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Effect of the Concentration of Various Polyunsaturated Fatty Acids in Human Follicular Fluid on Oocyte Maturation and Fertilization

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Abstract

Objective: This study aimed to investigate concentrations of different polyunsaturated fatty acids (PUFAs) in follicular fluid and how they affect oocyte maturation and fertilization.

Methods: A prospective study was conducted on 28 women eligible for *in-vitro* fertilization and embryo transfer. PUFA concentrations from 28 serum and 140 follicular fluid samples were analyzed using gas chromatography. Two mL of follicular fluid was collected from six consecutive follicles of each patient. Each follicle was aspirated independently and matched to an oocyte growing in the follicular milieu.

Results: Concentrations of 22 PUFAs were significantly lower within the follicles, approximately one-third their concentration in the serum. Linoleic acid concentration in the follicular fluid of metaphase II oocytes was significantly lower than in follicular fluid of immature oocytes. Additionally, γ-linolenic acid (GLA) concentration was significantly higher in the follicular fluid of fertilized oocytes (1.1 vs 0.9, $p = 0.007$). Conversely, eicosapentaenoic acid and docosapentaenoic acid were found to be significantly lower in follicular fluid of fertilized than nonfertilized oocytes.

Conclusion: Linoleic acid, GLA, eicosapentaenoic acid, and docosapentaenoic acid in follicular fluid may affect oocyte maturation and fertilization. However, PUFAs did not have any effect on post-fertilization embryo quality or pregnancy.

Keywords: polyunsaturated fatty acids; in vitro fertilization; embryo transfer; follicular fluid; oocyte

Abbreviations: ALA: α-linolenic acid, DHA: docosahexaenoic acid, DPA: docosapentaenoic acid, EPA: eicosapentaenoic acid. ET: embryo transfer, FA: fatty acids, GLA: γ-linolenic acid, HCG: human chorionic gonadotropin, HMG: human menopausal gonadotropin, ICSI: intracytoplasmic sperm injection, IVF: in vitro fertilization, PUFA: polyunsaturated fatty acids

Introduction

Diet and lifestyle affect embryo quality during *in vitro* fertilization (IVF) and embryo transfer (ET) [1]. In addition, the important role of fatty acids (FA) in promoting embryonic development has been shown [2]. Lass and Belluzzi et al. reviewed a few small prospective cohort studies and found conflicting results regarding the relationship between serum polyunsaturated fatty acids (PUFA) and IVF outcome measures and success rates [3]. Some studies showed that increased serum omega-3 PUFA levels provide a higher likelihood of live birth, while others failed to show a correlation. According to a study on human follicular fluid, linoleic acid (LA) was positively correlated with fertility percentages while arachidonic acid (AA) level was negatively correlated [4]. In another study, there were negative correlations between metaphase II oocytes and follicular fluid levels of stearic acid and LA [5]. In addition to the variation in these studies results, there is a dearth of information on the relationship between PUFA and the reproductive processes. Thus, the objective of this study was to determine the association between follicular PUFA concentrations and oocyte maturation, fertilization, embryonic development, and pregnancy success.

Materials and Methods

This prospective study enrolled 28 women eligible for IVF and ET and was conducted from August 2015 to December 2016. Twenty-eight serum and 140 follicular fluid samples were collected and analyzed.

