The adiponectin receptor agonist, AdipoRon, promotes reproductive hormone secretion and gonadal development via the hypothalamic-pituitary-gonadal axis in chickens

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ABSTRACT   Adiponectin is a key hormone secreted by fat tissues that has multiple biological functions, including regulating the energy balance and reproductive system by binding to its receptors AdipoR1 and AdipoR2. This study investigated the correlation between the levels of adiponectin and reproductive hormones in the hypothalamic-pituitary-ovarian (**HPO**) axis of laying hens at 4 different developmental stages (15, 20, 30, and 68 wk) and explored the effects of AdipoRon (an activator of adiponectin receptors) on the hypothalamic-pituitary-gonadal (**HPG**) axis and follicle and testicular Leydig cells in vitro and in vivo. The results demonstrated that the adiponectin level was significantly correlated with that of reproductive hormones in the HPO axis (e.g., GnRH, FSH, LH, and E2) in laying hens at 4 different ages. Moreover, AdipoRon could promote the expression of AdipoR1 and AdipoR2 and the secretion of reproductive hormones in the HPG axis, including GnRH, FSH, LH, P4, and T.

AdipoRon could also upregulate the expression of genes related to follicular steroidogenesis (*STAR, CYP19A1, CYP17A1,* and *CYP11A1*), hepatic lipid synthesis (*OVR, MTP*), follicular lipid uptake (*PPAR-g*), and follicular angiogenesis (*VEGFA1, VEGFA2, VEGFR1, ANGPT1, ANGPT2, TEK*) in the oviposition period, and all of these findings were consistent with the results obtained from in vitro experiments after the transfection of small white follicles (**SWFs**) with AdipoRon. Furthermore, the results suggest that AdipoRon increases the diameter of testicular seminiferous tubules, the number of spermatogenic cells and sperm production in vivo and enhances the expression of *AdipoR1, AdipoR2* and steroid hormones in vitro. Collectively, the findings suggest that AdipoRon could facilitate the expression and secretion of reproductive hormones in the HPG axis by activating its receptors and then improve the growth and development of follicles and testes in chickens.

Key words: adiponectin, AdipoRon, hypothalamic-pituitary-gonadal axis, follicular and testicular development, chicken

INTRODUCTION

Reproduction is an essential physiological process for species continuity, gonad growth, and development, which are precisely regulated by integration of the hypothalamus-pituitary-gonadal (**HPG**) axis. Gonadotropin-releasing hormone (**GnRH**) secreted from the hypothalamus promotes the synthesis and secretion of luteinizing hormone (**LH**) and follicle stimulating hormone (**FSH**) in the pituitary and eventually affects the fertility of animals. In chickens, the mature ovary contains multiple follicles of different sizes and at different developmental stages, and these include prehierarchical follicles (small white follicles [**SWFs**], diameter of 2−4 mm; small yellow follicles [**SYWs**], diameter of 4−8 mm; and large yellow follicles [**LYWs**], diameter of 8−12 mm), preovulatory follicles (F6-F1, diameter of 12−36 mm), and postovulatory follicles (**POF5-POF1**) (Gilbert et al., 1983; Lovell et al., 2003; Li et al., 2019). Diverse hormones in the HPG axis play pivotal roles in the regulation of cell proliferation, differentiation and degeneration and in secretion of hormones and cytokines and determine follicular development or atresia. Furthermore, during follicle growth and development, abundant capillaries in the thecal layer mediate the transport of yolk precursor material from the liver to the

developing follicle, which eventually leads to formation of an F1 follicle larger than 30 mm (Schneider, 2016). In roosters, the hypothalamic-pituitary-testicular (**HPT**) axis could affect spermatogenesis in the testis and modulate androgen synthesis in testicular Leydig cells (Deng et al., 2018; Barbe et al., 2019).

Adipose tissue-producing factors, such as adipokines and cytokines, exert systemic endocrine effects on multiple tissues (Wassie et al., 2019). Furthermore, adipokines can affect the gonad through the hypothalamic-pituitary axis (Tsatsanis et al., 2015). Adiponectin (AdipoQ) mainly secreted by mature adipocytes and plays an important role in energy homeostasis, vascular homeostasis, and immunity, which are also able to regulate reproduction by affecting gonad growth through the HPG axis (Ahima and Lazar, 2008; Stern et al., 2016; Barbe et al., 2019; Li et al., 2021a,b). The biological activity of AdipoQ is mediated by 2 receptors, namely, adiponectin receptor 1 (**AdipoR1**) and adiponectin receptor 1 (**AdipoR2**). To date, numerous studies have found that AdipoQ and its receptors are expressed in the HPG axis of chickens, which indicates that adiponectin and its receptors may play important roles in the HPG axis of chickens (Chabrolle et al., 2007a; Ocón-Grove et al., 2008; Li et al., 2021a). In recent years, growing evidence has shown that AdipoQ plays an important role in the reproductive system of mammals and birds. In rats, AdipoQ can inhibit the promoter activity and transcription of the KISS-1 gene through the activation of AMPK in hypothalamic GT1-7 neurons, and thereby affects the secretion of GnRH (Wen et al., 2012). In addition, in rat pituitary cells, AdipoQ can inhibit the release of LH (Rodriguez-Pacheco et al., 2007). In pigs at the luteal phase, AdipoQ affects the secretion of progesterone (**P4**) by luteal cells, estradiol (**E2**) by granulosa cells and testosterone (**T**) by intimal cells (Maleszka et al., 2014). In ruminants, AdipoQ can promote testicular development and sperm capacitation (Kasimanickam et al., 2013; Bai et al., 2018).

AdipoRon, a small-molecule adiponectin receptor (**AdipoR**) agonist, can activate the AMPK and PPAR-α pathways and then improve insulin resistance and glucose intolerance by binding to AdipoR1 and AdipoR2, and is involved in the processes of glucose metabolism, lipid metabolism, and the development of cardiac hypertrophy and ovarian cancer (Choi et al., 2018; Zhang et al., 2018; Ramzan et al., 2019). Furthermore, in humans, AdipoRon reportedly reduces aromatase expression and E2 production in luteinized granulosa cells by activating the AMPK and PPAR signaling pathways (Grandhaye et al., 2021). Our previous study showed that adiponectin (**AdipoRon**) could affect steroid production or hormone secretion in the follicular granulosa cells of chickens (Li et al., 2021b). However, there remains a lack of systematic research on AdipoQ or AdipoRon in the HPG axis of chickens.

Therefore, this study aimed to investigate the correlation between AdipoQ, including its receptors and reproductive hormones in the HPG axis, and the effect of AdipoRon on the secretion of reproductive hormones in the HPG axis and gonadal development in chickens. Additionally, we further evaluated the effect of AdipoRon on the secretion of steroid hormones (P4, E2, and T) by incubating testicular Leydig cells and SWFs in vitro.

MATERIALS AND METHODS

Ethics Statement

All sample collections and treatment procedures were conducted strictly in accordance with the protocol approved by the Institutional Animal Care and Use Committee (**IACUC**) of Henan Agricultural University, China (permit number: 19-0068).

Experimental Design, Animals, and Management

In this experiment, all experimental animals were collected from the poultry germplasm resource farm of Henan Agricultural University. The animals were fed in individual cages and provided free access to commercial feed and water. Forty healthy Hy-Line brown laying hens were selected and randomly divided into 4 groups, and blood samples were collected at 4 physiological periods of chickens, namely the expected oviposition period (15 wk), early oviposition period (20 wk), peak oviposition period (30 wk), and late oviposition period (68 wk).

Thirty healthy 30-wk-old Hy-Line brown laying hens of similar weight were randomly assigned to 3 treatment groups and injected intravenously with different dosages of AdipoRon (0, 5, and 15 mg/kg; MCE, Princeton, NJ). Blood was collected at 0 min before injection and 10, 20, 30, 60 and 120 min after injection. After 120 min, these birds were killed by cervical dislocation, and the hypothalamus, pituitary, and ovary tissues were then collected.

Thirty-six healthy 15-wk-old Hy-Line brown laying hens of similar weight were randomly assigned to 3 treatment groups and injected intravenously with different dosages of AdipoRon (0, 5, and 15 mg/kg; MCE). After continuous injection into the wing vein for 8 d, hypothalamus, pituitary, SWFs, and liver tissues were collected after euthanasia by cervical dislocation.

Eighteen 19-wk-old healthy Rhode Island Red roosters with similar weights were randomly allotted into 3 groups and injected intravenously with different dosages of AdipoRon (0, 5, and 15 mg/kg; MCE). Blood was collected at 0, 10, 20, 30, 60, 180, and 360 min after 7 d of injection. After 360 min, these birds were killed by cervical dislocation, and the hypothalamus, pituitary, and right testis tissues were then rapidly isolated, frozen in liquid nitrogen and stored at −80°C for the extraction of RNA. The left testis tissue was fixed in 4% formaldehyde fixative solution for histological examination.

SWF Culture and Chemical Treatment

The SWFs (2−4 mm) of 68-wk-old Hy-Line brown laying hens were transferred to high-glucose medium

Table 1. Primer sets for quantitative real-time PCR.

