Asian Journal of Andrology (2016) 18, 627–632

www.asiaandro.com; www.ajandrology.com

Open Access

ORIGINAL ARTICLE

Male Fertility

Potential role of punicalagin against oxidative stress induced testicular damage

Faiza Rao[1,2], Hui Tian[1,2], Wenqing Li[1,2], Helong Hung[1,2], Fei Sun[1,2]

Punicalagin is isolated from pomegranate and widely used for the treatment of different diseases in Chinese traditional medicine. This study aimed to evaluate the effect of Punicalagin (purity ≥98%) on oxidative stress induced testicular damage and its effect on fertility. We detected the antioxidant potential of punicalagin in lipopolysaccharide (LPS) induced oxidative stress damage in testes, also tried to uncover the boosting fertility effect of Punicalagin (PU) against oxidative stress-induced infertility. Results demonstrated that 9 mg kg^{-1} for 7 days treatment significantly decreases LPS induced oxidative damage in testes and nitric oxide production. The administration of oxidative stress resulted in a significant reduction in testes antioxidants GSH, T-SOD, and CAT raised LPO, but treatment with punicalagin for 7 days increased antioxidant defense GSH, T-SOD, and CAT by the end of the experiment and reduced LPO level as well. PU also significantly activates Nrf2, which is involved in regulation of antioxidant defense systems. Hence, the present research categorically elucidates the protective effect of punicalagin against LPS induced oxidative stress induced perturbation in the process of spermatogenesis and significantly increased sperm health and number. Moreover, fertility success significantly decreased in LPS-injected mice compared to controls. Mice injected with LPS had fertility indices of 12.5%, while others treated with a combination of PU + LPS exhibited 75% indices. By promoting fertility and eliminating oxidative stress and inflammation, PU may be a useful nutrient for the treatment of infertility.

Asian Journal of Andrology (2016) **18**, 627–632; doi: 10.4103/1008-682X.168792; published online: 12 January 2016

Keywords: fertility; oxidative stress; punicalagin; sperm parameters; testicular damage

INTRODUCTION

Infertility is a critical issue, influencing people psychosocially and medically. Approximately, 15% of couples undergo different forms of infertility, and 50% of these can be attributed to male factors. Oxidative stress has been identified as an important factor that influences male fertility status.[1–3] Oxidative stress is as an imbalance between reactive oxygen species (ROS) and antioxidants in the body. Some common factors that cause oxidative stress in testes are diabetes, cryptorchidism, radiation, and testicular torsion. Testicular infection also induces Oxidative stress that might have permanent effects on male fertility.[4,5]

Administration of lipopolysaccharide (LPS) *in vivo* can cause infection and in animals it has been observed in spermatogenesis disruption. Previous work proves that LPS injecting i.p. in mice cause sickness. The sickness depends on the dose and time of LPS injected in mice. Although sickness is due to the effect of LPS on the immune system that further leads to oxidative stress in mice testes.[4–6] A lot of studies can be found regarding LPS induced oxidative stress damage in testis, explaining how LPS induced oxidative stress in mice testes and damaged germ cells, Leydig cells, and reduced testosterone level.[7,9] It has been reported that administration of LPS leads to disruption of blood-testis barrier, ROS production and activation of nitric oxide (NO) synthase.[10]

Punica granatum belongs to family *Punicaceae* and is commonly known as pomegranate.[11] *Punica granatum* has been used extensively as traditional medicine in many countries[12] for the treatment of dysentery, diarrhea, acidosis, hemorrhage, and respiratory pathologies.[13,14] Pomegranate contains a number of polyphenol, including anthocyanins, minor flavonoids, and punicalagin, which is the most important member of ellagitannins family. The studies conducted before used pomegranate juice and punicalagin with a combination of other polyphenols.

Punicalagin (PU) is the largest polyphenol among the pomegranate ellagitannins, and it is responsible for most of the antioxidant activity.[15] Punicalagin with other polyphenols was found to be a potent antioxidant in protecting nitric oxide than grape juice, blueberry juice, red wine, Vitamin C, and Vitamin E.[16]

Very few studies can be found about the usage of punicalagin against oxidative stress induced testicular damage leading to infertility.[17,18] It has already been demonstrated that treatment of mice with Pomegranate juice for several days can prevent oxidative stress induced damage in testes.[8] However, whether punicalagin (PU, purity ≥98%) can also alone alleviate LPS induced oxidative stress damage in testis is unknown.

In this study, oxidative stress was induced in the testes by intraperitoneal injection of bacterial lipopolysaccharide (LPS), which resulted in disruption of spermatogenesis. We focused on the possible role of punicalagin in oxidative stress-induced damage and infertility in mice testes.

[1]Institute of Immunology and CAS Key Laboratory of Innate Immunity and Chronic Disease, Innovation Center for Cell Biology, School of Life Sciences and Medical Center, University of Science and Technology of China, Hefei, 230027, China; [2]Department of Life Science, Hefei National Laboratory for Physical Sciences at Microscale, Hefei, 230027, China.
Correspondence: Dr. F Sun (feisun@ustc.edu.cn)
Received: 13 March 2015; Revised: 02 May 2015; Accepted: 16 September 2015

MATERIALS AND METHODS

Chemicals

Reverse Transcription System kit was purchased from Takara Biotechnology; GSH (Glutathione) and LPO (Lipid peroxidation) T-SOD and CAT detecting kits were purchased from Nanjing Jiancheng Bioengineering; PCR (polymerase chain reaction) primers were synthesized by Sangon Biotech; PU (punicalagin), and LPS were purchased from Sigma Aldrich (St Louis, MO, USA) and TRIzol from Life Science Products (Invitrogen, Carlsbad, CA, USA).

