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Research Note

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Full title:

Leukocyte count in follicular aspirate samples could be a prognostic biomarker of the developmental competence of human metaphase II oocytes

Takayuki Ishikawa[1,2], Masakazu Doshida[1], Toshihiko Kyoya[1], Taeka Takisawa[1], Masahiro Sakurai[2], Yumi Hoshino[2], Kentaro Tanemura[2], Koichi Kyono[1], and Eimei Sato[2]

[1]Kyono ART Clinic, Sendai, Japan

[2]Laboratory of Animal Reproduction, Graduate School of Agricultural Science, Tohoku University, Sendai, Japan

Corresponding author:
Takayuki Ishikawa
Kyono ART Clinic, Reproductive Research Center, 3F
1-1-1, Honcho, Aoba-ku, Sendai 980-0014, Japan
Tel: +81 22-722-5991
Fax: +81 22-722-8840
E-mail: tishikawa@ivf-kyono.or.jp

Abstract

Multiple gestations markedly impact the health of children conceived via assisted reproductive technology (ART). The present study investigated the potential of leukocyte count in follicular aspirate (FA) samples as a follicular prognostic biomarker of the developmental competence of human metaphase II (MII) oocytes. FA samples were obtained from 27 ART patients during 27 natural menstrual cycles, Leukocytes were separated using density-gradient centrifugation and prepared the Diff-Quik stained smears. The 27 cycles were classified into 3 groups based on the developmental stage of the embryo (Group I, 7 oocytes (-); Group II, 6 unfertilized oocytes and 5 early cleavage embryos; Group III, 9 blastocysts) and evaluated estradiol, luteinizing hormone, and progesterone levels on the day of gonadotropin-releasing hormone agonist administration using enzyme immunoassay indicated that these levels did not correlate with developmental competence. The 27 cycles were divided into Categories A (19) and B (8) depending on the infiltrating leukocyte count in the FF (less than 2.5×10^4 cells/follicle, and the value or more, respectively). Further classification and analysis of the 19 cycles classified as Category A into 3 groups (Group I, 6 oocyte (-); Group II, 5 unfertilized oocytes and 3 early cleavage embryos; Group III, 5 blastocysts) showed no significant differences among the groups regarding infiltrating mononuclear leukocyte

(IML) count but a significantly increased infiltrating polymorphonuclear leukocyte (IPL) count in Group III compared to Groups I and II. These findings suggest that IPL count in FF is associated with the blastocyst formation of human MII oocytes, and thus it can be a predictor of the developmental competence of MII oocytes.

Key words: follicular fluid, human metaphase II oocytes, in vitro fertilization, infiltrating polymorphonuclear leukocyte

Introduction

Multiple gestations markedly impact the health and welfare of children conceived via assisted reproductive technology (ART). As twin pregnancies are associated with a higher risk of complications for both the mother and the fetus [1, 20, 21], reducing the incidence of multiple gestations is often desirable. One reasonable approach to reduce the disadvantage is single-embryo transfer (SET). With the development of SET, non-invasive and objective methods of oocyte/embryo selection have been required.

As oocyte maturation is controlled in the follicular microenvironment, age-related alteration of the microenvironment makes the human preovulatory oocyte susceptible to chromosomal disorders and cytoplasmic structural defects [8, 9, 25]. Because the follicular microenvironment is created by regulatory/metabolic factors present in the follicular fluid (FF) which is originated from follicular cells, detection of the existence of certain prognostic biomarkers in the FF might allow for prediction of oocyte viability. Higher levels of both follicular oxygen content [3, 23, 24] and vascular endothelial growth factor (VEGF) levels [14, 16] in FF have been correlated with the developmental competence of human oocyte/embryo during in-vitro fertilization (IVF) cycles. In addition, follicular concentrations of leptin [3, 7], nitric oxide (NO) [3], high-density lipoprotein (HDL) cholesterol, HDL component proteins, and HDL

micronutrients [5, 6] have been reported to play important roles for in human oocyte/embryo development.