Gene	GenBank number	Primer	Sequence of nucleotide (5-3)	Size (bp)
AdipoR1	NM_001031027.1	F	CCAGGAGAAGGTTGTGTTTG	149
		R	TGATCAGCAGTGCAATTCCT	
AdipoR2	NM_001007854.1	F	CTGCAACAACACAGACAGCC	171
		R	GGGCT TGTAGAAGGGGTGAC	
GnRH	NM_001080877.1	F	TTCACCGCATCTGTGGCAAT	165
		R	CTGGTAAGAGCCAGGGCATT	
GnRHR	NM_001012609.1	F	TCTGCTGGACCCCCTACTAC	127
		R	TCCAGGCAGGCATTGAAGAG	
FSH	NM_204257.1	F	TGCGGTGACCATCCTGAATC	144
		R	TTGATTGCTTCCATTGTGACTGA	
FSHR	NM_205079.1	F	ATGTCCTTGGGTCTCACCTG	202
		R	CTGTGAAAGCTCCCTTCGGA	
LH	NM_001030612.2	F	TCCCGTCTGTGCTACCAATG	146
		R	TCTGGAACTCACGGCAATGT	
LHR	NM_204936.1	F	GGGCTTTCCCAAGCCTACAT	133
		R	TGGTGTCTTTATTGGCGGCT	
PGR	NM_205262.1	F	TCTGGTTGCCACTACG	156
		R	CTTCCTCAAGCGACACG	
ESR	NM_205183.2	F	TATTACTGGACAGGAATCAAGGGA	137
		R	CAGGATGATGGACTTAAGGCA	
StAR	NM_204686.2	F	TCGGCACGAGCGCAT	147
		R	TGAAGGATGCTGGCTTGTCA	
CYP11A1	NM_001001756.1	F	GGTTGGCTCAACCTGTACCA	100
		R	CCCTGTAGATGGGCCCAAAG	
CYP19A1	NM_001364699.1	F	GAATTCTTCCCAAAACCGAATGAG	162
		R	GCACCGTCTCAGAAGAGTCACCAG	
CYP17A1	NM_001001756.1	F	CTGCTCCGCCACCTCAAC	99
		R	CCCGGTAAACGTTTGGATACA	
3BHSD	NM_205118.1	F	TTTGTTTAGCACTGAGGCAAGAG	255
		R	AGTCTTGCCCTGGAACTTGC	
VEGFA1	NM_205042.2	F	GTCGTACATATTCAGGCCATC	197
		R	GATTCTTTGGTCTGCAGTCAC	
VEGFA2	NM_001110355.1	F	GGGCCTAGAATGTGTCCCTG	169
		R	AGGCTCACAGTGATTTTCTTGT	
VEGFR1	NM_204252.1	F	TCCACATCGGCCATCATCTG	204
		R	CCACGGGCTCAATACCCTTT	
VEGFR2	NM_001004368.1	F	TTGTTTTTCCCGGCTGGACT	140
		R	TTAGGGTATTGGTGAGCGCC	
ANGPT1	NM_001199447.3	F	ACAAAAGCGGCGTCTACACT	187
		R	AGCCAGTGTTCACCTGATGG	
ANGPT2	XM_015284814.2	F	CGCTTGACTACGACGACTCC	90
		R	GGTTTAGTACCTTCAGGAGCCA	
TEK	XM_004949580.3	F	TTTTGACCAGGGGAAGGATACTC	277
		R	GGCTCTCGGTGTTGGTTTGT	
VTG	XM_015290869.1	F	CAGATGAACAGGACCCACGA	189
		R	CAAGCTGGTATCCTGTCGGC	
OVR	X95100.1	F	TGAAGCCTGCTGTGACTGTG	249
		R	AAGTGACTGACAGGAGTGAGC	
apoB	NM_001044633.1	F	AGGTAGAGGCAGGACGCATA	268
		R	AGAATGCTACGTCCCACACG	
MTP	NM_001109784.2	F	GCAGATGGACAGAGTTGGCT	93
		R	TTCCCTCTCCTCGCAGTGTA	
VLDLR	NM_205229.1	F	ATGGCCAGGATCGTAGACTT	292
		R	TCATTTATCTGAGGAGCAGG	
PPAR-g	NM_001001460.1	F	CAAGGCAGCGGCAAAATAAC	187
		R	GTGCCCATAAATGATGGCCTAA	
CCND1	NM_205381	F	ATAGTCGCCACTTGGATGCT	122
		R	AACCGGCTTTTCTTGAGGGG	
CCND2	XM_015292118.2	F	TCCGGAAACATGCACAAACG	257
		R	CCGGACTTGCCTAAGGTTGC	
CDK2	NM_001199857	F	ACGTGATCCACACGGAGAAC	132
		R	GCAGCTGGAACAGGTAGCTC	
CDK6	NM_001007892.4	F	CGGAGACATCGCCATGTGAAG	86
		R	CTGTGCGAAGGCAGCTAGA	
PCNA	NM_204170.2	F	GACAATGCGGATACGTTGGC	188
		R	TCACCAATGTGGCTGAGGTC	
Caspase8	NM_204592.3	F	GACATGTTGATGCCAGCCTT	115
		R	AGCAGGCAGGCTCCTAAAAC	
Caspase9	NM_001277932.1	F	GTCACAGACCTTGAGACCCG	155
		R	ACCAGGTGGTCTAGGGGTTT	
Bcl2	NM_205339.3	F	TCGGAAGCGATCTGCCTTTT	228
		R	CACCGACAGCTGCATTTACG	
GAPDH	NM_204305.1	F	GAACATCATCCCAGCGTCCA	132
		R	CGGCAGGTCAGGTCAACAAC	

supplemented with 5% fetal bovine serum, 100 IU/mL penicillin, 100 mg/mL streptomycin, and 1× insulin transferrin selenium (Gibco, San Diego, CA). The cultured SWFs were treated with AdipoRon (0, 5, 10 and 20 μg/mL). The follicles were cultured on a 0.45-μm filter membrane in 24-well culture plates (Corning Inc., Corning, NY) at 37°C under 5% CO_2 for 24 h. For the 5-bromo-2'-deoxyuridine (**BrdU**) incorporation assay, follicles were incubated with complete medium supplemented with 10 mg/mL BrdU (Mao Kang, Shanghai, China) for 24 h. Subsequently, the SWFs were fixed for histological observation or collected for Western blot and quantitative real-time PCR (qRT-PCR) experiments.

Morphological Observation

The testis and SWF tissues for histological testing were fixed in 4% formaldehyde fixative solution for more than 12 h and then dehydrated and embedded in wax. The tissue wax was cut into 4-μm sections. The testicular tissue sections were then stained with hematoxylin and eosin (**H&E**) according to the standard procedure. The SWF tissue sections were subjected to antigen retrieval in a microwave oven, mouse anti-BrdU monoclonal antibody (1:200, GB12051, Wuhan, China) was added, and the samples were incubated overnight at 4°C. Goat antimouse IgG (1:500, 115-165-003, Jackson) was then added, and the samples were incubated in the dark for 50 min. DAPI was added to counterstain the nucleus, and the slide was then mounted with an antifluorescence quencher. The slides were examined under a light microscope (Nikon, Tokyo, Japan).

Testicular Leydig Cell Culture

According to the above-described, roosters at the age of 19 wk were killed, and the left and right testicles were then placed in a solution containing 100 IU/mL penicillin and 100 μg/mL streptomycin D-Hanks. The testicular tissue was cut into small 1-mm^3 pieces and digested with Type II collagenase (Solarbio, Beijing, China) for 20 min. After termination of digestion, testicular Leydig cells were obtained by centrifugation at 1,000 rpm for 10 min after filtration through a 100-mesh filter, 200-mesh filter, and 500-mesh filter followed by centrifugation at 3,000 rpm in Percoll separation solution for

30 min. After the cell precipitate was diluted with culture medium (DMEM/F12 [Biological Industries, Kibbutz Beit Haemek, Israel]), 10% fetal calf serum (Biological Industries), 100 IU/mL penicillin, and 100 mg/mL streptomycin (Solarbio), it was inoculated on a culture plate for 48 h, placed in an incubator (set to 37°C and 5% CO_2) and subcultured, and the F1 generation was used for subsequent experiments.

Cell Proliferation and Apoptosis Assay

The cell counting kit-8 (**CCK-8**) assay was used for the measurement of testicular Leydig cell proliferation. F1 generation testicular Leydig cells were inoculated into 96-well plates, AdipoRon (0, 5, 10, 20, and 40 μg/mL) was added and incubated for 24, 48, or 72 h. The culture plate was removed from the incubator, the medium was replaced with 100 μL of medium containing 10 μL of CCK-8 solution, and the cells were cultured for 2 h. The optical density at 450 nm was then determined using a BioTek (Winooski, VT) microplate reader. The cell viability was calculated as described previously (Li et al., 2021b).

The treated cells were collected, stained with propidium iodide (**PI**) solution (50 g/mL PI and 100 g/mL RNase A in PBS) and then subjected to cell cycle analysis with a FACSCanto II (Becton Dickinson, Franklin Lakes, NJ). The data were collected and analyzed using ModFit LT 5.0 software. The extent of apoptosis was measured by Annexin V/PI double staining. Subsequently, 100 μL of binding buffer containing 5 μL of Annexin V-FITC and 5 μL of PI was added to the cell suspension, and the cells were then incubated for 15 min in the dark. The samples were subsequently analyzed using a FACSCanto II (Becton Dickinson), and the apoptosis rate was calculated with FlowJo software.

ELISA Assay for Hormones

The AdipoQ (MM-60262O1, detection range: 0.5−16 μg/mL), AdipoR1 (MM-2321O1, detection range: 6.25−200 ng/mL), AdipoR2 (MM-2318O1, detection range: 0.25−8 ng/mL), GnRH (MM-0920O1, detection range: 2.5−80 mIU/mL), FSH (MM-60262O1, detection range: 0.625−20 mIU/mL), LH (MM-1623O1, detection range: 5−160 ng/mL), T (MM-0786O1, detection range: 25−800 pg/mL), P4 (MM-60214O1 or MM-60214O1-1,

Table 2. Effects of different ages on serum AdipoQ, its receptors and reproductive-related hormones.