Ethics statement

All animal-based examinations were designed and performed with the recommendations in the Guide for the Care and Use of Laboratory Animals of National Institutes of Health. This study received ethical approval from Institutional Review Boards of University of Science and Technology of China (USTC).

Animals

Thirty-two adult male ICR mice (*Mus musculus*) weighing between 25 and 28 g were used for this study. Mice were obtained from the animal house of University of Science and Technology of China (USTC). Mice were kept in plastic cages; about four mice in each cage. Soft crushed wood shaving was used to cover the floor of all cages. The cages were kept clean, and mice were allowed an *ad libitum* approach to food and water. Mice were managed under standard laboratory conditions (22–24°C, 12-h light/12-h dark cycle).

Experimental design

Mice were randomized and divided into four groups (8 ICR mice in each group) as follows:

- **Group I** (control group): provided with water and fed with normal diet
- **Group II** (punicalagin group): the animals received punicalagin (9 mg kg^{-1} day^{-1}) orally by gavage daily for 7 days
- **Group III** (LPS group): the animals were injected with LPS (600 μg kg^{-1} i.p. daily) for 7 days
- **Group IV** (punicalagin + LPS co-administrated): the animals received punicalagin (9 mg kg^{-1} day^{-1}) orally by gavage concurrently injected with LPS (600 μg kg^{-1} i.p. daily) for 7 days. LPS and punicalagin doses were prepared in water. On the last day of treatment, male mice were exposed to female mice for 4 days. After 4 days of exposure to females, male mice were sacrificed under light anesthesia, then rapidly dissected and subjected to the following examinations.

LPS and PU doses were randomly chosen and on the basis of oxidative stress we chose the present doses. We also analyzed the toxicity effect of PU on different organs (data not mentioned in this paper) and selected this dose to be effective against oxidative stress damage in the testes.

Histological examination

Testes were removed and weighed with a digital balance. Small pieces of testes were fixed in Bouin's solution and 70% ethanol, were taken and dehydrated in graded ethanol, embedded in paraffin, and sectioned (5 μm thicknesses). The sections were stained with hematoxylin-eosin stain and observed under light microscope.

Biochemical analyses

Oxidative stress assessment

Lipid peroxidation estimation

The levels of lipid peroxidation in the testes homogenate were measured using a Lipid Peroxidation (LPO) Assay Kit (NJJCBio). The testes were first homogenized on ice using 0.2 g tissue in 1.8 ml physiological saline, centrifuged at 2500 rpm for 10 min. The supernatant was collected for the further process according to the prescription in the kit. 200 μl was collected to 96 orifice plate to detect OD at 586 nm.

Antioxidant status evaluation

The levels of GSH in the testes homogenate were measured using a Glutathione (GSH) Assay Kit (NJJCBio). The testes were first homogenized on ice in 9 ml of normal saline per gram tissue, centrifuged at 590 *g* for 10 min at 4°C. The supernatant was removed and used for assay, directed in the kit and absorbance of each sample at 420 nm in a glass cuvette was measured. T-SOD activity was determined by measuring absorbance at 560 nm. CAT activity was measured as absorbance at 405 nm by using an assay based on the consumption of H_2O_2.

Testicular nitrite assessment

Testicular nitrite was measured as described by Miranda *et al*.[19] In brief; testis homogenate 0.5 ml was added in 0.5 ml of absolute ethanol and centrifuged at 1520 *g* for 10 min. In 300 μl of Vanadium, chloride added in 300 μl of the supernatant. Further 300 μl of Griess 1 reagent and Griess 2 (were gifted by a friend in Nanjing University) was added and left for 30–35 min at room temperature. The absorption was calculated spectrophotometrically at 540 nm against blank.

RNA extraction and quantitative real-time PCR

Decapsulated testes were briskly frozen in liquid nitrogen and homogenized in TRIzol and total RNA was extracted according to instructions given by the manufacturer. RNA was estimated using 260/280 UV. Gene-specific primers were designed based on the corresponding mRNA sequences as, Nrf2 (F) 5'- CAGTGCTCCTATGCGTGAA -3' (R) 5'- GCGGCTTGAATGTTTGTC -3', β-Actin (F) 5'- TGTGATGGTGGGAATGGGTCAG -3' (R) 5'- TTTGATGTCACGCACGATTTCC -3'. PCR reactions (SYBR Premix Ex Taq, ROX Reference Dye, primers and cDNA template) were run in triplicates in 20-μl total reaction volume. The amplification conditions were as follows: DNA polymerase activation at 95°C for 30 s, followed by 42 amplification cycles of denaturation at 95°C for 5 s, annealing at 58°C for 30 s, and extension at 72°C for 30 s. The specificity of the RT-PCR product was verified with a melting curve and was conducted to size separation by electrophoresis on agarose gels in TAE buffer, after amplification. A standard DNA marker via electrophoresis was used to estimate product sizes; bands were envisioned under UV light and photographed.

