The effects of apelin on IGF1/FSH-induced steroidogenesis, proliferation, Bax expression, and total antioxidant capacity in granulosa cells of buffalo ovarian follicles

Borhan Shokrollahi

  Guangxi Buffalo Research Institute, Chinese Academy of Agricultural Sciences

Hai-Ying Zheng

  Guangxi Buffalo Research Institute, Chinese Academy of Agricultural Sciences

Ling-Yu Li

  Guangxi Buffalo Research Institute, Chinese Academy of Agricultural Sciences

Li-Ping Tang

  Guangxi Buffalo Research Institute, Chinese Academy of Agricultural Sciences

Xiao-Ya Ma

  Guangxi Buffalo Research Institute, Chinese Academy of Agricultural Sciences

Xing-Rong Lu

  Guangxi Buffalo Research Institute, Chinese Academy of Agricultural Sciences

An-Qin Duan

  Guangxi Buffalo Research Institute, Chinese Academy of Agricultural Sciences

Chen-Xi Huang

  Guangxi Buffalo Research Institute, Chinese Academy of Agricultural Sciences

Yuan-Yuan Xu

  Guangxi Buffalo Research Institute, Chinese Academy of Agricultural Sciences

Jiang-Hua Shang (✉ biotech@gxbri.com)

  Guangxi Buffalo Research Institute, Chinese Academy of Agricultural Sciences

---

Research Article

Abstract

Apelin (APLN) was believed to be an adipokine secreted from adipose tissue. However, studies demonstrate that it is a pleiotropic peptide and has several effects on the female reproductive system. In this study, the effect of different doses of IGF1 and FSH in the presence of APLN-13 on the production of estradiol and progesterone was evaluated in the follicular granulosa cells of buffalo ovaries, in addition, the effects of different doses of APLN isoforms (APLN-13 and APLN-17) on proliferation, the expression of Bax protein and total antioxidant capacity activity of the same cells were investigated. Granulosa cells of buffalo ovaries were cultured in the presence of different doses of IGF1 and FSH with or without APLN-13 (10^{-9}M) to evaluate its effect on the secretion of estradiol and progesterone that tested by ELISA assay. WST-1 method was used to survey the effect of APLN on granulosa cell proliferation and cytotoxicity. In addition, the antioxidant capacity of the cells in the presence of APLN was assessed using FRAP method. mRNA and Bax protein levels were measured in granulosa cells treated with APLN using real-time PCR and western blot techniques. APLN-13 stimulated the effect of IGF1 on the production of estradiol and progesterone, and the progesterone production levels were affected by APLN-13 dose-dependently. However, it did not significantly stimulate the effect of FSH on the secretion of estradiol or progesterone. APLN-13 (all doses) and − 17 (10^{-8} and 10^{-9} M) improved the proliferation of granulosa cells. Moreover, preincubation of the cells for an hour by APLN receptor antagonist (ML221, 10 µM) did not significantly affect the proliferation of cells. Neither APLN-13 nor APLN-17 were not cytotoxic for the cells compared to the control treatment. APLN-13 at the doses of 10^{-6} and 10^{-8} M substantially up and down-regulated Bax protein expression; however, such effects were not observed when the cells were preincubated with ML221. In addition, APLN-17 did not influence the expression amount of Bax. Furthermore, both APLN-13 and − 17 improved the total antioxidant capacity of the ovarian granulosa cells, but such effects were not seen when the cells were preincubated with ML221. These findings indicate that APLN enhanced the IGF1 induced steroidogenesis and improved the cell proliferation and antioxidant capacity of follicular granulosa cells of buffalo ovaries; however, its effect on Bax expression was divergent.

Introduction

As a new adipokine produced by adipose tissue, APLN is derived from prepro-APLN (77 amino acids). Prepro-APLN is cleaved into a 55-amino-acid fragment and later into different APLN isoforms such as APLN-36, APLN-17, APLN-13, and the pyroglutamyl APLN − 13 (Pyr- APLN-13; [1]). (Pyr) APLN-13 and − 17 are more active isoforms in more quantity in the bloodstream [2]. The receptor of APLN is APJ (an orphan G protein-coupled receptor) with a similar structure to the angiotensin II type 1 receptor (AT1; [3]). APJ can bind to different isoforms of APLN with various affinities and activate several signaling pathways that lead to the appearance of different functions in the body [4]. In addition to adipose tissue, APLN and APJ are extensively distributed in the various tissues of the body, such as the heart, brain, lung, blood vessels, spleen, intestine, breast, and reproductive tract [4−8]. Moreover, the known roles of APLN and APJ are participation in angiogenesis, cardiovascular function, fluid homeostasis, cell proliferation, food intake,

and regulation of energy metabolism [4, 9–11]. The reproductive roles of APLN have been reported in recent years, in addition to its localization in the gonads and the arcuate, supraoptic, and paraventricular hypothalamic nuclei [12]. By attempts from the last 15 years ago, the presence of APLN has been verified in the granulosa (GCs), theca luteal cells, and oocytes of bovine, human, and porcine [13–15]. The considerable effects of APLN have been reported on follicle selection in the bovine species [16], steroidogenesis in different species such as rats, humans, cattle, sheep, and pigs [13–15, 17, 18]. In our previous study, we defined the expression of APLN and APJ in the different developmental stages of ovarian GCs in buffalo [19]. Likewise, it is stated that APLN, 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 [13] and porcine [14] as well as buffalos [19] through various signaling pathways.

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 effects of APLN-13/-17 proliferation, cytotoxicity, Bax expression, and scavenging activity of GCs were studied.

Materials And Methods

Reagents and ethical statement

Unless otherwise stated, all chemicals and media used in the current study were obtained from Sigma-Aldrich (MO, USA). The Animal Ethics Committee at the Guangxi Buffalo Research Institute handled all aspects of the experiments and ensured they comply with animal research ethical regulations.

Hormones And Antibodies

Recombinant porcine FSH (Sigma F2293), recombinant human IGF1 (ab270062), APLN-13 (ab141010), APLN-17 (ab141011) were purchased from Abcam. Also, Bax (ab77566), and beta-Actin (ab8226) antibodies were obtained from Abcam. 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.

