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Di-(2-ethylhexyl) phthalate inhibits DNA replication leading to hyperPARylation, SIRT1 attenuation, and mitochondrial dysfunction in the testis

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Di-(2-ethylhexyl)-phthalate (DEHP) is a ubiquitously used endocrine disruptor. There is widespread exposure to DEHP in the general population which has raised substantial public concern due to its potential detrimental health effects. It is particularly pertinent to investigate the molecular mechanisms of its testicular toxicity which are largely unknown. By feeding male rats DEHP for 2 weeks, rat spermatogenesis became disrupted, resulting in a decreased number of spermatocytes and spermatids. Since rapidly dividing tissues appeared to be particularly vulnerable to DEHP toxicity we investigated the effect of DEHP on DNA replication. Intriguingly, DEHP appeared to inhibit DNA replication as evidenced by results of fiber tract analysis. This led to induction of the mitochondrial apoptotic pathways and increased ROS production. Furthermore, the toxicity of DEHP led to respiratory chain defects and attenuation of ATP level probably brought about by hyperPARylation and undermined SIRT1 activity. Our findings reveal a previously unknown mitochondrial dysfunction in DEHP-induced testicular toxicity and highlight the importance of SIRT1 in male reproduction.

D ndocrine disruptors (EDs) are a group of molecules capable of altering normal endocrine functions in animals and humans. These compounds are considered to mimic the effect of estrogen and other steroid hormones, deregulating the control of several hormone-dependent developmental processes¹. In recent years, newly discovered EDs have raised considerable concern due to their detrimental effects on human health. A variety of EDs have been shown to increase the disposition toward diabetes and cancer, disrupt bone turnover and reduce reproduction in both males and females with possible transmission of reproductive problems to the male offspring¹⁻⁵. Phthalates (or phthalate esters) are an important group of EDs with a diverse range of industrial applications, such as plasticized vinyl chloride (PVC), food packaging, clothing, cosmetics, medical products, personal care products, residential construction and automotive industries, leading to common exposure risk in humans^{6,7}. It is estimated that usage of phthalates exceeded 3 million metric tons annually worldwide in 2000⁸. Since phthalates are not chemically bound to the products, they can leak, migrate or evaporate resulting in significant environmental contamination, and human exposure mainly via oral, dermal, inhalation and intravenous routes^{9,10}.

One major phthalate is di-(2-ethylhexyl)-phthalate (DEHP), a widely used compound for which dietary exposure (food processing, packaging) likely represents the main source of contamination for the general population^{11,12}. After consumption, DEHP is rapidly hydrolyzed by lipases in the gut to its primary metabolite, mono-(2-ethylhexyl) phthalate (MEHP), which is further metabolized to mono-(2-ethyl-5-hydroxyhexyl) phthalate (MEHHP), mono-(2-ethyl-5-corobexyl) phthalate (MEOHP), and mono-(2-ethyl-5-corobexyl) phthalate (MECPP). Food contamination with DEHP in many countries including the UK and Germany has been reported¹³⁻¹⁵. A 2006 report showed that children (95% percentile, 21 ~ 25 µg/kg/day) were more highly exposed to DEHP than adults, scooped out the Reference dose (20 µg/kg/day), and the Tolerable Daily Intake (20 ~ 48 µg/kg/day)¹⁶. The European Food Safety Agency suggested not more than a maximal tolerable daily intake of

50 µg/kg body weight to prevent reproductive and developmental toxicity¹⁷. A recent issue about severe DEHP contamination in foods and beverages by illicit introduction of DEHP to clouding agents, a type of food additive, aroused heated public discussions¹⁸. Thus, various routes of exposure could lead to a daily intake of DEHP that greatly exceeds the established safety standard. Results from previous in vitro cellular and animal studies indicate that DEHP has a constellation of adverse effects, most notably hepatotoxicity, infertility, and teratogenicity^{1,19,20}. DEHP has been found to cause reproductive and developmental toxicities, such as apoptosis in germ cells which may contribute to male infertility^{19,20}. In rats, *in utero* and lactational exposure to DEHP reduced daily sperm production and induced reproductive tract abnormalities in the male offspring²¹. The reproductive toxicity of DEHP in males is well established, but a unifying mechanism explaining the male reproductive toxicity is lacking¹⁸ Additional experiments are therefore necessary to elucidate the toxicological and pathogenic pathways affected by DEHP.

