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Original Article

Association of Atorvastatin with GDF9 and BMP15 Expression and in vitro Maturation of Mouse Oocytes

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Abstract

Atorvastatin (ATV) is one of the hyperlipidemia treatment drugs with antioxidant and anti-inflammatory effects, growth differentiation factor 9 (GDF-9) and bone morphogenetic protein 15 (BMP-15) are two potent regulator factors of ovulation**.** This study investigated the effect of atorvastatin on GDF-9 and BMP-15 expression and explores its relationship with oocyte maturation in mice. A total of 200 oocytes from 40 mice were collected and divided into two groups (n=100 in each group), including the control group and the atorvastatin group (receiving 2 mg/kg atorvastatin). After oocyte culture in DMEM medium, GDF-9, and BMP-15 expression were evaluated by Real-time PCR. The rate of oocyte maturation was evaluated using fluorescent microscope scanning. Data were analyzed using an ANOVA test. GDF-9 and BMP-15 expression in control and atorvastatin groups were 2.3 and 4.8 ($P \leq 0.05$) and 1.2 and 3.4 fold respectively ($P \leq 0.001$). The oocyte maturation rate in the atorvastatin and control groups was 82% and 76%, respectively ($p > 0.05$). According to our findings, low-dose atorvastatin caused overexpression of GDF-9 and BMP-15 in mice oocytes and it could improve the quality and maturation of mouse oocytes.

Keywords: Atorvastatin; GDF-9; BMP-15; Oocyte.

1. Introduction

Statins are commonly used in hypercholesterolemia treatment [1, 2]. Atorvastatin (AT) is the most commonly used statin, AT is an effective inhibitor of 3-hydroxy-3-methyl-glutarylcoenzyme A (HMG Co-A) in the cell and reduces cholesterol as well as dolichol and coenzyme Q10 which is associated with low-density lipoprotein (LDL) cholesterol reduction [3, 4]. Also, statins have antioxidant effects and could improve the oocyte quality and accelerate the polycystic

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ovary syndrome (PCOS) treatment in women with poor ovarian response (POR) [5]. Growth differentiation factor-9 (GDF-9) and bone morphogenetic protein-15 (BMP-15), are two members of the transforming growth factor β (TGF-β) superfamily, they can increase the proliferation and metabolism rate of the granulosa cells and stimulate the expression of kit ligand (KL) in them. The granulosa cells have essential functions in oocyte maturation they provide nutrients, steroid hormones, and growth factors which are essential factors in the oocyte maturation process [6, 7].

BMP-15 and GDF-9 have similar structures and functions (**Figure 1**) and are critical regulators of ovarian function, they are secreted by oocytes through autocrine and paracrine mechanisms and are essential for ovarian differentiation, follicle growth, and granulosa cell proliferation [8, 9].

GDF-9 and BMP-15 have a high degree of amino acid similarity, they are similar in protein structure, and both are closely related to each other expression and function in the ovary [10, 11]. Recent evidence has shown a synergistic relationship between GDF-9 and BMP-15, GDF-9/BMP-15 heterodimers termed cumulin, and acts as a potent regulator of oocyte quality [12].

GDF-9 and BMP-15 induce the expression of the anti-mullerian hormone (AMH) and play an essential role in ovarian functions like folliculogenesis and oogenesis during an ovarian cycle [13]. Recent studies reported that GDF-9 is essential for oocyte maturation in the mouse model, while it seems that BMP-15 has a lesser role in oocyte maturation [14]. GDF-9 and BMP-15 treatment can increase the quality and maturity of in vitro maturated oocytes [15].

Considering the antioxidant effects of atorvastatin and its function in oocyte maturation and the function of GDF-9 and BMP-15 as two essential proteins in oocyte maturation, in this study we decided to investigate the relationship between atorvastatin treatment and GDF-9, BMP-15 expression in mice oocytes in-vitro maturation.

