RESEARCH

PACAP ameliorates the fertility of obese mice through PAC1/PKA/ERK/Nrf2 signal axis

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Abstract

Obesity is strongly linked to male infertility. Apoptotic inflammatory response caused by oxidative stress in testicular spermatogenic cells is one of the important causes of obesity-related male infertility. As bioactive peptide is secreted by the pituitary gland, pituitary adenylate cyclase activating polypeptide (PACAP) has a powerful triple role of anti-oxidation, anti-apoptosis, and anti-inflammation, and it is involved in the male reproduction regulation, but the specific mechanism remains unknown. The purpose of this study is to explore the role of PACAP in obesity-related male infertility. In cellular level experiments, mouse spermatocytes (GC-2) were treated with palmitate (PA) to establish an high-fat injury cell model *in vitro* and then treated with PACAP. In animal-level experiments, C57BL/6 male mice were fed with a high-fat diet (HFD) to induce obesity and subsequently treated with PACAP. The cell mechanism studies show that PACAP selectively binded to the PAC1 receptor to attenuate palmitic acid-induced mouse spermatogenic cell (GC-2) oxidative damage and apoptotic inflammatory response via the PKA/ERK/Nrf2 signaling axis. However, this mechanism was inhibited in GC-2 cells inhibiting the activity of Nrf2. The animal experiment studies show that PACAP treatment ameliorated obesity characteristics, including body weight, epididymal adipose weight, testes/body weight, serum lipids levels, and reproductive hormone levels *in vivo*. Additionally, PACAP was shown to improve the reproductive function of the obese mice, which was characterized by improved testis morphology and sperm parameters via Keap1/Nrf2/ARE pathway. These beneficial effects of PACAP were abolished in obese mice with testes-specific knockdown of Nrf2. Our data suggested that PACAP could ameliorate fertility in obese male mice and may be a promising candidate drug for obesity-induced male infertility.

Key Words

- ▶ obesity
- ▶ PACAP
- ▶ Nrf2
- ▶ oxidative stress
- ▶ male infertility

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Introduction

As the incidence of obesity induced by overnutrition continues to increase, the incidence of metabolic syndrome and some common metabolic diseases such as hypertension, coronary heart disease, and type 2 diabetes, which are often complicated or caused by obesity, are also

on the rise (Caballero 2007, Cabler *et al.* 2010). In addition to complications such as cardiovascular diseases, scientists have recently begun to realize that people with obesity or overweight showed an increasing incidence of infertility, and some manifestations of paternal obesity may have a

negative impact on basic sperm parameters, such as total sperm concentration and motility (Hammoud *et al.* 2008, Palmer *et al.* 2012).

Obesity can cause disturbance of the endocrine axis and affect male sex hormone secretion. In obese patients, the concentration of testosterone, gonadotropin and inhibin B are decreased and estrogen are increased (Kley *et al.* 1980). In addition, many studies have shown a relationship between fat abnormalities and infertility, but the detailed mechanism is still unclear (Ramírez-Torres *et al.* 2000, Shalaby *et al.* 2004). Excessive accumulation of fat in the testis and epididymis can directly lead to abnormal structure of the testis and epididymis and also release inflammatory factors, reactive oxygen species (ROS) and oxidative free radicals, affecting the endocrine function of testicular tissue, which have varying degrees of impact on both cell proliferation and spermatogenesis (Bachir & Jarvi 2014). More and more studies have shown that obesity has a significant negative effect on the male reproductive system (Galliano & Bellver 2013, Landry *et al.* 2013). Compared with those of a normal body weight, obese patients are more likely to accumulate ROS, leading to oxidative stress (Halliwell & Cross 1994). Oxidative stress is considered to play an important role in nutritional obesity-induced male infertility, which is one of the important reasons for obesity-induced male infertility, therefore, an important measure to prevent the occurrence of nutritional obesity-induced male infertility is to improve the antioxidant capacity of the patient's testicular tissue (Liu *et al.* 2020).

Therefore, there is an urgent need to find effective ways to prevent and treat obesity-induced male infertility. Many studies have found that pituitary adenylate cyclase activating polypeptide (PACAP), a potentially valuable neuroprotective peptide, has two forms: PACAP38 and PACAP37. They have similar biological activities and play an important role in anti-oxidation, anti-apoptosis and anti-inflammation (Dohi *et al.* 2002). PACAP and its receptors, PAC1, VPAC1 and VPAC2, are widely distributed in the CNS and peripheral tissues, especially in the brain, pancreas, fat, muscle and testis (Vaudry *et al.* 2009, Douiri *et al.* 2016). Moreover, testicular germ cells contain PAC1 receptors predominantly (Arimura 1992). Tanii *et al.* has found that PACAP could promote spermatogenesis in mice testes, improve sperm motility and ability to penetrate oocytes, and promote fertilization (Tanii *et al.* 2011). Reglodi *et al.* have found that PACAP promoted the expression of testosterone, collagen IV, and collagen IX through the PAC1-PKA-Sox9 axis and was involved in regulating the proliferation of spermatogenic cells,

sperm motility, and the formation of the blood-testis barrier (Reglodi *et al.* 2018). In short, PACAP is closely related to male infertility and can be used as a promising candidate drug to improve obesity-related male infertility.

The antioxidant signaling pathway Keap1/Nrf2/ARE is one of the important defensive transduction pathways for the body to resist internal and external environmental stress. The vicious cycle of oxidative stress and proinflammatory cytokine overexpression could be inhibited by activation of the Nrf2 signaling pathway (Itoh *et al.* 2010). Other experiments have shown that the antioxidant capacity of Nrf2 signaling pathway played a more important role in anti-apoptosis (Khodagholi & Tusi 2011). Besides, Keap1/Nrf2/ARE signaling pathway was activated in reproductive system diseases (Agarwal *et al.* 2018). Human sperm are particularly sensitive to oxidative stress, which is an important cause of male infertility. Enhancing Nrf2 expression helped reduce oxidative stress in patients with obesity (Liu & Ding 2017, Agarwal *et al.* 2018). Studies have shown that abnormal sperm morphology and function were related to increased production of reactive oxygen species (ROS) and decreased antioxidant status (i.e. oxidative stress) (Durairajanayagam *et al.* 2014). Nrf2 plays a key role in cellular antioxidant defense, thereby protecting the male reproductive tract from oxidative stress. The lack of Nrf2 function has a damaging effect on Nrf2^{-/-} mouse Sertoli cells and germ cells, as well as on the epididymal stage of sperm maturation, and exhibits decreased sperm count and sperm motility (Nakamura *et al.* 2010). Nrf2 controls the activity of enzymes and transporters in the blood-testis barrier and provides functional protection for the male reproductive system. Kang Chen *et al.* recruited 55 patients with weak and oligospermia, and 65 controls, and demonstrated low expression of Nrf2 mRNA in sperm of men with low sperm motility (Aitken *et al.* 1996). The Keap1/Nrf2/ARE signaling pathway plays a key role in the oxidative stress response, and Nrf2 is considered to be a transcription factor that promotes the expression of many important antioxidant genes (Kensler *et al.* 2007, Yu *et al.* 2013). Its downstream protein HO-1 is key in the male reproductive system and sexual dysfunction (Shamloul 2009). The Keap1/Nrf2/ARE signaling pathway and its regulated antioxidant enzymes have been shown to play a key role in the defense of cellular oxidative stress during spermatogenesis and fertilization (Kensler *et al.* 2007, Nakamura *et al.* 2010). The antioxidant enzymes and molecules regulated by this pathway, such as superoxide dismutase (SOD), glutathione (GSH), glutathione peroxidase (GPX) and catalase (CAT), are abundant in semen plasma or sperm cells (Jeulin *et al.* 1989, Aitken *et al.* 1996, Meseguer *et al.* 2007) and affect

