

Follicular fluid high density lipoprotein-associated micronutrient levels are associated with embryo fragmentation during IVF

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Received: 28 September 2009 / Accepted: 4 November 2009 / Published online: 18 November 2009
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Abstract

Purpose To investigate whether follicular fluid lipid-soluble micronutrients are associated with embryo morphology parameters during IVF.

Methods Follicle fluid and oocytes were obtained prospectively from 81 women. Embryo morphology parameters were used as surrogate markers of oocyte health. HDL lipids and lipid-soluble micronutrients were analyzed by high-pressure liquid chromatography. Non-parametric bivariate analysis and multivariable ordinal logistic regression models were employed to examine associations between biochemical and embryo morphology parameters.

Results Follicular fluid HDL cholesterol ($r=-0.47$, $p<0.01$), α -tocopherol ($r=-0.41$, $p<0.01$), δ -tocopherol ($r=-0.38$, $p<0.05$) and β -cryptoxanthine ($r=-0.42$, $p<0.01$) are negative-

ly correlated with embryo fragmentation. Ordinal logistic regression models indicate that a 0.1 $\mu\text{mol/L}$ increase in β -cryptoxanthine, adjusted for γ -tocopherol, is associated with a 75% decrease in the cumulative odds of higher embryo fragmentation ($p=0.010$).

Conclusion Follicular fluid HDL micronutrients may play an important role in the development of the human oocyte as evident by embryo fragmentation during IVF.

Keywords High density lipoprotein · Tocopherols · Carotenoids · Embryo fragmentation · In vitro fertilization

Introduction

Human ovarian follicular fluid (FF) contains high density lipoprotein (HDL) as the predominant lipoprotein with only traces of other lipoproteins [1, 2]. The importance of HDL in mammalian female reproduction is demonstrated by scavenger receptor class B group I, knockout (SR-BI KO) female mouse-generated embryos which arrest due to aberrant HDL metabolism [3–6]. In clinical studies, we have demonstrated negative correlations between FF HDL cholesterol and ApoAI levels with embryo fragmentation during in vitro fertilization (IVF) [7, 8]. These observations prompted us to further examine particular FF HDL components and examine their association with embryo morphology parameters during IVF. Since HDL is the major FF lipoprotein, we hypothesized that HDL is also the major carrier of lipid-soluble vitamins and micronutrients in FF. Our objective was to measure the lipid-soluble vitamin and micronutrient content of FF and to examine their associations with embryo morphology parameters in women undergoing IVF.

Presented in part at the American Society of Reproductive Medicine Annual Meeting in San Francisco, November 2008.

Capsule Follicular fluid lipid-soluble vitamins and micronutrients are correlated with HDL and may explain the observed association between HDL and embryo morphology parameters during IVF.

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Materials and methods

Subjects and samples

Eighty-seven female patients undergoing IVF treatment at the UCSF Center for Reproductive Health were recruited into this study with full informed consent. The study protocol and consenting process were approved by the UCSF Committee on Human Research. Table 1 describes the demographic characteristics of the study participants. Participants underwent gonadotropin-induced ovarian stimulation per clinic protocols as described previously [7]. FF from an individual mature 18–20 mm follicle was aspirated and processed immediately via centrifugation at 700 g for 10 min at room temperature and the straw-colored FF supernatant was cryopreserved in 1.8 cc aliquots at -80°C . Individual FF analytes were tracked to corresponding oocytes and embryos to examine the association between analyte levels and embryo outcomes.

IVF protocol

Intracytoplasmic sperm injection (ICSI) was performed in most cases (~87%) based on abnormal sperm parameters from prior semen analyses. All metaphase-II oocytes were fertilized on the day of oocyte collection with a previously-cryopreserved sperm specimen from the male partner or sperm donor. Zygotes were identified approximately 16–18 h following insemination, by the appearance of two pronuclei (2PN). All embryos were cultured individually in 5% CO_2 with filtered room air in 25 μL of G1™ culture media (Vitrolife, Inc. Englewood, CO USA) with 10% synthetic serum substitute (SSS) and Ovoil™ (Vitrolife Inc.) paraffin oil overlay. Embryo morphologic indices included individual embryo cell number (ECN) and individual embryo fragmentation score (EFS), each assessed on the day of embryo transfer. Experienced embryologists making

embryo morphology assessments were blinded to any biochemical results and the laboratory technicians were blinded to clinical outcome data. EFS was defined as: grade 1, 0% fragmentation; grade 2, 1–10% fragmentation; grade 3, 11–25% fragmentation, grade 4, 26–50% fragmentation; and grade 5, 51% or greater fragmentation.

Laboratory methods

FF HDL fractions were prepared by selective precipitation methods as previously described [7] since ultracentrifugation is known to alter lipoprotein structure [9]. Vitamin A, vitamin E (α , γ and δ tocopherol) and carotenoids (β -carotene, β -cryptoxanthin, lycopene, lutein and zeaxanthin) were measured simultaneously by HPLC [10]. Lutein and zeaxanthin were quantified together as a single peak and reported as a total lutein/zeaxanthin. Apolipoprotein-AI (ApoAI), Apo B-100, cholesterol, phospholipids and triglycerides were measured using diagnostic reagent kits from Wako Diagnostics Inc. (Richmond, VA, USA) adapted to the Cobas Fara II automated chemistry analyzer (Hoffmann–La Roche Banal, Switzerland). All analyses were performed in duplicate.

