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Variation of Anti-inflammatory Cytokines in Relationship with Menopause

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Abstract

Aim. The aim of this study was to assess serum levels of the key anti-inflammatory cytokines in women of reproductive age and in pre and postmenopausal women. *Material and Method.* 175 women were enrolled and were divided into 5 groups (1 – Fertile women; 2 – Pre- and perimenopausal women; 3 – Postmenopausal women; 4 – Surgically-induced menopause; 5 – Chronic inflammation). Multiplex cytokine kits were used to evaluate serum levels of interleukin-4, -10 and -13. We determined the serum levels of follicle stimulating hormone, of luteinizing hormone, 17β-estradiol, progesterone, dehydroepiandrosterone and dehydroepiandrosterone sulfate using sandwich ELISA. *Results.* IL-4, IL-10 and IL-17 present a statistically significant decrease ($p=0.00$, $p=0.00$, respectively $p=0.0053$) in women with natural or surgically induced menopause (groups 3 and 4), compared with fertile women and premenopausal women (Groups 1, 2 and 5). Serum levels of IL-4 and IL-10 are significantly higher in fertile patients with associated chronic inflammatory diseases (133.5 ± 1.314 pg/ml, respectively 6.406 ± 13.47 pg/ml) than in fertile patients without chronic inflammatory diseases or premenopausal women (84.67 ± 1.22 pg/ml, respectively 0.627 ± 0.714). *Conclusions.* IL-4 and IL-10, together with IL-17, show significantly lower serum values in patients with natural or surgically induced menopause compared with patients of childbearing age or in premenopause. IL-4 and IL-10 show significantly higher serum values for patients of childbearing age presenting chronic inflammatory pathology compared with patients of childbearing age without chronic inflammatory pathology or premenopausal patients.

Keywords: Menopause; Cytokine; Interleukin; Steroid hormones

Introduction

Menopause, a physiological process in a woman's life, is characterized by the stop of menstruation due to the loss of ovarian follicular activity. It occurs around the age of 50 years and is accompanied by hot flushes, decreased libido, changes in the corporeal composition. The changes occurring when entering menopause are a consequence of decreased estradiol levels, a decrease associated with aging and behavioural changes (lack of exercise, sedentary lifestyle) [1].

The onset of menopause hormone deficiency associates with the onset of a low systemic inflammatory status, inflammation manifested by increased serum levels of the key proinflammatory cytokines IL-1, IL-6 or tumor necrosis factor (TNF)α.

The relationship between hormonal decline installed with menopause and the increase of serum levels of proinflammatory cytokines is not fully understood, and Verthelyi and Klinman found that postmenopausal women had fewer cells actively secreting cytokines as compared to fertile women and the activity of these cells did not correlate with sex hormone levels [2]. Recent studies have shown a significant increase in menopause of other cytokines with proinflammatory effects, such as IL-2 or IL-8, which appear to be responsible for the occurrence of disorders associated with menopause, such as hot flashes and major depressive disorders [3,4].

Anti-inflammatory cytokines are immunoreglatory molecules controlling proinflammatory cytokine response and activity, whose main representatives are interleukin (IL)-1ra, IL-4, IL-10, IL-11 and IL-13. Cytokine receptors specific for IL-1, TNFα or IL-18, also works to inhibit proinflammatory cytokines [5]. IL-10 is a cytokine that is currently regarded as a potential therapy for inflammatory diseases involving T helper 1-type responses and it induces the differentiation of a subset of regulatory CD4+ T cells (Tr1). These cells were shown to inhibit Th1 and Th2-type inflammatory responses through the secretion of IL-10 [6].

Within the immune system there is a fragile balance between proinflammatory and anti-inflammatory cytokines, and this balance is responsible for regulating inflammation by these cytokines. On the other hand, except for IL-1ra, all anti-inflammatory cytokines present proinflammatory properties as well, but the effect of these cytokines is dependent on the time of their release, on the environment in which they are released, on cytokine receptor density and not in the least on the tissue response to these cytokines [7,8].

