Mitochondrial ferritin deficiency reduces male fertility in mice

Running title:

Mitochondrial ferritin and male fertility

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Abstract

Mitochondrial ferritin (FtMt) is a functional ferritin targeted to mitochondria that is highly expressed in the testis. To investigate the role of FtMt in the testis, we set up a series of controlled mating between mice deleted of the FtMt gene ($FtMt^{-/-}$) with $FtMt^{+/+}$ mice. We found that the number of newborns per litter and the fertility rate were strongly reduced for the $FtMt^{-/-}$ males, but not for the females, indicating that FtMt has an important role for male fertility. The morphology of the testes and of the spermatozoa of $FtMt^{-/-}$ was normal, and we did not detect alterations in sperm parameters and increase of oxidative stress indices. In contrast we observed that cauda epididymis of $FtMt^{-/-}$ mice were significantly lighter and contained less spermatozoa than those of controls, and that the ATP content of the $FtMt^{-/-}$ sperm was lower than that of the $FtMt^{+/+}$ one. We concluded that FtMt contributes to spermatogenesis and to male fertility.

Introduction

Mitochondrial ferritin (FtMt) is a functional ferritin targeted to mitochondria that is highly expressed in testes of many species, such as human, mouse and drosophila (Levi and Arosio 2004), where protects from iron-induced damage through the regulation of mitochondrial iron availability (Campanella*, et al* 2009). Mitochondria play an important role in spermatogenesis, as indicated by the findings that in drosophila defects in mitochondrial fusion processes (Hales and Fuller 1997) or mutations of the spermatogenesis-specific cytochrome gene cyt-c-d (Arama*, et al* 2006) lead to male sterility. In addition, the finding that the inactivation in drosophila of the dmfrn gene, which encodes the homolog of human mitoferrin2 responsible for mitochondrial iron incorporation, caused a male sterility and was rescued by a low iron diet (Metzendorf and Lind 2010) indicated that mitochondrial iron metabolism has an essential role in spermatogenesis, The testes are very rich in mitochondria and express high levels of proteins involved in mitochondrial iron transport and metabolism, such as frataxin and mitoferritin2. Testicular transferrin is the major secretory product of Sertoli cells with a critical role in the iron delivery to the germinal cells (Skinner and Griswold 1980) and transferrin receptor was detected mainly on early spermatocytes, supporting a need for these cells to take up iron (Leichtmann-Bardoogo*, et al* 2012). In addition, seminal transferrin concentration was found to be correlated with the sperm count in human sperm (Orlando*, et al* 1985) and severe iron overload, as it occurs in homozygous β-thalassemia patients, leads to oxidative damage and reduces male fertility (Perera*, et al* 2002). Thus, abnormalities of the expression of iron metabolism proteins in the testes and particularly in the spermatozoa may contribute to male infertility. A previous communication indicated that FtMt is highly expressed in the human sperm and that its concentration is significantly reduced in asthenospermic samples (Calzi*, et al* 2003). Moreover, a proteomic study of the sperm of fertile and unfertile subjects aimed to identify protein biomarkers, found FtMt to be the most prominent protein of interest since it was the only one among the 128 proteins

identified that was present in the control sperm samples but totally absent in all the abnormal sperms (Behrouzi*, et al* 2013). This stimulated the interest in proteins involved in mitochondrial iron metabolism, particularly FtMt, with regard to male infertility. Mouse strains deficient in the FtMt have been described (Bartnikas*, et al* 2010, Maccarinelli*, et al* 2014) and they were found to have normal hematological indices (Bartnikas*, et al* 2010) and to be more sensitive to the cardiotoxic drug doxorubicin (Maccarinelli*, et al* 2014). After the testis, the heart is one of the organs richest in FtMt and the finding that its absence makes the heart more sensitive to oxidative damage, supports the hypothesis that FtMt has a cytoprotective activity (Arosio and Levi 2010). FtMt-null mice are fertile, but the role of FtMt in the testis, where its concentration is the highest, remains unexplored. In this work we analyzed these mice in more detail, showing a significant decrease in male fertility that was not apparently associated to defect in sperm motility but to a lower level of ATP.

Materials and Methods

Animals. All mice analyzed were 3-month-old in the C57BL/6J background. All the procedures followed animal protection laws and institutional guidelines of the European Convention for the Protection of Laboratory Animals. The study was approved by the Institutional Animal Care and Use Committee of the University of Brescia and the Italian Ministry of Science and Research.

