Follicular fluid A β 40 concentrations may be associated with ongoing pregnancy following in vitro fertilization

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Abstract

Purpose To determine whether A β 40 levels in the follicular fluid (FF) of infertile women undergoing IVF demonstrate a relationship with IVF cycle parameters and outcome.

Methods FF A β 40 levels were compared between patients achieving ongoing pregnancy and those with unsuccessful cycles. Clinical data such as ongoing pregnancy rate, implantation rate, number of oocytes retrieved, number of 8 cells embryos with ≤ 5 % fragments, ratio of 8 cells embryos with ≤ 5 % fragments to total embryos per patient and cleavage rate were compared among three percentile groups of A β 40. CCK-8 method was used to measure the effect of A β 40 on rat granulosa cells proliferation in vitro. RT-PCR was used to detect the mRNA expression levels of steroidogenesis related genes.

Results Patients achieving ongoing pregnancy ($n=26$; 50.98 %) demonstrated significantly higher FF A β 40 levels compared to those with unsuccessful cycles ($n=25$; 49.02 %; $P=0.024$). No significant differences were observed in APP (amyloid precursor protein) and its other proteolysis products including sAPP α , sAPP β and A β 42 between the two groups. Statistically significant differences between the three percentile groups of A β 40 were observed only in the implantation rates and ongoing pregnancy rates. There were no statistically significant differences between the three percentile groups in the age, No. oocytes retrieved, No. 2 pronucleus, No. embryos transferred, No. 8 cells embryos with ≤ 5 % fragments and cleavage rate. Significantly

negative correlation exists between APP and AFC (antral follicle count) ($R=-0.360$, $P=0.005$) and oocytes retrieved ($R=-0.378$, $P=0.004$). There were also significantly positive correlations between A β 40 and A β 42 ($R=0.407$, $P=0.000$), between AFC and oocytes retrieved ($R=0.476$, $P=0.000$). Rat granulosa cells treated with A β 40 of different concentrations have improved their proliferative ability. Cells treated with 200 pg/ml A β 40 have the strongest ability of proliferation. 200 pg/ml A β 40 enhanced the expression of key molecules during steroidogenesis such as IGF-1, IGF-1 receptor (IGF-1R), FSH receptor (FSHR), P450 aromatase (P450arom), steroidogenic acute regulatory protein (StAR) and cholesterol side-chain cleavage cytochromes P450 (P450scc).

Conclusions A β 40 levels in follicle fluid may be associated with ongoing pregnancy and the moderate expression level of A β 40 is important for oocytes and embryos development.

Keywords A β 40 · Amyloid precursor protein (APP) · Ongoing pregnancy

Introduction

A β 40 is an proteolysis product of amyloid precursor protein (APP), which is widespread in nervous system and thoroughly investigated in Alzheimers' Disease (AD) [1]. APP is metabolized mainly by two pathways: one is non-amyloid pathway generating soluble amyloid precursor protein α (sAPP α), another is amyloid pathway generating soluble amyloid precursor protein (sAPP β), A β 42 and A β 40 [2]. Although oligomers form of A β 42 and A β 40 are major components of senile plaque that is one of the pathological hallmarks of AD, monomers of A β 42 and A β 40 have neurotrophic and neuroprotective effects, as well as properties of stimulation of neural-progenitor proliferation [3]. Numerous studies have suggested that monomers of A β 40 in cerebrospinal fluid

Capsule Patients achieving ongoing pregnancy demonstrated significantly higher FF A β 40 levels compared to those with unsuccessful cycles. Rat granulosa cells treated with A β 40 have improved their proliferative ability and enhanced the expression of key molecules during steroidogenesis.

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decline in AD patients compared with controls [4]. Other proteolysis products of APP, sAPP α and sAPP β , also promote neural progenitors differentiation apart from their neuroprotective characteristics [5]. APP involved in a variety of cell processes, including neurite outgrowth [6], neuron proliferation, cell adhesion [7] and cell migration [8], however, over expression of APP could induce neural apoptosis, thus deleterious [9]. Other studies suggested that APP levels reversely related to estrogen and could be reduced using estrogen [10].

The earliest study of APP in ovary could be found in Kimura et al. research [11], in which he demonstrated that APP expressed in the granulosa cells and APP soluble isoforms in follicular fluid. Whereas, to date no studies have been performed about the relationship between APP and its proteolysis products and ovarian status, and the purpose of the present study is to identify APP and its proteolysis products expression in human ovaries and explore the correlation between APP and its proteolysis products and stimulation parameters as well as pregnancy outcome in women undergoing in vitro fertilization (IVF).

