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CHAPTER
The anti-inflammatory function of follicular fluid HDL and outcome of modified natural cycle-in vitro fertilization

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(letter to the editor)
INTRODUCTION

Cholesterol homeostasis plays an important role in oocyte development and fertility (1). In follicular fluid (FF), the environment surrounding the developing oocyte, high density lipoproteins (HDL) are the predominant carriers of cholesterol (2, 3). Within the cardiovascular field the focus of research is currently changing from measuring the static biomarker HDL cholesterol (HDL-C) towards determining functional properties of HDL particles (4). With respect to the field of reproductive medicine, an interesting recent study in mice showed that a decreased cholesterol efflux function of FF-HDL results in impaired fertility (5). However, next to promoting cholesterol efflux HDL particles also have potent anti-inflammatory properties (4, 6). Accumulating evidence indicates that inflammatory disorders alter the composition of FF and result in infertility due to reduced oocyte quality (7, 8). Conceivably, HDL present in FF might modulate the local inflammatory state within the follicle. However, currently, to the best of our knowledge, no data exist exploring such a hypothesis either in humans or preclinical models. Therefore, the present study was designed (i) to characterize anti-inflammatory properties of FF-HDL in relation to systemic HDL and (ii) to determine if the anti-inflammatory function of FF-HDL is associated with outcomes of modified natural cycle-in vitro fertilization (MNC-IVF), a procedure close to normal human reproductive physiology (9).

METHODS

The study included 326 MNC-IVF cycles from 198 patients collected during either routine medical care (August 2013 – July 2014) or an observational cohort study to relate nutrition, biomarkers and MNC-IVF outcomes (October 2014 – March 2018, Netherlands Trial Register number NTR4409). For further details on cohort and MNC-IVF procedure, please see (9) and supplementary methods. Inclusion criteria were: growth of a single dominant follicle; retrieval of one oocyte; minimal macroscopic blood contamination; maximum cycle number of six. Medical ethics committee approval was requested, but waived, since FF is considered waste material. Patients consented to blood draws. Top quality embryos were defined as two pronuclei on day 1 and four cells, no multinucleated blastomeres and less than 20% fragmentation on day 2. Single embryo transfer took place on day 2 and the occurrence of pregnancy was confirmed by a positive serum hCG test two weeks later. The anti-inflammatory capacity of HDL was assessed as the ability to inhibit tumor necrosis factor-α induced VCAM-1 mRNA expression in endothelial cells in vitro (higher values indicate higher anti-inflammatory capacity), as previously published (6) using 2% (v/v) individual apoB-depleted plasma
or FF samples or phosphate buffered saline (PBS) (Online supplement). Results are expressed as median [interquartile range]. Wilcoxon Signed Ranks test, Spearman correlations and multilevel analysis using generalized estimating equations (GEE) were applied as appropriate (SPSS 23) (Supplementary methods). A P value <0.05 was considered statistically significant.

RESULTS AND DISCUSSION

Median age throughout all cycles was 31.9 [29.3-33.8] years and BMI 23.3 [21.1-26.1] kg/m² (27% overweight, 4.9% obese). Prior to IVF treatment, in 50.3% of cycles alcohol was consumed regularly and in 9.8% the patient was a smoker. Median subfertility duration was 35.6 [22.3-50.4] months. In 19 randomly selected first cycle MNC-IVF patients, FF-HDL anti-inflammatory capacity was comparable to that of matched plasma HDL (FF: 13.8% [3.4-24], plasma: 17.4% [8.9-25], P=0.872). However, no significant correlation between the anti-inflammatory capacity of the two matrices was found (r=0.098, P=0.689). In order to explore to what extent FF-HDL contributes to the whole FF anti-inflammatory capacity, eight patients undergoing first cycle MNC-IVF were randomly selected. Compared with whole FF, FF-HDL isolated from these samples by fast protein liquid chromatography had a significantly higher anti-inflammatory capacity (15% [12-19] versus 20% [16-23] reduction in VCAM-1 mRNA related to the full TNF-α-induced induction, P=0.012) indicating that HDL might be a physiologically relevant contributing factor to the protective function of FF against inflammation. In all 326 MNC-IVF cycles a higher FF-HDL anti-inflammatory function was related to an increased chance of the oocyte to develop into a top-quality embryo in unadjusted analyses (table). This result remained significant also after adjustment for age, BMI, smoking, alcohol and fertilization method (odds ratio per % increase in anti-inflammatory function: 1.02 [95% confidence interval: 1.00-1.03], per standard deviation (16.62%) increase in anti-inflammatory function: 1.33 [1.06-1.68], P=0.016; table). No significant relationship with the occurrence of pregnancy was found.