male fertility in different ways. We hypothesized that Nrf2 may be a promising therapeutic target for obesity-related male infertility. Taken together, PACAP and Nrf2 have been shown to alleviate obesity-related comorbidities. Nevertheless, there are no studies that investigate whether Nrf2 mediates the effects of PACAP in obesity-related male infertility.

Although there is growing evidence that obesity is an important cause of male infertility, its exact mechanism of action remains unclear. In this study, we demonstrated that activation of Nrf2 by PACAP in the form of PACAP38 alleviates impairment of spermatocyte (GC-2) induced by high fat at the cellular level. However, these beneficial effects are abolished in GC-2 cells with specific inhibition of Nrf2 activity. Moreover, we show that the protective effects of PACAP in the mouse spermatocyte cells (GC-2) were also present on spermatogenic damage identified in the obese mouse model. These helped us to explain the pathogenesis of reproductive injury in obese male mice, which provide theoretical basis and experimental reference for the diagnosis of clinically obesity-related male infertility and future drug treatment.

Materials and methods

Cell culture

The mice spermatocyte GC-2 cells (ATCC) were cultivated in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco) containing 10% fetal bovine serum (FBS) (Thermo Fisher Scientific) and 1% penicillin streptomycin (Thermo Fisher Scientific) in an incubator supplied with 95% air and 5% CO₂ at 37°C. The medium was replaced every 3 days, and cells were subcultured by treatment with 0.05% trypsin-EDTA (Invitrogen) for 1 min. Cells were cryopreserved in a frozen solution containing 600 µL of medium, 300 µL of FBS, and 100 µL of DMSO (Sigma-Aldrich).

Cell viability assay

The GC-2 cell line was derived from mouse spermatocyte and has been widely used as a source of spermatocyte for *in vitro* studies. It is well known that PA is one of the most abundant free fatty acids (FFAs) in serum. Due to the close relationship between rising serum FFA levels and obesity (Manco *et al.* 2000), PA injured *in vitro* cell models were established to mimic the conditions of obesity *in vivo*, as previously reported (Kuwabara *et al.* 2015). To mimic the *in vivo* obesity conditions *in vitro*, a damaged GC-2

cell model was established by using palmitic acid (PA) (Kunchuang Technology Development Co., Ltd., Xian, China). The GC-2 cells were seeded onto a 96-well plate at a density of 1.5×10^4 cells/well in DMEM medium. After 70% of the cells adhered to the wall, they were incubated with various concentration of PA (0, 100, 200, 300, 400, 500 and 600 µM) for 1, 3, 6, 12, 18, 24 and 48 h, respectively. The cells which were not treated with PA were used as normal control (NC). The cell viability in each experimental group was determined via the CCK-8 assay (Sigma-Aldrich). The groups with cell viability of approximately 60% were further used to establish the PA-injury GC-2 cell model. After the PA-injured GC-2 cell model was established, the cells were incubated with phosphate buffered saline (PBS) or various concentrations of PACAP (0.0001, 0.001, 0.01, 0.1, 1, and 10 µM) (GL Biochem Ltd, Shanghai, China) for an additional 24 h. The cells incubated without PA and PACAP were used as normal control (NC). The proliferation rate in each experimental group was determined using a CCK-8 assay. For the receptor blocking experiments, GC-2 cells were preincubated with 1 µM of MAX.D4 (a specific PAC1 receptor antagonist) (Sigma Aldrich), 1 µM of PG97-269 (a specific VPAC1 receptor antagonist) (Sigma Aldrich), and 1 µM of PG99-465 (a specific VPAC2 receptor antagonist) (Sigma Aldrich) at 37°C for 30 min before being treated with 100 nM of PACAP. The cell viability rate in each experimental group was also determined using the CCK-8 assay, that is, after the cell treatment time, 10 µL CCK-8 solution was added to each well (four replicates per group), incubated in a 37°C incubator for 30 min, and then the absorbance was measured at 450 nm with a microplate reader. Finally, the proliferation rate of GC-2 cells was calculated according to the OD value of each group.

Detection of ROS in cells

The levels of intracellular ROS were detected by the fluorescent probe 2',7'-dichlorofluorescein-diacetate (DCFHDA) kit (KeyGEN BioTECH, Jiangsu, China) according to the manufacturer's instructions. The GC-2 cells were seeded onto a six-well plate at a density of 5×10^5 cells/well in DMEM medium. After 70% of the cells adhered to the wall, they were incubated with 400 µM of PA. After exposure to PA for 12 h and treatment with PACAP for 24 h, the cultured GC-2 cells were washed twice with phosphate-buffered saline (PBS), and then incubated with 10 µM DCFH-DA in medium at 37°C for 20 min in darkness. After cells were washed three times with serum-free medium, the fluorescence intensity was immediately measured using a flow cytometer (excitation at 488 nM, emission at 525 nM)

(Becton, Dickinson and Company, USA) by digesting and collecting the cells (about 20,000 cells per group) or directly via a fluorescence microscope (Nikon Ti).

Measurement of malondialdehyde (MDA) and antioxidant enzymes

After exposure to PA for 12 h and treatment with PACAP for 24 h, the cultured GC-2 cells were harvested, sonicated and centrifuged to collect the supernatant. The levels of malondialdehyde (MDA), antioxidant enzymes such as superoxide dismutase (SOD), and glutathione peroxidase (GPx) were then measured using their respective assay kits (Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's instructions. MDA was evaluated by thiobarbituric acid (TBA) method as commercially recommended (Nanjing Jiancheng Bioengineering Institute, China). The method was based on the spectrophotometric measurement of the color produced during the reaction to TBA with MDA. MDA concentrations were calculated by the absorbance of TBA reactive substances (TBARS) at 532 nm. SOD activity was assayed using the xanthine/xanthine oxidase method based on the production of $O_2^{\cdot-}$ anions. GPX activity was estimated based on its catalyzation by the oxidation of reduced glutathione in the presence of cumene hydroperoxide. The generation of NAD phosphate was measured spectrophotometrically at 340 nm.

