Original Article

The involvements of intracellular basal calcium and membrane potential in para-phenylenediamine-impaired sperm function

Yannan Chen[1#], Tingting Yang[2#], Xiaoling Gu[1], Yuqi Chen[1], Qingxin Wang[1*^], Xia Wang[1*^]

[1]Center for Reproductive Medicine, Affiliated Hospital of Nantong University, Nantong, China; [2]Department of Gynecology and Obstetrics, Shanghai Baoshan Hospital of Integrated Traditional Chinese and Western Medicine, Shanghai, China

Contributions: (I) Conception and design: X Wang, Q Wang; (II) Administrative support: X Wang, Q Wang; (III) Provision of study materials or patients: Yannan Chen, T Yang; (IV) Collection and assembly of data: Yannan Chen, T Yang; (V) Data analysis and interpretation: X Gu, Yuqi Chen; (VI) Manuscript writing: All authors; (VII) Final approval of manuscript: All authors.

[#]These authors contributed equally to this work as co-first authors.

[*]These authors contributed equally to this work.

Correspondence to: Qingxin Wang, MD; Xia Wang, PhD. Center for Reproductive Medicine, Affiliated Hospital of Nantong University, No. 20 Xisi Road, Nantong 226001, China. Email: cuixiself@163.com; tdfywangxia@163.com.

Background: Para-phenylenediamine (PPD) is a crystalline solid that belongs to the aromatic amine group, widely used in the manufacturing of various dyes. PPD exhibits toxic effects on female hormone stability, ovarian function, and embryo development. Although studies have shown that PPD exposure can damage oocyte quality in female mice, research on its effects on male reproductive capability, particularly on human sperm quality and function, is limited. The purpose of this study was to investigate the effect of PPD on male semen and explore its mechanism.

Methods: Computer-assisted sperm analysis system and eosin-aniline black method were conducted to detect sperm motility and viability; sperm function was analyzed by tyrosine phosphorylation immunofluorescence staining, sperm mucus penetration capacity assay, and sperm acrosome reaction incidence; reactive oxygen species (ROS) and DNA damage were analyzed by specific kits; the transient calcium, intracellular basal calcium, and membrane potential were detected by multi-functional microplate reader after Fluo-4, Fura-10 AM and DiSC3(5) staining.

Results: PPD was shown to have a dose-dependent impact on both the motility and viability of human sperm. Furthermore, the ability of sperm to capacitate, penetrate viscous substances, and undergo acrosome reaction exhibited significant impairments in various aspects of sperm function. The impact of PPD on sperm is comparable to its effects on other bodily systems. Spermatozoon toxicity caused by PPD was found to be associated with increased levels of ROS and DNA damage, which indicated that oxidative stress plays a role in this process. Although the transient calcium response to PPD and progesterone was not disturbed, intracellular basal calcium was increased and membrane potential was depolarized after exposure of human sperm to PPD.

Conclusions: In summary, our findings suggest that increased intracellular basal calcium, hyperpolarization in the membrane potential of damaged sperm, and oxidative stress might be the underlying reasons for the decline in semen quality and dysfunction of sperm following PPD exposure.

Keywords: Para-phenylenediamine (PPD); reproductive toxicity; sperm function; calcium signal; membrane potential

Submitted Jul 29, 2024. Accepted for publication Nov 07, 2024. Published online Dec 28, 2024.
doi: 10.21037/tau-24-374
View this article at: https://dx.doi.org/10.21037/tau-24-374

^ ORCID: Qingxin Wang, 0000-0002-1932-4588; Xia Wang, 0000-0002-4338-9349.

Introduction

Background

Para-phenylenediamine (PPD) is a derivative of benzene, typically appearing as white or pale purple-red crystalline flakes at room temperature (1,2). It possesses oxidative properties and the ability to produce large colored molecules. Consequently, PPD is extensively applied in various industries, such as textile dyes, dark-colored cosmetics, tattoo inks, and photographic developers, and is a key component in permanent hair dyes (3,4). Currently, more than 66% of hair dyes contain PPD. In developed countries, the concentration of PPD that added to hair dyes is restricted to 2%, yet can range from 2% to 90% in some developing countries, due to the lack of standardized regulations (5).

Rationale and knowledge gap

After absorption through the skin and hair, PPD can enter the bloodstream, leading to severe adverse reactions (6). Acute PPD poisoning can result in significant facial and neck edema, acute respiratory distress, rhabdomyolysis, and even acute renal failure (7). A series of allergic reactions may also occur, including allergic syncope and severe shock (8,9). The cumulative toxicity of PPD in the body may induce various forms of cancer (10). PPD exposure can cause changes in the reproductive system. Women exposed to PPD for 10 years or more have an average plasma total testosterone level 14% higher than that of women who have never been exposed to PPD, with a significant positive correlation between exposure duration and plasma total testosterone level (11). PPD can also disrupt the spindle structure and chromosomal integrity of oocytes, impair the dynamics of the cortical granule protease ovastacin, and reduce the meiotic division ability and fertilization potential of oocytes (12). The PPD metabolite N-monacetyl-para-phenylenediamine (MAPPD) induces hormone disruption through the oxidative stress pathway, resulting in abnormal follicular development, damaged oocyte quality, and thus affecting embryo development (13). Although the toxic effects of PPD exposure on the reproductive system have been studied, its influence on male sperm function and potential underlying mechanisms have not been investigated.

Highlight box

Key findings

- Increased intracellular basal calcium, impaired sperm membrane potential hyperpolarization, and oxidative stress might be the underlying reasons for the decline in semen quality and dysfunction of sperm following para-phenylenediamine (PPD) exposure.

What is known and what is new?

- PPD exposure can damage oocyte quality in female mice.
- Research of PPD effects on male reproductive capability, particularly on human sperm quality and function, is limited.

What is the implication, and what should change now?

- Exposure to PPD led to a significant decrease in total motility, progressive motility (PR), and viability of sperm. Sperm capacitation, the ability to penetrate mucus, and acrosome reaction capabilities were significantly inhibited, whereas intracellular reactive oxygen species (ROS) overload led to an increase in DNA fragmentation index (DFI). This research offers a novel perspective on the toxicity of PPD on male reproductive system, highlighting that the health risk caused by PPD deserves more attention.

