Original Article

Argirein alleviates stress-induced and diabetic hypogonadism in rats via normalizing testis endothelin receptor A and connexin 43

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Aim: Argirein (rhein-arginine) is a derivative of rhein isolated from Chinese rhubarb (*Rheum Officinale* Baill.) that exhibits antioxidant and anti-inflammatory activities. In the present study we investigated the effects of argirein on stress-induced (hypergonadotrophic) and diabetic (hypogonadotrophic) hypogonadism in male rats.

Methods: Stress-induced and diabetic hypogonadism was induced in male rats via injection of isoproterenol (ISO) or streptozotocin (STZ). ISO-injected rats were treated with argirein (30 mg·kg⁻¹·d⁻¹, po) or testosterone replacement (0.5 mg·kg⁻¹·d⁻¹, sc) for 5 days, and STZ-injected rats were treated with argirein (40–120 mg·kg⁻¹·d⁻¹, po) or aminoguanidine (100 mg·kg⁻¹·d⁻¹, po) for 4 weeks. After the rats were euthanized, blood samples and testes were collected. Serum hormone levels were measured, and the expression of endothelin receptor A (ET_A), connexin 43 (Cx43) and other proteins in testes was detected. For *in vitro* experiments, testis homogenate was prepared from normal male rats, and incubated with ISO (1 μmol/L) or high glucose (27 mmol/L).

Results: ISO injection induced hyper-gonadotrophic hypogonadism characterized by low testosterone and high FSH and LH levels in the serum, whereas STZ injection induced hypogonadotrophic hypogonadism as evidenced by low testosterone and low FSH and LH levels in the serum. In the testes of ISO- and STZ-injected rats, the expression of ET_A, MMP-9, NADPH oxidase and pPKCε was significantly increased, and the expression of Cx43 was decreased. Administration of argirein attenuated both the abnormal serum hormone levels and the testis changes in ISO- and STZ-injected rats, and aminoguanidine produced similar actions in STZ-injected rats; testosterone replacement reversed the abnormal serum hormone levels, but did not affect the testis changes in ISO-injected rats. Argirein (0.3–3 μmol/L) exerted similar effects in testis homogenate incubated with ISO or high glucose *in vitro*.

Conclusion: Two types of hypogonadism of male rats exhibit increased expression of ET_A and depressed expression of Cx43 in testes, despite different patterns of serum FSH and LH. Argirein alleviates the two types of male hypogonadism via normalizing ET_A and Cx43 in testes.

Keywords: argirein; rhein; male hypogonadism; hypergonadotrophic; hypogonadotrophic; ET_A; connexin 43; aminoguanidine; testosterone

Acta Pharmacologica Sinica (2016) 37: 246–254; doi: 10.1038/aps.2015.125; published online 18 Jan 2016

Introduction

The incidence of male hypogonadism is increasing in modern society; approximately 37.8% of males who are at least 45 years of age and visit a physician in the USA exhibit low testosterone in the serum (<300 ng/dL)^[1]. Male patients, especially patients with obesity, diabetic mellitus^[2] or hypertension,

manifest a significant increase in the incidence of hypogonadism relative to the age-matched population. Stress caused by endurance physical training substantially contributes to male hypogonadism, in part, as a result of an overactive sympathetic nervous system^[3].

One feedback system, referred to as the hypothalamic-pituitary-gonadal axis (HPG axis), is critical in the maintenance of the normal functions of the testis; however, these functions may be functionally perturbed in cases of stress or hyperglycemia, which results in insufficient testosterone levels in association with altered follicle stimulating hormone (FSH) and

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Received 2015-05-09 Accepted 2015-10-08

luteinizing hormone (LH) in serum^[4-6].

Defects in testes that result from many causal factors and induce low serum testosterone require drug interventions to improve physical, psychological and social activities; current treatments have depended on testosterone replacement therapy (TRT)^[7, 8], which relieves the symptoms and signs by increasing testosterone in serum. It has not been fully elucidated whether TRT relieves the abnormal changes in a damaged testis.

