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Pycnogenol Improves the Destructive Results of Prenatal Bacterial Lipopolysaccharide Exposure in Adult Male Pups NMRI Mice

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ABSTRACT

Introduction: Prenatal exposure to lipopolysaccharide (LPS) can lead to structural damage and CNS dysfunction. The present study aimed to investigate the protective effects of prenatal administration of pycnogenol (PYC) against the negative effects of bacterial LPS on anxiety-like behavior, gonadotropin and sex hormone serum levels, and sperm quality and quantity in the adult male offspring of NMRI mice.

Methods: Pregnant mice were randomly divided into four groups (n = 10 per group): 1. Saline group: received a single dose of saline as solvent of pycnogenol by gavage for 3 days on gestation days 16-18. 2. LPS group: received a single dose of LPS (20 µg/kg, subcutaneously) on gestation day 20. 3. PYC: received 200 mg/kg/day of pycnogenol by gavage for 3 days, intraperitoneally, on gestation days 16-18. 4. LPS + PYC: received a single dose of LPS (20 µg/kg) on gestation day 20 and pycnogenol (200 mg/kg/day) by gavage for 3 days on gestation days 16-18. After maturity/puberty in male pups (60 days old), the anxiety-like behavior test was performed. After the behavioral test, serum levels of gonadotropins (luteinizing hormone, LH, follicle stimulating hormone, FSH), testosterone hormone and sperm quality were assessed.

Results: LPS administration increased anxiety-like behaviors and decreased serum LH and testosterone levels; however, PYC treatment reversed the negative effects of LPS to normal levels.

Conclusion: PYC treatment improves anxiety-like behavior and gonadotropin and testosterone secretions. Therefore, this substance can be used as a protectant and an aphrodisiac agent.

Keywords: pycnogenol, lipopolysaccharid, anxiety, gonadotropin, testosterone, mice.

INTRODUCTION

Lipopolysaccharide (LPS) is a toxic component of the cell wall of gram-negative bacteria and is widely used to establish a well-known model of bacterial infection (1). Human are frequently exposed to low levels of LPS through infection (2). Recent epidemiological studies and animal experiments have demonstrated that prenatal exposure to LPS could lead to structural damage and dysfunction of the cerebral cortex and hippocampal neurons, thereby inducing autism, schizophrenia, anxiety, and cerebral palsy in adulthood (3, 4). However, there is little information on the effects of maternal LPS exposure during pregnancy on reproductive function in male offspring. Prenatal exposure to LPS increases the levels of lipid peroxidation and nitric oxide and decreases the level of glutathione in the maternal liver, embryo, and placenta (5).

The use of medicinal herbs has increased in recent years because of the side effects of chemical drugs. Interest in herbal remedies has paved the way for a plethora of research. However, willful and excessive use of herbal medicines is not recommended because of their specific side effects. One of the most recently used substances in drug production is pine tree extract. More precisely, it is the bark extract of a pine native to southern France, where it is mainly known as pycnogenol and is marketed in the form of pills and supplements (6). This extract contains a mixture of flavonoids, procyanidins, and phenolic acids. Flavonoids are polyphenolic compounds found mainly in plants and appear as potent antioxidant and antiradical compounds. The benefits of this extract include reducing the risk of heart attacks, side effects of chemotherapy, and symptoms of erectile dysfunction, as well as improving blood flow, likely immune stimulation, mitigation of swelling, prevention of infection, and most importantly, a powerful antioxidant (7).

Given the above descriptions and the importance of pycnogenol, the present study aimed to investigate the protective effects of pycnogenol against the negative effects of bacterial endotoxins on anxiety-like behavior, gonadotropin and sex hormones, and sperm quality and quantity in adult male offspring of NMR mice.

MATERIALS AND METHODS

Animals

Forty adult female NMRI mice weighing 30 g were obtained from the Pasteur Institute of Iran and were kept under standard conditions (temperature range of 22±1 °C, humidity 40-45 %, and illumination conditions of 12 h of light and 12 h of darkness). The mice had free access to food and water. This experimental randomization study was conducted according to the National Committee for Ethics in Animal Study of the Islamic Azad University, Karaj Branch.

