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Original Article

The cytokine profile of follicular fluid changes during ovarian ageing

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ABSTRACT

Objective: Ovarian ageing is one of the commonest causes of infertility in patients consulting for assisted reproductive technology. The composition of the follicular fluid (FF), which reflects the exchanges between the oocyte and its microenvironment, has been extensively investigated to determine the metabolic pathways involved in various ovarian disorders. Considering the importance of cytokines in folliculogenesis, we focused on the cytokine profile of the FF during ovarian ageing.
Material and methods: Our cross-sectional study assesses the levels of 27 cytokines and growth factors in the FF of two groups of women undergoing in vitro fertilization. One group included 28 patients with ovarian ageing clinically characterized by a diminished ovarian reserve (DOR), and the other group included 29 patients with a normal ovarian reserve (NOR), serving as controls.
Results: With univariate analysis, the cytokine profile was found to differ significantly between the two groups. After adjustment of the *p*-values, platelet-derived growth factor-BB (PDGF-BB) was the only cytokine with a significantly lower concentration in the DOR group (7.34 ± 16.11 pg/mL) than in the NOR group (24.39 ± 41.38 pg/mL) ($p = 0.005$), independently of chronological age.
Conclusion: Thus, PDGF-BB would seem to be implicated in the physiopathology of DOR, potentially in relation to its role in folliculogenesis or in the protection against oxidative stress.

Introduction

Ovarian ageing is characterized by the gradual qualitative and quantitative deterioration of the ovarian oocyte reserve, resulting in impaired oocyte competence associated with decreased natural fertility, poor response to ovarian stimulation, and reduced in vitro fertilization (IVF) pregnancy rates [1–5].

A diminished ovarian reserve (DOR), the clinical consequence of ovarian ageing, is found in 10 % of women enjoying natural maternity compared to 25 % of women having recourse to assisted reproductive technology (ART) and being subject to poor fertility outcomes. The assessment and management of women affected by DOR-related infertility is of increasing importance today and a better understanding of the pathophysiological mechanisms of DOR would contribute to improving the implementation of ART [2–4,6].

In the ovarian follicle, the communication between the oocyte and the somatic cells, i.e. the granulosa and cumulus cells, governs the evolution of the follicle from the preantral to the final stage of ovulation. This intrafollicular paracrine dialogue promotes and regulates the specialized microenvironment required to fully support the meiotic and developmental competence of the oocyte, thus ensuring the viability of the embryo [7–11]. This dialogue

Abbreviations: AFC, Antral Follicle Count; AMH, Antimullerian Hormone; ARTAssist, ed Reproductive Technologies; CCL11, Eotaxin; DOR, Diminushed Ovarian Reserve; FF, Follicular Fluid; FGF, Fibroblast Growth Factor; FSH, Follicle Stimulating Hormone; GC, Granulosa Cells; G-CSF, Granulocyte-Colony Stimulating Factor; GM-CSF, Granulocyte-Macrophage Colony-Stimulating Factor; ICSI, Intra-Cytoplasmic Sperm Injection; IFN, Interferon; IL, Interleukin; IVF, In Vitro Fertilization; LH, Luteinizing Hormone; MIP, Macrophage Inflammatory Protein; NOR, Normal Ovarian Reserve; OCC, Oocyte-Cumulus Complex; PDGF-BB, Platelet-Derived Growth Factor BB; TNF-α, Tumor Necrosis Factor- α; VEGF, Vascular Endothelial Growth Factor.

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notably involves cytokines, the small proteins responsible for cell signaling under various physiological or pathological conditions. Cytokines, locally secreted within the ovarian follicle, regulate the ovarian functions, i.e. cell proliferation, steroidogenesis, angiogenesis, and oocyte development [7,12].

