Superoxide dismutase (SOD) is a ubiquitous antioxidant enzyme that catalytically converts the superoxide radical to hydrogen peroxide (H$_2$O$_2$). In mammals, high SOD activity is detectable in sperm and seminal plasma, and loss of SOD activity has been correlated with male infertility; however, the underlying mechanisms of sperm infertility remain to be clarified. Here we report that the deletion of two major SOD genes in Caenorhabditis elegans, sod-1 and sod-2, causes sperm activation defects, leading to a significant reduction in brood size. By examining the reactivity to the sperm activation signals Pronase and triethanolamine, we found that sod-1; sod-2 double mutant sperm cells display defects in pseudopod extension. Neither the content nor oxidative modification of major sperm protein, an essential cytoskeletal component for crawling movement, were significantly affected in sod-1;sod-2 mutant sperm. Surprisingly, H$_2$O$_2$, the dismutation product of SOD, could activate sod-1; sod-2 mutant sperm treated with Pronase. Moreover, the H$_2$O$_2$ scavenger ebselein completely inhibited pseudopod extension in wild-type sperm treated with Pronase, and H$_2$O$_2$ could directly induce pseudopod extension in wild-type sperm. Analysis of Pronase-triggered sperm activation in sod-1 and sod-2 single mutants revealed that sod-2 is required for pseudopod extension. These results suggest that SOD-2 plays an important role in the sperm activation of C. elegans by producing H$_2$O$_2$ as an activator of pseudopod extension.

In aerobic organisms, incomplete reduction of molecular oxygen constantly generates superoxide radicals (O$_2^-$). Although O$_2^-$ is considered to have low reactivity, it can act as a precursor for highly toxic reactive oxygen species, including hydroperoxyl radical, hydroxyl radical, and peroxynitrite. Approximately 1% of O$_2^-$ molecules are protonated at physiological pH, forming hydroperoxyl radical, which is sufficiently reactive to initiate lipid peroxidation and protein oxidation (1, 2). O$_2^-$ readily reacts with nitric oxide, forming peroxynitrite, which also reacts with a variety of biomolecules, such as proteins, DNA, and lipids (3). Additionally, by the iron-catalyzed Haber–Weiss reaction, O$_2^-$ assists with the formation of highly toxic hydroxyl radicals that non-selectively oxidize and decompose most organic compounds (4, 5). Therefore, elimination of O$_2^-$ is very important in biological systems. Superoxide dismutase (SOD)$^2$ is a ubiquitous antioxidant enzyme that protects organisms from oxidative stress by catalytically converting O$_2^-$ to hydrogen peroxide (H$_2$O$_2$) (6, 7). Eukaryotic cells have three types of SOD enzymes: cytosolic SOD containing Cu$^{2+}$ and Zn$^{2+}$ (CuZn-SOD) (8, 9), mitochondrial SOD containing Mn$^{2+}$ (Mn-SOD) (10, 11), and extracellular SOD containing Cu$^{2+}$ and Zn$^{2+}$ (EC-SOD) (12, 13).

Sperm and seminal plasma are known to contain several enzymatic antioxidants, such as glutathione peroxidase 4, peroxiredoxin 4, and CuZn-SOD (14–17), as well as non-enzymatic antioxidants, such as glutathione, ascorbic acid, and α-tocopherol (18–20). High SOD activity is detected in human sperm and seminal plasma compared with other cells and blood plasma, respectively (17). Several reports have shown that SOD activity is significantly decreased in the semen of infertile men compared with fertile control groups (21–23). In addition, O$_2^-$ contents are increased in the semen of male infertility patients compared with fertile normospermic subjects (24, 25). These previous reports suggest that SOD acts as an important enzymatic antioxidant to protect sperm from oxidative stress.

