Aqueous extract of *Lespedeza cuneata* improves male menopause by increase of nitric oxide and dihydrotestosterone

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Received: 3 May 2018 / Revised: 20 June 2018 / Accepted: 4 July 2018 / Published online: 11 July 2018

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Abstract This study aimed to evaluate the effect of aqueous extract of *Lespedeza cuneata* (ALC) on menopause induced erectile dysfunction (ED) and testosterone deficiency syndrome (TDS) in vitro and in vivo. Nitric oxide (NO) and cyclic guanosine monophosphate (cGMP) have been fundamental factors of ED, and free testosterone such as dihydrotestosterone (DHT) has a crucial role in mitigation of TDS. ALC increased the production of NO in a dose-dependent manner in vitro and in vivo. The level of neuronal nitric oxide synthase and cGMP was increased in the ALC group. The level of DHT and 17-beta hydroxysteroid dehydrogenases (17 β -HSD) was also increased in the ALC group. There was no significant change of hepatotoxicity, renal toxicity, lipid metabolism, and glucose. These results suggest that ALC enhanced NO, cGMP and free testosterone which could be developed as a health supplement for improving male menopause.

Keywords Aqueous extract of *Lespedeza cuneata* (ALC) · NO · cGMP · Free testosterone · Male menopause

Introduction

Male menopause is a clinical, biochemical syndrome with typical symptoms such as sexual instability, decreased vitality and erectile dysfunction, decreased muscle mass and bone density, passive attitude, and lowered serum testosterone level as the age increases in men (Burnett, 2006a; Jouannet et al., 2001). Modern middle-aged generations desire to maintain their vitality and health, and to prevent or retard aging with a healthy life. Increased lifespan lead to changes in the endocrine system of men such as women's menopause, which resulted in erectile dysfunction (ED) and testosterone deficiency syndrome (TDS). ED is a phenomenon in which blood does not flow normally into the corpus cavernosum tissue (Bivalacqua et al., 2000), which means that when the penis is not sufficiently erected, it is not persistent and occurs more than 25% of the total sex life (Bivalacqua et al., 2005; Goldstein et al., 1998). Penile erection is caused by intricate physiological interactions including the central nervous system, peripheral nervous system, and smooth muscle (Brixius et al., 2007; Lee et al., 2007). The induction of smooth muscle relaxation leads to expansion of the cavernosal volume. Therefore, NO production and cGMP degradation inhibition are very important factors for ED improvement. NO acts as a signaling molecule to the central and peripheral nervous system released from the non-adrenaline and non-cholinergic nerve endings of the penile vascular endothelium (Davies, 2015; Dashwood et al., 2011). In addition, the smooth muscle of the penis directly expanding the penile artery and indirectly promoting the activity of guanylate cyclase induces smooth muscle relaxation and erection (Fukuhara et al., 2011; Gao et al., 2017). Meanwhile, TDS has become the subject of academic interest and debate around the world. In the past

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decade, male hormone replacement therapy in male hypogonadism has been clinically proven to be beneficial to various organs (Shores and Matsumoto, 2014). Many of the males aged 60 and over are below the lower limit of young males in their 20 s and 30 s. Studies have shown that serum testosterone levels are lowered in men with hypogonadism, suggesting that hormone replacement therapy may contribute to improving quality of life (Gray et al., 1991; Harman et al., 2001; Wu et al., 2008). Despite studies of the usefulness of hormone replacement therapy, however, there is a controversy over the long-term effects, safety, and functional benefits of delaying geriatrics (Gruenewald and Matsumoto, 2003; Muller et al., 2003; Rhoden and Morgentaler, 2004). Therefore, it is required to develop health functional food materials which can relieve of TDS using natural materials. *Lespedeza cuneata* has been reported to improve aluminium toxicity and phosphorus deficiency (Sun et al., 2008), blood circulation (Lee et al., 2012), and hepatoprotective effect against oxidative stress (Kim et al., 2011). However, little is known about the mechanism of treatment and prevention for such disease.

