

## Protocatechualdehyde improves antineoplastic cyclophosphamide-induced mouse testicular damage

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### ABSTRACT

**We studied the effects of protocatechualdehyde on mouse sperm survival and sperm membrane integrity *in vitro* and on cyclophosphamide-induced mouse testicular damage *in vivo*. Protocatechualdehyde (0.01, 0.1, and 1 mg/mL) improved sperm survival rate and sperm membrane integrity in separated mouse sperm (all  $P < .05$ ). In cyclophosphamide-treated male mice (60 mg/kg/d by intraperitoneal injection, 5 days of treatment), protocatechualdehyde (40 mg/kg/d by intragastric gavage, 35 days of treatment) increased the testis index, epididymis index, and sperm nuclear maturity (all  $P < .05$ ). Protocatechualdehyde also improved testis morphology characterized by orderly arranged layers of spermatogenic cells, numbers of sperm in the lumen, normal mesenchymal cells, and close and tidy arrangement of the seminiferous tubules. Protocatechualdehyde also increased testicular superoxide dismutase activity and elevated DJ-1 expression. In addition, it decreased expression of ICAM-1 and enhanced the expression of VCAM-1, PEDF, VEGF, and PPAR $\gamma$ . These findings indicate that protocatechualdehyde increases mouse sperm survival and sperm membrane integrity *in vitro* and reduces cyclophosphamide-induced mouse testicular damage via DJ-1 and other targets *in vivo*. Protocatechualdehyde may thus be useful for treating reproductive damage and antineoplastic cyclophosphamide-induced reproductive toxicity.**

### INTRODUCTION

Male infertility is increasing as a result of various causes, such as environmental exposure, genetic predisposition, and lifestyle changes [1–3]. Drugs such as cyclophosphamide (CYP) can also contribute to male infertility. CYP is an extensively used anticancer drug and immunosuppressive agent. Male cancer patients treated with CYP have been reported to show a higher incidence of oligospermia and azoospermia [4, 5]. CYP is also used to produce male reproductive damage in animal models [6, 7].

Although L-carnitine can improve energy metabolism of sperm, no clearly effective drugs currently exist to treat male reproductive damage [8]. In China, doctors prescribe traditional Chinese medicine (TCM) to treat male reproductive damage with good results [9]. However, to date, no monomer compound rooted in TCM has been used to treat male reproductive damage.

Protocatechualdehyde (PAL; Figure 1), a phenolic acid compound, exists in many components of TCM, such as the leaves of *Stenoloma chusanum* (L.) Ching, *Ilex chinensis* Sims, and the roots of *Salvia miltiorrhiza*. PAL has demonstrated free radical scavenging activity

[10, 11] and antioxidant effect [12–14]. PAL has also been reported to have an antitumor effect [10, 15, 16], by inducing apoptosis and DNA damage of tumor cells, and a neuroprotective effect [17]. In addition, PAL has shown anti-inflammatory activity *in vitro* [11] and has inhibited ovalbumin-induced airway inflammation [18] and cisplatin-induced renal inflammation [13]. PAL also inhibits migration and proliferation of vascular smooth muscle cells and intravascular thrombosis [19] and protects against endothelial dysfunction [20]. However, thus far, the effects of PAL on male reproductive damage and antineoplastic CYP-induced reproductive toxicity have not been confirmed. Therefore, we investigated the effects of PAL on mouse sperm survival *in vitro* and CYP-induced mouse testicular damage.

## RESULTS

### PAL improved mouse sperm survival rate and total swelling rate *in vitro*

Sperm survival rate and total swelling rate were higher in animals treated with PAL *in vitro* (0.01, 0.1, and 1 mg/mL) than in animals treated with normal saline (control group) (all  $P < .05$ ). The elevated total swelling rate indicates the enhanced integrity of the sperm membrane (Table 1).

### PAL increased mouse testis index, epididymis index, and sperm nuclear maturity

CYP (60 mg/kg) treatment reduced the testis index, epididymis index, and sperm nuclear maturity (all  $P < .01$ ; Table 2), but PAL (40 mg/kg) increased the testis index ( $P < .05$ ), epididymis index ( $P < .05$ ), and sperm nuclear maturity ( $P < .01$ ).

### PAL improved the morphology of mouse testis

As shown in Figure 2, testicular morphology in the control group exhibited normal features: layers of spermatogenic cells were orderly arranged, the lumen

was filled with many sperm, the mesenchymal cells were normal, and arrangement of the seminiferous tubules was close and tidy. By contrast, the CYP-treated group showed disordered arrangements and decreased layers of spermatogenic cells, reduced sperm counts and thinned epithelium in the lumen, and increased inside diameter and augmented internals of the seminiferous tubules. Compared to the group treated with CYP alone, layers of spermatogenic cells and sperm numbers in the lumen were improved in the 10 mg/kg PAL group. The morphologic features in the 40 mg/kg PAL group were nearly normal, with orderly arranged layers of spermatogenic cells, normal numbers of sperm in the lumen, normal mesenchymal cells, and close and tidy arrangement of the seminiferous tubules.

