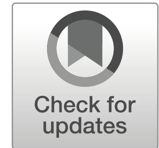
Reproductive Sciences (2020) 27:681–689
https://doi.org/10.1007/s43032-019-00074-9

ORIGINAL ARTICLE



Role of Visfatin in Restoration of Ovarian Aging and Fertility in the Mouse Aged 18 Months

Byung-Kyoo Park[1] · Min Jung Park[2] · Hwi Gon Kim[1] · Si Eun Han[1] · Chang-Woon Kim[3] · Bo Sun Joo[2,4] · Kyu-Sup Lee[1]

Received: 3 April 2019 / Accepted: 26 July 2019 / Published online: 15 January 2020
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Abstract

The activation of dormant primordial follicles and ovarian angiogenesis has been attempted as a new treatment strategy for age-related ovarian aging. This study examined whether visfatin rescues age-related fertility decline in female mice aged 18 months, and whether this effect relates to the mTOR/PI3K signaling pathways for activation of primordial follicles and ovarian angiogenesis. Female mice were intraperitoneally injected with 0.1 ml of 500 ng/ml or 1000 ng/ml of visfatin three times at intervals of 2 days, and both ovaries were provided for H&E staining. In another experiment, the mice were superovulated with pregnant mare's serum gonadotropin and human chorionic gonadotropin, and were mated with males. After 18 h, zygotes were collected and cultured for 4 days, and numbers and embryo developmental competency of zygotes retrieved were evaluated. The expression of mTOR/PI3K signaling pathway regulated genes (4EBP1, S6K1, and RPS6) and angiogenic factors (VEGF, visfatin, and SDF-1α) in the ovary were examined. As well, visfatin-treated mice were mated with male mice for 2 weeks, and the pregnancy outcome was monitored up to 3 weeks. Visfatin significantly increased the total numbers of follicles compared with control. Numbers of zygotes retrieved, blastocyst formation rate, and pregnancy rate were significantly increased at 500 ng/ml of visfatin (2.83%, 40.0%, and 80%, respectively) compared with control (0, 0, and no pregnancy). Ovarian expressions of S6K1, RPS6, VEGF, visfatin, and SDF-1α were significantly stimulated at 500 ng/ml of visfatin. These results show that visfatin treatment of an optimal dose rescues age-related decline in fertility, possibly by stimulating mTOR/PI3K signaling.

Keywords Visfatin · Ovarian aging · Fertility · Primordial follicle activation · mTOR/PI3K

Introduction

Female fertility decreases with advancing age, and ovarian aging has been suggested as a major cause of age-related decline in female fertility because it decreases oocyte quality and greatly reduces the total number of ovarian follicles [1]. There is a consensus that the decrease in oocyte quality rather than endometrial receptivity is a primary cause of ovarian aging [2, 3].

Byung-Kyoo Park, Min Jung Park, Bo Sun Joo and Kyu-Sup Lee contributed equally to this work.

✉ Bo Sun Joo
bosunjoo@hanmail.net

✉ Kyu-Sup Lee
kuslee@pusan.ac.kr

Byung-Kyoo Park
pimod@naver.com

Min Jung Park
ohnsa@naver.com

Hwi Gon Kim
bislsan@gmail.com

Si Eun Han
hansieun@gmail.com

Chang-Woon Kim
kcwoon@naver.com

1 Department of Obstetrics and Gynecology, Pusan National University School of Medicine, Gudeok-Ro 187, Seo-Gu, Busan 49241, Republic of Korea

2 The Korea Institute for Public Sperm Bank, Busan, Republic of Korea

3 Department of Obstetrics and Gynecology, Samsung Changwon Hospital, Sungkyunkwan University School of Medicine, Changwon, Republic of Korea

4 Infertility Institute, Pohang Women's Hospital, Posco-daero 269, Buk-Ku, Pohang, Gyeongsangbuk-do 37754, Republic of Korea

The decrease in oocyte quality has been reported mainly due to dysfunction of mitochondria and chromosomal abnormalities of oocytes, and defective ovarian angiogenesis [4–8], but the precise mechanism is not clear yet. As a result, there is no a practically effective treatment to improve oocyte quality.

Recently, a lot of studies have been conducted to improve the quality of oocytes by activating the early follicular development or ovarian microenvironment. One of them is the activation of ovarian angiogenesis during follicular development [9–12]. Ovarian angiogenesis plays a crucial role in a series of events of folliculogenesis, such as follicular growth and the selection of dominant follicle [13, 14].