Breeding study. Every male mouse (n=8 $FtMt^{-/-}$; n=8 $FtMt^{+/+}$) was housed with two virgin $FtMt^{+/+}$ females and, in parallel, 10 $FtMt^{-/-}$ and 4 $FtMt^{+/+}$ male mice were housed each with two virgin $FtMt^{-/-}$ females. After two weeks, all mice were housed separately for other 4 weeks during which we evaluated the pregnancy rate expressed as a percentage of pregnant females in respect to the total number of mating females (fertility rate), and the number of pups per litter (litter size).

Testes and sperm collection. Mice were anesthetized with Avertine (23 µL/g mouse; Sigma) and perfused with a physiological saline solution containing 2% heparin. The testes were removed and fixed in Bouin's Solution (Sigma) for 3 hours, gradually dehydrated and embedded in paraffin. The cauda epididymis were removed, weighted and placed in 500 µL of Dulbecco's modified Eagle's medium (D-MEM Life Technologies; 2% Fetal Bovine Serum, Sigma; 0.45% glucose; 0.6% HEPES) for sperm isolation. The medium was pre-warmed at 37°C, 5% CO_2 for 2 h. Each cauda was punctured several times with a 26G needle and the spermatozoa were allowed to swim out with the help of gentle squeezing with surgical scissors. Spermatozoa were allowed to disperse in the medium for 60 min at 37°C, 5% CO_2 with gentle agitation every 10 min and then transferred to micro centrifuge tubes for collection and analysis.

Sperm parameters analysis: concentration, morphology and motility. Sperm concentration of 14 $FtMt^{-/-}$ mice and 9 controls was determined by counting in a Burker's chamber 20 µL of the sperm isolate diluted 10-fold. For morphological analysis $2x10^6$ spermatozoa (n=3 $FtMt^{-/-}$; n=3 $FtMt^{+/+}$) were re-suspended in 4% paraformaldehyde (PFA) and incubated at 4°C for 30 min. Fixed spermatozoa were mounted on polarized slides, counterstained with Hematoxylin for 5 min and observed by optical microscopy using 20X and 40X magnification. Moreover, two $FtMt^{-/-}$ mice were sent to the Embryology Laboratory of Charles River (Lyon, France) where the sperm was analyzed with Computer Assisted Sperm Analysis tool (CASA) to detect cells number and motion.

Mitochondrial staining. Sperm isolate was re-suspended 10^4 cells/µL in 200 µL of 250 nM MitoTracker Red (Invitrogen) and incubated at 37°C for 30 min to stain live mitochondria. Then, samples were fixed in 4% PFA, mounted and incubated with DAPI 0.1 µg/mL for 15 min to counterstain the nucleus. The slides were observed using standard fluorescence microscopy.

ATP assay. ATP concentration in sperm was measured using ATP luminescence assay kit (CellTiter-Glo® Luminescent Cell Viability Assay, Promega), according to manufacturer's instructions. Sperm isolate (n=3 $FtMt^{-/-}$; n=3 $FtMt^{+/+}$) was re-

suspended 400 cells/μL in 1 mL of D-MEM. Luminescence of 100 μL of sample was measured with the EnSight Multimode Plate Reader (PerkinElmer). ATP luminescence was normalized to the concentration of the protein extracts.

Histology of testis and of epididymis. Paraffin embedded testes of 12-week-old mice (n=3 $FtMt^{-/-}$; n=3 $FtMt^{+/+}$) were sectioned and the 4 μm thick slices then stained with Hematoxylin for 1 min.

Western Blots. Sperm isolate (n=3 $FtMt^{-/-}$; n=3 $FtMt^{+/+}$) was centrifuged and lysed in 100 μL of homogenization lysis buffer with protease inhibitors (Complete Protease Inhibitor Cocktail; Roche: 200 mM Tris-HCl pH 8, 100 mM NaCl, 1 mM EDTA, 0.5% NP-40, 10% glycerol, 1 mM sodium orthovanadate, 1 mM sodium fluoride). Protein concentration was measured by Bradford assay. The FtMt was detected by western blotting: 20 μg of soluble proteins were heated at 70°C for 10 min, centrifuged at 13,000 rpm at 4°C and the supernatants were loaded on 8% denaturing-PAGE. For detection, a rabbit antibody for mouse FtMt was used (Santambrogio*, et al* 2007). Band intensity was revealed by enhanced chemiluminescence (ECL; GE Healthcare) and visualized with the Kodak Image Station 440CF (Kodak).