Analysis of PUFAs

We measured n-6 PUFAs (linoleic acid, γ-linolenic acid [GLA], eicosadienoic acid, dihomo-GLA, AA, and docosatetraenoic acid) and n-3 PUFAs (ALA, eicosapentaenoic acid [EPA], docosapentaenoic acid [DPA] and docosahexaenoic acid [DHA]). Gas chromatography was used to analyze PUFA concentration in both serum and follicular fluid. PUFA analysis was performed at SRL Inc. (Tokyo, Japan). Total lipid was extracted from 0.5 ml serum and 0.5 ml follicular fluid into chloroform/methanol (2:1 vol/vol). FAs were removed from complex lipids by incubating the lipid extract with 5% potassium hydroxide/ethanol solution at room temperature for 5 minutes and thereafter extracting into hexane (Kokusan Chemical Co., Ltd., Tokyo, Japan). FA methyl esters were formed by incubation with 5% hydrogen chloride methanol solution (Wako Pure Chemical Corporation, Japan) at 100°C and thereafter extracting into hexane. The FA methyl esters prepared were analyzed using a gas chromatograph equipped with a flame ionization detector. A capillary column (TC-70, GL Sciences Inc., Tokyo, Japan) was used with nitrogen as carrier gas and a flow rate of 25 ml/min. Each methyl ester was identified by comparing retention time with authentic standards purchased from Sigma-Aldrich Corporation (Tokyo, Japan). FA methyl esters were quantified by relative peak area against C23:0 internal standard. Equipment used included Shimadzu GC-17A and GC-2010 gas chromatographs (Shimadzu Corp., Kyoto, Japan), a Shimadzu AOC-20i autosampler (Shimadzu Corp., Kyoto, Japan), a C-R7A data processer, and an OPGU-2200S hydrogen generator.

IVF-ET and follicular fluid collection

Controlled ovarian stimulation was performed using the gonadotropin-releasing hormone agonist, Buserelin acetate (Suprecur®; Mochida Pharmaceutical Co., Ltd., Tokyo, Japan), administered intra-nasally at a dose of 900 μg/day from day 2 of the stimulation cycle to the day when human chorionic gonadotropin (HCG) was injected (10,000 IU; Gonatropin, Asuka Pharmaceutical Co., Ltd., Tokyo, Japan). An initial dose of 300 IU/day of human menopausal gonadotropin (HMG) (Teizo, Tokyo, Japan) was administered on day 3 of the cycle, followed by 225 IU/day of HMG. HCG was administered when diameters of the two ovarian follicles were ≥18 mm. Peak estradiol (E2) levels were measured before HCG administration.

Transvaginal oocyte retrieval and serum collection was performed 35 hours after HCG administration. Two mL of follicular fluid were collected from six consecutive follicles in each patient. Each follicle was aspirated independently and matched to the oocyte growing within it. Oocytes were fertilized either by conventional IVF-ET methods or by intracytoplasmic sperm injection (ICSI), 4 hours after retrieval. ICSI was performed for couples experiencing infertility due to a male factor. Embryos were cultured in early cleavage medium™ (IS Japan Co., Ltd., Saitama, Japan) in an atmosphere containing 5% CO_2, 5% O_2, and 89% N_2 at 37°C in an incubator (Astec Co., Ltd., Fukuoka, Japan). Embryo quality was evaluated based on Gardner's criteria, with embryos graded as >3BB classified as good-quality embryos (Gardner et al., 2000). All the embryos were cultured for five days, and blastocyst cryopreservation was performed.

After 2 months, a thawed single ET was performed. In the thawed ET cycle, a combination of transdermal E2 (Estrana®; Hisamitu Tokyo, Japan) and a progesterone (P) suppository (Chlormadinone Acetate, FUJI Pharma, Toyama, Japan) was used for hormonal replacement. Preparation of the endometrium started on day 2 of the hormonal replacement cycle and was achieved using a step-up regime (1.44–2.88 mg). The P suppository (12 mg/day) was commenced on day 15. The single embryos were transferred using ET catheters (Kitasato Supply, Shizuoka, Japan) under ultrasound guidance. A resultant clinical pregnancy was defined as presence of a gestational sac and fetal heartbeat.

Statistical analysis

Statistical analysis was conducted using JMP software (SAS Institute, Inc., Cary, NC, US). Proportions were compared using Fisher's exact test or chi-square test. Continuous variables were analyzed and compared using the student t-test or Mann-Whitney U test. A p-value of <0.05 was considered statistically significant.

Ethical approval statement

This study was conducted in accordance with the Declaration of Helsinki guidelines and was approved by the Institutional Review Board of the University of Ryukyus (#498). All patients gave written informed consent prior to their participation in the study.