Materials and methods

Patient population

The present study was a retrospective, laboratory-based study, conducted at the Center for Reproductive Medicine, Department of Gynecology and Obstetrics, Nanfang Hospital, affiliated with Southern Medical University between December 2012 and April 2013, and it was approved by the Ethics Committee of Nanfang Hospital and written informed consent was obtained from each participant. The main outcome measures of the present study were ongoing pregnancy rate and implantation rate, the secondary outcome measures include number of oocytes retrieved, number of 2 pronuclei, number of 8 cells embryos with ≤ 5 % fragments, ratio of 8 cells embryos with ≤ 5 % fragments to total embryos per patient, cleavage rate and cancelled transfer rate. A total of 51 patients treated with a routine GnRH agonist long protocol during IVF were included in the study. Exclusion criteria were PCOS, endometriosis and severe male factor.

Ovarian stimulation

All patients were treated with a routine GnRH agonist long protocol. 1.875 mg or 1.25 mg triptorelin depot (Diphereline, IPSEN, Signes, France) were administered on 21 day of the previous cycle for pituitary downregulation. A starting dose of FSH 150–300 IU (rFSH; Gonal F, Merck Serono, Modugno, Italy) was given depending on BMI, age and anticipated ovarian response from cycle day 2. Follicle growth was monitored by vaginal ultrasound and serum estradiol level. When

at least three follicles were ≥ 17 mm, 10,000 IU hCG was administered for oocyte retrieval 36 h later. 2 (women ≤ 35 years old) or 3 embryos (women >35 years old) were replaced into uterus transcervically on day 3 of embryonic development. The luteal phase was supported with 60 mg of progesterone in oil, starting on the day of oocyte retrieval and continuing until 8 weeks of gestation in the presence of a positive hCG test. An ultrasound and the presence of an intrauterine gestational sac at 12 weeks of gestation after embryo transfer defined the ongoing pregnancy.

Follicular fluid collection

The follicle fluid samples were collected from the first aspiration follicle (≥ 16 mm in diameter) of each ovary, and only follicle fluid samples which did not contain any visible blood contamination were used in this study, and samples that looked cloudy or blood stained were discarded. The collected follicular fluids were processed by centrifugation at 10,000 g for 3 min at 4 °C to eliminate cellular elements and subsequently frozen at -80 °C until Elisa analysis. All assays of follicular fluid for each patient were performed in duplicate.

Immunohistochemistry

The ovarian tissues were from patients undergoing ovariectomy because of endometrial cancer or cervical cancer. A total of ten patients were chosen and the mean age of them was 45 ± 4.3 years old. After dehydration with 5 % paraformaldehyde and then embedding with paraffin, the tissue was cut into slices of 5- μ m in thickness. Then slices of ovaries were pretreated for retrieving antigens with citrate buffer, removing endogenous peroxidase with 1.5 % peroxide. Followed by these procedures, slices were blocked in 5 % BSA (Bovine Serum Albumin) for 60 min and then incubated with polyclonal rabbit anti-human Amyloid Precursor Protein antibody (ab2072) (Abcam, Cambridge, UK) at a 1:200 dilution overnight at 4 °C. Slices were incubated with horseradish peroxidase -conjugated anti-rabbit secondary antibodies (Santa Cruz Biotechnology Inc., Santa Cruz, United States) for 1 h at 37 °C after three washes. Signals were developed using the diaminobenzidine kit (Santa Cruz Biotechnology Inc., Santa Cruz, United States), and cell nuclei were stained using hematoxylin solution (Sigma-Aldrich Inc., St. Louis, United States). For negative controls, the primary antibodies were replaced by PBS.

Western blot

Granulosa cells derived from follicle fluid are isolated by density gradient centrifugation. firstly, follicular fluids were pooled and centrifuged (400 g, 10 min) to remove tissue debris and most of the red blood cells. Then 50 % percoll (GE Healthcare, Uppsala, Sweden) was added above the

resuspended pellet carefully. Following centrifugation for 20 min at 400 g, three layers could be distinguished, the middle layer was collected, washed by centrifugation for 10 min at 600 g. Granulosa cells collected were splitted in RIPA cell lysis buffer (Beyotime, China) for half an hour and then centrifuged at 10,000 g for 5 min. The supernatant was collected and its protein concentration was detected using Bicinchoninic Acid (BCA) method. Equal amounts of total protein boiled with SDS-PAGE loading buffer were separated on a 8 % SDS-polyacrylamide gel and transferred onto PVDF membrane. After being blocked with 5 % skim milk powder solution for 1 h, the membrane was incubated with primary antibodies (ab2072, Abcam, UK) over night at 4 °C. The membrane was washed in Tris-Buffered Saline and Tween (TBST) for 5 min three times and then at room temperature incubated for 2 h with fluorescence-labeled secondary antibodies. Signals were developed using the Odyssey Infrared Imaging System from LI-COR. The grey value of the band was analysed by the soft ware of Quantity One. And APP relative level was defined as the ratio of APP to corresponding β -actin grey value.