Data analysis

Non-parametric statistical tests and models were employed using SAS 9.1.3 (SAS Institute, Inc. Cary, NC, USA). Statistical significance was defined as $P < 0.05$ for a two-tailed test. No correction for potential inflation of type-1 error due to the conduct of multiple statistical tests was incorporated. Spearman rank correlation coefficients were used to assess associations among and between FF HDL components and embryo morphologic indices. Using a stepwise procedure and a model building algorithm, to accommodate potential interactions and confounders, individual multivariable ordinal logistic regression models were

Table 1 Characteristics of study participants by demographic, treatment, and outcome ($n=81$)

Demographic covariates	Mean	SD	Median	Min.	Max.	Treatment covariates	<i>n</i>	%
Age (years)	36.2	5.9	37.7	20.9	44.0	Prior IVF cycle ^b	39	50.0
BMI (kg/m^2) ^a	23.5	3.4	22.6	18.1	35.4	Diagnosis ^c		
Patient ethnicity	<i>n</i>	%				Unexplained	20	25.3
Caucasian	45	55.6	–	–	–	Male factor	23	29.1
Asian (East Asia)	22	27.2	–	–	–	DOR	15	19.0
Unknown	9	11.1	–	–	–	Donor oocyte	10	12.7
Hispanic	2	2.5	–	–	–	Tubal Factor	4	5.1
African American	1	1.2	–	–	–	Endometriosis	4	5.1
Asian (Indian)	2	2.5	–	–	–	Recurrent SAB	2	2.5
						Anovulation	1	1.3
						ICSI	66	81.5

^a $n=5$ missing values; ^b $n=3$ missing values; ^c $n=2$ missing values; BMI body mass index; IVF in vitro fertilization; DOR diminished ovarian reserve; SAB spontaneous abortion; ICSI intra-cytoplasmic sperm injection

Table 2 Distributions of follicular fluid HDL components and correlations with HDL cholesterol, embryo fragmentation score (EFS) and embryo cell number (ECN)

Measured analytes	Units	<i>n</i>	Median	IQR	r_{HDLc}	<i>n</i>	r_{EFS}	r_{ECN}^a
HDLc	mmol/L	81	0.57	0.34	–	39	–0.47**	–0.02
ApoAI	g/L	81	1.05	0.22	0.25*	39	–0.25	0.13
HDL-PL	mmol/L	80	0.78	0.15	0.55**	39	–0.18	0.03
HDL-TG	mmol/L	79	0.07	0.06	–0.01	38	0.07	–0.13
Retinol	umol/L	80	0.01	0.03	0.47**	39	–0.08	–0.01
α -tocopherol	umol/L	80	5.50	3.31	0.66**	39	–0.41**	0.09
γ -tocopherol	umol/L	67	1.00	0.85	0.23	31	–0.34	–0.01
δ -tocopherol	umol/L	74	0.34	0.58	0.40**	38	–0.38*	–0.04
β -carotene	umol/L	80	0.11	0.14	0.16	39	–0.22	0.07
β -cryptoxanthin	umol/L	80	0.07	0.07	0.37**	39	–0.42**	0.08
Lycopene	umol/L	80	0.21	0.14	0.52**	39	–0.24	–0.16
Lutein and zeaxanthin	umol/L	80	0.12	0.07	0.42**	39	–0.26	0.18

^a Adjusted for day of embryo transfer; * $p < 0.05$; ** $p < 0.01$ (2-tailed). r (Spearman rank correlation coefficient with HDLc, EFS or ECN); *IQR* interquartile range; *ApoAI* (apolipoprotein AI); *HDLc* (high density lipoprotein cholesterol); *PL* (phospholipids); *TG* (triglycerides)

generated [11]. Outcomes comprised tertiles of EFS (1/ 2/ 3–5; $n = 13/ 15/ 11$) or ECN limited to day-three transfers (4–5 cells/ 6–7 cells/ 8–11 cells; $n = 4/ 10/ 16$). Model fit and the proportional odds assumption were evaluated throughout multivariable modeling, and missing values were imputed using an EM algorithm.

Results

Oocytes were retrieved from the sampled ovarian follicle in 83 of the 87 study participants undergoing IVF. FF volume collected from two participants was insufficient for any analysis therefore 81 participants with harvested oocytes and sufficient FF volume for analysis comprised the final study sample. Among all study oocytes 83.9% ($n = 68$) were arrested in Metaphase-II and 66.2% ($n = 45$) of these mature oocytes were normally fertilized (2PN) by either conventional IVF or intracytoplasmic sperm injection (ICSI). Sample size varied for certain analytes due to the limited volume of FF collected during this pilot study. The complete demographic, treatment and outcome variables for the study sample are described in Table 1. Table 2 presents the distributions of the absolute levels of all study analytes in FF as well as the correlation of each variable

with FF-HDL cholesterol, EFS and ECN adjusted for day of transfer.

