

A novel antioxidant formulation designed to treat male infertility associated with oxidative stress: promising preclinical evidence from animal models

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STUDY QUESTION: Does a novel antioxidant formulation designed to restore redox balance within the male reproductive tract, reduce sperm DNA damage and increase pregnancy rates in mouse models of sperm oxidative stress?

SUMMARY ANSWER: Oral administration of a novel antioxidant formulation significantly reduced sperm DNA damage in glutathione peroxidase 5 (GPX5), knockout mice and restored pregnancy rates to near-normal levels in mice subjected to scrotal heat stress.

WHAT IS KNOWN ALREADY: Animal and human studies have documented the adverse effect of sperm DNA damage on fertilization rates, embryo quality, miscarriage rates and the transfer of *de novo* mutations to offspring. Semen samples of infertile men are known to be deficient in several key antioxidants relative to their fertile counterparts. Antioxidants alone or in combination have demonstrated limited efficacy against sperm oxidative stress and DNA damage in numerous human clinical trials, however these studies have not been definitive and an optimum combination has remained elusive.

STUDY DESIGN, SIZE, DURATION: The efficacy of the antioxidant formulation was evaluated in two well-established mouse models of oxidative stress, scrotal heating and *Gpx5* knockout (KO) mice, ($n = 12$ per experimental group), by two independent laboratories. Mice were provided the antioxidant product in their drinking water for 2–8 weeks and compared with control groups for sperm DNA damage and pregnancy rates.

PARTICIPANTS/MATERIALS, SETTING, METHODS: In the *Gpx5* KO model, oxidative DNA damage was monitored in spermatozoa by immunocytochemical detection of 8-hydroxy-2'-deoxyguanosine (8OHdG). In the scrotal heat stress model, male fertility was tested by partnering with three females for 5 days. The percentage of pregnant females, number of vaginal plugs, resorptions per litter, and litter size were recorded.

MAIN RESULTS AND ROLE OF CHANCE: Using immunocytochemical detection of 8OHdG as a biomarker of DNA oxidation, analysis of control mice revealed that around 30% of the sperm population was positively stained. This level increased to about 60% in transgenic mice deficient in the antioxidant enzyme, GPX5. Our results indicate that an 8 week pretreatment of *Gpx5* KO mice with the antioxidant formulation provided complete protection of sperm DNA against oxidative damage. In mouse models of scrotal heat stress, only 35% (19/54) of female mice became pregnant resulting in 169 fetuses with 18% fetal resorption (30/169). This is in contrast to the antioxidant pretreated group where 74% (42/57) of female mice became pregnant, resulting in 427 fetuses with 9% fetal resorption (38/427). In both animal models the protection provided by the novel antioxidant was statistically significant ($P < 0.01$ for the reduction of 8OHdG in the spermatozoa of *Gpx5* KO mice and $P < 0.05$ for increase in fertility in the scrotal heat stress model).

LIMITATIONS, REASONS FOR CAUTION: It was not possible to determine the exact level of antioxidant consumption for each mouse during the treatment period.

WIDER IMPLICATIONS OF THE FINDINGS: Recent clinical studies confirm moderate to severe sperm DNA damage in about 60% of all men visiting IVF centers and in about 80% of men diagnosed with idiopathic male infertility. Our results, if confirmed in humans, will impact clinical fertility practice because they support the concept of using an efficacious antioxidant supplementation as a preconception therapy, in order to optimize fertilization rates, help to maintain a healthy pregnancy and limit the mutational load carried by children.

STUDY FUNDING/COMPETING INTEREST(S): The study was funded by the Clermont Université and the University of Madrid. P.G. is the Managing Director of CellOxess LLC, which has a commercial interest in the detection and resolution of oxidative stress. A.M. and A.P. are employees of CellOxess, LLC. J.R.D., A.G.-A. and R.J.A. are honorary members of the CellOxess advisory board.

Keywords: sperm DNA damage / reactive oxygen species / oxidative stress / antioxidants / infertility / glutathione peroxidase 5 / scrotal heat stress

Introduction

Sperm DNA damage is a significant determinant of semen quality (Aitken, 1999; Zenzes, 2000; Aitken and Krausz, 2001; Lewis and Aitken, 2005; Fernandez-Gonzalez et al., 2008; Lewis et al., 2013) and yet it is not routinely diagnosed or adequately treated in couples receiving infertility treatment in the form of assisted reproductive technology (ART). Men with this condition are likely to experience subfertility or infertility, expose their female partners to a greater risk of miscarriage, and pass on *de novo* genetic and epigenetic changes to their offspring, potentially compromising the health of future generations (Aitken et al., 2013). The present situation is concerning, as clinical studies confirm moderate to severe sperm DNA damage in about 60% of all men visiting IVF centers (Cohen-Bacrie et al., 2009) and in about 80% of men diagnosed with idiopathic male infertility (Simon et al., 2013).

The induction of DNA damage in spermatozoa could theoretically involve enzymatic cleavage of the DNA backbone as a result of enhanced endonuclease activity or fragmentation of the DNA as a consequence of free radical attack. For reasons that have been carefully set out in previous publications, most DNA damage in the male germ line appears to be oxidatively induced (Koppers et al., 2011; Aitken et al., 2013, 2015). Oxidative damage to all cell types, including gametes, results primarily from chemical insult via a surplus of short-lived, highly reactive metabolites designated by their chemically reactive heteroatom, such as reactive oxygen species (ROS) and reactive nitrogen species (RNS) (Halliwell and Whiteman, 2004). These metabolites consist of free radicals such as the hydroxyl radical (OH^\cdot), ionic species such as superoxide anion (O_2^-) or peroxytrite (ONOO^-) and neutral but reactive molecules such as hydrogen peroxide (H_2O_2). Given the molecular diversity of such reactive species, the type of damage sustained by DNA is equally diverse. The most recognized types of sperm oxidative DNA damage are: (i) single and double DNA strand breaks, (ii) abasic site generation, (iii) inter- or intra-strand DNA cross linkage, (iv) DNA-protein cross-linkage and (v) chemical modification of bases including DNA base adduct formation.

Sperm oxidative stress not only disrupts the integrity of sperm DNA but also limits the fertilizing potential of these cells through collateral damage to proteins and lipids in the sperm plasma membrane (Aitken et al., 1989). Spermatozoa are particularly vulnerable to oxidative attack due to a relative deficiency in the availability of intracellular antioxidant enzymes consequent to their lack of cytoplasm. Moreover, unlike many other cell types, sperm lipid membranes contain a particularly high percentage of polyunsaturated fatty acids (PUFAs) such as

docosahexaenoic acid (Jones et al., 1978, 1979; Koppers et al., 2010). These highly unsaturated fatty acids give the plasma membrane the fluidity it needs to participate in the membrane fusion events associated with fertilization. However, PUFAs oxidize readily to generate malondialdehyde and a variety of highly reactive α , β -unsaturated hydroxyalkenals, such as 4-hydroxynonenal and 4-hydroxyhexanal (Pizzimenti et al., 2013; Spickett, 2013; Moazamian et al., 2015). Once generated, these reactive aldehydes may attack other nearby PUFAs, thus propagating a chain reaction that eventually disrupts membrane fluidity. At high concentrations, these aldehydes also react with amino acids and nucleic acids to form stable protein and DNA adducts, which further disrupt sperm function (Luczaj and Skrzydlewska, 2003; Aitken et al., 2012).

Since many observational studies now confirm decreased levels of seminal antioxidant(s) and/or increased levels of oxidants in infertile versus fertile men (Aitken and Clarkson, 1987; Alvarez et al., 1987; Lewis et al., 1995, 1997; Fraga et al., 1996; Aitken, 1999; Balercia et al., 2000), it should come as no surprise that dietary supplementation with antioxidants has gained much attention in recent years (Lanzafame et al., 2009; Ross et al., 2010; Gharagozloo and Aitken, 2011; Showell et al., 2011, 2014). In this paper, we report the detailed scientific rationale behind the design of a novel antioxidant formulation (Fertilix[®]) and present preliminary *in vivo* data as evidence of its efficacy in reducing sperm oxidative DNA damage and improving pregnancy rates in mouse models of oxidative stress. A detailed explanation of the composition of Fertilix[®] is provided (see Supplementary Data) but the fundamental constituents are: carnitines, folic acid, lycopene, selenium, vitamin C, full-spectrum vitamin E and zinc.