Results

Patient Characteristics and Pregnancy Outcome

The average patient age was 36.0 ± 4.6 years, and anti-Mullerian hormone was 3.7 ± 3.0 ng/ml. Infertility was caused by either male factor (n = 15), tubal factor (n = 4), diminished ovarian reserve (n = 3) or unexplained infertility (n = 6). Average number of retrieved and fertilized oocytes per patient was 14.2 ± 8.4 and 7.0 ± 6.4, respectively. There were 90 fertilized oocytes, including 53 cryopreserved embryos and 37 thawed ETs. The biochemical pregnancy rate was 54.1% (20/37), and the live birth rate per oocyte retrieval was 35.1% (13/37).

The concentrations of PUFAs in the serum and follicular fluid samples

The concentrations of the six n-6 and four n-3 PUFAs in the 23 serum and 140 follicular fluid samples were analyzed and are presented in Table 1. Serum PUFA levels were three times higher than those of the follicular fluid. There was a correlation between AA follicular fluid and serum concentrations (r = 0.795, 95% CI 0.33–

Table 1: Concentrations of PUFAs in the serum and follicular fluid.

PUFAs		Concentration in serum (μg/ml)	Concentration in follicular fluid (μg/ml)
Linoleic acid	C18:2ω6	914.5 ± 149.1	238.2 ± 34.0
γ-Linolenic acid	C18:3ω6	4.8 ± 1.7	0.93 ± 0.42
Eicosadienoic acid	C20:2ω6	8.1 ± 2.0	4.6 ± 0.87
Dihomo-γ-linolenic acid	C20:3ω6	38.9 ± 13.9	16.5 ± 4.3
Arachidonic acid	C20:4ω6	209.3 ± 43.8	77.9 ± 12.2
Docosatetraenoic acid	C22:4ω6	5.5 ± 1.9	5.3 ± 2.2
α-linolenic acid	C18:3ω3	20.1 ± 7.0	3 ± 0.65
Eicosapentaenoic acid	C20:5ω3	27.6 ± 10.7	8.1 ± 3.1
Docosapentaenoic acid	C22:5ω3	14.0 ± 3.5	5.6 ± 1.5
Docosahexaenoic acid	C22:6ω3	124.7 ± 31.3	43.4 ± 8.7

0.95) and EPA (r = 0.723, 95% CI 0.17–0.9). No other FA concentrations correlated with serum concentrations.

Follicular PUFA concentrations and oocyte maturation, fertilization, embryonic development, and the success of pregnancy.

In Table 2, The concentration of PUFAs in the follicular fluid of MII oocytes, immature oocytes (metaphase I or Germinal vesicle stage), fertilized oocytes (2PN) and unfertilized oocytes (metaphase II, metaphase I, or Germinal vesicle stage oocytes) are shown. LA concentration was significantly lower in follicular fluid of metaphase II oocytes than in immature oocytes. GLA concentration in follicular fluid of fertilized oocytes was significantly higher than in nonfertilized oocytes. EPA and DPA concentrations were significantly lower in follicular fluid of fertilized oocytes compared to nonfertilized oocytes. DHA in the follicular fluid of fertilized oocytes was lower than in nonfertilized oocytes, but the difference was not statistically significant. There was no significant difference in PUFA concentration between good-quality and poor-quality blastocysts. PUFA concentrations in follicular fluids of pregnancy-positive and pregnancy-negative embryos were similar (Table 3).

Table 2. The concentration of PUFAs in the follicular fluid of MII oocytes, immature (MI or GV) oocytes, fertilized oocytes and Unfertilized oocytes

