Copper-zinc superoxide dismutase deficiency impairs sperm motility and *in vivo* fertility

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Abstract

Oxidative stress, overproduction of reactive oxygen species (ROS) in relation to defence mechanisms, is considered to be a major cause of male infertility. For protection against the deleterious effects of ROS, animals have a variety of enzymatic antioxidants that reduce these molecules to less reactive forms. The physiological role of these antioxidants *in vivo* has been explored extensively through genetic inhibition of gene expression; surprisingly, many of these animals remain fertile in spite of increased oxidative stress. Copper-zinc superoxide dismutase-deficient (*Sod1*^{-/-}) male mice are one such example for which *in vivo* fertility has been repeatedly reported as normal, although examination of fertility has consisted of simply pairing animals of the same strain and checking for litters. This is a fairly low criterion by which to assess fertility. Herein, we show that *Sod1*-deficient males have zero fertilisation success in sperm competition trials that pit them against wild-type males of an otherwise identical genetic background and are almost completely infertile when mated singly with females of a different genotype. We also show that various aspects of sperm motility and function are impaired in *Sod1*-deficient mice. Testing the breeding capabilities of mice under more ecologically relevant conditions and with females of different genotypes may help reveal additional physiological causes of infertility.

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Introduction

Reactive oxygen species (ROS) are produced from a variety of sources in biological systems (Beckman & Ames 1998). ROS have important signalling roles, but when unregulated, they can impair cellular function, cause oxidative damage to biomolecules and limit the ability to regulate gene expression (Droge 2002). For protection against the deleterious effects of ROS, organisms have a variety of enzymatic and non-enzymatic defences. Endogenous enzymatic antioxidant defences with an established role *in vivo* include superoxide dismutase (SOD), catalase and glutathione peroxidase (GPX; Halliwell & Gutteridge 1999). Dietary molecules such as vitamin E, vitamin C and carotenoids may also help protect against the overproduction of ROS, although their antioxidant role *in vivo* is less well established (Halliwell & Gutteridge 1999).

Spermatozoa are especially susceptible to damage caused by ROS. The plasma membranes of spermatozoa have high levels of polyunsaturated fatty acids, making them very susceptible to ROS-induced damage (Alvarez & Storey 1995). This damage can impair sperm function (Alvarez *et al.* 1987) and also cause further production of ROS. Spermatozoa contain relatively low levels of

antioxidant enzymes (Agarwal *et al.* 2003) and are largely transcriptionally silent (Aitken & Graves 2002), giving them less scope for protection against the damaging effects of ROS. In relation to ROS production, spermatozoa produce ATP to facilitate their motility, and this energy metabolism is a major source of ROS (Beckman & Ames 1998, Koppers *et al.* 2008). Spermatozoa, therefore, seem especially prone to ROS-induced damage, and this damage could be particularly detrimental due to the heritable modification of the germ line through uncontrolled oxidation (Aitken *et al.* 2003).

A plethora of correlative evidence shows that spermatozoa are negatively affected by uncontrolled ROS production and insufficient antioxidant defence (reviewed in Sikka *et al.* (1995), Saleh & Agarwal (2002) and Agarwal *et al.* (2003)). To determine the direct role of antioxidant defence in sperm function, genetic modification of gene expression has been used to impair the synthesis of antioxidant defence enzymes (Koppers 2012). Surprisingly, many knockout models of classic antioxidant defence enzymes have remained fertile, although additional oxidative challenges can sometimes reveal an effect on sperm motility or morphology (Koppers 2012). In *Gpx* knockout animals, fertility is

dependent on the isoform in question, with *Gpx1* and nuclear *Gpx4* male mice apparently retaining normal fertility (Ho *et al.* 1997, Conrad *et al.* 2005). However, disruption of *Gpx4* expression in the mitochondria leads to infertility in males when mated with a female of the same strain (Schneider *et al.* 2009).