Currently, there are few studies that evaluate serum changes of anti-inflammatory cytokines in relation with menopause, given that increased serum of proinflammatory cytokines has been demonstrated in menopause. Thus, an alteration in the serum levels of anti-inflammatory cytokines would be expected to occur as a counter-reaction to the above mentioned increase.

Our study aims to assess serum levels of the key anti-inflammatory cytokines in women of reproductive age and in women in pre and postmenopause.

Material and Method

Subjects

The study was conducted between 01.02.2011 – 31.12.2011 at "Dominic Stanca" Clinic of Obstetrics and Gynaecology, Cluj-Napoca.

The study included 175 patients admitted to the clinic, who were divided into five groups, as follows: **Group 1** (control group) - 35 healthy non-pregnant women of reproductive age (20-40 years old); **Group 2** (premenopausal women) - 40 healthy non-pregnant women in pre-and perimenopause (aged 46-53 years old), with regular menstruation or who had been without menstruation for no more than 6 months; **Group 3** (postmenopausal women) - 40 women in natural menopause (amenorrhea for at least 12 months) aged 54-65 years old, excluding patients with surgical or radiation induced menopause; **Group 4** (surgically-induced menopause) - 35 women in surgically-induced menopause (total hysterectomy with bilateral adnexectomy or simple bilateral adnexectomy) for at least 6 months, regardless of their age; none of the patients included in groups I-IV presented any acute or chronic inflammatory disease or were under recent corticosteroid treatment or under local oestrogen therapy or hormone replacement therapy; **Group 5** (Chronic inflammation) - 25 non-pregnant women of reproductive age (20-40 years old) with chronic inflammatory disease associated with low-grade systemic inflammation (psoriasis – 8 patients, systemic lupus erythematosus – 8 patients, antiphospholipid syndrome – 3 patients, alopecia aerata – 2 patients, rheumatoid polyarthritis – 2 pateints, sclerodermia – 2 patients, endometriosis – 1 patient), excluding patients with natural or surgically induced menopause before the age of 40, patients under local oestrogen therapy or hormone replacement therapy in the past 12 months.

Before enrolment all patients had been explained the purpose of this study and they gave their informed consent. The study was approved by the Ethics Committee of the "Iuliu Haţieganu" University of Medicine and Pharmacy, Cluj-Napoca.

A form was filled in for each patient included in the study containing general and anthropometric data (weight, height), the heredo-collateral history, personal pathological history, and data on the age and onset of menopause, data on the symptoms that appeared after the onset of menopause. The body mass index (BMI) was calculated as the ratio between the weight (kg) and the squared height (in meters). 5 ml of venous blood were harvested from each patient before breakfast, which was used to determine the complete blood count. Blood was centrifuged and the serum obtained was stored at-20oC for future determinations.

Determination of Steroid Sex Hormones

The steroid sex hormones were determined based on the samples stored at -20° C on the EDTA according to the technical specifications provided by the manufacturer.

Luteinizing hormone (LH) and follicle stimulating hormone (FSH) serum values were evaluated using immunoenzymatic assays by sandwich ELISA, according to the specifications provided by the manufacturer (NovaTec Immunodiagnostica GmbH, Dietzenbach, Germany). The sensitivity of the tests was 0.22 mIU/ml in the case of LH, and respectively 0.22 mIU/ml in the case of the FSH, while the intratest variation coefficients were ≤ 9.21% and intretest variation coefficients were ≤ 7.91% in the case of the LH, and respectively ≤ 5.1% for intratest and ≤ 7.6% for intertest in the case of the FSH.

