Heliyon 9 (2023) e21148

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon

Prophylaxis of decidual CD68$^+$/CD163$^+$ macrophage disbalance in extracorporeal fertilized women

Volodymyr Likhachov [a], Yanina Shimanska [a], Oleh Akimov [b,*], Viktoriya Vashchenko [a], Olena Taranovska [a], Iryna Zhabchenko [a], Igor Kaidashev [c]

[a] Department of Obstetrics and Gynecology No2, Poltava State Medical University, Shevchenko 23, Poltava, ZIP code 36011, Ukraine
[b] Department of Pathophysiology, Poltava State Medical University, Shevchenko 23, Poltava, ZIP code 36011, Ukraine
[c] Department of Internal Medicine No3 with Phthysiatry, Poltava State Medical University, Shevchenko 23, Poltava, ZIP code 36011, Ukraine

ARTICLE INFO

Keywords:
In vitro fertilization
Threat of premature birth
Arginase
Cytokines
Decidual macrophages
iNOS

ABSTRACT

The demographic crisis that prevailed in Ukraine in recent years, the state of war as a result of the aggression of the Russian Federation, reproductive losses among women of childbearing age are one of the most urgent problems in modern obstetrics and gynecology. One of the most effective methods of correcting impaired reproductive function is in vitro fertilization.

The purpose of this work: is to develop a pharmacological complex for the prevention of imbalance of CD68$^+$/CD163$^+$ decidual macrophages in vitro fertilized women.

Materials and methods: 105 pregnant women who were divided into 3 groups took part in the study. The first group included 20 women whose pregnancy occurred and is proceeding physiologically. The second group consisted of 85 women who became pregnant as a result of in vitro fertilization, including 37 pregnant women who refused prophylactic correction of the threat of premature birth, and 48 pregnant women who received prophylactic correction of the threat of premature birth: complex prescription of vitamin D3 2000 IU orally 2 times a day, micronized progesterone 200 mg 2 times a day and L-arginine aspartate 1000 mg 4 times a day, starting from 18 to 20 weeks of pregnancy.

Results: In women who refused prophylactic correction of the threat of premature birth, a local increase in the activity of inducible NO-synthase and concentration of tumor necrosis factor-α, and a decrease in the activity of arginase and in the level of interleukin-10 were observed in the cervical mucus. They have a lower expression of CD163$^+$ on placental decidual macrophages and an increased expression of CD68$^+$, which indicates a shift in the polarization of macrophages from an anti-inflammatory to a pro-inflammatory phenotype. The use of prophylactic treatment brings the studied parameters closer to the results of women in whom pregnancy occurred physiologically.

Conclusions: In women who became pregnant as a result of in vitro fertilization, at 28–30 weeks of pregnancy, changes specific for pro-inflammatory phenotype of decidual macrophages were observed. Complex administration of vitamin D3, micronized progesterone and L-arginine aspartate lead to restoration of anti-inflammatory phenotype of decidual macrophages.

* Corresponding author.
E-mail address: o.akimov@pdmu.edu.ua (O. Akimov).

https://doi.org/10.1016/j.heliyon.2023.e21148
Received 13 July 2023; Received in revised form 15 October 2023; Accepted 17 October 2023
Available online 18 October 2023

1. Introduction

The risk of premature birth during in vitro fertilization (IVF) is significantly higher than during spontaneous pregnancies [1,2]. According to the results of our previous studies, it was established that one of the factors of failure in the onset and bearing of pregnancy is hyperandrogenemia, which may be the reason for the use of IVF [3]. At the same time, the birth of children with an abnormally low weight (less than 1000 g) is also observed. The course of childbirth in such women is more often complicated by premature discharge of amniotic fluid, preeclampsia, fetal distress during pregnancy and childbirth, and delayed fetal development [4].

A change in the polarization of endometrial macrophages can cause impaired fertility [5]. Polarization of endometrial macrophages according to the M_1 (proinflammatory) phenotype is necessary to ensure physiological implantation of the zygote during IVF [6]. At the same time, to ensure the further carrying of the embryo and fetus, the polarization of decidual macrophages should be mainly according to the M_2 (anti-inflammatory) phenotype [7].

The desired influence on the polarization of uterine decidual macrophages can be achieved in several ways. Systemic administration of L-arginine may be one such way, as it may promote a shift in macrophage polarization towards the M_2 phenotype [8]. However, the role of L-arginine in macrophage polarization remains a matter of debate, as it is used by both phenotypes (for the formation of polyamines in M_2 and for the synthesis of nitric oxide in M_1) [9]. Also, systemic administration of L-arginine can prevent the development of amino acid imbalance and endothelial dysfunction [10]. Also, L-arginine can shift the balance of $CD68^+/CD163^+$ macrophages towards the predominance of $CD163^+$, which also helps to reduce the polarization of macrophages according to the M_1 phenotype [11].

Vitamin D3 can also affect the polarization of macrophages. Administration of $1,25(OH)_2D3$ increases the expression level of Tim-3 in macrophages, which promotes cell polarization to M_2 and inhibits polarization to M_1 [12]. Administration of $1,25(OH)_2D3$ also reduces inflammation in experimental models [13].

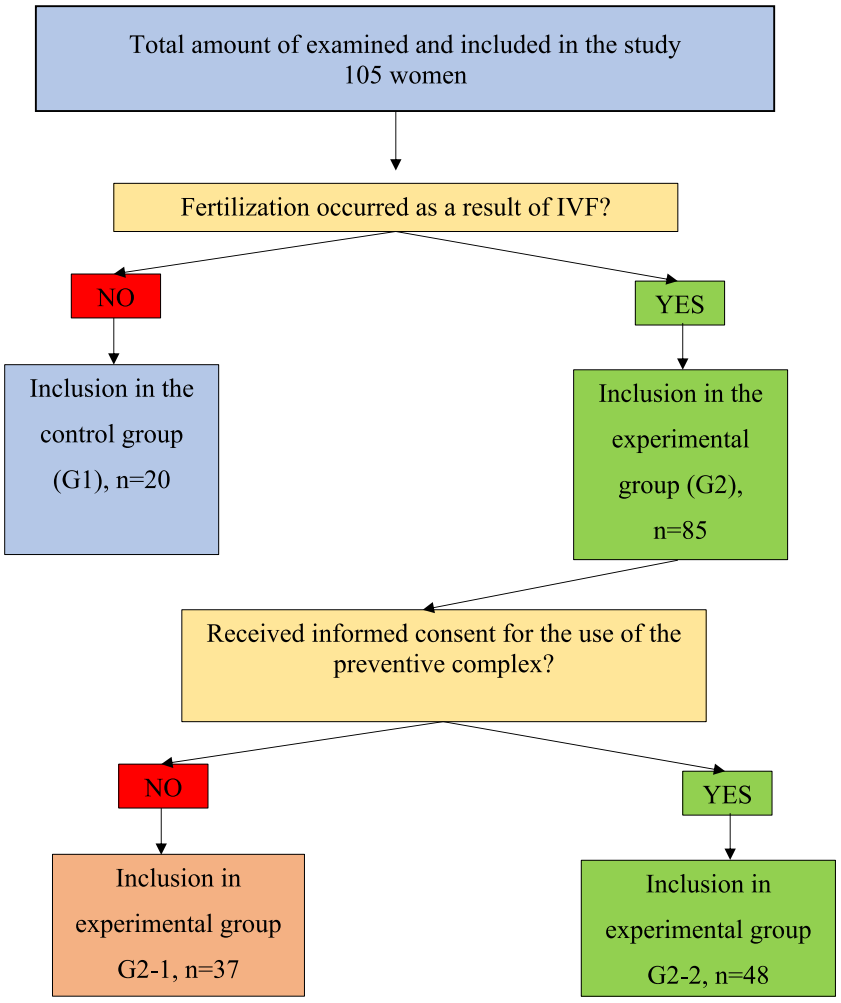

Fig. 1. Graphic structure of the study.

