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Reproduction, Fertility and Development
http://dx.doi.org/10.1071/RD16348

Mitochondrial ferritin deficiency reduces male fertility in mice

Federica Maccarinelli[A], *Maria Regoni*[A], *Fernando Carmona*[A], *Maura Poli*[A], *Esther G. Meyron-Holtz*[A,B] *and Paolo Arosio*[A,C]

[A]Molecular Biology Laboratory, Department of Molecular and Translational Medicine DMMT, University of Brescia, Viale Europa 11, 25123 Brescia, Italy.
[B]Laboratory for Molecular Nutrition, Faculty of Biotechnology and Food Engineering, Technion-Israel Institute of Technology, Technion City, 32000 Haifa, Israel.
[C]Corresponding author. Email: paolo.arosio@unibs.it

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 matings between *FtMt* gene-deletion mice ($FtMt^{-/-}$) 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 testis and of the spermatozoa of $FtMt^{-/-}$ mice was normal and we did not detect alterations in sperm parameters or in oxidative stress indices. In contrast, we observed that the cauda epididymides of $FtMt^{-/-}$ mice were significantly lighter and contained a lower number of spermatozoa compared with the controls. Also, the ATP content of $FtMt^{-/-}$ spermatozoa was found to be lower than that of $FtMt^{+/+}$ spermatozoa. These data show that *FtMt* contributes to sperm epididymis maturation and to male fertility.

Additional keywords: ATP, spermatogenesis, sperm motility.

Received 20 July 2016, accepted 3 December 2016, published online 9 January 2017

Introduction

Mitochondrial ferritin (*FtMt*) is a functional ferritin targeted to mitochondria that is highly expressed in the testis of many species, such as human, mouse and drosophila (Levi and Arosio 2004), where it 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* lead to male sterility (Arama *et al.* 2006). In addition, the inactivation in drosophila of the *dmfrn* gene, which encodes the homologue of human mitoferrin2 (alias SLC25A28) responsible for mitochondrial iron incorporation, caused male sterility that was rescued by a low-iron diet (Metzendorf and Lind 2010), indicating 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 for iron delivery to the germinal cells (Skinner and Griswold 1980) and transferrin receptor was detected mainly on early spermatocytes, suggesting 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 human sperm count (Orlando *et al.* 1985) and severe iron overload, as occurs in homozygous β-thalassemia patients, leads to oxidative damage and reduces male fertility (Perera *et al.* 2002). Thus, abnormalities in the expression of iron metabolism proteins in the testis and particularly in the spermatozoa may contribute to male infertility. A previous study indicated that *FtMt* is highly expressed in human spermatozoa and that its concentration is significantly reduced in asthenospermic samples (Calzi *et al.* 2003). Moreover, a proteomic study of the spermatozoa of fertile and unfertile subjects that aimed to identify protein biomarkers found *FtMt* to be the only one of 128 proteins identified that was present in the control sperm samples but totally absent in all the abnormal ones (Behrouzi *et al.* 2013). This stimulated interest in *FtMt* and in proteins involved in mitochondrial iron metabolism with regard to male infertility. Mouse strains deficient in *FtMt* have been described (Bartnikas *et al.* 2010; Maccarinelli *et al.* 2014) and they were found to have normal haematological indices (Bartnikas *et al.* 2010) and to be more sensitive to the cardiotoxic drug doxorubicin (Maccarinelli *et al.* 2014). The heart is rich in *FtMt* and the finding that its absence makes the heart

more sensitive to oxidative damage supports the hypothesis that *FtMt* has 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 analysed these mice in more detail, showing a significant decrease in male fertility that was not apparently associated with defects in sperm motility but with a lower level of ATP.

Materials and methods

Animals

All mice analysed were 3 months old on 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 Health.

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 2 weeks, all mice were housed separately for other 4 weeks, during which we evaluated the fertility rate expressed as a percentage of pregnant females per male and the mean litter size per male. This was done by counting at birth the number of pups born from the two females mated with the same male mouse. Statistical analysis was performed using parametric analysis of variance (two-way ANOVA) followed by post hoc Tukey's test. The level of significance was set at 0.05.