Elisa

sAPP α , sAPP β , A β 42 and A β 40 levels were determined using the commercially available ELISA Kits (IBL, Hokkaido, Japan). The ELISAs were performed according to the manufacturer's protocols. Briefly, samples that have been diluted 5 times were put into test sample wells for 100 μ l each well and then Incubated overnight at 4 °C. After adding labeled antibody solution and incubating the plate for 30 min at 4 °C, plate was washed 9 times. Subsequently, chromogen was added and then incubated for 30 min and stop solution was used. The plate reader was run at 450 nm, and sAPP α , sAPP β , A β 42 and A β 40 concentrations were calculated by running a standard curve. The detection limit of the assay was 0.09 ng/ml for sAPP α , 0.05 ng/ml for sAPP β , 0.29 pg/ml for A β 42, 2.77 pg/ml for A β 40. Intra- and interassay variations were 5.9 and 6.7 % for sAPP α , 2.8 and 8.7 % for sAPP β , 4.9 and 5.0 % for A β 42, 3.0 and 4.4 % for A β 40.

Granulosa cell culture and treatment with A β 40

Immature (21-day-old) female Sprague–Dawley rats were provided by the Experimental Animal Center of Southern Medical University (Guangzhou, China), and kept in sterile cages in a room under a 12-h light:12-h dark schedule, and were fed with sterile food and water ad libitum. All of the experiments involving animals were performed according to the guidelines of the Experimental Animals Management Committee (Guangdong Province, China). These immature female rats were injected subcutaneously with 50 IU PMSG

(Sigma, St. Louis, MO) to synchronize follicular development. Forty-eight hours later, these rats were killed and ovaries were collected aseptically. Granulosa cells were released from follicles to DMEM/F12 (Gibco Invitrogen, Paisley, UK) by using sterile syringe with needle. Granulosa cells were collected by centrifugation, and washed twice with culture medium, and then seeded in 6-well culture dishes (for RT-PCR) or 96-well culture dishes (for CCK-8 test) in DMEM/F-12 medium supplemented with 10 % fetal bovine serum (Gibco Invitrogen) at 37 °C in an atmosphere of 5 % CO₂. The cells were initially cultured for 48 h with no other treatment and then incubated in fresh culture medium with different final concentrations of A β 40 (Sigma-Aldrich) (0 pg/ml, 100 pg/ml, 200 pg/ml, 300 pg/ml, 400 pg/ml) for 24 h. Granulosa cell proliferation was measured with CCK-8 method in these five groups. The mRNA levels of FSHR, IGF-1, IGF-1R, P450arom, P450scc and StAR were detected by RT-PCR method in the three groups of 0 pg/ml, 200 pg/ml and 400 pg/ml.

RT-PCR

Total RNA was isolated as described (Hawkins et al., 2011; Plante et al., 2012). Potential genomic DNA contamination was deleted with gDNA eraser (Takara, Japan). The density of RNA was determined and reverse transcription was performed immediately with prime script RT kit (Takara, Japan). RT assays were carried out in a volume of 20 μ l, comprising 10 μ l total RNA, 1 μ l prime script RT enzyme mix 1 μ l RT primer mix, 4 μ l prime script buffer, 4 μ l rnase free water with the following conditions: 37 °C for 15 min, 85 °C for 5 s and 4 °C for storage. The specific primers used for quantitative polymerase chain reaction are listed in Table 1. Each real time PCR reaction (Takara, Japan) had the following components: 10 μ l of SYBR, 0.4 μ l of forward primer, 0.4 μ l of reverse primer, 0.4 μ l of ROX dye II, 6.8 μ l of water and 23 μ l of RT product. Real time PCR was performed on ABI 7500 Real-Time PCR System for 40 cycles (95 °C, 5 s; 60 °C, 30s) after an initial 30 s incubation at 95 °C. Data was analyzed with $\Delta\Delta$ Ct method. The fold change in the expression of each gene was normalized to the endogenous control GAPDH.