Clinical studies prove that the use of micronized progesterone in women who became pregnant as a result of IVF promotes the physiological course of pregnancy and reduces the risk of miscarriage [14]. The mechanism underlying this effect may be the selective inhibitory effect of progesterone on the polarization of decidual macrophages according to the M_1 phenotype and its simultaneous stimulating effect on the polarization of decidual macrophages according to the M_2 phenotype [15].

Several markers of macrophage polarization according to the M_1 phenotype are described in the scientific literature: CD68, CD80, CD86 [16]. CD68 and CD86 markers can be found in decidual macrophages in case of development of inflammatory diseases of the endometrium or placenta [17,18]. According to Ye Y. et al. increased expression of the CD68 receptor on decidual macrophages is associated with the threat of pregnancy loss [19]. CD163 and CD206 predominate among the polarization markers of decidual macrophages according to the M_2 phenotype [19,20]. Why is the predominance of CD206 expression on endometrial decidual macrophages necessary at the stage of implantation, and the predominance of CD163 expression is necessary to ensure bearing during pregnancy [19,20]. Therefore, the study of the expression of CD68 and CD163 can be prognostic for determining possible complications and premature birth in women with IVF.

Currently, the scientific literature provides a limited amount of information on changes in the functional state of decidual macrophages when using IVF, and there are no data on pathogenetically justified correction of these changes.

The purpose of this work is to develop a pharmacological complex for the prevention of imbalance of $CD68^+/CD163^+$ decidual macrophages in vitro fertilized women.

2. Research materials and methods

2.1. Patient population

The study was conducted on the basis of the Perinatal Center of the communal enterprise "Poltava Regional Clinical Hospital named after M.V. Sklifosovsky of the Poltava Regional Council" and the Department of Obstetrics and Gynecology No. 2 of the Poltava State Medical University, Poltava, Ukraine. 105 women who became pregnant using IVF were selected. All patients were thoroughly informed about the study program, about the nature and potential risks and benefits of their participation in the study, voluntarily signed the informed consent.

The conduct of the research was approved by the Commission on Ethical Issues and Bioethics of the Poltava State Medical University (Protocol No. 159 dated November 22, 2017).

2.2. Research design

Our study is an open, non-randomized controlled study.

2.3. Inclusion and exclusion criteria and treatment protocol

The first group (G1) included 20 women with a physiological pregnancy that occurred without in vitro fertilization and without risk factors for premature birth (Fig. 1). The course of pregnancy was without complications and the birth took place after 37 weeks of gestation. Pregnant women from this group were enrolled in experiment within a year. A separate control group for immunohistological research was made up of 5 healthy women.

In the second group (G2), the selection of women was carried out among pregnant women with the female infertility factor of tubal genesis, frequent inflammatory diseases of the lower genital organs in the anamnesis, pregnancy that occurred as a result of in vitro fertilization with embryo transfer (IVF + ET), in whom genetic pathology was ruled out after pre-implantation genetic diagnosis (PIGD), which involved screening for following genetic pathologies: cystic fibrosis, spinal muscular atrophy, Huntington's disease, sickle cell anemia and a number of others. Their body mass index (BMI) was low (<25 kg/m^2) and they had a high risk of the threat of premature birth, namely a decrease in the blood content of β-chorionic gonadotropin (β-hCG), free estriol, placental lactogen below the lower limit norms, cervicometry data on shortening of the cervix ≤ 25 mm. Involvement of women in the study was carried out at 18–20 weeks + 6 days of pregnancy at the stage of the second biochemical screening. Pregnant women from this group were enrolled in experiment within timeframe of 3 months (September–November).

Criteria for exclusion of women from the study: women who did not undergo PIGD; detection of chromosomal pathologies at the stage of PIGD; infection of the lower parts of the genital tract; body mass index ≥ 25 kg/m^2, presence of extragenital pathology in the acute stage at the time of the study, infectious diseases, allergies, congenital or acquired thrombophilias; bad habits (alcohol, smoking, drugs); rhesus immunization; signs of fetal intrauterine infection (FII).

The second group (G2) included 85 women who became pregnant as a result of IVF and had prognostic signs of a high risk of premature birth.

The second group of women was divided into two subgroups.

- G2-1 consisted of 37 pregnant women who did not receive prophylactic treatment; when the threat of premature birth is realized in them before 34 weeks of pregnancy and the opening of the cervix <3 cm, the absence of amnionitis, preeclampsia, bleeding, satisfactory condition of the woman; they underwent tocolysis with micronized progesterone per os 400 mg, with its repeated reception after 4 h, after which this therapy was continued at 200 mg after 8 h until the tone of the uterus normalized; at the same

time, for 48 h, antenatal prophylaxis of fetal respiratory disorders syndrome was performed with glucocorticoids (dexamethasone 6 mg after 12 h intramuscularly 4 times).

- G2-2 consisted of 48 pregnant women who, from the moment of inclusion in the study, received our proposed prevention of the threat of premature birth, which included: vitamin D3 (cholecalciferol) 2000 IU orally 2 times a day (before childbirth); micronized progesterone orally 200 mg 2 times a day until 34 weeks of pregnancy; L-arginine aspartate 1000 mg 4 times a day. Such prevention was started from 18 to 20 weeks + 6 days of pregnancy; L-arginine aspartate was prescribed in cycles of 15 days, repeated every 15 days until delivery. In the event that such pregnant women are at risk of premature birth, standard antenatal prophylaxis of fetal respiratory disorders syndrome with glucocorticoids was performed.

In order for the results of the immunohistochemical study of the prevalence of $CD68^+$ and $CD163^+$ cells in the placentas of healthy women to be comparable with other groups, the placentas of 5 healthy women who were delivered by planned cesarean section at 34 weeks of pregnancy due to the presence of monochorionic monoamniotic twins were additionally examined.

2.4. Clinical and laboratory monitoring

2.4.1. Collection of anamnesis, general clinical examination, analysis of the course of pregnancy and childbirth

Collection of somatic, obstetric and gynecological, infectious and reproductive anamnesis was carried out. Anthropometric indicators were studied. Height was determined in meters; body weight - in kilograms; body mass index (BMI) was calculated using the formula: BMI = body weight (kg)/height (in m^2). Overweight and obese women were not included in the study.

An analysis of the course and complications of pregnancy and childbirth, as well as the condition of newborns, was carried out.

All patients underwent general clinical laboratory examinations (general blood test, general urinalysis, bacterioscopic examination of vaginal secretions).

2.4.2. Collection of cervical mucus

Cervical mucus was collected during examination of the cervix in mirrors using a sterile brush (so that the mucus completely covers the brush); then the brush with mucus was immersed in 2 ml of 0.2 M Tris-buffer solution (pH = 7.4) and stored frozen at a temperature of −40 °C until laboratory examination.

2.5. Determination of cytokine content by ELISA method

At 32 weeks of pregnancy, cervical mucus was collected from patients of all groups for enzyme-linked immunosorbent assay (ELISA). The content of pro- and anti-inflammatory cytokines (TNF-α, IL-10) was studied using the appropriate standard commercial reagent kits of the company "Vector BEST" according to the manufacturer's instructions. In addition, the TNF-α/IL-10 index was calculated by dividing the concentration of TNF-α in the patient by the concentration of IL-10 in the same patient.