Cell apoptosis assay

Cell apoptosis was measured using the annexin V-FITC apoptosis detection kit (Ebioscience, USA) according to the manufacturer's recommendations. The GC-2 cells were seeded onto a six-well plate at a density of 5×10^5 cells/well in DMEM medium. After 70% of the cells adhered to the wall, they were incubated with 400 μ M of PA. After exposed to PA for 12 h and treated with PACAP for 24 h, the GC-2 cells were collected by trypsinization and washed with cold PBS. After centrifugation at 100 g for 3 min, the cells were resuspended in binding buffer and then incubated with FITC-labeled Annexin V and propidium iodide (PI) for 15 min in the dark, and 20,000 cells were taken from each group for flow analysis within 1 h.

Protein extraction and western blotting

Protein was extracted from frozen testicular samples or cultured GC-2 cells by using RIPA lysis buffer (50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1% NP-40, 0.5% sodium

deoxycholate and 0.1% SDS) (Beyotime) supplemented with 1% protease inhibitor (Beyotime), 1% phosphatase inhibitor (Beyotime) and 1% phenylmethanesulfonyl fluoride (PMSF) (Beyotime). It was then quantitated by BCA protein assay kit (Thermo). In total, 20–30 μ g of protein was loaded onto a 12% SDS-polyacrylamide gel, and separated proteins were transferred to PVDF membranes (Pall, USA). After blocking with 5% BSA at room temperature for 1 h, membranes were incubated with specific antibodies as follows: GAPDH (1:8000, CST), Nrf2 (1:1000, CST), ERK (1:1000, Abcam), p-ERK (1:1000, Abcam), HO-1 (1:1000, Abcam), NQO1 (1:1000, Abcam), Keap1 (1:1000, Santa Cruz), IL-6 (1:1000, Santa Cruz), TNF α (1:1000, Santa Cruz), PKA (1:1000, CST), p-PKA (1:1000, CST), cleaved caspase 3 (1:1000, CST), Bcl-2 (1:1000, Abcam) and Bax (1:1000, CST). The membranes were then incubated with the appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies for an additional 1 h at room temperature. The protein bands were visualized using West Pico Chemiluminescent Substrate (Thermo Fisher Scientific). Grayscale analysis of Western blot results was performed using ImageJ software (NIH) (Liu *et al.* 2014). Extraction of Nrf2 nuclear protein with nuclear protein extraction kit (Cell Signaling Technology, Inc.).

Animals and treatment

All animal experiments were in accordance with the use of laboratory animals. The housing and related procedures were approved by the Laboratory Animal Ethics Committee of Jinan University, China. C57BL/6J mice (SPF grade, male, approximate 6 weeks of age, weighing about 20 g) were purchased from Pengyue Experimental Animal Breeding Co., Ltd (Jinan, China). All mice were housed in individually ventilated cages and maintained on a 12 h light: 12 h darkness cycle. The mice were fed standard chow (control diet, CD; catalog number: TP2330055AC, fat calories 10%, carbohydrate calories 75%, and protein calories 15%; Trophic Animal Feed High-tech Co., Ltd, Nantong, China) ($n=10$ per group) or high-fat diet (HFD, research diets; catalog number: TP2330055A, fat calories 60%, carbohydrate calories 25%, and protein calories 15%; Trophic Animal Feed High-tech Co., Ltd, Nantong, China) ($n=40$ per group) at random for 4 weeks, and changes in body weight and food intake were monitored weekly. When the mice in the HFD group weighed 20% more than the body weight of the CD group, it was determined that the obese mice were successfully modeled (Sato *et al.* 2002, Yoo *et al.* 2011,

Liu *et al.* 2016). Subsequently, part of mice fed with HFD were intraperitoneally injected with different concentrations of PACAP (0.1 mg/kg, 0.2 mg/kg, 0.4 mg/kg) or sterile saline (control) once a day for 4 weeks (Yada *et al.* 2000, Wang *et al.* 2014). The specific groups were as follows: (1) CD+Vehicle ($n=10$ per group); (2) HFD+Vehicle ($n=10$ per group); (3) HFD+0.1 mg/kg PACAP ($n=5$ per group); (4) HFD+0.2 mg/kg PACAP ($n=5$ per group); (5) HFD+0.4 mg/kg PACAP ($n=20$ per group). A portion of PACAP-treated obese mice was reserved for Nrf2 lentivirus injection. Retro-orbital blood was collected from the mice, naturally coagulated at room temperature for 10–20 min, and centrifuged at 200–300 g for 20 min, and the supernatant was carefully collected, taking care to avoid hemolysis. Four blood lipids of triglyceride (TG), cholesterol (TC), low density lipoprotein cholesterol (LDL-C) and high density lipoprotein cholesterol (HDL-C) and four sex hormones of testosterone (T), estradiol (E2), follicle stimulating hormone (FSH) and luteinizing hormone (LH) in separated serum were measured using an automatic biochemical detector (Chemray 240, Rayto Life Technology, Shenzhen, China). The epididymal adipose and testes were taken, and epididymal adipose weight and the testicular weight/body weight ratio were calculated. All animals were euthanized by cervical dislocation, and effort was made to minimize pain and suffering.

Lentiviral transfection for downregulation of Nrf2 *in vivo*

Lentiviral vectors carrying the Nrf2 short-hairpin RNA (LV-Nrf2-shRNA) and scramble short-hairpin RNA (LV-ctrl-shRNA), which was used as a negative control, were purchased from Cyagen Biosciences (Suzhou, China). For testis-specific knockdown of Nrf2, HFD mice received 10 μ L lentiviral of supernatant (10^8 transducing units/mL) by intratesticular injection under pentobarbital anesthesia (50 mg/kg, i.p.) for 1 week. The procedural details of the intratesticular injection for lentiviral transfection were as follows: mice were anesthetized via pentobarbital sodium injection (50 mg/kg, i.p.). After the scrotums were sterilized, the lower abdomen was gently pressed in the direction of the genitals to expose the testes. Next, the testes were injected with the lentiviral supernatant using gentle syringe pressure and a sharp syringe (30 G). After the injection, the needle was kept in the testes for 30 s and then was slowly removed to prevent the injected material from spilling. Finally, the mice were put in the specific-pathogen-free animal room and allowed to awaken naturally.