Experimental design

After mating and ensuring pregnancy (by checking the vaginal plaque one day after mating), the female mice were randomly divided into four groups (10 pregnant mother mice in each group), saline group received a single dose of saline as a solvent of pycnogenol by gavage for 3 days (16-18 days of gestation), and the LPS group (LPS) received a single dose of LPS (Sigma-Aldrich, St Louis, MO, USA) (20 µg/kg body weight) after 18 days of gestation, subcutaneously; pycnogenol group received 200 mg/kg/day of pycnogenol (Puritan Co. France) by gavage for 3 days (16-18 days of gestation) pycnogenol + LPS group received 200 mg/kg/day of pycnogenol by gavage for 3 days (16-18 days of gestation) and a single dose of LPS (20 µg/kg body weight) in the 18th day of gestation. Then, in each group, 22 days old male offspring were separated from mothers, and after maturity/puberty (60 days old), the anxiety-like behavioral tests were performed. After the behavioral tests, gonadotropin and sex hormone serum levels and sperm quality of the male mice were measured as follows.

Pup tests

The postnatal number of offspring was counted and their birth weights and lengths were recorded per group. Male and female offspring were separated 22 days after birth. Following maturity (60 days of age), anxiety-like behavioral tests, serum gonadotropin and testosterone levels, and sperm quality tests were performed on the male offspring.

Body weights and lengths of pups

The body weights and lengths of the offspring were measured on the first day after birth using a scale and ruler, respectively.

Elevated plus maze (EPM)

The EPM was used to measure anxiety in mice. EPM contained two open and two closed arms (35×35 cm) and a central platform (5×5 cm) elevated 50 cm above the floor. The maze was placed in a test room that was completely alike for all mice in terms of sound, temperature, light, objects, and the experiment was carried out in full silence. At the time of the test, mice were placed in one of the open arms. The *time* spent in the open arm as well as the closed arm and the number of entries into the open arm and the closed arm were recorded using a ceiling camera for 5 min.

Using a ceiling camera, behavioral data were measured for 5 min including the duration the animal remained in the open arm, the duration the animal remained in the closed arm, the number of times the animal entered the arm, the number of times the animal entered the closed arm, and the locomotor activity of mice equaling the number of times the mice entered different arms.

$$OAT\ \% = [OAT / (OAT+CAT)] * 100$$

OAT (open arm time), which is the percentage of time when the mice pass the open arm is calculated as:

CAT (closed arm time) is the time spent in the closed arm, and OAE% is the percentage of time the mice entered the open arm.

To analyze the open arm activity, the percentage of open arm time (OAT) and open arm entries (OAE), as well as the rate of motor activity of the mice (locomotor activity), which is equivalent to the number of entries into different arms, was also calculated (8).

Sperm quality and quantity

The epididymis was isolated, and sperm were counted. Sperm motility, progression, and morphology were evaluated using a computer-assisted system of analysis (CASA) (Hoshmand Fannavar Company).

Measurement of serum gonadotropins and sex hormone levels

After the behavioral tests, mice in different groups were anesthetized using chloroform and blood samples were collected from the orbital sinus of the eye using a hematocrit tube. After half an hour, the blood samples were centrifuged at 5000 rpm for 4 min. Serum samples were then separated using a sampler and stored in microtubes at −21 °C. At the time of use, the serum was removed from the freezer half an hour earlier to cool down to ambient temperature for hormone assays. Serum testosterone, LH, and FSH levels were measured using ELISA kits (Fine Test Co., China), according to manufacturer's instructions.

Statistical analysis

Graph software was used to compare the between-group results. Data were analyzed using one-way ANOVA with post-hoc test. The significance level was set at $P < 0.05$. Data are represented as mean ± SEM.

RESULTS

Pups Body weights and lengths of pups

The body weights and lengths of the offspring were measured on the first day after birth. Offspring body weights and lengths displayed no significant differences among the studied groups ($P > 0.89$ and $P > 0.67$, respectively).

Evaluation of EPM test

In the EPM test, LPS significantly decreased the time spent in the open arm compared with the saline group ($P < 0.05$). A comparison of the LPS + PYC treatment with the LPS group showed a significant increase in the open arm times (OAT %) of the LPS + PYC ($P < 0.05$) groups compared to the LPS treatment (Fig. 1A).

The results showed that LPS led to a significant decrease in the number of open arm entries compared with the saline group ($P < 0.05$). A comparison between the LPS + PYC with the LPS group revealed significance differences ($P < 0.05$) in the number of open arm entries (OAE %) in the LPS + PYC group compared to the LPS group (Fig. 1B). Results obtained from locomotor activity indicated no significant differences