Cell proliferation test

Granulosa cells were seeded in a 96-well culture dish at a density of 10⁵/ml (10⁴ cells per well or 100 μ l cell suspension per well). Before and after cells were incubated for 24 h, CCK-8 test was performed for the five treatment groups (final A β 40 concentration of 0 pg/ml, 100 pg/ml, 200 pg/ml, 300 pg/ml, 400 pg/ml). 10 μ l CCK-8 solution (Beyotime, China) was added to each well for an additional 4 h. Then, the absorbance at 450 nm was measured on a multiwell plate reader (Spectra Max M5 from Molecular Devices). The

Table 1 Sequence of the primers used for real-time RT-PCR

Gene	Forward primer	Reverse primer
IGF-1	5'ACAAAGTCAGCTCGTTCCAT	5'GAGGCTCCTCCTACATTCTG
IGF-1-R	5'TCCTGATTCTCCGAGCTTA	5'TCTTCAGCTACCATGCAGTTC
StAR	5'GCTGGAAGTCCCTCAAAGA	5'CGAACTCTATCTGGGTCTGTGA
P450 _{scc}	5'CAGCTTCTTTCCCAATCCAA	5'GCATCAGGATGAGGTTGAAC
P450 _{arom}	5'CACTTCTAACACGCTCTTCCT	5'AGTCCTTGACGGATCTCTCA
FSHR	5'TGCAAGCCCAGATTACAG	5'GATTAAGAGGGACAAGCACGTA
GAPDH	5'CAAGGATACTGAGAGCAAGAGA	5'AGGCCCTCCTGTTGTTAT

proliferation rate of each group was defined as the ratio of the mean OD value at time point of 24 h to the mean OD value at time point of 0 h.

Statistical analysis

Continuous variables were tested for normal distribution using method of moment. Results are expressed as means and SD. Comparisons between the groups were calculated by *t*-test or One Way ANOVA if the data were normally distributed and by Wilcoxon Sign Rank test or Kruskal–Wallis test if the data were abnormally distributed. Analysis of covariance (ANCOVA) was performed to exclude the potential effects of some covariates. Correlations between APP and its proteolysis products and oocytes retrieved were determined by using bivariate correlation statistics and are expressed as Spearman correlation coefficients. Statistical analysis was performed by SPSS 16.0. Statistical significance was set at $P < 0.05$.

Results

Table 2 describes the participant and IVF cycle characteristics by outcome of IVF cycle (i.e., pregnant vs. not pregnant). Those achieving ongoing pregnancy ($n=26$; 50.98 %) demonstrated significantly higher FF A β 40 levels compared to those with unsuccessful cycles ($n=25$; 49.02 %; $P=0.024$), and received a significantly higher number of follicles ≥ 10 mm on hCG day and oocytes retrieved compared with those whose IVF cycles were unsuccessful; the remaining IVF cycle characteristics did not differ significantly (Table 2).

Although significant differences were observed in FF A β 40 levels between patients achieving pregnancy and those with failed outcomes as specified in Table 2, no significant differences were observed in APP and its other proteolysis products including sAPP α , sAPP β and A β 42 between the two groups (Table 3).

Percentile analysis to define percentile groups of A β 40 was performed. Table 4 show patients characteristics and

Table 2 Differences in patients' characteristics and stimulation data among pregnant and not pregnant women

	Not pregnant ($n=25$)	Pregnant ($n=26$)	<i>P</i>
A β 40 (pg/ml)	178.90 $\pm$ 76.59	228.60 $\pm$ 53.21	0.024
Age (years)	33.19 $\pm$ 5.26	31.73 $\pm$ 4.53	0.289
BMI (kg/m ²)	22.13 $\pm$ 2.69	21.74 $\pm$ 2.93	0.906
Duration of stimulation (days)	11.23 $\pm$ 1.94	11.23 $\pm$ 2.19	1.000
Total FSH administered (IU)	2,958.17 $\pm$ 1,083.44	2,613.46 $\pm$ 871.21	0.212
Antral follicle count (AFC)	12.36 $\pm$ 5.33	13.92 $\pm$ 4.99	0.292
Basal FSH (IU/L)	8.14 $\pm$ 4.39	6.55 $\pm$ 1.12	0.091
Basal LH	4.58 $\pm$ 2.38	4.90 $\pm$ 1.87	0.600
Basal E ₂ (pg/ml)	37.49 $\pm$ 18.06	42.69 $\pm$ 22.84	0.374
E ₂ on hCG day (pg/ml)	1,714.88 $\pm$ 962.33	2,109.96 $\pm$ 1,059.35	0.174
LH on day of hCG (IU/L)	1.99 $\pm$ 1.90	1.47 $\pm$ 1.30	0.272
Progesterone on day of hCG (ng/ml)	0.93 $\pm$ 0.48	0.93 $\pm$ 0.40	0.996
No. follicles ≥ 14 mm	6.42 $\pm$ 3.44	8.69 $\pm$ 4.88	0.058
No. follicles ≥ 10 mm	9.31 $\pm$ 5.84	13.58 $\pm$ 7.55	0.027
No. oocytes retrieved	6.88 $\pm$ 4.32	10.27 $\pm$ 5.78	0.021
No. embryos transferred	1.35 $\pm$ 1.09	1.81 $\pm$ 0.84	0.095
No. total embryos	4.12 $\pm$ 3.55	5.65 $\pm$ 3.37	0.116