Objective

Semen quality and sperm function are often seen as markers of male fertility. Reactive oxygen species (ROS) play a 2-fold function in the process of sperm capacitation. Appropriate ROS is involved in many physiological processes, such as phosphorylation of key proteins required for sperm capacitation. However, excess ROS inhibits sperm capacitation and increases sperm nuclear DNA damage (14,15). The DNA integrity of sperm is significantly correlated with its motility, and increased DNA fragmentation index (DFI) correlates with fertility and miscarriage rates and is an important predictor of pregnancy outcome (16). CatSper and KSper are 2 important sperm-specific ion channels (17). CatSper is predominantly distributed in the sperm flagellum. It dominates Ca^{2+} inward flow and is an essential prerequisite for initiating sperm capacitation (18). Ca^{2+} is extensively involved in a wide range of sperm physiological activities and even influences the outcome of fertilization in a calcium-shocked manner during sperm-egg binding (19). KSper is highly pH-sensitive and critical for male fertility, and the absence of this channel results in impaired sperm motility (20). In this study, we examined how exposure to PPD affected the motility and viability of human sperm, as well as key physiological processes involved in fertilization, which contributes to a deeper understanding of the influence of PPD on male reproductive health. We present this article in accordance with the MDAR and ARRIVE reporting

checklists (available at https://tau.amegroups.com/article/view/10.21037/tau-24-374/rc).

Methods

Human sperm collection and preparation

The Ethics Committee of the Affiliated Hospital of Nantong University granted approval for this study (Approval No: 2020-k069-01). Informed consent was taken from all the patients. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). We collected sperm samples and selected 6 cases of sperm with normal motility for each experiment. Semen was obtained through self-stimulation after abstaining for 3–7 days. All samples were collected from healthy males of reproductive age, who did not engage in occupations affecting fertility. Semen samples that were used for sperm motility analysis, human sperm viability test, evaluation of the mucus-penetrating ability of human spermatozoa, and DNA fragmentation of human sperm nuclei were collected using a high salt (HS) buffer after being washed twice, which consisted of 135 mM NaCl, 5 mM KCl, 1 mM $MgSO_4 \cdot 7H_2O$, 2 mM $CaCl_2 \cdot 2H_2O$, 20 mM HEPES, 5 mM glucose, 10 mM lactic acid, and 1 mM Na-pyruvate at pH 7.4 with NaOH. Alternatively, the samples that were used for sperm phosphorylation assay, evaluation of acrosome reaction, assessment of ROS, determination of intracellular calcium concentration ($[Ca^{2+}]i$), and detection of sperm membrane potential were obtained through swim-up in human tubal fluid (HTF; Nanjing Aibei Biotechnology Co., Ltd., Nanjing, China). The sperm sample was cultured in a 5% CO_2 incubator at a temperature of 37 ℃ (21).

Sperm motility analysis

PPD was dissolved in dimethyl sulfoxide (DMSO) and diluted with HS to achieve the desired experimental concentrations, with a final DMSO concentration maintained below 0.1%. We divided the sperm sample equally into 5 portions, 1 of which was the control group (0 μM) and the remaining 4 portions were the groups treated with different concentrations of PPD (5, 25, 100, and 200 μM). The samples were examined using a computer-assisted sperm analysis (CASA) system (WLJY-9000, WeiLi. Co., Ltd., Beijing, China) at designated time points. The following parameters were recorded: total motility and progressive motility (PR). Each count included more than 200 sperm. An eosin-nigrosin kit was purchased from Beijing Soleberg Technology Co., Beijing, China to assess sperm viability.

Sperm phosphorylation assay

The pretreated sperm samples were resuspended in HTF solution after a 1-hour incubation in HS. The samples were incubated for 4 hours after the addition of varying concentrations of PPD. Subsequently, the specimens were treated with 0.5% Triton X-100 for 10 minutes to make them permeable, and then left overnight at 4 ℃ while being exposed to an anti-phosphotyrosine antibody (Cat# 05-321; Sigma, St. Louis, MO, USA). Following the removal of the excess primary antibody, the samples were exposed to a fluorescent secondary antibody targeting mouse antigens for 1 hour, and then subjected to 4',6-diamidino-2-phenylindole (DAPI) counterstaining. Ultimately, the specimens were examined using a fluorescence microscope (ZEISS, Oberkochen, Germany) and quantified by Image J (National Institutes of Health, Bethesda, MD, USA).

Evaluation of acrosome reaction

Sperm samples after drug addition were diluted in HTF to achieve a concentration range of 1×10^6 to 20×10^6/mL, followed by an incubation period of 3 hours. Progesterone (Sigma) was added to the progesterone (P4) group, and incubation was continued for 1 hour. After staining with pisum sativum agglutinin (PSA, Sigma) in the dark for 40 minutes, samples were viewed and tallied using a fluorescence microscope (ZEISS) and quantified by the fluorescence signal of sperm head, with each group counting ≥200 sperm. Sperm with no or weak fluorescence of the head had acrosomal reaction ability, whereas sperm with complete fluorescence of the head had impaired acrosomal reaction ability.

Assessment of ROS and DNA damage

Pretreated sperm samples were resuspended in HTF solution after 1 hour incubation in HS and subsequently exposed to different concentrations of PPD for 3 hours and 30 minutes. The levels of ROS in each group of sperm were detected using a commercial assay kit (Jiancheng, Nanjing, China). Firstly, fluorescence intensity was initially measured using a multifunctional microplate reader, with excitation and emission wavelengths set at 488 and

525 nm, respectively. The fluorescence signal values were adjusted based on the values obtained from untreated controls that ran in parallel, in order to conduct further analysis. Secondly, the production of ROS was assessed by analyzing fluorescence microscopy images (ZEISS). DFI was estimated by sperm chromatin dispersion (SCD) test (Shenzhen Huakang Biomedical Engineering Co., Shenzhen, China) to assess DNA damage according to the product manual.

