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Effects of recombinant goose adiponectin on steroid hormone secretion in Huoyan geese ovarian granulosa cells

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ABSTRACT

Adiponectin is an adipokine associated with the regulation of reproductive processes. To determine whether recombinant goose adiponectin contributes to the goose ovarian steroidogenesis, the native and then purified goose recombinant adiponectin protein was produced using recombinant DNA technologies. The effect of recombinant adiponectin on the progesterone (P_4) and estradiol (E_2) production in Huoyan geese ovarian granulosa cells was examined. Furthermore, the effect of recombinant adiponectin (2.5 μg/mL) on the abundance of *StAR* (steroidogenic acute regulatory protein), *CYP11A1* (cytochrome P450scc, cholesterol side-chain cleavage enzyme) and *CYP19A1* (cytochrome P450 aromatase) mRNA and protein in granulosa cells was evaluated. Results indicate that a 24-h treatment with recombinant adiponectin (2.5 μg/mL) affected P_4 and E_2 production by geese ovarian granulosa cells by stimulating P_4 production ($P < 0.01$) and weakly inhibiting E_2 production ($P > 0.05$). Furthermore, when the results with treatment were compared to when there was not adiponectin treatment, the abundance of *StAR* and *CYP11A1* mRNA was greater ($P < 0.05$) while the *CYP19A1* mRNA slightly decreased ($P > 0.05$). In addition, the fluorescence intensity of *StAR* tended to be greater compared to PBS-treated ($P < 0.05$) and control groups ($P > 0.05$) and *StAR* protein abundance was greater ($P < 0.05$) compared to the other two groups. The fluorescence intensity and protein abundances of *CYP11A1* increased ($P < 0.05$) while those for *CYP19A1* tended to decrease ($P > 0.05$) after adiponectin treatment. The results indicate recombinant goose adiponectin affects steroidogenesis and/or hormone secretion of geese ovarian granulosa cells. There may, therefore, be important functions of adiponectin in goose reproductive physiology.

1. Introduction

Adiponectin is one of the cytokines mainly secreted by adipose tissue, which was identified as an important factor involved the regulation of reproductive processes (Chabrolle et al., 2007a; Smolinska et al., 2014), and associated with reproductive traits (Houde et al., 2008). Experimental results indicate adiponectin affects the reproductive system through autocrine, paracrine and endocrine effects on the hypothalamus-pituitary-gonadal (HPG) axis (Hoyda et al., 2007; Rodriguez-Pacheco et al., 2007; Wen et al., 2008; Psilopanagioti et al., 2009; Ramachandran et al., 2013; Kaminski et al., 2014; Kiezun et al., 2014).

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Adiponectin has an important function in ovarian steroid hormone secretion and steroidogenesis. Adiponectin affects hormone secretions of ovarian granulosa cells mainly by autocrine or paracrine actions (Chabrolle et al., 2007a; Cao et al., 2015). Adiponectin can increase progesterone secretion of ovarian granulosa cells of chickens that have been stimulated with insulin-like growth factors 1 (IGF-1), whereas adiponectin reduced progesterone production in the presence of follicle-stimulating hormone (FSH) or luteinizing hormone (LH) (Kadowaki and Yamauchi, 2005). Adiponectin has specific effects on the ovarian steroidogenesis process. When there is adiponectin treatment of granulosa cells from medium-sized follicles of prepubertal gilts and comparison made to non-treated control cells, there is a greater abundance of *StAR* and lesser abundance of *CYP19A1* mRNA (Ledoux et al., 2006). Likewise, there is regulation of the expression of steroidogenic genes such as *CYP11A1*, *StAR*, and *CYP19A1* by adiponectin in the ovary of rats, cattle and humans (Chabrolle et al., 2007b; Lagaly et al., 2008). These findings indicate that adiponectin can affect secretion of steroid hormones in ovarian cells through the regulation of steroidogenic gene expression.

Adiponectin was identified to being in differential abundance in ovarian and hypothalamic tissues of Huoyan geese during different stages of the egg-laying cycle (Cao et al., 2015, 2018). These findings indicate adiponectin is an important factor involved the regulation of reproductive processes of geese. In poultry, ovarian granulosa cells are similar to steroidogenic endocrine cells of mammals, and mainly produce progesterone, which is further converted in theca cells to androgens and estrogens (Huang et al., 1979). There are no other reports where there has been evaluation of the effects of adiponectin on hormone secretion in goose ovarian granulosa cells. Consequently, the present study was conducted in geese to assess the effects of recombinant adiponectin protein *in vitro* on cultured Huoyan geese granulosa cells. The aim of this study was to investigate, *in vitro*, whether adiponectin affected steroid hormone production in the granulosa cells of geese.

2. Materials and methods

2.1. Animals and sample collection

Huoyan geese were used in this study and reared on the Liaoning Huoyan Goose Stock Breeding Farm (Liaoyang, Liaoning province, China) using the typical practices used for managing geese at this farm (Detailed in Supplementary Methods). Fifteen healthy female geese at 12 months of age and with regular laying sequences were selected to sample. The average body weight (mean ± S.E.M.) was 3.5 ± 0.6 kg. Geese were sacrificed by exsanguination at 2 h after oviposition. All ovarian follicles were collected using sterilized scissors and tweezers from the abdominal cavities of the geese and placed in PBS (pH 7.4). Then the follicles were classified according to sizes (< 2, 2–4, 4–6, 6–8 and 8–10 mm) and stages of differentiation (follicle hierarchy: F_5, F_4, F_3, F_2 and F_1) according to previously reported nomenclature (Gilbert, 1971). The granulosa cells were separated from the F_4–F_2 ovarian follicles using the method described previously (Gilbert et al., 1977). The residual ovarian tissue was used to extract total RNA for reverse transcribing to adiponectin cDNA. All experimental procedures were approved by the animal welfare committee at the College of Animal Science and Veterinary Medicine of Shenyang Agricultural University (No.2011036).