Term	Week (wk)			
	15	20	30	68
AdipoQ (μg/mL)	10.71 ± 1.73[d]	11.77 ± 1.72[c]	14.31 ± 1.83[a]	13.13 ± 1.78[b]
AdipoR1 (ng/mL)	102.28 ± 23.08[c]	125.96 ± 19.47[b]	145.59 ± 21.80[a]	133.59 ± 22.02[ab]
AdipoR2 (ng/mL)	4.19 ± 0.70[d]	4.74 ± 0.73[c]	5.73 ± 0.81[a]	5.22 ± 0.79[b]
E2 (pg/mL)	255.05 ± 58.63[d]	299.52 ± 43.41[c]	426.01 ± 59.88[a]	357.92 ± 49.53[b]
FSH (mIU/mL)	10.84 ± 1.88[c]	13.55 ± 1.87[b]	15.83 ± 2.23[a]	13.84 ± 2.12[b]
GnRH (mIU/mL)	53.40 ± 7.93[c]	54.16 ± 7.48[c]	73.36 ± 9.62[a]	61.85 ± 8.46[b]
LH (mIU/mL)	100.73 ± 16.57[c]	124.59 ± 19.34[b]	151.72 ± 19.98[a]	132.31 ± 15.84[b]
P4 (ng/mL)	14.42 ± 3.21[d]	17.98 ± 2.98[c]	23.35 ± 2.69[a]	21.01 ± 3.08[b]

[a,b,c,d]Different superscript letters indicate significant differences at $P < 0.05$.

detection range: 0.9375−30 ng/mL or 50−1600 pmol/L), and E2 (MM-0787O1, detection range: 15−480 pg/mL) levels in serum or cell supernatant were determined using ELISA kits (Jiangsu Meimian Industrial Co., Ltd., Yancheng, China) according to the manufacturer's instructions. Six replicates of the samples in each treatment were analyzed.

RNA Extraction, cDNA Synthesis, and qRT-PCR

According to the manufacturer's instructions, total RNA was extracted from hypothalamic, pituitary, testicular, ovary, and liver tissues, SWFs and testicular Leydig cells using TRIzol reagent (Vazyme, Nanjing, China). The integrity of all RNA samples was determined by 1.5% agarose gel electrophoresis, and the concentration was determined by measuring the 260/280-nm absorbance ratio using a Nano Photometer spectrophotometer (Implen, Westlake Village, CA). The primers were designed by Primer 5.0 and synthesized by Sangon Biotech (Shanghai, China) (Table 1), and GAPDH was selected as the internal standard. cDNA synthesis was performed using a HiScript II 1st Strand cDNA Synthesis Kit (Vazyme). The qRT-PCR volume was 10 μL, which included 5 μL of ChamQ SYBR qPCR Master Mix (Vazyme), 0.5 μL each of the forward and reverse primers, 1 μL of cDNA and 3 μL of RNase free water. The qRT-PCR conditions were as follows: predenaturation at 95°C for 5 min; 35 cycles of denaturation at 95°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 30 s; and extension at 72°C for 10 min. The relative expression levels of genes were calculated using the $2^{-\Delta\Delta Ct}$ method (Schmittgen and Livak, 2008).

Western Blotting Analysis

For protein extraction, the treated testicular Leydig cells and SWFs were washed with PBS, and a total protein extraction kit (Solarbio) was then used for protein extraction. The protein concentrations were measured using a BCA Protein Assay Kit (Beyotime Biotechnology, Shanghai, China). Exactly 25 μg of protein was separated on a 10% SDS-PAGE gel and transferred to PVDF membranes (Millipore, Billerica, MA). The membranes were blocked in 5% nonfat milk in TBS with 0.1% Tween 20 and incubated overnight with rabbit anti-StAR (1:500, bs-3570R, Bioss, Beijing, China), rabbit anti-CYP11A1 (1:100, CSB-PA006389LA01HU, Cusabio, Wuhan, China) and rabbit anti-HSD3B1 (1:500, bs-3906R, Bioss, Beijing, China) at 4°C and then with an HRP-conjugated goat antirabbit (1:3,000, E-AB-1003, Elabscience, Wuhan, China) antibody at room temperature for 1 h. Rabbit monoclonal anti-GAPDH and HRP-conjugated IgG (ab181602, Abcam, Cambridge, UK) antibodies at a dilution of 1:1,000 were used as internal controls. The bands representing each sample were densitometrically quantified using the AlphaEaseFC program.

Statistical Analysis

All the data are presented as the mean ± SEM. The results were analyzed by one-way analysis of variance (**ANOVA**), and Duncan's method was used to compare the values with significant differences. A two-way ANOVA design for time and dose was used, and their interactions were examined as the main effects. Moreover, the Kolmogorov-Smirnov (**K-S**) test was used to ensure that the data were normally distributed. All statistical analyses were performed using Statistical Package for the

Table 3. Correlation analysis of serum levels of AdipoQ, its receptor and reproduction-related hormones.

Term		AdipoQ	AdipoR1	AdipoR2	E2	FSH	GnRH	LH	P4
AdipoQ	Pearson coefficient	1	0.187*	0.253**	0.410**	0.256**	0.391**	0.404**	0.411**
	P value		0.036	0.004	0.000	0.004	0.000	0.000	0.000
	Sample		126	126	126	126	126	126	126
AdipoR1	Pearson coefficient		1	0.146	0.392**	0.292**	0.331**	0.316**	0.310**
	P value			0.102	0.000	0.001	0.000	0.000	0.000
	Sample			126	126	126	126	126	126
AdipoR2	Pearson coefficient			1	0.381**	0.181*	0.348**	0.406**	0.318**
	P value				0.000	0.042	0.000	0.000	0.000
	Sample				126	126	126	126	126
E2	Pearson coefficient				1	0.371**	0.542**	0.467**	0.539**
	P value					0.000	0.000	0.000	0.000
	Sample					126	126	126	126
FSH	Pearson coefficient					1	0.310**	0.223*	0.380**
	P value						0.000	0.012	0.000
	Sample						126	126	126
GnRH	Pearson coefficient						1	0.396**	0.440**
	P value							0.000	0.000
	Sample							126	126
LH	Pearson coefficient							1	0.452**
	P value								0.000
	Sample								126
P4	Pearson coefficient								1
	P value								
	Sample								126

*$P < 0.05$ was considered significant differences and **$P < 0.01$ was considered extremely significant differences.
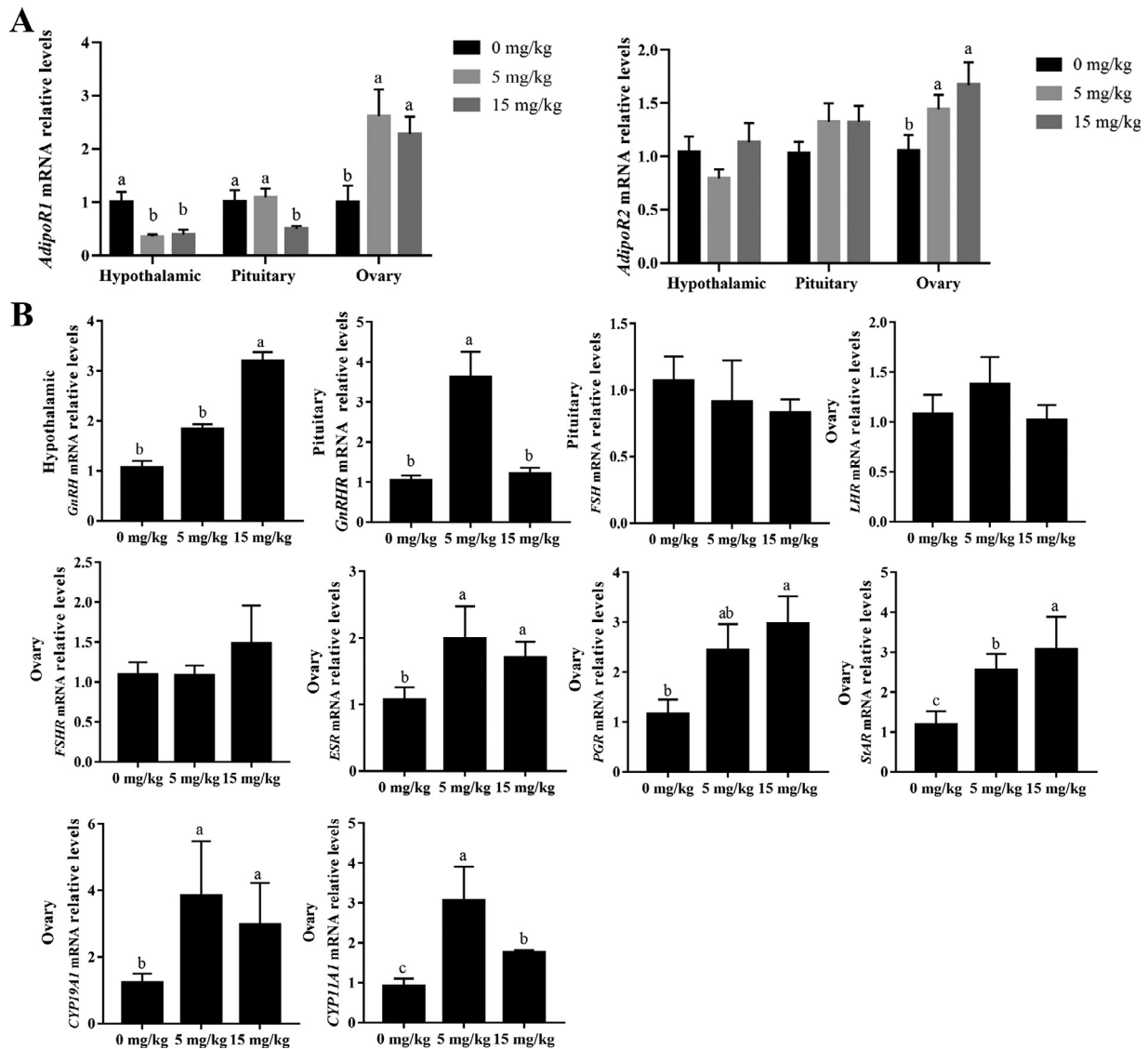
Figure 1. Expression of adiponectin receptor and reproduction-related genes in the HPO axis in laying hens during the peak oviposition period after the injection of AdipoRon. (A) Expression levels of $AdipoR1$ and $AdipoR2$ in the HPO axis. (B) Expression levels of reproduction-related genes in the HPO axis. Values marked with different superscripted letters are significantly different ($P < 0.05$).