SOD Activity. SOD analysis was performed separating 20 μg of sperm protein extracts on 12% non-denaturing PAGE. Protein level in? the gel was blotted and probed with rabbit anti-MnSOD antibody (Merk Millipore). For SOD activity (Cavadini*, et al* 2007) the polyacrylamide gel was incubated at room temperature in the dark with 50 mL of 50 mM phosphate, 0.2 mM NBT, 0.03 mM riboflavin and 0.1 mM EDTA for 15 min. After adding tetramethylethylenediamine (TEMED) to 15 mM final concentration, the gel was incubated for another 20 min at room temperature in presence of light.

Results

Male and female FtMt$^{-/-}$ mice are viable and do not show an evident phenotype. They are fertile, but we found some difficulties in maintaining a homozygous FtMt$^{-/-}$ colony, since the number of pups per litter and the number of litters were generally very low. Thus, we set up a series of controlled mating between FtMt$^{+/+}$ and FtMt$^{-/-}$ mice to verify whether the fertility problems were associated with the females or the males. Each male was mated with two virgin females for two weeks and then each mouse housed separately for other 4 weeks during which we evaluated the percentage of pregnant females in respect to the total number of mating females (fertility rate), and the number of pups per litter (litter size). The experiment consisted in the crossing for two consecutive weeks of 8 FtMt$^{-/-}$ and 8 FtMt$^{+/+}$ males with two FtMt$^{+/+}$ females/each, and 10 FtMt$^{-/-}$ and 4 FtMt$^{+/+}$ males with two FtMt$^{-/-}$ females/each and in analyzing the newborns. The results showed that the males with the FtMt$^{-/-}$ genotype had a mean number of pups per litter that was about half of that of males with the FtMt$^{+/+}$ genotype, irrespective of the female genotype, while no differences were observed between FtMt$^{-/-}$ and FtMt$^{+/+}$ females (Fig. 1A). Furthermore, the fertility rate of the crossings with FtMt$^{-/-}$ males was significantly lower compared to that obtained crossing FtMt$^{+/+}$ males with FtMt$^{-/-}$ females (Fig. 1B). We concluded that the deletion of the FtMt gene impaired significantly the male fertility but not the female fertility, in keeping with the observation that FtMt is highly expressed in testis, particularly in the spermatozoa.

Next we performed histological analyses of Bouin's fixed testis sections from FtMt$^{-/-}$ and FtMt$^{+/+}$ mice. No evident morphological abnormalities were found (Fig. 2A) and the weight of the testis and the number of spermatocytes were apparently normal in the presence or absence of FtMt. In contrast the cauda epydidymis of FtMt$^{-/-}$ mice were significantly lighter and contained a lower number of spermatozoa than those of FtMt$^{+/+}$ mice (Fig. 2B and 2C). For sperm analysis, spermatozoa were collected from the cauda epididymis, counted and analyzed by light microscopy before and after hematoxylin staining. We could not detect any evident difference in the number and the morphology of the FtMt-null spermatozoa and also after

staining with MitoTracker no differences were observed between the two murine strains. A more detailed analysis of sperm is normally obtained with automated capture video system using a Computer Assisted Sperm Analysis system (CASA), a technique not available near our laboratory. For this reason, we sent two $FtMt^{-/-}$ mice to the Embryology Laboratory of Charles River (Lyon, France). Compared with the in-house controls, no significant alterations were found in the total number of spermatozoa or in the percentage of spermatozoa with progressive motility (data not shown). A greater number of bent-head spermatozoa was observed in the $FtMt^{-/-}$ mice (data not shown), but it was not statistically significant.

Finally, we performed biochemical analyses. Western blotting of the spermatozoa extracts confirmed the absence of FtMt in the $FtMt^{-/-}$ mice, but no evident differences were detected in the level of mitochondrial manganese SOD2 protein (Fig. 3A) and SOD activity (not shown), confirming an equal number of mitochondria in the $FtMt^{-/-}$ and $FtMt^{+/+}$ spermatozoa and a normal oxidative stress status. In contrast, the measured ATP concentration of freshly isolated spermatozoa was significantly reduced in the $FtMt^{-/-}$ mice (Fig. 3B).

