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REPRODUCTIVE PHYSIOLOGY AND DISEASE

Correlation between follicular fluid levels of sRAGE and vitamin D in women with PCOS

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Abstract
Purpose The pro-inflammatory advanced glycation end products (AGEs) and their anti-inflammatory soluble receptors, sRAGE, play a role in the pathogenesis of PCOS. There is a correlation between vitamin D (vit D) and sRAGE in the serum, whereby vit D replacement increases serum sRAGE levels in women with PCOS, thus incurring a protective anti-inflammatory role.
Objective This study aims to compare levels of sRAGE, N-carboxymethyl-lysine (CML; one of the AGEs), and 25-hydroxy-vit D in the follicular fluid (FF) of women with or without PCOS, and to evaluate the correlation between sRAGE and 25-hydroxy-vit D in the FF.
Material and methods Women with (n = 12) or without (n = 13) PCOS who underwent IVF were prospectively enrolled.
Results Women with PCOS had significantly higher anti-Mullerian hormone levels, higher number of total retrieved and mature oocytes, and higher number of day 3 and day 5 embryos formed. Compared to women without PCOS, women with PCOS had significantly lower FF sRAGE levels. In women with PCOS, in women without PCOS, and in all participants together, there was a significant positive correlation between sRAGE and 25-hydroxy-vit D. sRAGE positively correlated with CML in women without PCOS but not in women with PCOS.
Conclusions In women with PCOS, the low ovarian levels of the anti-inflammatory sRAGE suggest that sRAGE could represent a biomarker and a potential therapeutic target for ovarian dysfunction in PCOS. Whether there is a direct causal relationship between sRAGE and vit D in the ovaries remains to be determined.

Keywords PCOS · Advanced glycation end products · RAGE · sRAGE · Vitamin D

Abbreviations

AGEs	Advanced glycation end products
sRAGE	Soluble receptor for advanced glycation end products

Introduction

Polycystic ovary syndrome (PCOS) is one of the most common endocrine disorders and affects up to 15% of reproductive-aged women [1]. PCOS is the most common cause of anovulation in reproductive-aged women [2] and has been associated with different adverse outcomes in women undergoing assisted reproduction, such as low fertilization rate, low number of good quality embryos, and high spontaneous abortion rates after ovulation induction and after in vitro fertilization (IVF) treatments [3–5]. Advanced glycation end

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products (AGEs) are highly reactive pro-inflammatory molecules that are physiologically formed by non-enzymatic alteration of proteins, lipids, and nucleic acids by glucose or by ingestion of a variety of fast foods [6, 7]. AGEs constitute a heterogeneous group of compounds of more than 20 members. N-carboxymethyl-lysine (CML) is a well-characterized AGE and has been used as a marker for AGE accumulation in various tissues [8]. Chronic diseases such as diabetes, insulin resistance, aging, oxidative stress, and PCOS accelerate the generation of endogenous AGEs [9–12]. In particular, women with PCOS have elevated levels of serum and ovarian AGEs regardless of their weight or insulin resistance status [13, 14].

AGEs act by binding to the extracellular matrix (receptor-independent) or by binding to a cellular receptor called RAGE (receptor for advanced glycation end products) [6]. After binding to RAGE, AGEs produce a sequence of events leading to cellular inflammation and apoptosis [15]. It has been shown that there is a marked induction of the pro-inflammatory RAGE in the ovaries of women with PCOS [16]. Soluble receptor for AGEs (sRAGE) is another form of receptor and is secreted extracellularly. sRAGE can be detected in the blood and follicular fluid [17]. sRAGE is considered an anti-inflammatory receptor because it binds circulating AGEs, thus preventing their binding to RAGE, leading to the alleviation of the adverse intracellular events of the pro-inflammatory AGE-RAGE axis [16, 18, 19]. Recent studies have shown that serum and follicular fluid sRAGE levels were lower in women with PCOS patients and inversely correlated with body mass index (BMI) and insulin resistance [17, 19, 20]. We have shown in women without PCOS that follicular fluid sRAGE levels positively correlated with follicular fluid anti-Mullerian hormone (AMH) levels, one of the best markers of ovarian reserve, and also predicted the number of oocytes retrieved following controlled ovarian stimulation (COS), indicating that sRAGE could potentially be used as a marker of ovarian reserve [21]. Higher sRAGE levels in the follicular fluid have been shown to be associated with favorable IVF outcome in terms of requiring lower doses of gonadotropins for COS, higher number of retrieved oocytes, and better pregnancy rates [19]. On the other hand, another study demonstrated that higher follicular fluid sRAGE levels were associated with poor embryo quality after IVF [22].