Although several studies have examined the presence of leukocytes in follicular aspirate (FA) samples obtained at oocyte retrieval in IVF cycles to determine their leukocyte populations [4, 12, 13, 15, 22], these studies used different methods of preparing the FA samples, estimating the leukocyte subset, and stimulating ovaries of patients, making the comparison of their results, which often differed, a difficult task. On the other hand, in their investigation of the leukocyte/erythrocyte ratio in FA samples and the leukocyte profile of both FA samples and peripheral blood in patients on the day of ovum pick up (OPU), Smith *et al*. (2005) concluded that leukocytes infiltrate the pre-ovulatory follicles [22]. Despite this finding, the origin of leukocytes remains unclear as a consequence of blood vessel damage during OPU.

Due to the mixed nature of the findings and the challenges faced in IVF research, no definitive predictor of oocyte viability has become widely used in clinical settings. This study evaluated infiltrating leukocyte count in FA samples as a prognostic biomarker of the developmental competence of human metaphase II (MII) oocytes.

The purpose of this experimental study is to evaluate the infiltrating leukocyte count in FF by examining FA samples obtained in natural cycles and to assess the

potential of this variable as a predictor for developmental competence of human MII oocytes.

Materials and Methods

Patients

FA samples were obtained during the 27 natural menstrual cycles of 27 patients with an unknown infertility factor undergoing an IVF cycle at the study site between December 2008 and October 2009 with the patients' informed consent.

Semen preparation

Semen samples were prepared using Sil-Select Plus (FertiPro N.V., Beernem, Belgium) in accordance with the manufacturer's protocol. After gradient centrifugation, washing and swim-up preparation were performed. Routine semen analysis was then performed according to World Health Organization guidelines [26].

Oocyte retrieval

Patients underwent transvaginal ultrasound and serum hormone analysis for estradiol (E_2), luteinizing hormone (LH), and progesterone (P_4) levels using enzyme

immunoassay (Eclusys LH, E2 II and Progesterone II; Roche Diagnostics K.K, Tokyo, Japan). Hormone levels were measured from Day 13 to 16 of the menstrual cycle (Day 1: the first day of the menstrual cycle) on the day of gonadotropin-releasing hormone (GnRH) agonist (Buserecure; Fuji Pharma, Tokyo, Japan) administration. The schedule of oocyte retrieval was determined according to serum E_2, P_4, and LH levels. Final oocyte maturation was intranasally triggered with a GnRH agonist when the mean diameter of the dominant follicle was >18 mm under ultrasonography.

OPU was performed transvaginally at 36 h after GnRH agonist administration. For each cycle, the only dominant follicle was gently aspirated using a sterile syringe by a physician; no mechanical aspirators were used.

Conventional in-vitro fertilization and embryo culture

Conventional in vitro fertilization (cIVF) was performed with subsequent embryo culture [10]. In brief, all incubations were performed at 37°C in 6% CO_2, 5% O_2, and 89% N_2. Oocyte-cumulus complexes (OCCs) were inseminated at a final concentration of 1×10^5 motile sperm/ml at approximately 40 h post-trigger and incubated in Medi-Cult IVF medium (MediCult a/s, Jyllinge, Denmark) for 20 h. Inseminated oocytes were cultured in Global medium (Life Global, Guilford, CT, USA) from day 2

to 6 post-fertilization. Blastocysts were assessed by Gardner's criteria [11] and vitrified on day 5 or 6 using Cryotop (KITAZATO Bio Pharma, Fuji, Japan).

Leukocyte preparation

Leukocytes were separated from the FA samples using Polymorphrep (Axis-Shield, Oslo, Norway) in accordance with the manufacturer's protocol and centrifuged 2 times at 500 × *g*. After the leukocytes had been suspended in 100 μl of PBS, 1 μl suspensions were applied to prepare smears. The smears were fixed in methanol and stained with Diff-Quik (DQ) (CYSMEX, Kobe, Japan) in accordance with the manufacturer's protocol, with 2 separate smears prepared for each cycle. The stained smears were examined microscopically at × 400 or × 1,000 magnification and the leukocytes were classified as lymphocytes, neutrophils and eosinophils.