Herein we present evidence that the testicular toxicity may be caused by direct inhibition of DNA replication leading to activation of the DNA damage response enzyme PARP1. This in turn leads to SIRT1 attenuation, apoptosis and mitochondrial dysfunction. Our findings provide a novel mechanistic insight into the pathogenesis of DEHP toxicity and highlight the importance of SIRT1 in male fertility.

Results

DEHP exhibits differential organ toxicity. The SD rats were fed with various doses of DEHP, ranging from 20, 100, 500, to 1000 mg/ kg, daily for 14 days. There were no deaths during the treatment period, and no significant changes of body weight among all the groups (Table S1). All rats were sacrificed 24 h after the last treatment and the weights of various organs, including liver, heart, spleen, kidneys, thymus, adrenal glands, and testes, were measured (Fig. S1). At a dose of 500 mg/kg, only the weights of liver (Fig. S1a) and testes (shown below) were significantly changed which were consistent with previous work showing increased sensitivity of these organs to DEHP toxicity²². At the very high dose of 1000 mg/kg, atrophy of the heart, spleen and kidneys was also noted, while the thymus and adrenal glands appeared unaffected (Fig S1). This effect of DEHP appeared to mimic the toxicity of the positive control flutamide (an endocrine disruptor with testicular toxicity, 50 mg/kg) as reported elsewhere²³.

DEHP induces testicular toxicity via DNA damage and germ cell apoptosis. Although studies on the testicular toxicity of DEHP have been published, a comprehensive investigation of the 14-day toxic effect of DEHP on rat testis, epididymides, anogenital distance, and androgen-dependent accessory reproductive glands (seminal vesicles, ventral prostate, levator ani plus bulbocavernosus muscles/ LABC) appeared to be lacking. We thus measured the weights of the above mentioned tissues. DEHP exhibited a trend of reducing the testicular weight at 100 mg/kg, and the reduction reached statistical significance at 500 mg/kg compared with the negative control (Fig. 1a). Furthermore, DEHP at different doses decreased the weights of all of the other organs as well as the anogenital distance (Fig. 2 a-f). Specifically, anogenital distance, seminal vesicle weight, and prostate weight appeared to be sensitive to DEHP toxicity at a dose of 100 mg/kg. To further unravel the testicular histopathology of DEHP-fed rats, testicular sections from the different groups were stained with H&E and observed under a light microscope. In control samples, spermatogonia (denoted G), spermatocytes (C), and both spermatids and mature spermatids (T) were sequentially well organized, with sperms (S) distributed around (Fig. 1g, first panel). In DEHP-treated rats, there was a significant dose-dependent decrease of spermatocytes (C) and spermatids (T). Specifically, at the dose of 500 mg/kg, spermatids were exceedingly rare and

mature spermatocytes were scarce. The testicular atrophy elicited by DEHP may be ascribed to increased cell death and/or decreased cell proliferation. Thus *in situ* detection of cell death in testicular tissues using TUNEL was employed. As shown in Fig. 1h, an increased number of TUNEL-positive staining cells (indicating DEHP induced more fragmented DNA in these tissues) was noted in DEHP-exposed rat tissues in a dose-dependent way, consistent with previous work²⁴. Interestingly, DEHP also exhibited antiproliferative activity towards testicular germ cells with the details shown below. Taken together it appears DEHP induced testicular atrophy through germ cell apoptosis and ablated proliferation.

DEHP inhibits DNA replication. Cell cycle arrest has been linked with DEHP-induced pathology, including induced G2/M arrest, and increased expression of some cell cycle proteins, including PCNA, cyclins D3, p53, and cyclin G1^{25,26}. Since DEHP appears to be severely toxic for replicating tissues we speculated that this compound may interfere with DNA replication *in vivo*. We therefore measured the effect of DEHP on replication using DNA fiber tract analysis. NIH3T3 and TM4 sertoli cells were treated with/without 70 μ M DEHP for 48 h after which we added a pulse of CldU (purple) for 20 min, followed by a 20 min pulse of IdU (Green, Fig. 2a). Strikingly, DEHP exposure strongly inhibited DNA replication in both NIH3T3 cells and TM4 sertoli cells (Fig. 2b–c).

