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CHANGES IN THE EXPRESSION OF ADIPONECTIN AND ITS RECEPTORS (ADIPOR1 AND ADIPOR2) IN SEXUALLY IMMATURE AND SEXUALLY MATURE SHEEP TESTES

Man Bai[1], Limin Sun, Jia Zhao, Guishan Zhang, Wei Ma, Lujie Xiang, Jiarong Li, Chao Jia, and Huaizhi Jiang[*].

Collage of Animal Science and Technology, Jilin Agricultural University, Changchun 130118, China.
[*]Corresponding Author: Huaizhi Jiang, E-mail: sheepandgoatgbr@126.com

ABSTRACT

Recent evidences have indicated that adiponectin and its receptors have an effect on male reproduction. The objective of this study was to detect the expression of adiponectin, AdipoR1 and AdipoR2 in sexually immature and sexually mature sheep testes using qRT-PCR and Western blot. The results showed that adiponectin, AdipoR1 and AdipoR2 were all expressed in sheep testes. The expression of *AdipoR1* and *AdipoR2* mRNAs ($p<0.05$ and $p<0.01$) as well as the expression of AdipoR1 and AdipoR2 proteins ($p<0.01$ and $p<0.05$) in sexually mature testes were significantly higher than that in sexually immature testes. However, there was no significant difference in the expression of *adiponectin* mRNA ($p>0.05$) in sexually immature and sexually mature testes, the expression of adiponectin protein in sexually mature testes was significantly higher than that in sexually immature testes ($p<0.01$). It is the first time to compare the mRNA and protein expression of adiponectin and its receptors in sexually immature and sexually mature sheep testes.

Key words: adiponectin, AdipoR1, AdipoR2, sheep, testes, qRT-PCR, Western-blot

INTRODUCTION

Adiponectin is an adipocytokine playing a dominant role in energy metabolism (Zhang *et al.* 2016), it is also termed Acrp30 (Philipp *et al.* 1995), apM1 (Maeda *et al.* 1996), Adipo Q (Hu *et al.* 1996) or GBP28 (Nakano *et al.* 1996). Adiponectin is a 25-30kDa protein, which consists of a C1q-like globular domain, an N-terminal collagen domain, a signal peptide, and a variable region. Adiponectin exists in two forms of full length and globular protein, which exerts its biological functions by acting with two distinct receptors, AdipoR1 and AdipoR2. In plasma, adiponectin mainly existed in full-length form. Different from the topology of G-protein coupled receptors, both of AdipoR1 and AdipoR2 contain seven transmembrane domains with a carboxyl terminus and an amino terminus, which is in extracellular and intracellular, respectively (Yamauchi *et al.* 2014). AdipoR1 has a high affinity with globular adiponectin, and AdipoR2 shows a medium affinity with both globular and full-length adiponectin (Yamauchi and Kadowaki 2013). Activation of the receptors can activate many important pathways, such as the AMPK pathway, PPAR pathway and MAPK pathway (Iii *et al.* 2013). Adiponectin regulates energy homeostasis by promoting fatty acid oxidation, reducing plasma triglycerides, increasing insulin sensitivity, and improving glucose metabolism (Lindberg *et al.* 2015). Reproduction cannot be separated from energy availability.