SOD is sometimes described as the first and most important line of antioxidant defence (Zelko *et al.* 2002) and occurs in several different isoforms. While fully deficient manganese Sod mice (*Sod2*^{-/-}) die prior to weaning, heterozygous *Sod2*-deficient mice (*Sod2*^{+/-}) suffer from increased oxidative stress, but are apparently fertile (Koppers 2012). One of the most frequently used mouse models of oxidative stress and ageing is the copper-zinc Sod fully deficient mouse (*Sod1*^{-/-}). *Sod1* knockout animals (*Sod1*^{-/-}) exhibit an elevation in the levels of various markers of oxidative stress (Elchuri *et al.* 2005, Muller *et al.* 2006, Perez *et al.* 2009), but otherwise appear relatively normal under standard laboratory conditions (Reaume *et al.* 1996, Elchuri *et al.* 2007). The fertility of female *Sod1*^{-/-} mice is greatly reduced, but three independent studies have reported that males are fully fertile when mated with C57BL/6 females (Ho *et al.* 1998, Matzuk *et al.* 1998, Tsunoda *et al.* 2012), the genetic background on which this knockout is usually maintained.

These studies, similar to virtually all of the above-cited examples, have simply paired males with a female of their own strain and allowed them to breed in isolation over several months. In the wild, males will mate with females of different genotypes and will also be required to compete with other males for fertilisation, as female mice regularly mate with more than one male in an oestrous cycle (Dean *et al.* 2006, Firman & Simmons 2008). It has recently been demonstrated that *Sod1*^{-/-} mice have a reduced fertility rate *in vitro*, due, at least in part, to an inability of sperm to penetrate the zona pellucida (Tsunoda *et al.* 2012). This suggests that SOD expression may influence fertility under particular conditions. Herein, we show that *Sod1*^{-/-} mice are almost entirely infertile when mated with the CBA strain of laboratory females, even though wild-type males (*Sod1*^{+/+}) mated at the same time with CBA females exhibit normal fertility. This is apparent when a male of each genotype (both *Sod1*^{-/-} and *Sod1*^{+/+}) is mated with the same female and sperm compete to fertilise a female's eggs (Parker 1970) and when each male is mated singly with separate females. This reduction in fertility is accompanied by various impairments in sperm motility and function.

Materials and methods

Experimental animals

Mice that did not express *Sod1* (*Sod1*^{-/-}) and their wild-type littermates (*Sod1*^{+/+}) were maintained on a C57BL/6

background. The generation of this knockout strain has been reported previously (Muller *et al.* 2006, Kostrominova *et al.* 2007). The line of *Sod1*-deficient mice used in these experiments was derived from three pairs of *Sod1*^{+/-} mice imported from the Jackson Laboratories (Bar Harbor, ME, USA) and used to create a SPF breeding colony at the Australian BioResource Centre in Mossvale, NSW, Australia. When 6–8 weeks old, experimental *Sod1* mice were transported and housed in conventional facilities at the University of New South Wales (UNSW). The mice were maintained at 22 ± 2 °C under a 12 h light:12 h darkness cycle. All experimental procedures and mating trials were conducted in the dark period under dim red light. Males were housed singly in cages (53 × 35 × 18 cm) and were regularly exposed to the odour and presence of males and females of the CBA strain to ensure the development of normal reproductive behaviour. Food (stock feed from Gordon's Specialty Stockfeeds, Yanderra, NSW, Australia) and water were provided *ad libitum*. CBA females (CBA/CaHAusb) of 6–7 weeks of age were purchased from the Australian BioResource Centre, transported to the UNSW and then housed in groups of two to three until the start of the experiments. The CBA strain of females was used in these experiments because previous studies have shown that matings between CBA females and C57BL/6 males produce viable offspring (Hager & Johnstone 2003, 2006) and these strains are derived from two different mouse lineages (Beck *et al.* 2000). All experimental procedures were approved by the UNSW animal ethics committee (approval number: 12/30A).