The structure of the ovarian follicle evolves during folliculogenesis. From the tertiary stage, the follicle comprises an antrum filled with follicular fluid (FF) surrounding the oocyte-cumulus complex (OCC) [13]. The FF is produced by the transfer of blood plasma components across the blood-follicle barrier, and by the secretions of the oocyte and the ovarian somatic cells, i.e. the theca cells and the granulosa cells (GC). The biochemical and immunological composition of the FF reflect the exchanges contributing to the acquisition of oocyte competence [10,12,14–17]. In the context of IVF, the FF has been explored in the search of biomarkers of oocyte and embryo quality. Various studies have shown some cytokines and growth factors of the FF to be positively correlated with oocyte quality, fertilization rate, embryo development and IVF outcomes, i.e. implantation potential and pregnancy rates [12,18–20]. Studies of the FF should also allow identification of specific markers or pathways underlying certain pathologies. Ageing and DOR are correlated with alterations of GCs and the microenvironment. Some authors have reported variations in the cytokine concentrations of the FF in poor ovarian responders and in older patients [20,21]. However, to our knowledge, only a few cytokines have been evaluated so far in the context of ovarian ageing. To explore the possible impact of DOR on the cytokine profile of the FF, we performed a targeted quantitative analysis of 27 cytokines in the FF from 28 women with ovarian ageing characterized by DOR, and 29 women with a normal ovarian reserve (NOR) serving as controls.

Material and methods

Characteristics of the patients

Fifty-seven women (aged 23–42 years) undergoing in vitro fertilization, with or without intracytoplasmic sperm injection (ICSI), were included in this descriptive, cross-sectional, monocentric study, carried out at the Angers University Hospital between November 2015 and March 2016. Patients were enrolled at our ART center, on the morning of oocyte retrieval, and divided into two groups according to the results of tests on their ovarian reserve. Patients were included in the DOR group (28 patients) if they had AMH levels below 2 ng/ml, or an AFC below 5 per ovary. In our center, we rarely offer patients IVF if their initial total AFC is less than 5 or if the AMH level is less than 0.5 ng/mL Patients were included in the NOR group (29 patients) with the following criteria: anti-Müllerian hormone (AMH) levels above 2 ng/mL, follicle stimulating hormone (FSH) levels below 8 IU/L, estradiol levels below 60 pg/mL and an antral follicle count (AFC) above 4 per ovary. Except for the AMH assays, all the other dosages and ultrasonography examinations were performed on day-3 of a spontaneous ovarian cycle.

Patients who had only one ovary or a medical history that might have contributed to a DOR, as well as patients with the polycystic ovary syndrome, or endometriosis, were excluded.

The characteristics of the patients and procedures used for the suppression of pituitary gonadotrophin release [using Cetrorelix (Cetrotide®; Merck-Serono, Geneva, Switzerland); Ganirelix (Orgalutran®; Organon, Oss, Netherlands); Triptorelin (Decapeptyl®; Ipsen Pharma, Paris, France)], and follicular growth stimulation [using uFSH + uLH (Luteinizing Hormone): Menotropine (Menopur®; Ferring Pharmaceuticals, Copenhagen, Denmark); rFSH and rLH: Follitropine alpha + Lutropine alpha (Pergoveris®; Merck-Serono, Geneva, Switzerland); rFSH: Follitropine alpha (Gonal-f®; Merck-Serono, Geneva, Switzerland) or Follitropine beta (Puregon®; Organon, Oss, Netherlands)] are summarized in Table 1. In each case, ovulation was induced with Ovitrelle® (Choriogonadotropine alfa, Merck-Serono, Geneva, Switzerland).

Collection of follicular fluid

Follicular fluid (FF) was aspirated during vaginal oocyte retrieval under ultrasound guidance 36 h after administration of human chorionic gonadotropin. All patients were in the fasting state. Immediately following the ovarian puncture, FF aliquots were examined to detect and remove OCCs, and oocytes were isolated for evaluation and culture. The remaining FF samples, collected from the same patient, were pooled and immediately centrifuged at 800 g for 10 min to remove residual cells. FF supernatants were collected and stored at −80 °C until analysis.

Quantification of cytokine

The concentrations of 27 cytokines, involved in different cell signaling pathways, i.e. the basic fibroblast growth factor (FGF),

Table 1
Characteristics of the patients.