Taken together, the results of the present study indicate that, in addition to a role in cholesterol transport, FF-HDL have anti-inflammatory properties that, at least under the assay conditions used in the present work, positively associate with certain early developmental parameters of the oocyte. FF-HDL are considered to be largely derived from plasma (1, 2, 5), however, they differ in size and composition compared to plasma HDL by containing less cholesterol and more phospholipids (2). In our study, the lack of a relationship between the anti-inflammatory capacities of FF and matched plasma HDL indicates that FF may contain anti-/pro-inflammatory factors specific to the ovarian environment.
Table 1: Generalized estimating equations analysis of the relationship between embryo development in Modified Natural Cycle-IVF/ICSI and follicular fluid anti-inflammatory capacity

| Follicular fluid anti-inflammatory capacity (%) | Unadjusted model | Adjusted model |
|-----------------------------------------------|------------------|----------------|
|                                               | Odds ratio [95% CI] | P value     | Odds ratio [95% CI] | Upper: P value |
|                                               | Upper: per 1% increase | Lower: per SD increase | Upper: per 1% increase | Lower: per SD increase |
| Top quality embryo                           |                  |               |                  |               |
| Yes (n = 106)                                | 15.28 [5.38 – 26.04] | 1.01 [1.00-1.03] | 0.031            | 1.02 [1.00-1.03] | 0.016 |
| No (n = 199)                                 | 12.36 [0.14 – 24.64] | 1.26 [1.02-1.55] |                  | 1.33 [1.06-1.68] |
| Pregnancy                                    |                  |               |                  |               |
| Yes (n = 55)                                 | 13.53 [4.33 – 30.14] | 1.00 [0.99-1.02] | 0.741            | 1.01 [0.99-1.03] | 0.342 |
| No (n = 161)                                 | 13.60 [4.83 – 24.60] | 1.05 [0.77-1.44] |                  | 1.17 [0.85-1.62] |

Adjusted models include age, BMI, smoking, alcohol, method (for top quality embryo); smoking, alcohol, duration of subfertility (for pregnancy). One standard deviation equals 16.62% anti-inflammatory capacity. Bold values: P<0.05. CI, confidence interval. SD, standard deviation.
It is unclear at present, if the differences in anti-inflammatory function between the matrices is due to remodelling occurring during the passage of plasma HDL into the follicle, or if anti-/pro-inflammatory factors are produced locally by granulosa cells, theca cells or the oocyte, which then associate with FF-HDL particles. Important advantages of our study are the prospective design and the use of MNC-IVF, which is closer to normal physiology compared with classical hyperstimulation IVF and allows for an individual correlation of FF composition with single oocyte and embryo characteristics. Potential limitations are that patients were from a single centre and that only one selected anti-inflammatory function was tested and not complete pro/anti-inflammatory profiles generated. However, the current study indicates that such studies would be worthwhile to be carried out. Further, no relationship between FF-HDL anti-inflammatory capacity and pregnancy was found, likely due to the fact that pregnancy is the result of multiple factors, including endometrial and spermatozoa function. Additional research is necessary to explore the mechanism behind the FF-HDL anti-inflammatory capacity (e.g., quantify FF-HDL components that exert anti-inflammatory function), since such an approach offers the potential to identify not only clinically relevant biomarkers for natural and assisted reproduction but also potential targets for therapeutic intervention.