Determination of $[Ca^{2+}]i$

When we measured the transient intracellular calcium signal, Fluo-4 AM (Molecular Probes, Eugene, OR, USA) was added to pretreated sperm samples at a final concentration of 2 μM, and F-127 (Molecular Probes) was added at a final concentration of 0.05%. After incubating in the dark for 30 minutes, the excess dye was washed away with HS, and fluorescence intensity was assessed using a multifunctional microplate reader, which had excitation and emission wavelengths at 488 and 525 nm, respectively. Baseline fluorescence (F0) was recorded upon the addition of the sperm sample. The fluorescence produced after drug addition (F) was measured. The calculation formula for changes in calcium signals was ΔF/F0 (%) = (F – F0)/F0 * 100%.

When we measured the intracellular basal calcium, sperm samples were resuspended in HS solution and incubated for 5 hours and 25 minutes after drug addition. Fura-10 AM (AAT Bioquest, Pleasanton, CA, USA) was added at a final concentration of 2.5 μM, and F-127 was added at a final concentration of 0.05%. After continued incubation in the dark for 35 minutes, the excess dye was washed away with HS, and fluorescence intensity was assessed using a versatile microplate reader with excitation/emission wavelengths of 354/524 nm and 415/524 nm. F340/F380 indicated the baseline calcium levels in sperm cells.

Detection of sperm membrane potential

Pretreated sperm samples were resuspended in HTF solution after 1-hour incubation in PPD. The sperm suspension was treated with DiSC3(5) dye at a concentration of 2 μM and then incubated in darkness for 10 minutes. Fluorescence values were measured using a multifunctional microplate reader, and membrane potential values were obtained by calibrating readings with valinomycin (MedChemExpress, Newark, NJ, USA) and a certain concentration gradient of KCl (22).

Animals

All animals in this study were obtained from the Nantong University Laboratory Animal Centre. Experiments were performed under a project license (No. S20220717-001) granted by Nantong University Laboratory Animal Centre, in compliance with the institutional guidelines for the care and use of animals. A protocol was prepared before the study without registration. Briefly, the study utilized 8-week-old male Institute of Cancer Research (ICR) mice, which were kept in standard housing conditions at a temperature of 20–23 ℃ and a light/dark cycle of 12 hours. Male ICR mice were divided into 3 groups by random assignment: a control group (0 mg/kg/day), a group receiving a low dose (1 mg/kg/day), and a group receiving a high dose (10 mg/kg/day). PPD (Rhawn Chemical Ltd, Shanghai, China) was first dissolved in DMSO and then further diluted with water. DMSO concentration was less than 0.1%. Mice received daily intraperitoneal injections of PPD for 35 consecutive days.

Statistical analysis

Statistical data analysis was conducted using GraphPad Prism 9.0 (GraphPad Software, San Diego, CA, USA). Each experiment was repeated at least 3 times, and the results were expressed as mean ± standard error of the mean (SEM). Unless otherwise stated, *t*-tests were used for comparisons during variance analysis. *P<0.05, **P<0.01, ***P<0.001, versus the control.

Results

PPD exposure impaired sperm motility and viability

In order to evaluate the impact of PPD on human sperm motility, various concentrations of PPD were added to sperm samples and incubated for 0, 3, and 6 hours. In comparison to the control group, there was a decrease in both total motility and PR of sperm that were exposed to PPD, which occurred in a time- and dosage-dependent manner (*Figure 1A*,*1B*). In addition, after incubation of different concentrations of PPD with human sperm samples for 3 hours, the PPD exposure group showed a higher increase in the number of dead sperm (*Figure 1C*,*1D*). The trend of decreased sperm viability was dose-dependent on PPD exposure concentration (*Figure 1E*).