Serum levels of 17β-estradiol (17β-E_2), progesterone (P) and dehydroepiandrosterone sulfate (DHEAS) were evaluated by competitive ELISA immunoenzymatic assays, according to the specifications provided by the manufacturer (NovaTec Immunodiagnostica GmbH, Dietzenbach, Germany). The sensitivity of the tests was 8.68 pg/ml for 17β-E2, 0.05 ng/ml for P, 0.03 mg/ml DHEAS. The intratests variation coefficients was ≤ 9% and ≤ 10% for intretests variation coefficients in the case of 17β-E2, ≤ 4% for intratests and ≤ 9.3% for intertests in the case of P, and ≤ 5.7% for intratests and ≤ 9.6% for intertests in the case of DHEA.

To determine dehydroepiandrosterone (DHEA) we used ELISA competitive immunoenzymatic assay, in accordance with the manufacturer's specifications (DRG Instruments GmbH, Marburg, Germany). The sensitivity of the test was 0.108 ng/ml, with the variation coefficients ranging between 3.84% and 6.92% in the case of the intratests, and 3.75% and 9.96% in the case of the intertests.

Cytokine Determination

To determine the serum levels we used multiplex cytokine kits (Fluorokine MAP Human MultiAnalyte Kit; 2 pieces) with the help of which we measured the serum levels of 11 cytokines: IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-17, IL-20 and TNFα. Dose measurements were performed with the help of a Luminex 200 system (Luminex Corporation, Austin, TX, USA) in accordance with the manufacturer's specifications (R&D Systems, Minneapolis, MN, USA).

The average sensitivity of the test for IL-4 was 1.75 pg/ml, and the intratest and intertest coefficients of variation ranged from 3.0-4.3%, and 9.4-15.9% respectively. The average sensitivity of the test for IL-10 was 0.13 pg/ml, and the intratest and intertest coefficients of variation ranged from 5.2-6.4%, and 7.3-10.1% respectively. The average sensitivity of the test for IL-17 was 0.39 pg/ml, and the intratest and intertest coefficients of variation ranged from 3.6-5.3%, and 7.6-8.1% respectively.

Statistical Analysis

Data is presented as the group mean (SD) and median (1st quartile – 3rd quartile). We compared baseline data using a t test for continuous variables. Pearson's simple correlation allowed studying the association between two variables. Statistical analyses were performed using SPSS software

(version 15.0, SPSS Inc, Chicago, IL) and STATA software (version 9.1, StataCorp, 4905 Lakeway Drive, College Station, Texas 77845 USA).

Results

Table 1 presents the descriptive statistics of the variables under study, which shows significantly lower values of 17β-E2 and significantly increased values of the FSH in postmenopausal groups (0.115±0.411 pg/ml, respectively 61.7±30.4 mUI/ml) and respectively in surgically-induced menopause women group (1.68±6.41 pg/ml, respectively 80.3±35.9 mUI/ml), compared with the fertile women group (22.7±31.1 pg/ml, respectively 2.22±1.91 mUI/ml), which confirms the hormonal changes occurring menopause.