The other research area for improving oocyte quality is the activation of primordial follicles. Primordial follicles are dormant in ovaries, and the pool of primordial follicles continues to provide developing follicles and fertilizable oocytes for the entire reproductive lifespan [15]. Dysfunctions of primordial follicles activation are a cause of premature ovarian failure (POF) and female infertility. Therefore, the regulation of primordial follicle activation is very important in the maintenance of female reproductive potential. The dormant primordial follicles are activated by various intracellular signaling pathways and intraovarian factors, such as vascular endothelial growth factors (VEGF), bone morphogenetic protein (BMP) series, and leukemia inhibitory factor [16, 17]. In this reason, studies on improving oocyte quality through the activation of dormant primordial follicles are becoming a hot issue as a new treatment strategy for ovarian aging, poor response, and POF [18–22].

Phosphatidylinositol 3-kinase (PI3K) signaling plays regulatory roles not only in a variety of physiological and pathological processes including cell proliferation, survival, migration, and metabolism, but also in oogenesis, folliculogenesis, ovulation, and carcinogenesis in mouse ovary [23]. Mechanistic target of rapamycin (mTOR) belongs to the PI3K-related kinase family, and recent several studies have strongly shown that the mTOR and PI3K signaling pathways play a critical role in the activation and survival of primordial follicles [24]. Indeed, the treatment with PI3K activators stimulated murine and human dormant primordial follicles [25, 26].

Visfatin is originally known to be a pre-B cell colony-enhancing factor, but nowadays it is more famous as an adipokine released by adipose tissues [27]. It is also expressed in a variety of cells including amniotic cells, macrophage, and human granulosa cells [28–30]. In addition, it stimulates angiogenesis by influencing the proliferation, migration, and invasion of vascular endothelial cells [31, 32]. It does not only induces the production of VEFG, representative angiogenic factor, in human endothelial cells via MAPK and PI3K/Akt signaling pathways, but also exerts angiogenic effects on human umbilical vein endothelial cells through the mTOR signaling pathway [33, 34]. Our previous study reported that administration of visfatin during superovulation improves developmental competency of oocytes and fertility potential in female mice aged around 30 weeks [12]. In these reason, this study investigated whether visfatin rescues fertility potential in very old female mice aged 18 months, and whether this effect relates to the mTOR/PI3K signaling pathways for activation of primordial follicles and ovarian angiogenesis.

Methods

This study was approved by the Institutional Review Board of Pusan National University Hospital, Korea. All animal experiments were conducted under the guidance for the Care and Use of Laboratory Animals of the National Institutes of Health, approved by the Pusan National University Hospital Institutional Animal Care and Use Committee.

Animals

We chose an age of 18 months, which corresponds to the early 70s of humans (http://www.age-converter.com/mouse-age-calculator.html; [36]). An 18-month-old C57BL/6 mice were obtained from the Korea Institute of Toxicology (Jinju, Gyeongsangnam-do, Korea) and housed in an animal facility under 12 h of light, dark cycle with food and water available ad libitum.

Treatment of Visfatin

Recombinant mouse visfatin (Adipogen Life Science, San Diego, CA, USA) was intraperitoneally injected into female mice aged 18 months at a concentration of 500 and 1000 ng/mL three times at intervals of 2 days. The control group was not treated with visfatin.

The dose and time of visfatin treatment was determined based on our preliminary experiments. In our previous study using mice aged 26 to 31 weeks [12], visfatin had the best effect at 500 ng/ml among the treated concentrations of 5, 10, 100, and 500 ng/ml. So the present study initially treated 500 ng/ml and 1000 ng/ml of visfatin once. However, there was no effect (data not shown). Then, this study treated the same concentration of visfatin three times at intervals of 2 days considering advanced female age.

Histological Hematoxylin and Eosin Staining and Ovary Follicle Counting

In the first experiment, 12 female mice aged 18 months were randomly divided into three groups ($n = 4$ per group), i.e., one control group and two visfatin treatment groups. After visfatin treatment, both ovaries were isolated and fixed in 4% paraformaldehyde at 4 °C overnight, and dehydrated using ethanol series, cleared in xylene, embedded in paraffin, and sectioned

for hematoxylin and eosin (H&E) staining. After mounting, sections were analyzed histologically under a light microscope. Follicles were counted in all sections from each ovary, and results were corrected for double counting. Follicles were classified into primordial follicle (an oocyte surrounded by one layer of flattened granulosa cells), primary follicle (an oocyte surrounded by one layer of cuboidal granulosa cells), secondary follicle (two or three layers of cuboidal granulosa cells with no antral space), antral follicle, and mature follicle. Follicles containing degenerated oocytes were deemed atresia based on the presence of apoptotic bodies in the granulosa cell layer, disorganized granulosa cells, or a degenerating oocyte.

