Abstract

Apelin was identified to be an adipokine secreted from adipose tissue [illegible]

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previous study, we defined the expression of Apelin and APJ in the different developmental stages of ovarian GCs in buffalo. Likewise, it is stated that apelin, in the presence of different factors such as IGF1 and FSH, has meaningful effects on the secretion of Progesterone (P4) and Estradiol (E2) in cattle and porcine as well as buffaloes through various signaling pathways [1].

The effects of APLN on IGF1/FSH-induced steroidogenesis and cell proliferation, apoptosis, and scavenging activity of ovarian follicular GCs in buffaloes are unmasked. Therefore, in this study, for the first time, we surveyed these functions. Thus, the effect of APLN-13 on IGF1/FSH-induced steroidogenesis was evaluated. In addition, the

e ects of APLN-13/-17 proliferation, cytotoxicity, Bax expression, and scavenging activity of GCs were studied [2].

Materials and Methods

Reagents

Unless otherwise stated, all chemicals and media used in the current study were obtained from Sigma-Aldrich (MO, USA).

Hormones and Antibodies

Recombinant porcine FSH, recombinant human IGF1 (ab270062), APLN-13 (ab141010), APLN-17 (ab141011) were purchased from Abcam. Also, APLN (ab141011), Bax (ab77566), and beta-Actin (ab8226) antibodies were obtained from Abcam. APJ (20341-1-AP), anti-rabbit IgG (SA00001-2), and anti-mouse IgG (SA00001-1) were purchased from Proteintech. Primary and secondary antibodies were used at 1: 500 to 1:1000 and 1/3000 for western blotting [3].

Follicle Collection and Granulosa Cells Culture

Bu alo ovaries were collected from a local slaughterhouse and transported on ice within 2 h a er slaughter to the laboratory in Phosphate-Bu ered Saline (PBS) supplemented with 0.05 mg/mL streptomycin and 0.06 mg/mL penicillin. In the laboratory, the ovaries were washed adequately with physiological saline solution.

To assess the e ect of the APLN on IGF1/FSH-induced steroidogenesis, cell proliferation, apoptosis, and cell redox status, the GCs culture model was established. erefore, all the healthy (well vascularized and having transparent follicular wall and uid) and visible follicles were aspirated by a 17-gauge needle attached with a 10-mL syringe. e aspirates were transferred to a 60-mm dish under sterile conditions with PBS, and all cumulus-oocyte complexes were removed. e remaining cells and liquids were centrifuged in 15-mL conical tubes at 700 g for 5 min. en, GCs were resuspended in Dulbecco's Modi ed Eagle Medium (DMEM) medium containing 10% Fetal Bovine Serum (FBS) and antibiotics and antimycotic solution (penicillin 100 U/mL, streptomycin 100 mg/mL, amphotericin B 0.25 mg/mL). Cell viability was evaluated using trypan blue exclusion dye, exceeding 80%. e cells were then seeded in a 48/96-well plate in a humidi ed CO_2 (5%) incubator at 37.5°C and having approximately 1.5 × 105 viable cells per well. e cells were allowed to attach and grow (75%-80% con uence) for 48 h. en cells were treated with fresh media (FBS free) containing di erent doses of porcine FSH or human recombinant IGF-I (0, 10^{-6}, 10^{-7}, 10^{-8}, 10^{-9}, and 10^{-10} M) singly or in the presence of 10^{-9} M APLN-13 and were maintained for 48 h. Control cells were grown in similar conditions as other cells except for the addition of the peptides. For each experimental condition, six replicates were tested. A er 48 h, the spent media were collected and stored for E2 and P4 assay. APLN-13 or APLN-17 were applied to GCs a er culturing for 48 hours with 10% FBS to investigate whether they a ected the mRNA and protein expression of Bax, the cells were treated with di erent doses of APLN-13 and APLN-17 (0, 10-6, 10-8, 10-9 M) for additional 48 hrs and then mRNA and protein were extracted from cells [4].

Total RNA Extraction, cDNA Synthesis

Total RNA was extracted from GCs of follicles using TRIZOL reagent by the manufacturer's instruction, and a xed amount of RNA (100 ng) was directly reverse-transcribed into a 20 µL rst-strand cDNA using a PrimeScript RT reagent Kit with gDNA Eraser (Perfect Real Time, TAKARA BIO INC, Japan) following the manufacturer's instructions [5].