2.2. Construction of recombinant expression vector

Total RNA was extracted from ovarian tissue using Trizol reagent (Invitrogen Corporation, Carlsbad, CA, USA) following the manufacturer's instructions. The RNA quality and concentration were determined using formaldehyde denaturing gel electrophoresis and NanoDrop 8000 spectrophotometry (NanoDrop, Thermo Scientific, Waltham, MA, USA). The RT-PCR (Reverse Transcription-Polymerase Chain Reaction) was performed as described in Supplementary Methods. The PCR product was gel-purified (Axygen Biosciences, Hangzhou, China) and cloned into a pMD18-T vector (TaKaRa, Dalian, China), then sub-cloned into the expression vector pET-28a (TaKaRa, Dalian, China) to generate a recombinant expression plasmid pET-28a-*adiponectin* (see Supplementary Methods). The construction strategy of recombinant expression plasmid pET-28a-*adiponectin* is depicted in Supplementary Fig. 1.

2.3. Expression and purification of recombinant protein

The pET-28a-*adiponectin* plasmid was induced in *E.coli* BL21 (DE3) bacterial liquid with isopropyl β-D-thiogalactopyranoside (IPTG) (DINGGUO, Beijing, China), then the expressed recombinant protein was purified using Ni-Agarose Resin (CWBIO, Beijing, China) (See Supplementary Methods). The protein concentration was determined using the BCA Protein Assay Kit (Applygen Technologies Inc. Beijing, China). The purified recombinant protein was stored at −80 °C for use.

2.4. Identification of recombinant adiponectin protein

The recombinant adiponectin protein sample was separated using a 12% SDS-PAGE and transferred electrophoretically onto a nitrocellulose membrane. Following blocking in 5% skimmed milk for 1 h at 4 °C, the membrane was incubated with anti-His mouse monoclonal antibody (1:800) (Beyotime, Shanghai, China) overnight at 4 °C and subsequently with AP-labeled Goat Anti-Mouse IgG (1:4000) (Beyotime, Shanghai, China) for 1 h at 37 °C. The immunoreactive bands were visualized using BCIP/NBT Alkaline Phosphatase Color Development Kit (Beyotime, Shanghai, China) according to the manufacturer's instructions.

2.5. Granulosa cell culture and identification

The harvested granulosa cells were washed with PBS (pH 7.4) and dispersed with 0.1% type II collagenase (Sigma, St Louis, USA) at 37 °C for 5 min. The cell suspension was filtered through a 200 micron nylon mesh to remove large clumps of cells and debris. The filtered cell suspension was centrifuged twice at 188 × *g* for 8 min and re-suspended in M199 medium (HyClone, Logan, UT, USA) containing 10% fetal bovine serum (FBS) (HyClone, Logan, UT, USA) and 100 U/mL of penicillin/streptomycin (Gen-view scientific Inc., Galveston, TX,USA). Cell viability and concentration was assessed as described in Supplementary Methods by an automatic cell counter (BodBoge, Shenzhen, China). After that, the cells were diluted with the medium to a concentration of 1×10^6/mL and plated in cell culture flasks and, 96-well and 24-well culture plates with M199 medium (HyClone, Logan, UT, USA) containing 5% FBS (HyClone, Logan, UT, USA), and then incubated at 37 °C under an atmosphere of 95% air and 5% CO_2. Granulosa cells were identified using FSHR (follicle-stimulating hormone receptor) as a molecular marker using the previously described method (Lou et al., 2017) (See detailed procedure in Supplementary Methods). The cell growth was examined every 24 h as described in Supplementary Methods using CellTiter96® AQueous One Solution Reagent (Promega, Madison, WI, USA).

2.6. Granulosa cell treatment experiment

To reduce overall variability by minimizing individual heterogeneity, granulosa cells collected from follicles of five different individual geese were pooled to generate one sample pool (Peng et al., 2003; Karp and Lilley, 2009). Five animals were randomly selected from 15 geese used in this study. A total of three biological sample pools were generated. All cells from three sample pools were divided into five groups (control group, PBS group, 1 μg/mL dose group, 2.5 μg/mL dose group, and 5 μg/mL dose group). Thus, each group was replicated three times. After 72 h pre-incubation (M199 medium with 5% FBS), the medium was replaced with fresh M199 medium without (control group, PBS group) or with recombinant adiponectin protein (1, 2.5, and 5 μg/mL dose group). The doses of adiponectin used in this experiment were selected based on its physiological concentration in avian plasma (Hendricks et al., 2009). Subsequently, the ovarian granulosa cells continued to incubate for 24, 48 and 72 h. The P_4 and E_2 concentrations in cultured samples were determined as described in Supplementary Methods using a goose progesterone and estradiol ELISA kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The concentration of P_4 and E_2 indicated there were hormone secretions of granulosa cells and response to adiponectin. Consequently, according on the concentration of P_4 and E_2, the optimal dose of recombinant adiponectin protein and treatment time was defined.

2.7. Semi-quantitative real-time PCR

To evaluate the effect of adiponectin on the relative abundance of mRNA for *StAR*, *CYP11A1* and *CYP19A1* in ovarian granulosa cells, semi-qRT-PCR was performed as described in Supplementary Methods. The primers used for semi-qRT-PCR (Supplementary Table 1) were designed and synthesized by Sangon Biotech Co., Ltd (Shanghai, China). The relative abundances of mRNAs of all target genes were calculated using methods previously described (Livak and Schmittgen, 2001).