Superovulation, Zygotes Collection, and Embryo Culture

In the second experiment, twelve female mice aged 18 months were intraperitoneally injected with 500 ng/ml (n = 6) and 1000 ng/mL (n = 6) of visfatin. Then, these mice superovulated by intraperitoneal injection with 0.1 mL of 5 IU pregnant mare's serum gonadotropin (PMSG; Sigma, St. Louis, MO, USA) followed by injection of 5 IU of human chorionic gonadotropin (hCG; Sigma) approximately 48 h later. Then, the mice were immediately paired with an 8- to 12-week-old individual male. The following morning, the mice were inspected, and those with a confirmed vaginal plug were considered fertilized. The control group (n = 5) was superovulated with PMSG and hCG without visfatin.

18 h after hCG injection, female mice with a confirmed vaginal plug were killed by cervical dislocation, and zygotes were retrieved from the oviductal ampulae, and only healthy zygotes were cultured in 20-μL drops of Gl-plus medium (Vitrolife, Göteborg, Sweden) with 10% SSS for the first 2 days, and then G2-plus medium (Vitrolife) with 10% SSS for the latter 2 days at 37 °C, 5% CO_2 incubator.

RNA Extraction and Quantitative Real-Time PCR

To understand whether the effect of visfatin relates with the activation of primordial follicle and angiogenesis, we examined ovarian mRNA expression of downstream molecules of the mTOR/PI3K signaling pathway (4EBP1 (eukaryotic initiation factor 4E binding protein 1), S6K1 (ribosomal protein S6 kinase beta-1), and RPS6 (ribosomal protein S6)) and angiogenic regulatory molecules (VEGF, visfatin, and SDF-1α (stromal cell derived factor-1α)). Just after the retrieval of the zygotes, both ovaries of each mouse were collected, and mRNA expressions of these molecules were measured by quantitative real-time polymerase chain reaction (PCR). Total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA, USA). Complementary DNA was synthesized from 1 μg of total RNA using AMV Reverse Transcriptase (Promega, Madison, WI, USA) and a random hexamer (Takara Bio, Inc., Otsu, Japan) at 42 °C for 1 h followed by inactivation of the enzyme at 95 °C for 5 min. Real-time PCR was performed using TOPreal™ qPCR 2X PreMIX SYBR (Enzynomics, Daejeon, Korea). Reaction mixtures were prepared using TOPreal™ qPCR 2X PreMIX, 0.5 pmol/μl of each primer, 100 ng of cDNA, and sterile water (RNase-free). The reaction conditions consisted of denaturation at 95 °C for 10 min, followed by 30 cycles of 95 °C for 10 s, 60 °C for 30 s. Each cDNA was subjected to PCR amplification using gene-specific primers (Table 1).

Protein Expression of 4EBP1, S6K1, and RPS6 in Ovarian Tissues

Total proteins were extracted from ovaries with RIPA lysis buffer (ProEX™ CETi lysis buffer; TransLab, Seoul, South Korea) according to the manufacturer's instructions. After lysis, total protein concentrations were measured using Pierce BCA Protein Assay Kit. The tissue lysates (30 μg protein/lane) was subjected to 10% SDS-PAGE gel, separated by

Table 1 Primers sequences used for real-time PCR amplification

Gene	Sequence (5' → 3')	
	Forward	Reverse
4EBP1	CTAGCCCTACCAGCGATGA G	CCTGGTATGAGGCCTGAATG
S6K1	GTAGTCCACGAACACCTGTC	TGAGGATTTGCCGTGCTGG
RPS6	GGACGCTAATCTCAGTGTTCTC	CCTGGGCTTCTTACCTTCTTTG
VEGF165	AGGCTGCTGTAACGATGAAG	GTCTGCATTCACATCTGCTG
Visfatin	CTTGTTCAGTCCTGGTATCC	GCGAAGAGACTCCTCTGTAA
SDF-1α	GTCTAAGCAGCGATGGGTTC	GAATAAGAAAGCACACGCTGC
GAPDH	TCAACGGCACAGTCAAGGC	CTCCACGACATACTCAGCAC

4EBP1, eukaryotic initiation factor 4E (eIF4E) binding protein 1; S6K1, ribosomal protein S6 kinase beta-1; RPS6, ribosomal protein S6; VEGF, vascular endothelial growth factor; SDF-1α, stromal cell derived factor-1α; GAPDH, glyceraldehyde 3-phosphate dehydrogenase

electrophoresis, and transferred to a PVDF membrane. The membranes were blocked with the ProNA™ General-block solution (Translab, Seoul, South Korea) for 1 h and incubated with diluted primary antibody solution overnight at 4 °C. After washing, the membranes were incubated with horseradish peroxidase (HRP)–conjugated monoclonal goat anti-rabbit IgG (TransGen Biotech, Beijing, China) for 1 hour at room temperature. Signals were detected using ProNA™ ECL Ottimo Western Blot Detection Kit (Translab, Seoul, South Korea). The following antibodies were purchased from cell signaling and used at the indicated dilution for western blot analysis—S6K1 (#2708, 1:2000), RPS6 (#2217, 1:5000), 4E-BP1 (#9644, 1:2500).