### PAL increased superoxide dismutase (SOD) activity in mouse testis

Testicular SOD activity was lower in the CYP group than the control group ( $P < .01$ , Table 2). However, administration of 40 mg/kg PAL to CYP-treated animals significantly increased testicular SOD activity ( $P < .05$ , Table 2).

### PAL increased the expression of DJ-1 in mouse testis

As shown in Figure 3, testicular DJ-1 expression was lower in the CYP group than the control group ( $P < 0.01$ ). However, administration of 40 mg/kg PAL to CYP-treated animals significantly increased testicular DJ-1 levels ( $P < .01$ ).

### PAL decreased the expression of ICAM-1 and enhanced the expression of VCAM-1, PEDF, VEGF, and PPAR $\gamma$ in mouse testis

Figure 4 shows that testicular expression of ICAM-1 was greater in the CYP group than the control group ( $P < .01$ ), whereas it was decreased by administration of 40 mg/kg PAL to CYP-treated animals ( $P < .01$ ). CYP inhibited the expression of VCAM-1, PEDF, and PPAR $\gamma$  in

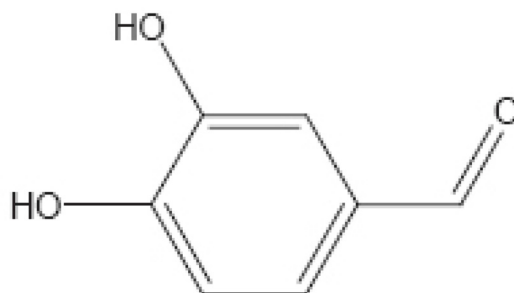


Figure 1: Chemical structure of PAL.

**Table 1: PAL (0.01, 0.1, and 1 mg/mL) significantly improved mouse sperm survival rate and sperm membrane integrity *in vitro***

| Group          | Sperm survival rate (%)         | Total swelling rate (%)          |
|----------------|---------------------------------|----------------------------------|
| Control        | 21.58 ± 5.98                    | 20.90 ± 3.38                     |
| PAL 0.01 mg/mL | 32.40 ± 1.40 <sup>#</sup>       | 28.12 ± 0.78 <sup>#</sup>        |
| PAL 0.1 mg/mL  | 38.23 ± 4.85 <sup>##</sup>      | 31.51 ± 2.51 <sup>##,*</sup>     |
| PAL 1 mg/mL    | 46.23 ± 3.47 <sup>##,**,△</sup> | 44.17 ± 7.95 <sup>##,**,△△</sup> |

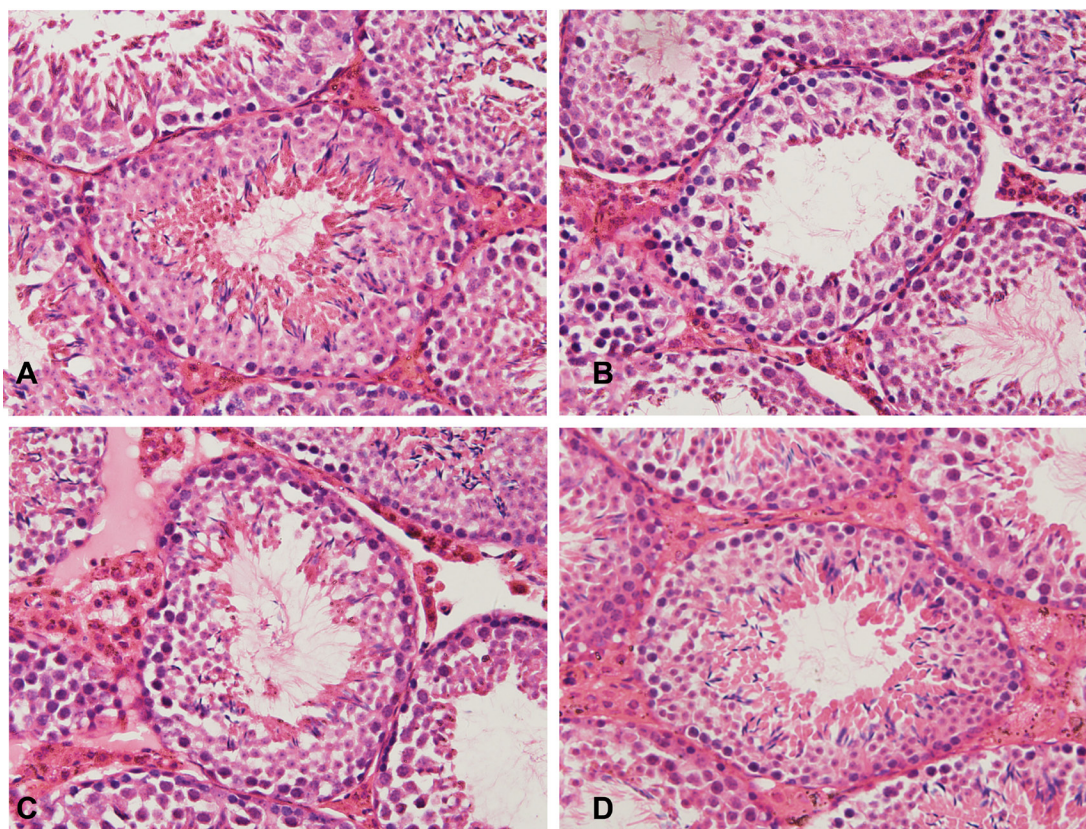
Data are expressed as means ± standard deviations ( $n = 6$ ). <sup>#</sup> $P < .05$ , <sup>##</sup> $P < .01$  versus control group. <sup>\*</sup> $P < .05$ , <sup>\*\*</sup> $P < .01$  versus PAL 0.01 mg/mL group. <sup>△</sup> $P < .05$ , <sup>△△</sup> $P < .01$  versus PAL 0.1 mg/mL group.