Testes are more susceptible to mitochondrion-associated apoptotic signaling upon exposure to DEHP. Inhibition of replication may lead to cell death and increased TUNEL staining. However, the TUNEL assay cannot distinguish between apoptosis, necrosis and autolytic cell death²⁷.We therefore systematically investigated both extrinsic ('initiator' is activated caspase-8) and mitochondrionassociated intrinsic (activated caspase-9' as initiator') apoptotic pathways in testicular tissues. The liver is also DEHP-sensitive and was included in this experiment. Tissues from animals treated with DEHP at 100 mg/kg and 500 mg/kg were selected for the assay based on the histopathological results. By immunoblotting, we detected the activation of both extrinsic and intrinsic apoptotic pathways as shown by an increased cleaved form of caspase-8 (p43, C-caspase-8) and processed form p37 of caspase-9 pro-form (C-caspase-9) in both liver and testes, respectively (Fig. 3a). Consistently, the shared downstream critical executioner of apoptosis, caspase-3, was activated in DEHP-treated tissues as evidenced by cleavage of its inactive zymogen (35 kDa) into activated p17 and p19 fragments (Fig. 3a). A substrate of activated caspase-3 is poly (ADP-ribose) polymerase-1 (PARP-1) and its cleavage induces cellular disassembly and apoptosis²⁸. As expected, in DEHP-exposed rat tissues, we detected increased levels of the signature 89 kDa apoptotic fragment of PARP-1 (Fig. 3a).

The Bcl-2 family of proteins is involved in the regulation of the mitochondrion-associated intrinsic apoptosis by governing mitochondrial damage, and we therefore speculated that they may be altered after DEHP treatment. Thus, we detected the protein levels of antiapoptotic Bcl-2 and Bcl-xL as well as the pro-apoptotic Bid, Bax and Bak²⁹. Though there were no significant changes of Bcl-2 (data not shown) in the liver and testes, DEHP upregulated the protein levels of Bax, Bak and Bid (Fig. 3 a-c). Accordingly, we detected a decreased expression of Bcl-xL in the DEHP-exposed group (Fig. 3). In comparison with the testes, the liver was less sensitive to intrinsic apoptotic cell death, as evidenced by a smaller increase in pro-apoptotic Bcl-2 members (Fig. 3 b-c), e.g. in 100 mg DEHP/Kg treated rats, changes in Bcl-xL, Bid, Bak, and Bak were all statistically significant in the testes, while no statistically significant difference was found in the liver. Unexpectedly, we detected an elevated Bcl-xL in DEHP-exposed liver which may be due to a secondary protective cellular response. In conclusion, in comparison with a dominant extrinsic apoptosis in the rat liver

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Figure 1 | DEHP induced mouse testicular toxicity by acceleration of apoptosis. (a–f) Rats were treated orally with different doses of DEHP for 14 days in a row, and were sacrificed on day 15. Major organs were collected and weighed. Data are shown as mean \pm SD, n=12. Corn oil was used as negative control and flutamide (50 mg/kg) was used as positive control. (g) Immunohistochemistry on testicular tissues of different groups. Tissues were stained with H&E, and spermatogonia ("G"), spermatocytes ("C"), and spermatids ("T"), as well as sperms ("S") were visualized using light microscopy. (h) Testicular tissues were stained with TUNEL for apoptosis, and TUNEL-positive cells were shown as green dots (arrowheads). Scale bars in G–H, 500 µm. See also Table S1 and Figure S1.



Figure 2 | DEHP inhibited DNA fiber replication. (a) Schematic representation of the experiment showing cells sequentially stained with CldU (purple, 20 min) and IdU (green, 20 min), and IdU tract length was measured. (b) Effect of DEHP on IdU tract length in mouse TM4 Sertoli cells and mouse fibroblast NIH3T3 cells. Cells were treated with/without 70 μ M for 48 h before staining, and over 120 events were calculated (error bars defining the 25th and 75th percentiles). (c) A representative set of DNA fiber pictures is shown. Scale bar, 20 μ m.

under DEHP exposure, the testicular apoptosis appeared to be caused by a mitochondrion-associated apoptotic signaling.