Mating of Visfatin-Administered Female Mice and Monitoring for Pregnancy Outcome

In the third experiment, a total of 14 female mice were treated with or without visfatin, 500 ng/ml ($n = 5$), 1000 ng/ml ($n = 5$), and control ($n = 4$). Then, the mice were immediately paired with an individual male in individual cages for 2 weeks. Each of the breeder males had one to two successful pregnancies before mating. After 2 weeks, the male mice were removed, and pregnancies were monitored for an additional 1 week. On 21 days after, all female mice were dissected, and the pregnancy was confirmed by the presence of the fetuses.

Statistical Analysis

An SPSS program (ver. 12.0) was used for statistical analysis. All comparisons of numbers of follicles at each developmental stage, the number of zygotes retrieved, blastocyst formation rate, ovarian mRNA and protein expression level, and numbers of fetuses were analyzed by Student's t test because comparisons were only between the tested dose and the control. A P value of $< .05$ was considered statistically significant.

Results

Effect of Visfatin on Ovarian Function, Ovarian Response, and Oocyte Quality

To investigate whether administration of visfatin improve ovarian function in aged female mice of 18-month-old, H&E staining was performed in ovaries. The histological characteristics of ovaries in each treatment group were shown in Fig. 1a. The total number of follicles was significantly increased in visfatin treatment (58 ± 0.5 at 500 ng/ml and 60 ± 0.6 at 1000 ng/ml) compared with the control (36 ± 0.5), with the increase rate of 61.1% and 66.7%, respectively. Of these, the number of surviving follicles including primordial, primary, secondary, and antral follicles was increased more than two times in visfatin treatment (48.0 at 500 ng/ml and 44.0 at 1000 ng/ml) compared with the control (21.0). In contrast, the number of atretic follicles was decreased more than half in visfatin treatment (6.0 at 500 ng/ml and 7.0 at 1000 ng/ml) compared with the control (13.0) (Fig. 1b). In detail, mean (±) number of primary, secondary, and antral follicles were 14.0(± 1.5), 6.0(± 1.0), and 1.0(± 0.6), respectively, in the control; 26.0(± 1.5), 11.0(± 0.6), and 6.0(± 0.5) in 500 ng/ml visfatin; and 26.0(± 2.1), 10.0(± 0.6), and 4.0(± 0.6) in 1000 ng/ml visfatin (Fig. 1c).