testis, whereas 40 mg/kg of PAL promoted the expression of VCAM-1, PEDF, and PPAR $\gamma$  (Figures 4, 5, and 6). Testicular VEGF expression was lower in the CYP group than the control group, but was increased by administration of 10 mg/kg and 40 mg/kg of PAL (Figure 5).

## DISCUSSION

In recent years, drugs made from natural sources have attracted increased attention for their potential use in disease [21, 22]. The ability of these products to relieve

treatment-related side effects has also been investigated [22]. In this study, the natural plant-derived product PAL was investigated. The results of *in vitro* study indicate that PAL improves the mouse sperm survival rate by eosin-Y staining and the total swelling rate by the hypo-osmotic swelling test (HOST). HOST was initially introduced as an assay to assess the functional integrity of the sperm membrane [23]. Now, HOST is also considered as an easy, inexpensive, and reliable test for predicting male fertility potential [24, 25] and can help with the sperm selection for intracytoplasmic sperm



**Figure 2: PAL improved testis morphology in CYP-treated male mice.** The results were representatives of three independent replications. The images were captured using an IX83 microscope (400 $\times$ ). (A) Control group. (B) Model group. (C) 10 mg/kg PAL group. (D) 40 mg/kg PAL group.

**Table 2: PAL (40 mg/kg) increased testis index, epididymis index, sperm nuclear maturity, and testicular SOD activity in CYP-treated male mice**

| Group        | Testis index (mg/g)         | Epididymis index (mg/g)     | Sperm nuclear maturity (%)                 | Testicular SOD activity (U/mg protein) |
|--------------|-----------------------------|-----------------------------|--|--|
| Control      | 3.573 ± 0.461               | 0.625 ± 0.179               | 87.17 ± 3.73                               | 5.099 ± 0.723                          |
| Model        | 2.701 ± 0.435 <sup>##</sup> | 0.510 ± 0.053 <sup>##</sup> | 30.14 ± 12.68 <sup>##</sup>                | 2.075 ± 1.406 <sup>##</sup>            |
| 10 mg/kg PAL | 2.637 ± 0.646               | 0.443 ± 0.074               | 25.94 ± 6.61                               | 3.750 ± 1.841                          |
| 40 mg/kg PAL | 3.282 ± 0.59 <sup>*</sup>   | 0.613 ± 0.143 <sup>*</sup>  | 71.06 ± 3.94 <sup>**</sup> , <sup>△△</sup> | 5.591 ± 2.580 <sup>*</sup>             |