Follicle Collection And Granulosa Cells Culture

Buffalo ovaries were collected from a local slaughterhouse and transported on ice within 2 h after slaughter to the laboratory in phosphate-buffered 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 effect of the APLN on IGF1/FSH-induced steroidogenesis, cell proliferation, apoptosis, and cell redox status, the GCs culture model was established. Therefore, all the healthy (well vascularized and having transparent follicular wall and fluid) and visible follicles were aspirated by a 17-gauge needle attached with a 10-mL syringe. The aspirates were transferred to a 60-mm dish under sterile conditions with PBS, and all cumulus-oocyte complexes were removed. The remaining cells and liquids were centrifuged in 15-mL conical tubes at 700 g for 5 min. Then, GCs were resuspended in Dulbecco's Modified Eagle Media (DMEM) media 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%. The cells were then seeded in a 48/96-well plate in a humidified CO_2 (5%) incubator at 37.5 ^{C} and having approximately 1.5×10^5 viable cells per well. The cells were allowed to attach and grow (75−80% confluence) for 48h. Then cells were treated with fresh media (FBS free) containing different doses of porcine FSH (0, 10^{-6}, 10^{-7}, 10^{-8}, $10^{-9,}$ and 10^{-10} M) and/or human recombinant IGF1 (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 mentioned peptides. After 48 h, the spent media were collected and stored for E2 and P4 assay. APLN-13 or APLN-17 were applied to GCs after culturing for 48 h with 10% FBS to investigate whether they affected the mRNA and protein expression of Bax, the cells were treated with different doses of APLN-13 and APLN-17 (0, 10^{-6}, 10^{-8}, 10^{-9} M) for additional 48 h and then mRNA and protein were extracted from cells.

Total Rna Extraction, Cdna Synthesis

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

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. The gene expression levels were analyzed with the $2^{-\Delta\Delta CT}$ method described previously by Livak and Schmittgen [20] concerning the housekeeping genes. The details of the selected genes and the primer pairs used in the study are provided in Table 1.

Table 1
Gene transcript, primer sequence (5'-3'), and the resulting fragment

Gene	Primer Sequence (5'-3')	Amplicon size (bp)	Accession No.
β-actin	F: TCTCACGGAGCGTGGCTACAG	100	NM_001290932.1
	R: CTGCTCGAAGTCCAGGGCCACGTA		
RPL15	F: TGGGCTACAAGGCCAAACAA	140	MG969348
	R: GCTTCGAGCAAA CTTGAGCTGG		
Bax	F: AACATGGAGCTGCAGAGGAT	84	[49]
	R: CCAGCTCTTGGTCGCTGTAGAG		

Western Blot Analysis

Total proteins were obtained from cultured GCs of different experiments by lysing in RIPA buffer 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. The pellet was eliminated, and lysates were diluted with 6X protein loading buffer (DL101-02; TransGen, China) and heated to 100°C for 5 min. After 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-buffered saline containing 0.1% (vol/vol) Tween-20 (TBST) for 2 h. Overnight incubation with the primary antibody (Bax and anti-beta-Actin) was performed. Then, after four washes, 10 min each, with TBST, membranes were incubated for 1 h at 37°C with goat anti-mouse IgG for beta-Actin and with goat anti-rabbit IgG for Bax. The membranes were washed four more times in TBST for 10 min each, then developed using ECL Plus (P0018, Beyotime, China) followed by detection with a multi-function imager (Syngene, Cambridge, UK). The intensities of individual bands were normalized to the expression of beta-Actin. Western blots were performed three times [17].

The Proliferation And Cytotoxicity Effects Of Gcs

The effects of APLN-13/-17 on GCs proliferation and cytotoxicity were evaluated by WST-1 ((4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolium]-1,3-benzene disulfonate) method. Briefly, $4×10^4$ 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 10μM) for 1 h and incubated for 48 h. According to the product manual, 10 μL of WST-1 was added to the cells during the last 4 h of incubation. Then, the absorbance was detected by a plate reader machine at a wavelength of 440 nm.

The 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

The FRAP method is a colorimetric assay based on the ability of the antioxidant molecules to reduce ferric-tripyridyl-triazine (Fe^{3+}TPTZ) to a ferrous form (Fe^{2+}TPTZ). Fe^{2+} is assessed spectrophotometrically through determination of its colored complex with 2,4,6-Tris(2-pyridyl)-s triazine (Fe^{2+} TPTZ). TPTZ reagent was prepared before use, mixed with 25 mL of acetate buffer, 2.5 mL of 2,4,6-Tris (2- pyridyl)-s-triazine (TPTZ) 10 mM in HCl 40 mM, and FeCl36H2O solution. In total, 4×10^4 cells per 200 µL of culture media were cultivated in a 96-well plate and treated with APLN-13/-17 for 48 h. Then, 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 incubated on ice for 30 min. The 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. The absorbance of Fe^{2+} TPTZ was detected at 595 nm. The ferric reducing ability of cell lysates was estimated by plotting a standard curve of absorbance against $FeSO^{4-}$7H2O standard solutions [21].

Steroids Elisa Assay

E2 and P4 concentrations were estimated in serum-free media from buffalo GCs after 48h of culture using ELISA kits supplied by Jiangsu Meibiao Biotechnology Co (E2, MB-2163A; P4, MB-4772A), China. Cells were plated in 48-well plates (10^5 viable cells/well), and six replicates were tested for each experimental condition (IGF1 and FSH in the presence or absence of APLN-13 or -17). The results were expressed as the concentration of the steroid (pmol/L). The intra- and inter-assay coefficients of variation for progesterone were less than 10%. Results are given as mean ± SEM. Data were obtained from 3 independent cultures that were done separately.

Statistical analysis

All the data have been shown as mean ± SEM. One or two-way ANOVA followed by Duncan's multiple range test was used to test the differences among groups by GLM procedure of SAS software (Version 9.4). A significant difference was considered if P < 0.05. The data were checked for normality and homogeneity of variance between the groups.

Results

The effect of varying IGF1 and FSH doses, with or without APLN-13, on the secretion of E2 and P4 from GCs of buffalo ovaries

Figure 1 portrays the effect of different doses of IGF1 and FSH, with and without APLN-13, on the secretion of E2 and P4 in buffalo ovarian GCs. To understand the effects of APLN with two main hormones influencing GCs biological function, we treated the GCs with different doses of IGF1 or FSH with or without APLN-13 in a sera-free DMEM culture media for 48 h following the incubation of the cells in the same media with 10% FBS. The results showed that different doses of IGF1 significantly increased the secretion of E2 and P4 from buffalo GCs ($P < 0.01$). The effects of IGF1 on E2 and P4 were substantially triggered when combined with APLN-13. There were no significant differences between the effects of IGF1 at 10^{-7} versus 10^{-6} M on the secretion of E2 ($P < 0.05$); nevertheless, IGF1 affected P4 production from GCs in a dose-dependent manner, increasing with higher doses. A 10^{-10} M FSH dose had no significant effect on E2 secretion. Despite this, other doses led to increased production of E2, with the highest effect being 10^{-8} M. Furthermore, treatment of GCs with FSH increased the secretion of P4, even though there was no substantive difference between various doses of FSH. In addition, we observed no significant differences between FSH alone and combined with APLN-13 (except for 10^{-9} M of FSH on E2) on E2 and P4 concentration production in the treated buffalo GCs.