PUFAs		MII (n = 127)	MI/GV (n = 13)	p	Fertilized oocytes (n = 90)	Unfertilized oocytes (n = 40)	p
Linoleic acid	C18:2ω6	230.3 ± 40.2	257.9 ± 50.1	0.019	231.1 ± 48.1	233.9 ± 37.1	0.71
γ-Linolenic acid	C18:3ω6	1.05 ± 0.45	0.96 ± 0.31	0.27	1.12 ± 0.45	0.9 ± 0.39	0.007
Dihomo-γ-linolenic acid	C20:3ω6	15.2 ± 3.8	14.3 ± 4.4	0.39	15.5 ± 3.6	14.4 ± 4.4	0.16
Arachidonic acid	C20:4ω6	75.3 ± 17.5	80.5 ± 21.2	0.34	75.4 ± 18.1	77.6 ± 2.6	0.51
Docosatetraenoic acid	C22:4ω6	5.2 ± 2.3	5.7 ± 4.7	0.7	5.4 ± 2.6	5.3 ± 3.1	0.8
Eicosadienoic acid	C20:2ω6	4.34 ± 0.88	4.4 ± 0.94	0.78	5.4 ± 2.6	5.3 ± 3.1	0.8
α-Linolenic acid	C18:3ω3	3.04 ± 0.86	3.26 ± 0.26	0.24	3.0 ± 0.89	3.0 ± 0.81	0.96
Eicosapentaenoic acid	C20:5ω3	8.6 ± 3.6	9.2 ± 3.5	0.54	8.2 ± 3.5	10.1 ± 3.6	0.004
Docosapentaenoic acid	C22:5ω3	5.2 ± 1.3	6.1 ± 2.3	0.12	5.1 ± 1.3	5.7 ± 1.8	0.036
Docosahexaenoic acid	C22:6ω3	40.2 ± 7.4	42.9 ± 11.1	0.34	39.9 ± 6.4	42.9 ± 10.7	0.09

Table 3: The concentration of PUFAs in the follicular fluid of good and poor-quality blastocysts, pregnancy-positive and pregnancy-negative embryos.

PUFAs		Good-quality blastocysts (n = 15)	Poor-quality blastocysts (n = 28)	p	Pregnancy-positive (n = 21)	Pregnancy-negative (n = 14)	p
Linoleic acid	C18:2ω6	234.3 ± 60.3	211.3 ± 35.5	0.2	215 ± 43.1	231.6 ± 43.7	0.2
γ-Linolenic acid	C18:3ω6	1.4 ± 0.5	1.1 ± 0.5	0.1	1.2 ± 0.54	1.0 ± 0.2	0.08
Dihomo-γ-linolenic acid	C20:3ω6	16.9 ± 3.3	15.4 ± 3.1	0.2	17.2 ± 4.2	15.1 ± 5.4	0.17
Arachidonic acid	C20:4ω6	80.5 ± 13.5	71 ± 20.2	0.1	75.9 ± 20.4	75.1 ± 14.9	0.89
Docosatetraenoic acid	C22:4ω6	6.2 ± 3.2	5.5 ± 2.8	0.5	5.9 ± 3.3	5.3 ± 2.5	0.5
Eicosadienoic acid	C20:2ω6	4.7 ± 1.25	4.57 ± 1.12	0.8	4.4 ± 0.1	4.4 ± 1.2	0.47
Alpha-Linolenic acid	C18:3ω3	2.9 ± 1.1	2.9 ± 1.0	1	3.0 ± 0.9	2.8 ± 0.9	0.41
Eicosapentaenoic acid	C20:5ω3	8.6 ± 3.9	7.2 ± 3.2	0.5	7.2 ± 2.6	8.1 ± 4.1	0.47
Docosapentaenoic acid	C22:5ω3	5.5 ± 1.5	5.1 ± 1.3	0.4	5.3 ± 1.5	5.1 ± 1.8	0.79
Docosahexaenoic acid	C22:6ω3	36.9 ± 35.9	39.7 ± 5.0	1	38.2 ± 7.3	42.8 ± 8.6	0.17

Discussion

Notably, our study discovered that the concentrations of PUFAs in follicular fluid were only one-third of those in the serum. In studies on dairy cows, follicular fluid FA levels were lower but correlated significantly with serum FA levels [6, 7]. Similarly, serum triacylglycerol and free FA concentrations correlated with follicular fluid concentrations, but their values were lower in follicular fluid than in serum [8]. Valcyx et al. also found significant associations indicating that metabolic changes in the serum are reflected in follicular fluid, potentially affecting oocyte quality. A previous study reported weak correlations between serum and follicular fluid levels of palmitoleic acid, EPA, DHA, and AA [5]. Similarly, our results showed a correlation between follicular fluid and serum concentrations of EPA and AA. This suggests that serum collection could replace highly invasive follicular fluid collection for these FAs.