In this study, the efficacy of Fertilix[®] has been examined in two animal models of oxidative stress within the male reproductive tract. The first model employed in this study was the *Gpx5* knockout (KO) (*Gpx5*^{-/-}) mouse (Aitken, 2009; Chabory et al., 2009). In these mice, the H_2O_2 -scavenging activity of GPX5 in the epididymis has been nullified, leading to increased exposure of mature spermatozoa to H_2O_2 -mediated oxidative stress during epididymal transit and storage (Chabory et al., 2009). Because of the targeted reduction of antioxidant capacity in the reproductive tracts of these mice, sperm nuclei exhibit high levels of oxidative DNA damage as revealed by accumulation of the oxidized guanine residue 8-hydroxy-2'-deoxyguanosine (8OHdG), a well-known biomarker of oxidative DNA damage (Noblanç et al., 2013). This model was therefore employed to determine the ability of oral antioxidant supplementation to correct oxidative stress within the epididymal lumen. However, in this model, fertilization and pregnancy rates are not statistically different from controls even though the incidence of miscarriages

and developmental defects is elevated (Chabory *et al.*, 2009). Thus in order to study impacts of antioxidant supplementation on fertility, a second model was employed.

The second system used in this study was the scrotal heat stress model, in which male mice are subjected to transient testicular thermal stress, resulting in diminished sperm quality, DNA damage, germ cell apoptosis, and impaired fertility (Jannes *et al.*, 1998; Zhu *et al.*, 2004; Banks *et al.*, 2005; Pérez-Crespo *et al.*, 2008; Paul *et al.*, 2009). These effects are accompanied by changes in testicular architecture as well as reduced populations of spermatids and mature spermatozoa in the seminiferous tubules (Hourcade *et al.*, 2010). Sperm DNA damage is evident as early as 6 h following stress induction and persists for up to 28 days, while female mice mated with heat stressed males exhibit significantly lower pregnancy and higher embryo resorption rates compared with those mated with unstressed controls (Pérez-Crespo *et al.*, 2008). In the present study, this model was used to assess the capacity of Fertilix[®] to protect not only sperm cells but also the testicular architecture from oxidative damage following a transient, acute heat stress. Thus, both of these well-established models were utilized to provide complementary information demonstrating the efficacy of Fertilix[®] against a wide range of biological end-points.

Materials and Methods

Animals

The present study was approved by the Regional Ethic Committee for Animal Experimentation (CEMEA-Auvergne; Authorization CE2-04) and the Animal Care and Ethics Committee (Informe CEEA 2014/025) of the Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA, Madrid, Spain) and adhered to the current legislation on animal experimentation in France and Spain according to the Guide for Care and Use of Laboratory Animals as adopted by the Society for Study of Reproduction. The *Gpx5*^{-/-} mice were derived as described originally from the C57BL/6 genetic line (Chabory *et al.*, 2009) while scrotal heat stress was realized as previously described (Pérez-Crespo *et al.*, 2008) on CD-1 mice (Harlan, Oxford, UK). The doses of the ingredients used for *in vivo* experiments correspond to the upper limit of the dose range given in Table 1.

Gpx5 KO

Sexually mature *Gpx5*^{-/-} mice and wild type (WT) controls were administered Fertilix[®], dissolved in water, which was available *ad libitum* for a period of 2 months. Control *Gpx5*^{-/-} and wild type mice were provided unsupplemented water during the 2 month period. Appropriate dosage, corresponding to the highest human dose of Fertilix[®] recommended by the manufacturer (CellOxess, Princeton, NJ, USA), was calculated as previously described (Reagan-Shaw *et al.*, 2007). After 2 months of treatment, supplemented *Gpx5*^{-/-} and WT mice and unsupplemented *Gpx5*^{-/-} and WT controls were sacrificed by CO₂ inhalation and cervical dislocation.

Scrotal heat stress model

After 1 week of Fertilix[®] oral administration in drinking water (or solely water for the control group), adult male CD-1 mice (8–12 weeks old) were anesthetized using xylazine-ketamine (0.1 ml/10 g body weight, i.p. injection) and were subjected to transient scrotal heat stress as previously described (Pérez-Crespo *et al.*, 2008). In short, males were suspended on a polystyrene raft such that their testes, which were prevented from retracting to an abdominal position, were immersed in water at 42°C for 30 min. Control mice were immersed in water at standard testicular temperature (33°C) for 30 min. Epididymal mature sperm samples and testes were

Table 1 Primers used to monitor the accumulation of H₂O₂-recycling enzymes in caudal epididymal extracts of wild type and *Gpx5*^{-/-} mice via quantitative RT-PCR.

Gene transcript	5'–3' primer sequences	Amplicon size (bp)
<i>Catalase</i>	Fw – GCAGATACCTGTGAACTGTC Rv – GTAGAATGTCCGCACCTGAG	229
<i>Gst</i> μ	Fw – GAAGTTCAAGCTGGGCCTGG Rv – GCATGATGAGCTGCATGCGG	194
<i>Sod3</i>	Fw – GCTCTCAGAGAACCCCTCT Rv – GTGCTATGGGGACAGGAAGA	170
<i>Cyclophilin B</i>	Fw – GGAGATGGCACAGGAGGAA Rv – GCCCGTAGTGCTTCAGCTT	76

Gpx5^{-/-}: glutathione peroxidase 5^{-/-}; *Sod3*: superoxide dismutase; *Gst* μ: glutathione S transferase μ.

obtained from at least 8 animals per treatment and 8 controls at two time points, 6 h and 15 days post heat stress, for analysis of the spermatozoa and histological examination of the testes. Scrotal heat stress and control males were each mated with 3 superovulated virgin female CD-1 mice at 7 days and 14 days post heat stress, a timeframe that corresponded to the period where maximum testicular and sperm damage were recorded (present work and Pérez-Crespo *et al.*, 2008). Females were caged overnight with a single male, and the presence of a vaginal plug was considered indicative of successful mating. Females were sacrificed by cervical dislocation on Day 14 of gestation, and pregnancy rate, as well as fetal number and resorption rate were determined.

Sperm preparation

Epididymides were removed, freed of connective tissues and fat, and were further divided into caput, corpus and caudal regions. Caudal tissues were transferred to a small glass dish containing 1 ml of M2 medium (Sigma-Aldrich, Saint-Quentin Fallavier, France) and punctured repeatedly with a 26-gauge needle to recover the spermatozoa. After 5 min incubation, to allow sperm cell dispersion, the suspensions were centrifuged at 500 × g for 5 min and pellets were resuspended into 200 μl of M2. Sperm counts were determined using a Malassez hemocytometer. Sperm motility was determined immediately after collection via computer-aided semen analysis (CASA) during which sperm tracks (300 frames) were captured for each sample using a CEROS sperm analysis system (Hamilton Thorne, Lisieux, France; software version 12).

Sperm DNA integrity

DNA compaction was studied using the modified protocol of Conrad *et al.* (2005) for toluidine blue (TB) staining, whereby spermatozoa were stained with 1% TB in McIlvaine's buffer (200 mM Na₂HPO₄ and 100 mM citric acid, pH 3.5) for 17 min at room temperature. Slides were dehydrated in ethanol and mounted with Cytoseal[™]60 medium (Thermo Scientific, Waltham, MA, USA). Three smears *per* sample were deposited on glass plates and at least 300 spermatozoa *per* smear were counted (duplicate slides for each animal). Assessment of sperm DNA fragmentation was carried out using the staining protocol of the Halomax[™] kit (Chromacell, Madrid, Spain), a modified sperm chromatin dispersion assay (Fernández *et al.*, 2003). Four smears *per* sample were deposited on glass plates and at least 300 spermatozoa *per* smear were counted (duplicate slides for each animal). Detection of 8OHdG was carried out on spermatozoa as described

previously (Chabory et al., 2009; Noblanc et al., 2013). Cauda spermatozoa were collected as described above and resuspended in a decondensing buffer (2 mM dithiothreitol and 0.5% triton X-100 in 1 × phosphate-buffered saline (PBS)) and incubated for 30 min at room temperature. After centrifugation at $500 \times g$ for 5 min at room temperature, spermatozoa were resuspended in 1 ml 1 × PBS, counted, and deposited onto a glass plate at a density of 30 000 cells per plate. Incubations with the primary antibody (N45.1; Gentaur, Euro-medex) were conducted overnight at 4°C. After two washes in 1 × PBS (5 min each) the secondary antibody was applied for 30 min at room temperature [dilution 1/1000]. Antibody binding was detected using the Vectastain R ABC kit incorporating peroxidase labeled Immunoglobulin G (Vector Laboratories, Abcys, Paris, France). Signal amplification was obtained by the use of the Vector Nova Red substrate kit for peroxidase (Vector Laboratories, Abcys, Paris, France). At least 300 spermatozoa per slide were counted (duplicate slides for each animal).