2.6. Determination of the activity of inducible NO-synthase and arginase

The activity of iNOS in cervical mucus was calculated by subtracting the activity of constitutive isoforms (cNOS) from the total activity of NO synthase. Total arginase activity was determined by the difference in L-ornithine concentrations before and after incubation at t = 37.0 °C. The obtained indicators were expressed in μmol/min per 1 g of cervical mucus protein [21].

2.7. Determination of $CD68^+$ and $CD163^+$ cells

For immunohistochemical analysis, samples were taken from 10 placentas of women of group G2-1 who did not receive prophylactic treatment and gave birth prematurely (at the 32-34th week of pregnancy) and from 8 placentas of women who received our proposed prophylactic treatment (G2-2) and were delivered at 34 weeks of pregnancy for various reasons (fetal distress, fetal growth retardation, etc.). As a control (G1), an immunohistochemical study of 5 placentas of healthy women was performed. Counting of decidual macrophages of $CD68^+$ and $CD163^+$ subpopulations was performed in 10 fields of view in each immunohistochemical preparation.

In the study, pieces of the placenta were used after spontaneous delivery or caesarean section. In all cases, the placenta separated and was born by itself, manual intervention was not performed. Samples measuring 10 × 10 × 25 mm were cut from the placenta and fixed in a 10 % neutral formalin solution. Next, paraffin blocks were prepared, from which sections with a thickness of 5–7 μm were made, those blocks were selected in which the chorionic villus tissue and decidual tissue were clearly visualized. Immunohistochemical study of subpopulations of decidual macrophages was carried out in the pathomorphological laboratory "CSD Health Care" (Kyiv), which is part of the European quality control system NordiQC.

Endogenous peroxidase depletion was performed in placental sections, after washing, primary monoclonal antibodies to CD68 and CD163 were applied to the sections (NovaCastra, United Kingdom). After incubation and washing of the sections, secondary antibodies to CD68 and CD163 markers were added. Next, sections were treated with extravidin conjugated with horseradish peroxidase with the addition of a developing mixture with 3-amino-9-ethylcarbazole. Dosage staining of drugs was carried out with hematoxylin according to Mayer's method.

The number of cells was counted by the morphometric method of standard areas in 10 fields of view at a magnification of 400 times

(eyepiece 10; objective 40), which allows to find out the number, typical localization and mutual arrangement of immunocytes. Documentation of the obtained preparations was carried out with a digital camera.

The $CD68^+/CD163^+$ ratio was calculated by dividing the number of $CD68^+$ cells by the number of $CD163^+$ cells.

2.8. Statistical analysis

Statistical processing was carried out using the Microsoft Excel software package and the Real Statistics 2019 extension to it. Minimal group size was determined by usage of "Statistical Power and Sample Size" function in Real Statistics 2019 with following parameters: effect size = 1; power = 0.8; number of tails (for two sample comparisons) = 2; alpha = 0.05. According to calculations minimal group size was 17 (Noncentrality = 2.915475947423; Critical value = 2.03693334346). We did not exclude patients above minimal required number, who volunteered to participate in experiment.

The Shapiro-Wilk test was used to determine the normal distribution of traits. With a normal distribution of signs, the Student's t-test was used to assess the statistical significance of the difference in indicators between groups. The Mann-Whitney *U* test was used for distribution of signs that differed from normal. The difference was considered statistically significant at $P < 0.05$.

3. Research results

3.1. Clinical examination results

In 85 pregnant women of the experimental group (G2), who became pregnant as a result of IVF before being divided into two subgroups compared to 20 healthy pregnant women of the control group (G1), anamnestic risk factors for the occurrence of premature birth were found. General clinical characteristic of study groups is given in Table 1. Thus, in G2: women from 31 to 35 years of age predominated (while in G1 - women aged 21–30); they more often had a complicated infectious anamnesis (chronic diseases of the genitourinary system, gall bladder, etc.); early and late menarche prevailed (85.1 %) (while in G1 women only 27 %); women G2 more often had a complicated obstetric and gynecological history (chronic inflammatory processes of the internal genital organs, long-term infertility, spontaneous abortions, frozen pregnancies, premature births, delayed fetal development, premature rupture of fetal membranes, intrauterine infection of the fetus); also, G2 patients with previous pregnancies had a higher frequency of surgical treatment of isthmic-cervical insufficiency, use of obstetric pessaries, surgical interventions in childbirth.

When comparing pregnancy complications in women of the G2 group by subgroups, we found that premature birth occurred 1.8 times more often in pregnant women of the G2-1 subgroup (89.2 % of cases against 50.0 % of cases in the G2-2 subgroup). Moreover, the vast majority of PP (59.5 %) in subgroup G2-1 occurred before 34 weeks of pregnancy (and in subgroup G2-2 this indicator was only 8.3 %).

Surgical correction of isthmic-cervical insufficiency was performed in 10.2 % of cases in subgroup G2-1 and in 4.2 % of cases in subgroup G2-2. Premature rupture of the fetal membranes occurred with almost the same frequency in both studied subgroups: in G2-1 - in 38 %, and in G2-2 - in 33.3 %.

In 8.9 % of cases in women of the G2-1 subgroup, the course of pregnancy was complicated by the syndrome of delayed fetal development and premature detachment of the normally located placenta, chorioamnionitis was diagnosed in 4.7 % of cases, partial dense attachment of the placenta - in 8.1 %. Pregnant women of the G2-2 subgroup did not develop the aforementioned complications at all.

The total number of children born in G2 due to the large number of twins (36 cases in both subgroups) amounted to 121 children, and almost equally in both groups - 60 and 61. Among women of subgroup G2-2, 55 children (90.2 %) were born without asphyxia, and 6 children (9.8 %) were born with mild degree of asphyxia in the complete absence of cases of severe asphyxia. In women of subgroup G2-1, asphyxia occurred in 16 children (26.7 % of cases), and severe asphyxia was observed in 2 children (3.4 %). The perinatal mortality rate in subgroup G2-1 was 16.7‰, and there was no perinatal mortality in subgroup G2-2.

Table 1
General clinical characteristics of the examined women.

Groups	Number of studied women	Average age (years)	BMI (kg/m^2)	Blood pressure syst./diast. (mm Hg)	Pulse (beats/min)	Daily diuresis (ml)	Concentration of hemoglobin in the blood (g/L)	Concentration of total protein in blood plasma (g/L)
G1 (physiological pregnancy)	20	25.4 ± 3.0	23.3 ± 0.9	112.2 ± 2.6 / 70.5 ± 1.4	78.7 ± 2.2	925.1 ± 36.2	126.6 ± 4.4	69.7 ± 2.9
G2-1 (untreated women with IVF)	37	32.7 ± 4.3	22.0 ± 1.1	115.1 ± 3.0 / 75.0 ± 2.2	80.8 ± 2.9	890.3 ± 29.1	118.0 ± 4.8	66.5 ± 3.4
G2-2 (treated women with IVF)	48	33.4 ± 3.7	24.2 ± 1.5	110.9 ± 4.1 / 68.7 ± 2.9	79.6 ± 3.1	905.2 ± 33.4	121.2 ± 3.9	71.4 ± 2.3

Note: no statistically significant difference was found between all the given indicators between groups.

3.2. Immunohistochemical result

The content of $CD68^+$ cells in the placentas of healthy pregnant women was 56.5 ± 4.5 per 10 visual fields, and in untreated women with IVF whose pregnancy ended prematurely (G2-1), it was 99.3 ± 6.6 per 10 visual fields, which is 1.76 times more than in healthy women ($p < 0.001$) (Fig. 2 A).