Covariates	Overall ($n = 57$)	NOR ($n = 29$)	DOR ($n = 28$)	P-value
Age of patients (years) Mean (SD)	33.16 (5.19)	29.76 (4.16)	36.68 (3.58)	< 0.001* Mann-Whitney
Body mass index Mean (SD)	23.56 (5.15)	23.17 (5.48)	23.97 (4.84)	0.40 Mann-Whitney
Addiction to smoking				
Non-smoker (n)	36	17	19	0.342
Smoker (n)	18	10	8	Khi-square
Former smoker (n)	2	2	0	
Information missing (n)	1	0	1	
E2 baseline (pg/mL) Mean (SD)	40.51 (20.35)	41.54 (16.62)	39.44 (23.88)	0.380 Mann-Whitney
FSH baseline (UI/L) Mean (SD)	8.07 (2.97)	7.07 (1.99)	9.11 (3.47)	0.01* Mann-Whitney
LH baseline (UI/L) Mean (SD)	5.05 (2.26)	4.62 (1.96)	5.53 (2.49)	0.186 Mann-Whitney
AMH baseline (ng/mL) Mean (SD)	2.38 (1.70)	3.73 (1.23)	0.94 (0.56)	< 0.001* Mann-Whitney
Antral follicle count Mean (SD)	14.26 (7.56)	19.74 (6.57)	8.58 (2.82)	< 0.001* Mann-Whitney
Total dose of FSH (UI) Mean (SD)	2605 (1165)	1746 (548)	3496 (942)	< 0.001* Mann-Whitney
LH suppression				
Agonist	3	2	1	0.57
Antagonist	54	27	27	Khi-square
Stimulation				
FSH	46	25	21	0.28
FSH + LH	11	4	7	Khi-square
Number of retrieved OCCs Mean (SD)	8.67 (6.22)	11.72 (6.80)	5.30 (3.46)	< 0.001* Mann-Whitney

eotaxin (CCL11), granulocyte-colony stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), interferon (IFN) γ, the interleukins IL-1β, IL-1RA, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8 (CXCL8), IL-9, IL-10, IL12 (p70), IL-13, IL-15, IL-17A, IP-10 (CXCL10), MCP-1 (CCL2), macrophage inflammatory protein (MIP) 1α (CCL3), MIP-1β (CCL4), platelet-derived growth factor BB (PDGF-BB), RANTES (CCL5), tumor necrosis factor- α (TNF-α), and the vascular endothelial growth factor (VEGF) A, were determined in the FF by multiplex fluorescent-bead-based technology (Luminex 200™, Austin, TX) using a commercial Luminex screening assay kit (Bio-Plex Pro™ Human Cytokine 27-plex Assay; Bio-Rad, Marnes-la-Coquette, France). Briefly, the FF samples were diluted two-fold before incubation with specific antibody-coated fluorescent beads according to the manufacturer's recommendations. After washing, 50 beads were analyzed with the Luminex 200™ and the cytokine concentrations of the samples were estimated through a serial dilution of cytokine standards.

Statistical analysis

Univariate statistical analysis of the data of the patients was done using the non-parametric Mann-Whitney test for quantitative variables, and the chi-squared test (χ^2) for qualitative variables. All the calculations were performed with Systat software, version 15.0 (SPSS Inc., Chicago, IL, USA). Differences were considered significant at $p < 0.05$.

The statistical analysis of cytokine concentrations was done after log transformation to normalize data. Critical *p*-values were calculated using an approach based on the Benjamini-Hochberg procedure [22] to correct the multiple-comparison false-discovery rate.

Ethical approval

All patients gave their written informed consent, and the collection of samples was approved by the Ethical Committee of the University Hospital of Angers, France (Number CB2015-06).

Results

Characteristics of the NOR and DOR patients

The characteristics of the patients are summarized in Table 1. As expected, since DOR is correlated with ageing, the mean age was significantly lower in the NOR group compared with the DOR group ($p < 0.001$). No statistical difference was found in terms of the body mass index (BMI) or addiction to tobacco smoking.