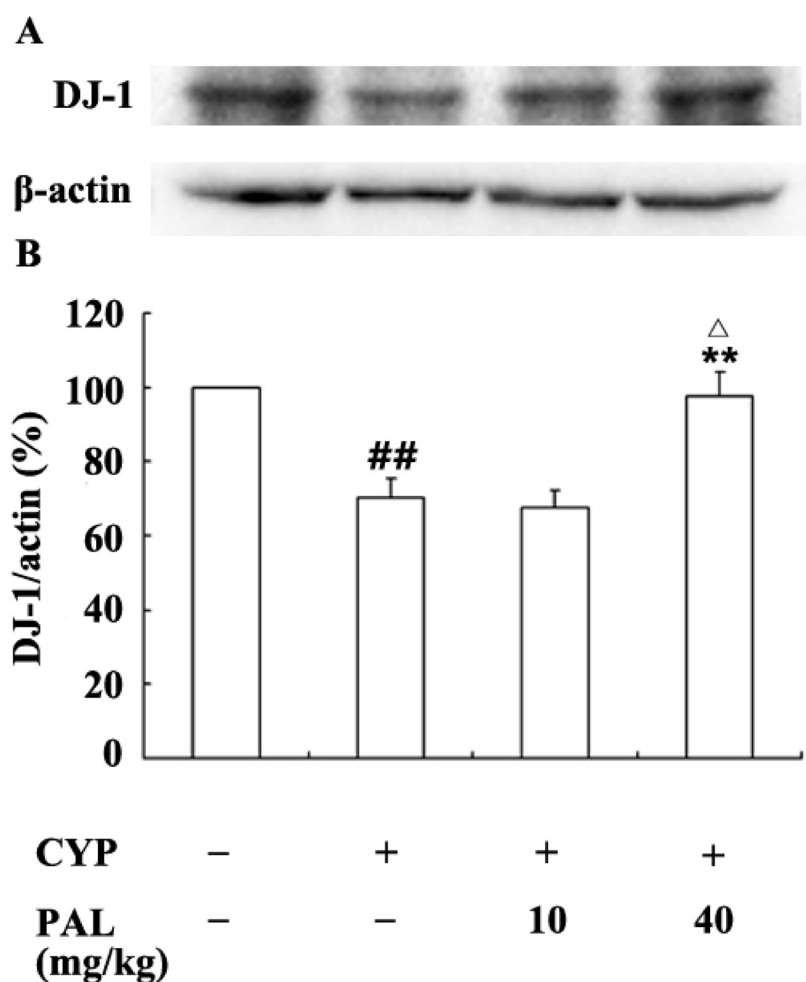
Data are expressed as means ± standard deviations (n = 12). <sup>##</sup>P < .01 versus control mice. <sup>\*</sup>P < .05, <sup>\*\*</sup>P < .01 versus CYP only mice. <sup>△△</sup>P < 0.01 versus 10 mg/kg PAL mice.

injection [26]. PAL increased total the swelling rate by HOST, which indicates that PAL protects mouse sperm membrane integrity and functional activity. The results of *in vitro* study prompted us to explore the effects of PAL on testicular damage *in vivo*.

In CYP-treated mice, PAL significantly increased the testis index and epididymis index, which indicates

that PAL decreased CYP-induced damage to testis and epididymis. Hematoxylin and eosin staining of testis further proved the protective effect of PAL against CYP-induced testicular damage, since the testis of PAL-treated mice showed normal features.

During spermatogenesis, most of the sperm DNA-binding protein histones are replaced by transition proteins