The free-living nematode Caenorhabditis elegans is a useful model organism to examine the effects of gene depletion on reproduction because it is genetically tractable, easily manipulated, and has a unique reproductive system. In C. elegans, there are two sexes: self-fertilizing hermaphrodite, in which the germline produces both sperm and oocytes, and male, in which the germline produces only sperm (26). C. elegans sperm consists of amoeboid cells lacking flagella that move by extending the pseudopod (27, 28). The reproductive system of the hermaphrodite consists of two tubular oviducts that are connected to a central uterus through two spermathecae. Fertilization occurs when the oocyte is pushed into the spermatheca that stores sperm. Because the fertilized eggs push out the sperm into the uterus simultaneously when they exit the spermatheca, sperm must crawl back into the spermatheca to fertilize the subsequent ovulated oocytes. C. elegans has five SOD genes encoding two cytosolic CuZn-SOD enzymes (sod-1 and sod-5), two mitochondrial Mn-SOD enzymes (sod-2 and sod-3), and one extracellular CuZn-SOD enzyme (sod-4). Van Raamsdonk et al. (29) reported that single-gene deletion mutants of sod-1

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$^2$The abbreviations used are: SOD, superoxide dismutase; TEA, triethanolamine; MSP, major sperm protein; AMS, 4-acetamido-4’-maleimidylstilbene-2,2’-disulphonic acid; H$_2$DCFDA, 2’,7’-dichlorodihydrofluorescein diacetate; 2-APB, 2-aminoethoxydiphenylborate; NGM, nematode growth medium; CBB, Coomassie Brilliant Blue.

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Role of SOD-2 in C. elegans sperm activation

Because the brood size was remarkably reduced by depletion of sod-1 and sod-2 in C. elegans, the reason for infertility in the sod-1;sod-2 mutant was investigated. In C. elegans, the self-fertilizing hermaphrodite produces both sperm and oocytes. In the hermaphrodite gonad, 300 sperm are produced during the late larval stage, and thereafter, oocytes are produced over the subsequent lifetime (26). Because the quality and quantity of sperm limit the brood size of C. elegans, we first focused on defects of sperm in sod-1;sod-2 mutants (Fig. 2). A round, non-motile spermatozoid yielded by meiosis undergoes a dynamic morphological change, so-called sperm activation, forming nonflagellated ameboid cells for crawling movement (Fig. 2A and Refs. 27, 28). The activation signal is transduced through SPE-8–group proteins, and the sperm can be activated in vitro by several factors, including a protease (Pronase), an ionophore (monensin), and a weak base (triethanolamine, TEA) (31, 32). To examine the process of pseudopod formation, we performed in vitro activation of sperm isolated from wild-type and sod-1; sod-2 mutant males (Fig. 2B). As Pronase serves as an SPE-8–group protein activator, 75% of wild-type sperm were fully activated following Pronase treatment, showing an actively extended pseudopod (Fig. 2C). The remaining 25% were composed of incompletely activated sperm with a partially extended pseudopod or a non-activated sperm that can move as an ameboid cell (Fig. 2C). Next, we tested the response of sod-1; sod-2 mutant sperm to TEA treatment. Activating sperm with TEA bypasses the requirement for Spe-8–class signaling components (33). With TEA treatment, 40% and 30% of wild-type sperm were fully and incompletely activated, respectively (Fig. 2D). The remaining 30% was composed of non-activated sperm that can move as an ameboid cell (Fig. 2D). These data indicate that sod-1;sod-2 mutant sperm have defects in pseudopod extension.

Oxidative modification of major sperm protein (MSP) is not responsible for sperm activation defects in the sod-1;sod-2 mutant

Here we reveal that sod-1;sod-2 mutant sperm are defective in pseudopod extension. In the activated ameboid sperm, the assembly of MSP at the leading edge of the pseudopod and disassembly at the pseudopod–cell body interface provide the protrusive force for ameboid movement (Fig. 2A and Refs. 34, 35). To obtain insight into the pseudopod extension defects in sod-1;sod-2 mutant sperm, we first examined the change of content and localization of MSP in wild-type and sod-1;sod-2 mutant sperm. To isolate large amounts of sperm sufficient for biochemical analysis, a temperature-sensitive mutation of the fem-3 gene q20 was introduced to sod-1;sod-2 mutants (36).