2.8. Cellular IF

To evaluate the effects of adiponectin on protein abundances of *StAR*, *CYP11A1* and *CYP19A1* in ovarian granulosa cells, cellular IF was performed as described in Supplementary Methods.

2.9. Western blotting

To further evaluate the effects of adiponectin on the protein abundances of *StAR*, *CYP11A1* and *CYP19A1* in ovarian granulosa cells, western blotting was performed as described in Supplementary Methods.

2.10. Statistical analysis

The SPSS 17.0 for Windows (SPSS Inc. Chicago, Illinois, USA) program was used to analyze the experimental data. The data of P_4 and E_2 concentration were analyzed using a two-way ANOVA with Bonferroni's *post-hoc* test. Other data were analyzed using a one-way ANOVA followed by use of the LSD *post-hoc* test. Before performing the ANOVA, a data normality test and data variance homogeneity test were conducted using the Levene's test. The results were reported as the Mean ± SEM. The $P < 0.05$ and $P < 0.01$ was considered to be significant and highly significant, respectively.

3. Results

3.1. Construction of recombinant plasmid

The ORF (open reading frame) sequences of *adiponectin* gene were amplified and cloned into pET-28a to produce plasmid pET-28a-*adiponectin*. The plasmid sequence was validated by PCR amplification and restriction enzyme digestion (Fig. 1) and DNA sequencing analysis (Supplementary Figs. 2 and 3).

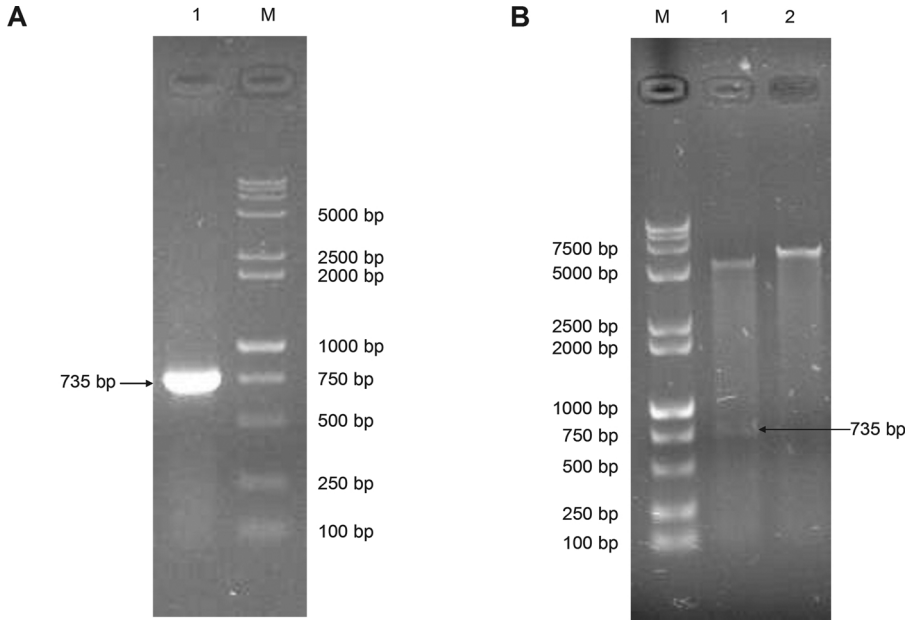

Fig. 1. PCR amplification and double enzyme digestion of recombinant plasmid (A) PCR amplification of adiponectin from pET-28a-*adiponectin*; Lane M, DNA Marker; Lane 1, PCR-amplified pET-28a-*adiponectin*; (B) Double enzyme digestion of recombinant plasmid; Lane M, DNA Marker; Lane 1, pET-28a-*adiponectin* digested with *Eco*R I and *Xho* I; Lane 2, pET-28a-*adiponectin*.

3.2. Expression and purification of recombinant adiponectin protein

Adiponectin protein can be expressed in the pET expression system with IPTG induction (Fig. 2A). Because of the presence of the His-tag, the protein was successfully purified using Ni-Agarose Resin. The results indicate that the 32 kDa purified recombinant adiponectin protein bands were present after SDS-PAGE (Fig. 2B) and western blotting (Fig. 2C) detection, which was consistent with the expected molecular weight of the adiponectin protein.