The content of $CD163^+$ cells in the group of women without treatment was 1.8 times lower (76.1 ± 4.8 per 10 visual fields) than in the placentas of women in the control group (135.0 ± 6.4 per 10 visual fields; $p < 0.001$) (Fig. 2B). Accordingly, the ratio of $CD68^+$/$CD163^+$ in women with IVF and premature birth was significantly higher (1.30 ± 0.07) than in healthy patients (0.42 ± 0.043) ($p < 0.001$) (Fig. 3).

In women who received our proposed preventive treatment, which included cholecalciferol, micronized progesterone, L-arginine aspartate, the number of $CD68^+$ cells in the placental tissue decreased by 1.61 times compared to that of women who did not receive treatment (61.5 ± 5.8 per 10 visual fields in the group of women with IVF who received prophylactic treatment against 99.3 ± 6.6/10 visual fields in women who did not receive treatment; $p < 0.001$), which is close to the indicator in healthy pregnant women (56.5 ± 4.5 per 10 visual fields; $p > 0.05$) (Fig. 3). The number of $CD163^+$ cells increased 1.55 times (118.4 ± 7.1 per 10 visual fields in the group of women with IVF who received prophylactic treatment vs. 76.1 ± 4.8 per 10 visual fields in women who did not receive treatment; $p < 0.002$), approaching the indicator in women without IVF (135.0 ± 6.4 per 10 visual fields; $p > 0.1$) (Fig. 3). The ratio of $CD68^+$/$CD163^+$ in pregnant women from the group of women with IVF who received prophylactic treatment significantly decreased to 0.52 ± 0.05 compared to women who did not receive treatment (1.30 ± 0.07; $p < 0.01$), not significantly different from the indicator in healthy pregnant women (0.42 ± 0.043; $p > 0.5$) (Fig. 4B).

3.3. Biochemical result

At 28–30 weeks of pregnancy, G2-1 women showed an increase in iNOS activity in cervical mucus from 1.20 ± 0.20 μmol/min per g of protein in healthy women to 3.08 ± 0.32 μmol/min per g of protein ($p < 0.001$ when compared with healthy women) (Fig. 5A). Arginase activity decreased from 2.16 ± 0.29 μmol/min per g of protein in healthy women to 1.13 ± 0.10 μmol/min per g of protein in the G2-1 group ($p < 0.001$ when compared with healthy women) (Fig. 5B). The use of preventive therapy for the threat of premature birth in women whose pregnancy occurred as a result of IVF reduced the activity of iNOS in cervical mucus to 1.66 ± 0.18 μmol/min per g of protein ($p < 0.001$ when compared with G2-1), and the activity of arginase increased to 1.98 ± 0.17 μmol/min per g of protein ($p < 0.001$ when compared with G2-1).

3.4. Immunological results

At 28–30 weeks of pregnancy, in women who became pregnant as a result of IVF and did not receive prophylactic therapy due to the threat of premature birth, there was an increase in the concentration of TNF-α in the cervical mucus to 6.15 ± 0.39 pg/ml compared to the indicators of women with a physiological pregnancy, which was 3.43 ± 0.22 pg/ml ($p < 0.001$) (Fig. 6A). The concentration of IL-10 in this group was reduced to 5.02 ± 0.38 pg/ml, and in the control group it was 9.35 ± 0.56 pg/ml ($p < 0.001$) (Fig. 6B). The TNF-α/IL-10 ratio was 1.23 ± 0.11 in women who became pregnant as a result of IVF and did not receive prophylactic therapy due to the threat of preterm birth, and in the control group this ratio was 0.36 ± 0.02 ($p < 0.001$) (Fig. 4A), which indicates the predominance of the profile of pro-inflammatory cytokines in G2-1.

The use of prophylactic therapy for the threat of premature birth in women whose pregnancy occurred as a result of IVF reduced the concentration of TNF-α in cervical mucus to 3.98 ± 0.23 pg/ml; $p < 0.001$ when compared with group G2-1 (6.15 ± 0.39 pg/ml); the concentration of IL-10 also increased to 8.37 ± 0.45 pg/ml in women of group G2-2; $p < 0.001$ when compared with group G2-1 (5.02 ± 0.38 pg/ml) (Fig. 6B), and the TNF-α/IL-10 ratio decreased to 0.48 ± 0.10; $p < 0.001$ when compared with group G2-1 (1.23 ± 0.11)

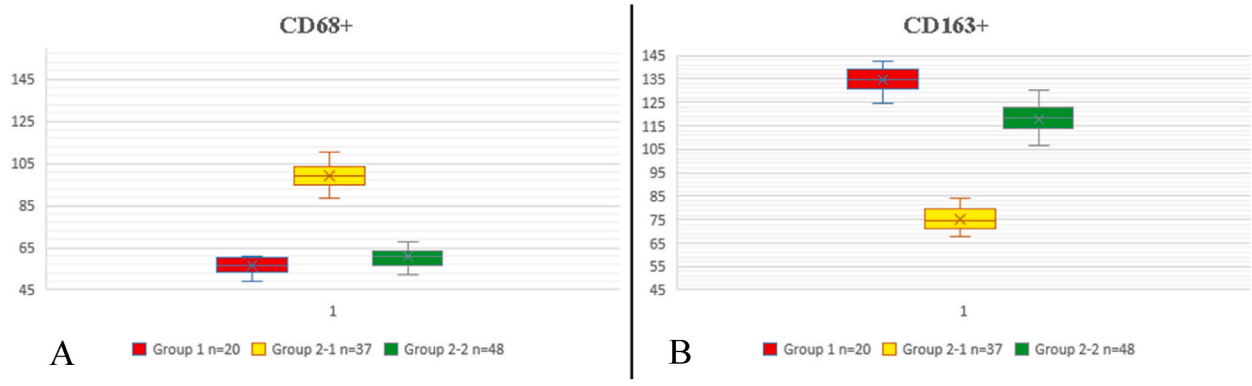

Fig. 2. Expression of CD68 and CD163 receptors on decidual macrophages of the placenta in the examined women after delivery: A – expression of CD68; B – expression of CD163 (mean in 10 fields of view, M ± m).