Testes and sperm collection

Mice were anesthetised with Avertine (23 μL g^{-1} 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 h, gradually dehydrated and embedded in paraffin. The cauda epididymides were removed, weighed and placed in 500 μL Dulbecco's modified Eagle's medium (DMEM, Life Technologies; 2% fetal bovine serum, Sigma; 0.45% glucose; 0.6% HEPES) for sperm isolation. The medium was pre-warmed at 37°C in 5% CO_2 for 2 h. Each cauda was punctured several times with a 26-guage 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 microcentrifuge tubes for collection and analysis.

Sperm parameter analysis: concentration, morphology and motility

Sperm cell number and motion of 14 $FtMt^{-/-}$ mice and nine controls were assessed by counting 20 μL of 10-fold diluted sperm isolate in a Burker's chamber. For morphological analysis 2×10^6 spermatozoa ($n = 3$ $FtMt^{-/-}$; $n = 3$ $FtMt^{+/+}$) were re-suspended in 4% paraformaldehyde and incubated at 4°C for 30 min. Fixed spermatozoa were mounted on polarised slides, counterstained with haematoxylin for 5 min and observed by optical microscopy (Olympus BH2; Olympus) using 20× and 40× magnification. The morphology of at least 50 spermatozoa was evaluated in eight different randomly selected fields, for a total of over 300 spermatozoa per mouse. Moreover, two $FtMt^{-/-}$ mice were sent to the Embryology Laboratory of Charles River (Lyon, France) where their spermatozoa and that of an in-house control mouse of the same background and age were analysed with a computer-assisted sperm analysis (CASA) tool to detect cell number, motion and morphology. The cells counted for each mouse were more than 220 for motility and more than 70 cells for morphology analysis.

Mitochondrial staining

Sperm isolate was suspended 10^4 cells μL^{-1} in 200 μL 250 nM MitoTracker Red (Invitrogen) and incubated at 37°C for 30 min to stain active mitochondria. Then, samples were fixed in 4% paraformaldehyde (PFA), mounted and incubated with 0.1 μg mL^{-1} 4',6-diamidino-2-phenylindole (DAPI) for 15 min to counterstain the nucleus. The slides were observed by standard fluorescence microscopy (Olympus BH2-RFC; Olympus) using 20× and 40× magnification. The morphology of at least 50 spermatozoa was evaluated in eight different randomly selected fields, for a total of over 300 spermatozoa per mouse.

Adenosine triphosphate assay

Adenosine triphosphate concentration in spermatozoa was measured using an ATP luminescence assay kit (CellTiter-Glo Luminescent Cell Viability Assay; Promega), according to the manufacturer's instructions. Briefly, sperm isolates ($n = 3$ $FtMt^{-/-}$; $n = 3$ $FtMt^{+/+}$) were suspended 400 cells μL^{-1} in 1 mL DMEM, loaded in a 96-well plate (100 μL per well) and added to 100 μL of the reagent CellTiter-Glo. This caused cell lysis and generation of a luminescent signal that was measured with the EnSight Multimode Plate Reader (PerkinElmer). The luminescence signal is proportional to ATP concentration and was normalised to the protein content of extracts obtained by lysing 500 000 spermatozoa in 100 μL of lysis buffer with protease inhibitors (Complete Protease Inhibitor Cocktail; Roche: 200 mM Tris-HCl pH 8, 100 mM NaCl, 1 mM ethylenediamine tetraacetic acid (EDTA), 0.5% NP-40, 10% glycerol, 1 mM sodium orthovanadate, 1 mM sodium fluoride). Protein concentration was measured by Bradford assay. Statistical analysis was performed using *t*-test, with $P < 0.05$ considered as significant.

Histology of testis and of epididymis

Paraffin-embedded testes and epididymides of 12-week-old mice ($n = 3$ $FtMt^{-/-}$; $n = 3$ $FtMt^{+/+}$) were sectioned (4 μm), deparaffinised in xylene, rehydrated through a series of alcohol gradients and stained with haematoxylin for 1 min. Sections were then dehydrated, covered with coverslips and evaluated using an optical microscope (Olympus BH2) at a final magnification of 20× and 60×. The number of spermatozoa in each epididymis was evaluated in eight different randomly selected fields by an investigator unaware of the genotype and counted with ImageJ software.