Social Sciences (**SPSS 26**) statistical software. $P < 0.05$ or $P < 0.01$ was considered to indicate statistical significance, and significant differences are indicated by different superscripted letters.

RESULTS

Correlation Analysis of the Serum Levels of AdipoQ, AdipoRs, and Reproduction-Related Hormones in the HPO Axis of Laying Hens at Different Physiological Periods

The concentrations of AdipoQ and its receptors and reproduction-related hormones in the HPO axis in laying hens of 4 different ages were determined by ELISA. The results showed that age had a significant impact on these indicators, which exhibited a similar trend of first increasing and then declining after 30 wk (Table 2). We then analyzed the correlation between AdipoQ and its receptor and reproduction-related hormones in the HPO

axis and found a significant correlation ($P < 0.05$ or $P < 0.01$) among all the indicators except AdipoR1 and AdipoR2 (Table 3).

Effect of AdipoRon on Reproductive Gene Expression and Hormone Secretion in the HPO Axis of Laying Hens During the Peak Oviposition Period

The effect of AdipoRon expression and secretion on reproduction-related genes and hormones in the HPO axis was explored by qRT-PCR and ELISA. The results showed that different doses of AdipoRon significantly increased the expression of $AdipoR1$ and $AdipoR2$ in the ovary ($P < 0.05$, Figure 1A), the expression of $GnRH$ in the hypothalamus, the expression of $GnRHR$ in the pituitary gland, and the expression of ESR, PGR, $STAR$, $CYP19A1$, and $CYP11A1$ in the ovary ($P < 0.05$, Figure 1B). Different doses of AdipoRon and different post-treatment times had significant effects on the levels

of AdipoQ, AdipoRs and reproduction-related hormones in the HPO axis in serum ($P < 0.05$, Table 4). AdipoQ, AdipoR1, AdipoR2, GnRH, LH, FSH, and P4 revealed a significant increase with increase in the AdipoRon dose and time after injection, whereas E2 showed a conditional decrease (Table 4).

Effect of AdipoRon on the Follicle Growth and Yolk Deposition of Laying Hens During the Expected Oviposition Period and Late Oviposition Period

The effects of AdipoRon on the HPO axis, liver yolk deposition and lipid synthesis- and follicular angiogenesis-related genes were determined by qRT-PCR. The results showed that AdipoRon significantly affected the expression of *GnRH* in the hypothalamus, *GnRHR*, *FSH*, and *LH* in the pituitary gland, *LHR* and steroid hormone production-related genes (*STAR, CYP19A1, CYP17A1,* and *CYP11A1*) ($P < 0.05$, Figure 2A: a−d). Moreover, oocyte vitellogenin receptor (**OVR**) and microsomal triglyceride transfer protein (**MTP**) mRNA expressions were significantly upregulated in the AdipoRon-treated group compared to those in the control ($P < 0.05$, Figure 2A: e), as well as the key genes involved in lipid uptake and adipogenesis (*VLDLR* and *PPAR-g*) and the angiogenesis-related genes (*VEGFA1, VEGFA2, VEGFR1, ANGPT1, ANGPT2,* and *TEK*)

were also shown high mRNA expressions in SWFs after injected AdipoRon ($P < 0.05$, Figure 2A: f and g).

To further explore the effect of AdipoRon on the follicles of hens at the late oviposition period, SWFs collated at this period were cultured in vitro, and AdipoRon was added at the same time. First, the survival of SWFs at different time points (24, 48, and 72 h) was assessed by the BrdU assay and qRT-PCR. The results showed that follicle cells proliferated at different time points (Figure 2B), and a significant increase in the expression of proliferation-related (*CCND1, CDK2, CDK6,* and *PCNA*) and antiapoptotic genes (*Bcl2*) genes and significant decreased in the expression of apoptotic genes (Caspase8 and Caspase9) were observed with gradual increases in the culture time indicated that SWFs were successfully cultured in this experiment ($P < 0.05$, Figure 2B). The SWFs were treated in vitro with different concentrations of AdipoRon (0, 5, 10, and 20 μg/mL) for 24 h, and the expression of steroid hormones, lipid uptake and formation-related genes, and angiogenesis-related genes was measured by qRT-PCR and Western blot. The results showed that AdipoRon, particularly at the concentration of 10 μg/mL, could upregulate the expression of these genes and the difference was significant ($P < 0.05$, Figure 2C). Western blot analysis showed that the expression of steroid hormone production-related proteins (CYP11A1) was markedly upregulated by treatment with AdipoRon at a concentration of 10 μg/mL ($P < 0.05$, Figure 2C).

**Table 4.** Effects of AdipoRon on serum hormone secretion in laying hens during the peak oviposition period.

Dose (mg/kg)	Time (min)	AdipoQ (μg/mL)	AdipoR1 (ng/mL)	AdipoR2 (ng/mL)	GnRH (mIU/mL)	LH (mIU/mL)	FSH (mIU/mL)	P4 (pmol/L)	E2 (pg/mL)
0	0	5.93 ± 1.29	127.04 ± 14.59	5.29 ± 0.76	47.50 ± 8.57	58.19 ± 12.02	10.85 ± 1.29	1032.46 ± 85.08	395.07 ± 56.72
	10	5.91 ± 0.90	124.20 ± 11.36	5.84 ± 0.66	56.35 ± 7.99	69.90 ± 12.35	11.97 ± 0.93	1069.76 ± 62.12	393.21 ± 38.47
	20	6.61 ± 1.30	133.33 ± 19.50	6.66 ± 0.47	56.87 ± 7.66	77.88 ± 18.13	14.33 ± 1.93	1164.39 ± 61.45	409.80 ± 30.82
	30	6.79 ± 1.57	127.74 ± 20.75	6.22 ± 0.92	58.74 ± 5.45	75.38 ± 10.06	15.61 ± 1.24	1176.21 ± 120.75	393.83 ± 45.25
	60	8.34 ± 1.31	145.93 ± 14.6	6.60 ± 0.58	67.11 ± 4.90	92.06 ± 18.79	16.51 ± 2.07	1254.55 ± 52.20	372.05 ± 55.02
	120	8.40 ± 1.55	147.19 ± 11.75	6.96 ± 0.77	67.55 ± 6.98	99.73 ± 16.51	16.56 ± 2.08	1267.06 ± 91.89	365.54 ± 50.44
5	0	6.27 ± 1.43	137.00 ± 11.69	7.47 ± 0.55	57.35 ± 6.90	70.86 ± 12.24	13.64 ± 1.30	1020.32 ± 58.42	402.80 ± 49.84
	10	8.28 ± 1.38	161.74 ± 20.78	8.54 ± 0.77	67.33 ± 8.05	94.39 ± 10.95	18.49 ± 1.92	1324.52 ± 114.36	388.41 ± 37.28
	20	8.58 ± 1.30	158.90 ± 17.20	7.92 ± 0.77	68.20 ± 6.65	100.63 ± 10.92	17.78 ± 1.93	1360.38 ± 95.58	385.79 ± 28.17
	30	9.67 ± 1.32	169.48 ± 21.44	9.12 ± 1.02	72.01 ± 5.55	109.45 ± 16.94	19.39 ± 2.28	1480.59 ± 144.22	348.78 ± 39.94
	60	11.19 ± 1.50	191.48 ± 14.87	9.08 ± 0.85	80.43 ± 6.31	120.56 ± 13.75	21.74 ± 2.03	1516.83 ± 126.33	257.05 ± 39.88
	120	11.27 ± 1.23	195.10 ± 23.23	8.95 ± 0.81	84.92 ± 4.65	131.13 ± 11.78	20.60 ± 1.69	1603.81 ± 137.32	186.87 ± 53.56
15	0	8.14 ± 1.35	133.47 ± 16.66	6.89 ± 0.70	56.84 ± 7.69	69.14 ± 10.56	14.11 ± 1.66	1141.28 ± 44.85	436.13 ± 35.37
	10	10.18 ± 1.32	176.56 ± 24.73	7.98 ± 0.18	74.82 ± 7.31	108.35 ± 12.67	18.94 ± 1.38	1413.18 ± 116.39	327.92 ± 48.86
	20	11.04 ± 1.17	179.02 ± 19.21	8.45 ± 0.99	72.49 ± 7.89	120.98 ± 14.09	19.45 ± 2.05	1590.52 ± 125.76	313.19 ± 40.50
	30	11.87 ± 1.42	217.08 ± 6.36	9.21 ± 0.73	81.23 ± 9.10	122.79 ± 21.79	19.72 ± 1.43	1628.46 ± 152.02	255.59 ± 43.55
	60	11.65 ± 0.95	219.85 ± 21.42	9.26 ± 0.66	81.48 ± 5.72	141.67 ± 14.21	22.07 ± 1.87	1790.50 ± 163.82	241.73 ± 34.81
	120	12.51 ± 1.61	207.19 ± 13.22	9.22 ± 0.83	87.12 ± 4.74	154.81 ± 9.84	23.01 ± 1.80	1900.67 ± 111.96	206.33 ± 26.06
0		7.00 ± 1.63[c]	134.24 ± 17.48[c]	6.26 ± 0.87[b]	59.02 ± 9.59[c]	78.86 ± 19.77[c]	14.30 ± 2.71[c]	1160.74 ± 116.80[c]	388.25 ± 46.59[a]
5		9.17 ± 2.21[b]	168.69 ± 26.97[b]	8.50 ± 0.99[a]	71.60 ± 11.12[b]	104.17 ± 23.36[b]	18.55 ± 3.18[b]	1380.40 ± 222.74[b]	328.15 ± 91.07[b]
15		10.90 ± 1.90[a]	188.86 ± 34.73[a]	8.50 ± 1.14[a]	75.66 ± 11.90[a]	119.62 ± 30.58[a]	19.55 ± 3.29[a]	1577.44 ± 277.28[a]	296.82 ± 84.01[c]
	0	6.74 ± 1.62[d]	132.90 ± 14.19[d]	6.63 ± 1.14[c]	54.20 ± 8.60[d]	66.48 ± 12.50[e]	12.93 ± 1.96[d]	1060.83 ± 82.16[e]	410.59 ± 49.31[a]
	10	8.13 ± 2.07[c]	154.82 ± 28.76[c]	7.55 ± 1.38[b]	66.27 ± 10.52[c]	91.19 ± 19.26[d]	16.64 ± 3.48[c]	1273.97 ± 173.16[d]	371.46 ± 49.31[b]
	20	8.73 ± 2.18[bc]	157.16 ± 25.74[c]	7.69 ± 1.05[b]	65.96 ± 9.64[c]	99.87 ± 22.37[c]	17.21 ± 2.84[c]	1371.25 ± 197.43[c]	370.33 ± 51.99[b]
	30	9.45 ± 2.49[b]	171.35 ± 40.25[b]	8.22 ± 1.64[a]	70.72 ± 11.30[b]	102.85 ± 25.71[c]	18.29 ± 2.51[b]	1430.79 ± 230.81[c]	333.46 ± 70.60[c]
	60	10.43 ± 1.92[a]	186.01 ± 34.56[a]	8.34 ± 1.40[a]	76.52 ± 8.55[a]	118.21 ± 25.20[b]	20.19 ± 3.19[a]	1520.46 ± 248.29[b]	288.77 ± 71.93[d]
	120	10.78 ± 2.18[a]	184.20 ± 30.56[a]	8.43 ± 1.26[a]	80.31 ± 10.13[a]	128.78 ± 25.25[a]	20.11 ± 3.15[a]	1591.67 ± 276.67[a]	247.17 ± 91.65[e]
P value	Dose	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	Time	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	Dose*Time	0.08	0.00	0.08	0.38	0.02	0.07	0.00	0.00