DEHP induces mitochondrial malfunction. To further investigate the potential role of mitochondria in DEHP-induced testicular toxicity, a series of mouse testicular cell lines were examined, including TM3 Leydig cells (CRL-1714, epithelial), TM4 Sertoli cells (CRL-1715, epithelial), GC-1 spg spermatogonia cells (CRL-2053), and GC-2spd(ts) spermatocytes (CRL-2196), as well as a non-testicular cell line NIH3T3 (fibroblast). All cells were treated with 70 µM DEHP for 48 h, and ROS in both intracellular and mitochondrial levels were increased as determined by flow cytometry (Fig. 4 a-b). Notably, mitochondrial ROS production appeared to be particularly prominent as compared with intracellular ROS. Among all the five cell lines, levels of the intracellular/mitochondrial ROS in TM3 Leygid cells and NIH3T3 were among the highest, indicating that DEHP may elicit it toxicity in various cell types. To validate a time-dependent manner of DEHP-induced cellular damage, we further performed the following experiments. Mouse TM4 sertoi cells were treated with 70 µM DEHP for 12, 24, or 48 h, followed by detection of mitochondrial ROS, and cell apoptosis. As shown in Fig. S2 b-c, values of both endpoints were increased in a time-dependent fashion. In combination with the results in Fig. 1G-H, Fig 3, and Fig 4C, our results strongly support DEHP induces damage of testicular cells in a dose- and timedependent manner.

The finding that DEHP inhibited DNA replication and induced cellular oxidative stress is reminiscent of previous work suggesting that DEHP treatment may induce DNA damage^{30–32}. Synthesis and conjugation of poly(ADP-ribose) polymers (PARs) to proteins are common response to DNA damage. The enzyme that is predominantly responsible for this is PARP-1 that PARylates itself and other

proteins in a reaction that utilizes NAD⁺³³. Our immunoblot data showed that DEHP induced hyperPARylation in a dose-dependent fashion, and concomitantly decreased NAD⁺ levels in both 100 and 500 mg/kg DEHP-treated groups (Fig. 4c, e). NAD⁺ is a key factor for the deacetylation activity of the mitochondrial regulator SIRT1 and this protein may play a crucial role in male fertility^{33,34}. Since NAD⁺ consumption by hyperPARylation may impair SIRT1 activity³³, we further asked whether DEHP treatment could also reduce SIRT1 activity and cause mitochondrial malfunction. Intriguingly, DEHP decreased the SIRT1 protein level as well as blunted its deacetylation activity, as evident from increased acetylated p53 levels in DEHP- treated testes (Fig. 4c). In addition, total p53 levels were increased supporting the idea that DEHP induces DNA damage.

We next examined the changes in the ATP production machinery, oxidative phosphorylation (OXPHOS) complexes in testicular tissues. Immunoblotting showed a substantial decrease in protein levels of subunits of OXPHOS complex II, III, IV and V (Fig. 4 c–d). At the DEHP dose of 100 mg/kg, the levels were almost half of all tested subunits of each complex. Consistent with the abovementioned data showing a decreased intrinsic (mitochondrial) apoptotic pathway in DEHP-exposed rat liver tissues, we did not detect significant changes in the OXPHOS complex in liver tissues from the same groups (Fig. S2). The decreased OXPHOS expression correlated with attenuated ATP levels in testes of DEHP-treated rats at both 100 and 500 mg/kg (Fig. 4f). Taken together the data support the idea that DEHP treatment attenuates mitochondrial biogenesis by impairment of the SIRT1 deacetylation via hyperPARylation-induced overconsumption of NAD⁺.

Discussion

Through both studies of mouse cell lines and *in vivo* investigations in rats, we systematically measured the effects of DEHP and found a



Figure 3 | Differential induction of extrinsic and intrinsic/mitochondrial apoptotic pathways by DEHP in rat liver and testes. (a) Rat liver/testicular tissues of different groups were applied for immunoblotting, and expression levels of designated members in both extrinsic and mitochondrial-associated intrinsic apoptotic pathways were detected. Experiments were done in triplicate and a representative set is shown. (b–c) Quantification of expression of designated proteins in liver (b) and testes (c). Values are expressed as mean \pm SEM (n=3). All the gels were run under the same experimental conditions as detailed in the Methods section, and full-length blots were cropped for final display.