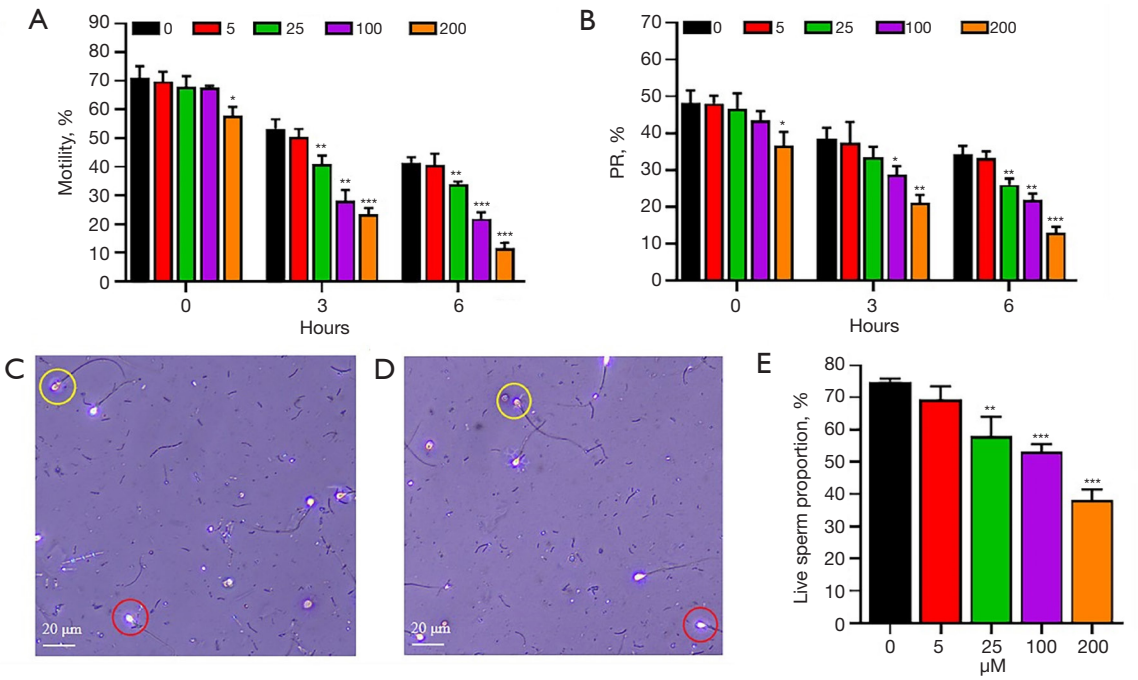

Figure 1 Effects of PPD on human sperm total motility, PR, and viability. (A) Statistical evaluations of total sperm motility in the control group (0 μM PPD) and PPD exposure group (5, 25, 100, and 200 μM). (B) Statistical evaluations of sperm PR in the control group and PPD exposure group (5, 25, 100, 200 μM). (C) Illustration of eosin-nigrosin staining to assess sperm viability in the control group. (D) illustration of eosin-nigrosin staining to assess sperm viability in PPD exposure group (5, 25, 100, and 200 μM). Scale bar =20 μM. (E) Statistical evaluation of sperm viability in the control group and PPD exposure group (5, 25, 100, and 200 μM). (A,B,E) Data represent the results of at least 3 independent experiments, presented as mean ± SEM. *, P<0.05; **, P<0.01; ***, P<0.001. Red circles: surviving sperm. Yellow circles: dead sperm. PPD, para-phenylenediamine; PR, progressive motility; SEM, standard error of the mean.

PPD exposure suppresses human sperm function

Next, we investigated whether PPD affected sperm function. Tyrosine phosphorylation has an impact on the process of sperm capacitation, acrosome reaction, and sperm–egg fusion, and especially reflects sperm capacitation levels (23,24). The degree of tyrosine phosphorylation from the control group was notably elevated compared to the 100 μM PPD group, which made no difference from the 25 μM PPD group (*Figure 2A-2C*). When the concentration of PPD exposure was 100 μM, there was a reduction in the degree of tyrosine phosphorylation (*Figure 2D*). PPD was found to suppress the penetration of human sperm through mucus, with a stronger inhibitory impact observed at 3 cm of glass capillary and a promoting effect in P4 group (*Figure 2E*). The occurrence rate of sperm acrosome reaction significantly decreased when exposed to PPD at concentrations of 25, 100, and 200 μM, without an inhibitory effect in the P4 group (*Figure 2F-2H*). These findings indicated that PPD negatively affected capacitation, penetration through mucus, and acrosome reaction of sperm.

PPD exposure increases DNA damage and ROS levels in human sperm

Given that PPD reduced sperm motility and impaired sperm function, that DFI and ROS are significantly correlated with sperm motility, and that excess ROS affects sperm nuclear DNA integrity, the assessment of DFI and ROS levels after PPD exposure is necessary (16,25). Levels of ROS in sperm from the PPD exposure groups (5, 25, 100, and 200 μM) were elevated, with ROS levels increasing with both the concentration and duration of PPD exposure (*Figure 3A-3C*). The PPD groups (100 and 200 μM) exhibited a notable elevation in DFI compared to the control group (*Figure 3D-3F*).

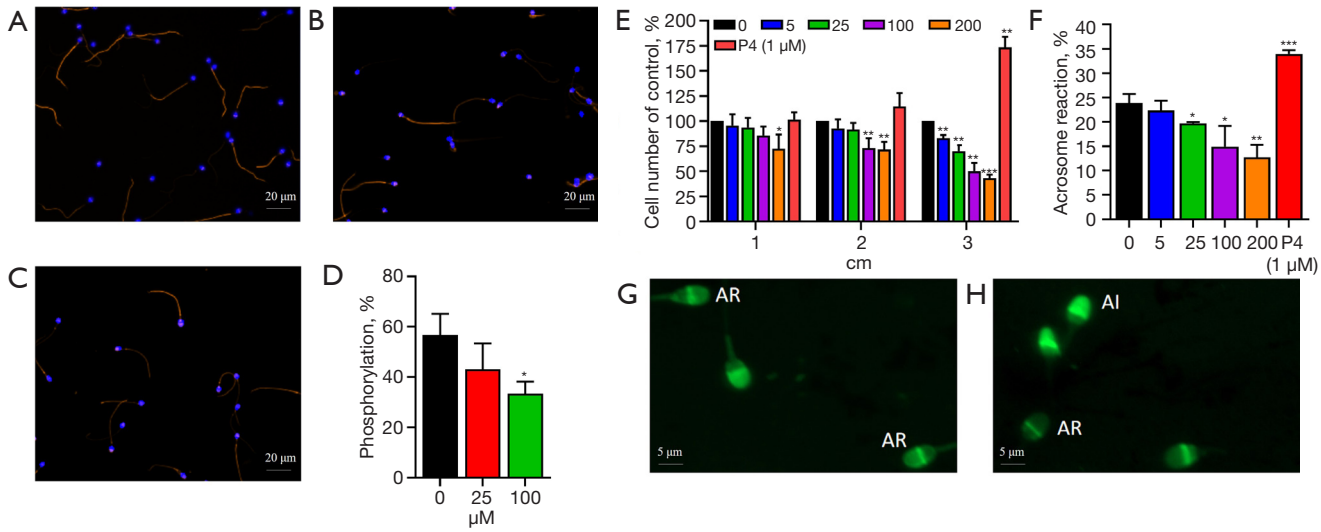

Figure 2 PPD affects human sperm capacitation, the ability to penetrate viscous media, and acrosome reaction. (A) Illustration of tyrosine phosphorylation staining in human sperm from the control group. (B) Illustration of tyrosine phosphorylation staining in human sperm from 25 μM PPD group. (C) Illustration of tyrosine phosphorylation staining in human sperm from 100 μM PPD group. Scale bar =20 μM. (D) Statistical evaluation of tyrosine phosphorylation levels in human sperm from the control group and PPD exposure group (25, 100 μM). (E) Statistical evaluation of mucus penetration ability in human sperm from the control group and PPD exposure group (5, 25, 100, 200 μM). (F) Statistical evaluation of acrosome reaction occurrence rate in human sperm from the control group and PPD exposure group (5, 25, 100, 200 μM). (G) Illustration of PSA staining in human sperm from the control group. (H) Illustration of PSA staining in human sperm from 100 μM PPD group. AR: human sperm undergoing acrosome reaction; AI: human sperm not undergoing acrosome reaction. Scale bar =5 μM. Data in (D-F) represent the results of at least 3 independent experiments, presented as mean ± SEM. *, P<0.05; **, P<0.01; ***, P<0.001. PPD, para-phenylenediamine; PSA, pisum sativum agglutinin; SEM, standard error of the mean.