Up to 85% of women with PCOS have vitamin D deficiency [23]. Vitamin D constitutes a contributing factor to several aspects of PCOS such as insulin resistance, obesity, and metabolic syndrome [24, 25]. Supplementation of 1,25-dihydroxy-vitamin D3 (vit D3) to vitamin D-deficient women with PCOS improves their serum androgen level and their insulin resistance index [26, 27]. We have shown that vit D3 supplementation to vitamin D-deficient women with PCOS elevates their serum sRAGE levels [28], suggesting that vit D3 could have an anti-inflammatory effect via the sRAGE and AGE-RAGE system. To the best of our knowledge, the relationship between sRAGE and vitamin D in the follicular fluid of women with or without PCOS undergoing IVF has not been studied. Given that both sRAGE and vitamin D could act as anti-inflammatory agents, we hypothesized that there is a positive correlation between follicular fluid sRAGE and vitamin D levels and that women with PCOS have lower levels of follicular fluid sRAGE and vitamin D.

Material and methods

Participants

Twenty-five women with (n = 12) and without (n = 13) PCOS undergoing controlled ovarian stimulation (COS) for IVF between June 2015 and October 2016 were prospectively enrolled. Rotterdam criteria were used for the diagnosis of PCOS [29]. Women aged between 21 and 40 with regular menstrual cycles who underwent gonadotropin-releasing hormone (GnRH) antagonist suppression protocols were eligible for the study. All women with PCOS had anovulation and oligomenorrhea. Exclusion criteria included (1) women with diminished ovarian reserve as defined by day 3 follicle-stimulating hormone (FSH) ≥10 IU/L and/or serum AMH <1 ng/mL and/or total antral follicle count (AFC) <10 on transvaginal ultrasound; (2) women on hormonal medications other than the IVF medications; and (3) women with hypothyroidism, hyperthyroidism, hyperprolactinemia, or adrenal dysfunctions. Institutional review board (IRB) approval was obtained from Maimonides Medical Center, and all participants signed appropriate consent for participating in the study.

Follicular fluid collection

For women without PCOS (controls), baseline blood samples were collected between days 3 and 5 of the menstrual cycle. For women with PCOS, baseline blood samples were collected prior to starting COS because of oligomenorrhea. Conventional ovarian stimulation was achieved with injections of gonadotropins such as Menopur (Ferring, Parsippany, NJ, USA) and either Follistim (Merck, White House Station, NJ, USA) or Gonal F (EMD Serono, Rockland, MA, USA), at a total starting dose of 150–300 IU daily. During COS, participants were closely monitored by transvaginal ultrasound and blood levels of estradiol (E2), progesterone (P4), and luteinizing hormone (LH) levels. When at least two follicles reached a diameter of 18 mm or greater, oocyte maturation was induced with the human chorionic gonadotropin Ovidrel (EMD Serono). The retrieved oocytes were fertilized by either conventional IVF or intracytoplasmic sperm injection (ICSI) as clinically indicated. All embryos were subsequently cultured until the blastocyst stage and one or two blastocysts were transferred in a

fresh cycle. The remaining surplus good-quality blastocysts were frozen by vitrification while the poor quality embryos were discarded.

Follicular fluid is composed partly of plasma exudates and partly of secretions from the ovarian follicle. Its composition reflects changes in processes occurring at the levels of granulosa and theca cells as well as plasma alterations in response to physiologic or pathological processes. Thus, follicular fluid has been used as a tool for studying ovarian physiology. In order to avoid blood contamination and dilution from rinsing, follicular fluid was collected from the first large (>18 mm) blood-free aspirate during oocyte retrieval. After oocyte collection, follicular fluid was centrifuged at 15,000*g* for 5 min in order to remove any vaginal mucosal cells.

Clinical characteristics of the participants

The clinical characteristics of the participants included age, BMI, day 3 FSH levels, serum AMH levels, AFC, total dose of gonadotropins used per cycle, and peak serum E2 levels on the day of Ovidrel trigger. The main outcome measures of the study were follicular fluid levels of sRAGE, CML, and 25-hydroxy-vitamin D levels. The secondary outcome measures included the total number of oocytes retrieved, number of mature oocytes in metaphase II (MII), number of fertilized oocytes, number of embryos formed on day 3 and day 5 post-fertilization, number of cryopreserved and discarded embryos, and clinical pregnancy rate (CPR) as defined by the presence of an intrauterine gestational sac on transvaginal ultrasound with positive fetal heart beat.