Discussion

It was shown that FtMt has a cytoprotective activity in human cells in culture (Arosio and Levi 2010, Campanella*, et al* 2009) and that its presence protects mice from doxorubicin cardiotoxicity (Maccarinelli*, et al* 2014). Thus, it was expected that its absence could affect also the functionality of the testis, the organ with the highest expression of FtMt (Santambrogio*, et al* 2007). Surprisingly an initial report of $FtMt^{-/-}$ mice showed no evidence for impaired fertility (Bartnikas*, et al* 2010), but the more careful analysis of the present study reveals a lower than normal fertility rate and a decrease to about half in the number of pups per litter from $FtMt^{-/-}$ males compared to that from $FtMt^{+/+}$ males. In this study, our analyses of the male reproductive tract and sperm of FtMt-null mice identified a few significant differences compared to the control mice, consisting in a lower weight of the cauda

epididymis containing fewer spermatozoa and in a decreased sperms ATP level. However, all major parameters of sperm morphology and motility did not show evident differences compared to C57BL reference values, in agreement with the fact that these mice are not infertile, but clearly, the mature sperm was less functional in the $FtMt^{-/-}$ mice. The spermatozoa we analyzed were those that spontaneously moved out from the cauda epididymis, a procedure that selects the fastest from the ones with low motility. Moreover, these cells have to undergo the transport on ductal system for full maturation and acquisition of complete motility. It is conceivable that the lack of FtMt and the reduced level of ATP may have a negative effect on these last steps of maturation resulting in a reduction of their motility and activity. This hypothesis is supported by two recent studies. One of them showing a reduced level of the mitochondrial ferritin in all asthenospermic sperms analyzed (Calzi*, et al* 2003), and the other showing FtMt to be the only one protein present in the control sperms but totally absent in all the abnormal samples, characterized by a low motility and/or DNA fragmentation (Behrouzi*, et al* 2013). In conclusion this work supports the hypothesis that FtMt contributes to spermatogenesis and thus to male fertility, with mechanisms that remain to be elucidated that may involve mitochondrial protection from oxidative damage. It also supports the suggestion that FtMt may be used as a marker of sperm motility and quality.

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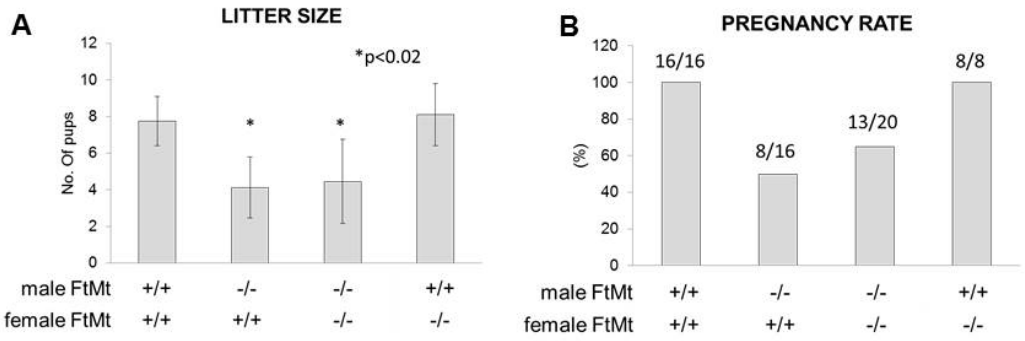

Figure 1: Breeding study. Each mouse male (n=8 $FtMt^{-/-}$; n=8 $FtMt^{+/+}$) was housed with two virgin $FtMt^{+/+}$ females and, in parallel, 10 $FtMt^{-/-}$ and 4 $FtMt^{+/+}$ male mice were housed each with two virgin $FtMt^{-/-}$ females. After two weeks all mice were housed separately for other 4 weeks during which we evaluated the number of pups per litter (litter size) (A) and the fertility rate expressed as percentage of pregnant females respect the total number of mating females (B). The number of pregnant females and of total females analyzed is presented above the columns.