Evaluation of sperm characteristics

For normal testicular function, sperm count and motility were considered as markers. Cauda epididymis was placed in DMEM/F12 medium with FBS 5% and cut it in small pieces. The medium was balanced in an incubator at temperature 37°C and 5% CO_2. The suspension was used for analysis of sperm motility. Sperm motility was assessed with x40 magnification lens under microscope using basic procedure for human semen analysis and was divided into three levels:

1. Fast progressive
2. Nonprogressive or Slow progressive
3. Immotile.

Sperms from all four groups of mice were collected from their cauda epididymis, and sperm number was determined by hemocytometer following method of Majumder and Biswas[20] and WHO laboratory manual.[21,22]

Fertility performance

Adult ICR male mice were divided into four groups. Control, punicalagin, LPS and administrated with punicalagin + LPS for 1 week, after which each of them was caged with untreated female ICR mice and provided with standard food. The maximum duration of male exposure to female was 4 days. The presence of vaginal plugs was considered for positive copulation. While female mice with no vaginal plugs observed were also separated after 4 days of exposure. Fertility performance of four groups was analyzed on the basis of number of litters given birth by each female mouse.

Statistical analysis

Data represented mean ± s.e.m. of at least three independent experiments. One-way analysis of variance (ANOVA) was used for statistical comparison of groups and followed by Tukey–Kramer for a multiple comparisons test, $P < 0.05$ was considered to be statistically significant.

RESULTS

Testicular weight

Mice injected with 600 μg kg^{-1} LPS showed confirmation of illness by 72 h until the day they were dissected, as disheveled fur, lazy, and red eye lines.[4–7] Significant testicular weight loss was observed in the LPS injected mice group compared to control group (**Figure 1a**). The reduction in testis weight was the result of germ cell degeneration and vacuolation formation.[4–7] Mice in control and PU group showed no signs of illness and PU + LPS group mice as compared to LPS group were healthy.

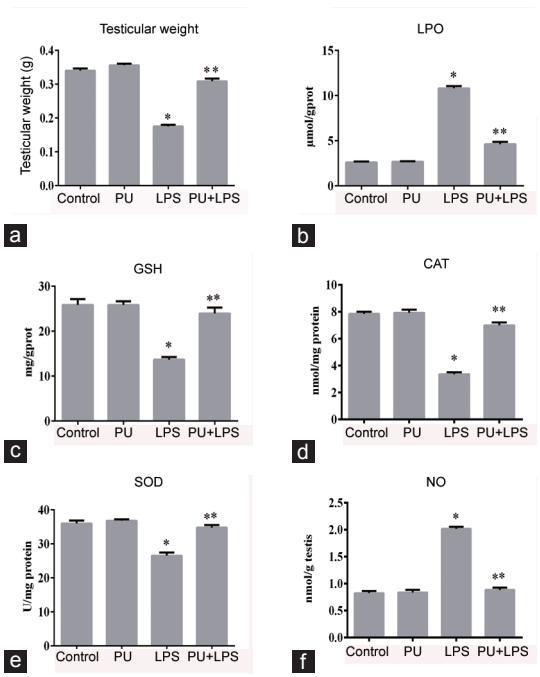


Figure 1: Testicular weight and oxidative stress parameters, measurement in mice testes. (**a**) Significant testicular weight loss in LPS injected group while PU + LPS co administration mice preserved testicular weight. Parameters as (**b**) Punicalagin (PU) prevented LPS induced testicular LPO content in mice; (**c**) Testicular GSH content in mice was reserved by Punicalagin (PU); (**d**) CAT (**e**) T-SOD (**f**) Punicalagin (PU) prevented LPS induced increase in testicular nitric oxide (NO) in mice. (*) indicate differences between control and LPS treated group and (**) indicate differences between LPS and punicalagin + LPS group respectively. Each column represents the mean ± s.e.m., $n = 8$. Statistical comparison between different groups were done by one-way (ANOVA) followed by Tukey–Kramer for a multiple comparisons test at $P < 0.05$.

Parameters of oxidative stress

A significant decrease in LPO concentration was observed in group treated with PU while an increase was observed in LPS group (**Figure 1b**). The GSH decreased significantly in LPS group and increased in PU + LPS group. This implies depletion of tissue GSH levels affiliated with ROS generation in LPS group and an increase of tissue GSH levels affiliated with ROS degeneration (**Figure 1c**). Activities of CAT and T-SOD were compared between control and LPS treated group, similarly between LPS and PU + LPS group (**Figure 1d** and **1e**). Concomitant PU supplementation normalizes testicular lipid peroxidation as well as T-SOD and catalase. Therefore, no changes could be seen of parameters discussed above in mice treated with PU alone.

Nitrite assessment in testicular tissue

LPS injection into mice extensively induced NO in testes that were comparatively higher than the control group mice. The other group PU + LPS showed a marked reduction in NO production and reached almost normal levels of NO (**Figure 1f**).