Growing evidences indicated that adiponectin can influence the male reproductive system by exerting central or peripheral effects on the hypothalamus-pituitary-gonadal (HPG) axis (Cao *et al.* 2015). *AdipoR1* and *AdipoR2* were expressed in human and rat pituitary and hypothalamus (Psilopanagioti *et al.* 2009; Kos *et al.*2007). In the pituitary, adiponectin regulated hormone secretion and gene expression in reproduction-related cells (somatotrophs and gonadotrophs) (Rodriguez-Pacheco *et al.* 2007). Adiponectin may exert its functions on hypothalamic and pituitary by autocrine/paracrine way. Adiponectin and its receptors were expressed in different testicular cells. In chicken, *adiponectin* and *AdipoR1* were expressed in peritubular and Leydig cells, whereas *AdipoR2* is expressed in Leydig cells, Sertoli cells and germ cells. Furthermore, the expressions of *AdipoR1* and *AdipoR2* mRNA in adult chicken testes were higher than prepubertal chicken testes (Ocón-Grove *et al.* 2008). AdipoR2 protein expression is also increased in Leydig cells during puberty in rat and the serum concentration of adiponectin in sexually mature mice was higher than sexually immature mice, at the same time, adiponectin concentration in seminal plasma are positively correlated with sperm concentration, sperm count, and normal morphology of spermatozoa (Combs *et al.*2013). The up-regulated expressions of testicular AdipoR1 and AdipoR2 could be related to sexual maturation. Adiponectin and its receptors play a key role in sperm morphology and function, which contributed to increased fertility. *Adiponectin* produced from testes and epithelium of male reproductive tract can influence sperm maturation (Martin. 2014). In bull, adiponectin was abundant in sperm tail, AdipoR1 was localized at acrosome, and AdipoR2 was primarily on sperm head, in high-fertility bulls, the serum adiponectin and the mRNA expression of *adiponectin* and its receptors in sperm were higher than normal bulls (Kasimanickam *et al.* 2013). In ram, *adiponectin*, *AdipoR1*, and *AdipoR2* mRNA were all

expressed in testis, epididymis (caput, corpus, and cauda), vesicular and bulbourethral glands, while adiponectin were not expressed in vas deferens (Rahmanifar and Tabandeh, 2012). *AdipoR1* and *AdipoR2* mRNA were also expressed in porcine epididymis (Dai *et al.*2006). The physiological role of adiponectin and its receptors in testicular function and male reproduction needs to be further investigated. It is necessary to understand the expression profile of adiponectin and its receptors in testis development.

Reproductive efficiency is an important indicator of animal husbandry income level. Sheep are typical species with seasonal reproduction (Weems *et al.* 2015), improving reproductive efficiency could bring a tremendous economic profitability to sheep industry. Sperm production is an important aspect of reproductive efficiency in rams. Furthermore, the use of artificial insemination (AI) in sheep needs for a better understanding of the molecular mechanisms, which regulate male sheep testes development and spermatogenesis. Testis is a special tissue producing sperms and secreting androgens (Wei *et al.* 2013). Spermatogenesis is a highly organized and complex process, which is dependent on normal testis development (Zhu *et al.* 2016). It has been confirmed that adiponectin, AdipoR1 and AdipoR2 were expressed in several species testes, but there was no report about the expression of adiponectin and its receptors in sexually immature and sexually mature sheep testes. The purpose of this study was to compare the expression of adiponectin, AdipoR1, and AdipoR2 in sexually immature and sexually mature sheep testes at mRNA and protein level. The results of this study can provide a better understanding in the effects of adiponectin and its receptors on sheep testes development and spermatogenesis.

MATERIALS AND METHODS

Ethics statement: The methods were carried out according to relevant guidelines from the Ministry of Agriculture in China. All experimental procedures were agreed by Jilin Laboratory Animal Specialized Committee.

Sample collection: Testes were collected from 3 sexually immature (2-month-old) and 3 sexually mature (12-month-old) male sheep in Qian'an Zhi Hua Sheep Breeding Co. Ltd (Jilin, China). All experimental sheep were raised under the same environmental conditions with natural light and free intake of food and water. All the samples were collected in autumn, and the testis of each ram was frozen and stored in liquid nitrogen.