In this study, we prepared the aqueous extracts from *Lespedeza cuneata* (ALC) and investigated the effect of ALC on ED by evaluating the vasodilatory factors such as NO, NOS, cGMP and PGE₂ in the corpus cavernosum and on TDS by evaluating the testosterone related hormone levels in the blood.

Materials and methods

Extraction of ALC

Lespedeza cuneata was collected in the area of Goksung, Jeonnam province Korea, in October 2016. A voucher specimen *L. cuneata* was authenticated by College of Pharmacy, Duksung Women's University. A 15 g dried preparation was extracted with 10 times volume (150 mL) of boiling distilled water at 95 °C for 12 ± 1 h. The aqueous extract was centrifuged at 516×g for 20 min at 4 °C and filtered through Whatman filter paper. The resulting supernatant was lyophilized to produce a powder (ALC, yield 15.3%), which was kept at 4 °C. The ALC was dissolved in normal saline (JW pharmaceutical, Korea) before administration to animals.

Animals and administration of samples

The animal procedures were in strict accordance with the National Institutes of Healthy Guidelines for the Care and Use of Laboratory Animals and were approved by the Animal Institutional Care and Utilization Committee of Dankook University (DKU-17-008). 12 weeks of male

Sprague–Dawley rats (weighing 350–380 g) were purchased from Orient Bio Co. (Daejeon, Korea). Rats were randomly divided into three groups, control group, 100 mg/Kg of ALC-treated group, and 250 mg/Kg of ALC-treated group (n = 10). The ALC was orally administered once a day for 30 days and the same amount of normal saline was administered to the control group. At the next day of last treatment, rats were anesthetized by intraperitoneal injection of 10 mg/kg zoletil and 5 mg/kg rompun. The lower abdomen of a rat was dissected to collect blood from the abdominal aorta and the spleen, liver, testes, and cavernosum were removed. The serum was collected from whole blood by centrifugation at 516×g and used for safety evaluation and vascular tone function evaluation. The weight of spleen and testis was measured for safety evaluation at the day of sacrifice. The cavernosum was homogenized by crushing Ultra-Turnax T25 (IKA-Lab, Germany) with 0.1 M potassium phosphate buffer (pH 7.4) and centrifuged with 516×g at 4 °C. After centrifugation, the supernatant was separated and used as a source of sexual function evaluation.

Safety evaluation

The body weight was measured once a week. After sacrifice, the weight of the spleen was compared with the control group. The hepatotoxicity (AST/ALT, Biovision, Milpitas, CA, USA), renal toxicity test (BUN, Mybioscience, Beverly, MA, USA), lactate dehydrogenase (LDH, Mybioscience) and gamma-glutamyl transferase transpeptidase (r-GT, Mybioscience) were evaluated using sandwich enzyme-linked immunosorbent assay (ELISA) as a manufacturers instruction. Total cholesterol, LDL-C, HDL-C, and triglyceride measurement (Mybioscience, Beverly, MA, USA) and glucose were also evaluated using enzyme immunoassay (EIA) kit (Biovision).

NO evaluation in vitro

For in vitro study, human umbilical vein endothelial cells (HUVECs) were purchased from LONZA (Walkersville, MD) and cultured in EGM-2 supplemented with 2% fetal bovine serum (FBS), 0.04% hydrocortisone, 0.4% human fibroblastic growth factor-B (hFGF-B), 0.1% vascular endothelial growth factor (VEGF), 0.1% insulin-like growth factor-1 (IGF-1), 0.1% ascorbic acid, 0.1% human epidermal growth factor (hEGF), 30 µg/mL gentamicin, 15 ng/mL amphotericin, and 1% heparin, obtained from LONZA. All media were also supplemented with 100 units/ml of penicillin and 100 µg/ml of streptomycin. HUVECs were seeded at a density of 1×10^6 onto 6-well plates and allowed to adopt for 24 h. After treated with various concentration of ALC, the supernatant was