Figure 4 | DEHP exposure induces NAD⁺/SIRT1 reduction via hyperPARylation that disrupts mitochondrial function. (a–b) Intracellular (a) and mitochondrial (b) ROS levels in a list of testicular cell lines and NIH3T3 cells, after treatment with 70 μ M DEHP for 48 h (mean \pm SEM, n=3). (c) Immunoblot for OXPHOS complex, SIRT1 expression and its deacetylation activity, as well as PAR expression in rat testes of designated groups. (d) Quantification on expression of designated proteins in control and two DEHP-treated groups (mean \pm SEM, n=4). (e) Relative NAD⁺ levels in control and two DEHP-treated groups (means \pm SEM, n=4). (f) Relative ATP levels in rat testes of control and two DEHP-treated groups (mean \pm SD, n=4). For Fig. 4 (c), all the gels were run under the same experimental conditions as detailed in the Methods section, and full-length blots were cropped for final display.

previously unknown toxicological mechanism. DEHP-induced testicular atrophy appeared to be caused by inhibition of DNA replication, activation of a response to DNA damage, SIRT1 attenuation and accelerated cell death via induction of mitochondrion-associated intrinsic apoptosis.

Using the DEHP rat model established in this study, we investigated the molecular mechanism underlying its testicular toxicity, and for the first time linked the testicular atrophy with a mitochondrionassociated intrinsic apoptotic cell death and defective Sirt1 activity. Previous work has linked decreased sex organ weights with an induction of apoptosis but a detailed molecular mechanism is lacking²⁴. To gain a holistic understanding of the mechanism, we investigated changes in a series of apoptosis-associated proteins in DEHPexposed testes. As expected, DEHP induced activation of both extrinsic and intrinsic/mitochondrial apoptotic pathways. But how does DEHP initiate cell apoptosis? Strikingly, DEHP inhibited DNA replication leading to activation of a DNA damage response. Consistent with this observation previous literature has suggested that DNA damage may accumulate after DHEP treatment³⁰⁻³² Thus, we postulate that DEHP induces apoptosis at least partially by stalling of replication forks leading to DNA strand breaks. Consistent with this idea, replication inhibitors are prominently used as chemotherapeutics and an extremely common adverse effect is testicular atrophy and sterility³⁵.

Mitochondria may play a role in male reproduction. For instance, both mitochondrial DNA mutations and defective ATP production may engender fertility problems^{36,37}. Consistent with this logic, our results show that DEHP treatment leads to mitochondrial damage perhaps aggravating its testicular toxicity. The activation of apoptosis in testes by DEHP could at least partially be attributed to its induction of mitochondrial damage and increased ROS production. Interestingly, activation of DNA damage leads to SIRT1 attenuation. SIRT1 is a well-known regulator of mitochondrial function. DEHP thereby suppresses testicular ATP levels due to a lower expression of OXPHOS complex. The DEHP-induced attenuation of ATP levels may be a crucial problem to male fertility since the motility of spermatozoa depends on sperm ATP, and sperm motility in DEHP-exposed workers was found to be reduced^{38,39}.