Based on emerging data, male hypogonadism may result due to an increase in pro-inflammatory cytokines, including activated endothelin receptor A (ET_A) and reactive oxygen species (ROS), and impaired intracellular communication via depressed connexin 43 (Cx43), which may result from the activation of NADPH oxidase (NOX) in the testis^[9-11]. Increasing evidence indicates that an overactivated ET_A is intimately linked to the activation of NOX, which produces more ROS and plays an important role in many cardiovascular diseases, as well as the pathologies of dysfunctional testes^[9]. Emerging data have suggested that the basal ET-1 level in patients with male hypogonadism is increased^[12], and our previous findings indicated that the ET-1, ECE (endothelin converting enzyme) and ROS levels are increased in thyrotoxicosis-induced testicular injury and altered in diabetic or adenine induced testopathy^[11, 13, 14]. Matrix metalloproteinase-9 (MMP-9) has a primary role in the modulation of the amount of extracellular matrix responsible for the normal structure and functions of the seminiferous tubules^[15]. Intracellular communication in the testis is unique for harmonizing testis function and is achieved by the normal expression of junctional protein connexin Cx43. Cx43 is severely affected by abnormal MMP-9, and its abnormality may participate in depressed steroidogenic acute regulatory protein (StAR) and 3 β -hydroxysteroid dehydrogenase (3 β -HSD) (two important enzymes responsible for testosterone biosynthesis); furthermore, increased Cx43 is associated with a recovery of low serum testosterone in male hypogonadism^[15]. Therefore, it is interesting to investigate interventions that may relieve an abnormal testis, using two models of hypogonadism, via the normalization of increased ET_A and decreased CX43 in testes.

Rhein is an effective ingredient that has been used in TCM (traditional Chinese medicine). It is isolated from Chinese rhubarb (*Rheum Officinale* Baill) and has been used alone^[16] or in a combined therapy^[17] in the treatment of diabetic nephropathy. The solubility of rhein is poor, and its $t_{1/2}$ is not sufficiently long; thus, chemical modification was conducted to improve its biological behaviors by connecting a moiety of *L*-arginine through a hydrogen bond. The novel compound exhibits antioxidant and anti-inflammatory activities with a prolonged $t_{1/2}$ ^[18, 19]. Following oral administration, the new compound argirein (rhein-arginine) releases both rhein and *L*-arginine, which may attenuate male hypogonadism with two patterns of gonadotrophic hormones.

We hypothesized that male hypogonadism is established by either sustained stress (as a result of isoproterenol administration) or diabetes (streptozotocin administration) in rats.

The two models of hypogonadism exhibit low testosterone and are different regarding the serum levels of FSH and LH. Upregulated ET_A, which is closely related to an activated NOX for ROS genesis^[20], may be upregulated to decrease the intracellular communication protein Cx43^[21]. In this study, we verified whether changes in ET_A and Cx43 may occur in the pathologies of the two models of male hypogonadotrophic hypogonadism, and the effects of argirein (rhein-arginine) was compared with TRT in the normalization of these changes in stress-induced male hypogonadism. In the treatment of diabetic male hypogonadism, aminoguanidine (AMG) was employed as a positive reference, which attenuates oxidative stress in diabetic mellitus^[22].

Materials and methods

Animals and chemicals

Male Sprague-Dawley (SD) rats, 220 $\pm$ 20 g in weight, were purchased from the Nanjing Experimental Animal Center and were maintained in a standard environment at a controlled temperature (20–25°C). Each rat was provided 15 g of standard chow and clean water each day. The restriction of food was useful for improving general conditions in the untreated rats^[14].

Argirein (rhein-arginine) (Figure 1) was synthesized at the Center of Traditional Chinese Medicine Processing, which is affiliated with Zhejiang Traditional Chinese Medicine University. Isoproterenol (ISO) injection, testosterone propionate and streptozotocin (STZ) were purchased from Shanghai Harvest Pharmaceutical Company (Shanghai, China) and Sigma (St Louis, MO, USA), respectively.