Fig. 1 Safety evaluation of ALC. The body weight was measured once a week (A) and the testicle weight (B) was measured finally after sacrifice. The hepatotoxicity (C, D), γ -GT (E), LDH (F), and renal toxicity test (BUN, G) were evaluated using ELISA as a manufacturers instruction. Each experiment was repeated three times and the values are expressed as mean $\pm$ SD. The comparisons between three groups were performed by analysis of one way ANOVA, Tukey test. There was no significant change of the weight, testicle, and hepatotoxicity in ALC administrated rat compared the control group

collected after 24 h. The supernatant was removed, mixed the same volume of Greiss solution (Sigma, St. Louis, MO), and measured using microplate reader (TECAN, Austria) at 450 nm.

NO, nNOS, cGMP, and PGE₂ evaluation in vivo

Nitric oxide (NO), nitric oxide synthase, cyclic guanosine monophosphate (cGMP), and prostaglandin E₂ (PGE₂) were assessed for vascular tone function evaluation. For NO evaluation, homogenized cavernosum supernatant was assessed by NO assay kit (Sigma, St. Louis, MO, USA).

For nitric oxide synthase evaluation, the homogenized cavernosum was treated with 1 μ M ionomycin and incubated for 2 h. Then, the supernatant was collected centrifuging at 516 $\times$ g transferred separately and measured using neuronal NOS kit (R & D, Minneapolis, MN, USA). For cGMP evaluation, a sample was prepared by adding 1:4 ethanol to homogenized cavernosum and centrifuging at 516 $\times$ g for 10 min to remove non-specific protein. The supernatant was lyophilized by vacuum, dissolved with the same volume of PBS, and measured using cGMP EIA kit (Cayman, Ann Arbor, MI, USA). For PGE₂ evaluation,

Fig. 2 Safety evaluation of ALC. Triglyceride (A), total cholesterol (B), LDL-C (C), HDL-C (D), and glucose (E) were evaluated using EIA as a manufacturers instruction. Each experiment was repeated three times and the values are expressed as mean $\pm$ SD. The comparisons between three groups were performed by analysis of one way ANOVA, Tukey test. A decrease of triglyceride and glucose was observed in 100 mg/Kg ALC treated group compared to the control group, however there was no significance on cholesterol, HDL, and LDL between control group and ALC treated group (* $p < 0.1$)

serum was removed from whole blood, and measured using a prostaglandin EIA kit (Cayman).

DHT, testosterone, SHBG, and 17 β -HSD evaluation in vivo

Dihydro-testosterone (DHT), testosterone, sexually-hormone-binding globulin (SHBG), and 17-beta hydroxysteroid dehydrogenases (17 β -HSD) were assessed for sexual function evaluation. The homogenized cavernosum was diluted 3 times with each assay buffer. The concentration of DHT, testosterone, and 17 β -HSD was measured by ELISA kit which was purchased from Mybioscience. The concentration of SHBG was measured by SHBG ELISA kit (Cusabio, Temecula, CA, USA).

Statistical analysis

The results are expressed as mean $\pm$ SD. Unpaired Student's t-tests were used for comparisons between two means. The comparisons between three groups were performed by analysis of one way ANOVA, Kruskal–Wallis test (Graph Pad, Prism®, La Jolla, CA). * $p < 0.1$, ** $p < 0.05$, and *** $p < 0.001$ were considered statistically significant.