[a,b,c,d,e]Different superscript letters indicate significant differences at $P < 0.05$.
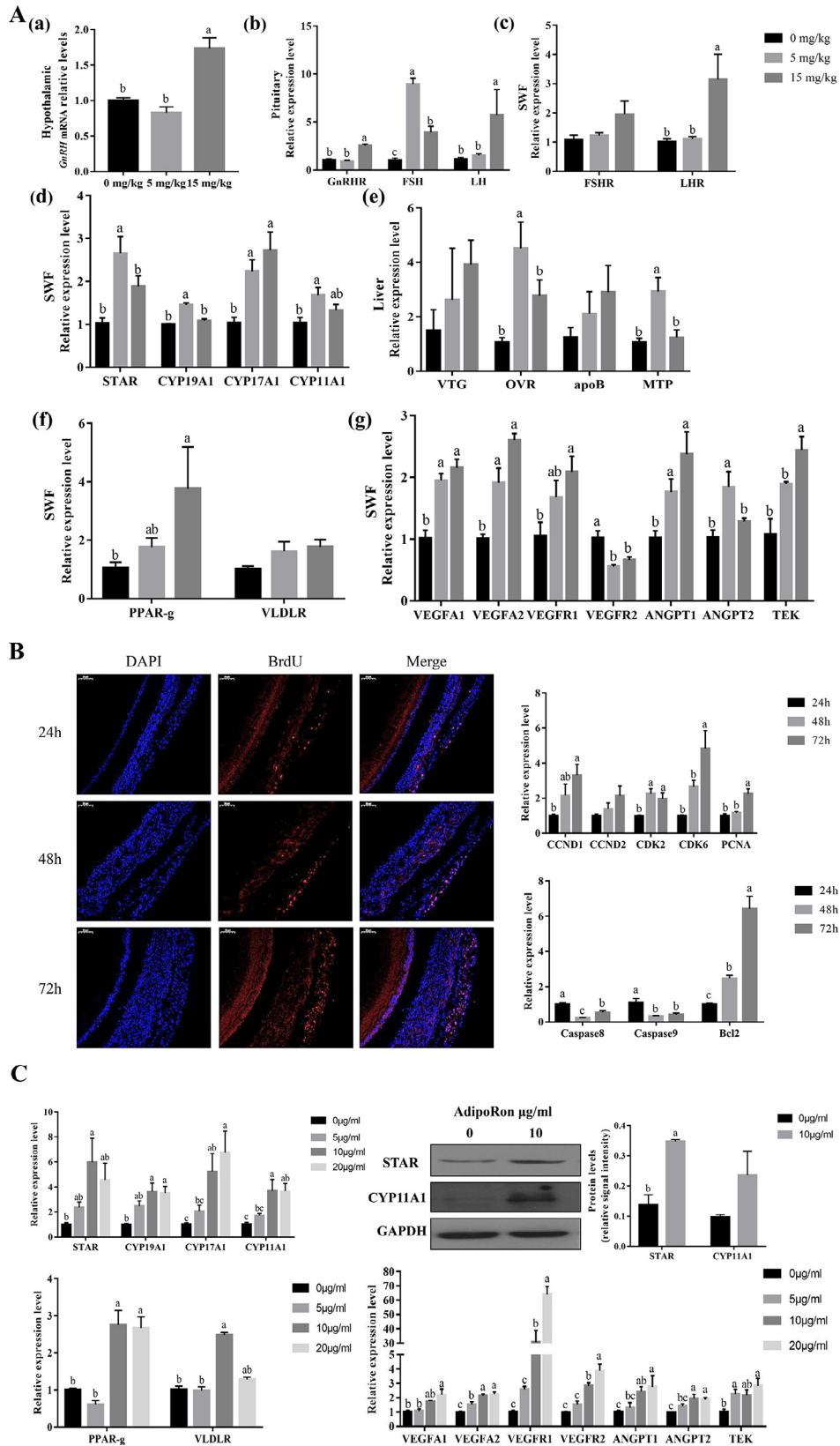
Figure 2. Effects of AdipoRon on the follicular growth of laying hens at the expected oviposition period (HPO axis) and late oviposition period. (A) Expression levels of reproduction-related genes in the HPO axis, liver lipid synthesis-related genes, follicle lipid uptake-related genes, and follicle angiogenesis-related genes in laying hens at the expected oviposition period 8 d after AdipoRon injection. (B) Analysis of the effect of AdipoRon on the proliferation and apoptosis of follicular cells in vitro by BrdU incorporation. Red fluorescence: BrdU-labeled cells, mainly distributed in the follicular theca externa. Blue fluorescence: DAPI staining. Scale bar: 20 mm. (C) Expression of steroidogenesis-related genes and proteins, follicle lipid uptake-related genes and angiogenesis-related genes in cultured follicles after treatment with AdipoRon for 24 h. Values marked with different superscripted letters are significantly different ($P < 0.05$).
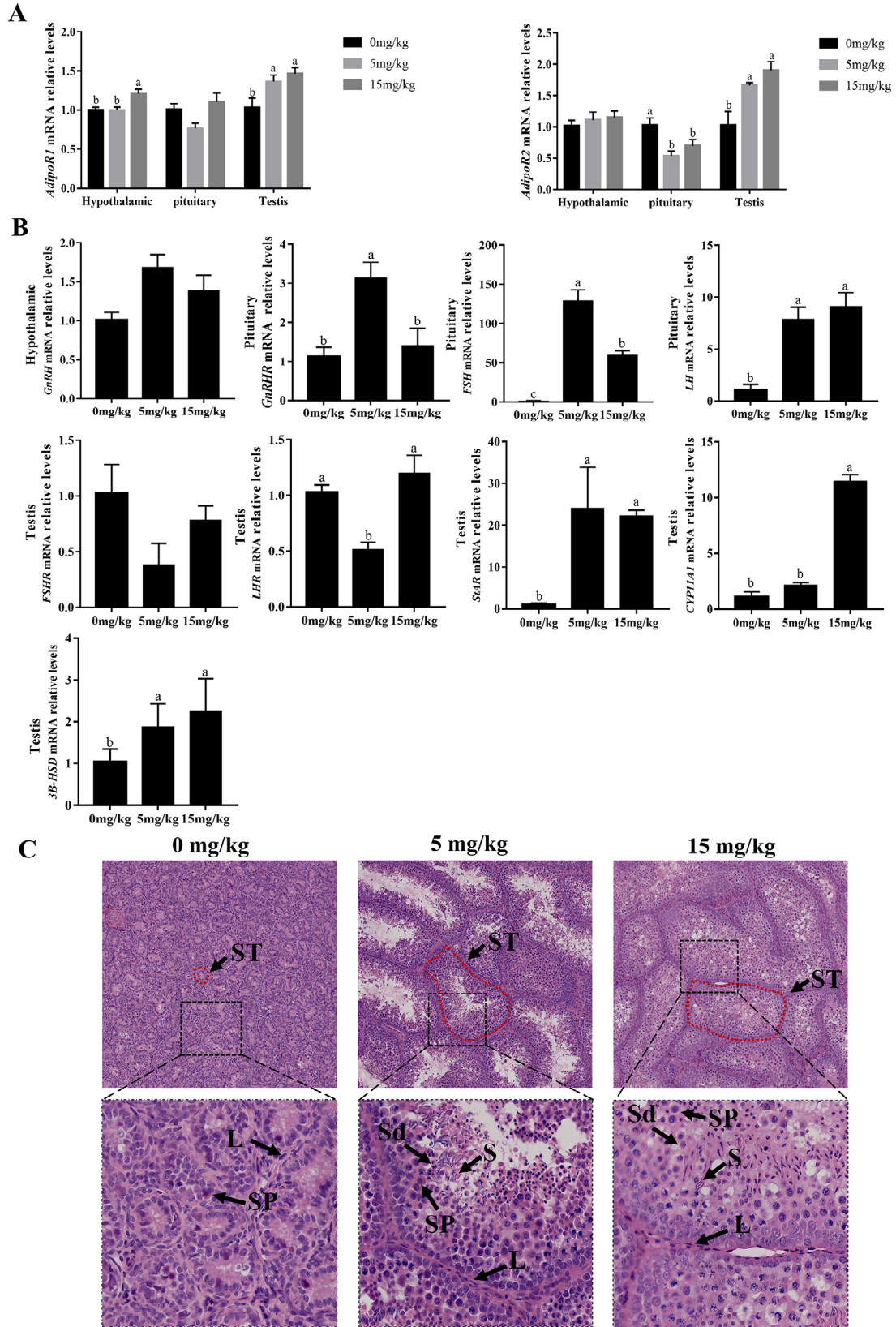
Figure 3. Effects of AdipoRon on adiponectin receptor genes, reproduction-related genes and testicle morphology in the HPT axis of roosters. (A) Expression levels of *AdipoR1* and *AdipoR2* in the HPG axis in roosters 7 d after injected AdipoRon. (B) Expression levels of reproduction-related genes in the HPG axis in roosters 7 d after injected AdipoRon. (C) Changes in the testicular morphology after treatment with different concentrations of AdipoRon for 7 d in vivo. Abbreviations: L, Leydig; S, sperm; Sd, spermatid; SP, spermatocyte; ST, seminiferous tubule (magnification: 400×). Values marked with different superscripted letters are significantly different (*P* < 0.05).