Two models of male hypogonadism *in vivo*

One hundred and four male SD rats, 180–210 g, were randomly employed into two experiments:

1. Stress induced hypogonadism: Thirty-two rats were divided into 4 groups ($n=8$): (1) normal; (2) ISO, 1 mg/kg, sc for 10 d; (3) and (4) ISO injected rats were administered (mg·kg⁻¹·d⁻¹, sc) testosterone (0.5) or argirein (30), respectively, on the last 5 days.

2. Diabetic hypogonadism: Sixty male rats were employed in the second experiment of hypogonadism, which was induced by a dose of STZ 65 mg/kg, ip^[14]. After 3 weeks of blood glucose monitoring at one week intervals, a diabetic rat model was confirmed when hyperglycemia was greater than 16.7 mmol/L^[14]. The biochemical and bioactive molecules were assessed at the end of 8 weeks. The intervention

Figure 1. The chemical structure of rhein-arginine (argirein) compound shows a hydrogen bond connecting the two moieties.

($\text{mg}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$, *po*) with argirein (40, 80 and 120) was compared with AMG (100) in the last 4 weeks. AMG has been demonstrated to be a positive drug to relieve diabetic lesions in rats^[22]. The experiment was conducted as follows: (1) normal; (2) STZ injected alone; (3) STZ injected and intervention with AMG; and (4), (5), and (6), intervened with multiple doses of argirein (40, 80 and 120 $\text{mg}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$). The normal and ISO or STZ treated rats were injected with 1% carboxymethylcellulose-Na (CMC-Na).

Serum biochemical measurements

The rats were anesthetized with urethane 1.5 g/kg *ip* and were euthanized via carotid artery bleeding on day 11 in experiment 1 or at the end of 8 weeks in experiment 2. The blood samples were collected, and the testes were harvested and stored in liquid nitrogen for subsequent analysis via PCR and Western blotting assays. Serum was collected to measure testosterone, FSH (follicle stimulating hormone) and LH (luteinizing hormone) with radioimmunoassays according to the manufacturer's instructions (Jiuding Medical Bio-engineering Company, Tianjin, China).

Two models *in vitro*

Twelve male rats were euthanized via cervical dislocation, and the testes were collected. The incubation of testis homogenate was produced at a ratio of 1:3 with KH solution, according to a previously reported method with modifications^[23]. In the experiment, the testis homogenate was employed to replace the isolated Leydig cells to investigate changes in the testis damaged by ISO or high glucose, and the beneficial effects were identified following argirein administration. An aliquot of the homogenate (0.3 mL) was distributed into two different experiments, which included RT-PCR and Western blotting assays, following incubation at 37°C for 4 h as follows:

1. ISO incubation: 1) Normal group; 2) ISO group (1 $\mu\text{mol/L}$); and 3) argirein (rhein-arginine) group (ISO+rhein-arginine 0.3, 1.0 and 3.0 $\mu\text{mol/L}$).

2. High glucose incubation: 1) Normal group; 2) High glucose group (27 mmol/L); and 3) Argirein group (high glucose + rhein-arginine 0.3, 1.0, and 3.0 $\mu\text{mol/L}$).

To further examine the efficacy of the argirein intervention in the two models *in vitro*, the effects were verified to occur in a dose-dependent and time-dependent manner.

RT-PCR

The mRNA expression of ET_A , NOX p47phox, MMP-9, Cx43 and PKC ϵ was determined with reverse transcriptase polymerase chain reaction (RT-PCR)^[11]. The process was performed as briefly described. Total mRNA was extracted from the homogenate of the testis or an aliquot of the incubated medium with TRIzol Plus (Biouniquer Technology Company, Nanjing, China). The reverse transcription was performed with a BU-Script KIT(50) (Biouniquer Technology Company, Nanjing, China) as follows (in 20 μL): 4 μL 5 $\times$ BU-Script buffer, 1 μL dNTP mix (10 mmol/L), 1 μL Oligo (dT)₁₈ (10 $\text{pmol}/\mu\text{L}$), 1 μL 20 U/ μL RNase Inhibitor, 1 μL 20 U/ μL BU-Script Reverse