The effects of various doses of APLN-13 and APLN-17 on the buffalo GCs proliferation and cytotoxicity

The effects of different doses of APLN-13/-17 on the GCs proliferation and cytotoxicity are presented in Fig. 2. APLN-13 caused a significant increase in cell proliferation compared to control cells; however, there were no substantial differences among various doses of APLN-13 in cell proliferation (Fig. 2A). APLN-17 also increased the proliferation of GCs at 10^{-8} and 10^{-9} M doses, but not at 10^{-6} (Fig. 2B). Neither APLN-13 nor APLN-17 did not have a substantially cytotoxic effect on the GCs (Fig. 2C, 2D). Results also showed that preincubation of the cells with APJ antagonist (ML221 10 μM) before APLN-13/-17 treatment did not affect the GC proliferation (Except APLN-17 at 10^{-6} M).

The effects of various doses of APLN-13 and APLN-17 on the expression abundance of Bax in the GCs of buffalo ovaries

The effects of various doses of APLN-13/-17 on the expression amount of Bax are shown in Fig. 3. APLN-13, when administered at 10^{-8} and 10^{-6} M, significantly decreased and increased the mRNA expression of Bax without preincubating the cells with ML221 (Fig. 3A). The same effects were not detected when the cells were preincubated with ML221 (10 μM; Fig. 3B) and APLN-13 at 10^{-6}M, significantly decreasing the mRNA and protein expression levels of Bax. On the other hand, different doses of APLN-17 did not significantly affect the abundances of Bax mRNA/protein (Fig. 3C, 3D).

The effects of various doses of APLN-13 and APLN-17 on the total antioxidant capacity of GCs

The effects of different doses of APLN-13/-17 on the antioxidant capacity of GCs are shown in Fig. 4. APLN-13 at the doses of 10^{-9}, 10^{-8} M significantly increased the reduction power of GCs compared to the control cells; however, it did not have a significant effect at the dose of 10^{-6} M (Fig. 4A). Contrastingly, all doses of APLN-17 had a statistically significant effect on the antioxidant power of GCs

(Fig. 4B). The cells treated with APLN-13 and APLN-17 at the doses of 10^{-8} had the highest impact on the antioxidant power of GCs. When cells were preincubated with ML221 (10 μM) for 1 h before being treated with APLN-13/-17, antioxidant capacity of the cells was not significantly affected.

Discussion

In recent years, adipokines have been the most focused peptides in the reproduction system. The effect of the APLN/APJ system on various aspects of reproduction has been studied in different species, but the findings are contradictory. There are no reported results regarding the effects of APLN on the reproduction of buffaloes. As part of the current study, we surveyed for the first time the effects of different FSH and IGF1 doses on E2 and P4 production in buffalo ovaries with and without APLN-13. Also, we evaluated the effects of APLN-13 and APLN-17 on cell proliferation, Bax protein expression, cytotoxicity, and antioxidant capacity in the same cells. Our findings showed APLN-13 stimulated the increasing effect of IGF1 on the secretion of E2 from GCs of buffalo ovaries; however, the effect of different doses was not the same. In addition, APLN-13 triggered the effect of IGFI on the production of P4 in a dose-dependent manner. In the presence of APLN-13, neither E2 nor P4 were significantly affected by FSH doses from buffalo GCs. These results agree with the literature [13, 15]. As one of the main adipokines, APLN has a crucial role in energy metabolism. It affects glucose transfer and mimics the insulin effect in skeletal muscles [22, 23]. As insulin and IGF1 have similar structures and receptors, APLN can enhance IGF1 responsiveness in GCs and increase E2 and P4 production [15]. Furthermore, previous studies showed that APLN enhances the phosphorylation of PI3K/Akt induced by IGF1 [15], which causes steroidogenesis regulation in GCs [24, 25]. Roche et al. [12] suggested that the effects of APLN on IGF1-induced steroidogenesis in GCs are exerted through PI3K/Akt signaling pathway.

The current results showed that APLN-13 and-17 significantly increased the proliferation of buffalo GCs; certainly, APLN-17 at 10^{-6}M did not substantially affect GCs proliferation compared to the control group. The same results were gained when the cells were preincubated with ML221 for 1 h. Additionally, we showed that APLN-13/-17 was not cytotoxic to buffalo GCs. Literature demonstrated that APLN restrains cell apoptosis and retina angiogenesis and improves the proliferation of vascular smooth muscle cells, osteoblasts, and microvascular endothelial cells [26–28]. Furthermore, the proliferative effect of APLN on various carcinoma cell lines has been verified in different studies [29–31]. In concurrence with our results, APLN promoted the GCs proliferation in the rat [32], bovine [13], and porcine [14]. However, the results of one study suggested that APLN does not affect human GCs proliferation, but in line with our findings, they explained that it does not affect the viability of GCs [15]. Another report declared that APLN prevents the proliferation and migration of pulmonary arterial smooth muscle cells [33]. We did not investigate the cellular pathways of APLN's effect on proliferation or apoptosis. However, the reported results showed that APLN promoted *in vitro* cell proliferation through activation of the ERK1/2 signaling pathway in porcine GCs [14], lung adenocarcinoma [34], MCF-7 (breast cancer cells) [35], and ovarian cancer cells [25]. On the other hand, in some other studies, it is illustrated that cell proliferation promotion can occur via the IP3/Akt signaling pathway [36, 37]. ERK1/2 signaling pathway activation is the main pathway for

cell proliferation improvement by APLN. ERK1/2, as an essential pathway to cell proliferation, triggers the expression of cyclin proteins (mainly cyclin D1) and improves cell cycle move on [38]. APLN also improves steroidogenesis and proliferation of GCs through increasing IGF1 sensitivity [15, 22].