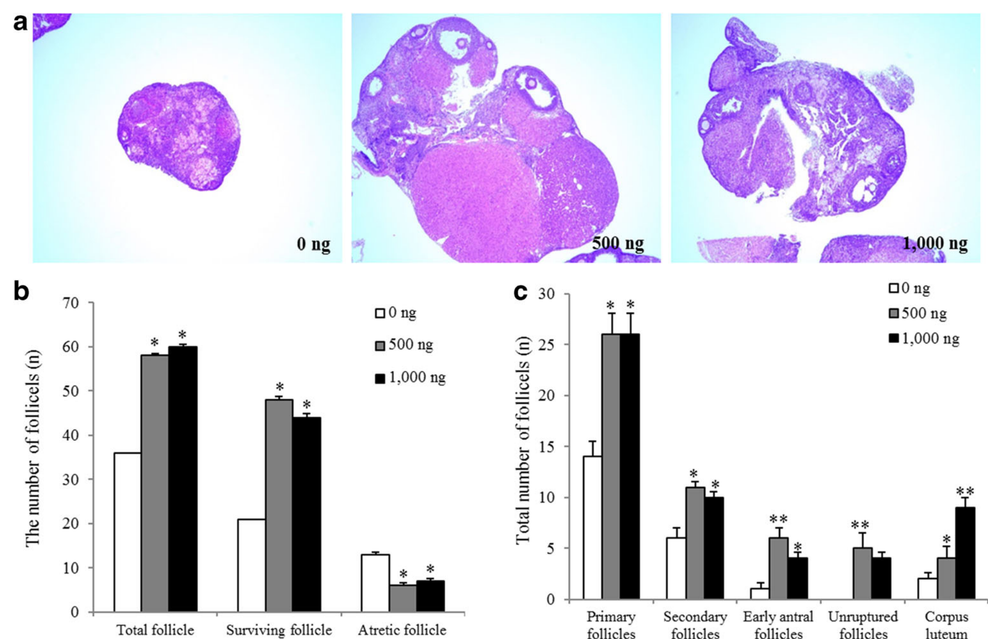

Fig. 1 Effect of visfatin on follicular development. Whole ovaries were collected the next day after three times visfatin treatments and provided in the histological analysis by H&E staining. **a** The histological characteristics of follicles at each development stage. **b** Comparison of the total number of follicles, surviving follicles, and atretic follicles. **c** The distribution of follicles at different stages. *$P < .05$, **$P < .01$ (vs control)

Table 2 Effect of visfatin treatment on the number and embryo development of zygotes retrieved

Visfatin (ng/ml)	No. of mice provided	No. of zygotes retrieved	No. of zygotes retrieved/mouse	No. of zygotes fragmented (%, mean ± SD)	No. of zygotes cultured (mean ± SD)	No. of 2-cell embryos (%, mean ± SD)	No. of blastocyst (%, mean ± SD)
0	5	1	0.20 ± 0.45	0	1	0	0
500	6	17	2.83 ± 1.16[a]	2 (11.7, 0.33 ± 0.50)	15 (2.50 ± 1.05)	10 (66.7, 1.67 ± 0.83)[a]	6 (40.0, 1.00 ± 0.63)[a]
1000	6	11	1.83 ± 1.16[a]	3 (27.3, 0.52 ± 0.83)	8 (1.33 ± 0.82)	5 (62.5, 0.83 ± 0.75)[a]	2 (25.0, 0.33 ± 0.52)[a]

[a] $P < .05$ (vs controls)

In addition, visfatin significantly increased the number of zygotes retrieved and embryo development rate to blastocyst compared with the control ($P < .05$), while the fragmentation rate was lower in the control group, but not significantly different from the experimental group. This effect of visfatin was more remarkable in 500 ng/ml than in 1000 ng/ml dose. The mean number of zygotes and development rates were 0 and 0% in the control group, 2.83 and 40.0% in 500 ng/ml, and 1.83 and 25.0% in 1000 ng /ml of visfatin (Table 2).

Effect of Visfatin on Fertility Potential

Fertility potential was enhanced by visfatin treatment. In detail, of each 5 mice treated with 500 ng/ml and 1000 ng/ml of visfatin, 4 in 500 ng/ml were pregnant, and a total of 12 fetus were produced (Fig. 2a); while in 1000 ng/ml, one was pregnant, and 3 fetuses were observed (Fig. 2b). As a result, an average of 2.2 and 0.6 fetuses was obtained in the 500 ng/ml and 1000 ng/ml visfatin treatment, respectively, whereas no pregnancies were found in the control group ($n = 4$) ($P < .05$) (Table 3).

RNA Expression of 4EBP1, S6K1, RPS6, VEGF, Visfatin, and SDF-1α in Ovarian Tissues

Ovarian mRNA expressions of S6K1 and RPS6 were significantly increased in 500 ng/ml of visfatin compared with the control ($P < .05$) (Fig. 3a). Expression of angiogenic factors in ovarian tissues was also examined in order to understand whether visfatin treatment results in a change in angiogenesis in the ovary. A total of 500 ng/ml visfatin treatment significantly increased the ovarian expression of three key angiogenic factors, VEGF, visfatin, and SDF-1α, compared with the control ($P < .01$) (Fig. 3b).

Protein Expression of 4EBP1, S6K1, RPS6 in Ovaries

To further verify the involvement of the mTOR/PI3K signaling pathway after visfatin treatment, the protein levels were examined in ovaries by western blotting. Similar to the results of mRNA expression, S6K1 and RPS6 protein expression was also significantly increased in 500 ng/ml visfatin treatment compared with the control (Fig. 4).