Transcriptase and 2 μL total RNA (1 $\mu\text{g}/\mu\text{L}$) and DEPC water were added to adjust the final volume to 20 μL . The prepared mixture was reversely transcribed to cDNA under the following conditions described in brief: 1) 30 °C, 10 min; 2) 42 °C, 60 min; 3) 99 °C, 5 min; and 4) 4 °C, 5 min. The cDNA products were subject to PCR reaction, and the PCR products were processed in parallel with β -actin. The band densities were analyzed using Labworks imaging acquisition and analysis software (GDS8000, Syngene, England).

Western blotting

The quantitative analysis of the ET_A , NOX p47phox, Cx43, PKC ϵ and peroxisome proliferator activated receptor α (PPAR α) proteins was performed as previously described^[13]. In brief, 200 mg of testis tissue was homogenized with 800 μL of lysis buffer (mmol/L) at pH 7.4, which comprised HEPES 20, 0.1% Triton X-100, NaCl 25, EGTA 2 and phenylmethylsulphonyl fluoride 1, and centrifuged at 12 000 r/min for 10 min. An aliquot of the supernatant or the incubated medium was processed for the subsequent analyses. The prepared protein solution was conducted on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), transferred onto nitrocellulose, blocked with nonfat milk (5% *w/v*), and subsequently incubated with primary and secondary antibodies sequentially: the primary antibodies included polyclonal goat anti- ET_A -IgG (Wuhan Boster Biological Technology, China), polyclonal rabbit anti-NOX p47phox-IgG (Affinity Bioreagents, USA), polyclonal rabbit anti-MMP-9-IgG (Wuhan Boster Biological Technology, China), polyclonal rabbit anti-connexin 43-IgG (Wuhan Boster Biological Technology, China), polyclonal rabbit anti-phosphorylated-PKC ϵ (Ser729)-IgG and anti-PPAR α (Upstate, USA), and polyclonal rabbit anti-Actin-IgG (Wuhan Boster Biological Technology, China); horseradish peroxidase-conjugated IgG was used as the secondary antibody (Wuhan Boster Biological Technology). Antigen was detected with a 3,3'-diaminobenzidine (DAB) kit (Wuhan Boster Biological Technology, China), and the samples were subsequently processed in parallel with β -actin. The density of the bands was analyzed using Labworks imaging acquisition and analysis software (GDS8000, Syngene, England).

Statistical analysis

Sigma Plot 9.0 (SPSS Inc, USA) was applied for the data processing, and the results were presented as the mean $\pm$ SD. Student's *t*-test was used to determine the statistical significance for differences between two mean values, and a one-way analysis of variance (ANOVA) was used followed by a Bonferroni test. The difference was considered statistically significant at $P<0.05$.

Results

Serum testosterone, FSH and LH following ISO administration

Following ISO administration for 10 days, the serum testosterone level was significantly decreased ($P<0.01$), which indicated a status of hypogonadism, and was accompanied by signifi-

Figure 2. Testis deficiency in ISO-injected rats: decreased testosterone and high FSH and LH in serum presented the characteristics of hypergonadotrophic hypogonadism. These changes were ameliorated by argirein (rhein-arginine) and a supplement of testosterone by testosterone propionate. (A) Low testosterone; (B) High FSH; (C) High LH. Mean±SD. $n=8$. ^c $P<0.01$ vs normal. ^e $P<0.05$, ^f $P<0.01$ vs ISO.

cantly increased LH and FSH ($P<0.01$) compared with normal. This finding indicated that the HPG axis responded well to a decrease in the serum testosterone by ISO, and a model of hypogonadotrophic hypogonadism was established. Following argirein (rhein-arginine) intervention for the last 5 days, these changes were substantially restored ($P<0.05$, $P<0.01$) compared with ISO alone (Figure 2). Testosterone propionate induced a remarkable increase in the serum testosterone and reduced the increased FSH and LH in the serum.