This study also revealed that APL-13 only at the doses of 10^{-8}M (without ML221) and 10^{-6}M (with ML221) decreased Bax protein expression; nonetheless, 10^{-6}M (in the absence of ML221) elevated the protein expression of Bax compared to control cell; therefore, the results were divergent. Bcl-22 protein family is a necessary governor for apoptosis in various types of cells [39]. Different members of the BCL2 family are expressed in growing follicles, such as anti-apoptotic and proapoptotic factors [40]. It is shown that Bax, as a proapoptotic protein, is engaged in the apoptosis of follicle cells under different conditions and, along with Bcl-2 as an anti-apoptotic factor, plays a critical role in the apoptosis of different cells. The anti-apoptotic effect of APLN has previously been shown in mouse [35] and human [26] osteoblasts, rat cardiomyocytes [41], and rat neurons [42]. Furthermore, Shuang et al. [32] showed that APLN decreases apoptosis of rat ovarian GCs. However, in contrast to parts of our study, they found that APLN was upregulated expression of Bax. In an older study, Shimizu et al. [16] found that the mRNA expression of APJ was increased in apoptosis-induced bovine GCs. They concluded that there is a correlation between GCs atresia and the expression of APJ. However, Roche et al. [13] did not find any effect of APLN-13/-17 on the apoptosis of bovine ovarian GCs. In line with our findings, APLN diminished mRNA and protein expression of proapoptotic proteins (including Bax) in BeWo cells [43]. APLN decreases apoptosis through PI3K/Akt and ERK1/2 signaling pathways, and activation of these pathways causes suppression of BAX/BAD proapoptotic proteins [44]. Moreover, it is demonstrated that APLN enhances Bcl-2 expression levels, reduces Bax protein's expression amount, and arrests the release of cytochrome C [44]. We did not assess the expression of Bax in luteinizing GCs. However, Shirasuna et al. [18] implied that down-regulation of the APLN−APJ system could be associated with cell apoptosis and leutolysis in bovine.

The present study results revealed that APLN-13/-17 had a significant effect on the antioxidant power of buffalo ovarian GCs, and APLN improved the antioxidant potential of GCs. Oxidative stress is a crucial factor causing reactive oxygen species (ROS) aggregation and subsequent cell death [45]. In human adipocytes, Than et al. [46] found APLN reduced ROS production and release, as well as oxidative stress and oxidative-induced cellular degeneration. Likewise, in agreement with the results of this study, APLN enhanced the response of rat adipocytes and adrenal medulla cells to oxidative stress and decreased the levels of oxidative stress indicators [47, 48]. Mlyczyńska et al. [43] outlined that APLN reduced oxidative stress by enhancing extracellular oxygen utilization. APLN increases GC antioxidant power and is directly correlated with improving cell viability and decreasing apoptosis. Another possible explanation for APLN's positive effect on cell death could be its antioxidant properties.

Conclusion

Our results showed that APLN stimulated the IGF1-induced production of estradiol and progesterone in the buffalo ovarian granulosa cells, improving the proliferation and antioxidant power of the same cells.

However, the effect of APLN on the Bax protein as a proapoptotic factor varied. These data demonstrate that APLN may have an essential role in steroidogenesis and proliferation of follicular granulosa cells of buffalos; nonetheless, these roles, in addition to Bax expression and antioxidant power of the cells are required more investigations.

Declarations

Availability of data

All data supporting the conclusions of this study are included in the article.

Conflict of interests

There are no conflicts of interest to declare

Funding

Acknowledgments

References

1. Pitkin SL, Maguire JJ, Bonner TI, Davenport AP (2010) International Union of Basic and Clinical Pharmacology. LXXIV. Apelin receptor nomenclature, distribution, pharmacology, and function. Pharmacol Rev 62:331-342

2. Tatemoto K, Hosoya M, Habata Y, Fujii R, Kakegawa T, Zou M-X, Kawamata Y, Fukusumi S, Hinuma S, Kitada C (1998) Isolation and characterization of a novel endogenous peptide ligand for the human APJ receptor. Biochem Biophys Res Com 251:471-476

3. O'Dowd BF, Heiber M, Chan A, Heng HH, Tsui L-C, Kennedy JL, Shi X, Petronis A, George SR, Nguyen T (1993) A human gene that shows identity with the gene encoding the angiotensin receptor is located on chromosome 11. Gene 136:355-360

4. Shokrollahi B, Shang J-H, Ahmad HI, Yang C-Y, Saadati N (2021) Reproductive roles of novel adipokines apelin, visfatin, and irisin in farm animals. Theriogenology

5. Estienne A, Bongrani A, Reverchon M, Ramé C, Ducluzeau P-H, Froment P, Dupont J (2019) Involvement of novel adipokines, chemerin, visfatin, resistin and apelin in reproductive functions in

normal and pathological conditions in humans and animal models. Int J Mol Sci 20:4431

6. De Falco M, De Luca L, Onori N, Cavallotti I, Artigiano F, Esposito V, De Luca B, Laforgia V, Groeger AM, De Luca A (2002) Apelin expression in normal human tissues. IN VIVO-ATHENS- 16:333-336

7. Palmioli E, Dall'Aglio C, Bellesi M, Tardella FM, Moscatelli S, Scocco P, Mercati F (2021) The apelinergic system immuno-detection in the abomasum and duodenum of sheep grazing on semi-natural pasture. Animals 11:3173

8. Mercati F, Maranesi M, Dall'Aglio C, Petrucci L, Pasquariello R, Tardella FM, De Felice E, Scocco P (2018) Apelin system in mammary gland of sheep reared in semi-natural pastures of the central apennines. Animals 8:223

9. Kasai A, Shintani N, Oda M, Kakuda M, Hashimoto H, Matsuda T, Hinuma S, Baba A (2004) Apelin is a novel angiogenic factor in retinal endothelial cells. Biochem Biophys Res Com 325:395-400

10. Heinonen M, Purhonen A, Miettinen P, Pääkkönen M, Pirinen E, Alhava E, Åkerman K, Herzig K (2005) Apelin, orexin-A and leptin plasma levels in morbid obesity and effect of gastric banding. Regul Peptid 130:7-13

11. Liu Q, Jiang J, Shi Y, Mo Z, Li M (2020) Apelin/Apelin receptor: A new therapeutic target in Polycystic Ovary Syndrome. Life Sci:118310

12. Sandal S, Tekin S, Seker FB, Beytur A, Vardi N, Colak C, Tapan T, Yildiz S, Yilmaz B (2015) The effects of intracerebroventricular infusion of apelin-13 on reproductive function in male rats. Neurosci Let 602:133-138

13. Roche J, Ramé C, Reverchon M, Mellouk N, Rak A, Froment P, Dupont J (2017) Apelin (APLN) regulates progesterone secretion and oocyte maturation in bovine ovarian cells. Reproduction 153:589-603

14. Rak A, Drwal E, Rame C, Knapczyk-Stwora K, Słomczyńska M, Dupont J, Gregoraszczuk E (2017) Expression of apelin and apelin receptor (APJ) in porcine ovarian follicles and in vitro effect of apelin on steroidogenesis and proliferation through APJ activation and different signaling pathways. Theriogenology 96:126-135

15. Roche J, Ramé C, Reverchon M, Mellouk N, Cornuau M, Guerif F, Froment P, Dupont J (2016) Apelin (APLN) and apelin receptor (APLNR) in human ovary: expression, signaling, and regulation of steroidogenesis in primary human luteinized granulosa cells. Biol Rep 95:104, 1-12