Detection of ROS in mouse sperm

The levels of ROS in mouse sperm were detected by the sperm cell oxidative stress reactive oxygen species (ROS) NBT colorimetric quantitative detection kit (MingXiu BioTECH, Shanghai, China) according to the manufacturer's instructions. Two millilitre of freshly collected (within 1 h) semen was pipetted into a 2 mL centrifuge tube and incubated in a 37°C incubator for 20 min. Then centrifuged in a mini-tabletop centrifuge for 7 min at a speed of 300 g . The supernatant was carefully removed, 2 mL of cleaning solution was added (Reagent A), the sperm cell pellets were mixed, and centrifuged for 7 min in a benchtop centrifuge at a speed of 300 g . The supernatant was carefully removed, added an appropriate amount of cleaning solution (Reagent A) was added, mixed well, sperm cells on the hemocytometer were counted, the sperm cell count was adjusted to 1×10^7 /mL, and put it in the 37°C incubator for use (within 1 h). Before the start of the experiment, 10 mL of cleaning solution (Reagent A) was removed into a bottle of staining solution (Reagent B). After mixing, it was marked as dyeing working solution and placed in the ice tank for later use. Hundred microliters of the pretreated mouse sperm cells was pipetted into a 1.5 mL centrifuge tube, 100 μ L of staining working solution was added, mixed well, and incubated in a 37°C cell incubator for 45 min. Then centrifuged in a mini-tabletop centrifuge for 10 min at a speed of 500 g . The supernatant was carefully removed, added 500 μ L of cleaning solution (Reagent A), and centrifuged for 10 min at a speed of 500 g in a micro bench top centrifuge. The cleaning solution was carefully removed and the previous experiment step was repeated once. Hundred microliters of dissolving solution (Reagent C) was added and mixed well, incubated for 5 min at room temperature, immediately transferred to a 96-well plate, put in a microplate reader and the active oxygen absorbance (OD) at a wavelength of 650 nm was measured.

Semen analysis in mice

The sperm was acquired by puncturing the cauda epididymides and squeezing the vas deferens into a petri dish containing 1 mL of G-IVF™ PLUS medium (Vitrolife, Sweden). The sperm of epididymis and vas deferens of each group of mice were tested with computer-assisted sperm analyzer system (CASA; Microptic Co., Barcelona, Spain). Ten microliters of sperm was added to a special sperm counting board, and the CASA system automatically calculated the semen concentration and analyzed the

sperm motility. At least five fields of vision was imaged with more than 200 sperm per test. The Diff-Quick Stain Kit (Huakang Co., Ltd., Shenzhen, China) recommended by WHO was used for sperm morphology analysis (Lu *et al.* 2010). Individual sperm morphology was scored as head defects, neck defects, tail defects or normal, as a percentage of each form. All of the previously mentioned methods were performed on at least 200 sperm cells and at least three repetitions of each sample were analyzed.

Histological analysis

For HE staining, the testis and epididymal adipose tissues of each group were fixed by 4% paraformaldehyde, embedded in paraffin, and cut into 5 μ m sections. Dewaxing, dextylene, dyeing, counterstaining, dehydration, solid sealing, etc., were carried out, and the pathological changes of testis and epididymal adipose tissues of each group were then observed under a light microscope (Nikon Ti).

Statistical analysis

Quantitative results are expressed as $\bar{x} \pm$ s.d. of at least three independent experiments. All data in this study were statistically analyzed using SPSS 19.0 and GraphPad Prism 6 software. Statistical differences between two groups were determined by Student's *t* test. One-way ANOVA was used for comparisons among different groups. *P*-value < 0.05 was considered statistically significant. **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

Results

PACAP promoted the proliferative activity of PA-injured GC-2 cells

We chose the GC-2 cell line to investigate whether PACAP was protected against PA-induced injury *in vitro*. Our experimental results indicated that the viability rate was approximately 60% when the cells were incubated with 400 μ M of PA for 12 h (Fig. 1A and B). Based on the principle of LD50, we chose 400 μ M PA for 12 h to establish a GC-2 high-fat injury cell model. PACAP has a protective effect on PA-injured cells. When the PACAP concentration was 0.1 μ M, the cell proliferation effect was the most obvious, and it returned close to the level of the NC group, which was significantly different compared with the PA group (Fig. 1C). Therefore, we chose 0.1 μ M

Figure 1

PACAP improved viability of PA-injured GC-2 cells. (A and B) Effects of PA on GC-2 cell viability. Cells were incubated with or without PA at various concentrations and times. Cell viability rate (%) = average OD₄₅₀ of each experimental group/average OD₄₅₀ of control group. (C) The cell viability of the PA-injured GC-2 cell model treated with PBS or 0.0001, 0.001, 0.01, 0.1, 1, and 10 μ M PACAP for 24 h. The cells without PA and PACAP were used as control. **P* < 0.05, ***P* < 0.01, ****P* < 0.001, vs NC or PA; ns, no significant difference. Data are presented as means $\pm$ s.d. PA, palmitic acid; PACAP, pituitary adenylate cyclase activating peptide.

PACAP for 24 h as the best repair condition for GC-2 cells with high-fat injury. Our data indicated that PACAP significantly promoted GC-2 cells proliferation after PA-induced injury.

PACAP alleviated PA-induced oxidative stress, apoptosis and inflammatory responses in GC-2 cells

Obesity induced oxidative stress and produced large amounts of ROS, which led to oxidative damage in spermatogenic cells (Meigs *et al.* 2007, Seo *et al.* 2014). The experimental results show that 100 nM of PACAP significantly reduced ROS levels in 400 μ M PA-induced GC-2 cells, and restored it to the NC level. The fluorescence detection results were consistent with flow cytometry results (Fig. 2A and B). The level of SOD activity and GPX level indirectly reflected the body's ability to scavenge free radicals, and the level of MDA indirectly reflected the severity of the body's cells being attacked by free radicals (Marzec-Wróblewska *et al.* 2011). The results show that PACAP increased SOD activity and GSH-PX content and decreased MDA levels in PA-injured GC-2 cells (Fig. 2C, D and E). The rate of apoptosis was determined using flow cytometry, while apoptosis-related protein levels were measured by Western blot analysis. The results show that PA markedly increased the apoptotic rate when compared to the control, while PACAP treatment significantly reversed PA-induced cell apoptosis (Fig. 2F). The Western blot analysis show that upregulation of cleaved caspase and Bax by PA were significantly attenuated by PACAP, meanwhile, PA-induced downregulation of Bcl-2 was also significantly ameliorated by PACAP (Fig. 2G and H). PA significantly enhanced IL-6 and TNF α expression compared with the normal control group, which could be significantly inhibited by PACAP treatment (Fig. 2I). These data proved that PACAP alleviated PA-induced oxidative stress, apoptosis and inflammatory responses in GC-2 cells.