Mating assays

The sperm competition mating procedure was based on a previously published method (Firman & Simmons 2011). A mixture of soiled bedding from the cages of both *Sod1*^{+/+} and *Sod1*^{-/-} males was placed into each female's cage three days prior to mating to induce oestrus (Marsden & Bronson 1964). On the morning of the trial (i.e. the beginning of the dark period), oestrus was confirmed by vaginal smear (Allen 1922). Each female was then placed into the cage of the first male with which it was to mate (either a *Sod1*^{-/-} or a *Sod1*^{+/+} male) and checked at least every hour thereafter for the presence of a copulatory plug. When a plug was observed, the mouse was gently scruffed and the plug was removed using a blunt probe. The female was then placed into the second male's cage (the other genotype from the first male with which it had just mated), and it was checked every hour until a second plug was observed. The female was then transferred into a clean cage. Half the females were mated with the *Sod1*^{-/-} male first and half with the *Sod1*^{+/+} male first.

Due to the relatively low propensity of male mating under such experimental conditions (Ramm & Stockley 2007), we conducted a total of 160 mating trials, of which 64 resulted in at least one successful copulation. For 33 of these successful matings, the female was not mated with a second male, but placed straight into a clean cage. This allowed us to assess the fertility of males when not competing under sperm competition.

Eight homozygous and ten age-matched wild-type males were used in these mating trials. Males were 2 months old at

the start of the experiments and ~4.5 months at completion. Each male was mated just once per week, and the experience that males accrued over the experiment was monitored to ensure that these two groups of males did not differ in their total sexual experience over the experiment.

Dissection

Females that successfully mated (to ejaculation) with a male were culled 14 days after mating using an overdose of carbon dioxide. Females were dissected, and the number of foetuses was counted and then a small tissue sample was taken from each foetus for genotyping. Paternity was assigned on the basis of whether foetuses were heterozygous for *Sod1* (i.e. must have been parented by the homozygous male and CBA female) or wild type (parented by the wild-type male and CBA female). Genotyping was conducted by the mouse genotyping service at the Australian Cancer Research Foundation (ACRF), the Garvan Institute, using a combination of real-time PCR and melting curve analysis. At the end of the experiments, all experimental males were culled by cervical dislocation. Males were quickly dissected, and the reproductive organs were removed and weighed.

Males used to assess sperm motility

As it was apparent that the fertility of males was deleteriously affected by the loss of *Sod1* expression, we examined sperm motility in a second set of males. These males were being used for a different experiment and were housed in a slightly different social environment. They were imported to the UNSW at the same age, housed in the same-sized cages and similarly exposed to CBA males and females. However, each mouse was housed in a cage with a *Sod1* heterozygous (*Sod1*^{+/-}) male. The cage was divided by a perforated plastic barrier so that each male was unable to physically touch the other, but olfactory and visual contact was permitted. On ten occasions over a 2-week period, the barrier between the males was removed, and the males were able to interact physically for a 15-min period to allow the development of aggressive behaviour. Sperm motility was assessed in ten *Sod1*^{-/-} males and nine *Sod1*^{+/+} males.

Sperm motility analysis

At the end of the experiments, males were culled by cervical dislocation. Males were quickly dissected, and the epididymides were removed and stored at 37 °C until analysis of sperm motility, which commenced 1–3 h after the dissection. Sperm samples were prepared for the analysis of motility using a previously published method (Koyama & Kamimura 1999). The right cauda epididymis was added to 100 µl of Biggers, Whitten and Whittingham (BWW) medium (Koyama & Kamimura 1999), and five cuts were made along its length. Sperm were allowed to swim out over a 5-min period, and then the epididymis was removed and an additional 400 µl of BWW medium was added. A 5.5 µl aliquot of the sperm suspension was added to a pre-warmed slide with a chamber depth of 20 µm (Cell Vu; Millenium Sciences Corp., New York, NY, USA),

and the motility parameters of 200–300 sperm were assessed with a computer-assisted sperm analyser (CASA; HTM-IVOS version 12: Hamilton Thorne, Beverly, MA, USA) using factory parameters (mouse 1) at a sampling frequency of 60 Hz. The means of the kinetic parameters were obtained by averaging the summary values from each sample.