16. Shimizu T, Kosaka N, Murayama C, Tetsuka M, Miyamoto A (2009) Apelin and APJ receptor expression in granulosa and theca cells during different stages of follicular development in the bovine ovary: Involvement of apoptosis and hormonal regulation. Anim Rep Sci 116:28-37

17. Mercati F, Scocco P, Maranesi M, Acuti G, Petrucci L, Cocci P, Renzi A, De Felice E, Dall'Aglio C (2019) Apelin system detection in the reproductive apparatus of ewes grazing on semi-natural pasture. Theriogenology 139:156-166

18. Shirasuna K, Shimizu T, Sayama K, Asahi T, Sasaki M, Berisha B, Schams D, Miyamoto A (2008) Expression and localization of apelin and its receptor APJ in the bovine corpus luteum during the estrous cycle and prostaglandin F2a-induced luteolysis. Reproduction 135:519-526

19. Shokrollahi B, Zheng H-Y, Li L-Y, Tang L-P, Ma X-Y, Lu X-R, Duan A-Q, Zhang Y, Tan X-H, Huang C-X (2022) Apelin and Apelin Receptor in Follicular Granulosa Cells of Buffalo Ovaries: Expression and Regulation of Steroidogenesis. Front Endocrinol 13

20. Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2− ΔΔCT method. Methods 25:402-408

21. Gigante P, Berni M, Bussolati S, Grasselli F, Grolli S, Ramoni R, Basini G (2018) Glyphosate affects swine ovarian and adipose stromal cell functions. Anim Rep Sci 195:185-196 DOI

22. Dray C, Knauf C, Daviaud D, Waget A, Boucher J, Buléon M, Cani PD, Attané C, Guigné C, Carpéné C (2008) Apelin stimulates glucose utilization in normal and obese insulin-resistant mice. Cell Met 8:437-445

23. Yue P, Jin H, Aillaud M, Deng AC, Azuma J, Asagami T, Kundu RK, Reaven GM, Quertermous T, Tsao PS (2010) Apelin is necessary for the maintenance of insulin sensitivity. Am J Physiol Endocrinol Metab 298:E59-67 DOI 10.1152/ajpendo.00385.2009

24. Zhou P, Baumgarten SC, Wu Y, Bennett J, Winston N, Hirshfeld-Cytron J, Stocco C (2013) IGF-I signaling is essential for FSH stimulation of AKT and steroidogenic genes in granulosa cells. Mol Endocrinol 27:511-523

25. Hoffmann M, Gogola J, Ptak A (2019) Apelin abrogates the stimulatory effects of 17β-estradiol and insulin-like growth factor-1 on proliferation of epithelial and granulosa ovarian cancer cell lines via crosstalk between APLNR and ERα/IGF1R. Mol Biol Rep 46:6325-6338

26. Xie H, Tang S-y, Cui R-r, Huang J, Ren X-h, Yuan L-q, Lu Y, Yang M, Wu X-p, Luo X-h (2006) Apelin and its receptor are expressed in human osteoblasts. Regul Peptid 134:118-125

27. Kasai A, Ishimaru Y, Kinjo T, Satooka T, Matsumoto N, Yoshioka Y, Yamamuro A, Gomi F, Shintani N, Baba A (2010) Apelin is a crucial factor for hypoxia-induced retinal angiogenesis. Arterioscl Thromb Vascul Biol 30:2182-2187

28. Olszanecka-Glinianowicz M, Madej P, Nylec M, Owczarek A, Szanecki W, Skałba P, Chudek J (2013) Circulating apelin level in relation to nutritional status in polycystic ovary syndrome and its association with metabolic and hormonal disturbances. Clin Endocrinol 79:238-242

29. Peng X, Li F, Wang P, Jia S, Sun L, Huo H (2015) Apelin-13 induces MCF-7 cell proliferation and invasion via phosphorylation of ERK1/2. Int J Mol Med 36:733-738

30. Yang L, Su T, Lv D, Xie F, Liu W, Cao J, Sheikh IA, Qin X, Li L, Chen L (2014) ERK1/2 mediates lung adenocarcinoma cell proliferation and autophagy induced by apelin-13. Acta Biochim Biophys Sin 46:100-111

31. Rene Gonzalez R, Watters A, Xu Y, Singh UP, Mann DR, Rueda BR, Penichet ML (2009) Leptin-signaling inhibition results in efficient anti-tumor activity in estrogen receptor positive or negative breast cancer. Breast Can Res 11:1-12

32. Shuang L, Jidong W, Hongjuan P, Zhenwei Y (2016) Effects of apelin on proliferation and apoptosis in rat ovarian granulosa cells. Clin Exp Obstet Gynecol 43:409-413

33. Zhang H, Gong Y, Wang Z, Jiang L, Chen R, Fan X, Zhu H, Han L, Li X, Xiao J (2014) Apelin inhibits the proliferation and migration of rat PASMC s via the activation of PI3K/Akt/m TOR signal and the inhibition of autophagy under hypoxia. J Cell Mol Med 18:542-553

34. Yang Y, Zhang X-J, Li L-T, Cui H-Y, Zhang C, Zhu C-H, Miao J-Y (2016) Apelin-13 protects against apoptosis by activating AMP-activated protein kinase pathway in ischemia stroke. Peptides 75:96-100

35. Tang S-Y, Xie H, Yuan L-Q, Luo X-H, Huang J, Cui R-R, Zhou H-D, Wu X-P, Liao E-Y (2007) Apelin stimulates proliferation and suppresses apoptosis of mouse osteoblastic cell line MC3T3-E1 via JNK and PI3-K/Akt signaling pathways. Peptides 28:708-718

36. Zhang J, Liu Q, Hu X, Fang Z, Huang F, Tang L, Zhou S (2015) Apelin/APJ signaling promotes hypoxia-induced proliferation of endothelial progenitor cells via phosphoinositide-3 kinase/Akt signaling. Mol Med Rep 12:3829-3834

37. Qin D, Zheng X-x, Jiang Y-r (2013) Apelin-13 induces proliferation, migration, and collagen I mRNA expression in human RPE cells via PI3K/Akt and MEK/Erk signaling pathways. Mol Vis 19:2227

38. Chen L, Tao Y, Feng J, Jiang YR (2015) Apelin protects primary rat retinal pericytes from chemical hypoxia-induced apoptosis. J Ophthalmol 2015

39. Greenfeld CR, Babus JK, Furth PA, Marion S, Hoyer PB, Flaws JA (2007) BAX is involved in regulating follicular growth, but is dispensable for follicle atresia in adult mouse ovaries. Reproduction 133:107-116