Table 3 Differences in APP proteolysis products among pregnant and not pregnant women

	Not pregnant (<i>n</i> =25)	Pregnant (<i>n</i> =26)	<i>P</i>
APP	2.16±2.00	1.24±1.08	0.105
sAPPα (ng/ml)	148.99±42.17	157.50±57.50	0.627
sAPPβ (ng/ml)	127.16±35.02	127.08±31.34	0.993
Aβ42 (pg/ml)	58.31±75.33	47.51±19.59	0.492
Aβ40 (pg/ml)	178.90±76.59	228.60±53.21	0.024

outcomes according to three groups of Aβ 40 values as follows: group 1, lower 25th percentile (<145.876 pg/ml); group 2, medium 50th percentile (145.876–270.989 pg/ml) and group 3, higher 75th percentile (>270.989 pg/ml). Statistically significant differences between the three groups were observed only in the implantation rates and ongoing pregnancy rates. There were no statistically significant differences between the three groups in the age, No. oocytes retrieved, No. 2 pronucleus, No. embryos transferred, No. 8 cells embryos with ≤5 % fragments and cleavage rate. Although medium (145.876–270.989 pg/ml) percentile demonstrated a relatively lower cancelled transfer rate compared with lower 25th percentile and higher 75th percentile, these difference was not statistically significant ($P>0.10$). A post-hoc analysis revealed that Ongoing pregnancy rates between medium (145.876–270.989 pg/ml) and higher (>270.989 pg/ml) percentile are comparable.

Table 5 showed that significantly negative correlation exists between APP and AFC ($R=-0.360$, $P=0.005$) and oocytes retrieved ($R=-0.378$, $P=0.004$). There were also significantly positive correlations between Aβ40

and Aβ42 ($R=0.407$, $P=0.000$), between AFC and oocytes retrieved ($R=0.476$, $P=0.000$).

Figure 1 showed APP expression in the ovarian tissue by immunohistochemistry. APP is widespread in the human ovary (Fig. 1a), including expression in the small pre-antral follicle (Fig. 1b), and expression in the granulosa cells and cumulus cells (arrow) of large antral follicle (Fig. 1c). The diffuse expression of APP in mouse brain is obviously stronger than human ovary (Fig. 1d). Figure 2 showed APP expression of eight patients detected in the granulosa cells of the ovaries from patients undergoing IVF by western blot. The relative APP value is calculated by the ratio of APP gray value to β-actin gray value.

Figure 3 showed that granulosa cells treated with Aβ40 of different concentrations have improved their proliferative ability. Cells treated with 200 pg/ml Aβ40 have the strongest ability of proliferation. The proliferation rate is highest (114.53 %) in the 200 pg/ml group, secondly (110.06 %) in the 300 pg/ml, next (106.77 and 106.86 %) respectively in the 100 pg/ml and 400 pg/ml group. Although proliferative ability of cells treated with 300 pg/ml Aβ40 is lower than 200 pg/ml, but still higher than 100 pg/ml.

Figure 4 showed that addition of Aβ40 have a significant effect on the mRNA expression of steroidogenesis related genes. IGF-1 and IGF-1R mRNA level have slightly increased in 200 pg/ml Aβ40 group than that in 0 pg/ml Aβ40 group. When the concentration of Aβ40 increased from 200 pg/ml to 400 pg/ml, IGF-1 and IGF-1R mRNA level did not further increase. Aβ40 have a significant impact on the mRNA expression of StAR, P450scc, P450arom and FSHR. The mRNA expression levels of StAR, P450scc, P450arom and FSHR in 200 pg/ml Aβ40 group are almost two times of the

Table 4 percentile groups of Aβ40 on the day of oocyte retrieval and their effects on different parameters and outcomes