Table 1. Descriptive statistics of the variables studied in the 5 groups

Variable	Calculated parameters	Group 1 (n=35)	Group 2 (n=40)	Group 3 (n=40)	Group 4 (n=35)	Group 5 (n=25)
Age	Mean (SD)	38.2 (5.4)	51.5 (0.7)	58.2 (3.6)	48.5 (3.1)	34.5 (5.6)
	Median	40.0	51.5	58.0	49.0	35.0
Weight	Mean (SD)	69.5 (14.2)	72.0 (4.6)	75.3 (13.5)	73.4 (8.7)	61.7 (5.8)
	Median	67	71.0	73.0	71.0	61.0
BMI	Mean (SD)	25.4 (5.3)	27.5 (3.9)	29.1 (5.2)	28.0 (3.4)	24.7 (1.3)
	Median	24.4	27.2	27.8	28.0	24.5
FSH	Mean (SD)	2.22 (1.91)	12.3 (17.5)	61.7 (30.4)*	80.3 (35.9)*	4.17 (3.39)
	Median	1.97	8.85	52.3	77.3	2.45
LH	Mean (SD)	5.47 (6.77)	15.6 (10.6)	20.5 (8.64)	32.0 (12.8)	6.75 (3.52)
	Median	3.72	14.5	19.3	30.2	5.66
17beta E2	Mean (SD)	22.7 (31.1)	60.8 (133.0)	0.115 (0.411)*	1.68 (6.41)**	7.61 (6.77)
	Median	8.09	18.7	0.007	0.15	6.92
Progesterone	Mean (SD)	4.04 (6.39)	1.39 (4.50)	0.137 (0.301)	0.225 (0.276)	2.98 (3.74)
	Median	1.42	0.24	0.05	0.12	1.16
DHEA	Mean (SD)	12.4 (5.81)	9.05 (4.08)	8.56 (6.37)	11.3 (7.96)	10.8 (4.35)
	Median	12.1	9.01	7.82	8.30	10.4
DHEAS	Mean (SD)	4.93 (3.60)	1,59 (1.37)	24.0 (55.5)	3.30 (2.73)	6.39 (4.30)
	Median	3.91	1,50	4.46	2.72	5.98

*p<0.001 compared to Group 1; **p≤0.05 compared to Group 1

To demonstrate the presence of menopause in the patients studied we performed a comparative analysis of the FSH and 17β-E_2 means on groups, which showed a statistically significant increase ($p < 0.001$) of the FSH in postmenopausal women (61.72±30.44 mIU/ml) compared with fertile women (2.218±1.912 mIU/ml), and a statistically significant decrease of 17β-E2 between the same groups (0.115±0.411 pg/ml, respectively 22.71±31.14). Mean values of the FSH and 17β-E2, as well as standard deviation (SD) in fertile women compared with those in menopause are presented in Table 2.

Table 2. Comparison of FSH and 17-B-Estradiol levels in group 1 and group 3

Group	Compared indicator	Mean	St. dev.	p - value
Group 1 : control vs. Group 3 : postmenopause	FSH	2.218	1.912	< 0.001
	FSH	61.72	30.44	
Group 1 : control vs. Group 3 : postmenopause	17β-E_2	22.71	31.14	< 0.001
	17β-E_2	0.115	0.411	

As for the three studied interleukins, serum levels of IL-4, IL-10 and IL-17 were detected in 56.57, 46.85, and 47.42% of the patients studied. Table 3 shows the detection rate of serum cytokines on the studied groups.

Table 3. Cytokine detection on study groups

Cytokine/Group	Gr. 1 (%)	Gr. 2 (%)	Gr. 3 (%)	Gr. 4 (%)	Gr. 5 (%)
IL-4	97.14	100	0	0	100
IL-10	88.57	87.5	0	0	64
IL-17	80	77.5	0	0	96

IL-4, IL-10 and IL-17 were detectable only in Groups 1, 2 and 5, as in Groups 3 and 4 their values were below the minimum detectable value. For the statistical interpretation of the results, based on the maximum undetectable value for each individual cytokine we simulated values after a continuous uniform distribution. This distribution takes values between 0 and the maximum undetectable value. In this way, the mean and SD values are obtained by simulations.

For all the three interleukins we observed a statistically significant decrease in women with natural or surgically induced menopause (groups 3 and 4), compared with fertile women and premenopausal women (Groups 1, 2 and 5). Table 4 shows the mean serum interleukin values as well as their statistically significance.