40. Hussein MR (2005) Apoptosis in the ovary: molecular mechanisms. Human Reprod Up 11:162-178

41. Zhang Z, Bo Y, Tao G-z (2009) Apelin protects against cardiomyocyte apoptosis induced by glucose deprivation. Chin Med J 122:2360-2365 DOI

42. Zeng XJ, Yu SP, Zhang L, Wei L (2010) Neuroprotective effect of the endogenous neural peptide apelin in cultured mouse cortical neurons. Exp Cell Res 316:1773-1783

43. Mlyczyńska E, Myszka M, Kurowska P, Dawid M, Milewicz T, Bałajewicz-Nowak M, Kowalczyk P, Rak A (2021) Anti-apoptotic effect of apelin in human placenta: studies on BeWo cells and villous explants from third-trimester human pregnancy. Int J Mol Sci 22:2760

44. Rastaldo R, Cappello S, Folino A, Losano G (2011) Effect of apelin-apelin receptor system in postischaemic myocardial protection: a pharmacological postconditioning tool? Antioxid Red Sig 14:909-922

45. Dawid M, Mlyczyńska E, Jurek M, Respekta N, Pich K, Kurowska P, Gieras W, Milewicz T, Kotula-Balak M, Rak A (2022) Apelin, APJ, and ELABELA: Role in Placental Function, Pregnancy, and Foetal Development—An Overview. Cells 11:99

46. Than A, Zhang X, Leow MK-S, Poh CL, Chong SK, Chen P (2014) Apelin attenuates oxidative stress in human adipocytes. J Biol Chem 289:3763-3774

47. Foroughi K, Khaksari M, Rahmati M, Bitaraf FS, Shayannia A (2019) Apelin-13 protects PC12 cells against methamphetamine-induced oxidative stress, autophagy and apoptosis. Neurochem Res 44:2103-2112

48. Niknazar S, Abbaszadeh H-A, Peyvandi H, Rezaei O, Forooghirad H, Khoshsirat S, Peyvandi AA (2019) Protective effect of [Pyr1]-apelin-13 on oxidative stress-induced apoptosis in hair cell-like cells derived from bone marrow mesenchymal stem cells. Eur J Pharmacol 853:25-32

49. Han P, Guerrero-Netro H, Estienne A, Cao B, Price CA (2017) Regulation and action of early growth response 1 in bovine granulosa cells. Reproduction 154:547-557
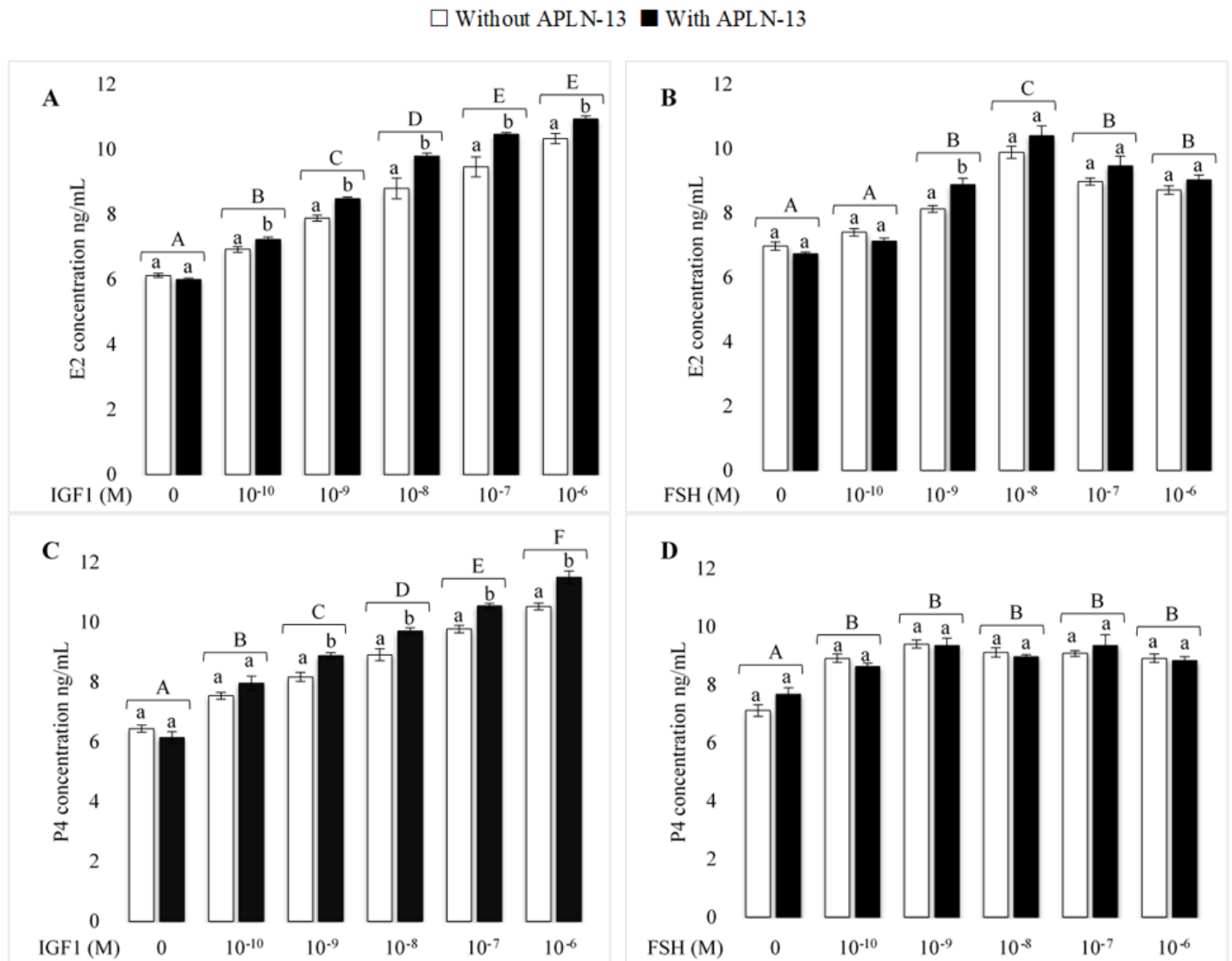
Figures