qRT-PCR: Total RNA was extracted from each testis lysates using RNAiso Plus reagent (TakaRa, Japan) according to the protocols. The RNA concentration was detected by Nanodrop 2000 (Thermo, USA). The RNA quality was judged by optical density (OD), when the value of OD260 nm/OD280 nm was between 1.8~2.0, the RNA can be used as template to synthesize the first strand cDNA. The cDNA was synthesized with 2μg template using ReverTra Ace qPCR RT Kit (TOYOBO, Japan) at 37°C for 15min, the procedure was terminated by incubation at 98°C for 5min. The specific primer information of *adiponectin*, *AdipoR1*, *AdipoR2*, and *β-actin* genes are showed in Table 1. *β-actin* was used as reference gene. Primers were designed and synthesized by Sangon Biotech (Shanghai, China). qRT-PCR was conducted using CFX-96 Manager Real-time PCR equipment (Bio-Rad, USA). Each qRT-PCR reaction contained 2μl cDNA, 0.4μM forward primer, 0.4μM reverse primer, 10μl SYBR Green PCR Master Mix (TOYOBO, Japan) and RNase free water added to 20μl. The thermo cycle conditions at 95°C for 30s, followed by 40 cycles of 95°C for 5s, 60°C for 10s and 72°C for 15s. qRT-PCR products were incubated at 65°C for 5s, then the temperature was increased to 95°C (at 0.5°C increment) for 10s to assess melting curves. No template control as negative control in the assay. All samples were amplified in three repetitions. The qRT-PCR products of all amplified genes were determined in an agarose gel (1%). The relative expressions of *adiponectin*, *AdipoR1* and *AdipoR2* genes were performed according to the $-\triangle\triangle C_T$ method (Livak and Schmittgen. 2001).

Table1. Primer information of *adiponectin*, *AdipoR1*, *AdipoR2* and *β-actin* genes.

Gene	Primers sequence	Gen Bank accession number	Product length (bp)
Adiponectin	Forward: AAGCGGCAGAGTTAGAAATCC	XM_004003053.1	122
	Reverse: TCAGATGAGTTGGTGGGAGAC		
AdipoR1	Forward: ATAGCCTGGTCCCATCTTTCT	XM_004013936.1	100
	Reverse: GGACACACTCCCATGATTAGC		
AdipoR2	Forward: CCTTGCTTCGTCTACTTGATTG	XM_004007604.1	114
	Reverse: AAACACTCCTGCTCTCACACC		
β-actin	Forward: GATCTGGCACCACACCTTCTA	U39357.1	115
	Reverse: GATCTGGGTCATCTTCTCACG		

Western blot: In western blot, a part of each frozen testis was cut and homogenized, from which total protein was extracted in testicular homogenates using lysis buffer with phosphatase inhibitor and protease inhibitor and centrifuged at 12000r/min for 5 min at 4°C to remove cell debris according to KeyGEN Whole Cell Lysis Assay manufacturer instructions (KeyGEN BioTECH, China). For each sample, 30ug of total protein was resolved by SDS-PAGE (10% gels) to separate adipoenctin, AdipoR1, AdipoR2, and β-actin proteins and then the proteins were transferred onto PVDF membranes (Millipore, USA) following standard procedures. The transferred protein was stained with Ponceau S to inspect membrane transfer. The membranes were then blocked for 3 hours in the mix liquid of 0.1% TBST and 5% skimmed milk powder and sequentially incubated overnight at 4°C with rabbit polyclonal anti-adiponectin (Santa Cruz Biotechnology, USA, diluted1: 250), rabbit polyclonal anti-AdipoR1 (Phoenix Pharmaceuticals, USA, diluted1:150), rabbit polyclonal anti-AdipoR2 (Phoenix Pharmaceuticals, USA, diluted 1:200), and polyclonal anti-β-actin (Sigma, USA, dilute 1:200). β-actin protein was used as aninternal control protein. After washing in 0.1% TBST, the membranes were then incubated for 1.5 hours at room temperature with mouse anti-rabbit IgG for adiponectin (diluted 1:2000) and AdipoR1 (diluted 1:1500) (Sigma, USA), goat anti-rabbit IgG for AdipoR2 (diluted 1:500) and for β-actin (diluted1:5000) (Santa Cruz Biotechnology, USA). Protein bands were visualized with BeyoECL Plus (Byeotime Biotechnology, China) after washing in 0.1% TBST. The band intensities were analyzed by the software of Image-Pro Plus 6.0 for windows. The results were presented as the ratio of target protein to β-actin protein.

