor protein p53 plays a role in Cu-induced death, but at the same time is part of the mechanism of Zn protection.

Metallothionein acts as an antioxidant able to bind to free copper in order to inhibit Cu toxicity. Furthermore, ascorbic acid may also be able to decrease the frequency of Cu depositions in the hypothalamus and any resulting oxidative damage [166].

Catechnis found in green tea directly scavenge diverse free radicals and their structure enables metal chelation including Fe^{3+} and Cu^{2+}[167]. At the same time, catechins are able to downregulate a variety of prooxidant enzymes (inducible nitric oxide synthase or xanthine oxidase) while upregulating antioxidant enzymes (including SOD, CAT and glutathione peroxidase) [168].

Kalpravith et al. [169] have shown that curcuminoids have the ability to allevaite OS in thalassemic patients, as shown by a decrease of LPO and activities of redox-sensitive redox enzymes (SOD and glutathione peroxidase), folowed by increased GSH levels. As bidenate chelators, curcuminoids were shown to effectively remove iron accumulated in diverse organs of thalassemic mice by inactivating the activities of Fe-regulatory proteins [170] and supressing hepcidin expression [171].

Recent studies in animals as well as humans demonstrate that resveratrol, a natural polyphenolic compound, has positive effects on the hypothalamic-pituitary-gonad axis, blood testosterone levels, sperm production and sperm motility [172, 173]. Furthermore, resveratrol may decrease germ

cell apoptosis [174, 175]. At the same time, Mojica-Villegas et al. [138] and Shin et al. [173] showed that resveratrol has the capacity to inhibit mitochondrial ROS production, disruption of membrane potential and permeability transition, thereby protecting the key intracellular organelle against the oxidative stress promoted by FeAA. Moreover, resveratrol has a cytoprotective effect against iron-related oxidative burst, as illustrated by the inhibition of apoptosis and alterations in apoptotic markers [173].

Conclusions and future directions

Iron and copper play indispensable roles in the physiology as well as pathology of male reproduction. Published studies highlight the crucial roles these micronutrients play in cellular respiration, spermatozoa development and metabolism as well as their ability to protect against oxidative stress in male gametes. On the other hand, pathologies connected to their defficiency on one hand or toxicities on the other, represent a crucial factor to be considered when focusing on complications of male infertility.

Unfortunately, clinical as well as research data drawing a clear line between the beneficial and toxic effects of both trace elements are still lacking. Thus, the assessment of iron and copper concentrations in both seminal fractions should become more routine in clinical settings to understand their complex two-sided roles in male infertility associated with diverse diseases. At the same time, more research is needed to be performed to understand the impact of different concentrations of both metals on the structural integrity and functional activity of male gonads as well as gametes. Last but not least, the potential of a variety of chelation and antioxidant substances deserves further exploration to eventually develop a targeted therapy to improve the fertilization potential of individuals suffering from diseases related to the "double-edged sword" activity of iron and copper.

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