PACAP ameliorated PA-induced GC-2 cell injury mainly via the PAC1/PKA/ERK/Nrf2 signaling pathway

Nrf2 acts as a transcription factor that promotes the expression of many key antioxidant genes, which play a key role in Keap1/Nrf2/ARE signaling pathways in response to oxidative stress, apoptosis and inflammatory response (Itoh *et al.* 2010). Therefore, we examined the expression of Nrf2 pathway-related proteins in GC-2 cells. The Western blot analysis show that in PA-injured GC-2 cells, PACAP could significantly increase Nrf2, HO-1 and

NQO1 expression and decrease Keap1 expression (Fig. 3A). To further investigate which PACAP receptor was involved in this protective effect, GC-2 cells were preincubated with three types of PACAP receptor antagonists before treatment with 100 nM of PACAP. We found that the suppression of cell damaging effect disappeared in PA-injured GC-2 cells preincubated with 1 μ M of MAX.D4 (PAC1 receptor antagonist) before treatment with 100 nM of PACAP. However, this protective effect was not nullified by PG97-269 (VPAC1 receptor antagonist) or PG99-465 (VPAC1 receptor antagonist). The results of the three PACAP receptor inhibitors on the expression of Nrf2 protein were consistent with the results on the cell proliferation (Fig. 3B). These data indicated that PACAP ameliorated PA-induced GC-2 cell damage mainly via the PACAP/PAC1/Nrf2 signaling pathway. The major downstream event following PACAP selectively binding to the PAC1 receptor was PKA phosphorylation (activation). This subsequently phosphorylated ERK, resulting in the upregulation of target genes/proteins (Dejda *et al.* 2008). Some studies have found that ERK could directly recognize the antioxidant regulator Nrf2 and activate Nrf2 to enter the nucleus. Our results show that PACAP increased the protein abundance of p-PKA and p-ERK in PA-injured GC-2 cells (Fig. 3C). Enhanced p-ERK further promoted the expression of downstream Nrf2 protein (Fig. 3D). Moreover, we blocked the PACAP/PKA/ERK signaling pathway with either MAX.D4 or H89 (a specific PKA inhibitor) or SCH772984 (a specific ERK inhibitor) before treatment with PACAP and subsequently measured the Nrf2 expression levels. As expected, MAX.D4, H89, and SCH772984 inhibited Nrf2 upregulation in PA-injured GC-2 cells after PACAP treatment. These data collectively demonstrated that PACAP specifically binding to PAC1 activated the PKA/ERK signaling pathway, resulting in Nrf2 upregulation, and the activation of Nrf2 further activated downstream targets, which might effectively promote cell proliferation and inhibit cell oxidative stress, apoptosis and inflammation, thus significantly facilitating the functional recovery of GC-2 cells after PA-injury.

Inhibition of partial Keap1/Nrf2/ARE pathway reversed PACAP-induced protective effect to GC-2 cells *in vitro*

Treating PA-injured GC-2 cells preincubated with 5 μ M of ML385 (a specific Nrf2 inhibitor) before treatment with 100 nM of PACAP blocked the antioxidant effect of PACAP, indicating that PACAP resisted oxidative

Figure 2

PACAP attenuated PA-induced GC-2 cell oxidative stress, apoptosis and inflammatory responses *in vitro*. (A and B) PACAP reduced ROS levels in PA-injured GC-2 cells. Qualitative analysis with fluorescence microscopy combined with flow quantitative analysis on ROS levels in GC-2 cells. (C, D and E) PACAP increased SOD and GPX activity and decreased MDA levels in PA-injured GC-2 cells. Corresponding kit was used to detect the SOD, MDA and GPX levels in GC-2 cells according to the manufacturer's instructions. (F) PACAP reduced PA-induced GC-2 cell apoptosis. Apoptosis rate of GC-2 cell were detected by flow cytometry using Annexin V-FITC/PI staining. In the images, Q4 represents normal cells, Q3 represents early apoptosis, Q2 represents late apoptosis, and Q1 represents cell debris. The apoptosis rate of cells is the combination of Q2 and Q3. (G) PACAP reduced expression of cleaved caspase3 protein in PA-injured GC-2 cells. * $P < 0.05$, *** $P < 0.001$, PA groups with different concentrations vs NC group, NC group or different concentrations of PACAP administration groups vs model group; ns, no significant difference. (H) PACAP reduced Bcl-2 and Bax protein expression ratio in PA-injured GC-2 cells. (I) PACAP reduced the expression of inflammation-related proteins in PA-injured GC-2 cells. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, NC or PA + PACAP vs PA. Data are presented as means $\pm$ s.d. A full color version of this figure is available at <https://doi.org/10.1530/JOE-20-0316>.

damage induced by PA in GC-2 cells by upregulating Nrf2 expression (Fig. 4A, B, C and D). ML385 partially offsetted the improvement effect of PACAP on PA-induced apoptosis and inflammatory response in GC-2 cells, indicating that the downregulation of Nrf2 could block the anti-apoptotic and anti-inflammatory effects of PACAP (Fig. 4E). To explore how upregulated Nrf2 regulated downstream target genes and inhibited oxidative stress, apoptosis and inflammatory responses in cells, we used

Western blot and from the results we saw that ML385 significantly decreased Nrf2 expression and increased Keap1 expression compared to PA + PACAP group, thereby attenuated the expression of downstream proteins HO-1 and NQO1, and blocked the activation of PACAP on the Keap1/Nrf2/ARE pathway. These data suggested that Nrf2 played a vital role in the antioxidant, anti-apoptotic and anti-inflammatory effects of PACAP on PA-injured GC-2 cells (Fig. 4F).

Figure 3

PACAP ameliorated PA-induced GC-2 cell injury through PAC1/PKA/ERK/Nrf2 signaling pathway. (A) PACAP activated Nrf2 signaling pathway in PA-injured GC-2 cells. (B) PACAP which selectively binds to PAC1 ameliorated GC-2 cell high-fat injury repair through regulating downstream signaling pathway. After incubating with or without 400 μ M of PA for 12 h, GC-2 cells were preincubated with 1 μ M MAX.D4, 1 μ M PG97-269, and 1 μ M PG99-465 for 30 min at 37°C prior to addition of 100 nM of PACAP. Max.D4, a specific PACAP type I receptor (PAC1) antagonist, was used for blocking PAC1 receptor; PG97-269, a specific PACAP type II receptor (VPAC1) antagonist, was used for blocking VPAC1 receptor; PG99-465, a specific PACAP type III receptor (VPAC2) antagonist, was used for blocking VPAC2 receptor. (C and D) PACAP promoted the expression of Nrf2 via activation of PKA/ERK pathway. Activation of PKA/ERK signaling pathway contributed to PACAP-induced Nrf2 upregulation. Western blot analysis of p-PKA, PKA, ERK, p-ERK, and Nrf2 expression in normal and PA-injured GC-2 cells. The cells were preincubated with 1 μ M MAX.D4, 1 μ M H89 and 2 μ M SCH772984 for 30 min at 37°C prior to addition of 100 nM of PACAP, then were cultured for another 24 h. H89 was used for blocking PKA activation, SCH772984 was used for blocking ERK activation. * P < 0.05, ** P < 0.01, *** P < 0.001, NC or PA + PACAP vs PA; # P < 0.05, ### P < 0.01, #### P < 0.001, PA + PACAP+MAX.D4 or PA + PACAP + PG97-269 or PA + PACAP + PG99-465 or PA + PACAP + H89 or PA + PACAP + SCH772984 vs PA + PACAP; ns, no significant difference. Data are expressed as mean $\pm$ s.d.