Figure 1

represents the effects of IGF1 (A, C) or FSH (B, D) given to buffalo GCs at 0, 10^{-10}, 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6} doses alone or combined with APLN-13 (10^{-9}). GCs were seeded in DMEM (with 10% FBS) for 48h and then in free sera DMEM media according to the above description. After collecting, ELISA examined the culture media for E2 and P4. Different capital and lower-case letters on the bars reveal the significant

effects of different concentrations of IGF1 or FSH and in combination with APLN-13, respectively. Data represent the mean ± SEM of 6 replicates/treatments repeated in 3 different experiments
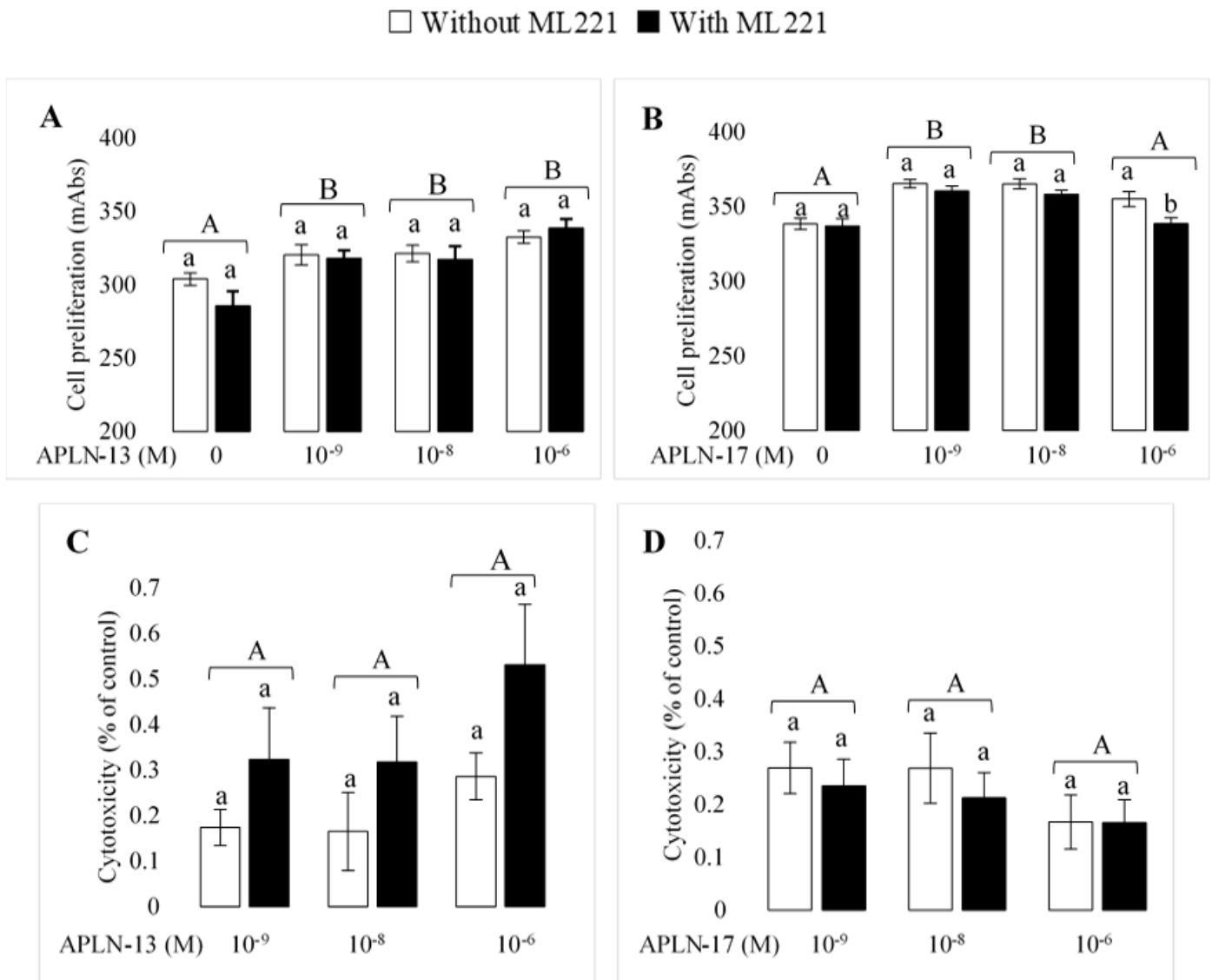

Figure 2

This figure shows the effect of APLN-13/-17 on buffalo GCs proliferation (A, B) and cytotoxicity (C, D). GCs were seeded in a DMEM media with 10% fetal bovine serum (FBS) in 96 wells plates and then in DMEM media with 1% FBS in the presence of APLN-13 or APLN-17 with concentrations 0, 10^{-9}, 10^{-8}, and 10^{-6} M for 48h with or without preincubation of the cells with APJ antagonist (ML221 (10μM)) for 1 h. At the last 4 h of incubation,10 μL of WST-1 was added to the cells. Then, the absorbance was detected by a plate reader machine at a wavelength of 440 nm against 620 nm. Data, expressed as milliAbs units, represent the mean ± SEM of 6 replicates/treatment repeated per each 3 different experiments. Different letters on the bars indicate a significant difference (P < 0.01) among treatments as calculated by ANOVA and Duncan test.
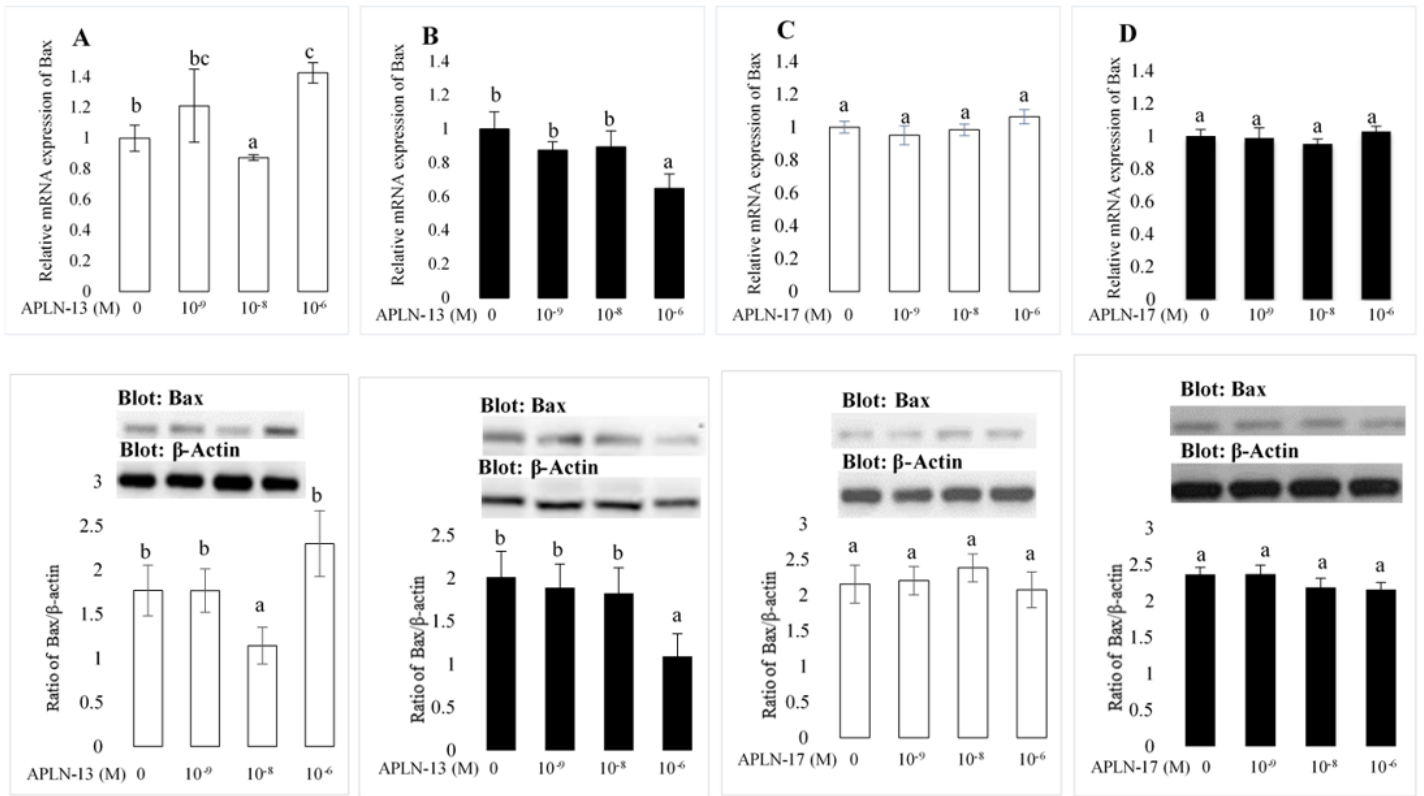
Figure 3