Quantitative RT-PCR

Total RNAs were isolated with the NucleoSpin[®] RNA II kit (Macherey-Nagel, France) and were reverse transcribed by moloney murine leukemia virus Reverse Transcriptase (Promega Corp., France) according to the manufacturer's instructions. Quantitative real-time PCR assays were performed using a RealPlex thermocycler (Eppendorf, Hamburg, Germany). During this procedure 2 µl of diluted cDNA template (1/20) were amplified using MESA GREEN qPCR MasterMix Plus (Eurogentec, France) according to the manufacturer's instructions. Primer sequences are given in Table 1. To ensure no genomic DNA contamination, primers were designed targeting distinct exons so that genomic DNA was unlikely to be amplified. A standard curve of amplification efficiency for each set of primers was generated via serial dilution of plasmids containing DNA of targeted genes. Melting curve analysis was carried out to confirm the specificity of primers. For quantification of transcripts, the relative method was used to calculate mRNA levels relative to the *Cyclophilin or/and 36B4* standard(s); chosen because they exhibited stable expression between the tissues and genotypes.

Microscopic observations

Observations and counts of TB staining and DNA fragmentation assays were made with an Axioskop transmitted light microscope (Carl Zeiss, Germany) at 400 × magnification. Spermatozoa positive for 8OHdG were counted by Axioplan2 imaging (Carl Zeiss, Germany) in transmitted light at 630 × magnification. Observations of fluorescent probes were made by Axioplan2 imaging (Carl Zeiss, Germany) at the following excitation and emission wavelengths: Alexa 488, BP 475/40 and BP 530/50; Hoechst 33342, BP 365/12 and LP 397, respectively (magnification: × 1000).

Histology

Scrotal heat stressed male mice and control mice were sacrificed by cervical dislocation, and the testes were fixed in modified Davidson fluid for 48 h (Latendresse et al., 2002). The testes were then rinsed with PBS and stored in 70% ethanol until the time of analysis. Testes were embedded in paraffin using routine histologic protocols for subsequent light microscopic evaluation. Serial 10 µm sections were cut from the paraffin blocks and selected for staining. All sections were stained with hematoxylin/eosin. Two investigators blinded to the groups interpreted the structural changes. At least eight animals per group were analyzed.

Statistical analyses

Kruskal–Wallis and Mann–Whitney nonparametric tests were performed with GraphPad Prism 5.02 software (GraphPad, CA, USA) to determine the significance of differences between samples. Chi-squared test was used to compare pregnancies and fetuses obtained in the treatment versus

control groups and z test was used to compare pregnancy rates (Microsoft Excel, Microsoft Corporation, Redmond, WA, USA). P-values of ≤ 0.05 were regarded as significant.

Results

Sperm parameters

Caudal epididymal spermatozoa from *Gpx5*^{-/-} mice exhibited similar counts and motility to WT spermatozoa as previously reported (Chabory et al., 2009). Fertilix[®] treatment did not significantly alter the total sperm motility of WT or *Gpx5*^{-/-} samples as measured by CASA (Fig. 1A). However, caudal sperm counts were significantly elevated in *Gpx5*^{-/-} animals supplemented with Fertilix[®] while the supplementation had no effect on caudal counts in WT animals (Fig. 1B).

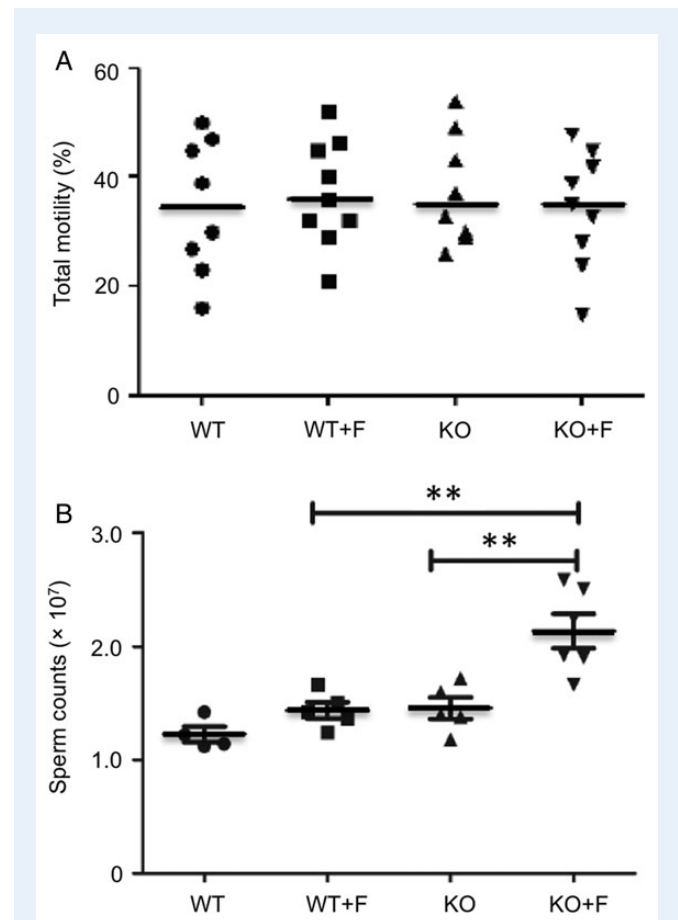


Figure 1 Evaluation of Fertilix[®] impact on mouse cauda sperm motility and counts. **(A)** The effect of Fertilix treatment on total sperm motility as estimated by computer-aided semen analysis in wild-type male mice (WT), wild-type male mice supplemented with Fertilix[®] (WT + F), *Gpx5*^{-/-}: glutathione peroxidase 5 (*Gpx5*)^{-/-} male mice (KO) and *Gpx5*^{-/-} male mice supplemented with Fertilix[®] (KO+F). **(B)** The effect of Fertilix[®] treatment on caudal sperm counts given in total number of cells retrieved respectively, in wild-type male mice (WT), wild-type male mice supplemented with Fertilix[®] (WT + F), *Gpx5*^{-/-} male mice (KO) and *Gpx5*^{-/-} male mice supplemented with Fertilix[®] (KO+F). Bars indicate mean values while error bars indicate SEM; *P ≤ 0.05; **P < 0.01 (Mann–Whitney statistical test).

Sperm DNA fragmentation and nuclear compaction

A sperm chromatin dispersion assay was used to estimate the level of sperm DNA fragmentation in each group. Fertilix[®] supplementation did not significantly change the level of DNA fragmentation recorded either in WT and KO samples (Fig. 2A). However, *Gpx5*^{-/-} caudal sperm samples did show significantly more nuclear fragmentation than WT caudal spermatozoa in line with previous findings (Chabory *et al.*, 2009). In addition, TB staining was used to evaluate the level of caudal sperm nuclear compaction in the various groups of mice. No significant differences were observed in the percentage of TB positive spermatozoa, suggesting that nuclear compaction was unaltered in these *Gpx5*^{-/-} mice (Fig. 2B).

8OHdG adduct formation

The prevalence of oxidative attack on DNA was directly measured in caudal spermatozoa through the formation of 8OHdG residues, a well-

established biomarker of DNA oxidation. Fertilix[®] supplementation significantly reduced 8OHdG formation to control levels (Fig. 3). Interestingly, Fertilix[®] treatment of WT animals did not diminish the percentage of caudal spermatozoa positive for 8OHdG, which remained around 30% (Fig. 3).

Impact of Fertilix[®] on the antioxidant status of the cauda epididymis

To investigate whether Fertilix[®] supplementation could protect the mouse epididymis against oxidative stress we monitored using real-time PCR, the accumulation of transcripts of primary antioxidant enzymes in the cauda epididymis, namely epididymal extracellular superoxide dismutase (eSOD3) and peroxide-processing enzymes including catalase and glutathione S transferase μ (GST μ) (Chabory *et al.*, 2009; Noblanc *et al.*, 2012). In Fig. 4, we confirm our previous studies by showing that transcripts for major (catalase) and accessory (GST μ) H₂O₂-recycling enzymes, are increased in *Gpx5*^{-/-} caudal epididymal extracts when compared with WT, while the transcript for the superoxide metabolizing enzyme (SOD3) is not significantly changed. By contrast, Fertilix[®] treatment reduced the accumulation of the antioxidant transcripts for catalase and GST μ , in both WT and *Gpx5*^{-/-} mice (Fig. 4).