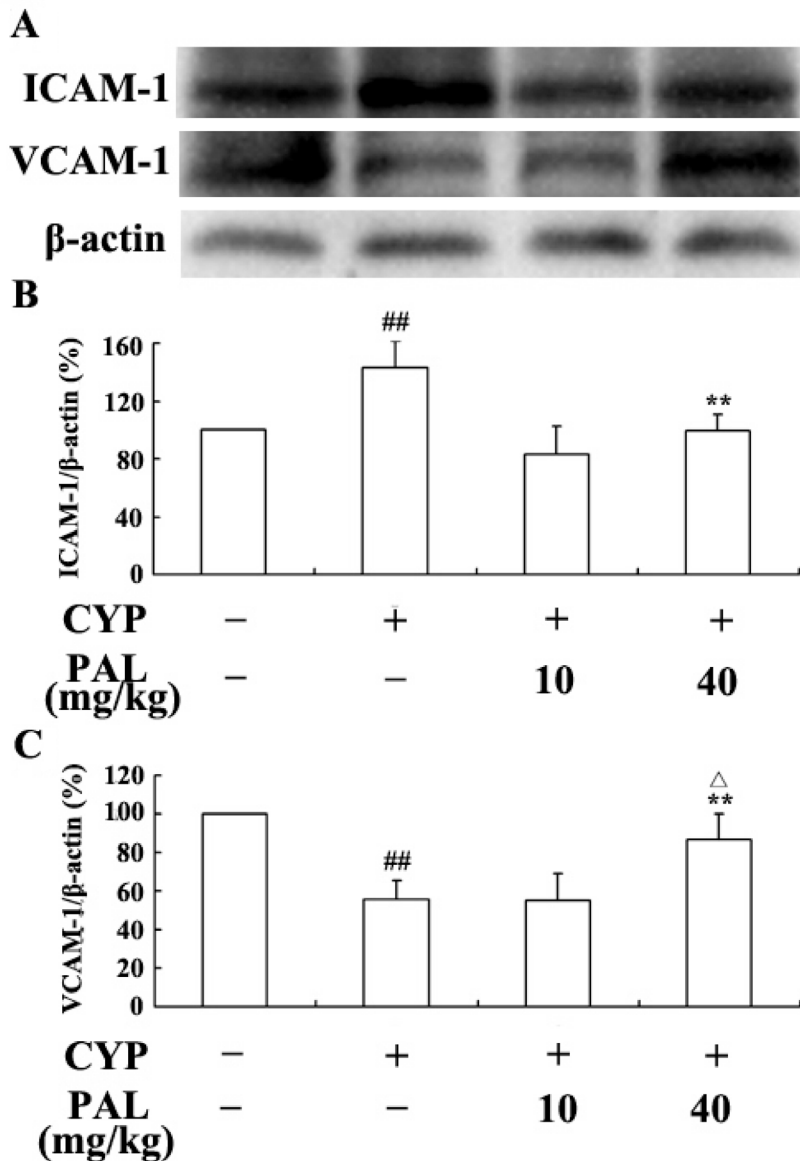


**Figure 3: PAL increased DJ-1 expression of testis in CYP-treated male mice by Western blotting.** The images are representatives of three independent replications. Data are expressed as means ± standard deviations (SD). <sup>##</sup>P < .01 versus control mice. <sup>\*\*</sup>P < .01 versus CYP only mice. <sup>△</sup>P < .05 versus 10 mg/kg PAL mice.

and then by protamines. Protamines cause the chromatin to condense tightly and thus generate mature sperm. Aniline blue (AB) preferentially binds to histone lysines; thus, AB staining can assess sperm nuclear maturity [27]. In our study, CYP dramatically increased the percentage of immature sperm. Immature sperm produce oxygen radicals, which can induce sperm death and reduce male fertility [28, 29]. Conversely, the correctly condensed chromatin of mature sperm can protect against CYP-induced oxidative damage to sperm DNA. In our study, PAL increased the percentage of mature sperm. Thus, our data indicate that PAL may promote the fertility of CYP-treated mice through increase in sperm maturity.

CYP reportedly causes male reproductive toxicity through generation of reactive oxygen species (ROS)

[30–32]. When ROS exceed the antioxidants in the body, oxidative stress takes place. Oxidative stress is a common pathology seen in approximately half of all infertile men [33]. Accumulated free radicals affect the activity of steroidogenic enzymes, produce lipid peroxidation, and thereby induce spermatogenic damage [34]. PAL has been demonstrated to have an antioxidant effect [10, 11, 14], which can protect against CYP-induced testicular damage. In addition, our study showed that the activity of SOD, an enzyme that cleans up superoxide anion radicals and other oxyradicals in the body, was inhibited by CYP in the testes of male mice, whereas PAL improved testicular SOD activity in CYP-treated mice. This result indicates that PAL antagonizes CYP-induced oxidative stress damage through increased testicular SOD activity.

