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Original Contribution

# Boronate probes for the detection of hydrogen peroxide release from human spermatozoa

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ABSTRACT

Human spermatozoa are compromised by production of reactive oxygen species (ROS), and detection of ROS in spermatozoa is important for the diagnosis of male infertility. The probes 2′,7′-dichlorohydrofluorescein diacetate (DCFH), dihydroethidium (DHE), and MitoSOX red (MSR) are commonly used for detecting ROS by flow cytometry; however, these probes lack sensitivity to hydrogen peroxide ($H_2O_2$), which is particularly damaging to mammalian sperm cells. This study reports the synthesis and use of three aryl boronate probes, peroxyfluor-1 (PF1), carboxyperoxyfluor-1, and a novel probe, 2-(2-ethoxyethoxy)ethoxyperoxyfluor-1 (EEPF1), in human spermatozoa. PF1 and EEPF1 were effective at detecting $H_2O_2$ and peroxynitrite ($ONOO^-$) produced by spermatozoa when stimulated with menadione or 4-hydroxynonenal. EEPF1 was more effective at detection of ROS in spermatozoa than DCFH, DHE, or MSR; furthermore it distinguished poorly motile sperm as shown by greater ROS production. EEPF1 should therefore have a significant role in the diagnosis of oxidative stress in male infertility, cryopreservation, age, lifestyle, and exposure to environmental toxicants.

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Reactive oxygen species (ROS) produced by human spermatozoa compromise sperm function [1–5], and as such their detection is important for the diagnosis of male infertility [6]. ROS are typically detected in human spermatozoa using fluorescent probes such as dihydroethidium (DHE), MitoSOX red (MSR), and 2′,7′-dichlorohydrofluorescein diacetate (DCFH) (Fig. 1) [7]. DHE is an intracellular ROS probe that fluoresces within both the head and the mitochondrial midsection of the spermatozoon upon oxidation. It is most commonly used for detection of superoxide ($O_2^{\bullet-}$), although it also reacts with hydrogen peroxide ($H_2O_2$) in the presence of peroxidases and with oxidases and cytochromes [8]. MSR is a charged variant of DHE that localizes in the mitochondrial matrix to predominantly respond to and measure the generation of $O_2^{\bullet-}$. DCFH is a fluorescein-based nonspecific probe that reportedly reacts with $H_2O_2$ [9] and other ROS, particularly hydroxyl radicals ($^{\bullet}OH$) and peroxynitrite ($ONOO^-$) [10]. This probe has some disadvantages, because it requires the concomitant presence of peroxidases to react with $H_2O_2$ [11], can undergo autoxidation, and is known to catalyze $O_2^{\bullet-}$ production [9]. An aryl boronate probe reported by Chang et al. [12], peroxyfluor-1 (PF1), reacts with both $H_2O_2$ and $ONOO^-$, but not $^{\bullet}OH$, $O_2^{\bullet-}$, nitric oxide (NO), or hypochlorite ($^-OCl$) [12,13]. This class of probe has found wide use in the in vivo detection of $H_2O_2$ [14], including research into ROS production in cryopreserved mouse spermatozoa [15]. The ability of aryl boronates to detect the low levels of ROS generated by mammalian spermatozoa suggests this class of probe as a potential diagnostic tool for the selective detection of ROS, particularly $H_2O_2$ in sperm cells. This would be of clinical significance as several independent studies have indicated that $H_2O_2$ is particularly damaging to mammalian sperm function [16–18].

A number of aryl boronates have been developed for use in a range of biological applications [14]. We chose to use PF1, carboxy-PF1 (CPF1), and a new probe, 2-(2-ethoxyethoxy)ethoxy-PF1 (EEPF1), for the study as they are structurally similar, to allow for direct and meaningful comparison, while being relatively easy to prepare on both small and larger scales. This is an important consideration for future work in this area. CPF1 is a variant of PF1

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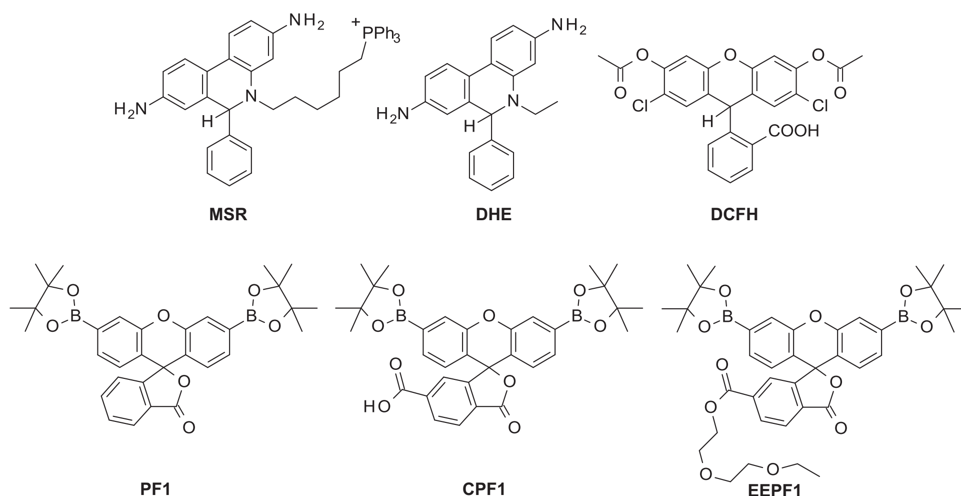