	Lower 25th percentile (<i>n</i> =12) (<145.87 pg/ml)	Medium 50th percentile (<i>n</i> =27) (145.87–270.98 pg/ml)	Higher 75th percentile (<i>n</i> =12) (>270.98 pg/ml)	<i>P</i>
Age (years)	33.17±4.87	32.37±4.65	31.25±5.32	0.627
E ₂ on hCG day (pg/ml)	2,118.37±875.29	1,924.60±1,144.92	1,703.94±938.06	0.637
No. follicles ≥14 mm	6.50±2.35	7.67±4.75	8.75±4.84	0.453
No. follicles ≥10 mm	9.67±3.75	12.07±7.72	12.42±7.97	0.559
No. oocytes retrieved	7.42±3.14	9.59±6.39	7.75±4.41	0.413
No. 2 pronucleus	4.67±2.67	5.85±4.10	4.75±3.19	0.534
No. embryos transferred	1.50±1.16	1.67±0.78	1.33±1.23	0.619
No. 8 cells embryos with ≤5 % fragments	1.36±1.28	1.57±2.15	1.50±1.67	0.95
Ratio of 8 cells embryos with ≤5 % fragments to total embryos per patient (%)	25±18	23±26	34±22	0.471
Cleavage rate (%)	62±21	71±20	68±29	0.565
Cancelled transfer rate (%)	8.3 (1/12)	0 (0/27)	8.3 (1/12)	0.180
Implantation rate (%)	21.0 (4/19)	49.0 (25/51)	33.3 (8/24)	0.031
Ongoing pregnancy rate (%)	16.6 (2/12)	59.2 (16/27)	58.3 (7/12)	0.014

* $p<0.05$ compared with lower 25th percentile

Table 5 Relationships between APP and its proteolysis products and AFC and number of oocytes retrieved

	A β 40	sAPP β	A β 42	AFC	Oocytes retrieved	APP	sAPP α
A β 40	R=1 P=0.000	R=-0.027 P=0.790	R=0.407 P=0.000	R=-0.079 P=0.430	R=-0.028 P=0.775	R=-0.067 P=0.598	R=0.187 P=0.115
sAPP β	R=-0.027 P=0.790	R=1.000 P=0.000	R=0.120 P=0.230	R=-0.195 P=0.061	R=-0.086 P=0.401	R=-0.024 P=0.852	R=-0.045 P=0.711
A β 42	R=0.407 P=0.000	R=0.120 P=0.230	R=1.000 P=0.000	R=-0.111 P=0.287	R=0.074 P=0.469	R=-0.110 P=0.386	R=0.216 P=0.073
AFC	R=-0.079 P=0.430	R=-0.195 P=0.061	R=-0.111 P=0.287	R=1.000 P=0.000	R=0.476 P=0.000	R=-0.360 P=0.005	R=-0.078 P=0.552
Oocytes retrieved	R=-0.028 P=0.775	R=-0.086 P=0.401	R=0.074 P=0.469	R=0.476 P=0.000	R=1.000 P=0.000	R=-0.378 P=0.004	R=0.026 P=0.830
APP	R=-0.067 P=0.598	R=-0.024 P=0.852	R=-0.110 P=0.386	R=-0.360 P=0.005	R=-0.378 P=0.004	R=1.000 P=0.000	R=0.115 P=0.444
sAPP α	R=0.187 P=0.115	R=-0.045 P=0.711	R=0.216 P=0.073	R=-0.078 P=0.552	R=0.026 P=0.830	R=0.115 P=0.444	R=1.000 P=0.000

mRNA expression levels of these four molecules in 0 pg/ml A β 40 group. When the concentration of A β 40 increased from 200 pg/ml to 400 pg/ml, P450scc mRNA level decreased and StAR and P450arom mRNA levels did not altered significantly, but FSHR mRNA levels increased.

Discussion

On the basis of the results that patients with ongoing pregnancy had significantly higher FF A β 40 level compared with non-pregnant patients, as well as that medium percentile group and higher percentile group of A β 40 have significantly higher ongoing pregnancy rate, we speculate that A β 40 level may be associated with pregnancy outcome during IVF. A β 40 peptide generation occurs during brain embryogenesis and seems to be required for normal brain development [12–14]. The A β 40 peptide may be neuroprotective: a peptide of the 28 first amino acids of A β 40 enhances the survival of hippocampal neurons in vitro [3]. Furthermore, A β 40 monomers induce the survival of developing neurons under trophic-factor deprivation and protect mature neurons against excitotoxic cell death [15]. The A β 40 peptide has also been reported to be neurotrophic when added at low concentrations to undifferentiated hippocampal neurons [16]. The properties of A β 40 monomers including the neurotrophic and neuroprotective effects, the proliferation of the neural progenitor cells, as well as the involvement in synaptic function imply that cleavage of the A β 40 peptide is of key physiological importance in the central nervous system. Huo et al. found a dramatical damage in seminiferous epithelium of testicular tissue after removal of the spermatic cord, suggesting that not only the hypothalamus pituitary testicular axis and paracrine regulation system but

also spermatic nerve participate in the regulation of spermatogenesis process [17]. Japanese researchers found that nerve cells in mouse brain promotes ovulation by an increased synthesis of a protein called “kisspeptin” [18]. Provided that both brain nerve cells and peripheral nerve cells contribute to germ cell development, we can speculate that A β 40 in different concentrations may lead to different microenvironments of nerve cells (including central nerve system and peripheral nerve embedded in ovary) to influence follicle development. All these previous research evidence may explain why patients with relatively higher A β 40 level are more likely to conceive compared with those with relatively lower A β 40 level.