Results and discussion

In this study, we performed vascular dilation, sexual function evaluation, and safety evaluation of ALC administered male rats. There was no correlation between body weight reduction in the control group and ALC treated group (Fig. 1A). There was no change in the weight of the

Fig. 3 Effect of ALC on the level of NO, NOS, cGMP, and PGE₂. **(A)** NO evaluation in vitro; the various concentration of ALC was added to the HUVECs and the level of NO was measured after 24 h incubating. The level of NO was significantly increased after ALC treatment. **(B)** NO evaluation in vivo; NO evaluation was assessed by NO assay kit using homogenized cavernosum supernatant. The level of NO in ALC treated group was significantly increased. **(C)** Nitric oxide synthase evaluation was assessed by nNOS kit using the homogenized cavernosum after 1 μ M ionomycin treatment for 2 h. The level of nNOS in ALC treated group was increased. **(D)** cGMP evaluation was assessed using cGMP EIA by kit. The level of cGMP in ALC treated group was increased. **(E)** PGE₂ evaluation was assessed by prostaglandin EIA kit. There was no change in PGE₂ levels. Each experiment was repeated three times and the values are expressed as mean $\pm$ SD. The comparisons between three groups were performed by analysis of one way ANOVA, Tukey test (* p < 0.1, ** p < 0.05, and p^{***} < 0.005)

testicle in ALC administrated group comparing the control group (Fig. 1B). We also assessed the level of AST/ALT (Fig. 1C, D), γ -GT (Fig. 1E) and LDH (Fig. 1F) as hepatotoxicity evaluation and BUN (Fig. 1G) as renal toxicity. There was no significant hepatotoxicity in ALC treated group. Whether to check the lipid metabolism abnormality after ALC administration, we assessed the level of triglyceride (Fig. 2A), cholesterol (Fig. 2B), HDL (Fig. 2C), LDL (Fig. 2D) and glucose (Fig. 2E). A decrease of triglyceride and glucose was observed in 100 mg/Kg ALC treated group compared to the control group, however there was no significance on cholesterol, HDL, and LDL between control group and ALC treated group.

In order to evaluate the NO production effect of ALC on the blood vessels, the various concentration of ALC was added to the HUVECs and the level of NO was measured

after 24 h incubating. As shown in Fig. 3(A), the level of NO was significantly increased as 6.89 ± 0.18 mM at 100 mg/mL, 6.97 ± 0.28 mM at 200 mg/mL, and 7.58 ± 0.19 mM at 500 mg/mL in a dose-dependent manner compared to control group (6.18 ± 0.29 mM). Penile erection refers to the phenomenon of blood stagnation in the corporal sinusoid due to the veno-occlusion of the penile artery (Burnett, 2006b; Muniz et al., 2013) and simultaneous relaxation of the penile acornosal helical arteries and the smooth muscle of the penile cavernosum through a complex renal artery inertia process (Dashwood et al., 2011; Derar et al., 2017; Groneberg et al., 2013). NO had been known to act as a major mediator in this process.

In vivo study, the level of NO in ALC treated group was significantly increased as 4.27 ± 0.66 mM at 100 mg/Kg and 5.37 ± 0.11 mM at 250 mg/Kg compared to control group (3.70 ± 0.65 mM) (Fig. 2B). The level of nNOS in

Fig. 4 Effects of ALC on the level of DHT, testosterone, SHBG, and 17 β -HSD. The homogenized cavernosum was diluted 3 times with each assay buffer. The concentration of DHT (A), testosterone (B), 17 β -HSD (C), and SHBG (D) was measured by ELISA kit as manufacturer's instructions. The level of DHT and 17 β -HSD was increased in 250 mg/Kg ALC treated group, but there was no significant change in testosterone and SHBG. Each experiment was repeated three times and the values are expressed as mean $\pm$ SD. The comparisons between three groups were performed by analysis of one way ANOVA, Tukey test (* $p < 0.1$)