Statistical analyses: All data was analyzed by T-test and presented as means ± SD. The statistical software SSPS18.0 was used to perform statistical analysis. It was considered statistically significant when the values of $P< 0.05$.

RESULTS

The expression of *adiponectin*, *AdipoR1* and *AdipoR2* mRNAs: The results of qRT-PCR revealed that *adiponectin* and its receptors (*AdipoR1* and *AdipoR2*) were all expressed in sexually immature and sexually mature sheep testes. There was no significant difference (Fig.1) in the expression of *adiponectin* mRNA in sexually immature and sexually mature testes. However, the expression of *AdipoR1* and *AdipoR2* mRNAs in sexually mature testes were significantly higher than sexually immature testes (Fig.1). The amplification fragments of *adiponectin*, *AdipoR1*, *AdipoR2*, and *β-actin* were shown in Figure 2.

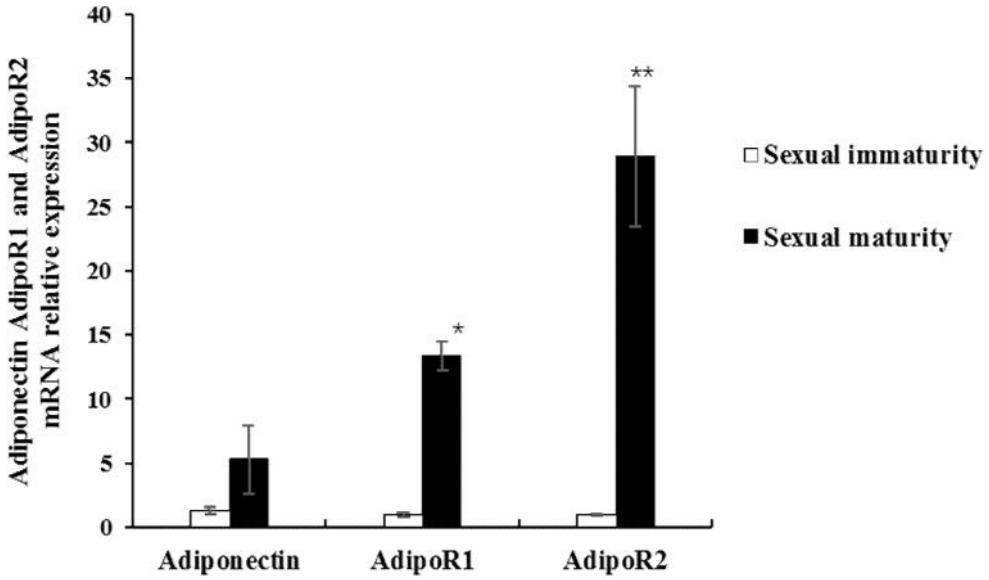

Figure 1. The mRNA expressions of *adiponectin*, *AdipoR1* and *AdipoR2* in sexually immature and sexually mature sheep testes. The results were presented as mean ± SD (n=3). Bars with different superscripts showed the significant difference. $^{*}p < 0.05$; $^{**}p < 0.01$.