Results

Deletion of SOD genes reduces brood size in C. elegans

We first examined the brood sizes of individual SOD gene deletion mutants (Fig. 1). Consistent with previous reports, the brood sizes of sod-1 and sod-2 mutants were reduced by 50% compared with the wild type; however, the brood sizes of sod-3, sod-4, and sod-5 mutants were not significantly affected (29, 30). In C. elegans, sod-1 and sod-2 encode the major isoforms of cytosolic CuZn-SOD and mitochondrial Mn-SOD, respectively. In contrast, sod-5 and sod-3 are minor inducible isoforms of CuZn-SOD and Mn-SOD, respectively. To avoid redundancy, we generated sod-1;sod-5 and sod-2;sod-3 double mutants and found that the brood sizes of both double mutants were slightly reduced compared with individual single mutants. To eliminate a large fraction of SOD activity in C. elegans, we generated sod-1;sod-2 double mutants. We found that the brood sizes of sod-1;sod-2 mutants were reduced to 15% that of the wild type. These data indicate that sod-1 and sod-2 serve as the primary SOD isoforms in the reproduction of C. elegans.

\[ \text{sod-1;sod-2 double-mutant sperm are defective in pseudopod extension} \]

and sod-2 cause a reduction in brood size in C. elegans. Furthermore, deletion of all SOD genes (sod-1;2;3;4;5 quintuple mutants) also decreases the brood size; however, the mechanism underlying this reduced fertility remains unclear (30). In this study, we generated C. elegans mutants lacking the genes encoding CuZn-SOD and Mn-SOD homologs (sod-1 and sod-2, respectively) and found that the deletion of sod-1 and sod-2 severely decreased brood size. sod-1;sod-2 double-mutant sperm was defective in pseudopod extension without suffering oxidative modification of sperm proteins. Pronase-triggered sperm activation analysis in sod-1 and sod-2 single mutants revealed that sod-2 is required for pseudopod extension. By stimulating the sperm with H$_2$O$_2$, we found that the production of H$_2$O$_2$ by SOD-2 is a key factor in pseudopod extension of C. elegans sperm.
Hermaphrodites of the fem-3 mutant and the sod-1;sod-2;fem-3 triple mutant produce only sperm at a restrictive temperature. Sperm isolated from /H11011 20,000 hermaphrodites of fem-3 and sod-1;sod-2;fem-3 mutants grown at restrictive temperature were subjected to Western blot analysis to determine MSP levels. In sod-1;sod-2;fem-3 mutant sperm, the MSP level was not significantly affected compared with fem-3 sperm (Fig. 3A).

Likewise, immunostaining of MSP did not show clear differences between fem-3 and sod-1;sod-2;fem-3 mutant sperm (Fig. 3B). C. elegans has 28 genes encoding MSP, and the degree of amino acid sequence identity is very high (97–100%) (37–39).

Of interest is that all of them contain two cysteine residues, at positions of 60 and 75 (Fig. 3C). Because the thiol moiety of cysteine is very sensitive to oxidation (40), we next examined an oxidative modification of cysteine residues in MSP. Sperm homogenates were reacted with 4-acetamido-4-/H11032maleimidylstilbene-2,2'-disulphonic acid (AMS), a free thiol alkylating reagent (41). The alkylated protein increases the molecular mass by 0.5 kDa per alkylated cysteine. When the alkylation of MSP was monitored over 4 h, the band intensity of the AMS-alkylated form increased in a time-dependent manner not only in fem-3 mutants but also in sod-1;sod-2;fem-3 mutants, suggesting that the cysteine residues in MSP exist as free thiol forms in both fem-3 mutant and sod-1;sod-2;fem-3 mutant sperm (Fig. 3, D and E).
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In vivo staining of isolated sperm by H$_2$DCFDA, an indicator of intracellular H$_2$O$_2$, revealed that the level of H$_2$O$_2$ was lower in sod-1;sod-2 mutant sperm than in wild-type sperm (Fig. 4A). Because SOD produces H$_2$O$_2$ by the dismutation of O$_2^-$, we examined the possibility that H$_2$O$_2$ promotes Pronase-triggered sperm activation. When treated with 0.3 mM H$_2$O$_2$, Pronase can activate sod-1;sod-2 mutant sperm, showing a level of activation comparable with that of the wild type (Fig. 4B). To confirm the involvement of H$_2$O$_2$ in Pronase-triggered sperm activation, we examined the effects of ebselen, a potent H$_2$O$_2$ scavenger (42, 43). The results showed that ebselen inhibited pseudopod extension in wild-type sperm treated with Pronase (Fig. 4C).