Table 4. IL-4, -10 and -17 serum levels comparison in groups 1 and 2 with groups 3 and 4

Comparison between	Compared indicator	No. of patients	Mean	St. dev.	p - value
Group 1,2 vs. Group 3,4	Interleukin IL-4	75	84.67	1.122	0.0000
	Interleukin IL-4	75	2.230	1.314	
Group 1,2 vs. Group 3,4	Interleukin IL-10	75	0.627	0.714	0.0000
	Interleukin IL-10	75	0.065	0.038	
Group 1,2 vs. Group 3,4	Interleukin IL-17	75	0.956	1.184	0.0053
	Interleukin IL-17	75	0.550	0.324	

At the same time, the results show that the serum levels of IL-4 and IL-10 are significantly higher in fertile patients with associated chronic inflammatory diseases (133.5±1.314 pg/ml, respectively 6.406±13.47 pg/ml) than in fertile patients without chronic inflammatory diseases or premenopausal women (84.67±1.22 pg/ml, respectively 0.627±0.714). Mean IL-4 and IL-10 values are presented in Table 5.

Table 5. Serum levels comparison of IL-4 and IL-10 in groups 1 and 2 with group 5

Comparison between	Compared indicator	No. of patients	Mean	St. dev.	p - value
Group 1,2 vs. Group 5	Interleukin IL-4	75	84.67	1.122	0.0136
	Interleukin IL-4	25	133.5	1.314	
Group 1,2 vs. Group 5	Interleukin IL-10	75	0.627	0.714	0.0424
	Interleukin IL-10	25	6.406	13.47	

In as far as IL-17 is concerned; there were no statistically significant differences between the 3 groups in which the values were detected. Table 7 shows the serum values obtained.

Table 7. Comparison of serum levels of IL-17 on study groups.

Comparison between	IL-17		
	Mean (s.d.)	Median	p-value
Group 1 vs. Group 2	0.959 (1.125) 1.245 (1.436)	0.349 0.349	0.394
Group 1 vs. Group 5	0.959 (1.125) 55.85 (213.4)	0.34 0.998	0.220
Group 2 vs. Group 5	1.245 (1.436) 55.85 (213.4)	0.349 0.998	0.223

Discussion

Cytokines are signalling protein molecules with important role in intercellular communication. Their source is represented by almost all cell types and tissues, which differentiates them from hormones [9,10]. Interleukins are a special group of cytokines, synthesized primarily by CD4 Th lymphocytes, but also by monocytes, macrophages and endothelial cells [11]. CD4 Th cells are divided into Th1 and Th2 depending on the cytokines produced. Th1 secretes mainly proinflammatory cytokines such as IL-2 or TNFα and interferon (IFN)-γ, while Th2 produces a variety of anti-inflammatory cytokines, including IL-4, IL-5, IL-10 and IL-13 [5]. Cytokines derived from Th2 cells (IL-4, IL-5, IL-10) support antibody production. Th2 cytokines induce an alternate activation with efficient antigen presentation to B cells. Transforming growth factor (TGF)β, corticosteroids, and IL-10 can induce an anti-inflammatory phenotype [12].

IL-4 and IL-10 are part of anti-inflammatory cytokines. There are studies showing an increase in inflammatory cytokines IL-4, IL-10 and IL-12, alongside TNFα after menopause as a compensatory mechanism, by which these pro-inflammatory cytokines TNFα to counteract [13].

IL-4 is a pleiotropic cytokine able to influence CD4 Th cell differentiation. This shows marked inhibitory effects on the expression and release of proinflammatory cytokines. It has the ability to block and suppress IL-1, TNFα, IL-6, IL-8 and macrophage inflammatory protein (MIP)-1a [14,15]. This is a key regulator in humoural and adaptive immunity. It seems that overproduction of IL-4 associates with allergic inflammation and asthma [16,17].

IL-10 is the most important anti-inflammatory cytokine involved in the human immune response. It is a potent Th1 proinflammatory cytokine inhibitor, such as IL-2 and IFN-γ [5]. A multitude of studies attest the anti-inflammatory role of this cytokine, as well as its role in preventing damage to the host and maintaining normal tissue homeostasis. Dysregulation of IL-10 is associated with enhanced immunopathology in response to infection as well as increased risk for development of many autoimmune diseases [18].