Quantitative Real-Time PCR analysis

Rt-qPCR was done in a total volume of 20 µL, containing equally distributed cDNA (100 ng), 10 mM each of the forward and reverse primers, and 10 µL of 2 × SYBR Green Master Mix (SYBR® Premix Ex Taq™ II (Tli RNaseH Plus, TAKARA, Japan). All reactions for all genes of interest were performed in triplicate and were run on the light Cycler 480 system (Roche Diagnostics) under the following conditions: 95°C for 30 s, followed by 40 cycles at 95°C for 5 s and 60°C for 30 s. β-actin and RPS15 were used as the internal control (reference genes) to normalize the relative gene expression levels. All reactions were performed in triplicate. e gene expression levels were analyzed with the $2^{-\Delta\Delta CT}$ method described previously by concerning the housekeeping genes. e details of the selected genes and the primer pairs used in the study are provided in Table 1.

Western Blot Analysis

Total proteins were obtained from cultured GCs of di erent experiments by lysing in RIPA bu er containing PMSF (R0010; Solarbio, China) at 4°C for 30 min followed by collection and centrifugation at 12,000 rpm for 5 min at 4°C. e pellet was eliminated, and lysates were diluted with 6X protein loading bu er (DL101-02; TransGen, China) and heated to 100°C for 5 min. A er cooling on ice, the samples were stored at -80°C until the western blotting. Western blotting was started by loading the samples on a 12% gradient polyacrylamide gel (P0012AC; Beyotime, China) and then transferred to a PVDF membrane (ISEQ00011; Millipore, China), followed by blocking in 8% (wt/vol) Difco Skim Milk in Tris-bu ered saline containing 0.1% (vol/vol) Tween-20 (TBST) for 2 h. Overnight incubation with the primary antibody was performed. en, a er four washes, 10 min each, with TBST, membranes were incubated for 1 h at 37

which in turn enhances the amount of formazan dye produced. e formazan dye produced from WST-1 by viable cells can be quanti ed by measuring the absorbance of the dye at OD=440 nm. Brie y, 4 × 104 cells per 200 µL of culture media were seeded in 96-well plates treated with APLN-13/17 (0, 10^{-9}, 10^{-8}, 10^{-6} M) with or without preincubation of cells with APJ antagonist (ML221 10 µM) for 1 hour and incubated for 48 hours. According to the product manual, 10 µL of WST-1 was added to the cells during the last 4 hours of incubation. en, the absorbance was detected by a plate reader machine at a wavelength of 44 Cytotoxicity 20 nm [7].

e rate of cytotoxicity of APLN-13/-17 was calculated according to the following formulae:

$$\% \text{ Cytotoicity} = \frac{100 \times (CTA - ATC)}{CTA}$$

Where CTA is the control group, and ATC is APLN treated cells.

Total Antioxidant Capacity Assessment by the FRAP Method

e FRAP method is a colorimetric assay based on the ability of the antioxidant molecules to reduce Ferric Tri Piridyl Tria Zine (Fe^{3+}TPTZ) to a ferrous form (Fe2+TPTZ). Fe2+ is assessed spectrophotometrically through determination of its colored complex with 2, 4, 6-Tris (2-Pyridyl)-Stria Zine (Fe^{2+} TPTZ). TPTZ reagent was prepared before use, mixed with 25 mL of acetate bu er, 2.5 mL of 2, 4, 6-Tris (2-Pyridyl)-s-Tria Zine (TPTZ) 10 mM in HCl 40 mM, and $FeCl_3 6H_2O$ solution. In total, 4 × 104 cells per 200 µL of culture media were cultivated in a 96-well plate and treated with APLN-13/-17 for 48 h. en, the plates were centrifuged for 10 min at 400 g, the supernatants were discarded, and cells were lysed by adding cold Triton 0.5% (v/v) + PMSF in PBS (200 mL/well) and incubating on ice for 30 min. e test was carried out on 40 mL of cell lysates added to Fe^{3+} TPTZ reagent and then incubated at 37°C for 30 min. e absorbance of Fe^{2+} TPTZ was detected at 595 nm. e ferric reducing ability of cell lysates was estimated by plotting a standard curve of absorbance against $FeSO^{4-} 7H_2O$ standard solutions [8].

Steroids ELISA Assay

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showed APLN-13 stimulated the increasing e ect of IGF1 on the secretion of E2 from GCs of bu alo ovaries; however, the e ect of di erent doses was not the same. In addition, APLN-13 triggered the e ect of IGFI on the production of P4 in a dose-dependent manner. In the presence of APLN-13, neither E2 nor P4 were signi cantly

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