Histological results

Control and PU groups

Testicular structures in control and PU groups were normal, without any difference in the cell associations located in the seminiferous tubules (**Figure 2a** and **2b**).

Normal spermatogenesis can be observed (**Figure 2e**). The sections showed normal seminiferous tubule, spermatogonia, primary spermatocyte, round spermatids, elongated spermatids, and spermatozoa.

PU + LPS group

Light microscopic study of hematoxylin and eosin-stained sections explained the nearly normal display of the seminiferous tubules, severity of testicular damage were lower than those of the LPS group. No vacuolization and loss of round spermatids was observed. The changes observed in this group were examined as moderate. The percentage of abnormal tubules was 30% (**Figure 2c**).

LPS group

In the LPS group, the testicular damage was observed, and 51% of tubules were abnormal (**Figure 2d**). Intratubular giant cell formation, degeneration of germ cells, and vacuolization among germ cells were some of the histopathological findings in testis tissue observed in LPS group. Degeneration of seminiferous epithelium, vacuolization, and loss of round spermatids nuclei and disordered epithelium were evidenced (**Figure 2f**).

PU assists Nrf2 activation

Nuclear factor erythroid-related factor 2 (Nrf2) plays a considerable role in defense against oxidative stress by stimulating the cellular antioxidant system. It has been reported that Nrf2 knockout mouse presented an Oxidative disruption in spermatogenesis.[23] We determined RT-PCR results to certify the activation of Nrf2 in mice testis (**Figure 3**). According to our results, the group treated with PU + LPS expressed a high level of Nrf2. Here we concluded that PU can be a powerful activator of Nrf2 to fight against Oxidative stress.

Motility and sperm count

Sperm motility in LPS group as compared to control group was significantly lowered. In LPS group, two types of motility were observed; nonprogressive and immotile (sperms were moving slowly in small circles or crooked line while others either did not change their

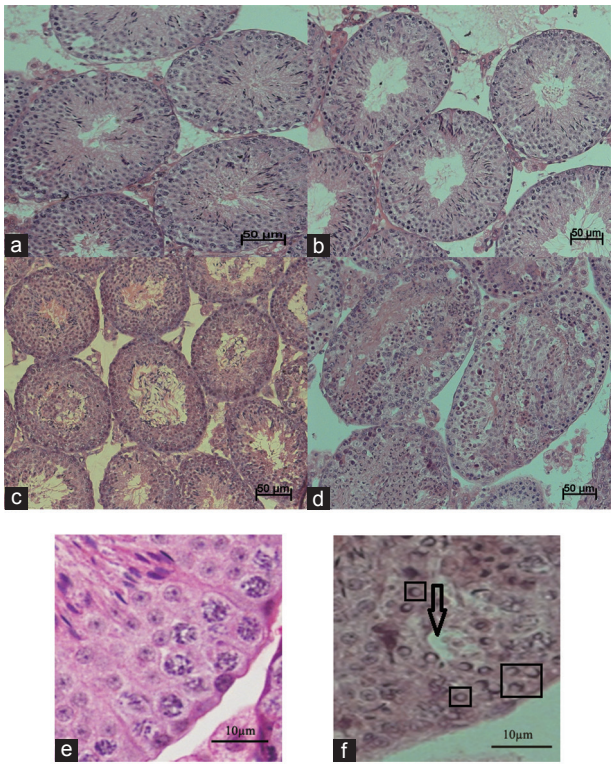


Figure 2: Histological examination of mice testes. (**a** and **b**) presents regular shape and normal spermatogenesis could be observed in control and Punicalagin (PU) injected mice testes. (**c**) Co-injection of Punicalagin (PU) prior to lipopolysaccharide (LPS) protected testes against oxidative injury; normal seminiferous tubules with presence of sperm and less degenerative germ cells could be observed. (**d**) An amount of 600 μg of lipopolysaccharide (LPS) administrated intraperitoneally in mice for 7 days causes Oxidative stress induced damage in mice testes. The figure shows degeneration of germ cells, absence of sperm and vacuolation formation in testicular tissue. (**e**) Normal spermatogenesis easily observed. (**f**) Degeneration of seminiferous epithelium (represented with arrow) vacuolization and loss of round spermatids nuclei was evidenced in LPS treated group (indicated by squares drawn).