Interestingly, the mitochondrial dysfunction in DEHP-treated cells may be ascribed to impairment of the SIRT1 deacetylation activity via overconsumption of NAD⁺ by hyperPARylation. The NAD⁺-dependent deacetylase SIRT1 is a multifaceted protein implicated in a wide spectrum of cellular processes encompassing anti-aging, anti-cancer, neuroprotection and regulation of cellular metabolism^{40,41}. Intriguingly this master regulator is also essential for animal fertility⁴². This is supported by the finding that only female Sirt1^{Y/Y} mice (harboring the point mutation H355Y that ablates its catalytic activity) are fertile, while female Sirt1^{-/-} mice, male Sirt1^{Y/Y} mice, and male *Sirt1^{-/-}* mice are all sterile³⁴. The regulation of cellular metabolism by SIRT1 is accomplished through a number of transcription factors. For example, SIRT1 expression correlates with the expression of several genes of the OXPHOS complex⁴³, and its downstream regulator PGC-1a (PPAR-gamma coactivator 1-alpha), with its activity depending on the deacetylated by SIRT1, upregulates several OXPHOS genes possibly through the transcription factors ERRa and GABPA⁴⁴. In this context it is important to notice that NAD⁺ is essential for the deacetylase activity of SIRT1. DNA damage leads to activation of PARP-1 that consumes NAD⁺ thereby attenuating the deacetylation activity of SIRT1. We also noticed a decreased protein level of SIRT1 in DEHP exposed rats, this may be due to a DNA-mediated SIRT1 expression decrease or a negative regulation by PPARγ which is a receptor of DEHP/MEHP⁴⁵. Importantly, the imbalance between PARP1 and SIRT1 is linked with several disorders, including mitochondrial diseases²⁹. Here we linked the NAD⁺/ SIRT1 reduction to its testicular atrophy in the DEHP rat model. Thus, our findings unveil the role of SIRT1 and DNA replication in



Figure 5 | Graphic abstract of a possible pathological mechanism of DEHP. A working model for DEHP to act as a DNA replication inhibitor to induce mitochondrial dysfunction and apoptosis, which may finally contribute to testicular atrophy. See manuscript for details.

DEHP-induced testicular toxicity, and highlight the importance of SIRT1 in male fertility.

In summary, we present new mechanistic insight into the pathogenesis of phthalate-induced testicular dysfunction through PARP activation (Fig. 5). Importantly, PARP1 is a druggable target with a number of inhibitors currently in clinical trials. It may therefore be possible to prevent DHEP-induced testicular atrophy by pharmacological interventions.

Methods

Materials and reagents. DEHP (#80030) and flutamide (#F9397) were purchased from Sigma-Aldrich. Primary antibodies used for immune blot were: anti-Caspase-8 (#9746), anti-Caspase-9 (#9502), anti-Caspase-3 (#9662), anti-PARP-1 (#9542), anti-Bcl-xL (#2764), anti-Bid (#2003), anti-Bax (#2772), anti-Bak (#6947), anti-acetyl p53 (Lys379, #2570) and anti-SIRT1 (#8469) antibodies from Cell signaling; Anti-p53 (#sc-71818) was from Santz Cruz; anti-actin (##ab6276) and mitoProfile total OXPHOS rodent Wb antibody cocktail (#ab110411) were from Abcam. All other reagents were from Sigma-Aldrich unless otherwise indicated.

Animals and housing environment. Male Sprague–Dawley (SD) rats (license number #SCXK(Hu)2008-0016) were purchased from SINO-BRITISH SIPPR/BK LAB Animal Ltd.(Shanghai, China). Rats were housed in an animal room under specific pathogen-free conditions (SPF), and maintained at $24 \pm 2^{\circ}$ C with a relative humidity of $50\% \pm 10\%$ and a 12:12 h light-dark cycle. Rats were fed on standard laboratory diet, with water *ad libitum*. All animal procedures had been approved by the Animal Care Committee of Shanghai Entry-Exit Inspection and Quarantine Bureau according to government guidelines for animal care.

The weanling rat assay. SD rats at postnatal days 21–35 were randomized into different groups with 12 rats/group, and treated with various doses of DEHP (20,100, 500, and 1000 mg/kg), corn oil (negative control) or flutamide (50 mg/kg, positive control) via oral gavage daily for 14 days. Body weight, clinical signs and abnormal behaviors were recorded daily during the experimental period. All animals were euthanized 24 h after the last treatment. Organs/tissues were carefully excised, trimmed free of fat and connective tissues, and weighed immediately. Specific tissues were saved for immunoblotting (flash frozen) or immunohistochemistry.

Immunohistochemistry. Immunohistochemistry was carried out as previously described²⁹. In general, the tissues were fixed in 10% neutral buffered formalin, then embedded in paraffin wax, followed by cutting into 5-µm histological sections and mounted on slides. Sections were stained with hematoxylin and eosin (H&E) or with a

terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) kit (Roche, #11684809910), and histopathological changes were examined under a light microscope or a fluorescence microscope.