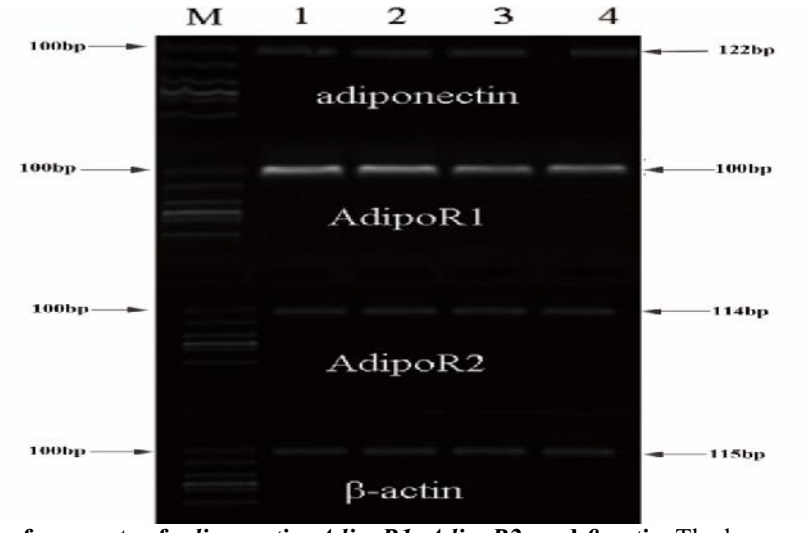

Figure 2. Amplification fragments of *adiponectin*, *AdipoR1*, *AdipoR2*, and *β-actin*. The lanes were as followed: M: DNA Marker (DL2000: 100bp, 250bp, 500bp, 750bp, 1000bp and 2000bp); 1, 2: sexually immature sheep testes; 3, 4: sexually mature sheep testes.

The expression of adiponectin, AdipoR1 and AdipoR2 proteins: The expression of adiponectin, AdipoR1 and AdipoR2 proteins in sexually immature and sexually mature testes were all significantly different (Fig.3). In sexually mature testes, there was about 5-times, 1-times and 2-times increase in the expression of adiponectin, AdipoR1 and AdipoR2 proteins than sexually immature testes, respectively. The expression of AdipoR2 protein is similar to AdipoR1 protein.

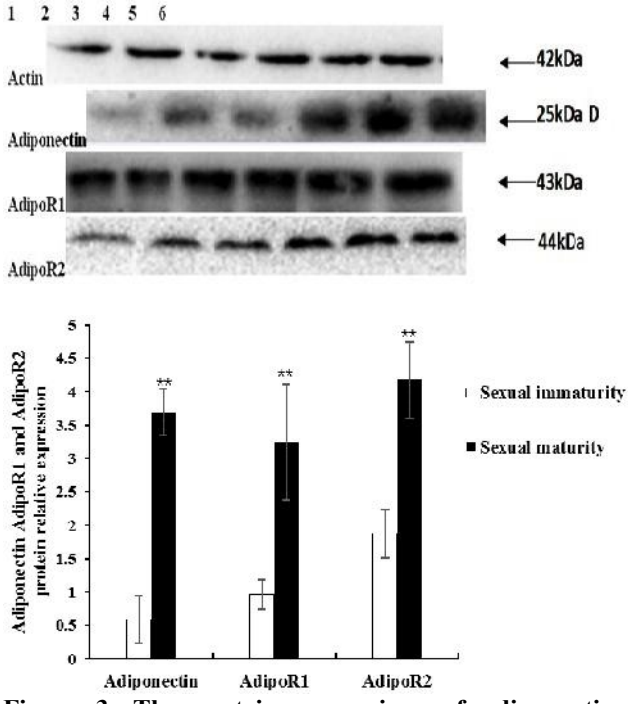

Figure 3. The protein expressions of adiponectin, AdipoR1 and AdipoR2 in sexually immature and sexually mature sheep testes.

Upper panels: representative immunoblots. 1,2,3 represent sexually immature testes; 4,5,6 represent sexually mature testes. lower panels: The relative expression of adiponectin, AdipoR1 and AdipoR2 proteins, respectively. The data was presented as mean ± SD (n=3). Bars with different superscripts indicate significant difference. * $p < 0.05$, ** $p < 0.01$.