Follicular fluid sRAGE, CML, 25-hydroxy-vitamin D, and insulin measurements

Levels of sRAGE (R&D Systems, Minneapolis, MN) and a well-characterized AGE, CML (Cell BioLabs, CA), were determined in follicular fluid using manual ELISA methods according to manufacturer protocols. The limits of sensitivity were 0.078 ng/mL and 0.43 μg/mL, respectively. 25-Hydroxy-vitamin D (Diasorin Liaison, Saluggia, Italy) and insulin (Beckman Coulter DxI, Chaska, MN) levels were measured using automated immunoassay methods. We tested linearity of follicular fluid samples in the vitamin D assay as a method of validation. All interassay and intraassay coefficients of variation were less than 15%.

Statistics

Data were not normally distributed so they were presented as medians (25th–75th percentiles). Mann-Whitney *U* test was used for comparison and Spearman correlation was used to assess the relationship between follicular fluid levels of sRAGE, CML, 25-hydroxy-vitamin D, and insulin. Fisher exact test was used for categorical data. $p < 0.05$ was considered statistically significant.

Results

Baseline characteristics

As seen in Table 1, both groups were similar in age, BMI, parity, baseline thyroid-stimulating hormone (TSH), and prolactin levels ($p > 0.05$ for all). However, as expected, serum AMH levels (6.4 vs. 2.1 ng/mL, respectively; $p = 0.0002$) and AFC (22 vs. 12, respectively; $p = 0.0003$) were significantly higher in women with PCOS compared to controls (Table 1).

Comparison of cycle characteristics and IVF outcome

Although women with PCOS received similar amounts of gonadotropins per cycle compared to control women, they had significantly higher peak E2 levels (3644 vs. 2371 pg/mL, respectively; $p = 0.046$) (Table 2). Women with PCOS also had higher number of oocytes retrieved (16 vs. 12.5, respectively; $p = 0.02$) and almost twice the number of mature oocytes (16 vs. 9, respectively; $p = 0.03$) compared to women without PCOS (Table 2). Women with PCOS had higher number of both day 3 embryos (10 vs. 6, respectively; $p = 0.049$) and day 5 embryos (9.5 vs. 6, respectively; $p = 0.02$) compared to controls; however, there were no statistically significant differences in cryopreserved and discarded embryos between both groups (Table 2). Although women with PCOS had higher number of mature oocytes and day 5 embryos, fewer embryos were transferred compared to controls (1.2 vs. 1.7, respectively; $p = 0.007$). And there was no difference in CPR between both groups (RR = 1.05, confidence interval [CI] 0.45–2.41).

Follicular fluid levels of sRAGE, 25-hydroxy-vitamin D, CML, and insulin

Compared to controls, women with PCOS had significantly lower levels of follicular fluid sRAGE (3.7 vs. 2.8 ng/mL, respectively; $p = 0.04$) (Table 3). There was no significant difference in the follicular fluid levels of CML, 25-hydroxy-vitamin D, or insulin between both groups ($p > 0.05$ for all) (Table 3). Among all the participants, there was a significant positive correlation between follicular fluid levels of sRAGE and 25-hydroxy-vitamin D ($r = 0.65$, $p = 0.0004$), and follicular fluid levels of sRAGE and CML ($r = 0.60$, $p = 0.001$) (Table 4). There was no significant correlation between sRAGE and BMI ($r = -0.2$, $p = 0.32$). In women with PCOS separately, there was a significant positive correlation between follicular fluid levels of sRAGE and 25-hydroxy-vitamin D ($r = 0.75$, $p = 0.007$) (Table 4). Similarly in women without PCOS separately, there was a significant positive correlation between follicular fluid levels of sRAGE and 25-hydroxy-vitamin D ($r = 0.72$, $p = 0.007$) and there was a

Table 1 Baseline characteristics of the study participants

	PCOS (n = 12)	Control (n = 13)	p value or RR (confidence interval)
Age (years)	31.5 [29.2–34.5]	35.0 [31.0–37.5]	0.07
BMI (kg/m^2)	24.5 [22.5–33.7]	25.0 [21.5–29.0]	0.5
Nulliparous	7/12	8/13	0.97 (CI 0.43–2.12)
TSH (u IU/mL)	1.3 [0.9–2.0]	1.7 [0.9–2.2]	0.5
Prolactin (ng/mL)	9.1 [6.1–15.5]	10.3 [6.8–14.6]	0.9
AMH (ng/mL)	6.4 [3.8–12.4]	2.1 [1.7–3.8]	0.0002
Day 3 estradiol (pg/mL)	76.1 [44.2–90.8]	69.6 [41.4–72.5]	0.3
Day 3 FSH (m IU/mL)	5.1 [2.8–6.0]	6.9 [5.9–8.0]	0.002
Antral follicular count	22.0 [17.5–24.0]	12.0 [11.5–15.5]	0.0003
ICSI	8/12	10/13	0.92 (CI 0.44–1.84)