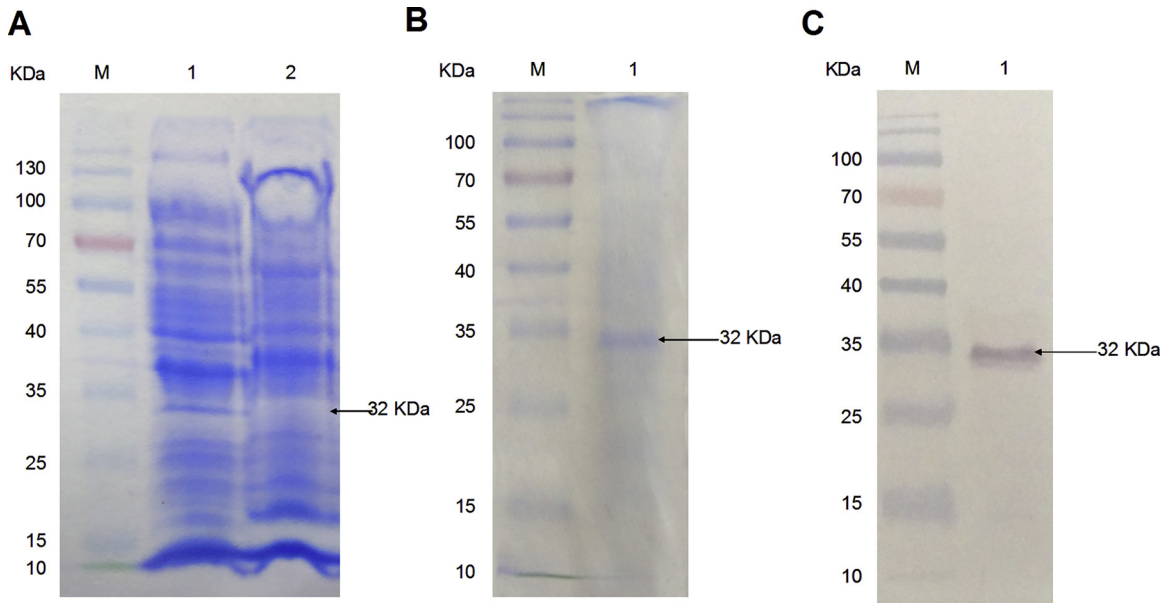

Fig. 2. Expression and purification of recombinant goose adiponectin protein; (A) SDS-PAGE of expressed recombinant adiponectin protein; Lane M, Protein Marker; Lane 1, recombinant adiponectin protein, Lane 2, control; (B) SDS-PAGE of purified recombinant adiponectin protein; Lane M, Protein Marker; Lane 1, recombinant adiponectin protein; (C) Western blotting of purified recombinant adiponectin protein; Lane M, Protein Marker; Lane 1, recombinant adiponectin protein; Predicted molecular weight of recombinant goose adiponectin protein is 32 KDa.

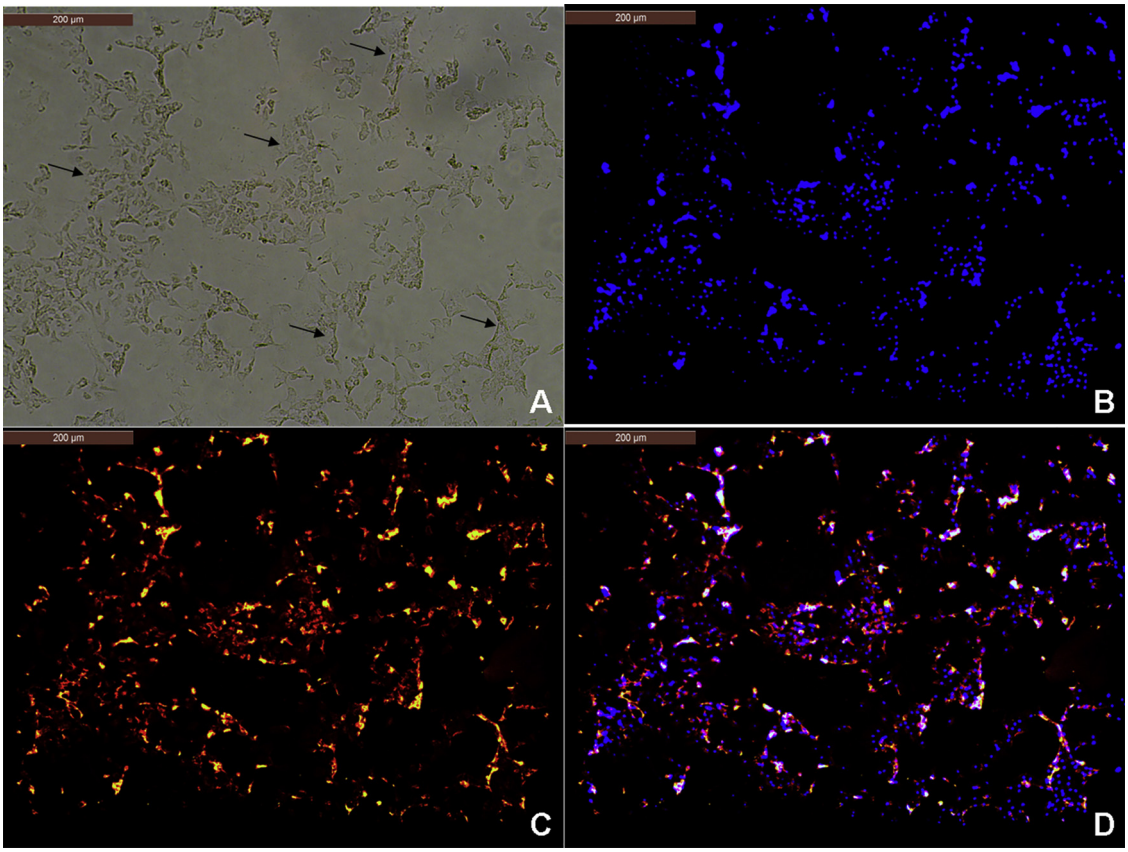

Fig. 3. Identification of primary goose ovarian granulosa cells. Cell membrane stained with FSHR in orange; Cell nuclei stained with DAPI in blue; Scale bar = 200 μm; (A) Bright-field primary ovarian granulosa cells, (B) DAPI, (C) FSHR and (D) Merge (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

3.3. Identification of ovarian granulosa cells

The granulosa cells isolated from the ovary were spherical. The concentration and percentage of live cells was 1.02×10^7/mL and 69%. When cultured for 24 h, some cells attached to the plate wall. As shown in Fig. 3, the immunofluorescence of the ovarian granulosa cell marker FSHR was positive, which meant that the cells isolated and cultured in this study were ovarian granulosa cells. The cell growth test results (Supplementary Fig. 4) indicated that the OD value of ovarian granulosa was the greatest when cultured for 3 days, indicating that the cell viability was greatest at this time and that this was a preferred time point for subsequent studies. Ovarian granulosa cells cultured for 72 h were, therefore, selected to conduct the recombinant adiponectin treatment experiment.