Establishment of a HFD-induced obesity mouse model

The body weight of the male mice which were fed with HFD for 4 weeks was significantly higher than that of mice fed with a CD (Fig. 5A), while the testis volume of the male mice fed with HFD for 4 weeks was smaller than that of male mice fed with a CD (Fig. 5B). The difference in body weight between the two groups became significant after 1 week of receiving their respective diet and was maintained for the following 3 weeks. After 4 weeks of feeding in each group, the average mice body weight in the HFD group was increased by 46.7% (P < 0.001) compared to that in the CD group, indicating that the obese mouse model was successfully established (Fig. 5C). The food intake in the

HFD group was always lower than that of the CD group. The differences could be attributed to the higher caloric content of HFD (5.5 kJ/g) when compared to that of CD (3.8 kJ/g) (Fig. 5D).

PACAP improved reproductive system damage of obese mice

After successfully establishing the obese mouse model, PACAP was injected intraperitoneally once a day for 4 weeks. As the results show, the body weight of mice in the HFD group mice was much higher than that of the CD group, whereas the high dose (0.4 mg/kg) PACAP-treated mice with HFD have a much lower average body

Figure 4

Partial inhibition of Keap1/Nrf2/ARE pathway reversed PACAP-induced spermatogenic cell protection *in vitro*. (A, B, C and D) ML385 reversed the inhibition effect of PACAP on oxidative stress in GC-2 cells induced by PA. (E) ML385 reversed the inhibition effect of PACAP on inflammation and apoptosis in GC-2 cells induced by PA. (F) ML385 reversed the activation effect of PACAP on Nrf2 signaling pathway in PA-injured GC-2 cells. After incubated with or without 400 μ M PA for 12 h, GC-2 cells were preincubated with 5 μ M ML385 for 24 h at 37°C prior to addition of 100 nM of PACAP. ML385 was used for blocking Nrf2 activation. * P < 0.05, ** P < 0.01, *** P < 0.001, NC or PA + PACAP vs PA; # P < 0.05, ## P < 0.01, ### P < 0.001, PA+PACAP+ML385 vs PA + PACAP. Data are expressed as mean $\pm$ s.d. A full color version of this figure is available at <https://doi.org/10.1530/JOE-20-0316>.

weight growth and also show a much lower food intake when compared to the other HFD groups (HFD + vehicle, HFD + PACAP 0.1 mg/kg, and HFD + PACAP 0.2 mg/kg) (Fig. 6A and B), indicating that PACAP has a weight loss effect by affecting the appetite of mice after it reached a certain concentration. The weight of epididymal adipose can reflect the degree of obesity. The high dose (0.4 mg/kg) PACAP-treated HFD mice were found to have a much lower epididymal adipose weight than that of HFD mice (Fig. 6C). Testicular index (testicular weight/body weight) is one of the criteria for judging

male infertility. It could be seen that high dose (0.4 mg/kg) PACAP could significantly increase testicular index compared to HFD + Vehicle group (Fig. 6D). Clinically obese patients are often accompanied by high blood lipids and high blood lipids can cause male sexual dysfunction. The results show that high dose (0.4 mg/kg) PACAP could effectively improve four lipids (TG, TC, LDL-C, HDL-C) in serum of high-fat diet mice (Fig. 6E). Obesity often leads to endocrine disorders, the lowered T, and increased E2 in the HFD group could destroy the negative feedback loop of

Figure 5

Description of the obese mouse model fed by HFD for 4 weeks. (A) Representative appearance of HFD and CD mice; (B) Representative testicular appearance of HFD and CD mice; (C) Body weight comparison between HFD and CD groups mice; (D) Weekly food intake between HFD and CD mice. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, HFD vs CD. Data are expressed as mean $\pm$ s.d. A full color version of this figure is available at <https://doi.org/10.1530/JOE-20-0316>.

the hypothalamus-pituitary-testis (HPT) axis, thus reducing sperm production. In this study, HFD mice exhibited the lowest levels of T, FSH and LH, and the highest levels of E2, while PACAP significantly increased T and decreased E2 and increased FSH and LH secretion in a dose-dependent manner (Fig. 6F). The improvement in the volume of epididymal adipose cells in the HFD+Vehicle group also show a PACAP dose dependency (Fig. 6G). Normal testicular tissue structure is a prerequisite for maintaining reproductive function. The testicular H&E staining results show that obese mice have an abnormal testicular structure compared to that of normal mice in the CD group. With the increase of PACAP concentration, the degree of testicular damage has gradually recovered, the thickness of spermatogenic epithelium has visibly restored, and the number of spermatogenic cells has increased (Fig. 6H). We have extracted the protein of the testis tissue of each group and the Western blot results indicated that PACAP could activate Nrf2 signaling pathway (Fig. 6I).

In summary, whether it was to slow down the mice weight gain or to improve the testicular oxidative stress damage induced by obesity, high dose (0.4 mg/kg) PACAP show the best effect. Therefore, we chose the concentration of 0.4 mg/kg PACAP to complete subsequent experimental studies.

PACAP promotes testicular Nrf2 expression and activates Nrf2/Keap1/ARE signaling pathway to protect mice testicular injury

To examine whether the protective role of PACAP in obesity-induced testicular oxidative stress was mediated via activation of Nrf2, the mice received an intratesticular injection containing LV-Nrf2-shRNA for Nrf2 knockdown. The protein expression level of Nrf2 in Nrf2 shRNA group transfected with target lentivirus was significantly lower than that in non-transfected group not transfected with lentivirus or in Ctrl shRNA group transfected with scramble lentivirus after injecting Nrf2 lentivirus in the mice testes (Fig. 7A). It indicated that lentivirus successfully transfected spermatogenic cells in mice testis and knocked down Nrf2 gene. We also collected semen samples from mice to detect the ROS levels. The results show that the ROS levels in the sperm of the HFD group were much higher than those in the NC group. After PACAP intervention, the ROS content in the sperm of the mice decreased, but the inhibitory effect of PACAP disappeared after knocking down Nrf2 (Fig. 7B). By using H&E staining, we found that the epididymal adipose volume in HFD+PACAP+shNrf2 group was larger compared with HFD+PACAP+shCtrl group after knocking down Nrf2 gene (Fig. 7C), and the testicular

Figure 6

PACAP improved reproductive system damage of obese mice. (A and B) Body weight and food intake curves of male mice fed with CD or HFD, and treated with or without 0.1, 0.2 and 0.4 mg/kg/day of PACAP throughout 4 weeks. $*P < 0.05$, $**P < 0.01$, HFD+PACAP 0.4 mg/kg vs HFD + Vehicle. (C and D) Epididymal adipose weight and the ratio of testes/body weight after a 4-week PACAP supplement. (E and F) Serum levels of TG, TC, LDL-C, HDL-C, T, E2, FSH, and LH were detected after mice received 4 weeks of PACAP treatment via i.p. injection. (G) Histological morphology of epididymal adipose stained with H&E staining. Scale bars = 100 μm. (H) Histological morphology of testes stained with H&E staining. Scale bars = 200 μm. (I) PACAP activates Nrf2 signaling pathway-related protein expression in testes of obese mice. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, CD + Vehicle or HFD + PACAP 0.2 mg/kg or HFD + PACAP 0.4 mg/kg vs HFD + Vehicle. Data are expressed as mean $\pm$ s.d. A full color version of this figure is available at <https://doi.org/10.1530/JOE-20-0316>.