It was further examined whether H$_2$O$_2$ directly promotes pseudopod extension in C. elegans (Fig. 5). Wild-type sperm responded to H$_2$O$_2$ in a dose-dependent manner, raising the possibility that H$_2$O$_2$ acts as an activator of pseudopod extension (Fig. 5A). Ebselen inhibited pseudopod extension in wild-type sperm treated with 1.25 mM H$_2$O$_2$ (Fig. 5B). In addition, ebselen treatment dramatically reduced the brood size of the wild-type in a dose-dependent manner (Fig. 5C). Taken together, these data strongly suggest that SOD is involved in sperm activation of C. elegans by producing H$_2$O$_2$ as an activator of pseudopod extension.

Deletion of sod-2 is responsible for pseudopod extension defects in sod-1;sod-2 mutants

To identify the gene that causes sperm activation defects in sod-1;sod-2 mutants, we compared Pronase-dependent pseudopod extension of sperm in sod-1 and sod-2 single mutants. In Pronase-treated sod-2 mutant sperm, fully activated sperm accounted for 40% of sperm cells, and the fraction of incompletely activated sperm was increased to 4-fold that of the wild type (Fig. 6A). Similar defects were also observed in sod-1;sod-2 mutant sperm treated with Pronase. In contrast, the fraction of activated sperm was not significantly affected in the sod-1 mutant compared with the wild type. In vivo staining of isolated sperm by H$_2$DCFDA revealed that the level of H$_2$O$_2$ was lower in sod-2 mutant sperm than in wild-type sperm (Fig. 6B). Moreover, in combination with H$_2$O$_2$, Pronase can activate sod-2 mutant sperm, showing a level of activation comparable with that of the wild-type (Fig. 6C). Sperm activation defects in sod-2 and sod-1;sod-2 mutant males were examined in vivo by crossing with dpy-11 hermaphrodites. Male sperm are known to out-compete hermaphrodite sperm; thus, the majority of embryos produced after mating are outcross progeny (44, 45). As shown in Fig. 6D, when crossed with sod-2 and sod-1;sod-2 mutant males, the number of outcross progeny was reduced.
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Although the degree of sperm activation defects was similar in sod-2 and sod-1;sod-2 mutants, the brood size of sod-1;sod-2 mutants was significantly reduced compared sod-2 mutants (Figs. 1 and 6A), suggesting that sod-1;sod-2 mutants have defects not only in sperm but also in oocytes. To investigate this possibility, sod-1;sod-2 mutant hermaphrodites were out-crossed with wild-type males (Fig. 6E). When crossed with wild-type males, sod-1;sod-2 mutant hermaphrodites produced ~250 outcross progeny, corresponding to 50% of that of wild-type hermaphrodites. In addition, the number of outcross progeny was also reduced in sod-1 mutant hermaphrodites when crossed with wild-type males, suggesting that sod-1 mutants have defects in the oocyte. These data indicate that deletion of sod-2 is responsible for the defects of pseudopod extension in sod-1;sod-2 mutant sperm.

Discussion

In C. elegans, several reports have shown that deletion of SOD genes reduces fertility; however, the mechanisms remain unclear. In this study, by comparing the reactivity of SOD gene–deleted C. elegans sperm with the activation signals, we demonstrated that simultaneous deletion of the sod-1 and sod-2 genes attenuated pseudopod extension, leading to a reduction in brood size (Figs. 1 and 2). Because O2 acts as a precursor of highly reactive oxygen species that modify cysteine residues in proteins, we first hypothesized that oxidative modification of cysteine in MSP causes sperm activation defects in sod-1;sod-2 mutants. However, significant differences were not observed between sod-1;sod-2 mutant and wild-type sperm (Fig. 3). In addition, carbonylation of sperm protein was not significantly affected in sod-1;sod-2 mutants compared with the wild-type (data not shown). These data suggest that oxidative modification of MSP would be negligible in sod-1;sod-2 mutant sperm.