Scrotal heat stress model

Representative histological testis sections of control mice or mice supplemented with Fertilix[®] at two time points following scrotal heat stress are shown in Fig. 5. Consistent with previous findings (Hourcade *et al.*, 2010), scrotal heat stress induced a loss of seminiferous tubule organization as well as abnormal tubules, which was especially visible 15 days post-stress (Fig. 5A and D). Conversely, testicular sections of Fertilix[®]-supplemented mice showed no signs of major abnormality (Fig. 5C and E) at the same time points compared with unstressed controls. These observations were quantified on large numbers of tubules representing all stages of spermatogenesis from several animals, as described in Table II. In control animals,

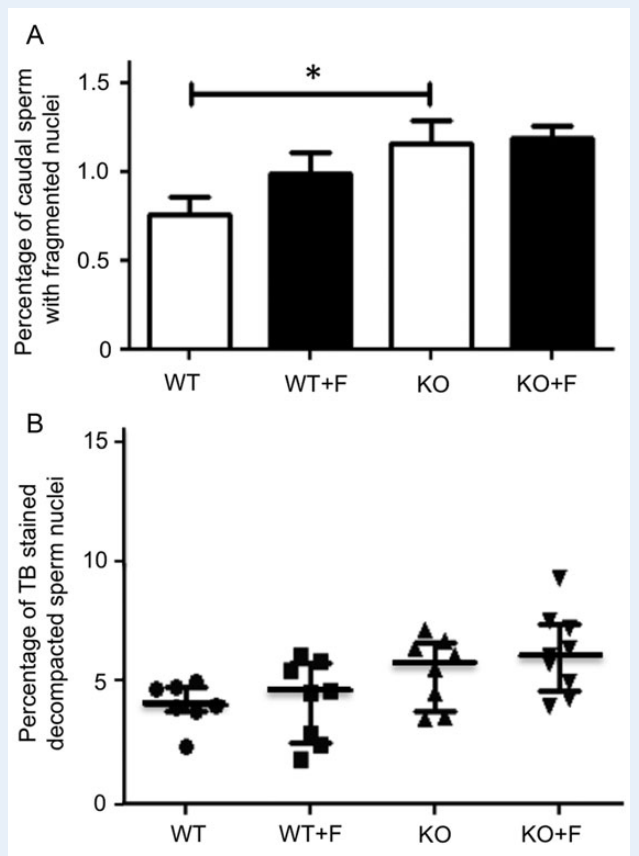


Figure 2 Evaluation of the impact of Fertilix[®] on caudal sperm DNA fragmentation and cauda sperm nuclear compaction in mice. (A) The effect of Fertilix[®] on the percentage of spermatozoa having a fragmented nucleus as revealed by the HalomaxTM assay, in WT, WT+F, KO and KO+F mice. Errors bars indicate SEM; * $P \leq 0.05$. (B) The effect of Fertilix[®] on the percentage of spermatozoa bearing a decondensed nucleus as revealed by the toluidine blue assay in WT, WT+F, KO and KO+F. Bars indicate mean values while errors bars indicate SEM (Mann–Whitney statistical test).

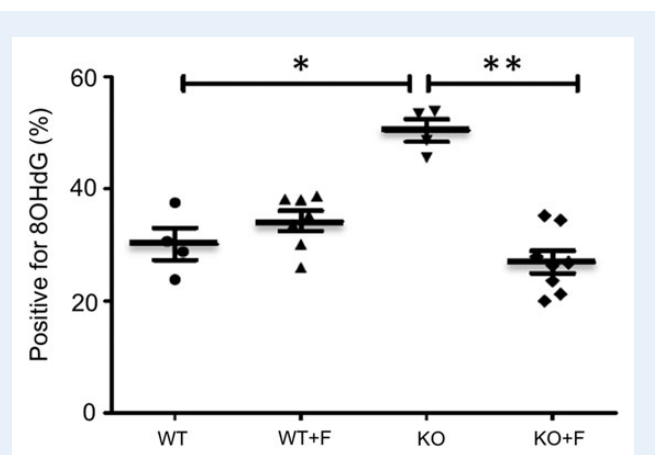


Figure 3 Evaluation of the impact of Fertilix[®] on caudal sperm DNA oxidation in mice. The effect of Fertilix[®] on the percentage of cauda spermatozoa showing high nuclear reactivity towards an 8-hydroxy-2'-deoxyguanosine (8OHdG) antibody in WT, WT+F, KO and KO+F mice. Bars indicate mean values while errors bars indicate SEM; * $P \leq 0.05$; *** $P < 0.01$ (Mann–Whitney statistical test).

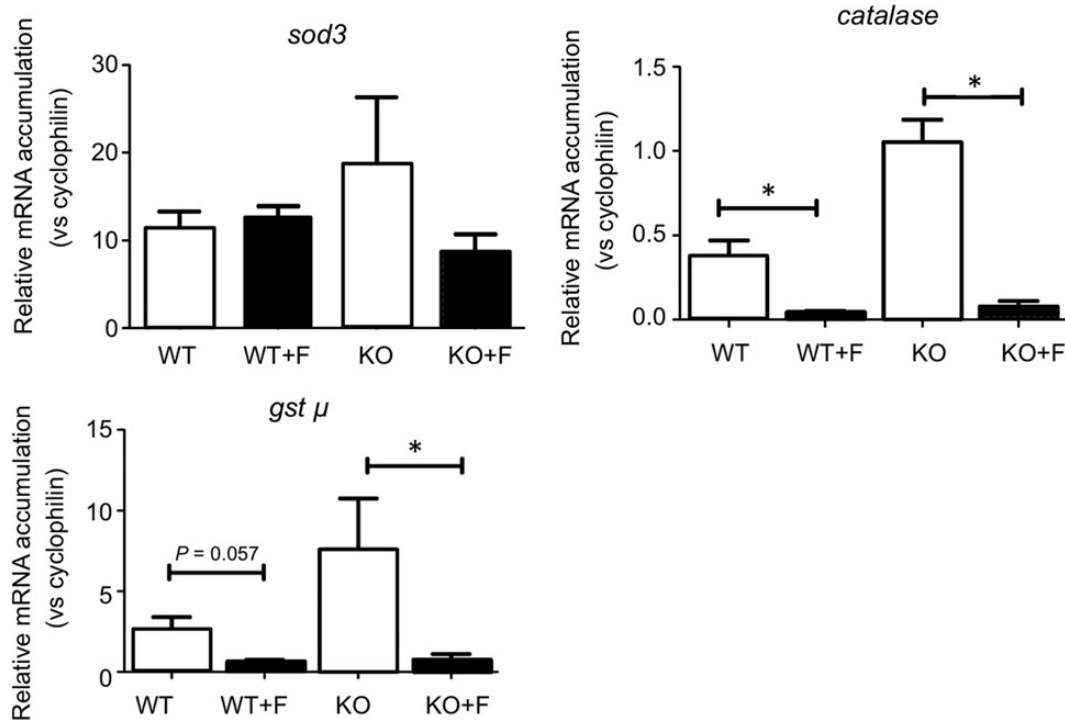


Figure 4 Quantitative RT–PCR estimations of *sod3*, *catalase*, and *gstμ* mRNA accumulation in cauda epididymal extracts from WT and KO male mice treated (F) or not treated with Fertilix[®]. For quantification of transcripts, the relative method was used to calculate mRNA levels relative to the *Cyclophilin* and *36B4* standards. Data presented for superoxide dismutase 3 (*sod3*), *catalase* and glutathione S transferase μ (*gstμ*) transcripts. Data presented as mean \pm SEM; * $P \leq 0.05$ (Mann–Whitney statistical test).

78% of observed tubules showed a normal organization while after scrotal heat stress only 30.8% of the tubules (Day 1 post-stress) and 22% of the tubules (Day 15 post-stress) were histologically normal (Table II). In Fertilix[®]-supplemented animals, the proportion of seminiferous tubules showing normal organization increased to 50.9% (Day 1 post-stress) and 64.7% (Day 15 post-stress). These data suggest that Fertilix[®] supplementation allowed the testicular tissue to better withstand acute transient scrotal heat stress ($P < 0.0001$).