Erythrocyte and leukocyte counts in FA samples

Based on the erythrocyte count in the FA samples and the blood cell populations in the peripheral blood of the patients on the day of OPU, the putative peripheral blood volume and peripheral leukocyte count in the FA samples were estimated. The infiltrating mononuclear leukocyte (IML) count in the FF was determined by

subtracting the peripheral blood mononuclear leukocyte count from the mononuclear leukocyte count in the FA samples. The infiltrating polymorphonuclear leukocyte (IPL) count in the FF was determined using a same method. As the FA samples contained varying amounts of flashing medium in each cycle, the leukocyte counts were expressed in terms of mean ± standard deviation (SD)/follicle.

Grouping of cycles

The 27 cycles were classified into Category A (less than 2.5×10^4 cells/ follicle) and Category B (greater than 2.5×10^4 cells/follicle) depending on peripheral blood leucocyte count in the FA samples. The cycles that were classified as Category A were further classified into 3 groups based on the developmental stage of the embryo (Group I; no oocyte was retrieved, Group II; an unfertilized oocyte or an embryo at the early cleavage stage, and Group III; a blastocyst).

Statistical analysis

Significant differences in hormone levels were evaluated by non-repeated measures ANOVA. On the other hand, infiltrating leukocyte counts in FF among the experimental groups were evaluated by Tukey-Kramer method. P values < 0.01 were

considered to indicate statistical significance.

Results

Patient age and oocyte retrieval

The mean patient age was 33.6 ± 3.1 years (range: 27–38 years). OPU was performed for 27 cycles.

Conventional IVF and embryo culturing

In 20 of the 27 cycles, 1 oocyte was retrieved and cIVF was performed. As the results of cIVF and subsequent in-vitro culturing, 14 fertilized oocytes (14/20, 70.0%) developed to the early cleavage stage and 9 blastocysts (9/14, 64.3%) were obtained on day 5 or 6. All blastocysts were classified as 6 Grade 4-blastocysts and 3 Grade 3-blastocysts, and vitrified them in LN_2.

Serum hormone levels

The 27 cycles in which OPU was performed were classified into 3 groups based on the developmental stage of the embryos and statistically evaluated. As shown in Table 1, evaluation of E_2, LH, and P_4 levels indicated that these levels did not correlate

with the developmental competence.

Peripheral blood leucocyte count in FA samples

Peripheral blood leucocyte count (peripheral blood volume) in the FA samples was less than 2.5×10^4 cells/ follicle (< 4 µl) in 19 cycles (Category A) but greater than 2.5×10^4 cells/follicle (> 6.8 µl) in 8 cycles (Category B).

Infiltrating leukocyte count in FA samples

The 19 cycles that were classified as Category A were further classified into 3 groups based on the developmental stage of the embryos. As shown in Table 2, although there were no significant differences between Group II and III with regard to IML count, there was a significant increase in the IPL count of Group III compared to that in Groups I and II. While IPL count ranged from 0.78 to 1.59×10^4 cells/follicle in Group III cycles, it was less than 0.29×10^4 cells/follicle in all Group I and II cycles. Due to the large number of peripheral blood leucocytes in the FA samples in all category B cases and the difficulty in determining IPL count on the stained smears, IPL count could not be determined accurately for this category.

Discussion

Oocyte maturation is known to be controlled a follicular microenvironment that is composed of follicular cells and regulatory/metabolic factors present in the FF. Based on this knowledge, several investigators have investigated levels of O_2, VEGF, NO, leptin, HDL cholesterol, HDL component proteins, and HDL component micronutrients in FF as predictors of the developmental competence of human oocytes/embryos in IVF cycles [3, 5, 6, 7, 14, 16, 23, 24]. In contrast, other investigators have focused on the presence of leucocytes in FF to determine leukocyte populations and the number of leukocytes in FA samples obtained at OPU in IVF cycles. However, the investigators have obtained mixed results due to their use of different methods and examination of different cycle characteristics patient populations, and could not determine the origin of leukocytes as a result of the presence of erythrocytes in the FA samples. Despite these challenges, in their investigation of the leukocyte/erythrocyte ratio in FA samples and the leukocyte profile of both FA samples and peripheral blood in patients on the day of OPU, Smith *et al*. (2005) concluded that leukocytes infiltrate the pre-ovulatory follicles [22].