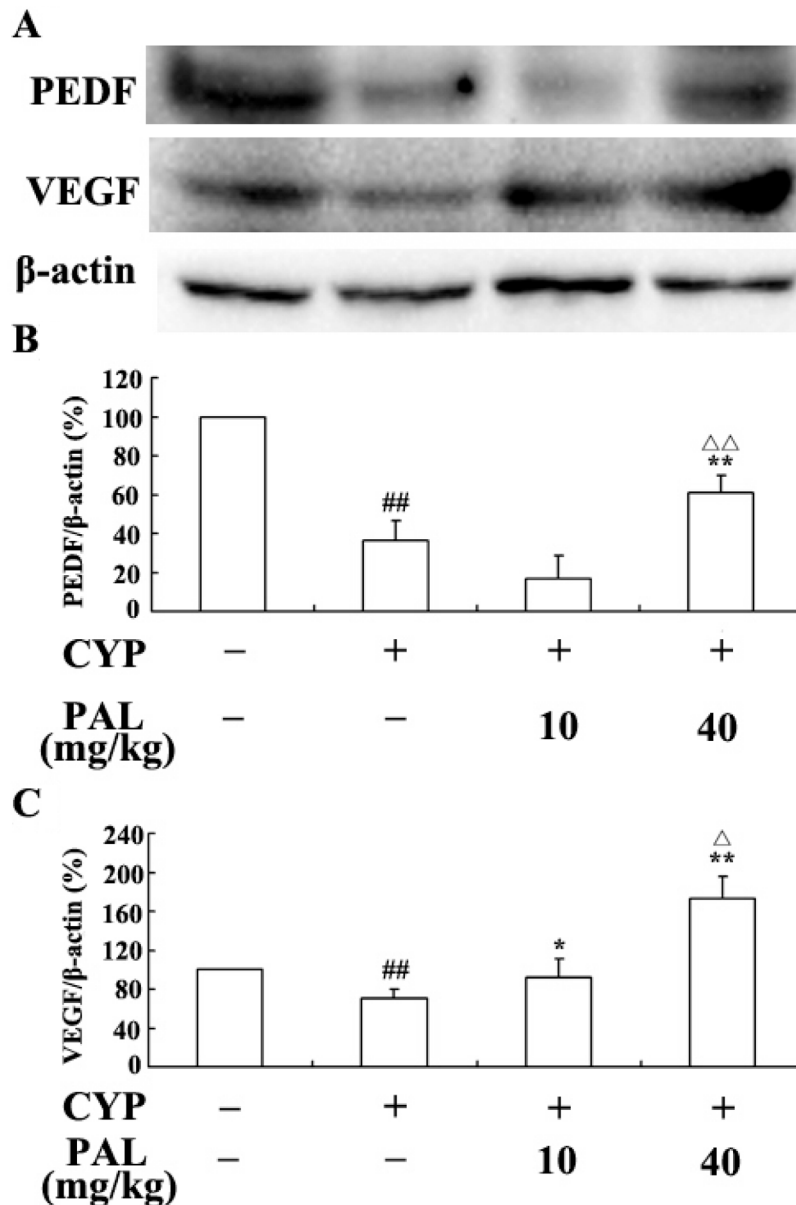


**Figure 4: PAL reduced ICAM-1 expression and enhanced VCAM-1 expression of testis in CYP-treated male mice by Western blotting.** The images are representatives of three independent replications. Data are expressed as means ± standard deviations (SD). <sup>##</sup>*P* < .01 versus control mice. <sup>\*\*</sup>*P* < .01 versus CYP only mice. <sup>△</sup>*P* < .05 versus 10 mg/kg PAL mice.

DJ-1 is reportedly related to infertility in male rodents exposed to toxicants [35–38]. In previous work, we showed for the first time that sperm DJ-1 levels in asthenozoospermia patients are lower than those in control subjects and that sperm DJ-1 levels are positively correlated with sperm motility [39]. These results indicate that DJ-1 is a key biomarker of spermatogenic damage. Because DJ-1 possesses anti-oxidative stress activity [40–42] and CYP induces reproductive damage through oxidative stress and because PAL binds with DJ-1 *in vitro* and has a protective effect in SH-SY5Y cells through DJ-1 [12], we explored whether DJ-1 is a target of PAL in CYP-treated male mice. We showed that CYP visibly suppresses the expression of DJ-1 in

testis of male mice, whereas PAL elevates the expression of testicular DJ-1 in CYP-treated male mice. As a result of these findings and the increase in SOD activity in testis induced by PAL in this study, we deduce that the protective effects of PAL against the testicular damage induced by CYP in male mice are mediated by DJ-1 through its anti-oxidative stress effect.

ICAM-1 was shown to be expressed in testis [43, 44] as a component of the blood-testis barrier (BTB) and can promote BTB integrity [44], and ICAM-1 expression is upregulated during cell stress [10]. In this study, the expression of ICAM-1 in testis of CYP-treated male mice was upregulated, which was possibly triggered by oxidative stress caused by CYP. PAL downregulated



**Figure 5: PAL increased PEDF and VEGF expression of testis in CYP-treated male mice by Western blotting.** The images are representatives of three independent replications. Data are expressed as means  $\pm$  standard deviations (SD). <sup>##</sup> $P < .01$  versus control mice. <sup>\*</sup> $P < .05$ , <sup>\*\*</sup> $P < .01$  versus CYP only mice.  <sup>$\Delta\Delta$</sup>  $P < .05$ ,  <sup>$\Delta\Delta\Delta$</sup>  $P < .01$  versus 10 mg/kg PAL mice.

ICAM-1 expression through an anti-oxidative stress effect, which may be one of the mechanisms by which PAL protects against testicular damage. However, the exact mechanism by which the upregulation and downregulation of ICAM-1 occurred should be further clarified. VCAM-1 is expressed in Leydig and Sertoli cells and promotes cell binding to lymphocytes [45, 46]. In cultured Sertoli cells, inflammatory cytokines such as TNF- $\alpha$ , IL-1 $\alpha$ , and IL-1 $\beta$  increase VCAM-1 expression [44, 47]. In our study, CYP reduced testicular VCAM-1 expression possibly due to the impairment of testis. Thus, PAL may increase testicular VCAM-1 expression by protecting the testis.

PEDF is localized in human testicular biopsy tissue, particularly the extracellular matrix and the cells of the tubular wall [48]. PEDF has been reported to be responsible for the development and maintenance of the avascular nature of seminiferous tubules, which are important for orderly spermatogenesis [48]. Our data show that PAL increased CYP-induced downregulation of testicular PEDF, which indicates that PAL contributes to the avascular nature of seminiferous tubules and spermatogenesis.

VEGF is expressed in testis [49] and increases testicular endothelial cell proliferation [50, 51]. In addition, VEGF-mediated endothelial migration is required for testis morphogenesis [52]. VEGFA isoforms are necessary for maintenance of undifferentiated spermatogonia and normal male fertility [53]. Our study

shows that PAL increases CYP-inhibited testicular VEGF expression, which may improve spermatogenesis and male fertility.