Statistical analysis

Generalised linear mixed-effects models were constructed to test for differences between *Sod1*^{-/-} and *Sod1*^{+/+} males using the lme4 package in R. When testing for a difference in the number of foetuses, the models were fitted with a Poisson distribution. When testing whether females were either pregnant or not, the models were fitted with a binomial distribution. To test for an effect of genotype on sperm competition success, we assessed the percentage of offspring sired by the male in the offensive sperm competition position (Mueller *et al.* 2008, Firman & Simmons 2011, Klemme & Firman 2013). A binomial distribution was used when examining this percentage, because all values were either 100 or 0% (effectively either 0 or 1) due to *Sod1*^{+/+} males siring all the offspring regardless of being the first or second to mate. Male genotype was added as a fixed effect and male ID was added as a random effect to control for the non-independence arising from using each male more than once. The significance of the genotype effect was tested by comparing models with and without the genotype term using a log-likelihood ratio test.

Differences between males with regard to the masses of reproductive organs were assessed in SPSS using General Linear Models. Differences in organ weights were assessed with body mass added as a covariate. Differences between males in sperm motility and related parameters were assessed using independent-samples *t*-tests.

Results

Reduced fertility in *Sod1*-deficient males

To ascertain whether *Sod1*^{-/-} males exhibit reduced fertility under more challenging mating conditions, we mated CBA females with both a *Sod1*^{+/+} and a *Sod1*^{-/-} male, randomising which male mated first. We hypothesised that sperm from *Sod1*^{-/-} males may exhibit lower competitive ability under sperm competition. On examining embryos 14 days after mating, it was apparent that 24 females were pregnant. Surprisingly, when the embryos were genotyped, every single offspring was found to be sired by a *Sod1*^{+/+} male, regardless of which male mated with the female first. When *Sod1*^{+/+} males were the second to mate, the offensive sperm competition position, they sired 100% of the embryos; when *Sod1*^{-/-} males were in the offensive sperm competition position, they did not sire a single embryo (effect of male genotype on the percentage of offspring sired in the offensive position: $\chi^2 = 29.47$, 1 d.f., $P < 0.0001$; Fig. 1A).

Figure 1 Reduced fertility rates in *Sod1*-deficient males. When compared with wild-type males (+/+), males deficient in *Sod1* (-/-) had greatly reduced fertility, both when competing directly with a wild-type male to fertilise one female's eggs (A) and when mated separately with a CBA female (B). Each dot on the graph represents the number of embryos sired from an individual mating attempt.

To further understand the dramatic difference in fertilisation success between *Sod1*^{+/+} and *Sod1*^{-/-} mice, males of each genotype were mated separately with CBA females. From 13 successful matings with *Sod1*^{-/-} males (e.g. where a copulatory plug was found), only one female was pregnant and carried just one embryo. This was a highly significant reduction in fertility when compared with the resulting embryos from 20 matings with equivalent *Sod1*^{+/+} males (effect of genotype on the number of embryos: $\chi^2=12.37$, 1 d.f., $P=0.0004$; Fig. 1B). This reduction in fertility was also apparent when conducting a binomial analysis to determine whether the mated females simply became pregnant or not (effect of male genotype on pregnancy: $\chi^2=5.86$, 1 d.f., $P=0.015$). Thus, the observed differences in fertilisation success may be attributable to the subfertility of *Sod1*^{-/-} male mice when mated with CBA females in the experiments in this study.

Mating behaviour

Sod1^{-/-} males did not differ in their time until copulation when compared with *Sod1*^{+/+} males (effect

of male genotype on time until copulation: $\chi^2=0.01$, 1 d.f., $P=0.91$). Over the 160 trials conducted, there was a tendency for *Sod1*^{-/-} males to be less likely to successfully ejaculate in the window of opportunity allowed, which was ~ 10 h, although this effect was not significant (effect of male genotype on success in mating in the first male position: $\chi^2=3.58$, 1 d.f., $P=0.059$). Thus, a difference in mating behaviour between the two male genotypes is unlikely to be the cause of such dramatic differences in fertility.