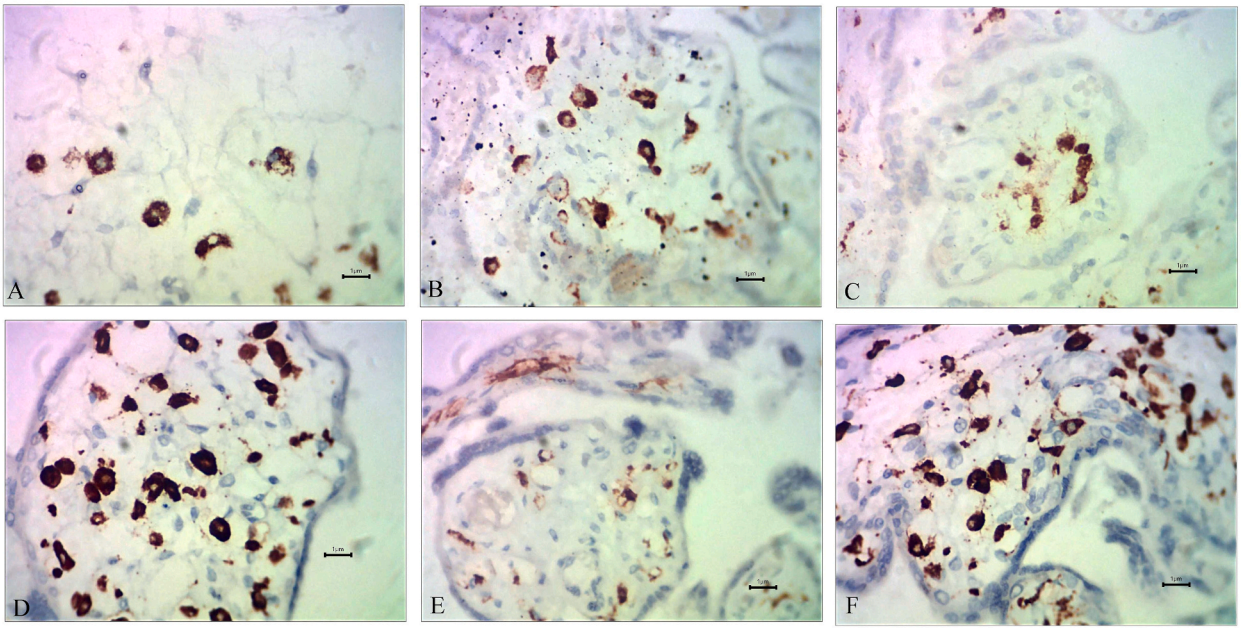

Fig. 3. Immunohistochemical staining of $CD68^+$ and $CD163^+$ macrophages with Mayer's hematoxylin staining. Magnification: Eyepiece ×10, Objective ×40. A – $CD68^+$ macrophages in control group; B – $CD68^+$ macrophages in group G2-1; C – $CD68^+$ macrophages in group G2-2. D – $CD163^+$ macrophages in control group; E – $CD163^+$ macrophages in group G2-1; F – $CD163^+$ macrophages in group G2-2.

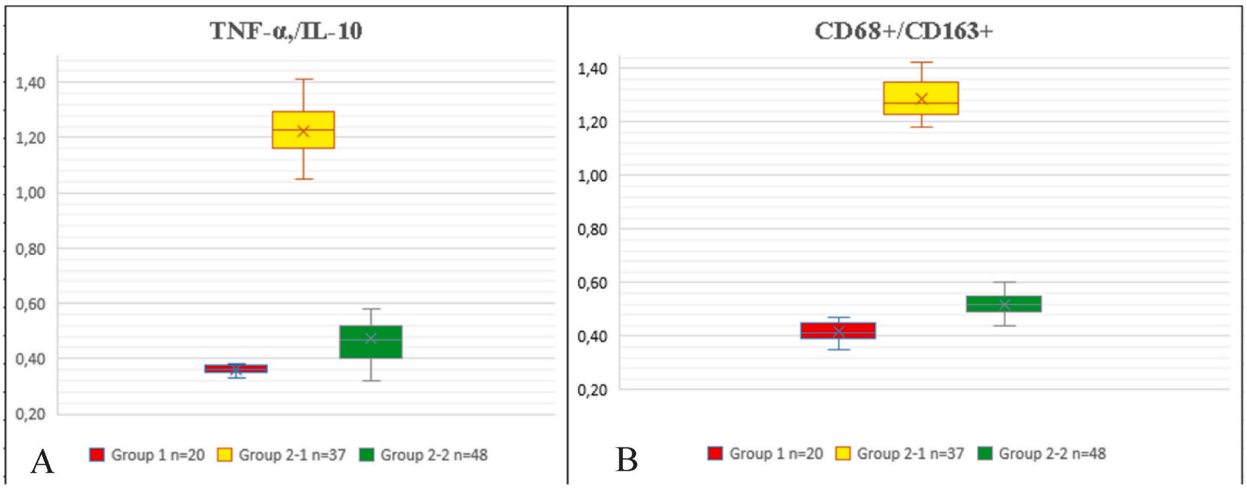

Fig. 4. The ratio of the concentration of pro- and anti-inflammatory cytokines in the cervical mucus of pregnant women at 28–30 weeks of gestation and the ratio of $CD68^+/CD163^+$ macrophages in the placenta after delivery: A – TNF-α/IL-10 ratio; B – $CD68^+/CD163^+$ ratio (M ± m).

(Fig. 4A).

There were no statistically significant differences in the investigated indicators of cervical mucus between pregnant women as a result of IVF who agreed to use prophylactic therapy for the threat of premature birth, and in the indicators of women with a physiological pregnancy. Thus, the use of prophylactic therapy for the threat of premature birth restores the cytokine profile of pregnant women as a result of IVF to the values of physiological pregnancy.

4. Discussion

A decrease in the number of $CD68^+$ and an increase in $CD163^+$ macrophages in women who became pregnant as a result of IVF after prophylactic treatment compared to women who refused treatment may indicate a decrease in inflammatory phenomena and a shift in the polarization of decidual macrophages towards the predominance of M_2 polarization, which is confirmed by the works of

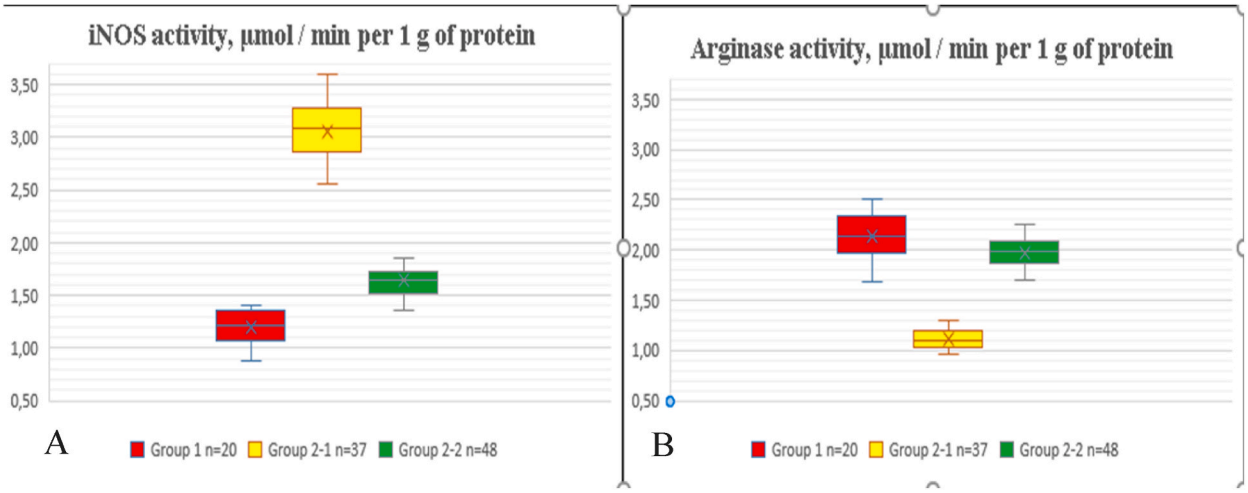

Fig. 5. Activity of marker enzymes of macrophage polarization in cervical mucus of pregnant women at 28–30 weeks of gestation: A – iNOS activity, µmol/min per g of protein; B – arginase activity, µmol/min per g of protein (M ± m).