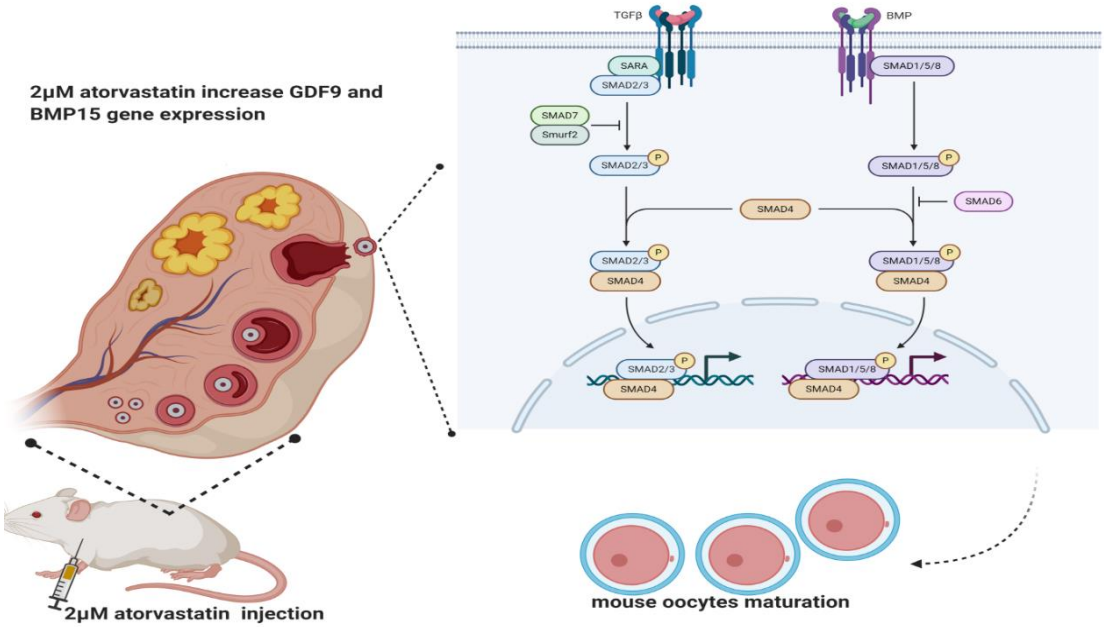

Figure 1. BMP and GDF-9 intracellular signaling pathway and oocyte maturation in mouse.

2. Materials and Methods

2.1. Animals and sample preparation

In this study, 40 Naval Medical Research Institute (NMRI) female mice, aged 4-5 weeks, were kept in the laboratory animal care center of Qazvin University of Medical Sciences. For ovulation stimulation, 7.5 permanent magnet synchronous generator (PMSG) units were used as an intraperitoneal injection for each mouse, Twenty-four hours after the PMSG injection, the mice were euthanized, and the ovaries were collected and immediately cultured in α- minimum essential medium (α-MEM). A total of 200 collected oocytes were divided into two groups including the control group: culture medium + growth factor, Atorvastatin group: culture medium + growth factor + 2μM atorvastatin, the oocytes of two groups for 24 hours cultured in α-MEM.

2.2. RNA extraction and cDNA synthesis

RNA was extracted using the RNeasy Mini Kit (Qiagen) according to the manufactory protocol. The purity and concentration of collected RNA were evaluated with Nanodrop-2000 (Thermo Fisher Scientific). The Quanti Nova Reverse Transcription Kit (Qiagen, Germany) was used to reversely transcribe the RNA, the cDNA was finally stored at -20 temperature.

2.3. Real-Time PCR

Primer design was performed using oligo7 and primer3 software (**Table 1**). The real-time PCR reaction was performed based on the Qiagen protocol. The PCR reaction was performed as follows: initial denaturation and activation of the polymerase for 10 min at 95°C, followed by 30 cycles of 30 sec. at 95°C, 30 sec. at 59°C, and 60 sec. at 72°C. The final extension was for 10 min at 72°C. β-actin was used as an internal control. The mRNA levels of the two target genes were calculated with the ΔΔCT method and evaluated as fold change relative to the β-actin internal control.

2.4. Oocytes Maturation

The oocytes maturation evaluation was performed by imaging using an inverted microscope (XDS-2ITALY), for this aim the oocytes that completed the first meiosis and showed the first mature polar body were counted the counted data of the two groups were analyzed using image j software.

Table 1: Sequences of GDF-9 and BMP-15 specific primers used in this study for qRT-PCR analysis.

Gene	Primer sequence	Direction
GDF-9	5'-CAACCA GGTGACAGGACC-3' 5'-AGGCAGAGTT GTTCAGAGTG-3'	Forward Reverse
BMP-15	5'-AGCCAAGAGGTAGTGAGGTT-3' 5'-TGCAATACTGCCTGCTTGAC-3'	Forward Reverse
β-actin	5'-TCGTGGGCCGCTCTAGGCAC-3' 5'-TGGCCTTAGGGTTCAGGGGG-3'	Forward Reverse

3. Results and Discussion

3.1. GDF-9 and BMP-15 expression

The GDF-9 gene expression was 2.35±0.1 and 4.84±0.3 fold in the control and atorvastatin groups respectively, in the atorvastatin group it was significantly increased compared to the control group (P≤0.05) (**Figure 2**). BMP-15 gene expression was 1.07±0.2 and 3.2±0.3 fold in the control and atorvastatin groups respectively, it was significantly increased in the atorvastatin group compared to the control group (P≤0.001).