Western blots

Sperm isolates ($n = 3$ $FtMt^{-/-}$; $n = 3$ $FtMt^{+/+}$) were centrifuged and lysed in 100 µL of lysis buffer with protease inhibitors (Complete Protease Inhibitor Cocktail; Roche). Protein concentration was measured by Bradford assay. For *FtMt* western blot 20 µg of soluble proteins were heated at 70°C for 10 min, centrifuged at 16 000*g* at 4°C for 20 min and the supernatants were loaded onto an 8% non-denaturing gel. The proteins were then electrophoretically transferred to a polyvinylidene fluoride (PVDF) membrane, which was subsequently incubated with a rabbit anti-mouse-FtMt antiserum at a dilution of 1 : 1000 for 1 h (Santambrogio *et al.* 2007). For superoxide dismutase 2 (SOD2) detection 20 µg of sperm protein extracts were separated by polyacrylamide gel electrophoresis (PAGE) on a 15% denaturing gel and the membrane was blotted with anti-manganese superoxide dismutase (Mn-SOD) rabbit antibody (Merk Millipore). The membranes were then washed and incubated with a horseradish peroxidase (HRP)-labelled anti-rabbit Ig antibody and the activity revealed by enhanced chemiluminescence (ECL; GE Healthcare) and visualised with the Kodak Image Station 440CF (Kodak). The measurements were performed in at least three independent experiments and analysed by *t*-test. The level of significance was set at 0.05.

SOD2 activity analysis

For SOD activity 20 µg of sperm protein extracts were separated on 12% non-denaturing PAGE (Cavadini *et al.* 2007) and the polyacrylamide gel was incubated at room temperature in the dark in 50 mL of 50 mM phosphate, 0.2 mM 4-nitro blue tetrazolium chloride (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 the presence of light. Band intensity was analysed with the Kodak Image Station 440CF (Kodak). The measurements were performed in at least three independent experiments and analysed by *t*-test. The level of significance was set at 0.05.

Results

Breeding studies

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 very low. Thus, we set up a series of controlled matings 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 2 weeks and then each mouse housed separately for other 4 weeks, during which we evaluated the percentage of pregnant females (fertility rate) and the number of pups per litter on the day of birth (litter size). The experiment consisted in crossing eight $FtMt^{-/-}$ and eight $FtMt^{+/+}$ males with two $FtMt^{+/+}$ females each and 10 $FtMt^{-/-}$ and four $FtMt^{+/+}$ males with two $FtMt^{-/-}$ females each and in analysing the pregnant females and the number of newborns. The results showed that the males with the $FtMt^{-/-}$ genotype had a mean number of pups per litter that was ~30% of that of males with the $FtMt^{+/+}$ genotype, irrespective of the female genotype, while no differences were observed between $FtMt^{-/-}$ and $FtMt^{+/+}$ females (Fig. 1*a*). Furthermore, the fertility rate of the crossings with $FtMt^{-/-}$ males was significantly lower compared with that obtained crossing $FtMt^{+/+}$ males with $FtMt^{-/-}$ females (Fig. 1*b*). We concluded that the deletion of the *FtMt* gene significantly impaired male, but not female fertility, in keeping with the observation that *FtMt* is highly expressed in the testes, particularly in the spermatozoa.

Testis and epididymis histological analysis

No evident morphological abnormalities were found (Fig. 2*a*) and the weight of the testis (99 ± 5 mg vs 84 ± 19 mg) and the number of spermatocytes were apparently normal in the presence or absence of *FtMt*. In contrast, the cauda epididymides of $FtMt^{-/-}$ mice were significantly lighter than those of $FtMt^{+/+}$ mice (Fig. 2*d*) and the morphology analysis and counting showed a lower number of spermatozoa (Fig. 2*b–c*).