Figure 7

PACAP does not attenuate testicular oxidative damage in HFD mice with testicular knockdown of Nrf2. (A) The knockdown efficiency of Nrf2 in LV-Nrf2-shRNA transfected mouse testis was examined by Western blot. $**P < 0.01$, non-transfected or Ctrl shRNA vs Nrf2 shRNA. (B) PACAP treatment reduced ROS levels in the sperm of the HFD mice via Nrf2. (C) HE staining to observe the effect of Nrf2 lentivirus on epididymal adipose. Scale bars = 100 μ m. (D) HE staining to observe the effect of Nrf2 lentivirus on testes. Scale bars = 200 μ m. (E, F and G) PACAP treatment improved sperm characteristics in HFD mice via Nrf2. (H) Western blot detection of Nrf2 pathway-related protein expression in testes after knocking down Nrf2. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, CD + Vehicle or HFD + PACAP vs HFD + Vehicle; $\#P < 0.05$, $\#\#P < 0.01$, $\#\#\#P < 0.001$, HFD + PACAP + shNrf2 vs HFD + PACAP. Data are expressed as mean $\pm$ s.d. A full color version of this figure is available at <https://doi.org/10.1530/JOE-20-0316>.

tissue structure was significantly destroyed (Fig. 7D) in HFD+PACAP+shNrf2 group, indicating that knockdown of Nrf2 reversed the protective effect of PACAP on testis and epididymis. Sperm parameters analysis show that the sperm concentration, sperm motility, and abnormal sperm ratio in the HFD group recovered after PACAP treatment. Knockdown of Nrf2 in the testis weakened that protective effect of PACAP, indicating that Nrf2 played an important role in improving the semen quality of obese mice by PACAP (Fig. 7E, F and G). In addition, knockdown of Nrf2 in the testis reduced the expression of Nrf2 pathway-related proteins and inhibited the antioxidant signaling

pathway in mice testis, suggesting that the Nrf2 pathway mediated the inhibitory effect of PACAP on testicular oxidative stress (Fig. 7H).

Mechanism of PACAP regulating obesity-induced spermatogenic disorders (Fig. 8).

Discussion

Excessive fat accumulation leads to obesity. In an endocrine organ, fat secretes inflammatory causing factors and produces ROS that attacks testicular spermatogenic

Figure 8

Schematic diagram of PACAP selectively binding to PAC1 inhibited obesity-induced cell oxidative injury and apoptosis via Keap1/Nrf2/ARE pathway. PACAP selectively binds to the PAC1 receptor and activates PKA/ERK and activated ERK promotes the expression of Nrf2. Nrf2 enters the nucleus and combines with the antioxidant response element (ARE) to activate the expression of downstream HO-1 and NQO1, increasing the level of SOD and GPX, thereby inhibiting ROS-induced oxidative stress damage, apoptosis and inflammation, and improving high-fat-induced germ cell damage. cAMP-response element-binding protein; PAC1, PACAP type I receptor; PACAP, pituitary adenylate cyclase-activating peptide; PKA, protein kinase A; ERK, extracellular regulated kinase; Nrf2, nuclear factor-erythroid 2-related factor-2. A full color version of this figure is available at <https://doi.org/10.1530/JOE-20-0316>.

cells and causes apoptosis of spermatogenic cells. Palmitic acid (PA) is one of the highest free fatty acids in serum, and elevated levels of serum free fatty acids are closely related to obesity. PA-induced cell model *in vitro* can mimic the high-fat environment in obesity-related or type 2 diabetes patients. In this study, PA was used to establish a GC-2 high-fat injury cell model to simulate the obese environment *in vivo*. Whereafter we found that PACAP can improve obesity-induced germ cell damage at the cellular level *in vitro*.

Oxidative stress plays an important role in nutritional obesity-related infertility. In the study of obesity and related diseases such as diabetes and cardiovascular disease, it was found that the level of oxidative stress markers in these patients is higher than that of normal population (Halliwell & Cross 1994), so it was speculated that oxidative stress may be an important pathogenesis of obesity-induced reproductive disorders. Obesity-related patients often produce more ROS, and the body's antioxidant, anti-apoptotic and anti-inflammatory abilities decline. When ROS accumulates in the body beyond the antioxidant defense capacity of spermatogenic cells, it induces spermatogenic cells to undergo oxidative stress damage, resulting in apoptosis of spermatogenic cells (Seo *et al.* 2014). During the process of spermatogenic cell apoptosis, many systems and substances are activated, which in turn leads to dysregulation of the inflammatory response (Matsumaru & Motohashi 2020). Studies have shown that nutritional obesity can significantly increase the apoptosis of testicular spermatogonia and spermatocytes in adolescent rats with conversion disorder, which reduces the generation of sperm cells

and sperm (Bieniek *et al.* 2016). In our experiments, high-fat (PA) increased ROS levels and cell apoptosis in spermatocyte GC-2, resulting in a decrease in the number of mature spermatozoa. It may be one of the reasons for poor spermatogenic ability of obesity-related patients. Therefore, improving the antioxidant capacity of the reproductive system in obesity-related patients can reduce ROS-induced apoptosis of spermatogenic cells and prevent the occurrence of obesity-induced infertility. As a multifunctional bioactive peptide secreted by the pituitary gland, PACAP has various biological functions such as regulating glycolipid metabolism, neuroprotection, anti-oxidation, anti-apoptosis and anti-inflammation (Dohi *et al.* 2002). Our results show that the intracellular ROS level decreased significantly after PACAP treatment, and GPX and SOD levels also increased, while the MDA level and the number of apoptotic cells decreased. In addition, PACAP could also reduce the expression of apoptosis-related proteins and inflammatory factors in cells. These results indicated that PACAP protected the normal function of germ cells by reducing oxidative stress in spermatogenic cells induced by high fat, inhibiting ROS-induced cell apoptosis and inflammatory damage.