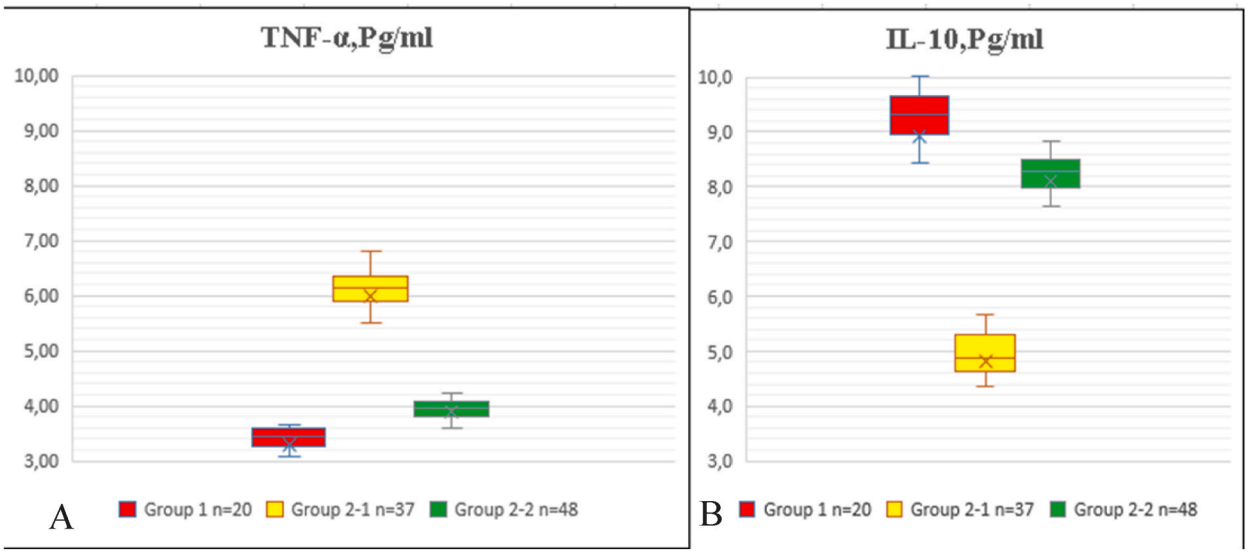

Fig. 6. The concentration of cytokines in the cervical mucus of pregnant women at 28–30 weeks of gestation: A – concentration of TNF-α, pg/ml; B – concentration of IL-10, pg/ml (M ± m).

Yemchenko Ya.O. and A.V. Aikyan [22,23]. According to Y. Li et al. an increase in $CD68^+$ macrophages in women is accompanied by endometritis and the inability to carry a child, while an increase in $CD163^+$ macrophages allows pregnancy to proceed normally [24]. This confirms the effectiveness of the prophylactic complex chosen by us to correct the imbalance of $CD68^+/CD163^+$ decidual macrophages in women with in vitro fertilization.

According to our research, the concentration of the pro-inflammatory cytokine TNF-α increases in women who became pregnant as a result of IVF and who refused preventive treatment, which is consistent with the data obtained by Yildizfer F. et al. [25]. Studies indicate that TNF-α levels in late pregnancy may be associated with IVF success and may be predictive of preterm birth risks [26]. According to Alijotas-Reig J. et al. high concentrations of TNF-α in the II and III trimesters of pregnancy can provoke miscarriage and premature birth [27].

According to our research, simultaneously with the increase in the concentration of TNF-α in women with IVF who refused prophylactic treatment, the concentration of IL-10 decreases. Such a decrease in the concentration of IL-10 against the background of an increase in TNF-α can be a risk factor for premature birth. According to Kaislasuo J. et al. the ratio of IL-10/TNF-α may have prognostic value for differentiating physiological or pathological (with the threat of premature termination) course of pregnancy [28].

We found that women with IVF who refused prophylactic treatment have an increase in the ratio of TNF-α/IL-10, which, according to Winger E.E. et al., has a negative prognostic value, especially for women who became pregnant as a result of IVF [29].

Nitric oxide is an important gaseous mediator, which encompasses many physiological functions during pregnancy. However, nitric oxide produced by inducible isoform of NO-synthase can be formed in excessive quantities or transformed into toxic peroxynitrite in reaction with superoxide anion. Excessive quantities of NO produced by iNOS can cause miscarriages [30]. Arginases form L-ornithine from L-arginine and they are competitors with NO-synthases for L-arginine. L-ornithine is then transformed by ornithine decarboxylase enzyme to putrescine, which is further converted to spermidine and spermine. Putrescine, spermidine and spermine are polyamines, which play crucial role in cell division, proliferation and differentiation in female reproductive organs [31].

An increase in iNOS activity in a group of women with IVF who refused prophylactic treatment is also a prognostic criterion that indicates possible complications of the course of pregnancy that occurred as a result of IVF [32]. In the scientific literature, there is a limited amount of data on changes in arginase activity in women whose pregnancy occurred as a result of IVF. However, arginase gene polymorphism can cause the development of preeclampsia [33].

Considering the fact that the expression of iNOS and TNF-α genes is characteristic for the polarization of macrophages according to the M_1 phenotype [34], as well as the predominance of $CD68^+$ cells in the placenta of women with IVF who refused prophylactic treatment may indicate an excessive level of polarization of macrophages according to the M_1 phenotype in this group.

According to our study, in women with IVF receiving prophylactic treatment, the concentration of IL-10 increases against the background of a decrease in the concentration of TNF-α, the activity of iNOS decreases against the background of an increase in the activity of arginase in the cervical mucus, and the number of $CD163^+$ positive cells in the decidual tissue also increases. Such changes may indicate the effectiveness of our proposed preventive treatment, which contributes to the prevalence of macrophage polarization according to the anti-inflammatory M_2 phenotype.

Progesterone has the ability to promote a shift in the polarization of macrophages, which were previously stimulated by bacterial lipopolysaccharide and switched to M1 polarization, towards their expression of $CD163^+$ markers and increased production of IL-10 [35]. According to Tsai Y.C. et al. the effect of progesterone consists not only in stimulating the polarization of macrophages according to the M_2 phenotype, but also contributes to their acquisition of a special phenotype characteristic of decidual endometrial macrophages [36].

The introduction of vitamin D3 has the property of reducing the production of pro-inflammatory cytokines (TNF-α, IL-6, granulocyte-macrophage colony-stimulating factor 2, etc.) by decidual macrophages and may be part of the autocrine-paracrine regulation of macrophage polarization during pregnancy [37].

Thus, the combination of progesterone and vitamin D3 may have a synergistic effect on stimulating the polarization of macrophages towards the predominance of the M_2 phenotype. Similar results were obtained by Chen Y. et al. when studying the effect of the progesterone-vitamin D3 complex on the immunological status and pregnancy outcomes in rats with an autoimmune model of spontaneous abortions [38].

The effect of L-arginine aspartate is probably related to the provision of a substrate for arginases, which are expressed during the polarization of macrophages according to the M_2 phenotype. The result of arginase-dependent cleavage of L-arginine is the formation of polyamines (putrescine, spermine, spermidine), which are integral factors in the formation of immunological tolerance during pregnancy [39].

Thus, the prophylactic complex proposed by us can not only contribute to the polarization of macrophages according to the M_2 phenotype due to progesterone effects and the influence of vitamin D3, but also provide a sufficient amount of substrate for the synthesis of polyamines.

Limitations of this study. The limitations of our study are the small number of patients in the studied groups, as well as the lack of separate control groups for drugs (vitamin D3, micronized progesterone, L-arginine aspartate), which are part of the complex preventive therapy of premature birth in women whose pregnancy occurred as a result of IVF. Another limitation of our study is the use of only two receptors $CD68^+$ and $CD163^+$ to identify and differentiate macrophage subpopulations.

Prospects for further research. The prospects of our research are the further study of the mechanisms that lead to a shift in macrophage polarization in women whose pregnancy occurred as a result of IVF and the improvement of methods of preventing such a shift. Further study of the role of genetic polymorphism of NO-synthase isoforms, the role of transcription factors NF-κB, STAT-3 and Nrf-2 is also appropriate.