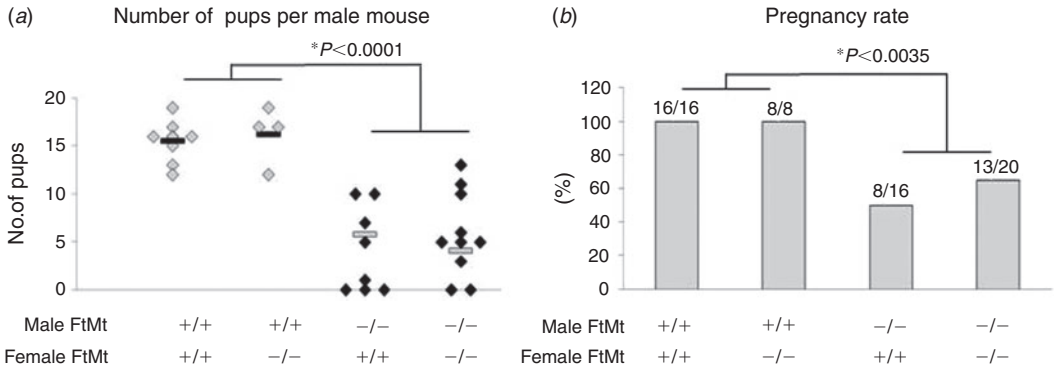

Fig. 1. Breeding study. Each mouse male ($n = 8$ $FtMt^{-/-}$; $n = 8$ $FtMt^{+/+}$) was housed with two virgin $FtMt^{+/+}$ females and, in parallel, 10 $FtMt^{-/-}$ and 4 $FtMt^{+/+}$ male mice were each housed with two virgin $FtMt^{-/-}$ females. After 2 weeks all mice were housed separately for other 4 weeks, during which we evaluated the number of pups per litter on the day of birth. (*a*) Dot plot showing the total number of pups born from two females mated with the same male mouse (litter size) and (*b*) the fertility rate expressed as a percentage of pregnant females with respect to the total number of females mated. The number of pregnant females and of total females analysed is presented above the columns.