3.4. Progesterone and estradiol concentrations

The concentration of P_4 and E_2 in the culture medium of ovarian granulosa cells treated with and without recombinant goose adiponectin was measured. As depicted in Fig. 4A, in comparison with the control and PBS group, a 24-h treatment with recombinant goose adiponectin at the 2.5 μg/mL dose stimulated P_4 secretion ($P < 0.01$), while the 5 μg/mL dose also stimulated P_4 secretion but to a lesser extent ($P < 0.05$). There was no difference ($P > 0.05$) among groups after treatment for 48 h and 72 h. As depicted in Fig. 4B, following culturing for 24 h, treatment with recombinant goose adiponectin (1, 2.5 and 5 μg/mL dose) had a small effect on E_2 secretion ($P > 0.05$) compared to control and PBS-treated group. There were no effects of any of the doses when there was culturing for 48 and 72 h ($P > 0.05$). In addition, the secretion of P_4 when cultured for 48 h was slightly increased compared to 24 h ($P > 0.05$), and subsequently there was a decrease at 72 h ($P < 0.01$). Furthermore, the secretion of E_2 decreased as culture time advanced ($P < 0.01$). Based on these findings, the 2.5 μg/mL dose of recombinant adiponectin and the 24 h treatment time was used for *in vitro* treatment experiments.

3.5. Change relative abundance of sterol/steroidogenic mRNA as a result of adiponectin treatment

The relative abundance of mRNA for *StAR*, *CYP11A1* and *CYP19A1* in ovarian granulosa cells with and without recombinant goose adiponectin treatment was examined using semi-qRT-PCR. As depicted in Fig. 5, the relative abundance of mRNA for *StAR* and

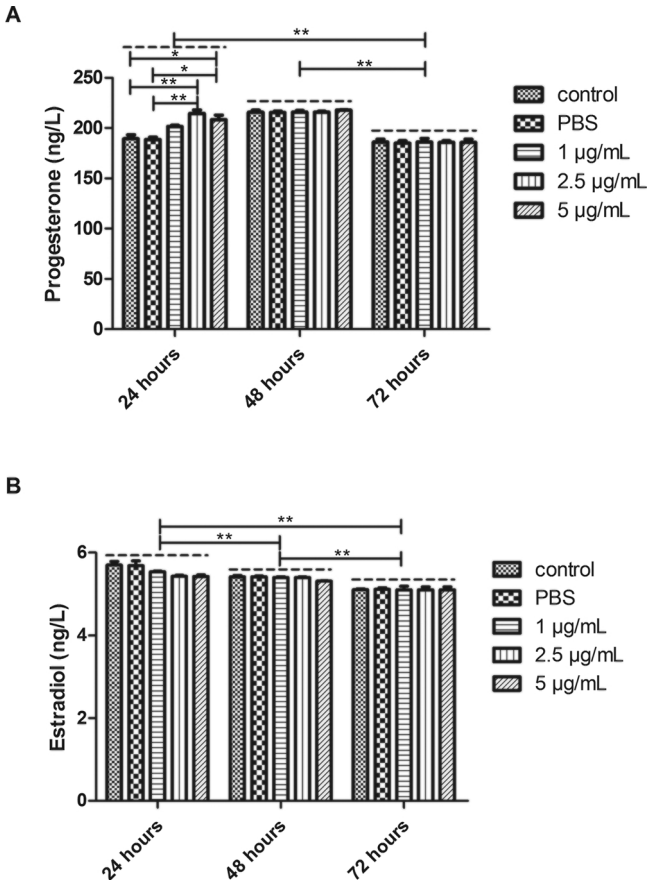

Fig. 4. Effect of recombinant goose adiponectin (1, 2.5, and 5 μg/mL) on the production of P_4 and E_2 in goose ovarian granulosa cells; (A) P_4 concentration after treatment with recombinant adiponectin for 24, 48 and 72 h, respectively; Y-axis represents P_4 concentration; (B) E_2 concentration after treatment with recombinant adiponectin for 24, 48 and 72 h, respectively; Y-axis represents E_2 concentration; Values of bars are expressed as Mean ± SEM ($n = 3$); Single and double asterisks indicate $P < 0.05$ and $P < 0.01$, respectively; Two-way ANOVA with Bonferroni's *post-hoc* test was performed.

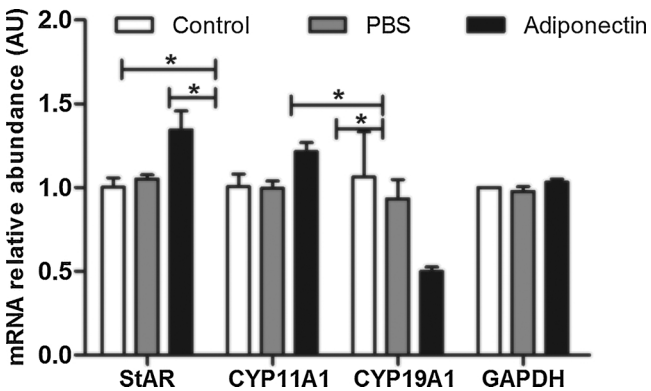

Fig. 5. Effect of recombinant goose adiponectin (2.5 μg/mL, 24 h treatment) on relative abundance of mRNA for *StAR*, *CYP11A1* and *CYP19A1* in goose ovarian granulosa cells; Y-axis represents the fold change of mRNA abundance which presented in AU (arbitrary units); Values of bars are expressed as Mean ± SEM ($n = 3$); Single asterisks indicate $P < 0.05$; One-way ANOVA followed by LSD *post-hoc* test was performed.