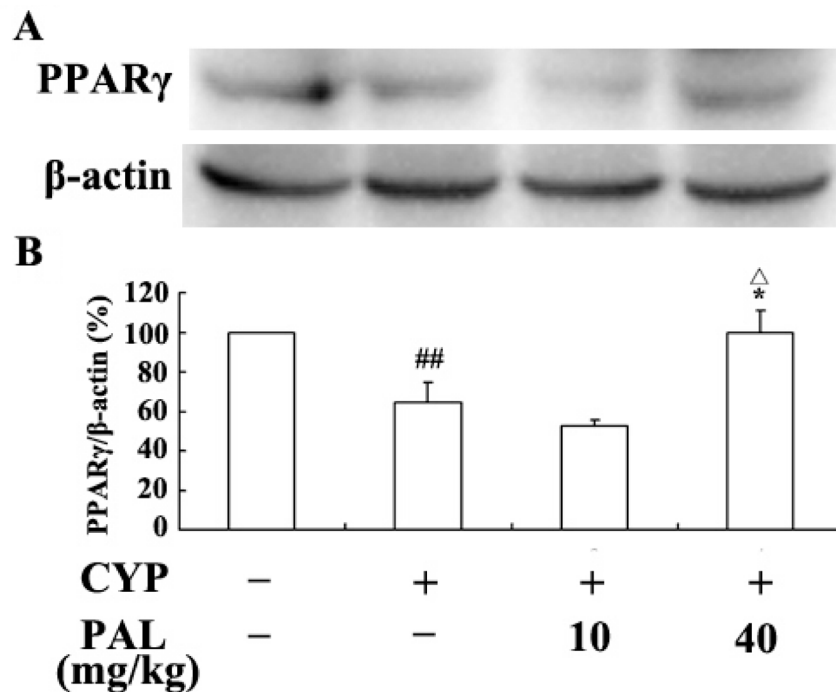
PPAR $\gamma$  is expressed in Sertoli cells [54]. It provides enough energy to support spermatogenesis by mediating lipid metabolism [55] and thus is associated with male fertility. In our research, reduced testicular PPAR $\gamma$  in CYP-treated mice implied destroyed spermatogenesis and reduced fertility; however, PAL normalized spermatogenesis and increased male fertility by increasing levels of PPAR $\gamma$ .

In summary, we demonstrate that PAL improves sperm survival and sperm membrane integrity *in vitro* and CYP-induced mouse testicular damage. PAL should be investigated as a potential drug to treat reproductive damage and antineoplastic CYP-induced reproductive toxicity.

## MATERIALS AND METHODS

### Drugs and chemicals

PAL (No. LC70N14, purity >98%) was purchased from J&K Scientific LTD. CYP (No. 13052125) was purchased from Jiang Su Heng Rui Medicine, Co., Ltd., China. Eosin-Y (No. 0409A15) and aniline blue (AB, No. 0409A15) were purchased from Beijing Leagene Biotech, Co., Ltd.



**Figure 6: PAL increased PPAR $\gamma$  expression of testis in CYP-treated male mice by Western blotting.** The images are representatives of three independent replications. Data are expressed as means  $\pm$  standard deviations (SD). <sup>##</sup> $P < .01$  versus control mice. <sup>\*</sup> $P < .05$  versus CYP only mice.  <sup>$\Delta$</sup>  $P < .05$  versus 10 mg/kg PAL mice.

## Animals

Male mice 6 to 7 weeks old (species: Kun-Ming; strain: Swiss) and weighing  $35.0 \pm 2.0$  g were purchased from the Experimental Animal Center, North China University of Science and Technology (Certificate No. SCXK [Ji] 2010-0038). They were housed at a constant temperature ( $22^{\circ}\text{C} \pm 1^{\circ}\text{C}$ ) and constant humidity ( $55\% \pm 10\%$ ), under 12-hour light/12-hour dark conditions with ad libitum access to food and water. All animal experiments were performed in accordance with relevant guidelines and regulations approved by the Institutional Review Board and Experimental Animal Research Committee of North China University of Science and Technology (No. 15012 and 201515, respectively).

## Sperm survival rate assay

Separated bilateral epididymides from normal male mouse were put into normal saline at  $37^{\circ}\text{C}$ , washed clean, and then placed in 1.5 mL of normal saline and cut into pieces. After sperm dissociated, the supernatants were filtrated with four-layer paper filters [56]. Sperm from six mice were mixed, counted, and matched to a suspension with a concentration of  $2 \times 10^7/\text{mL}$  and placed at  $37^{\circ}\text{C}$ . The sperm suspensions were equally divided into four groups and treated with normal saline or 0.01, 0.1, or 1 mg/mL of PAL at  $37^{\circ}\text{C}$  for 30 minutes. One drip of sperm suspension after treatment and one drip of eosin-Y solution were blended, dropped on slides, and covered. After laying for 30 to 60 seconds, the slides were observed under an IX83 microscope ( $400\times$ ; Olympus). The heads of dead sperm were stained pink, whereas the heads of live sperm were not stained. Survival rate was calculated in all 200 sperm. A total of 36 mice were used.