Pregnancy outcomes following scrotal heat stress

In order to evaluate the impact of Fertilix[®] on the reproductive potential of spermatozoa following transient heat stress, males were each mated with three female mice. The resulting numbers of pregnant mice, live fetuses and resorption sites were monitored (Table III). Scrotal heat stressed animals ($n = 18$) successfully impregnated only 19 of 54 dams (35.2%), giving birth to 169 fetuses, while non-stressed controls were significantly more successful, impregnating 34 of 36 dams (94.4%) and giving rise to 340 fetuses. Fertilix[®]-supplemented male mice ($n = 19$) subjected to scrotal heat stress approached the fecundity of unstressed controls, achieving pregnancy in 42 of 57 females (73.7%) and giving rise to 427 fetuses (Table III; $P < 0.05$). The number of fetal resorptions were also assessed as follows; 2.9% (10/340) in normal dams versus 18% (30/169) in heat stressed animals but was significantly reduced to 9%

(38/427) in Fertilix[®]-supplemented mice. The average litter weight was unchanged at about 42 g in all 3 groups.

Discussion

The purpose of this study was to determine whether a carefully formulated antioxidant preparation, Fertilix[®], could ameliorate the consequences of oxidative stress within the male reproductive tract using two animal models: (i) the *Gpx5* KO mouse which exhibits diminished antioxidant protection in the epididymal lumen in the absence of any systemic redox change and (ii) a mild scrotal heat stress. In both situations Fertilix[®] administration was shown to have a significant protective effect. In *Gpx5*^{-/-} mice, this antioxidant formulation reduced the levels of oxidative DNA damage in the spermatozoa and reduced the epididymal need to exhibit a compensatory increase in catalase or GST μ expression (Fig. 4). The strong reduction in catalase and GST μ expression levels recorded in cauda epididymal extracts of animals supplemented with Fertilix[®] suggests that hydrogen peroxide generation is efficiently controlled in that compartment by one or more of the ingredients present in the Fertilix[®] formulation. However, it is not possible to know which ingredient(s) is (are) responsible for this effect as many of the constituents of Fertilix[®], including lycopene, vitamin E, selenium and carnitines, can reduce H₂O₂-mediated oxidative alterations (as examples see: Salem et al., 2012; Salama et al., 2015). The increase in sperm counts recorded only in *Gpx5*^{-/-} mice treated with Fertilix[®] (Fig. 1) was

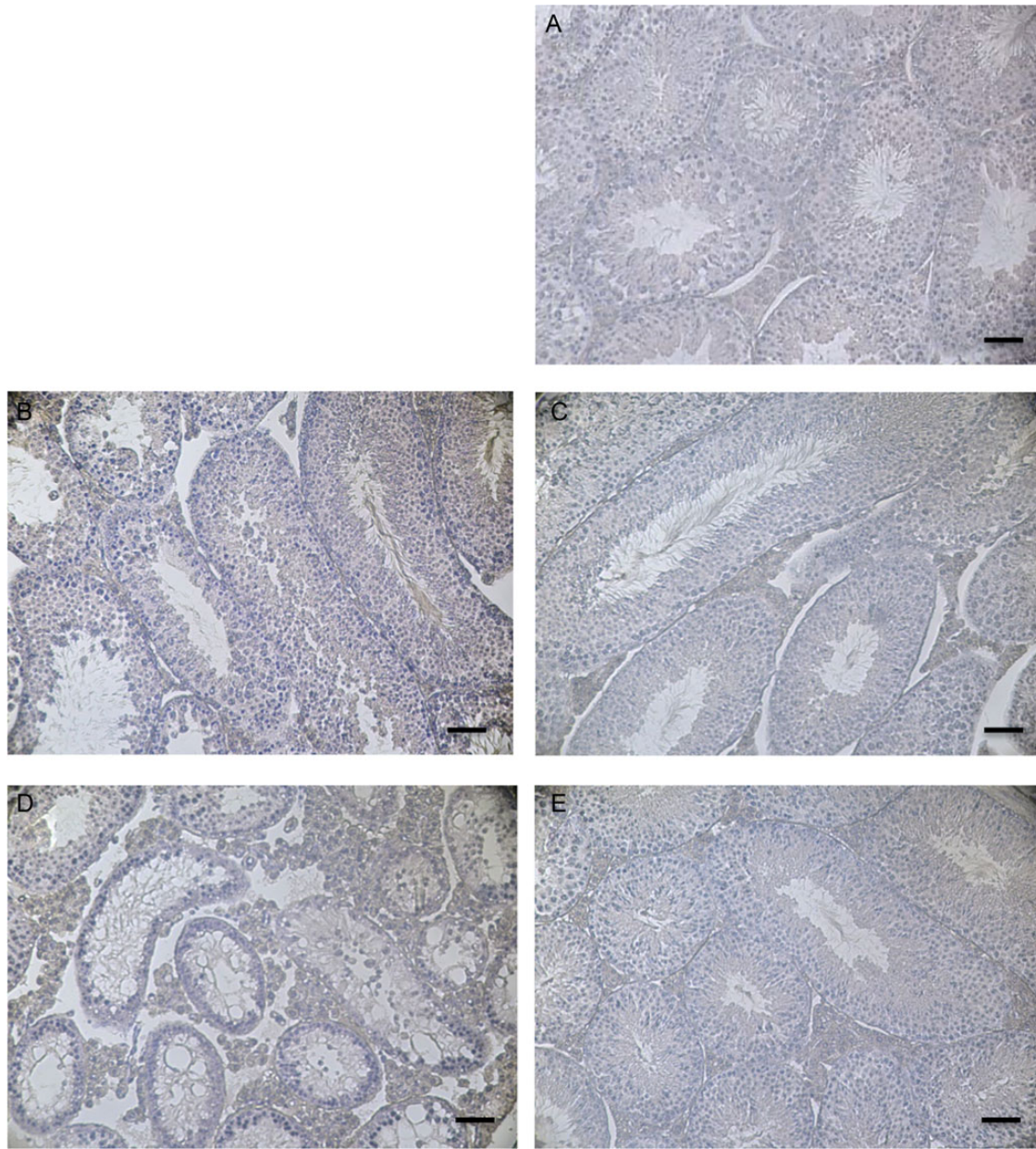


Figure 5 Evaluation of the impact of Fertilix[®] on testicular histology in the scrotal heat stress mouse model. Representative photographs showing sections of seminiferous tubules stained with hematoxylin/eosin in control mice (A), unsupplemented mice at 1 day (B) and 15 days (D) post scrotal heat stress, and mice supplemented with Fertilix[®] at 1 day (C) and 15 days (E) post scrotal heat stress. Scale bar = 50 μm .

unexpected and difficult to explain, given that the effects of *GPx5* depletion are confined entirely to the epididymis. One hypothesis may be that the antioxidant treatment associated with the epididymal activities that are switched-on in this KO model (Chabory *et al.*, 2009; Noblanc *et al.*, 2012) to compensate for the lack of *GPX5*, may act synergistically with the antioxidants in Fertilix[®] to protect spermatozoa transiting the epididymis from elimination by quality control processes which we have previously shown to be operative in this organ (Jrad-Lamine *et al.*, 2011), thereby increasing caudal sperm counts. In the scrotal heat stress model, Fertilix[®] exhibited a capacity to protect spermatogenesis from the adverse effects of increased temperature (Fig. 5; Table II) as clearly

indicated by the absence of abnormal spermatogenic tubules at Day 15 and the significant increase in overall fertility (Table III). This ability of antioxidants to protect the seminiferous tubules from structural damage is impressive and clearly indicates that the constituents of Fertilix[®] are able to gain access to the germinal epithelium. Spermatogenesis is a temperature-dependent process, and increases in scrotal temperature are known to induce DNA damage and a complex stress response, including induction of genes associated with oxidative stress and hypoxia resulting in germ cell death (Paul *et al.*, 2009). The significant rise in both pregnancy rates and reduction of fetal resorptions in scrotal heat stress model provide definitive evidence in support of the prophylactic use of oral

antioxidant administration in ameliorating oxidative stress in the male germ line.