Western blotting. Western blotting was performed as previously described²⁹. Briefly, rat tissues were cut into small fragments, and treated with RIPA buffer (Cell Signaling) containing protease inhibitor cocktail. Tissues were then sonicated, centrifuged, and the supernatant was collected and denatured by heating at 95°C for 5 min. Samples were loaded on SDS-PAGE gel for size fractionation, then transferred onto a PVDF membrane. Membranes were blocked with 5% blotting-grade milk for 0.5 h, incubated with primary antibodies overnight at 4°C, followed by washing with TBST, and incubated with secondary antibodies. Fluorescence was imaged and quantitative analysis of band intensities was performed using ImageJ.

Cell culture and assays for cell toxicity. The cell lines TM3 Leydig cells, and TM4 Sertoli cells from ATCC were cultured in Ham's F12 + DMEM (1:1) with 2 mM glutamine, 5% horse serum, and 2.5% foetal bovine serum (FBS). Balb/c 3T3 fibroblast cell line (clone A31) was obtained from the Cell Bank of Type Culture Collection of Chinese Academy of Science (Shanghai, China), as well as GC-1spg spermatogonia and GC-2spd(ts) spermatocytes were all maintained in DMEM medium supplemented with 10% FBS. All cells were cultured in an incubator with 5% CO₂ at 37°C. Cell toxicity of DEHP on TM3 Leydig cells was tested by MTT assay. It should be noticed that DEHP is not very soluble in the culture medium, and is often visible as an emulsion. Thus the concentration of DEHP added in cell culture was 500 μ M which resulted in a soluble concentration of approximately 70 μ M per previous report⁴⁶.

Flow cytometry. Intracellular ROS and mitochondrial ROS were measured with specific dyes⁴⁷. In detail, intracellular ROS was detected by 2',7'- dichlorodihydrofluorescein diacetate (DCFH-DA) at a concentration of 5 μ M for 30 min; and mitochondrial ROS was measured after staining cells with mitoSOX at a concentration of 3 μ M for 30 min. Fluorescence signals were finally detected FACS.

ATP and NAD⁺ levels. Cellular ATP levels were detected with an ATPliteTM Luminescence Assay kit (PerkinElmer) as per the manufacturer's instructions. In general, TM3 cells were pretreated with DEHP at the indicated concentrations for 24 h. ATP inhibitor cocktail was added to inhibit ATP consumption. Then 50 μ L of lysis buffer was added, followed by addition of substrate. Luminescence was measured in a luminescence counter (PerkinElmer). NAD+ assay was performed by using a commercial NAD/NADH assay kit (#ab65348).

DNA fiber experiment. In the DNA fiber experiments, cells were treated with 70 μ M DEHP for 48 h. Cells were then incubated in 20 μ M CldU for 20 min and then in 100 μ M IdU for 20 min. Cells were then harvested by trypsinization and washing with cold PBS. The cell pellets were mixed with lysis buffer (0.5% SDS in 200 mM Tris-HCl [pH 7.5], 50 mM EDTA) on a glass slide. After tilting for 5 min, the slides were air dried, fixed in 3 : 1 methanol/acetic acid, incubated in 2.5 M HCl for 60 min, neutralized in 0.4 M Tris-HCl (pH 7.5) buffer for 5 min, washed in PBS, and immunostained⁴⁸. Antibodies and the dilutions used were, respectively, rat anti-BrdU (CldU), 1 : 200; Dylight 649 goat anti-rat, 1 : 100; mouse anti-BrdU using a Zeiss Axiovert 200 M microscope with the Axio Vision software packages (Zeiss).

Statistics. SPSS16.0 Statistical System was used to analyze the data. All data were presented as mean \pm standard deviation (SD) or mean \pm standard error of the mean (SEM). Data were analyzed using an unpaired Student's *t* test for two groups, or one-way analysis of variance (ANOVA) for multiple groups. Differences were considered statistically significant when the *p* value was less than 0.05.

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Author contributions

X.L., E.F.F., T.B.N., H.G., designed all the experiments; X.L., E.F.F., M.S.K., H. C., L.Q., J.L., Y.H. and J.H., performed the experiments. X.L., E.F.F., M.S.K. and T.B.N. wrote the manuscript while V.A.B. provided suggestions for revision.

Additional information

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