In recognition of these findings and challenges, all FA samples examined in this study were obtained from patients in natural cycles, and contamination of the FA samples by peripheral blood due to blood vessel damage during OPU was considered.

The putative peripheral blood volume was estimated based on erythrocyte count in both the FA samples and the peripheral blood, and the leukocyte count in the FA samples determined by subtracting the putative peripheral blood leukocyte count from the leukocyte count in the FA samples. Analysis of FA samples containing varying amounts of peripheral blood in which the peripheral blood leucocyte count was less than 2.5×10^4 cells/follicle revealed a stronger correlation between blastocyst formation of MII oocytes and IPL count in FA samples obtained from follicles nurturing MII oocytes that developed to the blastocysts (Group III) compared with that of Groups I and II. On the other hand, the peripheral blood leucocyte count of category B exceeded 2.5×10^4 cells/ follicle, leading to difficulty in estimating the IPL count due to the large number of peripheral blood leucocytes on the DQ-stained smears.

As it involves the participation of both leukocytes and inflammatory mediators, ovulation, *th*e terminal stage of oocyte maturation, shares several similarities with a local inflammatory reaction. In fact, previous studies have found that several inflammatory genes are expressed in preovulatory granulosa cells (GCs) and cumulus cells (CCs) [17]. Specifically, the concentration of IL-8 (known as typical chemokine CXCL8) has been found to be higher in dominant follicles of the late follicular/ovulatory phase compared with those of the mid-follicular phase, and the

capability of GCs to synthesize IL-8 to be up-regulated [18, 19]. It is possible that the infiltrating polymorphonuclear leucocyte (IPML) populations of the FA samples examined in this study may have reflected the integrity of the microenvironment of the ovulatory follicle, specifically that of an environment that promoted oocyte maturation and ensured developmental competence of the human MII oocyte.

In conclusion, this is the first study to reveal that the IPL count of FA samples might be associated with the ability of human MII oocytes to develop to blastocysts. Although further studies are required using larger samples before IPL count can definitively defined a predictor in clinical settings and as a subset of IPL count populations in FA samples, the findings of this study indicate that IPL count may be a predictor of the developmental competence of human MII oocytes.

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Table 1. Preovulatory serum E_2, LH and P_4 levels in patient on the day of GnRH-agonist administration

	oocyte(-)	Unfertilized early cleavage stage	blastocyst	*P* value
Group (n)	I (7)	I (11)	III (9)	
E_2 (pg/ml)	304.6±89.8	306.0±95.0	242.8±53.8	NS
LH (mIU/ml)	7.8±8.7	12.8 ±6.7	8.2±3.0	NS
P_4 (ng/ml)	0.42±0.16	0.54±0.25	0.63±036	NS

E_2: estradiol; LH: luteinizing hormone; P_4: progesterone

Non-repeated ANOVA method; NS: no statistical significance.
Serum hormone levels are given as means±SD

Table 2. Comparison of accurate infiltrating leukocyte count in FF classified by developmental competence of MII oocytes

Developmental stage	Group	Cycles (n)	IML count (10^2 cells/follicle)	IPL count (10^2 cells/follicle)
oocyte(−)	I	6	28.5 ± 22.0^{a}	5.7 ± 7.2^{c}
unfertilized	II	2	111.9 ± 22.1^{b}	8.4 ± 9.2^{c}
early cleavage stage	II	6		
blastocyst	III	5	159.9 ± 62.6^{b}	106.6 ± 30.7^{d}

Note: IPL count: infiltrating polymorphonuclear leukocyte count; IML count: infiltrating mononuclear leukocyte count; IML count and IPL count are expressed as means±SD , oocyte (−): no oocyte was retrieved

Tukey-Kramer method, $^{a-b, c-d}P<0.01$