To confirm this possibility, sod-1;sod-2 mutants were treated with deferoxamine, a potent iron chelator, which did not restore the brood size (48 ± 5 broods in intact sod-1;sod-2 mutants versus 50 ± 3 broods in deferoxamine-treated sod-1; sod-2 mutants). Taken together, these results indicate that oxidative stress is not responsible for the reduction in brood size in sod-1;sod-2 mutants.

In this study, we found that H2O2 produced by SOD-2 is involved in sperm activation in C. elegans. H2O2 functions as a downstream signaling molecule for Pronase-dependent pseudopod extension because ebselen inhibited Pronase-dependent sperm activation in wild-type worms (Figs. 4 and 5). Liu et al. (46) showed that calcium signaling is required for Pronase-triggered sperm activation in C. elegans. In this previous report, both a phospholipase C inhibitor (U73122) and an inositol trisphosphate receptor antagonist (2-aminoethoxydiphenylbo-
rate, 2-APB) blocked Pronase-triggered sperm activation. The authors used calcium-free sperm medium to avoid extracellular calcium entry and concluded that 2-APB exerted its inhibitory effect by antagonizing calcium release from the smooth endoplasmic reticulum, an intracellular calcium pool. Interestingly, H$_2$O$_2$ is known to promote calcium mobilization in mammalian cells by interacting with the inositol triphosphate receptor on the endoplasmic reticulum membrane (47, 48). We propose that a Pronase/H$_2$O$_2$/calcium pathway mediates Pronase-triggered sperm activation. Furthermore, the possibility of an inhibitory effect of 2-APB on H$_2$O$_2$-induced sperm activation may be worthy of future investigation.

Much evidence suggests that SOD-2 is essential for the regulation of H$_2$O$_2$ signaling in Pronase-triggered sperm activation (Fig. 6); however, how Pronase promotes O$_2^-$ production for sperm activation remains to be determined. There are two possibilities for the mechanism of O$_2^-$ production in sperm activation signaling. One possibility is that globin directly generates O$_2^-$. Recently, De Henau et al. (49) demonstrated that O$_2^-$ produced by a globin protein (GLB-12) acts as a signaling molecule for reproduction in C. elegans. GLB-12 can convert molecular oxygen to O$_2^-$ in the gonadal sheath cells that surround germ cells, and cytosolic SOD-1 produces H$_2$O$_2$ by the dismutation of O$_2^-$. Because GLB-12 is a plasma membrane–bound globin, identification of mitochondrion-associated globin proteins is important to verify the possibility that the globin/SOD-2 pathway acts as an efficient source of H$_2$O$_2$ for sperm activation. The other possibility is that the mitochondrial electron transport chain provides O$_2^-$. During C. elegans spermatogenesis, mitochondria in the primary spermatocytes are segregated into the spermatids, whereas unnecessary cellular components such as actin and ribosomes are deposited in the residual body and are ultimately degraded (50). Additionally, Liu et al. (51) reported that partial deletion of the mitochondrial DNA, which removes several mitochondrial electron transport chain components, reduces sperm motility, resulting in a reduction in the egg-laying rate of C. elegans. It is necessary to analyze the underlying mechanism of how Pronase promotes O$_2^-$ production in mitochondria during sperm activation.

In conclusion, this study demonstrates for the first time that the defects in pseudopod extension of sperm are responsible for the reduced fertility of sod-1;sod-2 mutants. Unexpectedly, the level of oxidative stress was not increased in sod-1;sod-2 mutant sperm, and H$_2$O$_2$ promoted pseudopod extension not only in wild-type sperm but also in sod-1;sod-2 mutant sperm. SOD-2 and SOD-1 are required for pseudopod extension and oocyte function, respectively. On the basis of the above data, we propose that H$_2$O$_2$ produced by SOD-2 acts as a signaling molecule for sperm activation in C. elegans.