Discussions

This study used 18-month-old female mice as natural ovarian aging model to investigate whether visfatin could restore age-related fertility decline due to ovarian aging, because an age of 18 months in mice corresponds to the late 50s and early 70s of humans [35, 36], and it is difficult to expect a successful pregnancy due to severe ovarian aging and deterioration of oocyte quality. Nevertheless, the present study clearly shows that visfatin treatment increased the number of surviving follicle at four stages from primordial to antral follicle in folliculogenesis and improved ovarian function, oocyte quality(developmental competency of oocytes), and finally fertility potential. To our knowledge, this is the first report that the administration of visfatin could recover the declining

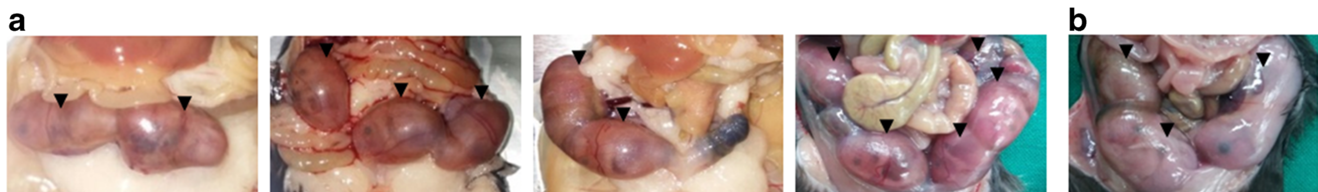

Fig. 2 The number and status of fetus on pregnant female mice after visfatin treatment. The female mice were intraperitoneally injected with visfatin of 500 ng/ml ($n = 5$) (**a**) and 1000 ng/ml ($n = 5$) (**b**) three times at intervals of 2 days. On the day after the last injection of visfatin, the mice were mated with male mice for 2 weeks. The number and status of fetus (arrow head) was examined by dissection on 21 days after mating. Control group ($n = 4$), without visfatin, were not pregnant

Table 3 Pregnancy outcome of female mice after visfatin treatment

Visfatin (ng/ml)	No. of females mated with male	No. of pregnant mice (pregnancy rate)	No. of total fetuses	No. of offspring/ female mice mated
0	4	0 (0%)	0	0
500	5	4 (80%)	12	*2.20 ± 1.30[a]
1000	5	1 (20%)	3	*0.60 ± 1.34

*Numbers of offsprings per female mice mated was presented as mean ± SD

[a] $P < .05$ (vs controls)

fertility at this age, even though it is a mouse experiment. This result suggests that the administration of visfatin could be applied as a new treatment strategy for natural ovarian aging (Table 1).

Numbers of dormant primordial follicles decrease as increased age, but primordial follicles still remain in female adult around menopause. These dormant primordial follicles are activated by various intraovarian activation mechanisms including various intracellular signaling pathways and intraovarian factors [16, 17]. In this respect, recent many studies have focused on improving ovarian aging and deteriorated oocytes quality by activating ovarian microenvironment such as the activation of primordial follicles [37]. The mTOR and PI3K signaling pathways are representative intracellular signaling pathways that have been reported to play a critical role in the activation and survival of primordial follicles [22–26]. Hence, the present study examined the number of primordial follicles and expression of mTOR/PI3K signaling pathway components (4EBP1, S6K1, and RPS6). As expected, the result shows that the number of primordial follicles and ovarian expressions of S6K1 and RPS6 increased at 500 ng/ml dose that enhanced ovarian function and fertility. This result shows for the first time that visfatin increased ovarian expression of main components of mTOR/PI3K signaling pathway. From these results, we suppose that stimulation of mTOR/PI3K signaling pathways by visfatin in aged ovary may produce good quality oocyte through the activation of primordial follicles.

The activation of ovarian angiogenesis has been studied as another way to improve oocyte quality because ovarian angiogenesis plays an important role in follicular