Abnormal ET_A , NOX, MMP-9, PKC ϵ and Cx43 *in vivo*

The administration of ISO led to a series of changes in the testis, including a remarkable upregulation of the mRNA and protein levels of ET_A , NOX p47phox, MMP-9, and mRNA of PKC ϵ and the protein ratio of pPKC ϵ /PKC ϵ , as well as a downregulation of Cx43 ($P<0.01$). These changes were alleviated following argirein administration in the last 5 d ($P<0.01$) compared with the ISO group. No response to testosterone supplementation was identified (Figure 3).

Serum testosterone, FSH and LH in diabetic testis

Eight weeks following a whole dose of STZ, the serum testosterone was significantly decreased; however, the FSH and LH responses were significantly decreased. The diabetic testis exhibited a hypogonadotrophic property, and the abnormalities were attenuated by AMG and argirein. In the serum, the insulin levels were significantly decreased, and there was no response to argirein or AMG (Figure 4).

Abnormal ET_A , Cx43 and PPAR α in diabetic testis *in vivo*

Diabetes led to male hypogonadism associated with changes in the testis as follows: upregulated mRNA and protein levels of ET_A and downregulated mRNA and protein levels of Cx43 were identified ($P<0.01$) relative to normal. Decreased PPAR α was also associated with these changes. These changes were relieved by argirein and AMG administration in the last 4 weeks ($P<0.01$) (Figure 5).

ET_A , NOX and Cx43 *in vitro*

In the present experiment, the testicular homogenate preparation was incubated with ISO or high glucose *in vitro* to mimic conditions under stress or diabetes, respectively. Following a short exposure for 4 h, there was a significant upregulation of mRNA and a protein abundance of ET_A and p47phox, whereas Cx43 was notably decreased in the two tests *in vitro* relative to normal ($P<0.05$, $P<0.01$, respectively). Significant relief was induced by argirein ($P<0.05$, $P<0.01$, respectively) compared with ISO or high glucose alone (Figure S1, Figure S2).

To test the effects induced by argirein *in vitro*, three doses were employed in the models of testis homogenate incubated with ISO or high glucose, respectively. Beneficial effects on upregulated ET_A and NOX p47phox and decreased CX43 occurred in a dose-dependent manner in the two models (Figure 6, 7). The experiment was conducted over time, and the variables were evaluated at three different time points. The results demonstrated a relief of abnormal CX43 in a time dependent manner in the two models (Figure 6, 7).

Discussion

The main findings indicated that the models of hypergonadotrophic and hypogonadotrophic hypogonadism were independently established by stress (ISO-injected) and diabetes (STZ-injected) in rats, respectively. The abnormal serum hormone levels were significantly corrected following the administration of testosterone, argirein, and AMG, respectively.

The compromised biosynthesis of testosterone was a result of oxidative stress, which was caused by an activated NOX involved in the testis. A subtype of p47phox is required as an “organizer” protein, which is necessary in the process of full NOX activation^[24]. The interaction of p47phox with other components, such as p22phox, further facilitates the full activation of the oxidase^[25]. An activation of NOX p47phox in diabetic nephropathy was identified in relation to ET_A activation and was suppressed by the endothelin receptor antagonist CPU0213, which indicates a close link between the ET system

Figure 3. Upregulation of ET_AR (ET_A), NADPH oxidase p47phox, MMP-9 and PKCε (pPKCε), and downregulation of Cx43 were found in the ISO injected rats *in vivo* and were significantly attenuated by arginine, but the hypergonadotrophic rats had no response to testosterone propionate. (A and B) ET_A; (C and D) NADPH oxidase p47phox; (E, F) MMP-9; (G and H) Cx43; (I and J) PKCε and pPKCε/pKCε. Mean±SD. *n*=8. °*P*<0.01 vs normal. °*P*<0.05, °*P*<0.01 vs ISO.

and NOX, as identified in the present study^[10].