DISCUSSION

This study is the first time to demonstrate the mRNA and protein expression of adiponectin and its receptors (AdipoR1 and AdipoR2) in sheep testes. In addition, it is also the first time to compare the expression variations of adiponectin, AdipoR1 and AdipoR2 in sexually immature and sexually mature sheep testes. The results shown that the expression of *AdipoR1* and *AdipoR2* mRNAs in sexually mature testes were obviously higher than sexually immature testes ($p<0.05$ and $p<0.01$), which is similar to the expression trends of AdipoR1 and AdipoR2 proteins ($p<0.01$ and $p<0.05$). Interestingly, the results shown that there was no significant difference in the expression of *adiponectin* mRNA ($p>0.05$) between sexually immature and sexually mature testes, but the expression of adiponectin protein in sexually mature testes was about 5-times higher than sexually immature testes ($p<0.01$). It demonstrated that the large variations in mRNA and corresponding protein expression. The Central Dogma holds that "DNA makes RNA makes proteins", the variations between protein expression and gene transcript maybe related to transcriptional and post-transcriptional regulation (Gry *et al.* 2009). At the same time, the difference in mRNA and protein stability and negative feedback regulation (high protein concentration suppresses mRNA expression) may

be also the reasons for this result (Smolinska *et al.* 2014). Compared with AdipoR1, the mRNA and protein expressions of AdipoR2 in sexually immature and sexually mature sheep testes were more abundant, which could be thought that the physiological role of AdipoR2 is more important in reproductive system than AdipoR1. It is verified by the previous research that testes weight of *AdipoR2*-knocked mice are lighter than wild-type mice and it is accompanied by atrophy of the seminiferous tubules, but there was no significant change in *AdipoR1*-knocked mice (Bjursell *et al.* 2007). The results of previous research that *Adiponectin*, *AdipoR1* and *AdipoR2* mRNA were expressed in chicken, rat and ram testes were similar to our results. Adiponectin and its receptors were expressed in different testicular cells, such as Leydig cells, Sertoli cells, spermatids and spermatozoa, which suggested that adiponectin possibly, regulated testicular function through endocrine and/or paracrine way.

Researchers have confirmed that adiponectin and its receptors were involved in regulating steroidogenesis (Paschke *et al.* 2010). The ability of adiponectin regulated steroidogenesis has been identified in different tissues, such as testis, ovary, placenta, granulosa cells and adrenal glands (Chabrolle*et al.*2007 a, b; Caminos *et al.*2005, 2008; Li and Sun 2009). Adiponectin immunoreactivity was mainly located at the cytoplasm of Leydig cells and weak to negligible immunostaining in germ cells of rat (Caminos *et al.* 2008). Testosterone, a steroid hormone, is mainly synthesized and secreted by Leydig cells, it can be inferred that adiponectin and its receptors may participate in regulating testosterone synthesis in Leydig cells to regulate testis development and spermatogenesis. In addition, previous study provided evidences that adiponectin promoted cell proliferation and stimulated cell growth by acting with AdipoR1 and AdipoR2 to activate Ras-ERK1/2 signal pathway (Lee *et al.* 2008). Adiponectin, AdipoR1 and AdipoR2 were all expressed in germ cells of testis, epididymis (caput, corpus, and cauda), vesicular and bulbourethral glands (Rahmanifar and Tabandeh 2012), they were also expressed before and after sperm capacitation (Kasimanickam *et al.*2013), which suggested that adiponectin might have a role in spermatogenesis and sperm capacitation. A recent study verified that adiponectin and its receptors were contributed to maintain ram sperm motility (Kadivar *et al.* 2016). Therefore, it can be speculated that adiponectin and its receptors potentially influence spermatogenesis and sperm motility by activating different signal pathways. Protein as direct executor of biological function, the change of the protein expression influences on life activities. In this study, the expression of adiponectin, AdipoR1 and AdipoR2 proteins in sexually mature testes increased 5-times, 1-times and 2-times than sexually immature testes, respectively. It can be thought that adiponectin and its receptors could promote sheep testes maturation

Taken together, these results indicate that adiponectin and its receptors could promote sheep testes development and spermatogenesis. This study will provide novel evidences for the physiological role of adiponectin and its receptors on testes development. However, the molecular mechanism of adiponectin and its receptors in testes development and spermatogenesis is not clear. Therefore, further studies should aim at elucidating the molecular mechanism of adiponectin and its receptors in testes to reveal their physiological role in male reproductive.

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Competing financial interests: We declare that there is no conflict of interest.

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