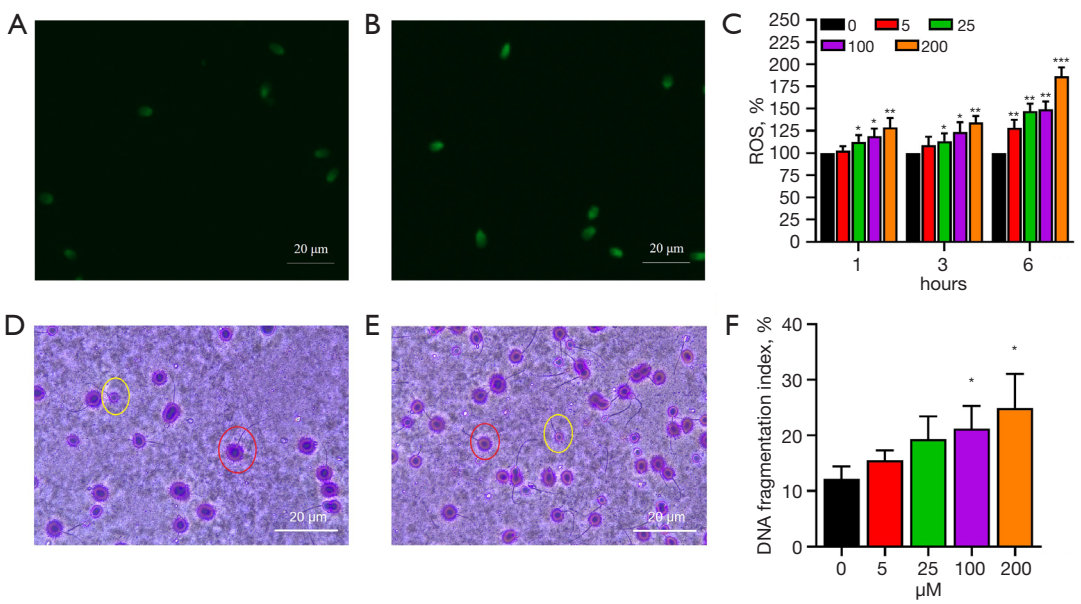

Figure 3 PPD Affects ROS levels and DNA damage in human sperm. (A) Illustration of ROS staining in human sperm from the control group. (B) Illustration of ROS staining in human sperm from 200 μM PPD group. Scale bar = 20 μM. (C) Statistical evaluation of ROS levels in human sperm from the control group and 200 μM PPD group. (D) Illustration of DFI staining in human sperm from the control

group. (E) Illustration of DFI staining in human sperm from 200 μM PPD group. Scale bar =20 μM. (F) Statistical evaluation of DFI in human sperm from the control group and 200 μM PPD group. Data in (C) and (F) represent the results of at least 3 independent experiments, presented as mean ± SEM. Red circles: spermatozoa that do not produce fragmented DNA. Yellow circles: spermatozoa that produce fragmented DNA. *, P<0.05; **, P<0.01; ***, P<0.001. PPD, para-phenylenediamine; ROS, reactive oxygen species; DFI, DNA fragmentation index; SEM, standard error of the mean.