Excess ROS negatively impact the reproductive system, which results in abnormal testis function and sperm quality^[26]. Oxidative stress heavily injures mitochondrial function, which in turn facilitates the abnormal reactions in the respiratory chain, ie, decreased energy provision that leads to reduced protein biosynthesis involved in both sperm generation and

testosterone biosynthesis in the testis^[26–27].

A normal ET system is essential to maintain testicular function and morphology. ET-1 rhythmically produced by the seminiferous epithelium and Sertoli's cells initiates rhythmic contractility of the muscle-like cells that surround the tubules, which transports sperm from the testis to the epididymis^[28]. In adenine medicated rats, the ET system was markedly

Figure 4. Diabetic testopathy in STZ-injected rats: Decreased testosterone was predominant in association with a decreased FSH and LH in serum, characterized as hypogonadotrophic hypogonadism in rats. The abnormalities were ameliorated significantly by arginine 40, 80, and 120 mg·kg⁻¹·d⁻¹ compared with AMG. (A) Low testosterone; (B) Low FSH; (C) Low LH; (D) Low insulin in serum. Mean±SD. *n*=8. °*P*<0.01 vs normal. °*P*<0.01 vs STZ.

Figure 5. Upregulation of ET_AR (ET_A) and downregulation of Cx43 and PPARα were found in STZ injected rats *in vivo* and were ameliorated significantly by arginine 40, 80, and 120 mg·kg⁻¹·d⁻¹. (A and B) ET_AR; (C and D) Cx43; (E) PPARα. Mean±SD. *n*=8. ^c*P*<0.01 vs normal. ^e*P*<0.05, ^f*P*<0.01 vs STZ.

Figure 6. The dose-dependent and time-dependent effects of arginine on isoproterenol (ISO) 1.0 μmol/L incubated testis homogenate *in vitro*. Up-regulation of mRNA and protein expressions of ET_AR (ET_A), NADPH oxidase p47phox and downregulation of Cx43 were dose-dependently ameliorated by arginine from 0.3 to 3.0 μmol/L. In addition, arginine time-dependently reversed downregulation of mRNA and protein expressions of Cx43 in isoproterenol incubated medium. (A and B) Dose-dependent effect of arginine on ET_AR; (C and D) Dose-dependent effect of arginine on Cx43; (E and F) Dose-dependent effect of arginine on p47phox; (G and H) Time-dependent effect of arginine on Cx43 expression. Mean±SD. *n*=6. ^c*P*<0.01 vs normal. ^e*P*<0.05, ^f*P*<0.01 vs ISO.

Figure 7. The dose-dependent and time-dependent effects of argirein on high glucose-incubated testis homogenate *in vitro*. The up-regulation of mRNA and protein expressions of ET_AR (ET_A), NADPH oxidase p47phox and downregulation of Cx43 were dose-dependently ameliorated from 0.3 to 3.0 μmol/L. In addition, argirein time-dependently reversed downregulation of mRNA and protein expressions of Cx43 in high glucose incubated medium. (A and B) Dose-dependent effect of argirein on ET_AR; (C and D) Dose-dependent effect of argirein on Cx43; (E, F) Dose-dependent effect of argirein on p47phox; (G and H) Time-dependent effect of argirein on Cx43 expression. Mean±SD. *n*=6. ^b*P*<0.05, ^c*P*<0.01 vs normal. ^e*P*<0.05, ^f*P*<0.01 vs high glucose.

suppressed and associated with serious damage to the testis^[11]. However, an overactivated ET system has also been demonstrated to cause testis malfunction, which is relevant to the potent contraction of capillaries in the testis and results in an insufficient blood supply^[29], the promotion of ROS generation and the subsequent induction of apoptosis of testis cells^[14]. As demonstrated in the present study, these changes were reproduced by a simple approach that includes the incubation of testis homogenate with ISO or high glucose *in vitro*, and NOX activation is likely involved independently. The responses to argirein were mild *in vitro* because the exposure to argirein was brief and occurred in only the last 2 h. In general, the effect of argirein on the diseased testes occurred in a dose-related and time-dependent manner.