Table 5. Effects of AdipoRon on the secretion of serum hormones in roosters.

Dose (mg/kg)	Time (min)	AdipoQ (μg/mL)	AdipoR1 (ng/mL)	AdipoR2 (ng/mL)	GnRH (mIU/mL)	FSH (mIU/mL)	LH (mIU/mL)	T (pg/mL)
0	0	6.29 ± 1.51	132.71 ± 18.60	5.16 ± 0.56	52.08 ± 9.83	9.35 ± 1.79	8.36 ± 1.65	435.85 ± 82.22
	10	6.29 ± 1.25	128.88 ± 17.47	5.20 ± 0.56	52.29 ± 6.86	11.42 ± 1.16	8.32 ± 1.69	398.61 ± 42.69
	30	6.43 ± 1.34	140.90 ± 15.52	5.46 ± 0.27	53.92 ± 5.71	10.35 ± 1.07	10.08 ± 1.07	610.36 ± 94.30
	60	6.74 ± 1.36	143.33 ± 17.38	5.47 ± 0.69	55.07 ± 7.98	12.67 ± 2.09	9.62 ± 1.53	601.36 ± 74.61
	180	6.49 ± 0.74	144.93 ± 20.24	5.46 ± 0.45	58.51 ± 7.26	12.72 ± 1.61	10.10 ± 2.20	631.55 ± 71.79
	360	8.74 ± 0.58	142.86 ± 16.38	5.34 ± 0.52	54.96 ± 8.78	11.54 ± 1.84	8.77 ± 1.99	553.60 ± 73.76
5	0	6.88 ± 0.81	138.29 ± 17.59	5.58 ± 0.74	56.74 ± 7.48	12.69 ± 2.06	9.70 ± 1.80	483.09 ± 64.89
	10	9.49 ± 0.87	174.41 ± 10.84	6.07 ± 0.87	68.43 ± 9.44	14.35 ± 1.69	11.14 ± 2.12	627.86 ± 23.12
	30	9.71 ± 1.84	160.65 ± 18.51	6.84 ± 0.49	69.09 ± 7.44	15.25 ± 1.83	10.84 ± 1.03	734.74 ± 74.74
	60	11.63 ± 1.38	194.16 ± 19.82	7.21 ± 0.63	75.74 ± 3.56	17.48 ± 2.03	13.86 ± 1.25	827.06 ± 87.12
	180	11.00 ± 1.02	174.65 ± 14.00	8.02 ± 0.92	75.81 ± 3.00	17.81 ± 1.65	16.96 ± 1.04	763.96 ± 92.80
	360	12.29 ± 1.26	178.99 ± 21.31	8.55 ± 0.45	82.70 ± 2.41	18.95 ± 1.26	17.96 ± 1.82	714.06 ± 62.86
15	0	8.57 ± 1.20	165.22 ± 17.50	5.73 ± 0.40	62.64 ± 6.86	15.01 ± 1.57	11.55 ± 1.38	550.83 ± 79.85
	10	9.38 ± 0.95	163.29 ± 16.43	6.75 ± 0.78	74.71 ± 9.10	16.06 ± 1.71	13.04 ± 1.42	620.95 ± 56.49
	30	10.94 ± 1.56	183.20 ± 18.21	7.19 ± 0.74	69.80 ± 7.13	17.02 ± 1.64	13.98 ± 1.93	808.15 ± 65.16
	60	11.77 ± 1.52	202.05 ± 16.44	7.82 ± 0.62	75.99 ± 8.39	18.91 ± 1.97	15.84 ± 1.66	890.29 ± 89.18
	180	11.06 ± 0.83	195.35 ± 21.96	7.64 ± 0.41	87.33 ± 5.35	19.82 ± 2.29	17.79 ± 2.59	781.50 ± 78.58
	360	12.10 ± 1.00	187.11 ± 17.42	9.31 ± 0.56	96.72 ± 6.27	18.95 ± 1.56	19.15 ± 1.16	813.00 ± 85.28
0		6.81 ± 1.42^c	138.33 ± 17.46^c	5.33 ± 0.51^c	54.24 ± 7.63^c	11.25 ± 1.94^c	9.14 ± 1.75^c	529.50 ± 115.32^c
5		9.87 ± 2.26^b	167.29 ± 24.97^b	6.91 ± 1.27^b	70.08 ± 10.67^b	15.78 ± 2.86^b	13.07 ± 3.54^b	672.82 ± 139.28^b
15		10.58 ± 1.72^a	182.23 ± 22.26^a	7.36 ± 1.26^a	77.45 ± 13.33^a	17.56 ± 2.44^a	15.12 ± 3.16^a	738.90 ± 141.78^a
	0	7.23 ± 1.49^e	145.08 ± 22.21^d	5.50 ± 0.62^e	57.14 ± 8.85^c	12.37 ± 2.91^c	9.86 ± 2.03^d	489.61 ± 85.94^c
	10	8.21 ± 1.86^d	153.00 ± 25.03^{cd}	5.96 ± 0.97^d	64.25 ± 12.83^b	13.78 ± 2.50^b	10.68 ± 2.64^{cd}	536.40 ± 120.45^c
	30	8.99 ± 2.50^{cd}	161.64 ± 24.50^{bc}	6.47 ± 0.94^c	63.99 ± 9.94^b	14.15 ± 3.30^b	11.68 ± 2.23^c	716.78 ± 113.90^b
	60	9.95 ± 2.79^b	179.01 ± 32.02^a	6.81 ± 1.22^{bc}	68.53 ± 12.25^b	16.29 ± 3.39^a	13.06 ± 3.08^b	769.72 ± 152.42^a
	180	9.61 ± 2.32^{bc}	173.12 ± 28.03^{ab}	7.08 ± 1.28^b	74.72 ± 13.32^a	16.97 ± 3.56^a	15.13 ± 4.02^a	729.16 ± 102.00^{ab}
	360	10.97 ± 1.92^a	169.11 ± 26.51^{ab}	7.68 ± 1.88^a	77.85 ± 19.37^a	16.33 ± 3.94^a	15.14 ± 5.12^a	692.35 ± 133.37^b
P value	Dose	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	Time	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	Dose*Time	0.00	0.06	0.00	0.00	0.21	0.00	0.04

a,b,c,d,eDifferent superscript letters indicate significant differences at $P < 0.05$.

Effect of AdipoRon on the Expression of Reproduction-Related Genes in the HPT Axis Hormone Secretion and Testicular Structure

The qRT-PCR and ELISA were performed to determine the effect of AdipoRon expression and secretion on reproduction-related genes in the HPT axis and hormones. The results showed that the different doses of AdipoRon significantly affected the expression of *AdipoR1* in the hypothalamus and testis and the expression of *AdipoR2* in the testis (Figure 3A), the expression of *GnRHR, FSH* and *LH* in the pituitary gland, and the expression of *LHR, STAR, CYP11A1*, and *3B-HSD* in the testis ($P < 0.05$, Figure 3B). As revealed by ELISA, the AdipoQ, AdipoR1, AdipoR2, GnRH, FSH, LH, and T levels in serum increased significantly in a dose-dependent manner after injected AdipoRon ($P < 0.05$, Table 5). The levels of these hormones in serum at different time points showed an upward trend overall. The interaction of dose and time exerted a significant effect on the AdipoQ, AdipoR2, GnRH, LH, and T levels in serum ($P < 0.05$, Table 5). In addition, by hematoxylin-eosin staining we observed morphological changes in testicular tissue after treatment with AdipoRon. The results indicated that AdipoRon increased the diameter of the seminiferous tubules, the abundance of spermatogenic cells and sperm production (Figure 3C).