There is no consensus concerning the definition of DOR. The diagnosis is suspected on the basis of a set of arguments (parameters such as AMH, AFC, FSH) varying from team to team and requiring the use of site-specific values (Practice Committee of ASRM, 2015). Our patients were initially classified according to their plasma AMH and FSH levels and to their AFC, thus, the AMH levels ($p < 0.001$) and the AFC ($p < 0.001$) were significantly lower in the DOR group, whereas the FSH levels were significantly higher than in the NOR group ($p = 0.01$). However, plasma E2 and LH baseline levels were not significantly different between the two groups. Only one patient in the DOR group had an AFC < 5. Moreover, this diagnosis is confirmed by a poor response to stimulation [23]. Thus in our cohort, the ovarian response to the stimulation, in terms of the total amount of gonadotrophin used and according to the number of oocytes retrieved, was lower in the DOR group than in the NOR group. The total dose of FSH used was significantly higher ($p < 0.001$) whereas that of the number of oocytes retrieved was significantly lower ($p < 0.001$) in the DOR group than in the NOR group. The quantities of the drugs used for the suppression of LH and ovarian stimulation were not significantly different between the two groups (Table 1).

Quantification of cytokines in the FF in the NOR and DOR patients

Of the total number of 27 cytokines studied in the FF, 7 were not detected in the DOR and the NOR groups. Using univariate analysis after log transformation, the levels of 4 cytokines were significantly different in the DOR group compared to the NOR group. The concentrations of PDGF-BB ($p = 0.005$) and RANTES ($p = 0.023$) were significantly lower in the DOR group than in the NOR group, whereas the concentrations of MCP-1 ($p = 0.030$) and the IL-1 receptor antagonist (IL-1RA) ($p = 0.041$) were significantly higher in the DOR group than in the NOR group of patients.

After correction of the false discovery rate, only the FF level of PDGF-BB was significantly lower in the DOR group (7.34 ± 16.11 pg/mL) than in the NOR group (24.39 ± 41.38 pg/mL) ($p = 0.005$) (Fig. 1).

No statistical differences were found in the FF concentrations of the other cytokines detected (Table 2).

Impact of the chronological age of the NOR and DOR patients

To examine the possibility of the patient's age being a confounding factor in the variation of the PDGF-BB level between the DOR and the NOR groups of patients, we carried out a supplementary statistical analysis using a Mann-Whitney test to compare the 15 younger DOR patients and the 15 older NOR patients. With univariate analysis, there was no significant age-related difference in the PDGF-BB concentration between the two groups ($p = 0.25$), whereas with the Mann-Whitney test, the PDGF-BB level was significantly lower in the 15 younger DOR patients compared to the 15 older NOR patients ($p = 0.0079$) (Table 3).

Discussion

Various cytokines play an essential role during folliculogenesis, i.e. in follicular growth and maturation as well as in the ovulation process [7,12,20,24–26]. Since the chemical composition of the FF reflects follicular metabolism during folliculogenesis, the study of cytokine profiles in the FF should help understand the processes underlying follicular physiology and pathology.

Some authors have reported variations in the levels of certain cytokines in the FF in the context of ageing in older women and in poor ovarian responders (POR). In these studies, IL-2, IL-6 and IL-8

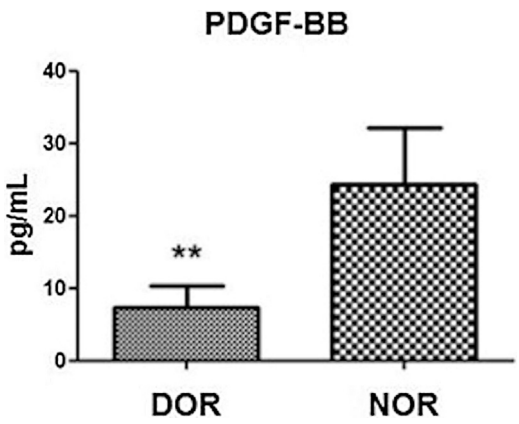

Fig. 1. Cytokines concentrations of platelet-derived growth factor (PDGF)-BB (pg/mL) in follicular fluid from diminished ovarian reserve (DOR) and normal ovarian reserve (NOR) patients. Data represent mean ± SEM. PDGF-BB:** P = 0.004 lower level in DOR patients compared with control.

Table 2
Concentrations of 23 cytokines showing no significant difference between patients with a normal ovarian reserve and patients with a diminished ovarian reserve.