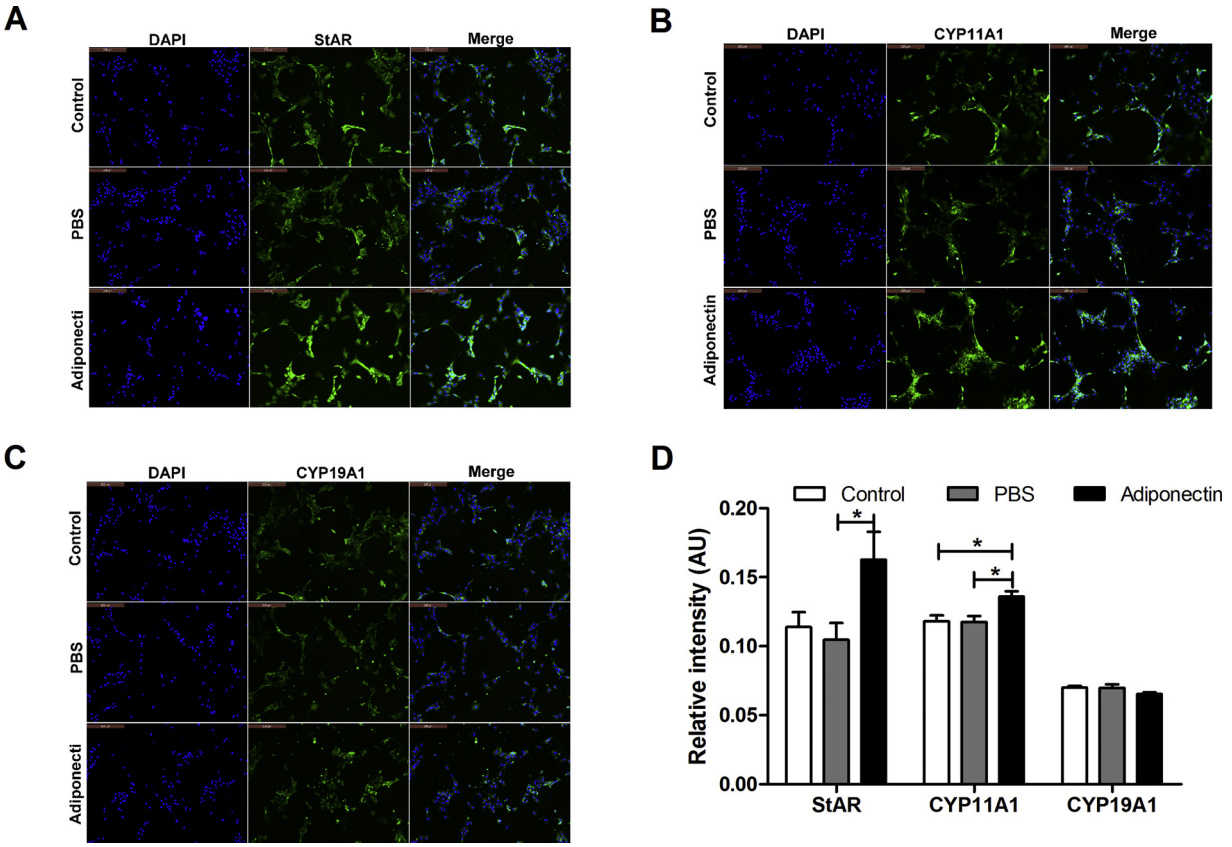

Fig. 6. Effect of recombinant goose adiponectin (2.5 μg/mL, 24 h treatment) on the immunofluorescence intensity of *StAR*, *CYP11A1* and *CYP19A1* protein in goose ovarian granulosa cells; (A), (B), and (C) are the representative photomicrographs of *StAR*, *CYP11A1* and *CYP19A1* protein, respectively. Cell membrane stained with *StAR*, *CYP11A1* and *CYP19A1* are green; Cell nuclei stained with DAPI are blue; Scale bar = 200 μm; (D) The semi-quantification of the immunofluorescence intensity in above groups; Y-axis represents the relative fluorescence intensity which expressed in AU (arbitrary intensity units); Values of bars are expressed as Mean ± SEM (n = 3); Single asterisks indicate $P < 0.05$; One-way ANOVA followed by LSD *post-hoc* test was performed (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

CYP11A1 in the adiponectin treated group was greater ($P < 0.05$) compared with the other groups, the relative abundance of mRNA for *CYP19A1* was marginally less ($P > 0.05$) with adiponectin treatment as compared to the control.

3.6. Change of sterol/steroidogenic proteins abundances as a result of adiponectin treatment

The abundance of protein for *StAR*, *CYP11A1* and *CYP19A1* in ovarian granulosa cells was examined. As depicted in Fig. 6, when ovarian granulosa cells were treated for 24 h with recombinant goose adiponectin (2.5 μg/mL), the fluorescence intensity of *StAR* protein tended to be greater than that in the PBS-treated ($P < 0.05$) and control group ($P > 0.05$). The abundance of *CYP11A1* protein was greater ($P < 0.05$) compared to control and PBS-treated group. The abundance of *CYP19A1* protein tended to less ($P > 0.05$) after treatment with adiponectin.