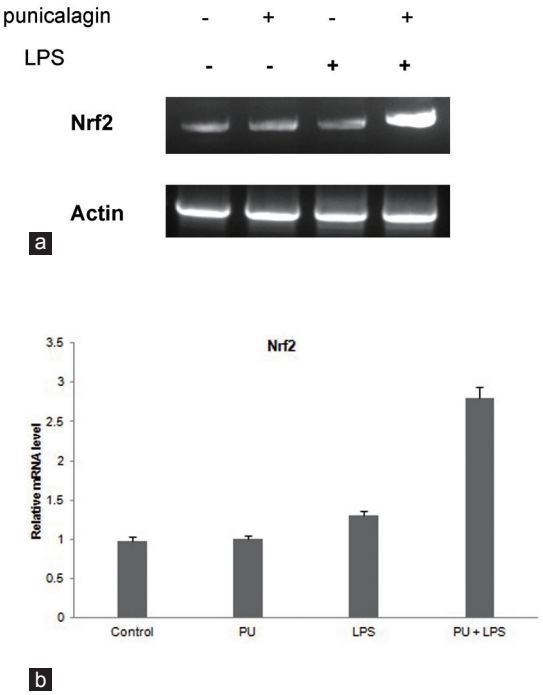


Figure 3: Punicalagin (PU) as powerful activator of Nrf2 to fight against Oxidative stress. (**a**) Electrophoresis agarose gel result for confirmation of RT-PCR results proved the activation of Nrf2 in PU + LPS group. (**b**) RT-PCR results certify the activation of Nrf2 in mice testis in PU + LPS group as compare to other groups. Increased mRNA level of Nrf2 observed in PU + LPS group and decreased in LPS proved PU to be an activator for Nrf2 against oxidative stress. Values are normalized using actin as standard endogenous.

position but had a moving tail or no movement at all). Group treated with PU + LPS showed fast progressive movement (sperms moved fast linearly), whereas few sperms showed slow or nonprogressive movement (sperms moving slowly in curved or crooked line). LPS treatment for 7 days significantly decreased epididymal sperm count than the control and PU supplemented LPS treated groups (**Table 1**).

Fertility test

After 4 days of exposure for fertility test, male mice were separated. Females from Control and punicalagin group had 12–14 l each. Females from LPS group had 6–7 l each and two of the females were not pregnant. Females from punicalagin + LPS group had 10–12 l each. The reason for the LPS group nonpregnant females could be unsuccessful copulation, which can also be a reason of oxidative stress on behavior.

DISCUSSION

This study to our knowledge is the first to highlight the role of PU (purity ≥ 98%) in oxidative stress induced testicular damage and infertility. LPS intraperitoneal administration in male ICR mice induced oxidative stress damage in testis. The treatment with PU resulted in decreased oxidative stress damage in testis and boosted fertility.

Table 1: Sperm count

Animal group	*Sperm count (10^6 ml^{-1})*
Control	5.8±0.1
PU	5.9±0.2
LPS	3.2±0.4^
PU + LPS	5.0±0.3

LPS treatment for 7 days significantly decreased sperm count while, group supplemented with PU (9 mg kg^{-1}) + LPS treatment for 7 days relevantly increased sperm count. ^Significant at $P<0.05$ LPS: lipopolysaccharide; PU: punicalagin

Previous studies explained intraperitoneal injection of LPS induces oxidative stress in rat testes and leads to a reduction in sperm counts, motility, and abnormalities in the histology of testis.[24] The present study determines that LPS administration of 600 μg kg^{-1} reduces testes weight and epididymal sperm count in 7 days, this indicates an obstruction with spermatogenesis. Further LPS disrupts germ cell population in seminiferous tubules. Oxidative stress induction by LPS is well-documented, causing the production of inflammatory cytokines and ROS generation.[25,26] ROS are known to cause testicular damage in different pathological conditions.[27,28] Germ cells are accessible to oxidative stress because of its affectionate federation with ROS generation.[29] Previous studies showed that i.p. injection of LPS increased NOS level in testes.[30,31] The present study also verifies the induction of NOS in testes as a result of LPS induced oxidative stress.

Antioxidants and herbal medicines proved to be favorable in working against oxidative stress.[32–34] Similarly polyphenols present in

pomegranate plays a significant antioxidant role *in vitro*.[35] Punicalagin is the major component of pomegranate and is known as a strong antioxidant in different diseases.[36,37] In this study, LPS induced oxidative stress in mice testes which caused a reduction in spermatogenic cell types as spermatocyte and spermatids while spermatogonia are displaced in some tubules. Oxidative stress induced in testes activated iNOS that resulted in excess NO generation. NO is powerful oxidant and play a major role in testicular damage that correlates with overproduction of NO. The administration of oxidative stress resulted in a significant reduction in testis GSH, T-SOD, CAT and raised LPO and NO production compared with the control. SOD is known to be the foremost line of the defensive system against oxyradicals, reduction in CAT by the end of 7 days reflects the inability of the testis to eliminate oxidative stress. Mice exposed to LPS and punicalagin for 30 days resulted decrease in LPO and increase in GSH contents in testes (**Supplementary Figure 1a** and **1b**) also proves the nontoxic effect of Punicalagin. Punicalagin with purity of 98% was given orally for 7 days to the mice that were injected with LPS. Punicalagin treatment prevented a reduction in testicular weight in PU + LPS group of mice when compared with those in LPS alone group. This protective effect of PU can be explained by the fact that PU prevents testicular damage as a result of oxidative stress in spermatogenic cells of seminiferous tubules. Similarly, the strong antioxidant property of PU resulted in significant decrease in LPO and NO production and increase in GSH, T-SOD, and CAT. Histopathologic study of testicular tissue of mice injected with LPS showed germ cells degeneration, reduction in seminiferous tubules, and vacuolation. When testes of mice injected with PU + LPS were compared to those injected with LPS alone, PU treatment restrained a reduction in testicular weight. This protective effect of PU can be explained by the fact that it prohibits cellular damage that develops as a result of oxidative stress in testes. Continuous, injecting a dosage of LPS generates extended ROS production.[38,39] In the present study, the histological and microscopic study revealed the reduction of sperm count in the epididymis of LPS injected mice while mice given PU for 7 days showed an increase in sperm count in the epididymis. These results indicated that PU can play an important role in boosting fertility. We also performed a fertility test for a longer exposure of punicalagin to male mice and found that the longer exposure of punicalagin (PU) 9 mg kg^{-1} for 30 days is nontoxic and resulted in the normal birth of litters (**Supplementary Table 1**).