Cytokines	NOR (*n*=29)	Valeur-*P*[a]	DOR (*n*=28)
FGF	60.92 (0–151.84)	0.23 (NS)	66.11 (25.07–130.09)
Eotaxine	10.86 (0–50.75)	0.21 (NS)	1665 (0–116.91)
G-CSF	0 (0)[b]	1 (NS)	0 (0)[b]
GM-CSF	218.23 (0–347.93)	0.95 (NS)	202.39 (30.48–345.97)
IFN-γ	41.98 (0–354.97)	0.65 (NS)	30.17 (0–162.23)
IL-1β	0 (0)[b]	1 (NS)	0 (0)[b]
IL-2	0 (0)[b]	1 (NS)	0 (0)[b]
IL-4	1.88 (0–6.58)	0.08 (NS)	0.97 (0–4.91)
IL-5	0 (0)[b]	1 (NS)	0 (0)[b]
IL-6	3.16 (0–81.61)	0.85 (NS)	4.25 (0–86.98)
IL-7	5.95 (0–36.19)	0.25 (NS)	7.90 (0–21.3)
IL-8	184.55 (50.96–384.37)	0.16 (NS)	267.30 (55-49-1885.99)
IL-9	9.80 (0–48.37)	0.91 (NS)	10.09 (0–39.8)
IL-10	1.72 (0–26.91)	0.94 (NS)	1.23 (0–17.44)
IL-12 (p70)	105.13 (0–244.41)	0.26 (NS)	13,506 (0–248.67)
IL-13	3.45 (0–14.92)	0.83 (NS)	3.08 (0–11.23)
IL-15	0 (0)[b]	1 (NS)	0 (0)[b]
IL-17A	0 (0)[b]	1 (NS)	0 (0)[b]
IP-10	222.13 (87.25–712.26)	0.21 (NS)	291.56 (78.51–988.7)
MIP-1α	0.95 (5.83)	0.43 (NS)	1.13 (0–2.76)
MIP-1β	46.57 (17.1–88.34)	0.57 (NS)	47.44 (3.79–105.73)
TNFα	0 (0)[b]	1 (NS)	0 (0)[b]
VEGF-A	1181.24 (456.53–2594.75)	0.16 (NS)	143,400 (174.39–2779.82)

NOR: normal ovarian reserve; DOR: diminished ovarian reserve; FGF: fibroblast growing factor; G-CSG: granulocyte colony stimulating factor; GM-CSF: granulocyte-macrophage colony-stimulating factor; IFN: interferon; IL: interleukin; MIP: macrophage inflammatory protein; TNF: tumor necrosis factor; VEGF: vascular endothelial growth factor; NS: not significant. [a]Level of significance is defined as $P < 0.05$. [b]Undetectable value. Data are median (pg/mL).

Table 3
Comparison between younger DOR patients and older NOR patients.

Covariates	Younger DOR (*n* = 15)	Older NOR (*n* = 15)	P-value
Age of patients (years) Mean (SD)	33.87 (2.2)	33.07 (1.49)	0.25 Mann-Whitney
Body mass index Mean (SD)	23.46 (5.09)	23.52 (6.68)	0.98 Mann-Whitney
Addiction to smoking			
Non-smoker (n)	9	9	0.42
Smoker (n)	5	4	Khi-square
Former smoker (n)	0	2	
Information missing (n)	1	0	
E2 baseline (pg/mL) Mean (SD)	41.1 (27.53)	43.97 (18.35)	0.74 Mann-Whitney
FSH baseline (UI/L) Mean (SD)	9.6 (3.75)	6.41 (1.30)	0.004* Mann-Whitney
LH baseline (UI/L) Mean (SD)	5.01 (2.11)	4.44 (1.78)	0.43 Mann-Whitney
AMH baseline (ng/mL) Mean (SD)	0.78 (0.41)	3.87 (1.45)	< 0.001* Mann-Whitney
Antral follicle count Mean (SD)	8 .23 (3.56)	21.75 (12.63)	< 0.001* Mann-Whitney
Total dose of FSH (UI) Mean (SD)	3497 (805)	1762 (488)	< 0.001* Mann-Whitney
LH suppression			
Agonist	1	1	–
Antagonist	14	14	
Stimulation			
FSH	10	14	0.17
FSH + LH	5	1	Khi-square
Number of retrieved OCCs Mean (SD)	5.53 (3.54)	13.2 (8.71)	0.003* Mann-Whitney
PDGF-BB level (pg/mL) Mean (SD)	6.14 (17.24)	23.63 (34.25)	0.0079* Mann-Whitney