With the onset of menopause, there is a marked decrease in oestrogen levels, decrease associated with a low level inflammatory condition responsible for many diseases associated with menopause. Numerous studies suggest that oestrogen deprivation associated with menopause is one of the main causes for the immune changes associated with this period [19]. However, most of these studies have focused on the main proinflammatory cytokines: IL-1, IL-6 and TNFα.

The relationship between anti-inflammatory cytokines and menopause is not fully understood. Some studies have found no statistically significant differences between serum levels of IL-4 and IL-10 before and after menopause, as well as no changes in serum levels of these cytokines after oestrogen replacement therapy (ERT) [20,21]. On the other hand, recent studies show that serum levels of IL-4 and IL-10 demonstrate a statistically significant increase in patients with natural or surgically induced menopause after ERT, even after short-term therapy [22,23]. This increase could result in decreased serum levels of the key proinflammatory cytokines, decrease observed after ERT

in women in menopause. A major study showed involvement of IL-4 and IL-10 in postmenopausal osteoporosis. This study showed that plasma TNFα, IL-4, IL-10, IL-12, urinary hydroxyproline and calcium Increase with menopause. The Increase of anti-inflammatory IL-10, IL-12 and IL-4 Especially is probably a compensatory mechanism, by which these pro-inflammatory cytokines TNFα to counteract, and thus balance and oxidative stress it is inducing osteoclast activating effects [24]. Regarding IL-4, another recent study demonstrated that oestrogen treatment to reproductive senescent rat females attenuated the expression of IL-4, IL-12p70, and IL-13, thus suggesting that after menopause there are a number of changes in the levels of these cytokines, changes that can be attenuated by oestrogen therapy [25].

In our study, we observed significantly lower serum levels of IL-4, IL-10 in women in natural or surgical menopause compared to women of childbearing age. This decrease could be the result of the oestrogenic deprivation appeared in menopause. On the other hand, it was observed that in women of childbearing age who associate chronic inflammatory diseases, the serum levels of IL-4 and IL-10 are significantly higher than in fertile patients without chronic inflammatory diseases. This increase can be attributed to the same aforementioned compensatory mechanism.

IL-17 is a strong pro-inflammatory cytokine that responds to the invasion of the immune system by extracellular pathogens and induces destruction of the pathogen's cellular matrix [26]. On IL-17, there are currently no studies to clarify its relation to menopause. Instead, there are numerous studies showing the pro-inflammatory role of IL-17 and its involvement in immune and autoimmune diseases such as rheumatoid arthritis, asthma, lupus or antitumor immunity [27,28,29]. Our study showed significantly lower values of IL-17 in women in natural and surgically induced menopause compared to those in fertile women and in fertile women presenting chronic inflammatory pathology, and there have been no statistically significant differences when compared to women without chronic inflammatory pathology .

Change in serum anti-inflammatory cytokine values after menopause, associated or not with the increase of the major proinflammatory cytokines, could be related to the function of monocytes and macrophages, a function that is impaired due to oestrogen deficiency. The implications of these cytokines in the development and progression after menopause of important diseases such as osteoporosis, cardiovascular diseases, hot flashes or depressive syndrome, have been extensively studied, but there are still quite a few gaps to be filled in.

Conclusion(s)

Our study examines the variation of the major anti-inflammatory cytokines in relation to menopause. Thus, it was shown that IL-4 and IL-10, together with IL-17, show significantly lower serum values in patients with natural or surgically induced menopause compared with patients of childbearing age or in premenopause. Meanwhile, IL-4 and IL-10 show significantly higher serum values for patients of childbearing age presenting chronic inflammatory pathology compared with patients of childbearing age without chronic inflammatory pathology or premenopausal patients. Further studies are needed to clarify the role of the two anti-inflammatory cytokines IL-4 and IL-1 in menopause as well as their relationship with the proinflammatory cytokines, including IL-17.

Conflict of Interest

The authors declare that they have no conflict of interest.

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