Effect of AdipoRon on the Proliferation and Apoptosis of Leydig Cells

To determine the effect of AdipoRon on the proliferation and apoptosis of testicular Leydig cells in vitro, we transfected testicular Leydig cells with 5 doses of AdipoRon (0, 5, 10, 20, and 40 μg/mL). The results showed that at 24 and 72 h, the cell viability of testicular Leydig cells decreased gradually when increased the doses of AdipoRon, and the viability of the cells treated with a dose that exceeded 10 μg/mL was significantly lower than that of the control group ($P < 0.05$, Figure 4A). However, all the doses of AdipoRon had no significant effect on the viability of testicular Leydig cells at 48 h (Figure 4A). The cell cycle results showed that compared with the control group, 5 μg/mL and 10 μg/mL AdipoRon had no significant effect on the G1 to S/G2 transition during cell cycle progression in testicular Leydig cells compared to those in the control group (Figure 4B). Furthermore, a flow cytometry assay demonstrated that 5 and 10 μg/mL AdipoRon exerted no significant effect on the apoptosis of testicular Leydig cells compared with that in the control group
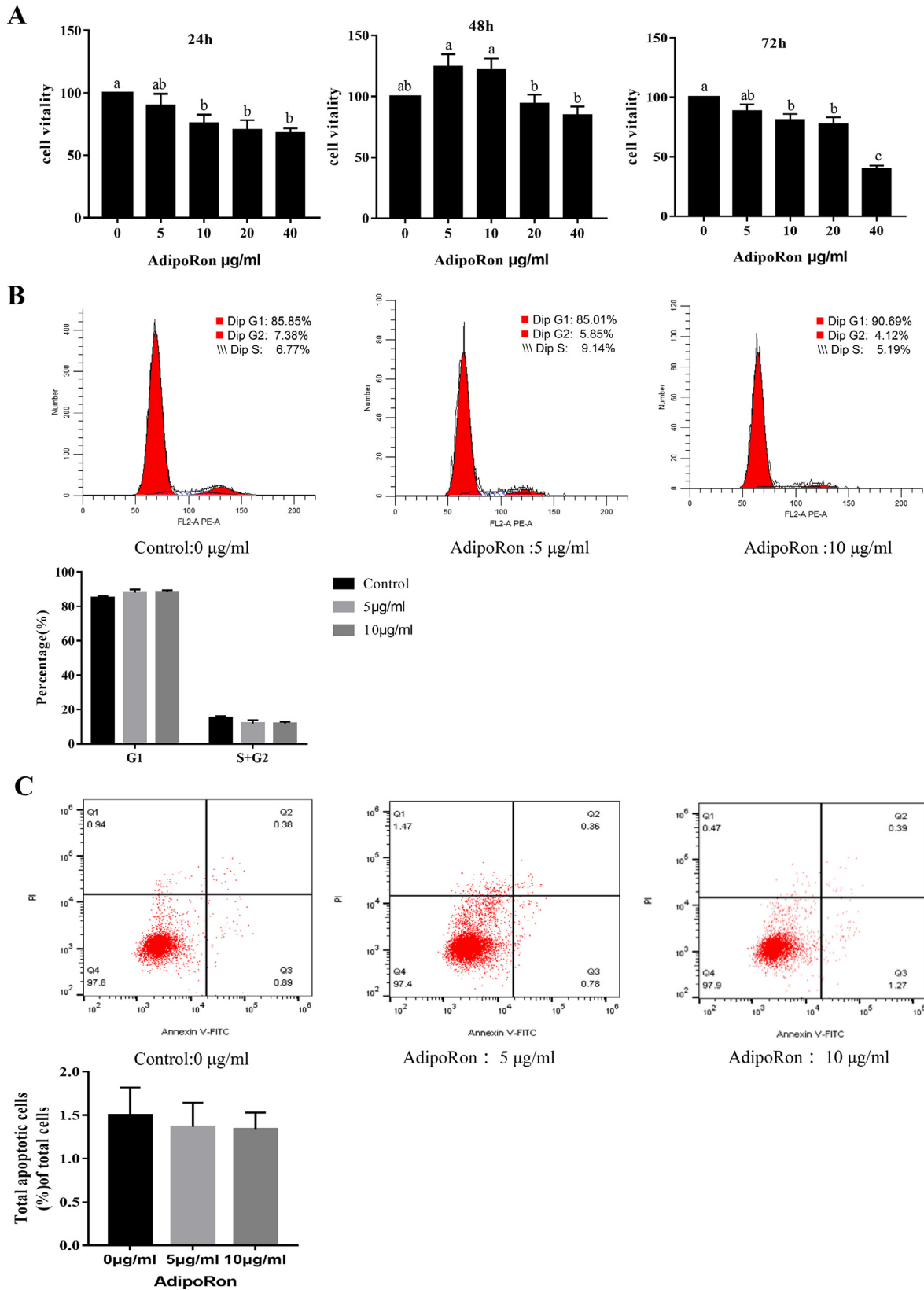
Figure 4. Effect of AdipoRon on the proliferation and apoptosis of testicular Leydig cells in vitro. (A) Testicular Leydig cell vitality after treatment with AdipoRon at different levels and for different incubation times. (B) PI cycle analysis of testicular Leydig cells treated with 0, 5, or 10 μg/mL AdipoRon. (C) Annexin-V/PI analysis of apoptosis of testicular Leydig cells treated with 0, 5, or 10 μg/mL AdipoRon. Values marked with different superscripted letters are significantly different ($P < 0.05$).
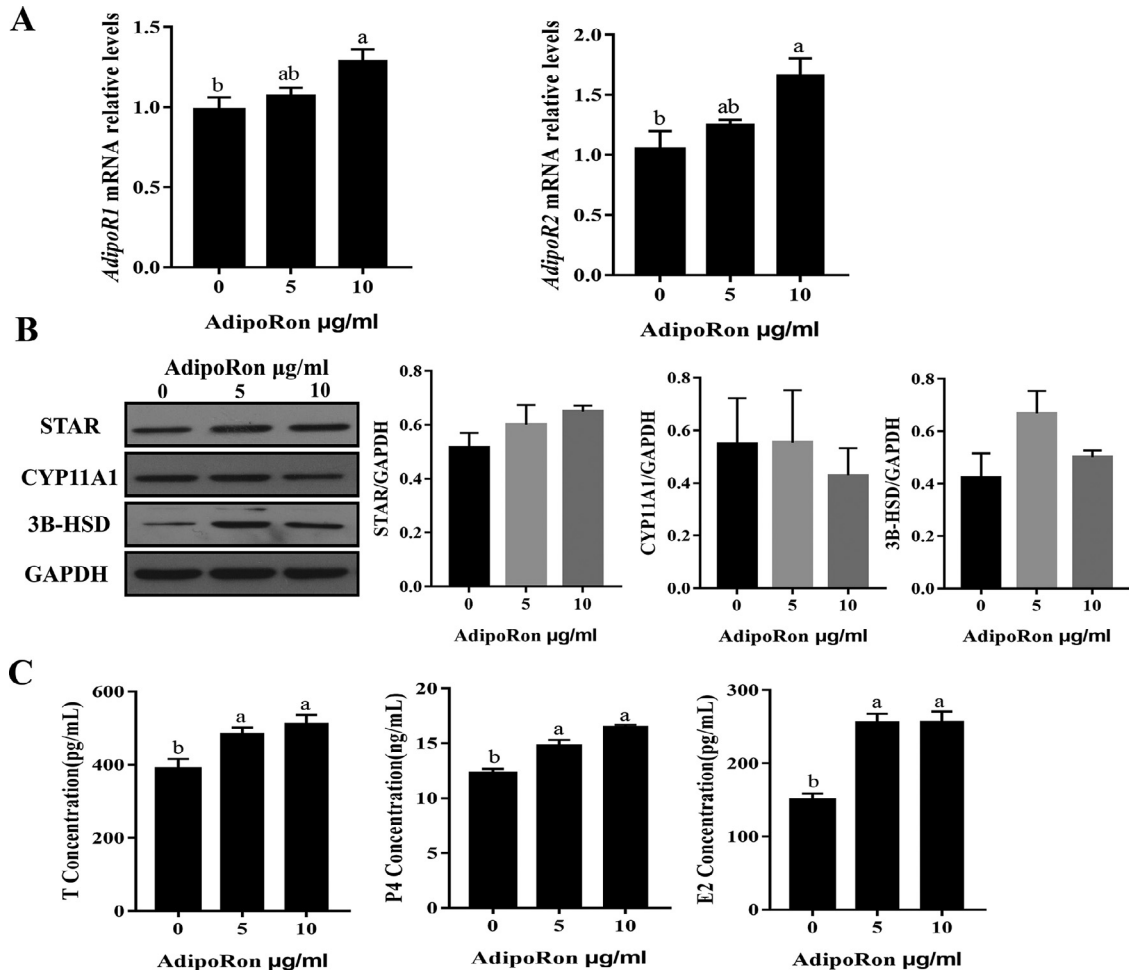
Figure 5. AdipoRon facilitated the expression of adiponectin receptor and steroid hormone secretion in Leydig cells. (A) AdipoRon increased the expression of adiponectin receptor mRNA (AdipoR1 and AdipoR2). (B) AdipoRon increased the expression of steroid hormone synthesis-related proteins (STAR and 3B-HSD). (C) AdipoRon increased the secretion of steroid hormones (T, P, and E2) in testicular Leydig cells. Values marked with different superscripted letters are significantly different ($P < 0.05$).

(Figure 4C). Therefore, an AdipoRon dose of 5 or 10 μg/mL at 48 h after transfection was selected for subsequent experiments.

### *Effect of AdipoRon on the Expressions of Steroidogenic Enzymes in Testicular Leydig Cells*

The effect of AdipoRon on steroidogenesis genes and proteins in testicular Leydig cells was further explored by qRT-PCR Western blotting and ELISA. The results demonstrated that AdipoRon (5 and 10 μg/mL) upregulated the expression of *AdipoR1* and *AdipoR2*, and significant upregulation was observed with a dose of 10 μg/mL ($P < 0.05$, Figure 5A). In addition, AdipoRon (5 and 10 μg/mL) upregulated STAR and 3B-HSD protein expression (Figure 5B) and significantly promoted the secretion of T, E2 and P4 in testicular Leydig cells ($P < 0.05$, Figure 5C).

DISCUSSION

Although AdipoQ is an important cytokine secreted by fat cells, many studies have proven that it plays a major role in animal reproduction and is widely expressed in the HPG axis of chickens (Ocón-Grove et al., 2008; Li et al., 2021a). In our study, AdipoQ expression was significantly correlated with chicken reproduction-related hormones in the HPG axis, which indicated that AdipoQ may play an important role in the HPG axis of chickens.