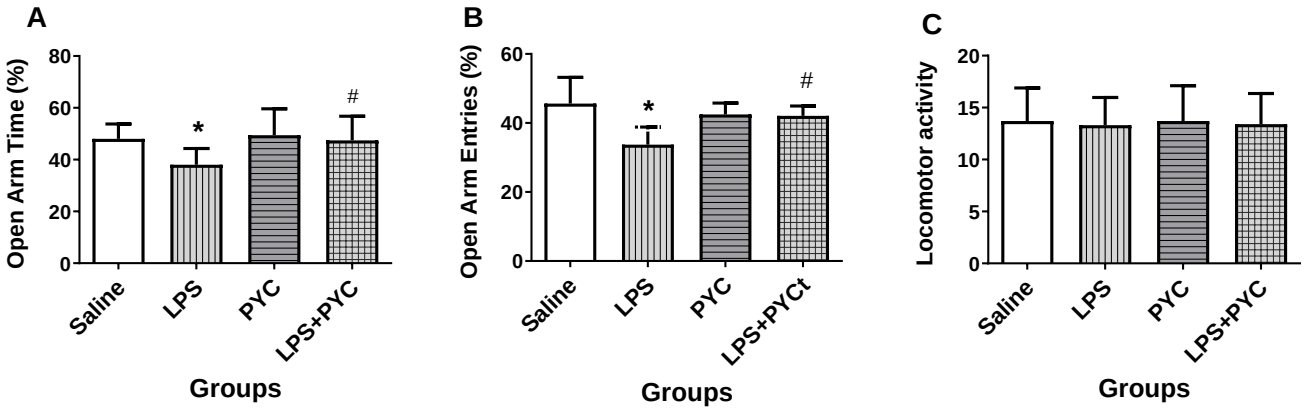

Figure 1. Prenatal effects of PYC administration on anxiety-like behavior of male pup mice observed in EPM (mean ± SEM). a, b: LPS led to a significant decrease in the open arm time (OAT%) and number of open arm entries (OAE%) compared to saline mice. A comparison between the LPS + PYC group with the LPS group revealed significant increases in OAT% and OAE% in the LPS + PYC group compared to the LPS group. c: Locomotor activity showed no significant differences between the studied groups. *P < 0.05, significant difference compared to the saline group, # P < 0.05, significant difference compared to the LPS group.

between the LPS and the LPS + PYC groups and the saline animals (Fig. 1 C).

The quality and quantity of sperm

According to the results of sperm count, the LPS group displayed significant differences from the saline group (P < 0.01). The PYC + LPS group was significantly increase compared to the LPS group at significance levels of P < 0.05.

The percentage of the motile sperm in the LPS group was lower than that in the saline group, but PYC reversed this decline in the LPS + PYC group (P < 0.05).

The results of progressive sperm showed a significant decrease between the LPS and saline groups (P < 0.01); however, PYC reversed the negative effect of LPS in the LPS + PYC group (P < 0.01).

The normal morphology of sperm in the LPS group was lower significantly than that in the saline group (P < 0.01), whereas PYC treatment in the LPS + AST group decreased the negative effect of LPS on sperm morphology (P < 0.01).

Serum gonadotropins and sex hormone levels

Serum LH level in the LPS group was significantly decrease from that in the saline group, but PYC treatment could reverse the negative effect of LPS in PYC + LPS group compare to the LPS group (P < 0.01) (Fig 2A).

Table 1. Pycnogenol and LPS effects on the indices of sperm analysis in 60-day old male offspring.

	Saline	LPS	PYC	LPS+PYC
Sperm count (million/ml)	6.5 ± 1.7	4.6 ± 1.01**	8.5 ± 1.1	6.4 ± 2.5$^{\#}$
Motile sperm (%)	40.3 ± 5.5	31.2 ± 3.5*	42.4 ± 4.6	39.4 ± 4.1$^{\#}$
Progressive sperm (%)	22.5 ± 3.5	12.5 ± 1.2**	27.6.1 ± 3.7	24.1 ± 2.6$^{\#\#}$
Normal morphology (%)	64.8 ± 7.7	41.8 ± 4.7*	74 ± 4.6	72.8 ± 7.1$^{\#\#}$

*P < 0.05 and **P < 0.01 significant difference compared to the saline group, # P < 0.05, ## P < 0.01 significant difference compared to the LPS group.