5. Conclusions

In women whose pregnancy occurred as a result of in vitro fertilization, at 28–30 weeks of pregnancy, there is an increase in the number of $CD68^+$ cells, which is accompanied by an increase in the activity of the inducible isoform of NO-synthase and the concentration of TNF-α in cervical mucus.

The complex administration of vitamin D3 2000 IU orally 2 times a day, micronized progesterone 200 mg 2 times a day and L-arginine aspartate 1000 mg 4 times a day to women whose pregnancy occurred as a result of in vitro fertilization leads to an increase in $CD163^+$ cells, against the background of increased arginase activity and IL-10 concentration in cervical mucus.

The article is a part of the research project at the Department of Obstetrics and Gynecology No.2 of Higher Education Institution Poltava State Medical University “The role of chronic infection of the uterus and lower genital tract in the formation of obstetric and gynecological pathology” (state registration No. 0117U005276, implementation period 2017–2022).

Data availability statement

Data will be made available on request.

CRediT authorship contribution statement

Volodymyr Likhachov: Conceptualization, Data curation, Formal analysis, Investigation, Project administration, Supervision, Writing – review & editing. **Yanina Shimanska:** Conceptualization, Formal analysis, Investigation, Methodology, Writing – original draft, Data curation, Visualization. **Oleh Akimov:** Data curation, Formal analysis, Methodology, Validation, Visualization, Writing – original draft, Investigation, Writing – review & editing. **Viktoriya Vashchenko:** Data curation, Formal analysis, Investigation, Methodology, Writing – original draft. **Olena Taranovska:** Conceptualization, Data curation, Formal analysis, Methodology, Writing – original draft. **Iryna Zhabchenko:** Data curation, Formal analysis, Project administration, Supervision, Writing – review & editing. **Igor Kaidashev:** Conceptualization, Project administration, Supervision, Validation, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

References

[1] P. Cavoretto, M. Candiani, V. Giorgione, A. Inversetti, M.M. Abu-Saba, F. Tiberio, et al., Risk of spontaneous preterm birth in singleton pregnancies conceived after IVF/ICSI treatment: meta-analysis of cohort studies, Ultrasound Obstet. Gynecol. 51 (1) (2018) 43–53, https://doi.org/10.1002/uog.18930.

[2] Y. Yao, X.H. Xu, L. Jin, Macrophage polarization in physiological and pathological pregnancy, Front. Immunol. 10 (2019) 792, https://doi.org/10.3389/fimmu.2019.00792.

[3] L.M. Semeniuk, V.K. Likhachov, T.Y. Yuzvenko, L.M. Dobrovolska, O.G. Makarov, Risk markers of reproductive loss in women with hyperandrogenism, Wiad. Lek. 71 (8) (2018) 1550–1553.

[4] Y.V. Shimanskaya, Peculiarities of the course of pregnancy and childbirth of women with in vitro fertilization from the high-risk group for the occurrence of premature birth, The Medical and Ecological Problems 25 (5–6) (2021) 20–23, https://doi.org/10.31718/mep.2021.25.5-6.05 [Ukrainian].

[5] M.G.D. Broi, R.A. Ferriani, P.A. Navarro, Ethiopathogenic mechanisms of endometriosis-related infertility, JBRA Assist Reprod 23 (3) (2019 Aug 22) 273–280, https://doi.org/10.5935/1518-0557.20190029.

[6] G. Sheikhansari, Z. Pourmoghadam, S. Danaii, A. Mehdizadeh, M. Yousefi, Etiology and management of recurrent implantation failure: a focus on intra-uterine PBMC-therapy for RIF, J. Reprod. Immunol. 139 (2020 Jun), 103121, https://doi.org/10.1016/j.jri.2020.103121.

[7] F.Y. Azizieh, R. Raghupathy, IL-10 and pregnancy complications, Clin. Exp. Obstet. Gynecol. 44 (2) (2017) 252–258.

[8] Y.L. Chen, M.T. Lin, W.H. Wang, S.L. Yeh, C.L. Yeh, Intravenous arginine administration attenuates the inflammatory response and improves metabolic profiles in diet-induced obese mice after sleeve gastrectomy, Metabolites 12 (2) (2022 Feb 7) 153, https://doi.org/10.3390/metabo12020153.

[9] M. Kieler, M. Hofmann, G. Schabbauer, More than just protein building blocks: how amino acids and related metabolic pathways fuel macrophage polarization, FEBS J. 288 (12) (2021 Jun) 3694–3714, https://doi.org/10.1111/febs.15715.

[10] V. Tsuber, Y. Kadamov, L. Brautigam, U.W. Berglund, T. Helleday, Mutations in cancer cause gain of cysteine, histidine, and tryptophan at the expense of a net loss of arginine on the proteome level, Biomolecules 7 (3) (2017 Jul 3) 49, https://doi.org/10.3390/biom7030049.

[11] V.I. Shynkevych, S.V. Kolomiiets, I.P. Kaidashev, Effects of l-arginine and l-ornithine supplementations on the treatment of chronic periodontitis: a preliminary randomized short-term clinical trial, Heliyon 7 (11) (2021 Nov 9), e08353, https://doi.org/10.1016/j.heliyon.2021.e08353.

[12] S. Liang, J. Cai, Y. Li, R. Yang, 1,25-Dihydroxy-Vitamin D3 induces macrophage polarization to M2 by upregulating T-cell Ig-mucin-3 expression, Mol. Med. Rep. 19 (5) (2019 May) 3707–3713, https://doi.org/10.3892/mmr.2019.10047.

[13] X. Zhu, Y. Zhu, C. Li, J. Yu, D. Ren, S. Qiu, Y. Nie, X. Yu, X. Xu, W. Zhu, 1,25-Dihydroxyvitamin D regulates macrophage polarization and ameliorates experimental inflammatory bowel disease by suppressing miR-125b, Int. Immunopharm. 67 (2019 Feb) 106–118, https://doi.org/10.1016/j.intimp.2018.12.015.

[14] H. Tournaye, G.T. Sukhikh, E. Kahler, G. Griesinger, A Phase III randomized controlled trial comparing the efficacy, safety and tolerability of oral dydrogesterone versus micronized vaginal progesterone for luteal support in in vitro fertilization, Hum Reprod. 2017 May 1;32(5):1019-1027, Erratum in: Hum. Reprod. 32 (10) (2017 Oct 1) 2152, https://doi.org/10.1093/humrep/dex023.

[15] F.M. Menzies, F.L. Henriquez, J. Alexander, C.W. Roberts, Selective inhibition and augmentation of alternative macrophage activation by progesterone, Immunology 134 (3) (2011 Nov) 281–291, https://doi.org/10.1111/j.1365-2567.2011.03488.x.

[16] B. Wen, Y.Y. Dang, S.H. Wu, Y.M. Huang, K.Y. Ma, Y.M. Xu, X.L. Zheng, X.Y. Dai, Antiatherosclerotic effect of dehydrocorydaline on ApoE-/- mice: inhibition of macrophage inflammation, Acta Pharmacol. Sin. 43 (6) (2022 Jun) 1408–1418, https://doi.org/10.1038/s41401-021-00769-3.

[17] J.H. Che, Z.M. Zheng, M.Q. Li, X. Yao, Macrophage polarization in placenta accreta and macrophage-trophoblast interactions, Am. J. Reprod. Immunol. 88 (6) (2022 Dec), e13611, https://doi.org/10.1111/aji.13611.