While clinical studies with a wide variety of different antioxidant preparations have been generally encouraging (Greco et al., 2005; Tremellen et al., 2007; Tunc et al., 2009; Gharagozloo and Aitken, 2011; Showell et al., 2011, 2014), most of these investigations have been flawed because they did not select the patients on the basis of oxidative stress but instead either randomly selected patients for such treatment or employed indirect markers of oxidative damage including poor sperm motility or DNA fragmentation (Kessopoulou et al., 1995; Moslemi and Tavanbakhsh, 2011; Ménéz et al., 2014). Similarly, the effectiveness of such treatment has rarely been examined in terms of the resolution of the oxidative stress but rather inferred from changes in semen quality, apoptosis or even fertility (Tremellen et al., 2007; Tunc et al., 2009). As a result of this lack of direct engagement with markers of oxidative stress, it has been difficult to determine the effectiveness of antioxidant therapy *in vivo* (Gharagozloo and Aitken, 2011) although the effectiveness of antioxidants such as α -tocopherol or resveratrol have been repeatedly demonstrated *in vitro* (Aitken et al., 1989; Mojica-Villegas et al., 2014). In this study, we have utilized animal models where the impacts on male reproductive function are definitively oxidative and have demonstrated effective reversal of the oxidative stress phenotype using an antioxidant preparation. Clearly, these

data suggest that the detailed formulation of Fertilix[®] (see [Supplementary Data](#)) is such that the constituents are bioavailable and bioactive *in vivo* in both the testis and the epididymis. These promising data now open the way for clinical trials with this antioxidant to determine whether this antioxidant formulation is able to reverse the oxidative stress encountered in the human male population.

Oxidative stress in the male reproductive tract originates from various pathophysiological stressors, such as age, infection or exposure to environmental toxicants, as well as unhealthy attributes of the modern lifestyle including smoking and obesity (Aitken et al., 2014; Wright et al., 2014). Of particular interest from the perspective of ART, is the observation that most DNA damage in the male germ line is oxidatively induced and strongly linked with adverse downstream events including increased rates of miscarriage as well as *de novo* mutations, birth defects, metabolic disease and obesity in the offspring (Aitken et al., 2009; Chabory et al., 2009; De luliis et al., 2009; Lane et al., 2014). In light of such findings, reducing oxidative stress in the germ line of males contemplating parenthood would seem advisable and would logically be deemed a matter of 'best practice' as far as ART clinics are concerned.

It is also important to stress the obvious point that antioxidant therapy will only influence pathologies that are induced by oxidative stress. Treating normal males, who are not suffering from oxidative stress, with an aggressive antioxidant preparation may even induce 'reductive stress' by suppressing redox reactions that are vital to normal physiology and inducing, rather than suppressing, DNA damage (Seifirad et al., 2014). In this context, it is undeniable that male infertility, and even DNA damage, in spermatozoa may have multiple etiologies many of which do not involve a contribution from oxidative stress. For example, several studies have indicated that DNA fragmentation in spermatozoa may also involve a contribution from nucleases potentially activated in sperm chromatin during the late stages of apoptosis (Smith et al., 2013b; Muratori et al., 2015). At present the molecular identity of this putative nuclease or the mechanisms associated with its activation are uncertain. However the fact that Fertilix[®] did not reduce DNA fragmentation levels as measured with a Halomax[™] assay in the *Gpx5*^{-/-} model (Fig. 2) suggests there are elements of the DNA fragmentation process that are beyond the reach of antioxidants. Importantly, however, Fertilix[®] did significantly decrease the incidence of 8OHdG positive cells in these mice (Fig. 3). This activity is particularly important clinically because 8OHdG is a mutagen and the germ line has a very limited capacity to remove such adducts from the DNA prior to the initiation of embryo development (Smith et al., 2013b).

Spermatozoa are unusual in that they only possess the first enzyme in the base excision repair pathway, OGG1 (8-oxoguanine DNA glycosylase) (Smith et al., 2013b). As a result, when oxidative DNA damage

Table II Characterization of mouse testicular cross sections at Day 1 and Day 15 following acute transient scrotal heat stress.

Samples [#]	Tubules analyzed	Tubules with spermatogenesis	Tubules with abnormal phenotype
Control	614	479 (78.0%)	0 (0%)
SHS (Day 1)	159	49 (30.8%)	0 (0%)
SHS+Fertilix [®] (Day 1)	267	136 (50.9%) ^a	0 (0%)
SHS (Day 15)	282	62 (22.0%)	126 (44.7%) ^c
SHS+Fertilix [®] (Day 15)	312	202 (64.7%) ^b	0 (0%)

SHS, Scrotal Heat Stress.

[#]Six mice were analyzed per group.

^aFertilix significantly increased number of tubules with spermatogenesis compared with SHS controls on the day of heat stress (Chi square statistic, $P < 0.001$).

^bFertilix significantly increased number of tubules with spermatogenesis compared with SHS controls 15 days following heat stress (Chi square statistic, $P < 0.001$).

^cFertilix significantly decreased number of tubules with abnormal phenotype compared with SHS controls (Chi square statistic, $P < 0.001$).

Table III Fertility rates of mice treated and not treated with Fertilix following acute transient scrotal heat stress.

	Treated males	Mated females	Successful pregnancies	Pregnancy rate (%)	Fetus number	Fetal resorption n (%)
Control	12	36	34	94.4	340	10 (2.9)
SHS	18	54	19	35.2	169	30 (17.8)
SHS + Fertilix [®]	19	57	42*	73.7*	427*	38 (8.9)*

Mating occurred between 1 and 2 weeks after SHS.

* $P < 0.05$ for difference between SHS and SHS + Fertilix[®], Chi-square and z test.

occurs, these cells can remove the adducted base but are unable to advance the repair process further because they do not possess the next components of the pathway, APE1 (apurinic/aprimidinic endonuclease 1) and XRCC1 (X-ray repair complementing defective repair in Chinese hamster cells 1). In contrast, the oocytes are characterized by high levels of APE1 and XRCC1 but possess limited supplies of OGG1 (Smith *et al.*, 2013b). Thus DNA repair involves the concerted action of both male and female gametes. If oxidative DNA damage in spermatozoa is severe then the oocyte will be presented with an abundance of abasic sites created by OGG1, which will destabilize the DNA backbone and increase the tendency for DNA fragmentation to occur. Furthermore, when this system is overwhelmed, which is frequently the case in the infertile patient population (Aitken *et al.*, 2010), then the oocyte is presented with unresolved 8OHdG residues, which this cell has a limited capacity to address, given the relative lack of OGG1 in the female germ line. It is therefore likely that these highly mutagenic DNA lesions will persist beyond the immediate post-fertilization round of DNA repair into S-phase of the first mitotic division, increasing the risk of *de novo* mutations being created as the embryo enters the cleavage stage of development. Evidence to support the link between oxidative DNA damage in the male germ line and the mutational load carried by the progeny can be found in studies of paternal ageing which clearly reveal that advanced paternal age is associated with increased oxidative DNA damage in spermatozoa (Weir and Robaire, 2007; Smith *et al.*, 2013a) and an increased incidence of *de novo* mutations in the offspring (Kong *et al.*, 2012).

Conclusions

Cells utilize a selection of antioxidants to protect themselves against the deleterious effects of oxidative stress. The development of antioxidant supplements to combat oxidative stress in the male germ line *in vivo* will therefore involve the development of an optimized combination of scientifically validated antioxidants. As set out in the [Supplementary Data](#), we used a set of stringent medicinal chemistry criteria to critically examine every antioxidant ingredient, herbal or plant extract reported in the literature for some measure of improvement of sperm quality or male fertility. We found compelling evidence for only seven ingredients. The doses and nature of ingredients (synthetic versus natural) used, were also critically examined to ensure little or no short or long-term side effects. The *in vivo* experiments using mouse *Gpx5*^{-/-} and scrotal heat stress models reported in this article provide the first robust evidence that antioxidants can access the male germ line *in vivo* and effectively address the consequences of oxidative stress at both testicular and epididymal levels. We do not currently understand the etiology of oxidative DNA damage in human spermatozoa and so cannot be certain that the results obtained in this study would be replicated in a clinical situation. Nevertheless these results provide an important objective basis for actively pursuing clinical trials in human subjects exhibiting evidence of oxidative damage to their spermatozoa. Large, well-designed clinical trials are now required to validate these findings in infertile couples.

Supplementary data

Supplementary data are available at <http://humrep.oxfordjournals.org/>.

Authors' roles

P.G. designed, developed and orchestrated the preparation of Fertilix[®] for this study and participated in the design of the experiments, the analysis of the data and the preparation of the manuscript. J.R.D., A.G.-A., A.C., A.N., A.K., A.C., S.P.-C. and E.P. designed and undertook the experimental work, contributed to the analysis and presentation of data and facilitated preparation of the manuscript. A.M. and A.P. were involved in the overall management of the project and contributed to the preparation of the manuscript. R.J.A. was involved in critically reviewing the initial draft of this publication and in redrafting the final version of the paper.