Nrf2 plays an extensive role in defense against oxidative stress by awakening the cellular antioxidant system while simultaneously suppressing inflammatory pathways.[40] To further verify our work, we examined mRNA expression of Nrf2 in samples of all four groups. Our results demonstrated activation of Nrf2 in the LPS-injected group compared to control group; however the treatment with PU triggered Nrf2, in comparison to the LPS group. These results showed that PU helps Nrf2-antioxidant signaling, which acts more strongly against oxidative stress. Antioxidants act as anti-terrorist forces of the body. They give up electrons to the free radicals through a system to neutralize these free radicals. This neutralization helps the body to make more antibodies without harm. However, the body is exposed to increasing amounts of harmful free radicals due to daily lifestyle and the environment. Therefore, supplements of antioxidants are needed to protect the body against diseases and aging.[41,42] A healthy supply of antioxidants can be obtained from natural sources, and, for that reason, previous studies demonstrated the potent role of PU against oxidative stress.[43]

In this study, we used PU to cure infertility caused by LPS induced oxidative stress. Our results demonstrated that PU can be a new light in the field of infertility. Regarding the complete results of this study, fertility tests were performed to evaluate the effect of oxidative stress on fertility. Groups of mice were set for mating tests on the same day as explained above. All mice in control groups and mice in the PU + LPS group gave birth to young ones with 1–2 days difference, while the two female mice from LPS group had no babies and number of litters given birth by other females were different as compared to control and punicalagin (PU) + LPS group. These results of mating tests show that PU can play an important role in curing sub-fertility. No previous data were found on using PU (purity ≥98%) for sub-fertility treatments; therefore, comparisons could not be made. Health benefits of pomegranate have been touted due to its bioactive compounds, one of them being PU, which is a large molecule found in pomegranate husks.

We report that PU could be the main active compound promoting fertility by reducing oxidative stress damage in testes, mitigating oxidative stress, promoting Nrf2 and increasing sperm count. Our results suggest that PU from Pomegranate could comprise an effectual dose for oxidative stress induced sub-fertility. This study verifies that activation of iNOS plays a crucial role in the chain of LPS events leading to an oxidative stress injury in mice testes. We also concluded that activating of Nrf2 in our body is very useful to fight against oxidative stress. So, PU can be a booster for Nrf2. PU has a potent protective effect against testicular toxicity and may be clinically useful. Nonetheless, additional studies are recommended on this subject before the clinical application can be endorsed.

AUTHOR CONTRIBUTIONS

FR, FS and WL designed the study. FR, WL, HT and HH carried out all experiments. FR, FS and WL analyzed the experimental data, drafted article and revised the paper. All authors read and approved the final manuscript.

COMPETING INTERESTS

The authors declare no competing interests.

ACKNOWLEDGMENTS

This work was supported by National Natural Science Foundation of China Grants 81125005 and 81430027 (to F.S.), the National Basic Research Program of China (2014CB943100).

Supplementary information is linked to the online version of the paper on the *Asian Journal of Andrology* website.

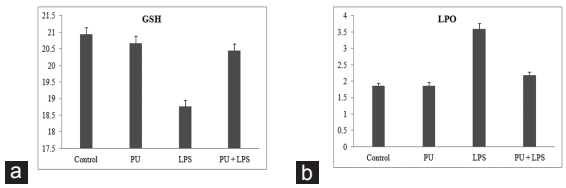
REFERENCES

1 Aitken RJ, Roman SD. Antioxidant systems and oxidative stress in the testes. *Oxid Med Cell Longev* 2008; 1: 15–24.
2 Wright C, Milne S, Leeson H. Sperm DNA damage caused by oxidative stress: modifiable clinical, lifestyle and nutritional factors in male infertility. *Reprod Biomed Online* 2014; 28: 684–703.
3 Apostoli P, Catalani S. Metal ions affecting reproduction and development. *Met Ions Life Sci* 2011; 8: 263–303.
4 Reddy MM, Mahipal SV, Subhashini J, Reddy MC, Roy KR, *et al*. Bacterial lipopolysaccharide-induced oxidative stress in the impairment of steroidogenesis and spermatogenesis in rats. *Reprod Toxicol* 2006; 22: 493–500.
5 Allen JA, Diemer T, Janus P, Hales KH, Hales DB. Bacterial endotoxin lipopolysaccharide and reactive oxygen species inhibit Leydig cell steroidogenesis via perturbation of mitochondria. *Endocrine* 2004; 25: 265–75.
6 Lei L, Ping M, Yongjun L, Chen H, Wai-Sum O, *et al*. Intermedin Attenuates LPS-induced Inflammation in the rat testis. *PLoS One* 2013; 8: e65278.
7 Mallikarjuna RM, Chandra MT, Reddy PR, Pallu R. Bacterial LPS mediated acute Inflammation-induced spermatogenic failure in rats: role of stress response proteins and mitrochondrial dysfunction. *Inflammation* 2010; 33: 235–43.
8 Al-Olayan M, El-Khadragy F, Metwally M, Moneim A. Protective effects of pomegranate (*Punica granatum*) juice on testes against carbon tetrachloride intoxication in rats. *BMC Complement Altern Med* 2014; 14: 164.
9 Adel RA, Gouda KH, Abdulaziz AA, Abdulaziz MA, Salim SA, *et al*. Pro-inflammatory and oxidative stress pathways which compromise sperm motility and survival may