Given the present evidence that the ongoing pregnancy rates between medium percentile group and higher percentile group are comparable, as well as our results that the medium percentile group have relatively higher oocytes retrieved, higher embryos number, higher good quality embryos number, higher ratio of good quality embryos, higher cleavage rate, and in contrast lower cancelled transfer rate (although there is no statistically significance), we speculate that moderate A β 40 levels are important for oocytes development and subsequent embryos development, and over-lower or over-higher A β 40 are both inappropriate, although the damage of over-lower A β 40 levels are more apparent. The optimal and accurate range of A β 40 levels need further investigation.

Among pregnant and non-pregnant patients, no significant differences in APP and other three proteolysis products were observed. However, APP levels exhibited a decreasing trend in pregnant patients. And combined our result that APP level significantly and negatively correlated to AFC and oocytes retrieved, over expression of APP may have a negative impact on oocytes generation perhaps by apoptosis promotion,

Fig. 1 APP expression in the ovarian tissue by immunohistochemistry. (a) APP is widespread in the human ovarian, especially in the follicle and around the follicle (arrows). (bar ~1 mm) (b) APP is expressed in the small pre-antral follicle (arrow), stronger than the tissue around. (bar ~50 μ m) (c) APP is expressed in the granulosa cells and cumulus cells (arrow) of large antral follicle. (bar ~200 μ m) (d) The diffuse expression of APP in mouse brain is apparently stronger than human ovarian. (bar ~200 μ m)

similar to the mechanism in nervous system. A significantly positive relationship exists between A β 40 level and A β 42 level, this may be regarding to they are generated by the same pathway (amyloid pathway). Indeed, of several species of A β peptides that have been identified in the brain [19], A β 40 is the major isoform. However, less abundant species, A β 42, and its N-terminally truncated isoforms are the more aggregant and neurotoxic [19]. Regarding to the similar properties of them, A β 40 and A β 42 isoforms stimulate proliferation of primary neural progenitor cells isolated from rat E18 cerebral cortices [20]. Furthermore, they also have their respective characteristics, A β 40 induces the differentiation of neural progenitor cells into neurons at the end of S-phase whereas A β 42 drives the differentiation towards the astrocyte lineage [21]. We also detected higher sAPP α level in follicle fluid on hCG day than in serum of menstrual cycle day 2 (about one time higher, data not shown).

sAPP α (non-amyloid pathway) and sAPP β (amyloid pathway) are important cleavage products of APP. they can protect neurons against against excitotoxic, metabolic, and oxidative insults [22]. And the former is approximately one hundred times more effective than the latter. With regard to these two

products, we failed to find any significant relationship between them and stimulation outcomes. Considering that the sample size is small, the effect of them on oocytes and embryos as well as fecundity could not be excluded.

Our results showed that A β 40 could promote proliferation of rat granulosa cells. The proliferation rate is highest (114.53 %) in the 200 pg/ml A β 40 group, secondly (110.06 %) in the 300 pg/ml, next (106.77 and 106.86 %) respectively in the 100 pg/ml and 400 pg/ml group. Cells treated with A β 40 at concentration of 200 pg/ml has the strongest proliferation-promoting ability. Although proliferative ability of cells treated with 300 pg/ml A β 40 is lower than 200 pg/ml, but still higher than 100 pg/ml. This phenomenon is consistent with our clinical result showing that the optimal pregnancy rate is present in the medium concentration group (145.876–270.989 pg/ml) and higher concentration group (>270.989 pg/ml).