**Fig. 1.** Chemical structures of the ROS sensors used in this study. MSR, DHE, and DCFH are oxidized by removal of the indicated hydrogens to produce a fluorescent aromatic structure. PF1, CPF1, and EEPF1 are oxidized by the deprotection of the pinacolatoboron groups to produce highly fluorescent structures.

originally synthesized for attachment to other functional groups [19,20]. EEPF1 contains a truncated polyethylene glycol (PEG) group with increased hydrogen bond acceptors to enhance the aqueous solubility relative to PF1. A series of comparative studies were performed to define the relative ability of all three probes to detect ROS generation by human spermatozoa in a sensitive and selective manner. This study examines the relative capacities of these probes to detect $H_2O_2$ and $ONOO^-$ spontaneously generated by human spermatozoa exhibiting impaired motility. The results have important diagnostic implications for the facilitated detection of oxidative stress in mammalian spermatozoa exhibiting signs of impaired functionality.

## Materials and methods

### *Materials*

Unless otherwise stated all chemicals were purchased from Sigma Aldrich. *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDC-HCl) was obtained from GL Biochem (Shanghai). 4-Hydroxynonenal (4HNE) was from Sapphire Biosciences; MitoSOX red, dihydroethidium, and Live/Dead fixable FAR red stain were from Life Technologies. 2′,7′-Dichlorofluorescein diacetate was from Molecular Probes. Freshly prepared Biggers, Whitten, and Whittingham (BWW) medium was used for all experiments, supplemented with 1 mg/ml polyvinyl alcohol (PVA), 5 units/ml penicillin, and 5 mg/ml streptomycin, and the osmolarity was kept between 290 and 310 mOsm/kg [21].

### *Semen samples*

The University of Newcastle Human Ethics Committee and the NSW State Minister for Health approved the use of semen samples for research. A cohort of unselected, normozoospermic donors, mainly university students of unknown fertility status, supplied semen samples for this study. Semen samples were produced into a sterile container and delivered to the laboratory within 1 h of ejaculation.

### *Sample preparation*

Spermatozoa were isolated by discontinuous Percoll gradient centrifugation using a simple two-step design incorporating 44 and 88% Percoll as described previously [22]. Purified spermatozoa were recovered and washed with Hepes-buffered BWW medium supplemented with 1 mg/ml PVA [21], centrifuged at 500*g* for 5 min, and resuspended at a concentration of $2 \times 10^7$ cells/ml.

### *Leukocyte removal*

Where indicated, all residual traces of leukocyte contamination in the sperm suspensions were removed using magnetic beads (Dynabeads, Dynal, Oslo, Norway) coated with a monoclonal antibody against the common leukocyte antigen CD45 (Invitrogen, Carlsbad, CA, USA). After Percoll isolation, $5 \times 10^6$ cells in 100 μl BWW medium were added to prewashed antibody-bound Dynabeads and then placed on a rotor for 30 min. After incubation, each sample was placed in a magnetic holder to separate leukocyte-bound Dynabeads from purified sperm cells in BWW medium. Luminol-peroxidase-mediated chemiluminescence was then used to confirm the removal of leukocytes from each sperm suspension; for this purpose 20 μl of zymosan opsonized with autologous serum was added to each 400-μl sample, 5 min after the beginning of the luminometry run [7].

### *Treatments*

Spermatozoa were treated with menadione (0–50 μM), arachidonic acid (AA; 0–50 μM), and $H_2O_2$ (0–4 mM) for 15 min at 37 °C. Treatments with 4HNE (0–400 μM) were for 30 min at 37 °C. Stock solutions of menadione were made up fresh daily in dimethyl sulfoxide (DMSO), with a minimum dilution of 1/100 in BWW medium before being added to spermatozoa.

### *Staining*

After spermatozoa were treated they were incubated with PF1, CPF1, and EEPF1 for 30 min at 37 °C at a final concentration of 10 μM. Stock solutions were made up using DMSO at a concentration of 10 mM.

### *Flow cytometry*

A FACS-Canto flow cytometer (Becton–Dickinson) was employed using a 488-nm argon laser coupled with emission measurements using the 530/30 band pass (green) FITC channel. Ten thousand sperm events were recorded after nonsperm events

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