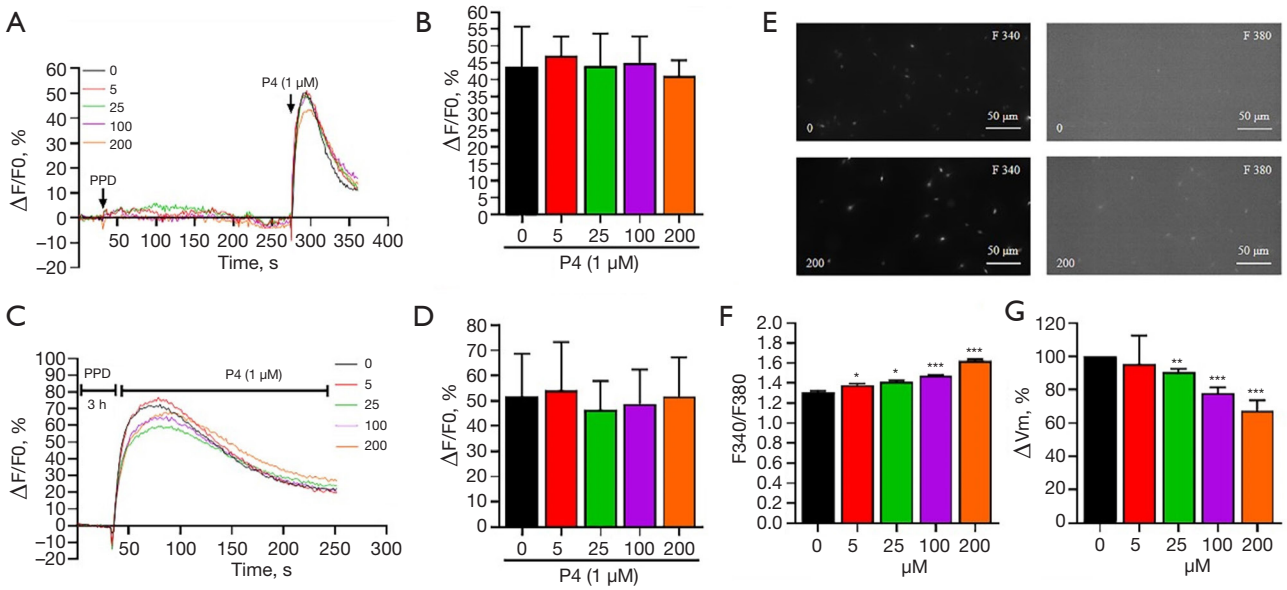

Figure 4 PPD affects intracellular basal calcium and sperm membrane potential hyperpolarization. (A) Changes in $[Ca^{2+}]i$ signal of human sperm pre-incubated with various concentrations of PPD (0, 5, 25, 100, 200 μM) at 0 hours and P4 induction. (B) Statistical evaluation of $[Ca^{2+}]i$ signal of human sperm pre-incubated with various concentrations of PPD (0, 5, 25, 100, 200 μM) at 0 hours and after P4 induction. (C) Changes in $[Ca^{2+}]i$ signal of human sperm pre-incubated with various concentrations of PPD (0, 5, 25, 100, 200 μM) at 3 hours and P4 induction. (D) Statistical evaluation of $[Ca^{2+}]i$ signal of human sperm pre-incubated with various concentrations of PPD (0, 5, 25, 100, 200 μM) at 3 hours and after P4 induction. (E) Illustrations of basal calcium staining in human sperm from the control group and 200 μM PPD group. Scale bar =50 μM. (F) Statistical evaluation of intracellular basal calcium levels in human sperm from different groups (0, 5, 25, 100, 200 μM). (G) Statistical evaluation of membrane potential levels in human sperm from different groups (0, 5, 25, 100, 200 μM). Data in (B,D,F,G) represent the results of at least 3 independent experiments, presented as mean ± SEM. *, P<0.05; **, P<0.01; ***, P<0.001. PPD, para-phenylenediamine; P4, progesterone; SEM, standard error of the mean.

PPD exposure increases intracellular basal calcium levels and disrupts sperm membrane potential hyperpolarization

Human sperm function is a $[Ca^{2+}]i$-dependent process, so we examined the impact of PPD on $[Ca^{2+}]i$ levels in sperm. Human sperm exposed to various concentrations of PPD (0, 5, 25, 100, and 200 μM) for 0 and 3 hours did not exhibit a significant variance in the transient calcium ion signal ($[Ca^{2+}]i$) compared to the control group (*Figure 4A-4D*). This finding suggested that PPD might not directly affect $[Ca^{2+}]i$ through CatSper in human sperm. Considering that PPD can induce apoptosis by affecting intracellular basal calcium (26), we employed Fura-10 AM staining to evaluate alterations of sperm's basal calcium levels following 6 hours of exposure to varying concentrations of PPD. Compared with the control group, exposure to PPD led to a rise in the basal calcium levels inside human sperm (*Figure 4E,4F*).

Human sperm membrane potential is closely related to sperm capacitation (27). Human sperm were co-cultured with varying levels of PPD for a duration of 1 hour. The PPD group showed a significant impact on sperm plasma membrane hyperpolarization compared with the control group, resulting in a decrease in sperm membrane potential (*Figure 4G*).

Discussion

Despite the toxicity of PPD on male reproductive having been demonstrated in mouse models, there is a limited number of relevant studies and the impact of PPD on human sperm remains uncertain. Our findings suggest that exposure to PPD led to a significant decrease in total motility, PR, and viability of sperm. Sperm capacitation, the ability to penetrate mucus, and acrosome reaction capabilities were significantly inhibited, whereas intracellular ROS overload led to an increase in DFI.

Comparison of studies and mechanism of effects

Research has indicated that local subchronic exposure to PPD can result in a notable reduction in testicular weight and sperm concentration, along with elevated levels of oxidative stress in testicular tissues (28). The impact of N-(1,3-Dimethylbutyl)-N'-phenyl-1,4-phenylenediamin (6PPD), a PPD analogue, on mouse semen quality and the outcomes of *in vitro* fertilization have been investigated (29). In this study, animal experiments revealed that PPD exposure reduced sperm PR and concentration in mice, increased sperm deformity rates, and caused degenerative changes in the seminiferous tubules of mouse testes (Figure S1), consistent with previous reports (28,30). These results indicated that PPD adversely affected spermatogenesis and sperm maturation, potentially rendering human sperm more vulnerable to its reproductive toxic effects.

We found that PPD affected human sperm motility and viability, and impaired sperm functions such as the ability to penetrate mucus and undergo an acrosome reaction. Benzo(a)pyrene affected sperm motility, ability to penetrate mucus, and acrosome reaction, but its viability was not affected (31). Similarly, the reproductive toxicity of phthalic acid esters is manifested as reduced acrosome responsiveness (32). Pentachloro-phenol does not impact the motility, viability, mucus penetration ability, or acrosome responsiveness of sperm, but it does impair the induction of P4 (33). Benzene derivatives have different toxic effects on human sperm, and the sperm toxicity of PPD might be unique, which is distinct from other benzene derivatives and deserves further research.

Protein phosphorylation, especially tyrosine phosphorylation, is of great significance in sperm capacitation and acrosome reaction (34,35). The function of human sperm may be compromised by the organic compound anethole's impact on the tyrosine phosphorylation of sperm (36). In our experiments, we found that exposure to PPD significantly reduced the phosphorylation level of human sperm, as well as its ability to penetrate mucus and undergo an acrosome reaction. PPD may decrease sperm motility and affect sperm function by inhibiting tyrosine phosphorylation, similar to the mechanism of sperm toxicity of anethole. Mature sperm produce large amounts of ROS and are more susceptible to oxidative damage than other cell types (37). We found that PPD exposure significantly increased ROS levels in sperm. Excessive ROS can inhibit sperm vitality and capacitation, reduce sperm membrane fluidity, and affect adhesion and fusion with oocytes (38). Normal fertilization and embryo development require the integrity of sperm DNA, and excessive ROS can affect the integrity of sperm nuclear DNA (25), consistent with our research findings.

Calcium signaling serves as an important intracellular second messenger in sperm, participating in various physiological processes, including spermatogenesis, sperm maturation, the ability to penetrate viscous substances, and acrosome reaction (39). Environmental disturbances may lead to a decline in the functionality of human sperm by influencing $[Ca^{2+}]i$ (36,40). No significant alterations were observed in $[Ca^{2+}]i$ of human sperm following exposure to PPD, implying that PPD might not impair sperm function by CatSper. Similarly, bisphenol A (BPA) reduces sperm motility, viability, hyperactivation, capacitation, and acrosome responsiveness by inhibiting tyrosine phosphorylation in sperm, but does not impact $[Ca^{2+}]i$ (41). We speculated that this might be related to the fact that BPA and PPD have similar structures. Salami *et al.* found that increased basal calcium levels in human lymphocytes play a crucial role in PPD-induced cell apoptosis (26). Therefore, we measured the basal calcium concentration in sperm cells after PPD exposure and found that PPD exposure significantly increased intracellular basal calcium concentration. Moreover, the higher the degree of PPD, the more pronounced the increase in intracellular basal calcium. The build-up of calcium inside the sperm could play a crucial role in triggering irreversible mechanisms that ultimately result in cellular demise. Impaired sperm function caused by PPD might be related to increased intracellular basal calcium accumulation rather than $[Ca^{2+}]i$ alteration.