It is well known that the HPG axis can regulate gonad development and reproduction in animals, and these processes are affected by multiple factors, such as reproductive hormones (GnRH, FSH, LH, E2, P4, and T) and cytokines. In the HPG axis, GnRH secreted by the hypothalamus can promote the secretion of FSH and LH by the pituitary gland. FSH and LH bind with FSHR and LHR, respectively, to promote follicle maturation from prehierarchical follicles to preovulatory follicles and to induce testicular development. Moreover, FSH and LH increased the levels of P4, E2, androgens, and T in plasma (Johnson and Lee, 2016; Guo et al., 2021). Cytokines, particularly AdipoQ, reportedly to regulate the expression of steroidogenesis genes (*StAR, CYP11A1, CYP19A1*, and *3BHSD*) and the secretion of steroid hormones in ovarian and testicular tissues of animals (Ledoux et al., 2006; Chabrolle et al., 2007b; Lagaly et al., 2008; Pierre et al., 2009; Ramanjaneya et al., 2011; Singh et al., 2014; Rak et al., 2017). In our study, we
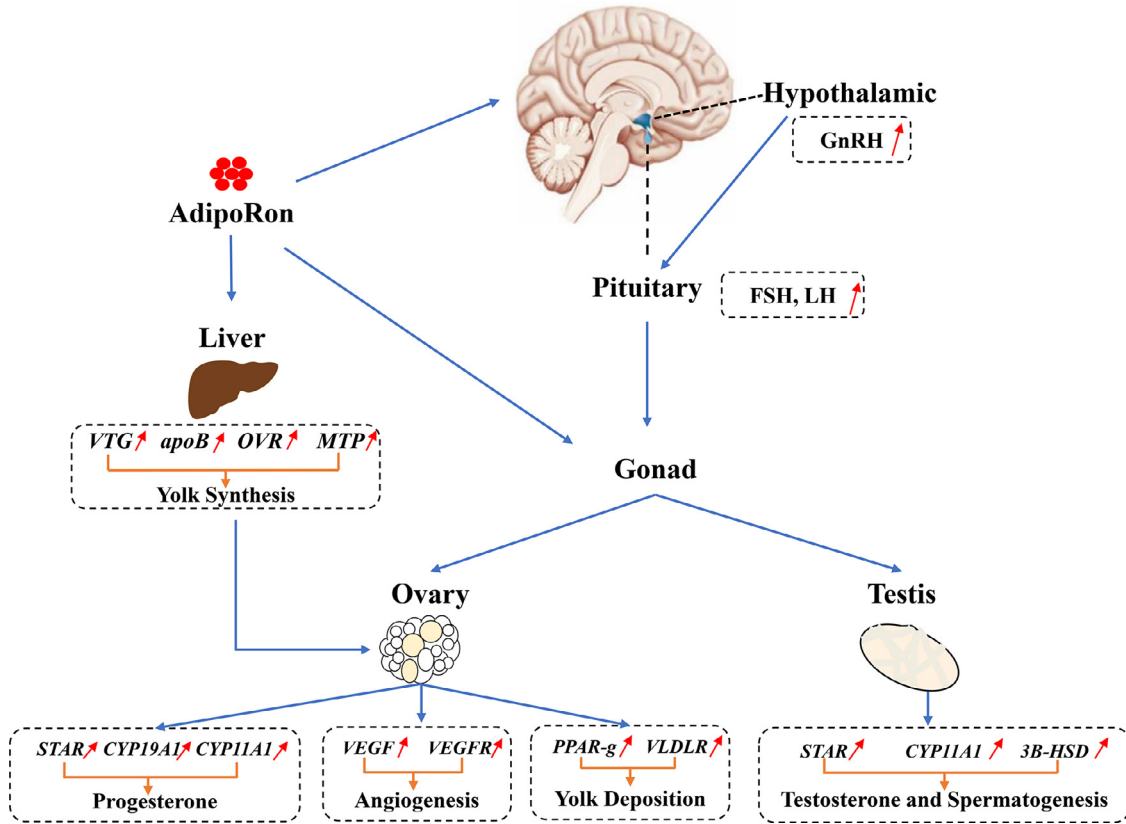
Figure 6. Pattern of AdipoRon regulation of the HPG axis and the promotion ovarian follicle and testicular development. AdipoRon promotes the synthesis of lipids in the liver of laying hens, the secretion of reproductive hormones through the hypothalamic-pituitary-gonadal axis, the formation of follicular blood vessels in laying hens and the generation of male sperm to promote the development of gonads.

found that in vitro injection of AdipoRon can promote the expression of *AdipoR1* and *AdipoR2* in the ovary and testis, and increase the expression of key reproductive genes in the HPG axis of chickens (i.e., *GnRH, FSH, LH, StAR, CYP11A1, CYP19A1*, and *3BHSD*). Moreover, the results demonstrated that AdipoRon promoted the secretion of adiponectin and its receptors, HPO axis-related hormones (i.e., GnRH, FSH, LH, and P) and HPT axis-related hormones (i.e., GnRH, FSH, LH, E2, P, and T). In contrast, AdipoRon decreased E2 secretion in the HPO axis of chickens, which was consistent with the results from previous studies (Singh and Krishna, 2012; Meng et al., 2019; Li et al., 2021b). The results indicate that the production of progesterone from cholesterol and pregnenolone in avian ovaries has the ability to convert progesterone to testosterone but not to estradiol. Thus, these findings indicate that AdipoRon could promote gonadal development and maturation by enhancing the secretion of reproductive hormones in the HPG axis.

In the ovarian cortex of chickens, primordial follicles are activated and gradually develop into growing follicles, which leads to the forming formation of prehierarchical follicles. The follicles contain yellow yolk, indicating their development to SWFs. The preovulatory stage of rapid yolk deposition is then initiated, and the follicles develop into enormous follicles within a short time (Ma et al., 2020). VLDL and vitellogenin are the main components of the egg yolk during yolk deposition and are transported to mature oocytes via

VLDLR-mediated endocytosis from the space between vessels and GCs in the oocyte membrane. Numerous studies have shown that PPAR-g regulates VLDLR expression by promoting lipid uptake and lipogenesis in adipose tissue (Tao et al., 2010; Ma et al., 2020). Furthermore, the ability of yolk precursor formation decreases during the aging process (Liu et al., 2018). This study showed that AdipoRon upregulated the expression of *VLDLR* and *PPAR-g* in SWFs and promoted the transport of yolk precursors.

Angiogenesis is a critical biological process for ovarian development and functions, and the promotion of angiogenesis in the thecal layers of prehierarchical follicles could facilitate yolk deposition (Lin et al., 2019), which is regulated by multiple endogenous and exogenous factors, such as VEGF (a pivotal regulator of angiogenesis in endothelial cells) and ANGPT. In chickens, the expression of VEGF and its receptors in healthy prehierarchical follicles (6−8 mm) is higher than that in atretic follicles (Kim et al., 2016). Furthermore, AdipoQ was recently described as a potential factor that regulates angiogenesis (Adya et al., 2015; Nigro et al., 2021). Our results demonstrated that AdipoRon markedly increased the expression of *VEGF, VEGFR*, and *ANGPT* in SWFs, which indicates that AdipoRon promotes follicular development by increasing angiogenesis of the outer membrane of laying hen follicles.

In mammals, studies have confirmed that adiponectin receptors are localized in the testicular Leydig cells and vas deferens of mice and that adiponectin can promote

spermatogenesis and testosterone production in the testes (Choubey et al., 2019). In our study of the HPT axis, we showed that AdipoRon not only promoted the secretion of HPT axis-related hormones (i.e., GnRH, FSH, LH, E2, P, and T) but also increased the lumen sizes of testicular seminiferous tubules and promoted spermatogenesis. Furthermore, high levels of AdipoRon (20 or 40 μg/mL) and incubation for 24 or 72 h could impact the proliferation and apoptosis of testicular Leydig cells; however, small amounts (5 or 10 μg/mL) of AdipoRon added for 48 h had no effect on cell vitality, which indicated that the effect of AdipoRon on the proliferation and apoptosis of testicular Leydig cells depended on the concentrations and processing duration (Li et al., 2021b). In male rats, AdipoQ inhibits testosterone production by reducing StAR protein expression and suppresses the transcription of the *KISS1* gene by reducing the nuclear translocation of SP-1 (a transcription factor known to be involved in steroidogenic gene regulation) in hypothalamic cells (Caminos et al., 2008; Pfaehler et al., 2012; Wen et al., 2012). Therefore, AdipoQ may play an important role in mesenchymal cells by inhibiting the transcriptional activity of SP-1 and thereby downregulating the expression of steroid genes (Momoi et al., 1992; Pena et al., 1999; Sugawara et al., 2000; Shih et al., 2011). In the present study, AdipoRon supplementation in Leydig cells of chickens not only promoted the expression of *AdipoR1* and *AdipoR2* but also promoted T, E2, and P4 expression by upregulating the production of the STAR and 3B-HSD proteins. These findings suggest that AdipoQ could enhance the secretion of steroid hormones by activating *AdipoR1* and *AdipoR2* and thereby promotes testicular growth and development.

CONCLUSIONS

In summary, this study showed that AdipoQ was significantly associated with the reproductive hormones of the HPG axis, which suggests that AdipoQ and its receptors play an important role in the reproductive process of chickens. Furthermore, AdipoRon could promote the expression of reproductive hormones, hepatic lipid synthesis and follicular angiogenesis in the HPO axis, the expression of reproductive hormones in the HPT axis and the formation of testicular sperm and steroidogenesis in Leydig cells in vitro. Collectively, these results suggest that AdipoRon could promote gonad growth and development through the HPG axis (Figure 6).

ACKNOWLEDGMENTS

DISCLOSURES

The authors declare that they have no conflict of interest. The funders had no role in study design, data collection, and interpretation or in the decision to submit the work for publication.

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