Western blotting was used to analyze the protein abundance of *StAR*, *CYP11A1* and *CYP19A1* in ovarian granulosa cells. As depicted in Fig. 7, when ovarian granulosa cells were treated for 24 h with recombinant goose adiponectin (2.5 μg/mL), the abundance of *StAR* and *CYP11A1* protein was greater ($P < 0.05$) compared to the group not treated with adiponectin. There was a slight trend for a decrease in abundance of *CYP19A1* protein ($P > 0.05$) compared to the group not treated with adiponectin.

4. Discussion

In recent years, the role of adiponectin in regulation of the ovarian functions has been investigated. Results of previous studies indicate that adiponectin and its receptors (AdipoR1 and AdipoR2) were present in ovary of various species including humans, mice, cows, pigs, chickens, turkeys, and bats (Chabrolle et al., 2007a, b; Chabrolle et al., 2009; Maillard et al., 2010; Maleszka et al., 2014b; Singh et al., 2014; Diot et al., 2015), and directly or indirectly affected ovarian steroidogenesis. In the current study, there was *in vitro* study, for the first time, of the function of adiponectin on steroid hormone secretion in geese ovarian granulosa cells.

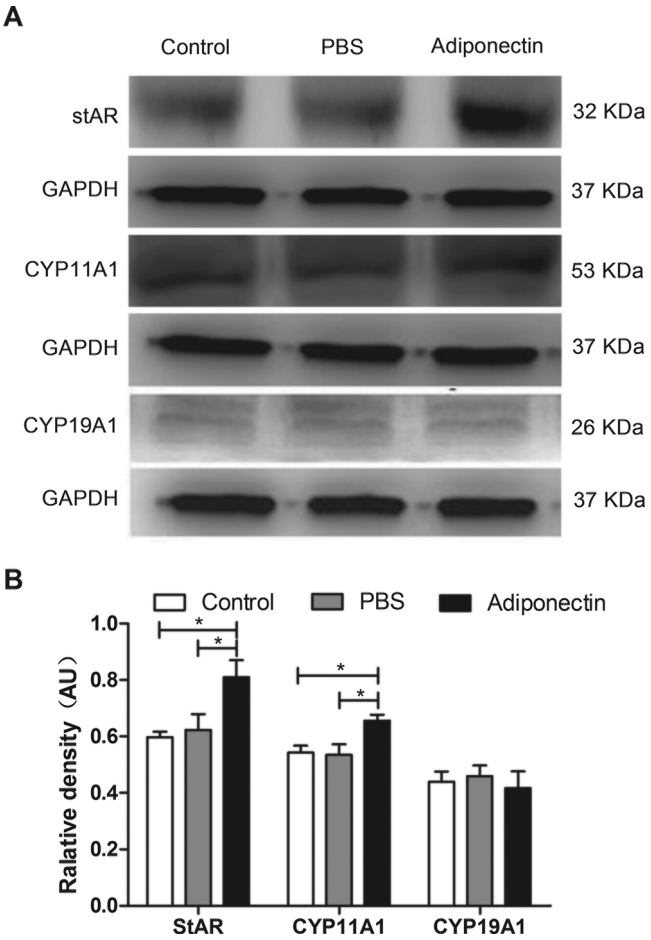

Fig. 7. Effect of recombinant goose adiponectin (2.5 μg/mL, 24 h treatment) on the protein abundance of *StAR*, *CYP11A1* and *CYP19A1* in goose ovarian granulosa cells; (A) Representative immunoblots; (B) Densitometric analysis of *StAR*, *CYP11A1* and *CYP19A1* protein relative to *GAPDH*; Y-axis represents the relative optical density which expressed in AU (arbitrary optical density units); Values of bars are expressed as Mean ± SEM ($n = 3$); Single asterisks indicate $P < 0.05$; One-way ANOVA followed by LSD *post-hoc* test was performed.

Consistent with results from previous studies, results of the present study indicate that adiponectin affects the production of steroid hormones including progesterone and estradiol. Treatment with recombinant goose adiponectin (2.5 μg/mL, 24 h) resulted in an increase and slight decrease of progesterone and estradiol production from goose ovarian granulosa cells, respectively. It might be inferred that the granulosa cells in ovarian follicles of birds produce progesterone from cholesterol and pregnenolone, and have the capacity to convert progesterone to testosterone but not to estradiol. Adiponectin was observed to affect steroidogenesis in the ovary of numerous mammalian and other species (Chabrolle et al., 2007a; Chabrolle et al., 2007b; Chabrolle et al., 2009; Anuradha and Krishna, 2014). In *in vitro* studies with cultured pig granulosa cells, recombinant human adiponectin at larger doses (10 μg/mL) in combination with insulin induced the increase in progesterone secretion in comparison to cells stimulated with insulin alone, and sustained a basal secretion of estradiol (Maleszka et al., 2014a). In primary rat granulosa cells, treatment with recombinant human adiponectin (5 μg/mL) in the presence of IGF-1 (10^{-8} M) resulted in an increase in progesterone secretion by about two-fold and estradiol production by about 1.6-fold (Chabrolle et al., 2007b). Regarding avian species, after treatment using recombinant human adiponectin (10 μg/mL) combined with IGF-1 (100 ng/mL), progesterone production was increased by about 3-fold in $F_{3/4}$ hen granulosa cells and 1.5-fold in F_2 and F_1 hen granulosa cells (Chabrolle et al., 2007a). These results indicate that adiponectin enhanced the IGF-1 effects on the production of steroid hormone secretion. Different species, combined factors, sources and concentration of recombinant protein and other factors may lead to different ovarian responses to adiponectin. In ovaries of the *Cynopterus sphinx*, treatment with mouse adiponectin (5 μg/mL) either alone or in combination with a large dose of glucose (25 mM/mL) resulted in increased progesterone synthesis as compared with the control group and when there were smaller doses of glucose (10 mM/mL) (Anuradha and Krishna, 2018). Inconsistent results were, however, observed in chicken granulosa cells where treatment with recombinant human adiponectin (10 μg/mL) along with LH and FSH inhibited progesterone production (Chabrolle et al., 2007a). Similarly, recombinant human adiponectin (3 μg/mL) had inhibitory effects on progesterone and androstenedione production that was induced by LH in combination with insulin in theca cells of cattle. The inhibitory effects of adiponectin on steroidogenesis were primarily localized to theca cells, but not granulosa cells (Lagaly et al., 2008). In rat granulosa cells, recombinant