Changes in sperm membrane potential are crucial for sperm capacitation, hyper-activation, acrosome reaction, and other physiological processes (42). CatSper and KSper are 2 important sperm-specific ion channels that affect sperm membrane hyperpolarization. KSper, the

most diverse class of ion channels, regulates intracellular K^+ concentration. In human sperm, there are subfamilies of SLO calcium-activated K^+ channels, voltage-gated K^+ channels, and inward-rectifying K^+ channels (20). After incubation of PPD with human sperm for 1 hour, sperm membrane potential decreased. Given that we excluded the association of CatSper channels with the decline in semen quality, we speculate that the impaired activation of KSper channels played a role in the mechanism of PPD toxicity, which requires further investigation.

Conclusions

In brief, exposure to PPD led to a significant decrease in total motility, PR, and viability of sperm. Sperm capacitation, the ability to penetrate mucus, and acrosome reaction capabilities were significantly inhibited, whereas intracellular ROS overload led to an increase in DFI. The increase in intracellular basal calcium accumulation and impaired sperm membrane potential hyperpolarization might be the reasons for the decrease in sperm motility, viability, and sperm function caused by PPD exposure. This research offers a novel perspective on the male reproductive toxicity of PPD; greater attention should be paid to the health risks caused by exposure to PPD.

Acknowledgments

This study was supported by the Institute of Reproductive Medicine, Medical School, Nantong University, Nantong. The authors are extremely grateful to the volunteers for their participation in this study.
Funding: This research was funded by the Research Project of Jiangsu Commission of Health (No. Z2022069), Postdoctoral Project of the Affiliated Hospital of Nantong University (No. BSH202315), and Scientific Research Project of Nantong Health Commission (No. MS2022003).

Footnote

Reporting Checklist: The authors have completed the MDAR and ARRIVE reporting checklists. Available at https://tau.amegroups.com/article/view/10.21037/tau-24-374/rc

Data Sharing Statement: Available at https://tau.amegroups.com/article/view/10.21037/tau-24-374/dss

Peer Review File: Available at https://tau.amegroups.com/article/view/10.21037/tau-24-374/prf

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://tau.amegroups.com/article/view/10.21037/tau-24-374/coif). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The Ethics Committee of the Affiliated Hospital of Nantong University granted approval for this study (Approval No: 2020-k069-01). Informed consent was taken from all the patients. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). Experiments were performed under a project license (No. S20220717-001) granted by Nantong University Laboratory Animal Centre, in compliance with the institutional guidelines for the care and use of animals.

References

1. More SL, Fung ES, Mathis C, et al. Dermal exposure and hair dye: Assessing potential bladder cancer risk from permanent hair dye. Regul Toxicol Pharmacol 2023;138:105331.
2. Alberola IA, Hernández EJ, Carracedo JFS, et al. Early presentation of allergic contact dermatitis due to paraphenylenediamine. Allergol Immunopathol (Madr) 2024;52:93-6.
3. Pot LM, Scheitza SM, Coenraads PJ, et al. Penetration and haptenation of p-phenylenediamine. Contact Dermatitis 2013;68:193-207.
4. He L, Michailidou F, Gahlon HL, et al. Hair Dye Ingredients and Potential Health Risks from Exposure to Hair Dyeing. Chem Res Toxicol 2022;35:901-15.
5. Bhagavathula AS, Bandari DK, Khan M, et al. A