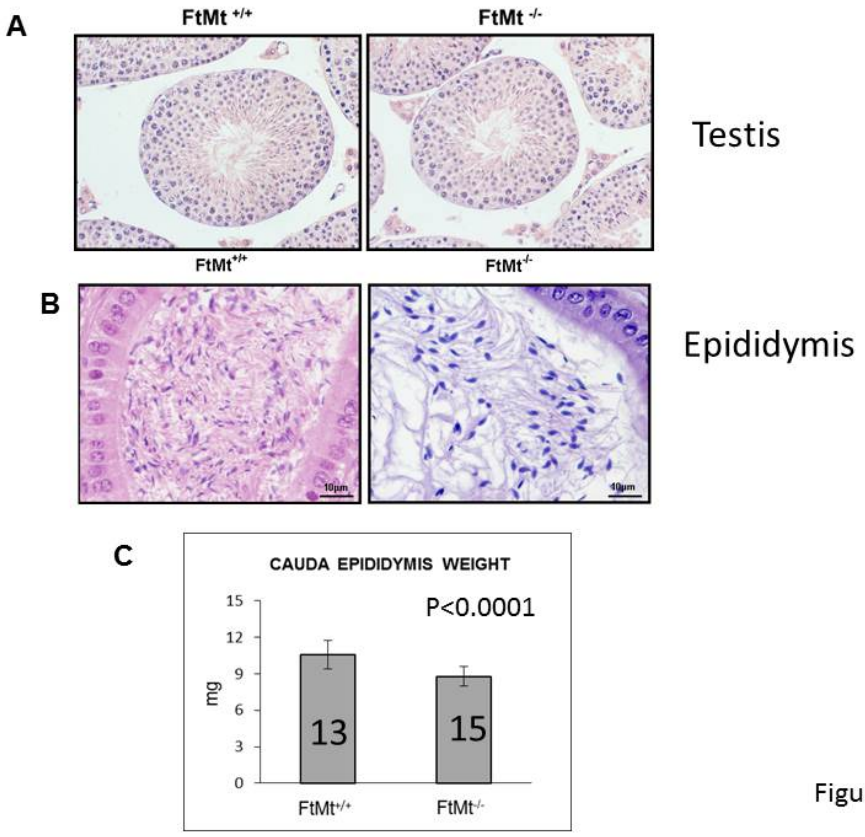

Figure 2

Figure 2. Morphological analysis of male reproductive tract. (A) Paraffin embedded testes of $FtMt^{-/-}$ and $FtMt^{+/+}$ mice were sectioned and stained with Hematoxylin. No evident difference in morphology or spermatocyte number/maturation is observed. (B) Hematoxylin stain of epididymis sections showed a lower density of spermatozoa in FtMt -/- mice. (C) Before sperm analysis cauda epididymis were collected and weighed. The data from 13 $FtMt^{+/+}$ and 15 $FtMt^{-/-}$ mice show that the mean cauda epididymis weight of $FtMt^{-/-}$ mice is significantly lower than those of the controls.

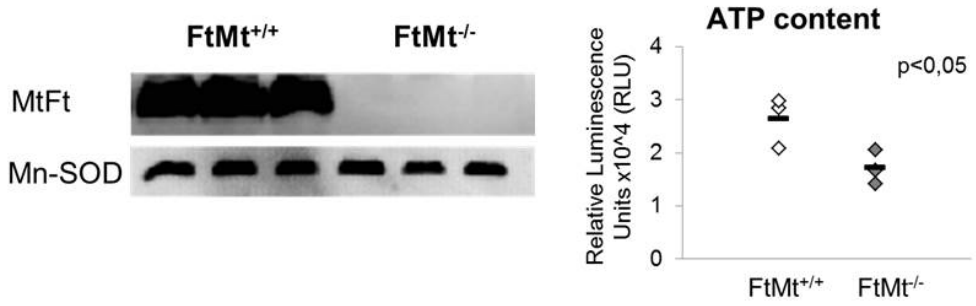

Figure 3: Biochemical analyses. (A) Immunoblotting of 20 µg protein extracts from spermatozoa of $FtMt^{+/+}$ and $FtMt^{-/-}$ with anti-FtMt antibody confirmed the absence of the protein in sperm $FtMt^{-/-}$. No differences were revealed in oxidative stress indices analyzed including manganese superoxide dismutase (Mn-SOD). (B) Evaluation of ATP content in the homogenate of freshly isolated sperm from 3 $FtMt^{+/+}$ and 3 $FtMt^{-/-}$ mice. The luminescence values were normalized to the concentration of the protein extracts obtained from the same volume of sperm isolate. ATP content showed to be significantly decreased in the absence of FtMt.