be altered by L-carnitine. *Oxid Med Cell Longev* 2009; 2: 73–81.
10 Wallgren M, Kindahl H, Rodriguez-Martinez H. Alterations in testicular function after endotoxin injections in the boar. *Int J Androl* 1993; 16: 235–43.
11 Voravuthikunchai SP, Sririrak T, Limsuwan S, Supawita T, Iida T, *et al*. Inhibitory effects of active compounds from Punica granatum pericarp on verocytotoxin production by enterohemorrhagic *Escherichia coli* O157:H7. *J Health Sci* 2005; 51: 590–6.
12 Singh RP, Chidambara MK, Jayaprakasha GK. Studies on the antioxidant activity of pomegranate (*Punica granatum*) peel and seed extracts using *in vitro* models. *J Agric Food Chem* 2002; 50: 81–6.
13 Ricci D, Giamperi L, Bucchini A, Fraternale D. Antioxidant activity of *Punica granatum* fruits. *Fitoterapia* 2006; 77: 310–2.
14 Sánchez-Lamar A, Fonseca G, Fuentes JL, Cozzi R, Cundari E, *et al*. Assessment of the genotoxic risk of *Punica granatum* (*Punicaceae*) whole fruit extracts. *J Ethnopharmacol* 2007; 115: 416–22.
15 Seeram N, Lee R, Hardy M, Heber D. Rapid large scale purification of ellagitannins from pomegranate husk, a byproduct of the commercial juice industry. *Sep Purif Technol* 2005; 41: 49–55.
16 Gil MI, Tomás-Barberán FA, Hess-Pierce B, Holcroft DM, Kader AA. Antioxidant activity of pomegranate juice and its relationship with phenolic composition and processing. *J Agric Food Chem* 2000; 48: 4581–9.
17 Sherif WM, Sibghatullah S, Sree H, Mueen AK, Ibrahim AR. Sensibility of male rats fertility against olive oil, *Nigella sativa* oil and pomegranate extract. *Asian Pac J Trop Biomed* 2013; 3: 563–8.
18 Tariq I, Piero S, Saeed A. Pomegranate peel and fruit extracts: a review of potential anti-inflammatory and anti-infective effects. *J Ethnopharmacol* 2012; 143: 397–405.
19 Miranda KM, Espey MG, Wink DA. A rapid, simple spectrophotometric method for simultaneous detection of nitrate and nitrite. *Nitric Oxide* 2001; 5: 62–7.
20 Majumder GC, Biswas R. Evidence for the occurrence of ecto (adenosine triphosphatase) in rat epididymal spermatozoa. *Biochem J* 1979; 183: 737–43.
21 World Health Organization. WHO Laboratory Manual for the Examination and Processing of Human Semen. 5th ed. Geneva: World Health Organization; 2010. p. 21–6.
22 Lu WH, Gu YQ. Insights into semen analysis. A Chinese perspective on the fifth edition of the WHO laboratory manual for the examination and processing of human semen. *Asian J Androl* 2010; 12: 605–6.
23 Nakamura BN, Lawson G, Chan JY, Banuelos J, Cortes MM, *et al*. Knockout of the transcription factor NRF2 disrupts spermatogenesis in an age-dependent manner. *Free Radical Biol Med* 2010; 49: 1368–79.
24 Abd-Allah AR, Helal GK, Al-Yahya AA, Aleisa AM, Al-Rejaie SS, *et al*. Pro-inflammatory and oxidative stress pathways which compromise sperm motility and survival may be altered by L-carnitine. *Oxi Med Cell Longev* 2009; 2: 73–81.
25 Jaworek J, Konturek SJ, Macko M, Kot M, Szklarczyk J, *et al*. Endotoxemia in newborn rats attenuates acute pancreatitis at adult age. *J Physiol Pharmacol* 2007; 58: 131–47.
26 Zhao L, Chen YH, Wang H, Ji YL, Ning H, *et al*. Reactive oxygen species contribute to lipopolysaccharide-induced teratogenesis in mice. *Toxicol Sci* 2008; 103: 149–57.
27 Selvakumar E, Prahalathan C, Mythili Y, Varalakshmi P. Protective effect of dl-alpha-lipoic acid in cyclophosphamide induced oxidative injury in rat testis. *Reprod Toxicol* 2004; 19: 163–7.
28 Jacobson CF, Miller MG. Species difference in 1, 3-dinitrobenzene testicular toxicity: *in vitro* correlation with glutathione status. *Reprod Toxicol* 1998; 12: 49–56.
29 Bauche F, Fouchard MH, Jegou B. Antioxidant system in rat testicular cells. *FEBS Lett* 1994; 349: 392–6.
30 O'Bryan MK, Schlatt S, Gerdprasert O, Phillips DJ, De Kretser DM, *et al*. Inducible nitric oxide synthase in the rat testis: evidence for potential roles in both normal function and inflammation-mediated infertility. *Biol Reprod* 2000; 63: 1285–93.
31 Jonsson CK, Setchell BP, Martinelle N, Svechnikov K, Soder O. Endotoxin induced interleukin 1 expression in testicular macrophages is accompanied by down regulation of the constitutive expression in Sertoli cells. *Cytokine* 2001; 7: 283–8.
32 Yuan H, De-Xiang X, Jian-Zhen W, Hua W, Ling-Zhen W, *et al*. Melatonin protects against lipopolysaccharide-induced intra-utrine fetal death and growth retardation in mice. *J Pineal Res* 2006; 40: 40–7.
33 Agarwal A, Shen H, Agarwal S, Rao AV. Lycopene content of tomato products: its stability, bioavailability and *in vivo* antioxidant properties. *J Med Food* 2001; 4: 9–15.
34 Narumi S, Takayuki A, Mikae O, Koji F. Anti and pro-oxidative effects of flavonoids on metal induced lipid hydroperoxide dependent lipid peroxidation in cultured hepatocyte loaded with α-Linolenic acid. *Free Radic Biol Med* 1999; 27: 1313–23.
35 Lorena T, Aneta W, Esther S. Antioxidant activity and protein-polyphenol interactions in a Pomegranate (Punica granatum L.) Yogurt. *J Agric Food Chem* 2014; 62: 6417–25.
36 Sala H. The power of the pomegranate: biblical fruit with medicinal properties. *Altern Complement Ther* 2006; 12: 121.
37 Seeram NP, Adams LS, Henning SM, Niu Y, Zhang Y, *et al*. *In vitro* antiproliferative, apoptotic and antioxidant activities of punicalagin, ellagic acid and a total pomegranate tannin extract are enhanced in combination with other polyphenols as found in pomegranate juice. *J Nutr Biochem* 2005; 16: 360–7.
38 Hensley K, Robinson KA, Gabbita SP, Salsman S, Floyd RA. Reactive oxygen species, cell signaling, and cell injury. *Free Radic Biol Med* 2000; 28: 1456–62.
39 Victor VM, Rocha M, De la Fuente M. Immune cells: free radicals and antioxidants in sepsis. *Int Immunopharmacol* 2004; 4: 327–47.
40 Li W, Khor TO, Xu C, Shen G, Jeong WS, *et al*. Activation of Nrf2 antioxidant signaling attenuates NFkappaB-inflammatory response and elicits apoptosis. *Biochem Pharmacol* 2008; 76: 1485–9.
41 Weber C, Sejersgard Jakobsen T, Mortensen SA, Paulsen G, Holmer G. Antioxidative effect of dietary coenzyme Q10 in human blood plasma. *Int J Vitam Nutr Res* 1994; 64: 311–5.
42 Whiting SJ, Calvo MS. Dietary recommendations for Vitamin D: a critical need for functional end points to establish an estimated average requirement. *J Nutr* 2005; 135: 304–9.
43 Xu X, Yin P, Wan C, Chong X, Liu M, *et al*. Punicalagin inhibits inflammation in LPS-induced RAW264.7 macrophages via the suppression of TLR4-mediated MAPKs and NF-kappaB activation. *Inflammation* 2014; 37: 956–65.