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Conflict of interest

P.G. is the Managing Director of CellOxess LLC, which has a commercial interest in the detection and resolution of oxidative stress. A.M. is an employee of CellOxess LLC, which has a commercial interest in the detection and resolution of oxidative stress. A.P. is an employee of CellOxess LLC, which has a commercial interest in the detection and resolution of oxidative stress. J.R.D., A.G.-A. and R.J.A. are honorary members of the CellOxess advisory board.

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The rationale behind the composition of Fertilix[®]

In this supplementary file we present a brief account of the rationale behind the composition of Fertilix[®], focusing initially on two of the major constituents, carnitine and vitamin E, and then going on to review the remaining components of the formulation including a consideration of the dosages employed.

Carnitines

In humans, the endogenous carnitine pool is made up of free L-Carnitine (LC) and a wide range of alkanoyl ester derivatives with Acetyl-L-Carnitine (ALC) and Propionyl-L-Carnitine (PLC) receiving the most attention (Evans and Fornasini, 2003). Carnitines are zwitterions with a quaternary ammonium group. Although LC is synthesized in the liver and kidneys from lysine and methionine (Steiber *et al.*, 2004; Crill and Helms, 2007), it is abundant in food with highest concentrations found in red meat (95 mg/100 g). LC transports long-chain acyl groups from fatty acids into the mitochondrial matrix, so they can be broken down through β -oxidation to acetyl-CoA to obtain usable energy via the citric acid cycle (Houten and Wanders, 2010). ALC is formed in the mitochondria by carnitine acetyltransferase, which combines LC with an acetyl group from CoA. ALC is then transported across the inner mitochondrial membrane by carnitine acetyltranslocase, where it diffuses out of the mitochondria into the cytoplasm and serves as a source of acetyl groups for cytosolic proteins (Lysiak *et al.*, 1988; Longnus *et al.*, 2001).

The antioxidant actions of carnitines are strongly supported by numerous studies but the specific mechanisms are not fully clear. Most of the studies suggest an indirect mechanism-of-action, with increased expression of antioxidant enzymes such as heme oxygenase-1, endothelial nitric oxide synthase (Calabrese *et al.*, 2005), superoxide dismutase and glutathione peroxidase (Rani and Panneerselvam, 2002) responding to carnitine intake. Other studies have shown significant increases in concentrations of low molecular weight antioxidants such as melatonin (Esposti *et al.*, 1994), vitamin C and vitamin E (Kumaran *et al.*, 2003). Alternatively, carnitines may reduce ROS levels by correcting toxic levels of acetyl-CoA or replacement of fatty acids into plasma membrane phospholipids. Some *in vitro* evidence also suggests that ALC possesses direct antioxidant capability (Schinetti *et al.*, 1987; Geremia *et al.*, 1988). Irrespective of the exact mechanism, the antioxidant action of carnitines is confirmed by many studies demonstrating a significant reduction of oxidative stress biomarkers of lipids, proteins and DNA (Juliet *et al.*, 2005; Zhai *et al.*, 2008).

In the male genital tract, carnitines are concentrated in the epididymis and taken up by sperm cells during epididymal transit (Bøhmer *et al.*, 1978). Interestingly, in seminal fluid, most LC and ALC are found in seminal plasma rather than the spermatozoa themselves. This arrangement facilitates protection of these cells from damaging extracellular

ROS generated by moribund spermatozoa and activated leukocytes in the ejaculate. Additionally, ALC has been shown to aid in post-injury recovery of mouse spermatogenesis *in vivo* after exposure of the testis to X-ray (Amendola *et al.*, 1989), magnetic field radiation (Ramadan *et al.*, 2002), or hyperthermic insult (Amendola *et al.*, 1991) relative to controls.

Several human clinical studies document significantly lower concentrations of carnitines in the semen of infertile relative to fertile men. These studies correlate concentrations of carnitine species with a rise in sperm count, motility and viability (Lewin *et al.*, 1976; Soffer *et al.*, 1981; Gürbüz *et al.*, 2003; Balercia *et al.*, 2005) highlighting their important role in spermatogenesis. It is therefore not surprising that LC and ALC supplementation, alone or in combination with each other, has been the subject of at least 22 clinical trials in male infertility, more so than any other antioxidant (Gharagozloo and Aitken, 2011). Although these trials were small and in general considered low quality, the consistency of the results and a meta-analysis of the published data overwhelmingly support the use of carnitines in reducing sperm oxidative stress with concomitant improvement of semen parameters, particularly sperm motility, and in some studies, improved clinical outcomes (Zhou *et al.*, 2007).

Thus, the collective evidence supporting the benefits of carnitine therapy in alleviating oxidative stress, boosting sperm 'preconception health' and preserving male fertility is compelling. However, there are some caveats concerning the routine use of LC. Studies from the Ames group, comparing the antioxidant efficacy of dietary LC and ALC reveal that only ALC is effective in decreasing oxidative damage to lipids and DNA (Liu *et al.*, 2004). Moreover, free LC has low oral bioavailability at levels commonly used in oral supplements, exhibiting a range of 5–18% bioavailability in normal healthy subjects (Evans and Fornasini, 2003). Unabsorbed L-carnitine is mostly degraded by microorganisms in the large intestine. One point of concern is that LC can alter cecal microbial composition leading to markedly enhanced synthesis of the proatherogenic metabolite, trimethylamine-N-oxide (TMAO). Human subjects with high TMAO levels and concurrently high plasma LC levels carry increased risks of both cardiovascular disease and major adverse cardiac events (Koeth *et al.*, 2013; Brown and Hazen, 2014). In light of these findings, long-term supplementation of high dose free LC should be avoided. Fortunately, propionyl-L-carnitine (PLC) is a natural prodrug for LC. Although stable in whole blood, PLC undergoes renal and hepatic hydrolysis by the enzyme carnitine acetyl transferase, readily converting into propionyl co-enzyme A and free L-carnitine (Pace *et al.*, 2000; Vanella *et al.*, 2000). Moreover, due to its higher lipophilicity, PLC is rapidly absorbed, thus allowing for significantly reduced doses in a dietary supplement formulation. PLC is also known to be a scavenger of superoxide anion, protector against DNA cleavage and effective inhibitor of the lipoperoxidation of polyunsaturated fatty acids (Vanella *et al.*, 2000). Due to such favorable properties, a combination of ALC and PLC was used as the choice carnitines in our formulation.

Vitamin E

Vitamin E is in fact a collective term given to a family of at least eight essential micronutrients classed as α -, β -, γ -, and δ -tocopherols and tocotrienols (Brigelius-Flohé and Traber, 1999). All eight isoforms are made up of a chroman head group and hydrophobic phytyl chain, which is unsaturated in tocotrienols. Vitamin E isoforms are the most potent inhibitor of lipid peroxidation known (Niki, 1987). Once in the cell, vitamin E can be found in the lipid bilayer, with the chroman head group towards the surface and the phytyl chain embedded in the hydrocarbon region, with the head group generally responsible for all antioxidant activity. As an antioxidant, vitamin E prevents the propagation of free radical reactions, and has been shown to provide protection to DNA, proteins, and lipids against chemical insult by ROS/RNS.

Although the evidence supporting the use of vitamin E as an effective antioxidant in ameliorating sperm oxidative stress is overwhelming, a detailed survey of the literature on vitamin E congeners cautions against the long-term use of any single component of the vitamin E family. Instead, our findings strongly favor the use of full spectrum natural vitamin E family in any adjuvant antioxidant dietary supplement. The following is a partial account of our findings.

Among infertile men, vitamin E appears to be the most widely used antioxidant recommended by fertility specialists. The most commonly recommended form of vitamin E is synthetic α -tocopherol. This is a racemic mixture of 8 stereoisomers, with seven being unnatural and serving no known biological benefit (Traber, 2006). Short-term clinical studies with the racemic mixture have not revealed any adverse effects, but concerns over high doses or long-term use have been expressed (Graat *et al.*, 2002; Miller *et al.*, 2005; Pizzorno and Neustadt, 2005; Hatfield and Gladyshev, 2009; Klein *et al.*, 2011; Albanes *et al.*, 2014). The isoform α -tocopherol is the most abundant one in nature and appears to have the highest biological activity of all isoforms (Brigelius-Flohé and Traber, 1999). It has a higher plasma and tissue concentration, and a much longer elimination half-life than that of its counterparts (Traber, 1994).