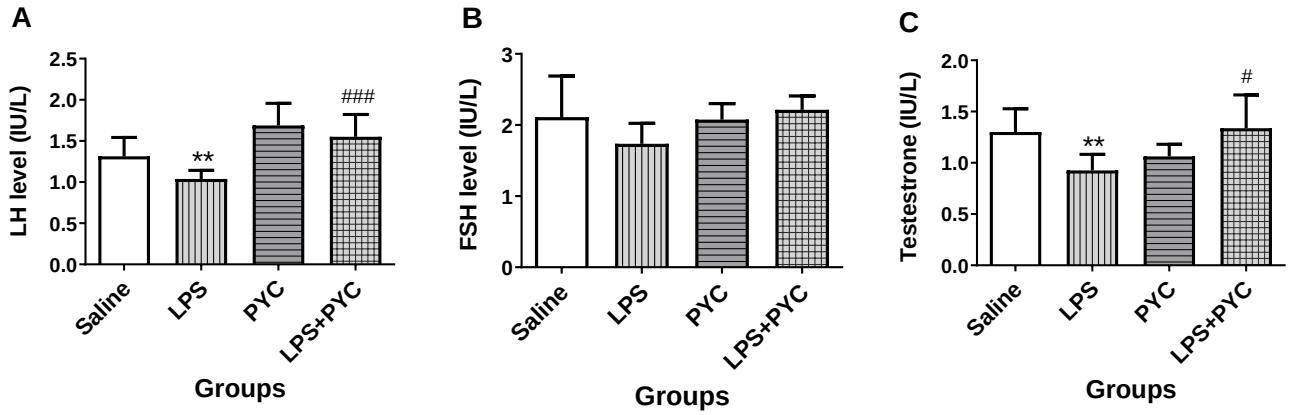

Figure 2. Serum LH, FSH, and testosterone levels in 60 days old male offspring mice following LPS and PYC treatment. a: Serum LH level was significantly lower in the LPS group compared to the saline group, however, PYC treatment increased it in the LPS + PYC group than in the LPS group. b: Serum FSH level was not significantly different among the studied groups. c: Serum testosterone level in the LPS group was significantly lower than that in the saline group, but PYC treatment could change it in the PYC + LPS group that in the LPS group. * $P < 0.05$ and ** $P < 0.01$ significant difference compared to the saline group, ≠ $P < 0.05$ and ### $P < 0.001$ significant difference compared to the LPS group.

The serum FSH level was not significantly different between the LPS - treated group and the saline group (Fig 2B).

The serum testosterone level in the LPS group was significantly lower than that in the saline group ($P < 0.01$); however, PYC treatment increased testosterone level in the PYC + LPS group compared to the LPS group ($P < 0.05$) (Fig. 2C).

DISCUSSION

This research was designed to address the preventive effects of PYC pretreatment against the negative effects of prenatal LPS exposure in male offspring of NMRI mice. Mice that received LPS at a dose of 20 µg/kg developed anxiety and spent less time in the open arm, indicating their increased stress levels. LPS reduced some anxiety-like behaviors (OAT and OAE %) in the EPM, the indices of sperm analysis (sperm count, motility, normal morphology, and progression), and serum LH and testosterone levels. However, administration of PYC reversed the negative effects of LPS to normal levels.

There are not enough studies in the literature to directly address the ameliorating effect of PYC on anxiety behaviors and fertility during LPS exposure; however, several studies have shown the protective effects of PYC on prenatal LPS-exposed reproductive and behavioral deficits in adult offspring (9).

Male fertility depends on the number of sperm used. A critical sperm counts of 20 million sperm/m^3 is required for pregnancy. Fertility rate decreases in men whose sperm count is less than the critical value. There are various causes for male infertility. Many studies have examined the effects of various substances, including both useful and harmful compounds, on various bodily systems and biochemical parameters (10).

In our experimental mice treated with 200 mg/kg pycnogenol, statistical analysis showed that pycnogenol treatment led to increased LH and testosterone levels, but not FSH, and the indices of sperm quality and quantity in the PYC and PYC + LPS groups, which can increase fertility in men. The increase in LH level stimulates Leydig cells; hence, this was followed by an increase in testosterone levels, thereby increasing the indices of sperm quality.

In our previous study, we demonstrated the destructive effect of LPS and protective effect of PYC on anxiety, and motor function (11, 12). Grimm et al. detected the antioxidant nature of pycnogenol that could help cell health by inhibiting and removing free radicals (13).

Similarly, the present study demonstrated that LPS-induced anxiety, leading to affected sexual behaviors and stress in NMRI mice, was significantly mitigated by treatment with

pycnogenol. In addition, findings of Ďuračková et al. also presented evidence that treatment with pycnogenol improved lipid metabolism, steroid levels, and erectile function in people with erectile dysfunction and poor sexual function (14, 15). Solati et al. investigated the effects of bacterial lipopolysaccharide during pregnancy and anxiety behaviors in pregnant mothers and male offspring undergoing treatment. They found that LPS caused anxiety and elevated the serum levels of cytokines and corticosterone in mothers (12).

CONCLUSION

It can be concluded that pycnogenol can alleviate anxiety caused by the negative effects of LPS, increase LH and testosterone levels, and increase sperm quality and quantity.

DECLARATIONS

Authors have no conflict of interest to declare.

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