Our results showed that A β 40 at the concentration of 200 pg/ml could up-regulate IGF-1 mRNA and IGF-1 receptor (IGFR) mRNA. IGF-1 could promote proliferation of granulosa cells and inhibit apoptosis of granulosa cells [23]. IGF-1 could also enhance the role of FSH to strongly improve the activity of aromatase and the production of estrogen, inducing the spontaneous maturation of immature human oocytes [24]. Therefore, the results that A β 40 up-regulated IGF-1 and IGF-1R mRNA suggest that A β 40 of appropriate concentration could promote proliferation of granulosa cells and improve the production of estrogen by increasing IGF-1 and IGF-1R, thus to promote the oocyte quality and ongoing pregnancy. However, IGF-1 mRNA in cells treated with

Fig. 2 APP expression is detected in the granulosa cells of the ovaries from patients undergoing IVF by western blot. The figure demonstrated the results of eight patients. The relative APP value is calculated by the ratio of APP gray value to β -actin gray value

Fig. 3 Granulosa cells treated with A β 40 of different concentrations have improved their proliferative ability (**a**). The proliferation rate is highest (114.53 %) in the 200 pg/ml group, secondly (110.06 %) in the 300 pg/ml, next (106.77 and 106.86 %) respectively in the 100 pg/ml and 400 pg/ml group (**b**)

400 pg/ml A β 40 is not higher than that in cells treated with 200 pg/ml A β 40, indicating that over-higher concentration of A β 40 is not beneficial.

FSHR and P450arom expressed in the granulosa cells directly dominate steroidogenesis and play a crucial role during the follicle development and maturation [25, 26]. Only after FSH combines to FSHR, P450arom could be activated by cAMP pathway. Under the catalyzation of P450arom, androstenedione (secreted by theca cells) is transformed into estrogen. Sufficient estrogen is essential for oocyte development and maturation. P450arom is just the rate-limiting enzyme that transforms androgen into estrogen. Because the production of estrogen is limited by the content of FSHR, the decreased FSHR expression could reduce the production of estrogen and hinder the oocyte development. As a result, the declined FSHR expression leads to fewer oocytes retrieved and poorer oocyte quality after ovarian stimulation. In the present experiment, we demonstrated that A β 40 could up-regulate the FSHR mRNA in a dose dependent pattern. This indicates that

A β 40 may promote ovarian response and oocytes retrieved by up-regulating FSHR mRNA, and simultaneously A β 40 may promote the final oocytes maturation and ongoing pregnancy by up-regulating FSHR and P450arom mRNA.

When steroid hormone is synthesized, firstly StAR transports free cholesterol from mitochondrial outer-membrane to mitochondrial inner membrane, then free cholesterol is hydroxylated by the P450scc located in mitochondrial intra-membrane, then producing pregnenolone [27]. This step is the main limiting site during the synthesise of steroid hormone. Our results showed that 200 pg/ml A β 40 up-regulated StAR and P450scc mRNA significantly but 400 pg/ml A β 40 did not further up-regulate StAR and P450scc mRNA, indicating that only appropriate concentration of A β 40 could promote the synthesise ability of steroid hormone. Appropriate concentration of A β 40 may strengthen the expression of steroid hormone to promote oocytes quality and ongoing pregnancy by strengthening these key rate-limiting enzymes.

Fig. 4 Addition of A β 40 have a significant effect on the mRNA expression of steroidogenesis related genes in rat granulosa cells. IGF-1 and IGF-1R mRNA level have slightly increased in 200 pg/ml A β 40 group than that in 0 pg/ml A β 40 group, IGF-1 and IGF-1R mRNA level did not further increase in 400 pg/ml A β 40 group (**a–b**). The mRNA expression levels of StAR, P450scc, P450arom and FSHR in 200 pg/ml A β 40 group are almost two times of the mRNA expression levels of these four molecules in 0 pg/ml A β 40 group (**c–f**). When the concentration of A β 40 increased from 200 pg/ml to 400 pg/ml, P450scc mRNA level decreased and StAR and P450arom mRNA levels did not altered significantly, but FSHR mRNA levels increased (**c–f**)

In summary, appropriate concentration of A β 40 could enhance these key molecules during steroidogenesis such as IGF-1, IGF-1R, FSHR, P450arom, StAR and P450scc to promote the survive of granulosa cells and production of estrogen, and as a result, the oocytes quality and pregnancy rate is promoted. These molecules did not further increase largely when the concentration of A β 40 increased from 200 pg/ml to 400 pg/ml. Our experiment data is consistent with clinical data, in which the optimal pregnancy rate exists in the medium

concentration group (145.876–270.989 pg/ml) and higher concentration (>270.989 pg/ml) group. The slight decrease in implantation rate in higher concentration (>270.989 pg/ml) group may be associated with the negative effect of higher concentration A β 40 on some key molecules (such as IGF-1 and P450scc).

In our clinical data, the sample is relatively small, so the selected sample may be lack of sufficient representativeness and difficult to ensure the accuracy and reliability. Large

sample is needed to explore the relationship between A β 40 and pregnancy in future.

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