## Hypo-osmotic swelling test

The mouse sperm suspensions were prepared as described above and equally divided into four groups that were treated with normal saline or 0.01, 0.1, or 1 mg/mL of PAL. The hypotonic solution was prepared as originally reported and contained fructose and sodium citrate with osmotic pressure of 150 mOsm and ionic strength of 0.15 [23]. All groups were treated with the hypotonic solution at  $37^{\circ}\text{C}$  for 30 minutes [23]. The precipitates were absorbed and dropped on slides. The slides were covered and observed under an IX83 microscope ( $400\times$ ). Tail-changed sperm were swelling sperm. Total swelling rate was calculated in all 200 sperm. In total, 36 mice were used.

## *In vivo* treatments

Forty-eight mice were randomly divided into four groups equally: control group, CYP only group, 10 mg/kg PAL group, and 40 mg/kg PAL group. The groups

were treated daily with normal saline (control group) or CYP 60 mg/kg [57] (CYP only group and 10 and 40 mg/kg PAL groups) by intraperitoneal injection for 5 days. At the same time, the control and CYP only groups were treated daily with normal saline, and the PAL groups were treated with 10 or 40 mg/kg of PAL by intragastric gavage for 35 days. The doses of PAL were determined by primary experiments. Twenty-four hours after the last treatment, mice were weighed and then sacrificed by spine dislocation, and the bilateral testes and epididymides were removed and weighed. Testis index and epididymis index of the mice were determined using the ratio of testis or epididymis weight to body weight (unit: mg/g).

## Sperm chromatin condensation evaluation by AB staining

The separated mouse bilateral epididymides were put into normal saline at  $37^{\circ}\text{C}$ , washed clean, and then placed in 1.5 mL of normal saline and cut in grain. After incubating for 3 to 5 minutes, the suspensions were filtrated using four-layer paper filters [56]. The filtrates were smeared. After being dried and fixed, the smears were stained with 5% AB solution diluted in 4% acetic acid (approximate pH, 3.5) [58, 59] and observed under an IX83 microscope ( $400\times$ ). The heads of mature sperm were stained pale, whereas heads of immature sperm were stained navy blue. The percentage of mature sperm was calculated in 200 sperm.

## Hematoxylin and eosin (HE) staining

The separated mouse testes were put into normal saline, washed clean, fixed in 10% neutral formalin, and then embedded with ceresin wax and continually cut into slices. The sections were stained by HE staining and observed under an IX83 microscope ( $400\times$ ).

## SOD activity assay

The separated mouse testes were put into normal saline, washed clean, and then placed in normal saline and cut in grain. After being made into homogenates, the testes suspensions were centrifuged at 3000 rpm,  $4^{\circ}\text{C}$  for 10 minutes. The supernatants were used to determine SOD activity according to the instructions of an SOD assay kit (No. A001-3; Nanjing Jiancheng Bioengineering Institute, China) by spectrophotometry.

## Western blotting

The separated mouse testes were put into normal saline, washed clean, cut in grain, and then placed in RIPA buffer. After being made into homogenates, the testes suspensions were centrifuged at 12,000 rpm,  $4^{\circ}\text{C}$  for 10 minutes. The supernatants were used as a total protein extracts. Total protein concentration of the lysates was

determined using a BCA protein assay reagent kit (Pierce). Equal amounts of protein extract were subjected to a 12.5% SDS-polyacrylamide electrophoresis gel and transferred to a PVDF membrane (Millipore) for 45 minutes at 250 mA. The PVDF membrane was blocked with 5% nonfat dry milk in TBST solution for 3 hours at room temperature under agitation and then processed for immunodetection. Anti-DJ-1, anti-ICAM-1, anti-VCAM-1, anti-PEDF, anti-VEGF, and anti-PPAR $\gamma$  polyclonal antibody (all 1:500, Santa Cruz) were used as the primary antibody, and HRP-conjugated IgG was used as the secondary antibody.  $\beta$ -Actin was used as an internal standard. An enhanced chemiluminescence detection system was applied to detect the target proteins. For quantification of protein expression levels, the optical density of each band was quantified by Quantity One software (Bio-Rad). Levels of protein were normalized against  $\beta$ -actin and expressed as fold change relative to the control.

### Statistical analysis

Data are expressed as mean  $\pm$  standard deviation. Statistical analysis was performed using Student's *t*-test or  $\chi^2$  test by SPSS 22.0 software, and differences were considered to be statistically significant when  $P < .05$ .

### Author contributions

C.-N.A. carried out all the experiments. X.-P.P. revised the manuscript. Q.-P.Y. and J.-T.D. helped to perform the experiments *in vitro*. S.-Y.H. supplied some antibodies. R.W. helped with Western blotting. H.-N.Z. designed the project, prepared the tables and figures, and wrote the manuscript.

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### CONFLICTS OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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