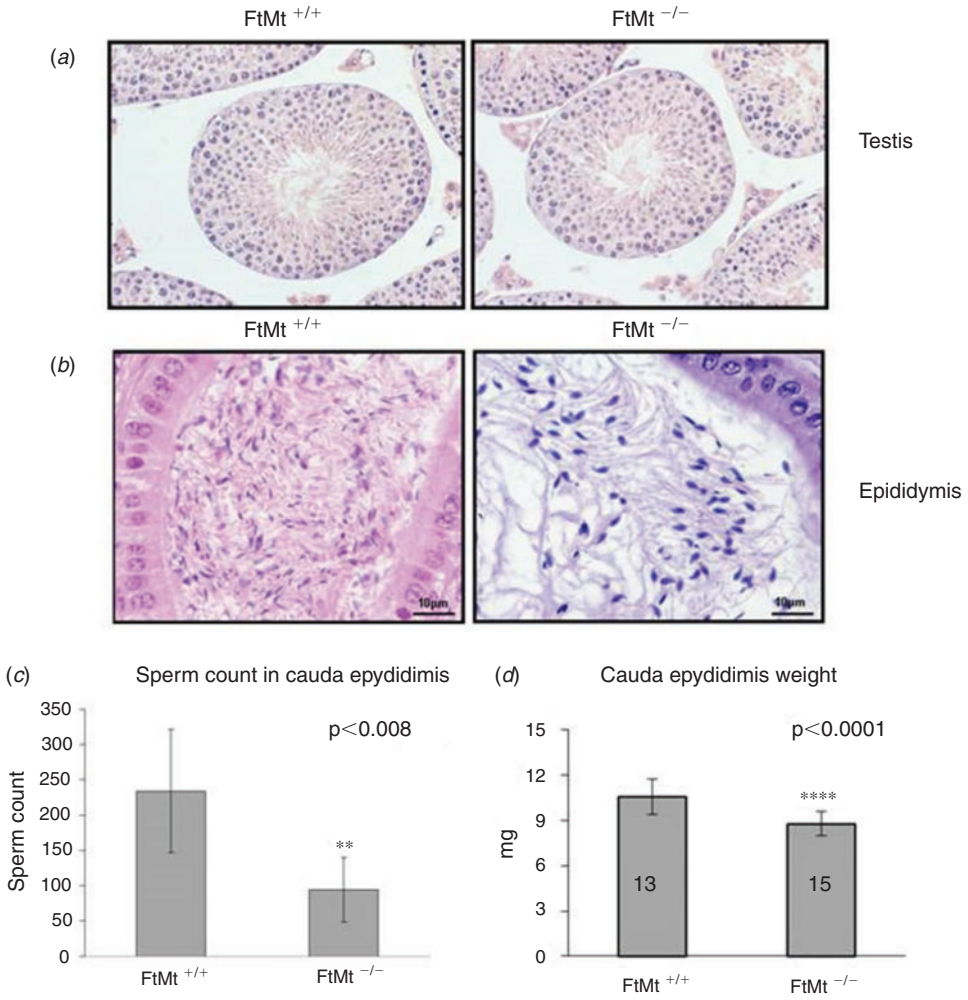

Fig. 2. Morphological analysis of male reproductive tract. (*a*) Paraffin-embedded testes of $FtMt^{-/-}$ and $FtMt^{+/+}$ mice were sectioned and stained with haematoxylin. No evident difference in morphology or spermatocyte number or maturation was observed. (*b*) Haematoxylin stain of epididymis sections showed a lower density of spermatozoa in $FtMt^{-/-}$ mice. (*c*) Sperm count in cauda epididymis. The number of spermatozoa was evaluated in eight different randomly selected fields per mouse by an investigator unaware of the genotype and counted with ImageJ software. (*d*) Before sperm analysis cauda epididymides were collected and weighed. The data from 13 $FtMt^{+/+}$ and 15 $FtMt^{-/-}$ mice show that the mean cauda epididymidal weight of $FtMt^{-/-}$ mice is significantly lower than that of the controls. Statistical analysis was performed using *t*-test, with $P < 0.05$ considered as significant. Error bars show standard deviation.

Sperm parameter analyses

For sperm analysis, spermatozoa were collected from the cauda epididymis, counted and analysed by light microscopy before and after haematoxylin staining. We could not detect any evident difference in the number, motion or morphology of the *FtMt*-null spermatozoa and also after staining with MitoTracker no differences were observed between the two genotypes. For a more detailed analysis of spermatozoa obtained with automated capture video using a CASA system, we sent two $FtMt^{-/-}$ mice to the Embryology Laboratory of Charles River. 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. A greater number of bent-head spermatozoa was observed in the $FtMt^{-/-}$ mice, but it was not statistically significant (Table 1).

Biochemical analyses

Western blotting of the sperm extracts confirmed the absence of *FtMt* in the $FtMt^{-/-}$ mice, but no evident differences were detected in the level of mitochondrial SOD2 protein and SOD activity (Fig. 3*a*), 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. 3*b*).

Table 1. CASA sperm analysis report of two *FtMt*$^{-/-}$ and one control mouse

Sperm counts in the range 80 – 160 $\times 10^6$ mL^{-1} are considered indicative of normal sperm production in C57BL/6J mice of ~3 months of age. Normal reference value for progressive motility is above 15%. For sperm morphology at least 5% of the spermatozoa should have a normal shape

Mouse genotype	Count		Motility		Morphology	
	Cells counted	Total concentration ($\times 10^6$ mL^{-1})	Total motility (%)	Progressive motility (%)	Cells counted	Normal (%)
FtMt$^{-/-}$ male 1	275	168	56	51	78	14
FtMt$^{-/-}$ male 2	274	167	43	38	76	38
FtMt$^{+/+}$ in-house control	229	140	69	56	70	37