**Experimental procedures**

**General methods and strains**

Maintenance and genetic manipulation of C. elegans were carried out as described previously (52). The Bristol strain N2 was used as the standard wild-type strain. In addition, the fol-
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Figure 6. sod-2 is responsible for pseudopod extension defects in sod-1;sod-2 mutants. A, quantitative analysis of sperm activation in the wild type, sod-1 mutants, sod-2 mutants, and sod-1;sod-2 double mutants treated with Pronase. The percentage of each type of sperm is shown. The data represent the mean ± S.D. of three independent experiments with 10 worms per genotype per trial. **, p < 0.01; *, p < 0.05. B, fluorescence and phase-contrast images of wild-type, sod-1 mutant, and sod-2 mutant spermatids stained with H$_2$DCFDA. The spermatids are outlined with dashed lines. Scale bars = 2 μm. Right panel, the fluorescence intensity of H$_2$DCFDA in wild-type, sod-1 mutant, and sod-2 mutant sperm. The data represent the mean ± S.D. of three independent experiments with 10 worms per genotype per trial. ***, p < 0.001. C, quantitative analysis of sperm activation in the wild type, sod-1 mutant, and sod-2 mutants treated with 200 μg/ml Pronase in combination with 0.3 mM H$_2$O$_2$. Asterisks indicate extended pseudopodia. Scale bars = 2 μm. The percentage of activated sperm is shown in the bottom panel. The data represent the mean ± S.D. of three independent experiments with 10 pairs per genotype per trial. *p < 0.05. D, brood sizes of wild-type, sod-1 mutant, sod-2 mutant, and sod-1;sod-2 double mutant males mated with a dpy-11 mutant hermaphrodite were examined. The data represent the mean ± S.D. of three independent experiments with 10 pairs per genotype per trial. ***, p < 0.001; **, p < 0.01; *, p < 0.05.

Flowing mutations were used: sod-1(tm776)II, sod-2(gk257)I, sod-3(gk235)X, sod-4(gk101)III, sod-5(tm1146)II, dpy-11(e224)V, and fem-3(q20)IV. Strains were maintained at 20 °C, except for the temperature-sensitive mutant fem-3, which was maintained at 15 °C. Each mutant was backcrossed seven times.

Fluorescence and Nomarski microscopy

Fluorescence images were obtained with a BZ-8000 fluorescence microscope (Keyence, Tokyo, Japan). Nomarski images were obtained with an IX71 microscope (Olympus, Tokyo, Japan) equipped with a cooled DS-5Mc charged-coupled device camera (Nikon, Tokyo, Japan).

Egg production

To measure self-brood size, L4-stage hermaphrodites were placed on individual nematode growth medium (NGM) plates. Worms were transferred daily to fresh NGM plates until egg laying ceased, and the total number of live progeny was counted. To measure cross-brood size, L4-stage males and hermaphrodites were placed on individual NGM plates and trans-
ferred daily to fresh NGM plates until egg laying ceased. The number of cross-progeny was measured by doubling the number of male progenies. In case of mating with dpy-11 hermaphrodites, the number of cross-progeny was measured by counting the number of non-dumpy progenies.

**Sperm activation**

Young adult males were dissected in a drop of sperm medium (50 mM HEPES (pH 7.0), 1 mM MgSO₄, 25 mM KCl, 50 mM NaCl, 5 mM CaCl₂, and 10 mg/ml polyvinylpyrrolidone K30) containing 200 µg/ml Pronase or 70 mM TEA on a poly-L-lysine–coated slide. For TEA treatment, 1 mg/ml bovine serum albumin was added instead of polyvinylpyrrolidone, and the pH level of the sperm medium was adjusted to 7.8. Pseudopod extension was observed within 10 min by Nomarski microscopy. Sperm with a long pseudopod extended more than the same length of the cell body were classified as activated sperm. Sperm with a short pseudopod that arrested in the extension process were classified as incompletely activated sperm. Sperm that did not show any extension of pseudopodia within 10 min were classified as non-activated spermats. To examine the effects of H₂O₂ and ebselen on sperm activation, the reagents were added to the sperm medium at the indicated concentrations.