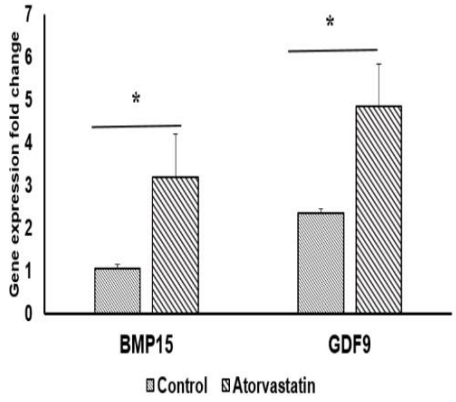

Figure 2. Expression of BMP-15 and GDF-9 genes in control and atorvastatin treatment groups*, P≤0.05.

3.2. Maturation rate of oocytes

After 24 hours of oocytes cultured in α-MEM, 77% and 82% of total oocytes were fully matured in the control group and the atorvastatin groups (culture medium+growth factor+2μM atorvastatin) respectively (**Figure 3**), the maturation of oocytes was increased about 5% in the atorvastatin group compared to the control group, but the difference of two groups was not statistically significant (p≥ 0.05) (**Figure 3**).

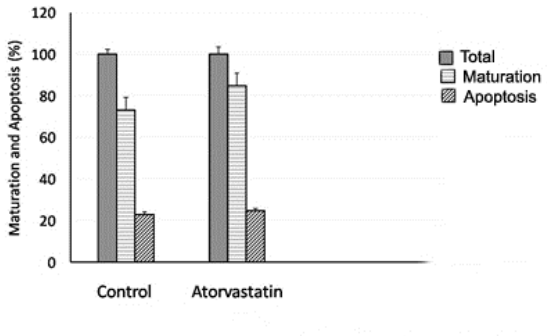

Figure 3. Oocyte maturation and apoptosis rate in control and atorvastatin groups.

This study investigated the relationship between atorvastatin treatment and GDF-9 and BMP-15 expression and the rate of oocyte maturation in mice. The results showed that 2μM atorvastatin treatment of immature mouse oocytes will increase the GDF-9 and BMP-15 gene expression in oocytes. This increased expression was related to the in-vitro maturation of mouse oocytes. There are some previous reports about the function of GDF-9 and BMP-15 in oocyte quality and fertility rate in mice, sheep, pigs, and humans [16, 17].

Matzuk et al. reported that GDF-9 knockout female mice did not have the natural differentiation of follicles and lacked ovulation and pregnancy (18]. In humans, mutations in GDF-9 and BMP-15 are mainly associated with infertility, ovulation defects, and defect in ovarian maturation [19, 20]. Ramirez et al. reported that the expression of mRNA transcripts of these genes was related to the maturation of dog oocytes, which findings are in line with our findings in mouse oocytes [21]. It was reported that GDF-9 and BMP-15 gene expression reduced in vitro-matured oocytes compared to intra-ovarian oocytes [22]; these results indicate a direct correlation between the expression of these

genes and oocyte maturation parameters. In another study, Parlacomes et al. showed that 10 mg/kg atorvastatin concentration increased the expression of AMH and vascular endothelial growth factor (VEGF) in the early follicles and increased the maturity and quality of mouse oocytes [23] These findings were similar to our result in mouse oocytes maturation at 2 mg/kg atorvastatin concentration.

GDF-9 and BMP-15 pathways are active during oocyte maturation, and the driving mechanisms for oocyte nuclear maturation involve GDF-9 and BMP-15 homodimers [24]. Gong et al. reported that for poor ovarian responders, in particular, those over 40, the expression of GDF-9 and BMP-15 declined along with increased age and it is related to poorer oocyte quality and in-vitro fertilization (IVF) outcome [25]. Based on the findings of previous studies and our findings, the high level of expression of GDF-9 and BMP-15 may effective on the maturation and quality of eggs, and as was reported in Gong et al.'s study the decreased amount of these two proteins with age will reduce the quality of oocytes and their fertility rate. In this study for the first time, we determined that a low dose of atorvastatin could increase the expression of GDF-9 and BMP-15 and improve the quality and maturation of in vitro mouse oocytes, it seems that atorvastatin can prevent the decrease in the expression of GDF-9 and BMP-15 in people over 40 years old and help maintain the quality and maturation of oocytes in old age.

4. Conclusion

A low dose of atorvastatin could increase the expression of GDF-9 and BMP-15 and thus lead to an increase in the quality and maturation of oocytes in the in vitro environment, and it seems that a low dose of atorvastatin can be used to increase the efficiency of IVF.

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Conflict of interest

The authors declare to have no conflict of interest.

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