SUPPLEMENTARY METHODS

HDL isolation by precipitation of apolipoprotein B-containing lipoproteins
HDL was isolated by precipitating apolipoprotein B-containing lipoproteins according to a previously published method used by us and others for HDL function studies (1-4). Briefly, 50 µl polyethylene glycol-6000 (Sigma-Aldrich, Germany) in 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) at pH 8.0 was added to 100 µl plasma or follicular fluid followed by mixing and incubation on ice for 30 min. Subsequently, samples were centrifuged at 2000 g at 4°C for 30 min. HDL-containing supernatants were transferred to clean tubes and kept on ice for immediate use in the HDL function assay. We and others established before that this method results is suitable to fully recover the HDL fraction (5, 6).

Modified natural cycle-IVF procedure
Modified natural cycle-IVF procedures took place following standard protocol (7). Beginning at menstrual cycle days 6-8, follicular growth was closely followed by ultrasound, in parallel with regular measurements of serum hormone levels (luteinizing hormone and estradiol). When the dominant follicle reached a diameter of 14 mm, the patients started with daily injections of 0.25 mg cetrorelix, a GnRH antagonist.
(Cetrotide®, Merck BV, The Netherlands) up to the day of ovulation triggering (including the day itself) and daily injections of 150 IU Follitropin-alpha, a recombinant form of FSH (Gonal-F®, Merck BV, The Netherlands) up to the day of ovulation triggering. When the dominant follicle reached a diameter of 18 mm or serum estradiol levels surpassed 0.8 nmol/L, patients were administered 10 000 IU hCG (Pregnyl®, Organon, The Netherlands) in order to induce ovulation. The oocyte was retrieved from the dominant follicle approximately 34 hours later by ultrasound-guided transvaginal follicle aspiration. A single-lumen aspiration needle was used and the follicle was not flushed. If an oocyte was obtained, it was subsequently inseminated within 6 hours following standard procedure (incubation with spermatozoa-containing culture medium or intracytoplasmatic sperm injection).

**HDL anti-inflammatory function assay**

The anti-inflammatory capacity of HDL was assessed as the ability to inhibit tumour necrosis factor-α induced VCAM-1 mRNA expression in endothelial cells in vitro (higher values indicate higher anti-inflammatory capacity), as previously published (1). Briefly, human umbilical vein endothelial cells, pooled from different donors, were provided by the Endothelial Cell Core Facility of the University Medical Centre Groningen. Then 2% (v/v) of individual apoB-depleted plasma or FF samples or phosphate buffered saline (PBS) was added. After 30 min, cells were washed and incubated with 10 ng/ml tumour necrosis factor-α (TNF-α; R&D systems, Abingdon, UK) for 5 h followed by RNA extraction and determination of VCAM-1 mRNA expression with quantitative real-time PCR. Then the percent reduction in VCAM-1 mRNA expression relative to the response of TNF-α stimulated cells without added HDL was calculated.

**Statistical analysis**

Results are expressed as median [interquartile range]. Differences in anti-inflammatory capacity between FF and matched plasma and between unfractionated FF and FF-HDL were analysed by Wilcoxon Signed Ranks test, and their association expressed as Spearman’s r.

Multilevel generalized estimating equations (GEE) was used in order to study the relationship between FF-HDL anti-inflammatory capacity and outcomes of MNC-IVF. Initial models were subsequently adjusted for confounders that were previously shown in literature to impact embryo quality and pregnancy (specifically maternal BMI, age and smoking status), as well as for MNC-IVF characteristics that were significantly related to anti-inflammatory function or fertility outcome variables (GEE P value cut-off: 0.15). In order to prevent overfitting of adjusted models, predictors were removed one-by-one.
based on P value until the model included a maximum of ten cases per predictor. The results of the GEE analysis are given as odds ratio (95% confidence interval).

All analyses were done with SPSS 23, a P value <0.05 was considered statistically significant.

**ADDITIONAL INFORMATION**

**Key words**
High density lipoprotein, anti-inflammatory activity, fertility, follicular fluid, embryo quality

**Abbreviations**
FF, follicular fluid; MNC-IVF, modified natural cycle-in vitro fertilization; HDL-C, high density lipoprotein cholesterol; CVD, cardiovascular disease; HUVECS, Human umbilical vein endothelial cells; TNF-α, tumour necrosis factor-α; VCAM-1, vascular cell adhesion molecule-1; GEE, generalized estimating equations; NO, nitric oxide; PBS, phosphate buffered saline.
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