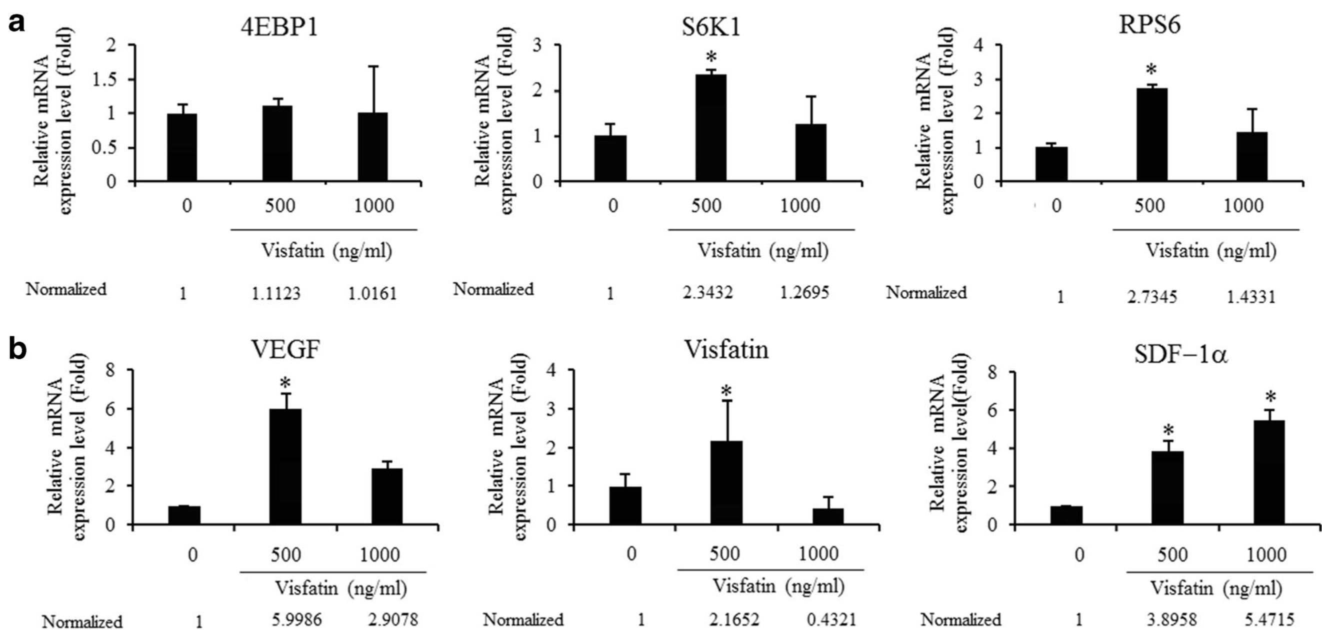

Fig. 3 Real-time PCR analysis of mTOR/PI3K signaling pathway components and angiogenic factors in the ovaries of female mice aged 18 months. The female mice were treated with visfatin, superovulated, and mated with male mice. Just after the retrieval of the zygotes, whole ovaries were collected and provided in real-time PCR analysis. **a** mRNA expression level for 4EBP1, S6K1, and RPS6 of mTOR/PI3K signaling pathway. **b** mRNA expression level for VEGF, visfatin, and SDF-1α of angiogenic factors. Each PCR was performed in duplicate on each sample. Relative gene expression levels were calculated versus GAPDH. Data were presented mean ± SD. *$P < .05$ (vs control). 4EBP1, eukaryotic initiation factor 4E (eIF4E) binding protein 1; S6K1, ribosomal protein S6 kinase beta-1; RPS6, ribosomal protein S6; VEGF, vascular endothelial growth factor; SDF-1α, stromal cell derived factor-1α