Data are expressed as median [25th–75th percentile] or n/total

BMI body mass index, *PCOS* polycystic ovary syndrome, *TSH* thyroid-stimulating hormone, *AMH* anti-Mullerian hormone, *FSH* follicle-stimulating hormone, *ICSI* intracytoplasmic sperm injection

positive correlation between follicular fluid levels of sRAGE and CML (r = 0.89, p = 0.0001) (Table 4).

Discussion

The aim of this study was not to compare CPR between women with or without PCOS. This pilot study aimed to compare sRAGE protein concentrations and 25-hydroxy-vitamin D levels in the follicular fluid of women with or without PCOS who underwent COS for IVF. It also analyzed the correlation between follicular fluid sRAGE with follicular fluid 25-hydroxy-vitamin D, CML (one of the most commonly studied AGEs), and insulin. The results showed that women with PCOS had significantly lower follicular fluid sRAGE protein but similar follicular fluid 25-hydroxy-vitamin D levels compared to control women. Higher follicular fluid sRAGE protein levels were associated with higher 25-hydroxy-vitamin D.

PCOS is considered a state of chronic low-grade inflammation, which has been implicated as a mechanism for the development of metabolic and reproductive dysfunctions observed in PCOS [30]. In women with PCOS, serum AGEs are elevated even in the absence of insulin resistance and AGE-RAGE interaction could be implicated in the underlying pathophysiology of PCOS [11, 31, 32]. Insulin resistance accelerates AGEs' formation [6], and ultimately, AGEs can deposit in various organs throughout the body including the ovaries. Theca and granulosa cell layers in the ovaries of women with

Table 2 Cycle characteristics and IVF outcome of the participants

	PCOS (n = 12)	Control (n = 13)	p value or RR (confidence interval)
Gonadotropin dose (IUs)	3263.0 [1838.0–3981.0]	3075.0 [2438.0–4436.0]	0.7
Total days of stimulation	11.0 [9.0–12.7]	10.0 [9.0–11.0]	0.2
Peak estradiol (pg/mL)	3644.0 [2695.0–4262.0]	2371.0 [1883.0–3222.0]	0.046
Number of oocytes retrieved	16.0 [13.2–35.5]	12.5 [9.5–16.0]	0.02
Number of mature oocytes	16.0 [10.2–27.7]	9.0 [7.0–13.0]	0.03
Number of fertilized oocytes	10.0 [6.7–25.7]	6.0 [4.5–10.0]	0.06
Number of day 3 embryos	10.0 [6.7–25.7]	6.0 [4.5–10.0]	0.049
Number of day 5 embryos	9.5 [6.7–18.7]	6.0 [3.5–8.0]	0.02
Number of transferred embryos	1.0 [1.0–1.0]	1.7 [1.0–2.0]	0.007
Number of cryopreserved embryos	4.0 [1.2–9.7]	2.0 [0.0–4.0]	0.09
Number of discarded embryos	5.0 [2.2–6.0]	2.0 [1.0–4.0]	0.2
Clinical pregnancy rate	7/12	7/13	1.05 (CI 0.45–2.41)

Data are expressed as median [25th–75th percentile] or n/total

IU/mL international unit/mL

Table 3 Follicular fluid AGE, sRAGE, 25-hydroxy-vitamin D, and insulin levels

	PCOS (n = 12)	Control (n = 13)	p value
sRAGE (ng/mL)	2.8 [2.2–3.6]	3.7 [3.0–4.7]	0.04
CML (μg/mL)	1.4 [1.2–1.6]	1.5 [1.3–1.7]	0.5
25-Hydroxy-vitamin D (ng/mL)	50.5 [39.8–63.8]	50.5 [40.7–69.2]	0.7
Insulin (m IU/L)	2.5 [1.5–5.1]	3.8 [1.9–7.0]	0.3

Data are expressed as median [25th–75th percentile];

sRAGE soluble receptors for advanced glycation end products, *CML* N-(carboxymethyl) lysine

PCOS have increased AGEs and RAGE expression irrespective of insulin resistance [13]. Furthermore, studies have shown that AGE-RAGE interaction interferes with the actions of LH and insulin on granulosa cells [33]. These alterations in polycystic ovaries could lead to the AGE-RAGE induced abnormal steroidogenesis in women with PCOS [14]. Elevated AGEs and their interaction with RAGE in PCOS could be associated with abnormal follicular development and defective steroidogenesis in these women [14].