Supplementary data

Fertility test performed after 30 days observation. Adult ICR male mice were divided into four groups, control, punicalagin, LPS and administration with punicalagin + LPS for 30 days, after which each of them was caged with untreated female ICR mice and provided with standard food. Male mice were separated from females after vaginal plugs were observed. Presence of vaginal plugs was considered for positive copulation.



Supplementary Figure 1: Evaluation of oxidative stress. GSH and LPO, considered as markers for Oxidative stress evaluation. After 30 days of observation these both markers were measured to conclude whether punicalagin is still ahead to protect testes from oxidative stress. (**a**) GSH contents in LPS group presented a decrease as compared to control and co-administrated group of LPS and punicalagin (PU + LPS). **(b)** LPO contents in LPS group presented increase as compared to control and punicalagin + LPS co-administrated group.

Supplementary Table 1: Fertility test after 30 days treatment

	Mice set for fertility test on same day			
	Control	*PU*	*LPS*	*PU + LPS*
Number of females pregnant	All 8 females were pregnant	All 8 females were pregnant	All 8 females were pregnant	All 8 females were pregnant
Number of pups	Number of pups each female range (12–14)	Number of pups each female range (12–14)	Number of pups each female range (10–14)	Number of pups each female range (12–14)
Difference	Females gave birth with 2–3 days difference		Females gave birth with 12–20 days difference as compared to control group	Females gave birth with 5–6 days difference as compared to control group

The results indicate LPS group litters birth difference in days. We here conclude that the day difference might be the result of oxidative stress on sexual behavior which actually leads to late birth. This may open a vast study of oxidative stress in late birth. LPS: lipopolysaccharide; PU: punicalagin