Masses of reproductive organs and sperm motility

At the end of the experiments, *Sod1*^{-/-} males exhibited minor reductions in the masses of their reproductive organs compared with *Sod1*^{+/+} males (Table 1). Their epididymides and seminal vesicles were of comparable size, but the mass of their testes was lower than that of the testes of *Sod1*^{+/+} males (Table 1).

To further ascertain the differences in reproductive physiology between *Sod1*^{+/+} and *Sod1*^{-/-} males, we examined sperm motility in a further set of these mice. The percentages of motile and progressively motile spermatozoa were much lower in epididymal sperm samples obtained from *Sod1*^{-/-} mice (Table 2). A similar finding has recently been reported, where the percentage of motile and progressively motile sperm of *Sod1*^{-/-} mice was lower after 7 h of incubation in a human tubal fluid medium when compared with that of sperm of *Sod1*^{+/+} males (Tsunoda *et al.* 2012). Furthermore, we report that average path velocity, curvilinear velocity and lateral head amplitude were also negatively affected in *Sod1*^{-/-} males (Table 2), which may further contribute to their reduced fertilising ability.

Discussion

Female mice mate multiply in the wild, and sperm from different males often compete to fertilise a female's eggs (Dean *et al.* 2006, Firman & Simmons 2008). Animals in natural populations also exhibit genetic heterogeneity, highlighting that males will mate with females that are genetically unrelated to them. In spite of this, previous studies assessing fertility in *Sod1*^{-/-} mice have been conducted under normal laboratory conditions, with genetically identical females of the same strain (Ho *et al.* 1998, Matzuk *et al.* 1998, Tsunoda *et al.* 2012). Our

Table 1 Differences in the weights of wild-type and *Sod1*-deficient males and their reproductive organs and differences between groups after correcting for body mass.

	+/+	-/-	d.f.	F	P value
Body mass	29.84 $\pm$ 0.82	29.72 $\pm$ 0.79	–	–	–
Seminal vesicles	0.226 $\pm$ 0.014	0.248 $\pm$ 0.013	1.19	2.43	0.14
Testes	0.192 $\pm$ 0.004	0.182 $\pm$ 0.004	1.19	4.99	0.039
Epididymis	0.099 $\pm$ 0.004	0.097 $\pm$ 0.003	1.19	0.40	0.54

Table 2 Sperm motility and related parameters in wild-type and *Sod1*-deficient males. Results in bold are significantly different between genotypes.

	+/+	-/-	d.f.	t	P value
Motility (%)	53.2±3.6	32.9±4.5	17	3.50	0.003
Progressive motility (%)	46.6±3.2	25.1±3.6	17	4.47	<0.001
Average path velocity (VAP; µm/s)	59.6±2.4	47.4±3.4	17	2.83	0.012
Straight line velocity (VSL; µm/s)	29.9±1.8	25.1±2.2	17	1.68	0.11
Curvilinear velocity (VCL; µm/s)	130.9±3.7	102.1±5.8	17	4.05	0.001
Lateral head amplitude (µm)	8.3±0.18	7.2±0.19	17	4.28	0.001
Beat cross frequency (Hz)	35.7±0.9	34.3±0.9	17	1.06	0.30
Straightness (VSL/VAP)	45.8±1.3	49.7±2.2	17	-1.52	0.15
Linearity (VSL/VCL)	22.3±0.7	24.4±1.4	17	-1.31	0.21
Head elongation (minor/major axis)	41.2±2.8	43.6±2.16	17	0.28	0.60
Head area (µm ²)	10.6±0.5	9.6±0.3	17	0.09	0.98

results indicate that more realistic mating procedures can reveal important phenotypic differences between *Sod1*^{-/-} mice and their *Sod1*^{+/+} siblings. When mated with the CBA strain of females, *Sod1*^{-/-} males are virtually infertile.