Except for β -carotene and γ -tocopherol, all FF fat-soluble vitamins and micronutrients are significantly positively correlated with FF HDL cholesterol concentration. With this expansion of the dataset from our prior report (8), FF total HDL cholesterol remains negatively correlated with EFS ($r = -0.47$, $P = 0.003$). FF concentrations of α -tocopherol ($r = -0.41$, $P = 0.009$), δ -tocopherol ($r = -0.38$, $P = 0.017$) and β -cryptoxanthine ($r = -0.42$, $P = 0.008$) are also negatively correlated with EFS. While other FF micronutrients also correlate negatively with EFS, these do not approach statistical significance ($P > 0.10$). No meaningful correlations are detected between ECN, adjusted for day of embryo transfer, and FF analytes ($P > 0.15$).

Table 3 presents the ordinal logistic regression model for EFS in which a 0.1 $\mu\text{mol/L}$ increase in FF β -cryptoxanthin concentration is associated with a statistically significant ($P = 0.010$) decrease of 75% in the cumulative odds (odds ratio = 0.25) for an increased EFS, conditional on FF γ -tocopherol. FF γ -tocopherol is itself significantly associated with a 66% decrease in the cumulative odds (odds ratio = 0.34) for an increased EFS ($P = 0.035$) for each one 1.0 $\mu\text{mol/L}$ increase in concentration. There are no significant predictors of ECN.

Table 3 Ordinal logistic regression of higher embryo fragmentation score (EFS) on follicular fluid β -cryptoxanthine and γ -tocopherol concentrations ($n = 39$)

	β	95% CI Lo	95% CI Hi	<i>P</i> -value	unit Δ	OR
Intercept 2	1.29	–0.32	2.90	0.117	–	–
Intercept 1	3.44	1.45	5.43	0.001	–	–
β -cryptoxanthine	–1.37	–2.41	–3.34	0.010	0.1 $\mu\text{mol/L}$	0.25
γ -tocopherol ^a	–1.07	–2.06	–0.07	0.035	1.0 $\mu\text{mol/L}$	0.34

^a $n = 8$ missing values imputed employing an EM algorithm. β , slope; *CI* confidence interval; *OR* odds ratio; Δ , unit of change

Discussion

In summary, we extend our initial observations [7, 8] with additional evidence in support of a role for the HDL particle in the developmental potential of the human oocyte during early embryogenesis. We have now demonstrated that multiple components of FF HDL particles including cholesterol, ApoAI, and the micronutrients, β -cryptoxanthine and γ -tocopherol, represent unique clinical predictors of embryo fragmentation during IVF. From these results it is unclear whether the HDL particles themselves or co-linear HDL particle components such as β -cryptoxanthin and α -tocopherol are responsible for the protective effect against embryo fragmentation. It is clear that the strong correlations between HDL and lipophilic micronutrient levels in FF prompt all future studies to consider adjusting for associated covariates during statistical analyses. While previous studies have not demonstrated significant clinical effects associated with carotenoids, retinoids, or tocopherols, it is not clear that either study analyzed the relationship with embryo morphology parameters [12, 13]. Consistent with Schweigert et al. we observe similarly reduced levels of micronutrients in FF compared to plasma [13]. Our observations suggest that numerous lipophilic components of HDL particles including micronutrients may be influencing the membrane and cytoplasmic properties of the oocyte with downstream effects on embryo fragmentation occurring during in vitro cytokinesis.

There are several notable limitations to this study: (1) we cannot make any conclusions regarding a role for sperm factors even though we considered total motile sperm count in our multivariate analyses; and (2) these findings are simply associations and do not necessarily imply causal effect. We acknowledge that more detailed human and mammalian studies need to be performed in order to assess causality.

To what extent the constitution of the FF HDL particle is a reflection of intra-follicular metabolic processing versus extra-follicular modeling is not known. Our understand of FF HDL metabolism during folliculogenesis and oocyte development is limited and further complicated by the multiple proteins and lipids that interact with and contribute to the biological roles of HDL particles [8]. Given the relative predominant existence of HDL particles in FF during folliculogenesis, it remains to be determined if these lipophilic micronutrients are influencing oocyte development independent of intra-follicular cholesterol and phospholipid transport mechanisms [8]. Our findings raise interesting questions regarding the biological mechanisms that are directing apparent effects of HDL-associated micronutrients on oocyte development and competence. Furthermore, the negative correlations between multiple FF HDL components and embryo fragmentation provide reasons to consider HDL particle composition as a useful biomarker of embryo fragmentation during IVF.

Acknowledgement We acknowledge the assistance of Julia Sandler, Giulia Conti, and Natasha Narayan and the support of the physicians and staff of the UCSF Center for Reproductive Health, San Francisco, CA.

Funding This work was not supported by any grant funding. Institutional discretionary research funds available to Drs. Browne and Fujimoto were used to support this work.

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