It has been reported that high-fat and high-calorie diets can cause nutritional obesity in rats, abnormal glucose and lipid metabolism, sex hormone disorders, oxidative stress in the body and local high inflammatory reaction of the testis, etc., caused by obesity destroy testicular spermatogenesis function of obese rats (Bieniek *et al.* 2016). Studies have shown that PACAP could suppress appetite and reduce the weight of rats (Iemolo *et al.* 2015). Another study confirmed that PACAP inhibited

obesity-induced apoptosis of testicular spermatogenic cells by inactivating p53-mediated mitochondrial apoptosis signaling pathway (Yan *et al.* 2020). Our study found that under the effect of PACAP, high-fat-induced mouse body weight and epididymal fat increase were effectively controlled, testicular weight increased significantly, blood lipids (TG, TC, LDL-C and HDL-C) and sex hormones (T, E2, FSH and LH) levels have been significantly improved, and fat and sex hormone metabolism disorders have been effectively improved. In addition, PACAP reduced the pathological damage of testicular tissue induced by obesity. These results indicated that PACAP could improve the testicular reproductive function of obese mice from multiple levels such as improving obese body weight, fat and sex hormone metabolism, epididymis and testicular structure.

PACAP could also activate Nrf2 pathway-related proteins expression. Nrf2 is a key regulator of oxidative stress in cells, and its activity is mainly negatively regulated by Keap1. Nrf2 regulates the transcription of genes containing antioxidants in the promoter by interacting with the antioxidant response element (ARE). Mice lacking Nrf2 reduced the constitutive and inducible expression of SOD, CAT, and GSH (Wild *et al.* 1999, Nguyen *et al.* 2003). Previous research have shown that Nrf2 gene knockout mice could accelerate the reduction of the testicular and epididymal antioxidant capacity, thereby generating oxidative stress, destroying spermatogenesis and affecting the reproductive capacity of mice (Nakamura *et al.* 2010). According to relevant literature reports, Nrf2^{-/-} male mice testis weight, testis and epididymal sperm counts were age-related declination, and compared with WT mice aged 2–7 months, Nrf2^{-/-} male mice have increased spermatogenic tubule vacuolation. In addition, GSH levels, antioxidant gene expression and enzyme activity in Nrf2^{-/-} male testes and epididymis were lower than that in Nrf2^{+/+} male (Chan & Kwong 2000). Yamamoto *et al.* found that decreased testicular antioxidant capacity led to increased testicular oxidative stress, resulting in germ cell apoptosis, reduced sperm production, and ultimately reduced Nrf2^{-/-} male fertility (Yamamoto *et al.* 2004). The previously mentioned research show that Nrf2 was an important factor for spermatogenesis, and the lack of transcription factor Nrf2 led to severe obesity-related spermatogenesis obstruction. Therefore, Nrf2 signaling could be used as a therapeutic target for obesity-induced infertility, so understanding the relationship between PACAP and Nrf2 signaling would promote the development of new pharmacological insights to treat infertility associated with nutritional obesity.

We have demonstrated that at the cellular level the Keap1/Nrf2/ARE pathway mediated the protective effect of PACAP on high-fat-induced germ cell damage. PACAP has three main receptor subtypes of PAC1, VPAC1 and VPAC2. PACAP binds to different receptors and triggers different signaling pathways to play different biological functions (Vaudry *et al.* 2009). However, it remains unclear which receptor is involved in the protective effect of PACAP on germ cells. Our receptor blocking experiment results indicated that PACAP selectively bound to the PAC1 receptor, activated the Nrf2 signaling pathway, and improved high-fat-induced GC-2 cell damage. More and more evidences indicated that PACAP was mainly mediated by PAC1 receptor and exerted antioxidant and antiapoptotic effects (Seaborn *et al.* 2011). PACAP selectively bound to the PAC1 receptor, activated adenylate cyclase (AC), and catalyzed the conversion of ATP to cAMP, which in turn activated cAMP-dependent protein kinase A (PKA). Phosphorylated PKA could make intracellular phosphorylation of various proteins, such as MEK, ERK, etc., which produced antioxidant and antiapoptotic effects (Gräs *et al.* 2000). It has been found that phosphorylated ERK could directly recognize the antioxidant regulatory factor Nrf2 and activate Nrf2 to enter the nucleus, thus activating Keap1/Nrf2/ARE signaling pathway (Wei *et al.* 2018). Our experimental results show that PACAP upregulated the expression levels of p-PKA and p-ERK in GC-2 cells with high-fat injury. The upregulated p-ERK further promoted the expression of downstream Nrf2 protein. Subsequently, we used MAX. D4, H89, and SCH772984 to pretreat cells and found that MAX.D4, H89, and SCH772984 all effectively inhibited PACAP-induced Nrf2 upregulation. These data indicated that PACAP selectively bound to PAC1 and activated the PKA/ERK/Nrf2 signaling pathway, thereby promoting the repair of high-fat injured cells.

In animal level experiments, our Western blot results show that PACAP could promote the expression of Nrf2 protein and downstream proteins HO-1 and NQO1 and downregulate the expression of Keap1 in the testis tissue of obese mice, indicating that PACAP might activate the expression of downstream proteins HO-1 and NQO1, activate the antioxidant signaling pathway, inhibit oxidative damage and apoptosis in testicular spermatogenic cells induced by obesity, and improve the fertility of obese mice by upregulating Nrf2 expression. These protective effects of PACAP were weakened after suppressing Nrf2 activity or knocking down the Nrf2 gene.

In summary, the current study revealed three main findings. First, we have demonstrated that obesity-

induced male reproductive damage was related to the decreased activity of Nrf2. Secondly, a decrease in Nrf2 levels led to an increase in ROS levels, induced oxidative stress, and thus enhanced the apoptotic and inflammatory effects in spermatogenic cells. Thirdly, PACAP was shown to selectively bind to the PAC1 receptor and activate the downstream PKA/ERK/Nrf2 signaling pathway to protect against obesity-induced male reproductive impairment (Fig. 8). Our findings collectively demonstrated that the Keap1/Nrf2/ARE signaling pathway played a crucial role in restoring the fertility of men with obesity after PACAP treatment. However, more research is necessary to understand the potential mechanisms of the anti-obesity, anti-oxidation, anti-apoptosis and anti-inflammation effects of PACAP. Nevertheless, our results laid the foundation for exploring PACAP as a promising candidate drug for male infertility caused by obesity.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

Yi Ma and Wailan Shan conceived and designed the research. Wailan Shan, Zixian Wang, Xiaohua Lu inquired and collected information. Wailan Shan, Shiyin Lu, Jia Feng researched and performed the research. Wailan Shan, Biqian Ou, Huixian Li processed and analysed the data. Wailan Shan, Jia Feng and Yi Ma wrote, reviewed and/or revised the manuscript. Wailan Shan, Jia Feng and Xiaohua Lu contributed equally to this work.

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