The reduced fertility observed in the experiments of this study could be partly attributable to the smaller testis mass of *Sod1*^{-/-} males. This trait, in particular, is expected to influence a male's ability to compete under sperm competition, due to its influence on the number of sperm that can be produced and transferred when mating (Parker *et al.* 1997, Ramm *et al.* 2005). Relative testis mass is strongly correlated with sperm competition risk across species in a variety of taxa (Parker *et al.* 1997, Ramm *et al.* 2005), with one theory predicting that testis mass and the consequential larger sperm number will evolve in situations of heightened sperm competition (Parker *et al.* 1997). Within species, testis size has been shown to correlate with reproductive success (Preston *et al.* 2003, Schulte-Hostedde & Millar 2004). Although relative testis mass was reduced, it should be noted that there was some overlap in testis size when compared with *Sod1*^{+/+} males. Indeed, we found that *Sod1*^{+/+} males with the smallest testes at the end of the experiments had still been capable of fertilising females in the last mating attempt. Therefore, some other aspect of reproductive function is likely to be impaired in *Sod1*^{-/-} mice to cause such a drastic reduction in fertility.

A potentially more important factor influencing the fertility of *Sod1*^{-/-} males may be their reduced sperm motility and path velocity. A relationship between average path velocity and litter size has been reported in another litter-bearing species (pigs), with sperm with low motility being predictive of a smaller litter size (Broekhuijsen *et al.* 2012). In one species of fish

(*Xiphophorus helleri*) where multiple paternity and sperm competition are prevalent, average path velocity was also found to be a predictor of paternity (Gasparini *et al.* 2010). In addition to this, there are indications that average path velocity is negatively correlated with oxidative stress in human spermatozoa (Aitken *et al.* 1993, 1998). This suggests that the fertility of *Sod1*^{-/-} mice may be impaired by decreased sperm swimming speed, which could be caused by an inability to protect spermatozoa against oxidative damage.

What may be the cause of the severely reduced fertility of *Sod1*^{-/-} mice reported here when previous studies have found these mice to be as fertile as *Sod1*^{+/+} males? It is likely that the reproductive physiology of CBA females differs from that of C57BL/6 females, which *Sod1* males have previously been mated with, and creates a more challenging environment for *Sod1*^{-/-} mouse spermatozoa. The CBA and C57BL/6 strains of mice are known to differ in fertility, and reciprocal mating crosses between these strains have demonstrated that the smaller litter sizes of the C57BL/6 strain are a consequence of the male genotype (Hager & Johnstone 2003). Mating C57BL/6 males with CBA females leads to litter sizes that are smaller than those observed when CBA females are mated with a male of their own strain (Hager & Johnstone 2003). The cause of this difference in fertility remains to be determined (Haig 2003), but increased oxidative stress with SOD1 deficiency could further reduce the ability of C57BL/6 males to successfully fertilise a CBA female's eggs, possibly due to the effects of elevated oxidative damage on sperm function.

Laboratory strains of mice differ greatly in a variety of aspects of their reproductive physiology. These include not only overall fertility rates and litter sizes (Silver 1995), but also specific differences in the composition, size and number of their cumulus cells and zonae pellucidae (Krzanowska 1972), which may influence the ability of spermatozoa to successfully reach the egg plasma membrane (Primakoff & Myles 2002). Compared with five other strains of laboratory mice, mice belonging to the CBA strain have a very tightly packed layer of cumulus cells surrounding their eggs and exhibit slow dispersal of these cells when treated with hyaluronidase (Krzanowska 1972, Wabik-Śliz 1997). It could, therefore, be expected that the reduced sperm motility of *Sod1*^{-/-} males may limit their ability to penetrate this layer and reach the zona pellucida.