systematic review and meta-analysis of the prevalence and complications of paraphenylenediamine-containing hair dye poisoning in developing countries. Indian J Pharmacol 2019;51:302-15.
6. Tseng A, Kovacs C, Wong DKH. When beauty is more than skin deep: Acute hepatitis secondary to topical para-phenylenediamine exposure from hair dye shampoo. Can Fam Physician 2023;69:403-5.
7. Güdeloğlu E, Erdur B. Retrospective evaluation of childhood paraphenylenediamine intoxication due to black henna. Turk J Pediatr 2020;62:387-93.
8. Tsimpidakis A, Katoulis A, Nicolaidou E, et al. Hair Dyes Sensitization and Cross-Reactions: Challenges and Solutions: A Systematic Review of Hair Dye Allergens' Prevalence. Dermatitis 2024;35:13-23.
9. Che D, Gao J, Du X, et al. p-Phenylenediamine induces immediate contact allergy and non-histaminergic itch via MRGPRX2. Chem Biol Interact 2022;351:109751.
10. Zhang Y, Birmann BM, Han J, et al. Personal use of permanent hair dyes and cancer risk and mortality in US women: prospective cohort study. BMJ 2020;370:m2942.
11. Nagata C, Wada K, Tsuji M, et al. Association of hair dye use with circulating levels of sex hormones in premenopausal Japanese women. Eur J Public Health 2015;25:895-9.
12. Wang X, Zhao X, Chen Y, et al. Para-phenylenediamine deteriorates oocyte quality by impairing mitochondrial function. Environ Toxicol 2022;37:1803-13.
13. Yu L, Zhai J, Wang Y, et al. Exposure to N-monoacetyl-p-phenylenediamine impaired ovarian function in mice. J Appl Toxicol 2021;41:2031-41.
14. Drevet JR, Hallak J, Nasr-Esfahani MH, et al. Reactive Oxygen Species and Their Consequences on the Structure and Function of Mammalian Spermatozoa. Antioxid Redox Signal 2022;37:481-500.
15. Xavier MJ, Nixon B, Roman SD, et al. Paternal impacts on development: identification of genomic regions vulnerable to oxidative DNA damage in human spermatozoa. Hum Reprod 2019;34:1876-90.
16. Du C, Tuo Y. Correlation of DNA fragments with routine semen parameters and lifestyle and their impact on assisted reproductive outcomes. Rev Int Androl 2023;21:100337.
17. Pinto FM, Odriozola A, Candenas L, et al. The Role of Sperm Membrane Potential and Ion Channels in Regulating Sperm Function. Int J Mol Sci 2023;24:6995.
18. He Y, Wang B, Huang J, et al. Environmental pollutants and male infertility: Effects on CatSper. Ecotoxicol Environ Saf 2024;277:116341.
19. Zhang X, Liang M, Song D, et al. Both protein and non-protein components in extracellular vesicles of human seminal plasma improve human sperm function via CatSper-mediated calcium signaling. Hum Reprod 2024;39:658-73.
20. Cong S, Zhang J, Pan F, et al. Research progress on ion channels and their molecular regulatory mechanisms in the human sperm flagellum. FASEB J 2023;37:e23052.
21. Chen C, Li B, Huang R, et al. Involvement of Ca2+ and ROS signals in nickel-impaired human sperm function. Ecotoxicol Environ Saf 2022;231:113181.
22. Baro Graf C, Ritagliati C, Stival C, et al. Determination of a Robust Assay for Human Sperm Membrane Potential Analysis. Front Cell Dev Biol 2019;7:101.
23. Lee WJ, Hwang JM, Jo JH, et al. Adverse Effects of Avobenzone on Boar Sperm Function: Disruption of Protein Kinase A Activity and Tyrosine Phosphorylation. Reprod Toxicol 2024;125:108559.
24. Luo L, Sun L, Li S, et al. miR-124-3p regulates the involvement of Ptpn1 in testicular development and spermatogenesis in mouse. Gene 2024;893:147967.
25. Andrabi SW, Ara A, Saharan A, et al. Sperm DNA Fragmentation: causes, evaluation and management in male infertility. JBRA Assist Reprod 2024;28:306-19.
26. Salami M, Pourahmad J, Zarei MH. Toxicity of para-phenylenediamine (PPD;1, 4 diaminobenzene) on isolated human lymphocytes: The key role of intracellular calcium enhancement in PPD-induced apoptosis. Toxicol Ind Health 2023;39:388-97.
27. Liang M, Ji N, Song J, et al. Flagellar pH homeostasis mediated by Na+/H+ exchangers regulates human sperm functions through coupling with CatSper and KSper activation. Hum Reprod 2024;39:674-88.
28. Bharali MK, Dutta K. Testicular toxicity of para-phenylenediamine after subchronic topical application in rat. Int J Environ Health Res 2012;22:270-8.
29. Yao K, Kang Q, Liu W, et al. Chronic exposure to tire rubber-derived contaminant 6PPD-quinone impairs sperm quality and induces the damage of reproductive capacity in male mice. J Hazard Mater 2024;470:134165.
30. Al-Shaikh TM, Alkaseb KS, Mudawi MME, et al. Toxicological studies on commercial hair dye brand commonly used in Saudi Arabia: histological study of the liver, kidney, heart, and testis. J Basic Appl Zool 2018. doi: 10.1186/s41936-018-0036-4
31. Traini G, Tamburrino L, Ragosta ME, et al. Effects of Benzo[a]pyrene on Human Sperm Functions: An In Vitro Study. Int J Mol Sci 2023;24:14411.

32. Cosci I, Garolla A, Cabrelle A, et al. Lipophilic phthalic acid esters impair human sperm acrosomal reaction through the likely inhibition of phospholipase A(2)-signaling pathway. Biochem Pharmacol 2022;205:115249.
33. Zhang X, Kang H, Peng L, et al. Pentachlorophenol inhibits CatSper function to compromise progesterone's action on human sperm. Chemosphere 2020;259:127493.
34. Liang Z, Dai C, He F, et al. AKAP3-mediated type I PKA signaling is required for mouse sperm hyperactivation and fertility†. Biol Reprod 2024;110:684-97.
35. Jung EJ, Jo JH, Uwamahoro C, et al. Ritonavir Has Reproductive Toxicity Depending on Disrupting PI3K/PDK1/AKT Signaling Pathway. Toxics 2024;12:73.
36. Luo T, Wang F, Weng S, et al. Anethole compromises human sperm function by affecting the sperm intracellular calcium concentration and tyrosine phosphorylation. Reprod Toxicol 2020;93:99-105.
37. Aitken RJ, Drevet JR. The Importance of Oxidative Stress in Determining the Functionality of Mammalian Spermatozoa: A Two-Edged Sword. Antioxidants (Basel) 2020;9:111.
38. Serafini S, O'Flaherty C. Redox Regulation to Modulate Phosphorylation Events in Human Spermatozoa. Antioxid Redox Signal 2022;37:437-50.
39. Finkelstein M, Etkovitz N, Breitbart H. Ca(2+) signaling in mammalian spermatozoa. Mol Cell Endocrinol 2020;516:110953.
40. Yang W, Ling X, He S, et al. Perturbation of IP3R-dependent endoplasmic reticulum calcium homeostasis by PPARδ-activated metabolic stress leads to mouse spermatocyte apoptosis: A direct mechanism for perfluorooctane sulfonic acid-induced spermatogenic disorders. Environ Pollut 2024;343:123167.
41. Li N, Kang H, Peng Z, et al. Physiologically detectable bisphenol A impairs human sperm functions by reducing protein-tyrosine phosphorylation. Ecotoxicol Environ Saf 2021;221:112418.
42. Zhang J, Zheng L, Chen Y, et al. LRRC52 is likely a functional component of human KSper. Biol Reprod 2024;110:711-21.

(English Language Editor: J. Jones)

Cite this article as: Chen Y, Yang T, Gu X, Chen Y, Wang Q, Wang X. The involvements of intracellular basal calcium and membrane potential in para-phenylenediamine-impaired sperm function. Transl Androl Urol 2024;13(12):2661-2671. doi: 10.21037/tau-24-374