levels were found significantly higher whereas the IL-10 level was significantly lower in older women than in younger women and good responders [20,27]. In our study, we assessed the concentrations of a large panel of cytokines ($n = 27$) in the FF of NOR and DOR patients. PDGF-BB was the only cytokine with a significantly different concentration in the two groups of patients. Although DOR and POR are two associated conditions, both leading to reduced ovarian responses to stimulation and poor fertility outcomes, no significant differences were found in the IL levels of the FF in the two groups of patients. This result, which is contrary to reports in the literature indicating that IL concentrations are related to ageing, may be due to the relatively small number of patients included in our study.

In addition, in order to control the possible impact of the patient's age as a confounding factor in the difference in the concentration PDGF-BB observed in the two groups of patients, we compared two sub-groups, comprising 15 younger DOR patients and 15 older NOR patients. There was no difference in the mean age of these two groups, whereas the concentration of PDGF-BB was significantly lower in the 15 younger DOR patients compared to the 15 older NOR patients ($p = 0.0079$). This result highlights the importance of the ovarian reserve in the decrease of the concentration of PDGF-BB in the FF, regardless of chronological age.

Several authors have discussed the function of PDGF in the FF. Interestingly, this cytokine seems to play a key role during folliculogenesis [28–30]. PDGF, a dimer of disulfide-linked polypeptide chains (PDGF-A, PDGF-B, PDGF-C and PDGF-D), mediates multiple cellular processes such as cell migration and survival, organogenesis and embryogenesis. PDGF paracrine

signaling, based on the interaction with two receptor tyrosine kinases, PDGFR-α and PDGFR-β, promotes their dimerization [31,32].

In the ovary, PDGF-A chains have been reported in the GCs of secondary follicles whereas PDGF-B chains have been reported in the GCs and theca cells of primary and secondary follicles as well as in oocytes [30,33]. Within the follicle, PDGFs mediate the activation of the primordial follicle and the growth of primary and secondary follicles via different pathways. PDGFs promote kit-ligand (KL) secretion by the GCs, leading to the intra-oocyte signalization PI3K (3-phosphoinositide-dependent protein kinase) pathway. Furthermore, PDGFs may directly promote the proliferation of theca cells by their fixation on theca PDGFRs or indirectly via the KL fixation [29,30,34,35]. The role of each PDGF isoform during folliculogenesis has not yet been identified. So far, only PDGFR-β has been detected in primordial follicles, suggesting the key role of this receptor. In our study, PDGF-BB was the only isoform assessed. Interestingly, in vivo, only the PDGF-BB/PDGFR-β interaction has been reported. Thus, the decreased concentration of PDGF-BB during ovarian ageing in the DOR patients may be linked to an alteration of the mechanisms involved in follicular activation [31,33]. Moreover, PDGFs promote secondary follicular growth as well, and the decreased concentration of PDGF-BB may have a global impact on folliculogenesis.

PDGF-BB may also act as an important antioxidative signaling factor as reported in neural tissues. The protective role of PDGF-BB has been evidenced in astrocytes in which oxidative damage is attenuated by the decreased formation of reactive oxygen species [36]. Ovarian ageing and DOR are associated with increased oxidative stress in the follicular environment. Indeed, several authors have reported variations of oxidative markers in the FF of older women and poor ovarian responders [27,37–39]. Thus, we may hypothesize that the decreased concentration of PDGF-BB in patients with DOR may be linked to increased oxidative stress.

In conclusion, our study shows the impact of ovarian ageing on the cytokine profile of the FF with a decreased level of PDGF-BB compared to control patients. Thus, DOR-related infertility may be linked to the alteration of metabolic pathways involving PDGF-BB, leading to the perturbation of folliculogenesis or antioxidative defenses.

Key message

PDGF-BB would seem to be implicated in the physiopathology of diminished ovarian reserve.

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Declaration of Competing Interest

The authors declare no conflicts of interest.

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