human adiponectin (1–10 μg/ml) had no effect on basal or FSH-induced progesterone and estradiol production (Chabrolle et al., 2007b). This inconsistency may be due to differential responses of species and different physiological stages at the time of cell collections or differences in types of gonadotropins and growth factors added to the culture medium. These factors could affect the action of adiponectin or affect its pathways, resulting in different responses.

Another important aspect of the present study was whether adiponectin affects steroidogenesis through the regulation of steroidogenic gene expression. Transcription and activation of genes encoding steroidogenic enzymes are required for successive conversion of cholesterol to other steroids during steroidogenesis. Among these enzymes, *StAR* is abundantly present in the steroid-producing cells, which is the first key mediator and rate-limiting step in the steroidogenic pathway, and required for the transportation of cholesterol across the mitochondrial membrane (an obligatory step for steroid production) (Stocco, 2001). In avian species, during the transition of the pre-hierarchical follicles to a pre-ovulatory hierarchy, the cells of the granulosa layer begin to produce *StAR* and *CYP11A1* proteins and as a consequence progesterone is produced as a result of the action the *HSD3Bs* (3β-hydroxysteroid dehydrogenases) (Li and Johnson, 1993; Johnson and Bridgham, 2001). The *CYP19A1* protein is responsible for estrogen biosynthesis. The relative expression of *StAR*, *CYP11A1* and *CYP19A1* genes can directly or indirectly affect the synthesis and secretion of progestogen and estrogen. Results for previous studies indicate that the elevated progesterone production in the granulosa layers of the $F_3 \rightarrow F_1$ follicles is predominantly associated with an increased abundance of *StAR*, *CYP11A1* and *HSD3B* mRNAs and enhanced HSD3B activity (Marrone and Sebring, 1989; Johnson et al., 2002; Sechman et al., 2011). Results of numerous studies indicate adiponectin regulates ovarian steroid hormones secretion as a result of changes in expression of steroidogenic pathway genes, including *StAR*, *CYP11A1*,*CYP19A1* and *HSD3B* (Ledoux et al., 2006; Chabrolle et al., 2007b; Lagaly et al., 2008; Pierre et al., 2009; Ramanjaneya et al., 2011; Singh et al., 2014). Likewise, the contribution of adiponectin to steroidogenesis and expression of related genes also was observed in the testes and adrenal glands. In MA-10 mouse Leydig cells, adiponectin treatment enhanced progesterone production through an increase of the *StAR* and *CYP11A1* steroidogenic enzymes (Landry et al., 2015). In human adrenocortical H295R cells, adiponectin treatment resulted in a significant increase in cortisol production, together with increases in abundance of mRNA for important steroidogenic genes including *StAR*, *CYP11A*, *HSD3B*, *CYP17* and *CYP11B1* (Ramanjaneya et al., 2011). In the present study, the increase in *StAR* and *CYP11A1* and decrease in *CYP19A1* after adiponectin treatment was consistent with results using the semi-qRT-PCR, immunofluorescence (IF) and western blotting. Furthermore, the concentrations of hormones detected are consistent with these other results. These findings indicate that adiponectin affected the production of steroid hormones by affecting transcription of steroidogenic related genes in geese ovarian granulosa cells.

In the present study there were some limitations. Firstly, animals used in this study were geese that were in their peak egg-laying period. Granulosa functions in geese that were at other stages of the egg-laying cycle were not investigated. Secondly, the purity of recombinant adiponectin protein was not determined even though it was purified using Ni-Agarose Resin. Thirdly, the percentage of ovarian granulosa cells was not confirmed, even though separated cells were identified to be ovarian granulosa cells using an FSHR marker. Furthermore, the recombinant adiponectin used in the present study originated from geese, yet adiponectin used in most previous reports was commercial recombinant human adiponectin. Whether the recombinant human adiponectin protein has a similar or different effect on goose granulosa cell function needs investigation in the future. There are also other considerations such as the physiological concentration of adiponectin in geese blood during different development stages. Furthermore, the *in vivo* effect of adiponectin function at the ovary of geese needs to be elucidated. If these results are confirmed in the future, endogenous and exogenous adiponectin and its analogues might be used to improve the reproductive performance of geese.

In summary, results of the present study are the first in which there was expression of the native goose adiponectin and subsequent purification. Furthermore, results of the present study are the first to indicate that adiponectin affected steroid hormone production that was associated with altered expression of the steroidogenic genes in goose ovarian granulosa cells. This research provides important information about adiponectin functions in goose reproductive physiology.

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Conflicts of interest

None

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.anireprosci.2019.03.019.

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