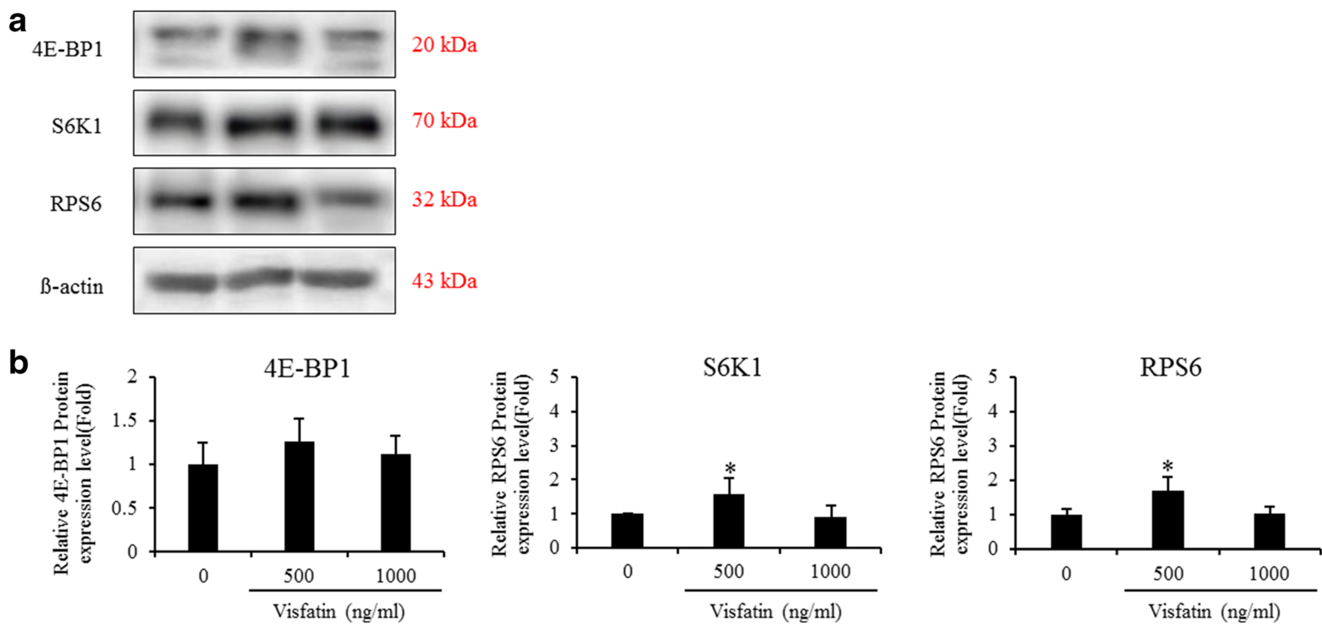

Fig. 4 Western blot analysis protein expression levels of mTOR/PI3K signaling pathway components in the ovaries of female mice aged 18 months. The female mice were treated with visfatin, superovulated, and mated with male mice. Just after the retrieval of the zygotes, whole ovaries were collected and provided in western blot analysis. **a** Representative western blot for 4EBP1, S6K1, and RPS6 protein expression in ovaries collected just after the retrieval of the zygotes. **b** Quantifications of western blot analysis for these markers. Data are mean ± SEM for three independent experiments. *$P < .05$ (vs control). 4EBP1, eukaryotic initiation factor 4E (eIF4E) binding protein 1; S6K1, ribosomal protein S6 kinase beta-1; RPS6, ribosomal protein S6

growth and the selection of dominant follicle by sullying active blood essential for the induction of oocytes with good quality [13, 14]. Several previous studies have shown that direct injection of VEGF gene fragments or VEGF into the ovary increases angiogenesis and the number of follicles to be ovulated [38–40]. Gene expression of these angiogenic factors are upregulated by visfatin in a dose-dependent manner via MAPK and PI3K/Akt signaling pathways [33]. Visfatin also has a role to exert angiogenic effects on human umbilical vein endothelial cells (HUVECs) through the mTOR signaling pathway [34]. Our previous study on mice aged 26–31 weeks showed an increase in expression of ovarian VEGF by co-treatment of visfatin during superovulation [12]. The present study also shows clearly that visfatin significantly increased ovarian expression of angiogenic factors (VEGF, SDF-1α, and visfatin) at a concentration of 500 ng/ml rather than 1000 ng/ml.

In fact, 1000 ng/ml of visfatin also slightly increased the expression of S6K1, RPS6, VEGF, and SDF-1α in a similar pattern to that of 500 ng/ml, although there was no significance. This increased gene expression may have contributed to the increase in the number of zygotes retrieved, embryo's developmental competency, and fertility potential. This result suggests that the dose of 500 ng/ml is the best optimal concentration to be effective than the dose of 1000 ng/ml.

The present study has a critical limitation that it does not know clearly how visfatin increases the fertility of aged mice. Supposedly, it seems that visfatin administered into the peritoneal cavity may enter the blood vessel and stimulate the ovary in two ways through the bloodstream. First, visfatin may stimulate the production of angiogenic factors, (VEGF, visfatin, and SDF-1α) from ovarian stromal cells and granulosa cells to activate ovarian angiogenesis. Second, visfatin may stimulate the PI3K/mTOR signaling pathway in the ovary and induce activation of the dormant primordial follicles. Therefore, it is presumed that if dormant primordial follicles are activated via the PI3K/mTOR signaling pathway, the development of the primary follicles proceeds, and development after secondary follicle may be induced by angiogenesis activated by visfatin. As a result, high quality oocytes with embryo developmental competency to blastocyst might be produced. Nevertheless, this study does not show direct evidence that the beneficial effects of visfatin on ovarian function and fertility are related to the activation of primordial follicles and ovarian angiogenesis. Therefore, additional research should be done to elucidate this direct relationship using follicle and/or granulosa cell cultures or knockout mice.

In conclusion, the present study shows that administration of visfatin improves ovarian function and fertility potential in aged female mice of 18-month-old. It increases ovarian expression of the mTOR/PI3K signaling pathways components and angiogenic factors. These results suggest that the

administration of visfatin could be applied as a new treatment strategy for ovarian aging by regulating ovarian signaling pathways for the activation of primordial follicles.

Funding Information This study was supported by a grant of the Basic Research Project, Ministry of Science and ICT and National Research Foundation, Republic of Korea (2017R1A2B4010859).

Compliance with Ethical Standards This study was approved by the institutional review board of Pusan National University Hospital, Korea. All animal experiments were conducted under the guidance for the Care and Use of Laboratory Animals of the National Institutes of Health, approved by the Pusan National University Hospital Institutional Animal Care and Use Committee.

Conflict of Interests The authors declare that they have no conflict of interest.

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