**H₂DCFDA staining**

Young adult males were dissected in sperm medium containing 50 µM 2′,7′-dichlorodihydrofluorescein diacetate (H₂DCFDA). After 10 min of incubation, sperm were observed by fluorescence microscopy. The fluorescence intensity of H₂DCFDA was quantified using ImageJ (National Institutes of Health, Bethesda, MD).

**Sperm isolation and Western blotting**

Approximately 20,000 young adult fem-3 or sod-1; sod-2; fem-3 mutant hermaphrodites grown at 25 °C were collected, washed with M9 buffer (0.3% KH₂PO₄, 0.6% Na₂HPO₄, 12H₂O, 0.5% NaCl, and 1 mM MgSO₄) five times, and squashed between two acryl plates by pressing the plates with a pressure of 0.5% NaCl, and 1 mM MgSO₄) to release sperm from the worm body. Two plates were detached and rinsed with ice-cold M9 buffer. The squashed sample was filtered through 10-µm nylon nets fitted to a Swinnex filter holder (Merck Millipore, Darmstadt, Germany), and the filter-passed sperm were collected by centrifugation (1500 × g, 10 min, 4 °C). Sperm suspended with ice-cold M9 buffer were sonicated, and sperm protein was quantified by BCA assay. Sperm lysates were boiled for 5 min in Laemmli sample buffer without 2-mercaptoethanol and subjected to 12.5% SDS-polyacrylamide gel electrophoresis. Proteins were blotted on a PVDF membrane (Merck Millipore), incubated with 4% skim milk in TBS-T (Tris-buffered saline with 0.05% Tween 20) for blocking and treated with the antibody. The following primary antibodies obtained from the Developmental Studies Hybridoma Bank were used: mouse anti-MSP 4A5 antibody at 1:200 and mouse anti-α-tubulin 12G10 at 1:100. The immunoreactive bands were detected by ECL reagents (GE Healthcare).

**Immunostaining**

Young adult males were dissected in a drop of sperm medium containing 1.5% paraformaldehyde on a poly-L-lysine–coated slide. The slide was incubated in a humid chamber for 5 min, freeze-crack for 10 min on dry ice, incubated in 95% ethanol for 1 min, and washed with PBS-T three times. The slides were treated with the primary antibody, mouse anti-MSP 4A5 antibody at 1:2.5, in a humid chamber overnight. After this, the slides were treated with the secondary antibody, goat anti-mouse IgG conjugated with FITC at 1:140 (F2012, Sigma-Alrich, St. Louis, MO), in a humid chamber for 4 h. The slides were then stained with DAPI and observed by fluorescence microscopy.

**Detection of oxidative modification of MSP**

Oxidation of cysteine residues in MSP was examined by AMS alkylation. Sperm protein was solubilized in buffer A (100 mM Tris-HCl (pH 8.0), 1% SDS, and 1 mM EDTA) containing 15 mM AMS and incubated for 4 h at 37 °C. Alkylated sperm proteins were boiled for 5 min in Laemmli sample buffer without 2-mercaptoethanol and subjected to 15% SDS-PAGE, and the gels were stained with CBB.

**Statistical analysis**

Significance was assessed by one-way analysis of variance and Tukey’s honest significant difference test in cases where multiple strains or conditions were examined. Student’s t test was conducted to assess significance in cases where only two strains were examined.

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**Role of SOD-2 in C. elegans sperm activation**

Oxidative stress in mammalian spermatozoa has been observed to be associated with a decrease in fertility, and the importance of maintaining sperm antioxidant levels has been highlighted. It is well established that mammalian spermatozoa are particularly susceptible to oxidative stress and have a limited ability to regenerate antioxidants. This raises the possibility that antioxidant deficiencies in spermatozoa could contribute to male infertility.

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