ALC treated group was also increased as 6.92 ± 0.48 U/L at 100 mg/Kg and 7.30 ± 0.21 U/L at 250 mg/Kg compared to control group (6.19 ± 0.35 U/L) (Fig. 3C). Meanwhile, the level of cGMP was increased as 0.034 ± 0.007 nM at 100 mg/Kg and 0.037 ± 0.015 nM at 250 mg/Kg compared to control group (0.022 ± 0.014 nM) (Fig. 3D). There was no change in PGE₂ levels (Fig. 3E). NO relaxation of smooth muscle promotes the formation of guanylate cyclase, which converts NO to 5-guanosine triphosphate (GTP) into 3,5-cyclin guanosine monophosphate (cGMP), followed by guanylate cyclase to activate cGMP- resulting in a decrease in intracellular calcium and consequent relaxation of the vascular smooth muscle. NO is mediated by the nitric oxide synthase (NOS) family of enzymes, which are made by converting L-arginine to L-citrulline via multiple steps of redox-reactions (Groneberg et al., 2013; Zheng et al., 2007). The isoforms of NOS include inducible NOS (iNOS), endothelial NOS (eNOS), and neuronal NOS (nNOS) (Andersen et al., 2010; Masuda, 2008). Although iNOS is produced by specific cytokines as a part of the immune response by macrophages, eNOS and nNOS are essentially produced in endothelial cells and neurons (Dashwood et al., 2011; Fukuhara et al., 2011; Liu et al., 2015). Activation of NO is activated by increasing concentrations of calcium and calmodulin (Derar et al., 2017; Zheng et al., 2007). Relatively, the concentration of PGE₂ did not change (Fig. 3E). Although PG receptors have been implicated in the erectile function of the corpus

cavernosum, penile artery, and penile smooth muscle (Davies, 2015; Fukuhara et al., 2011), ALC might inhibit the production of phosphodiesterase and lead to maintain or increase the production of cGMP independent of the PG receptor.

As shown in Fig. 4(A), the level of DHT was significantly increased as 400.03 ± 53.75 pg/mL at 100 mg/Kg and 495.06 ± 89.41 pg/mL at 250 mg/Kg compared to control group (316.71 ± 57.56 pg/mL), respectively. However, the level of total testosterone showed no difference (Fig. 4B). The level of HSD17B13 was increased as 0.634 ± 0.078 ng/mL at 100 mg/Kg and 0.753 ± 0.0056 ng/mL at 250 mg/Kg compared to control group (0.559 ± 0.073 IU/L) (Fig. 4C). The level of SHBG showed no difference (Fig. 4D). Sexual function is generally known to be independent of testosterone levels (Efesoy et al., 2018; Groneberg et al., 2013), but TDS due to this decrease results in psychological sexual dysfunction (Ho et al., 2016). Patients with impaired gonadal function cause fatigue, depression, weakness, and loss of confidence. Generally, if the free testosterone level is less than 70 ng/dL or the total testosterone level is less than 300 ng/dL, the patient is classified as hypogonadal (Efesoy et al., 2018; Ho et al., 2016). In this study, the total testosterone level in ALC group did not change, but DHT and HSD17B13 were increased which has been involved in androgenic activity (Celik and Yucel, 2013; Kataoka et al., 2013). This increase in androgenic activity related hormones such as DHT and HSD17B13 by ALC suggests that they not only

promote erection but also can lead to a significant improvement in TDS.

Phosphodiesterase type V (PDE5) inhibitors such as sildenafil such as Viagra (Pfizer) inhibit cGMP degradation by inhibiting PDE5 and induce penile erection (Aslan et al., 2014; Greenspan and Barkin, 2012). However, the use of Sildenafil has been associated with several side effects, including a headache, redness, and color confusion due to the non-selective effects on various tissues (An and Jeong, 2005; Milman et al., 2008). Many recent studies have aimed to search for natural remedies and herbal remedies for erectile dysfunction without side effects. Among them, extracts of Hawthorn (Kim et al., 2000), *Caesalpine sappan* (Xie et al., 2000), *Uncaria rhynchophylla* (Goto et al., 2000), *Cordyceps sinensis* (Chiou et al., 2000), and *Gynostemma pentaphyllum* (Tanner et al., 1999) exhibit an anti-hypertensive effect by endothelium-dependent vasorelaxation or direct stimulation of NO-cGMP release. However, since the reports on TDS improvement of these extracts are still insignificant, the significance of vascular function and sexual function of ALC is considered to be of novelty. As a result of the safety evaluation of ALC, there was no spleen abnormality, weight loss, liver morphology. The biochemical blood test also revealed no hepatotoxicity, no renal toxicity, and no lipid metabolism abnormality even at a high concentration of 250 mg/Kg.