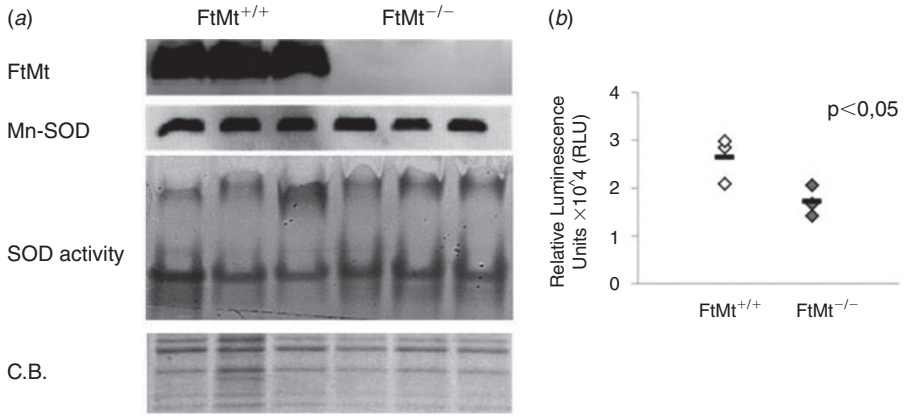

Fig. 3. Biochemical analyses. (*a*) Immunoblotting of 20-µg protein extracts from spermatozoa of *FtMt*$^{+/+}$ and *FtMt*$^{-/-}$ with anti-*FtMt* antibody confirmed the absence of the protein in *FtMt*$^{-/-}$ spermatozoa. Mitochondrial manganese superoxide dismutase (Mn-SOD) did not show differences in protein level or activity. Coomassie blue staining (C.B.) of total protein as loading control. (*b*) Evaluation of ATP content in the homogenate of freshly isolated spermatozoa from three *FtMt*$^{+/+}$ and three *FtMt*$^{-/-}$ mice. The luminescence values were normalised to the concentration of the protein extracts obtained from the same volume of sperm isolate. Adenosine triphosphate content was significantly decreased in the absence of *FtMt*.

Discussion

It was shown that *FtMt* has a cytoprotective activity in human cells in culture (Campanella *et al.* 2009; Arosio and Levi 2010) and, in fact, the deletion of its gene increases the cardiotoxicity of doxorubicin in the *FtMt*-null mice (Maccarinelli *et al.* 2014). The testis is the organ with the highest expression of *FtMt*, higher than the heart (Santambrogio *et al.* 2007), thus it was expected that the deletion of its gene would affect male fertility. However, in the initial description of a *FtMt*$^{-/-}$ mouse strain the fertility was considered, but no evident impairments were found (Bartnikas *et al.* 2010). Also, the *FtMt*$^{-/-}$ strain, independently produced in our laboratory, was fertile (Maccarinelli *et al.* 2014). However, a more careful observation performed in the present study revealed that the *FtMt*$^{-/-}$ mice have a lower fertility rate and the number of pups per litter from *FtMt*$^{-/-}$ males is ~30% of that from *FtMt*$^{+/+}$ males. This finding stimulated an analysis of the male reproductive tract and spermatozoa of *FtMt*$^{-/-}$ mice to identify the cause of this abnormality. However, no significant differences were found in the size and morphology of the testis or in the major parameters of sperm morphology and motility that we could measure. In fact, we used CASA on only two *FtMt*$^{-/-}$ mice in comparison with one C57BL/6J reference mouse of the same age and no significant differences were detected. The lack of strong abnormalities in sperm motility and morphology suggests that the functional defects are minor, which is consistent with the moderate effects on fertility. It should also be noted that the analysis of the mouse spermatozoa is restricted to the ones that spontaneously move out from the cauda epididymis, a procedure that selects the fastest and more active ones. In addition, to reach full maturation, motility and activity these cells have to undergo transport through the ductal system, thus they do not represent the actual cells responsible for fertility.

The significant differences we observed between the *FtMt*$^{-/-}$ and the control mice consisted of a lower weight of the cauda epididymis, of a lower number of spermatozoa stored in the cauda epididymis and also of a lower ATP concentration in the spermatozoa. It is conceivable that the lack of *FtMt* and the reduced level of ATP may have a negative effect on the last steps of maturation resulting in a reduction of their motility and activity. An important

role of mitochondrial ferritin in fertility is also indicated by two recent studies. One of them showed a reduced level of mitochondrial ferritin in all human asthenospermic spermatozoa analysed (Calzi *et al.* 2003) and the other showed *FtMt* to be the only protein present in control spermatozoa but totally absent in all abnormal ones, characterised by a low motility 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. It also supports the suggestion that *FtMt* may be used as a marker of sperm motility and quality.

Acknowledgements

We are grateful to Dr Andreas Meinhardt for suggestions and comments and to Dr Marco Spiller and Dr Daria Leali for support in sperm analysis. The work was partially supported by MIUR grant PRIN10–11 to P. A. and by Telethon grant GGP1099 to P. A.

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