It has recently been demonstrated that *Sod1*^{-/-} mice have a reduced capability to penetrate the zona pellucida during IVF (Tsunoda *et al.* 2012), and therefore differences in the permeability of the zona pellucida between strains may be responsible for the greatly reduced fertility of *Sod1*^{-/-} mice when mated with CBA females. CBA females tend to exhibit relatively rapid removal of the zona pellucida when treated with a pronase solution (Krzanowska 1972), although this does not necessarily mean that sperm will easily penetrate this

barrier. *In vitro*, CBA males are highly capable of fertilising the eggs of CBA females while the zona pellucida is intact, while males of various other strains of laboratory mice, including C57BL/6, have much lower fertility rates when mating with females of this strain (Kaleta 1977). If the zona pellucida is removed experimentally, the fertility rates of males of other strains of laboratory mice dramatically increase to levels observed in matings with CBA males (Kaleta 1977). This suggests that the interaction between C57BL/6 sperm and the CBA zona pellucida is somewhat impaired when compared with normal fertilisation with both sexes of the CBA mouse.

To fully understand the causes of reduced fertilisation success in this study, *in vitro* experiments are now required where the sperm of *Sod1* mice are allowed to fertilise eggs from both C57BL/6 and CBA females. Experimental removal of the cumulus cells and zona pellucida may help reveal which stages of fertilisation are causing the observed results. In a broader sense, it may be important to determine whether the impaired fertilisation ability of *Sod1*^{-/-} males is also apparent when males are mated with strains of females other than C57BL/6 and CBA. This would give an insight into whether it is a peculiarity of the CBA female reproductive phenotype that makes fertilisation challenging for males with SOD1 deficiency or whether fertilisation in C57BL/6 females is particularly easy. Ideally, experimental matings with recently wild-derived female mice, which are genetically representative of wild mice, would help to reveal the extent to which oxidative stress could impair fertility in natural populations.

The apparent normal fertility of *Sod1*^{-/-} males when mating with females of the same strain has been demonstrated in three different laboratories over the last 15 years (Ho *et al.* 1998, Matzuk *et al.* 1998, Tsunoda *et al.* 2012). Although the phenotypes of *Sod1*^{-/-} males seem consistent between laboratories and over time, we cannot exclude the possibility that some aspect of the environment in the experiments of this study caused their fertility to be lower than that reported by previous studies, and this contributed to their reduced fertility when mated with CBA females. Very little information regarding housing conditions has been provided by previous studies. In this study, it should be noted that males of both genotypes were singly housed in relatively large cages and were regularly exposed to the odour of other males and females, which could be different from the way that males were housed in the previous studies. We used this housing protocol to further create a more natural environment and also to stimulate normal reproductive behaviour. Exposing developing male mice to the odour of other males and females actually facilitates sperm production (Ramm & Stockley 2009), so it is unlikely that this had a deleterious effect. Wild-type males also maintained a normal level of fertility in these experiments. Nonetheless, it would be

interesting to experimentally test whether housing males in different environments influences the phenotypic effects of particular gene knockouts, particularly in relation to sperm function.

Antioxidant enzyme 'knockout' animals, such as the *Sod1*^{-/-} model employed here, have been used extensively and successfully to understand the causal effects of oxidative stress on health and ageing (Perez *et al.* 2009, Salmon *et al.* 2010). These animals have also been studied to ascertain the role of these antioxidants in both male and female fertility. Many of these knockout animals apparently remain fertile, although the amount of data obtained from these animals is, at present, insufficient (Koppers 2012). Our results highlight that the antioxidant enzyme SOD1 may have a more important role in influencing male fertility *in vivo* than realised previously. Perhaps more importantly, our use of a different strain of laboratory mouse in mating trials highlights that altered fertility may be revealed when males are mated with females of different genetic backgrounds. Most strains of laboratory mice have been selectively bred for hundreds of generations, and this is likely to have led to some degree of coevolution between male and female reproductive physiology. We predict that mating males with phenotypes of interest to females of several different genetic backgrounds may give a better indication of factors that influence fertility in natural populations of animals.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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