In conclusion, we experimentally proved that ALC had a potential to increase the production and secretion of NO, cGMP, nNOS, and free testosterone in penile tissues, and suggested that ALC could be developed as a health supplement for improving TDS.

Acknowledgements This research was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (2017R1D1A1B03030060) and the Korea Institute of Planning and Evaluation for Technology in Food, Agriculture, Forestry, and Fisheries (iPET) through the Technology Commercialization Support Program, funded by the Ministry of Agriculture, Food, and Rural Affairs (MAFRA) (816004-02-2), Republic of Korea.

Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

References

- An TG, Jeong JC. Effect of Ojawan on the ethanol-induced erectile-dysfunction in rats. Korean J. Orient Int. Med. 26: 605–614 (2005)
- Andersen ML, Guindalini C, Santos-Silva R, Bittencourt LR, Tufik S. Association analysis of endothelial nitric oxide synthase G894T gene polymorphism and erectile dysfunction complaints in a population-based survey. J. Sex. Med. 7: 1229–1236 (2010)
- Aslan Y, Guzel O, Balci M, Tuncel A, Yildiz M, Atan A. The impact of metabolic syndrome on serum total testosterone level in patients with erectile dysfunction. Aging Male Off. J. Int. Soc. Study Aging Male. 17: 76–80 (2014)
- Bivalacqua TJ, Champion HC, Mehta YS, Abdel-Mageed AB, Sikka SC, Ignarro LJ, Kadowitz PJ, Hellstrom WJ. Adenoviral gene transfer of endothelial nitric oxide synthase (eNOS) to the penis improves age-related erectile dysfunction in the rat. Int. J. Impot. Res. 12: S8–S17 (2000)
- Bivalacqua TJ, Musicki B, Usta MF, Champion HC, Kadowitz PJ, Burnett AL, Hellstrom WJ. Endothelial nitric oxide synthase gene therapy for erectile dysfunction. Curr. Pharm. Des. 11: 4059–4067 (2005)
- Brixius K, Middeke M, Lichtenthal A, Jahn E, Schwinger RH. Nitric oxide, erectile dysfunction and beta-blocker treatment (MR NOED study): benefit of nebivolol versus metoprolol in hypertensive men. Clin. Exp. Pharmacol. Physiol. 34: 327–331 (2007)
- Burnett AL. Nitric oxide in the penis—science and therapeutic implications from erectile dysfunction to priapism. J. Sex. Med. 3: 578–582 (2006a)
- Burnett AL. The role of nitric oxide in erectile dysfunction: implications for medical therapy. J. Clin. Hypertens. 8: 53–62 (2006b)
- Celik O, Yucel S. Testosterone replacement therapy: should it be performed in erectile dysfunction? Nephro Urol. Mon. 5: 858–861 (2013)
- Chiou WF, Chang PC, Chou CJ, Chen CF. Protein constituent contributes to the hypotensive and vasorelaxant activities of *Cordyceps sinensis*. Life Sci. 66: 1369–1376 (2000)
- Dashwood MR, Crump A, Shi-Wen X, Loesch A. Identification of neuronal nitric oxide synthase (nNOS) in human penis: a potential role of reduced neuronally-derived nitric oxide in erectile dysfunction. Curr. Pharm. Biotechnol. 12: 1316–1321 (2011)
- Davies KP. Development and therapeutic applications of nitric oxide releasing materials to treat erectile dysfunction. Future Sci. OA. (2015). <https://doi.org/10.4155/FSO.15.53>
- Derar D, Ali A, Tharwat M, Al-Sobayil F, Zeitoun MM. Erectile dysfunction in male dromedary camels: Clinical findings and changes in the nitric oxide metabolite, cardiac troponin I and testosterone concentrations. Theriogenology. 89: 201–205 (2017)
- Efesoy O, Cayan S, Akbay E. The effect of testosterone replacement therapy on penile hemodynamics in hypogonadal men with erectile dysfunction, having veno-occlusive dysfunction. Am. J. Men's Health. 12: 634–638 (2018)
- Fukuhara S, Tsujimura A, Okuda H, Yamamoto K, Takao T, Miyagawa Y, Nonomura N, Okuyama A. Vardenafil and resveratrol synergistically enhance the nitric oxide/cyclic guanosine monophosphate pathway in corpus cavernosal smooth muscle cells and its therapeutic potential for erectile dysfunction in the streptozotocin-induced diabetic rat: preliminary findings. J. Sex. Med. 8: 1061–1071 (2011)
- Gao L, Zhao Z, Guo F, Liu Y, Guo J, Zhao Y, Wang Z. Association of endothelial nitric oxide synthase polymorphisms with an increased risk of erectile dysfunction. Asian J. Androl. 19: 330–337 (2017)
- Goldstein I, Lue TF, Harin PN, Rosen RC, Steers WD, Wicker PA. Oral sildenafil in the treatment of erectile dysfunction. N. Engl J. Med. 338: 397–404 (1998)
- Goto H, Sakakibara I, Shimada Y, Kasahara Y, Terasawa K. Vasodilator effect of extract prepared from *Uncariae ramulus* on isolated rat aorta. Am. J. Chin. Med. 28: 197–203 (2000)
- Gray A, Feldman HA, McKinlay JB, Longcope C. Age, disease, and changing sex hormone levels in middle-aged men: results of the