Data to date suggest that vitamin D impacts sRAGE and the AGE-RAGE axis [34–36]. In cell culture studies, calcitriol blunted the AGE-induced upregulation of RAGE mRNA and protein and counteracted their stimulating effect on NF-κB pathway [37, 38]. In streptozotocin-induced diabetic rats, calcitriol attenuated the increased expression of cardiac RAGE, probably via modulating angiotensin II type 1 receptor [39]. RAGE expression was reduced in an in vitro blood-brain barrier model following vit D3 application [40]. Additionally, vitamin D supplementation provided protection against the vascular complications in diabetes via inhibiting the deposition of CML in the aortic wall, subsequently inhibiting the oxidative stress-mediated pathways [41]. Supplementation of vitamin D in hemodialysis patients increased serum sRAGE levels and exhibited a protective role by inhibiting interleukin-6 [42]. The role of vitamin D has been defined as a vascular protective agent that is mediated by counteracting the deleterious actions of AGEs on the endothelial cells (by inhibiting NF-κB pathway that is activated by AGEs) [37]. On the other hand, one study found no link between vitamin D and AGEs in diabetic patients [43]. Accumulating evidence has suggested that vit D3 replacement improves the clinical and metabolic outcomes in women with PCOS [26, 27, 44]. We have previously shown in vitamin D-deficient women with PCOS that supplementation with vit D3 increased serum sRAGE levels and caused a drop in the elevated serum AMH levels [28]. This suggests that vit D3 supplementation could play a role by altering follicular fluid levels of anti-inflammatory sRAGE and AGE-RAGE system and thus inhibiting the inflammation in PCOS women.

Table 4 Correlation between follicular fluid levels of sRAGE, 25-hydroxy-vitamin D, CML, and insulin

	r value	p value
All participants		
sRAGE vs. CML	0.60	0.001
sRAGE vs. 25-hydroxy-vitamin D	0.65	0.0004
sRAGE vs. insulin	−0.18	0.4
PCOS group		
sRAGE vs. CML	−0.37	0.2
sRAGE vs. 25-hydroxy-vitamin D	0.75	0.007
sRAGE vs. insulin	−0.10	0.7
Control group		
sRAGE vs. CML	0.89	0.0001
sRAGE vs. 25-hydroxy-vitamin D	0.72	0.007
sRAGE vs. insulin	−0.11	0.7

sRAGE soluble receptors for advanced glycation end products, *CML* N-(carboxymethyl) lysine

In this study, we found a positive correlation between follicular fluid sRAGE and follicular fluid CML in the control group but not in the PCOS group. Similarly, Willemsen [45] showed that serum CML and pentosidine (a well-characterized AGE) were positively correlated with serum sRAGE in patients hospitalized for heart failure. Additionally, Kerkeni et al. [46] showed that sRAGE serum levels positively correlated with serum AGEs in patients with diabetic retinopathy. In contrast, one explanatory hypothesis for this correlation is that as the pro-inflammatory CML increases in PCOS, there is lack of a counter-effect of anti-inflammatory response as reflected by lower sRAGE production leads to lower follicular fluid sRAGE levels in these women.

Our study has several limitations that include the following: (1) a small sample size that limited an ability to find any differences in CPR between both group if any difference existed, (2) sRAGE and 25-hydroxy-vitamin D levels in the serum were not evaluated as we have previously shown a relationship in the serum, and (3) although follicular fluid levels of only one of 20 members of the AGE family, CML, was measured, sRAGE has similar binding affinity to all of them and they exert similar bodily effects [32].

In conclusion, this pilot study presents a platform for studying the mechanisms by which AGEs could cause ovarian dysfunction in women with PCOS and calls for studies to evaluate the therapeutic efficacy of vitamin D supplementation in

women with PCOS. A proof of principle for the efficacy of vitamin D supplementation in treating and/or preventing ovarian dysfunction in patients with PCOS is likely to provide strong justification for the continued development of clinical trials of vitamin D as a cost-effective strategy to improving ovarian health via targeting the AGE-RAGE and sRAGE systems.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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