The Protein and mRNA expression of Bax in the buffalo GCs treated by different doses of APLN-13/-17 without (A/C) or with (B/D) preincubation of cells with APJ antagonist (ML221 (10μM)). GCs were seeded in DMEM (with 10% FBS) for 48h and then in free sera DMEM media containing different concentrations of APLN-13/-17 (0, 10^{-9}, $10^{-8,}$ and 10^{-6} M) for 48h in the presence or absence of APJ antagonist (ML221 (10μM)). Following the total protein and mRNA extraction from the treated buffalo GCs, they were used to assess the expression levels of Bax by western blot and quantitative real time-PCR, respectively. β-actin and RPL15 expression levels in the same mRNA samples were used to normalize the mRNA expression of Bax. The upper and lower parts of the panels show the relative levels of mRNA and illustrative immunoblots, respectively. In this regard, GCs protein extracts were immunoblotted by the Bax antibody. An anti-β-actin antibody then reprobed the membranes with the same protein loading for confirmation. By evaluating blots, the ratio of Bax to β-actin was calculated. Statistical analyses were done and the results are presented as the mean± SEM. The different letters on the bars show significant differences at P<0.05.
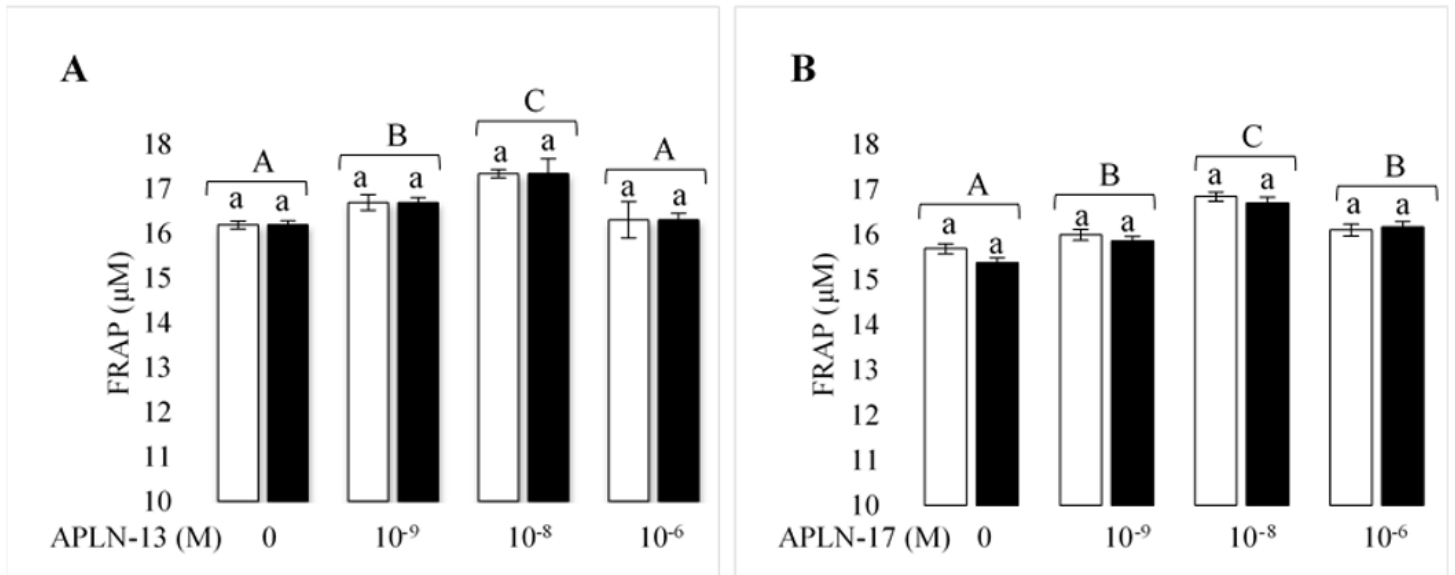
Figure 4

Effect of the treatment with APLN-13/-17 on nonenzymatic scavenging activity of buffalo GCs. GCs were seeded in a DMEM media with 10% fetal bovine serum (FBS) in 96 wells plates and then in DMEM media with 1% FBS in the presence of APLN-13 or APLN-17 with concentrations 0, 10^{-9}, 10^{-8}, and 10^{-6} M for 48h with or without preincubation of the cells with APJ antagonist (ML221 (10µM)) for 1 h. Then, 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. The test was carried out on 40 mL of cell lysates added to Fe3+ TPTZ reagent and then incubated at 37 ^{C} for 30 min. The absorbance of Fe2+ TPTZ was detected at 595 nm. The ferric reducing ability of cell lysates was estimated by plotting a standard curve of absorbance against FeSO4-7H2O standard solutions. Data represent the mean ± SEM of 6 replicates/treatments repeated in 3 different experiments. Different letters on the bars indicate a significant difference (P < 0.01) among treatments as calculated by ANOVA and Duncan test.