Increased MMP-9 expression may induce an abnormality of testicular remodeling, which adversely affects degradation and recombination of the extracellular matrix, and thus relates to fibro-proliferative reactions in testicular tissues^[30]. It is consistent with our previous findings that ROS and the activated ET_A regulate MMP-9 to mediate the fibrosis process in the vasculature, as well as diabetic testis^[31–33].

In addition to the PKA (protein kinase A) signaling pathway, the PKCε signaling pathway is activated in myocardial cells by ISO, ET-1 or H₂O₂^[33]. Increased phosphorylated PKCε

(a ratio of pPKCε/PKCε protein) may predicate an increase in the pro-inflammatory reactions as a result of ET_A activation, which may represent a potential target for drug intervention to relieve hypergonadotrophic hypogonadism.

Spermatogenesis is continuously subjected to the process of proliferation and differentiation of germ cells, which requires highly coordinated cellular interactions via intercellular junctions. Cx43 is the most abundant protein that communicates signals through gap junctions in the vertebrate testis, and gap junctions between Sertoli cells and spermatogonia are critically involved in controlling germ cell survival^[34–35]. Abnormal Cx43 participates in diabetic lesions, such as in diabetic nephropathy^[36]. In this study, the downregulated Cx43 caused by ISO or diabetes *in vivo* induces basic pathological changes in testis dysfunction. Thus, we confirmed that depressed Cx43 expression is as important as abnormal ET_A implicated in the defective testis that leads to male hypogonadism, which may occur under physical and mental stress or diabetes mellitus. These changes were relieved by argirein. The beneficial effect of argirein on the diabetic testis was as effective as AMG. Testosterone supplementation did not relieve these changes in the testis, with the exception of correcting serum hormones. Downregulated PPARα is a sign of pathological changes in the testis, and increased PPARα caused by PPARα agonists

is beneficial to diabetic vascular lesions^[37]. Furthermore, an upregulation of the decreased PPAR α by argirein may indicate an improved capillary circulation in the diabetic testis.

The induction of an abnormal testis via a whole dose of STZ injected in rats is a model of type 1 diabetes, with a significant decrease in serum insulin; the reduced serum insulin did not respond to AMG or argirein. In contrast, the rat model of type 2 diabetes caused by high fat and high sugar and a low STZ dose induced increased insulin in the serum as a result of insulin resistance, which was significantly alleviated by argirein^[35].

The theoretical consideration of male hypogonadism is complex, and in our previous study, the abnormal expression of the main genes related to testosterone biosynthesis was due to endoplasmic reticulum stress and NOX activation^[10, 35]. Here, we report that abnormal ET_A and CX43 are actively implicated in the diseased testes, which is consistent with the findings in our previous studies^[11, 38].

Limitations: the *in vitro* tests reproduce data that resemble the *in vivo* data; however, we did test in a simply way using testis homogenate instead of the incubation of cells, such as Leydig cells, isolated from the testis^[15, 35]. Thus, the results of the *in vitro* test do not indicate injury of the reproductive epithelial cells, Sertoli cells or Leydig cells individually; instead, the testis as a whole is considered affected under these conditions.

In summary, in male hypogonadism, an overactivation of the ET_A pathway with NOX activation is definitely involved in the molecular mechanisms of defective testes that contribute to the two types of male hypogonadism. Argirein is possibly promising in correcting the two types of hypogonadism via the normalization of abnormalities in ET_A, NOX and Cx43 in the affected testis.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (No 81570413 and 81070145).

Author contribution

De-zai DAI designed research; Ming XU, Chen HU, Hussein-hamed KHAN, Fang-hong SHI and Qing LI performed research; Xiao-dong CONG contributed new analytical tools and reagents; Yin DAI analyzed data; De-zai DAI and Ming XU wrote the paper.

Supplementary information

Supplementary materials were available on Acta Pharmacologica Sinica's web site.

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