- Massachusetts male aging study. *J. Clin. Endocr. Metab.* 73: 1016–1025 (1991)
- Greenspan MB, Barkin J. Erectile dysfunction and testosterone deficiency syndrome: the “portal to men’s health”. *Can. J. Urol.* 19: 18–27 (2012)
- Groneberg D, Lies B, König P, Jäger R, Friebe A. Preserved fertility despite erectile dysfunction in mice lacking the nitric oxide receptor. *J. Physiol.* 591: 491–502 (2013)
- Gruenewald DA, Matsumoto AM. Testosterone supplementation therapy for older men: potential benefits and risks. *J. Am. Geriatr. Soc.* 51: 101–115 (2003)
- Harman SM, Metter EJ, Tobin JD, Pearson J, Blackman MR. Longitudinal effects of aging on serum total and free testosterone levels in healthy men. *Baltim. Longitud. Study Aging J. Clin. Endocr. Metab.* 86:724–731 (2001)
- Ho CH, Wu CC, Chen KC, Jaw FS, Yu HJ, Liu SP. Erectile dysfunction, loss of libido and low sexual frequency increase the risk of cardiovascular disease in men with low testosterone. *Aging Male Off. J. Int. Soc. Study Aging Male.* 19: 96–101 (2016)
- Jouannet P, Wang C, Eustach F, Kold-Jensen T, Auger J. Semen quality and male reproductive health: the controversy about human sperm concentration decline. *APMIS.* 109: 333–344 (2001)
- Kataoka T, Hotta Y, Ohno M, Maeda Y, Kimura K. Limited effect of testosterone treatment for erectile dysfunction caused by high-estrogen levels in rats. *Int. J. Impot. Res.* 25: 201–205 (2013)
- Kim SH, Kang KW, Kim KW, Kim ND. Procyanidins in crataegus extract evoke endothelium-dependent vasorelaxation in rat aorta. *Life Sci.* 67: 121–131 (2000)
- Kim SM, Kang K, Jho EH, Jung YJ, Nho CW, Um BH, Pan CH. Hepatoprotective effect of flavonoid glycosides from *Lespedeza cuneata* against oxidative stress induced by *tert*-butyl hydroperoxide. *Phytother. Res. PTR.* 25: 1011–1017 (2011)
- Lee EJ, Kim HS, Kim BC, Hawang SW, Hwang SY. Effect of KH-305 on nitric oxide synthase activity and erectile dysfunction in young rats. *J. Korean Soc. Food Sci. Nutr.* 36: 305–310 (2007)
- Lee JK, Kang DG, Lee HS. Vascular relaxation induced by aqueous extract of *Lespedeza cuneata* via the NO-cGMP pathway. *J. Nat. Med.* 66: 17–24 (2012)
- Liu C, Lu K, Tao T, Zhang L, Zhang X, Jiang L, Huang Y, Guan H, Chen M, Xu B. Endothelial nitric oxide synthase polymorphisms and erectile dysfunction: a meta-analysis. *J. Sex. Med.* 12: 1319–1328 (2015)