In this study, follicular fluid of immature oocytes had a significantly higher LA concentration than metaphase II oocytes. LA has been reported to be associated with meiosis and oocyte maturation in bovine oocytes [9] and may play a role in regulating bovine oocyte maturation; high LA concentrations may be a contributing factor to oocyte arrest at the germinal vesicle stage [10]. Therefore, high LA levels indicate impaired egg maturation. In animals and humans, LA used in AA biosynthesis undergoes a biological reaction called the AA cascade, forming prostaglandins (PGs), leukotrienes and thromboxane [11]. LA supplementation resulted in a marked increase in prostaglandins E2 (PGE2) and F2α (PGF2α) concentrations in spent media [10], suggesting an ability of cumulus cells to metabolize LA and PGE2 [12].

The 2-series PGs derived from AA are generally considered to be more biologically active than 1- and 3-series PGs. PGE2 mediates many important reproductive processes, including ovulation, implantation, and luteal function. Increased availability of PGE2 during maturation leads to increased gene expression associated with oocyte competence and improves the quality of blastocysts produced. Additionally, PGE2 has an important role in oocyte protection against oxidative stress [13]. Therefore, investigating whether low LA levels in follicular fluid obtained from MII oocytes indirectly indicates that PGE2 was produced from consumed LA, is necessary.

Fertilized follicular fluid had significantly higher GLA levels, which is n-6 PUFA, and lower EPA and DPA levels, which are n-3 PUFA. Meanwhile, there was no significant difference in PUFA concentration in follicular fluid of good- and poor-quality blastocysts. Moreover, contrary to our expectations, our findings showed no significant difference in PUFA concentrations in follicular fluid of pregnancy-positive and pregnancy-negative embryos. In previous studies, n-3 PUFA intake has been shown to improve oocyte and embryo qualities in animal and human studies. Nehra et al. demonstrated that short-term dietary intake of an n-3 PUFA-rich diet is associated with improved murine oocyte quality [14]. Another study assessed the effect of preconception maternal diet, specifically the FA profile, on pregnancies and live births following IVF and reported that LA and n-6 PUFA intake are associated with improved pregnancy rates in overweight and obese women [15]. Chiu *et al.* reported that high levels of total n-3 PUFAs and EPA in serum are associated with a higher probability of pregnancy and live birth [16]. In two preconception cohort studies of dietary fat intake and fecundability, low n-3 FA intake was associated with reduced fecundity [17,18]. The PREPARE trial, a randomized controlled trial investigating whether a drink high in EPA, DHA and the recommended dose of vitamin D, in combination with increased intake of olive oil for 6 weeks before IVF, altered morphokinetic markers of early embryo development and showed that a short period of dietary omega-3 FAs and vitamin D supplementation improved embryo quality [18]. However, other studies have yielded conflicting results. Wakefield et al. reported that maternal dietary supply high in PUFAs during *in-vivo* fertilization in mice could alter the morphological appearance of the embryo and lead to decreased developmental ability in the blastocyst stage [19]. High PUFA levels resulted in altered mitochondrial distribution and calcium levels, and increased reactive oxygen species. The Wakefield study suggests that high maternal dietary n-3 PUFA exposure reduces normal embryo development in mice and is associated with altered mitochondrial metabolism. Jungheim et al. reported that elevated serum ALA levels are associated with a decreased chance of pregnancy after IVF. Addition of DHA in the ***in-vitro maturation*** (IVM) medium may benefit the development of pig oocytes, but EPA appears to be cytotoxic [20]. On the other hand, there are few reports on PUFA concentration in human follicular fluid and IVF [4, 5]. Shaaker et al. demonstrated that LA is positively correlated with fertilization percentage while the AA level is negatively correlated [4]. It is still unclear whether PUFAs in human follicular fluid have a positive impact on fertilization, embryo quality, and even pregnancy.

In our study, pregnancy outcomes were identified by single ET. Each individual follicle was aspirated independently and matched to an oocyte. The strength of this paper was that PUFA levels in follicular fluid directly correlated with pregnancy success of embryos obtained from the follicle. The limitation of this study was the small sample size.

Conclusions

LA in follicular fluid affected oocyte maturation, and EPA and DPA affected fertilization. However, PUFAs did not have any effect on post-fertilization embryo quality or pregnancy.

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Declaration of Interest

The authors declare no conflicts of interest.

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