More recently, infertile men were shown to have significantly lower concentrations of α - and γ -tocopherols in their blood plasma, as well as significantly lower concentrations of α -, γ -, and δ -tocopherols in seminal plasma when compared with fertile men (Benedetti *et al.*, 2012). However, α -tocopherol binds with about 10-fold higher affinity to α -tocopherol transport protein (α -TTP) relative to other isoforms, (Hosomi *et al.*, 1997; Galli *et al.*, 2007; Sylvester, 2007). Exclusive exogenous administration of α -tocopherol would therefore saturate α -TTP, thus significantly inhibiting the binding and transfer of other dietary tocopherols and tocotrienols potentially affecting the physiological balance of the vitamin E isoforms in the body (Handelman *et al.*, 1985; Huang and Appel, 2003; Stocker, 2004). Maintaining this balance is important as recent studies show that while α -tocopherol is a potent inhibitor of ROS it has little antioxidant activity towards RNS such as peroxynitrites. In contrast, γ -tocopherol has little ROS inhibitory activity but is a potent RNS inhibitor (Cooney *et al.*, 1993; Christen *et al.*, 1997; McCarty, 2007). Thus, the exclusive exogenous supplementation of α -tocopherol would inhibit the absorption of dietary γ -tocopherol resulting in the buildup of RNS. This is important as RNS such as peroxynitrite are known to decrease motility and mitochondrial membrane potential in human spermatozoa (Vignini *et al.*, 2006; Uribe *et al.*, 2015) and may induce *de novo* DNA mutation specific to this class of

reactive species (Tang *et al.*, 2012). Other tocopherols and tocotrienols found naturally in a variety of plant species also appear to have potent antioxidant activities, but a full discussion of their activity is beyond the scope of this paper and will be disclosed elsewhere. In conclusion, natural vitamin E isoforms appear to act synergistically to provide a more effective management of oxidative stress. These considerations were the basis for including a combination of all eight natural isoforms of vitamin E in Fertilix[®] formulation at doses that avoid saturation of the relevant transporters.

Overall composition of Fertilix[®]

At the heart of our design strategy to treat oxidative stress in the male tract was the premise that a well-balanced combination of antioxidants would be much more effective than any single antioxidant for three key reasons. First, oxidative stress is not a local phenomenon and can occur in both lipophilic and hydrophilic compartments of cells and tissues. Therefore, a combination of hydrophilic and lipophilic antioxidants offers a more effective inhibition of oxidative stress. This is irrefutable as both hydrophilic (e.g. Vitamin C) and lipophilic (e.g. Vitamin E) antioxidants have been clinically effective in reducing oxidative stress in sperm cells and indeed other cell types (Suleiman *et al.*, 1996; Greco *et al.*, 2005; Song *et al.*, 2006). Second, the structural diversity within ROS or RNS makes it impossible for any single antioxidant, or a single mode of antioxidant action, to reduce all oxidants. For example, as indicated above, while α -tocopherol is a potent inhibitor of ROS formation it has low reactivity towards RNS. Conversely, its isoform γ -tocopherol is a potent inhibitor of RNS but essentially unreactive towards ROS (Christen *et al.*, 1997). Finally, the combination of several antioxidants has the potential to allow for the use of lower doses of any one ingredient, mitigating known risks associated with high intake of individual antioxidant species (Donnelly *et al.*, 1999; Graat *et al.*, 2002; Miller *et al.*, 2005; Pizzorno and Neustadt, 2005; Ménéz *et al.*, 2007; Giustarini *et al.*, 2008; Hatfield and Gladyshev, 2009; Klein *et al.*, 2011; Koeth *et al.*, 2013; Albanes *et al.*, 2014; Brown and Hazen, 2014).

Given the wide range of antioxidant molecules to choose from, only evidenced-based antioxidants shown to alleviate oxidative stress in spermatozoa, improve semen parameters, or enhance male fertility were considered for further evaluation. A detailed search of the scientific literature revealed a large number of such antioxidants which are listed below in alphabetical order; arginine, carnitines, carotenoids and/or vitamin A (e.g. astaxanthin, lutein, lycopene, zeaxanthin), cobalamin, CoQ10 (or the reduced form ubiquinol), docosahexaenoic acid (DHA), flavonoids (e.g. quercetin, rutin, naringenin, epicatechin, epigallocatechin, pycnogenol), ellagic acid, folic acid, glutathione, lipoic acid, melatonin, magnesium, N-acetylcysteine, selenium-L-methionine, stilbenoid (e.g. resveratrol), taurine/hypotaurine, vitamin C, vitamin D, vitamin E, uric acid and zinc. Each ingredient on the list was critically assessed against literature reports evidencing their role in male infertility through human clinical trials, *in vivo* data documenting effects on semen parameters, fertilization or pregnancy, deficiency in the semen of infertile men relative to fertile counterparts, and efficacy in reducing sperm oxidative DNA damage. Some of these findings have been previously reported (Gharagozloo and Aitken, 2011). Efficacious ingredients were then further studied for favorable pharmacokinetics and pharmacodynamics, synergy or competition with other antioxidants and short and long-term side-effects.

Supplementary Table S1 Individual constituents of Fertilix®.

Antioxidant	Dose range
Carnitine blend	200–800 mg (1–4 mM)
Folic acid	400–500 µg (0.91–1.1 µM)
Lycopene	7.5–15 mg (14–28 µM)
Selenium	55 µg (0.7 µM)
Vitamin C	30–90 mg (0.15–0.51 mM)
Full spectrum natural vitamin E	104–290 mg (0.25–0.7 mM)
Zinc	7.5–11 mg (0.12–0.17 mM)

The net result of this intellectual exercise was to identify a cohort of natural antioxidants that are listed in [Supplementary Table S1](#), which then comprised the ingredients that make up Fertilix®.

Fertilix® formulations: a consideration of doses

The daily doses of each of the ingredients selected for the formulation could only be estimated, guided by a number of important considerations, such as the doses used in previous clinical trials, synergy of action between ingredients, oral bioavailability, duration of action or half-life of the ingredient. The dosages used were mainly in line with the Recommended Daily Intake (RDI) where this information was established by North American and European regulatory authorities ([Department of Health, 1991](#); [Commission of the European Communities, 1993](#); [Institute of Medicine, 1998, 2000, 2001](#); [World Health Organization and Food and Agriculture Organization, 1998](#)).

The doses for some of the ingredients were further refined to reduce or avoid the risk of possible long-term side-effects or known undesirable interactions with other cellular components. For example, such considerations were applied to selenium in light of its potential adverse cardiovascular results from the SELECT clinical trial ([Klein et al., 2011](#)) which set off a request from the European Commission to the European Food Safety Authority to provide a scientific opinion on the safety of L-selenomethionine doses in food supplements ([Scientific Opinion of the Panel on Food Additives, Flavourings, Processing Aids and Materials in Contact with Food, 2008](#)). Similar considerations were applied to doses used for vitamin C, which at high concentration is known to reduce interchain disulphide bridges in protamines, opening the cysteine net and subsequently promoting DNA decondensation in spermatozoa ([Donnelly et al., 1999](#); [Ménézo et al., 2007](#), [Giustarini et al., 2008](#)).

Additional considerations concerning the dose of individual constituents were deemed necessary since the degree of antioxidant deficiency in the male reproductive system or the magnitude of sperm DNA damage is highly variable in men and rarely determined in practice. Given this situation, inappropriate dosing of patients with only one formulation is highly likely. This is particularly worrying in men with little or no DNA damage in their spermatozoa. In these men with normal semen parameters, the infertility factor most likely resides with their female partner. In practice, a significant number of these men, believing antioxidants to be safe and wishing to boost their fertility potential, resort to self-medication with high dose antioxidant(s) or antioxidant formulations that may use aggressive doses of individual ingredients. This is very common in USA where

more than half the men attending IVF clinics use antioxidants prior to assisted conception. Unfortunately, administration of high doses of antioxidants in men with little or no sperm DNA damage may in fact impair their fertility by inducing reductive stress ([Chen et al., 2013](#)). Reductive stress occurs when over-treatment with exogenous antioxidant concentration depletes the natural levels of ROS and RNS critical for cell signaling and sperm function ([Ido, et al., 1997](#); [Ghycky and Boros, 2001](#); [Brewer et al., 2013](#)). To address the wide range of sperm DNA damage observed in men seeking ART (ref) and minimize or altogether avoid over-supplementation, three separate formulations using identical ingredients but substantially differing in the doses of certain constituents, were developed for commercial use ([Table S1](#)).

In conclusion the above rationale indicates that the antioxidant formulation described in this report, Fertilix®, represents the distillation of current knowledge in this field, combining optimal efficacy with minimal risk of reductive stress. The animal studies reported in the accompanying paper encourage belief that this formulation will be effective in human subjects suffering from oxidative stress in their germ line.

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