- Masuda H. Significance of nitric oxide and its modulation mechanisms by endogenous nitric oxide synthase inhibitors and arginase in the micturition disorders and erectile dysfunction. *Int. J. Urol. Off. J. Jpn. Urol. Assoc.* 15: 128–134 (2008)
- Milman N, Burton CM, Iversen M, Videbaek R, Jensen CV, Carlsen J. Pulmonary hypertension in end-stage pulmonary sarcoidosis: therapeutic effect of sildenafil? *J. Heart Lung Transpl.* 27: 329–334 (2008)
- Muller M, van der Schouw YT, Thijssen JHH, Grobbee DE. Endogenous sex hormones and cardiovascular disease in men. *J. Clin. Endocr. Metab.* 88: 5076–5086 (2003)
- Muniz JJ, Lacchini R, Rinaldi TO, Nobre YT, Cologna AJ, Martins AC, Tanus-Santos JE. Endothelial nitric oxide synthase genotypes and haplotypes modify the responses to sildenafil in patients with erectile dysfunction. *Pharmacogenomics J.* 13: 189–196 (2013)
- Rhoden EL, Morgentaler A. Risks of testosterone-replacement therapy and recommendations for monitoring. *N. Engl. J. Med.* 350: 482–492 (2004)
- Shores MM, Matsumoto AM. Testosterone, aging and survival: biomarker or deficiency. *Curr. Opin. Endocrinol. Diabetes Obes.* 21: 209–216 (2014)
- Sun QB, Shen RF, Zhao XQ, Chen RF, Dong XY. Phosphorus enhances Al resistance in Al-resistant *Lespedeza bicolor* but not in Al-sensitive *L. cuneata* under relatively high Al stress. *Ann. Bot.* 102: 795–804 (2008)
- Tanner MA, Bu X, Steimle JA, Myers PR. The direct release of nitric oxide by gypenosides derived from the herb *Gynostemma pentaphyllum*. *Nitric Oxide* 3: 359–365 (1999)
- Wu FCW, Tajar A, Pye SR, Silman AJ, Finn JD, O’Neill TW, Bartfai G, Casanueva F, Forti G, Giwercman A, Huhtaniemi IT, Kula K, Punab M, Boonen S, Vanderschueren D. Hypothalamic-pituitary-testicular axis disruptions in older men are differentially linked to age and modifiable risk factors: the European male aging study. *J. Clin. Endocr. Metab.* 93: 2737–2745 (2008)
- Xie YW, Ming DS, Xu HX, Dong H, But PPH. Vasorelaxing effect of *Caesalpinia sappan* involvement of endogenous nitric oxide. *Life Sci.* 67: 1913–1918 (2000)
- Zheng H, Bidasee KR, Mayhan WG, Patel KP. Lack of central nitric oxide triggers erectile dysfunction in diabetes. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 292: R1158–1164 (2007)