[18] K.C. Wheeler, M.K. Jena, B.S. Pradhan, N. Nayak, S. Das, C.D. Hsu, D.S. Wheeler, K. Chen, N.R. Nayak, VEGF may contribute to macrophage recruitment and M2 polarization in the decidua, PLoS One 13 (1) (2018 Jan 11), e0191040, https://doi.org/10.1371/journal.pone.0191040.

[19] Y. Ye, L. Peng, A. Chelariu-Raicu, C. Kuhn, X. Dong, U. Jeschke, V. von Schönfeldt, Prostaglandin E2 receptor 3 promotes M1 macrophages polarization in unexplained recurrent pregnancy loss, Biol. Reprod. 106 (5) (2022 May 17) 910–918, https://doi.org/10.1093/biolre/ioac030.

[20] Y. Ono, O. Yoshino, T. Hiraoka, E. Sato, Y. Fukui, A. Ushijima, A. Nawaz, Y. Hirota, S. Wada, K. Tobe, A. Nakashima, Y. Osuga, S. Saito, CD206+ M2-like macrophages are essential for successful implantation, Front. Immunol. 11 (2020 Oct 23), 557184, https://doi.org/10.3389/fimmu.2020.557184.

[21] A.M. Yelins'ka, OYe Akimov, V.O. Kostenko, Role of AP-1 transcriptional factor in development of oxidative and nitrosative stress in periodontal tissues during systemic inflammatory response, Ukrainian Biochem. J. 91 (1) (2019) 80–85, https://doi.org/10.15407/ubj91.01.080.

[22] Y.O. Yemchenko, V.I. Shynkevych, K.Y. Ishcheikin, I.P. Kaidashev, PPAR-gamma agonist pioglitazone reduced CD68+ but not CD163+ macrophage dermal infiltration in obese psoriatic patients, PPAR Res. 2020 (2020 May 1), 4548012, https://doi.org/10.1155/2020/4548012.

[23] A.Z. Aykian, V.I. Shynkevych, I.P. Kaydashev, Characterization of CD68 + and CD163 + infiltration by primary focus macrophages and metastatic lesions of regional lymph nodes in luminal invasive breast carcinomas, depending on their immunophenotype, World of Medicine and Biology 66 (4) (2018) 15–22, https://doi.org/10.26724/2079-8334-2018-4-66-15-22.

[24] Y. Li, S. Yu, C. Huang, R. Lian, C. Chen, S. Liu, L. Li, L. Diao, U.R. Markert, Y. Zeng, Evaluation of peripheral and uterine immune status of chronic endometritis in patients with recurrent reproductive failure, Fertil. Steril. 113 (1) (2020 Jan) 187–196.e1, https://doi.org/10.1016/j.fertnstert.2019.09.001.

[25] F. Yildizfer, O. Donma, M. Yen, O. Ekmekci, Z.A. Karatas Kul, Z. Keser, A. Esat Imal, E. Cagil, M. Mengi, H. Ekmekci, S. Sahmay, B. Nalbantoglu, M.M. Donma, In vitro fertilization, levels of pro-inflammatory factors and lipid peroxidation, Int J Fertil Steril 9 (3) (2015 Oct-Dec) 277–284, https://doi.org/10.22074/ijfs.2015.4541.

[26] M.E. Rahiminejad, A. Moaddab, M. Ebrahimi, S. Rabiee, A. Zamani, M. Ezzati, A. Abdollah Shamshirsaz, The relationship between some endometrial secretion cytokines and in vitro fertilization, Iran. J. Reproductive Med. 13 (9) (2015 Sep) 557–562.

[27] J. Alijotas-Reig, E. Esteve-Valverde, R. Ferrer-Oliveras, E. Llurba, J.M. Gris, Tumor necrosis factor-alpha and pregnancy: focus on biologics. An updated and comprehensive review, Clin. Rev. Allergy Immunol. 53 (1) (2017 Aug) 40–53, https://doi.org/10.1007/s12016-016-8596-x.

[28] J. Kaislasuo, S. Simpson, J.F. Petersen, G. Peng, P. Aldo, E. Lokkegaard, M. Paidas, L. Pal, S. Guller, G. Mor, IL-10 to TNFα ratios throughout early first trimester can discriminate healthy pregnancies from pregnancy losses, Am. J. Reprod. Immunol. 83 (1) (2020 Jan), e13195, https://doi.org/10.1111/aji.13195.
[29] E.E. Winger, J.L. Reed, S. Ashoush, T. El-Toukhy, S. Ahuja, M. Taranissi, Degree of TNF-α/IL-10 cytokine elevation correlates with IVF success rates in women undergoing treatment with Adalimumab (Humira) and IVIG, Am. J. Reprod. Immunol. 65 (6) (2011 Jun) 610–618, https://doi.org/10.1111/j.1600-0897.2010.00946.x.
[30] Y. Zhang, H. Wang, P. Qiu, J. Jiang, X. Wu, J. Mei, H. Sun, Decidual macrophages derived NO downregulates PD-L1 in trophoblasts leading to decreased Treg cells in recurrent miscarriage, Front. Immunol. 14 (2023 Jul 14), 1180154, https://doi.org/10.3389/fimmu.2023.1180154.
[31] J. Bodis, B. Farkas, B. Nagy, K. Kovacs, E. Sulyok, The role of L-arginine-NO system in female reproduction: a narrative review, Int. J. Mol. Sci. 23 (23) (2022 Nov 28), 14908, https://doi.org/10.3390/ijms232314908.
[32] Y. Lan, X. Yang, Y. Liang, L. Lei, Y. Li, S. Wang, Vasomotor tone-associated factors and pregnancy outcomes of women who undergo in vitro fertilization, Growth Factors 39 (1–6) (2021 Feb-Jul) 28–36, https://doi.org/10.1080/08977194.2021.2021899.
[33] C.C. Pinto-Souza, F. Coeli-Lacchini, M.R. Luizon, R.C. Cavalli, R. Lacchini, V.C. Sandrim, Effects of arginase genetic polymorphisms on nitric oxide formation in healthy pregnancy and in preeclampsia, Nitric Oxide 109–110 (2021 May 1) 20–25, https://doi.org/10.1016/j.niox.2021.02.003.
[34] C.F. Tsai, G.W. Chen, Y.C. Chen, C.K. Shen, D.Y. Lu, L.Y. Yang, J.H. Chen, W.L. Yeh, Regulatory effects of quercetin on M1/M2 macrophage polarization and oxidative/antioxidative balance, Nutrients 14 (1) (2021 Dec 24) 67, https://doi.org/10.3390/nu14010067.
[35] M.I. Mendoza-Cabrera, R.E. Navarro-Hernández, A. Santerre, P.C. Ortiz-Lazareno, A.L. Pereira-Suárez, C. Estrada-Chávez, Effect of pregnancy hormone mixtures on cytokine production and surface marker expression in naïve and LPS-activated THP-1 differentiated monocytes/macrophages, Innate Immun. 26 (2) (2020 Feb) 84–96, https://doi.org/10.1177/1753425919864658.
[36] Y.C. Tsai, J.T. Tseng, C.Y. Wang, M.T. Su, J.Y. Huang, P.L. Kuo, Medroxyprogesterone acetate drives M2 macrophage differentiation toward a phenotype of decidual macrophage, Mol. Cell. Endocrinol. 452 (2017 Sep 5) 74–83, https://doi.org/10.1016/j.mce.2017.05.015.
[37] K.N. Evans, L. Nguyen, J. Chan, B.A. Innes, J.N. Bulmer, M.D. Kilby, M. Hewison, Effects of 25-hydroxyvitamin D3 and 1,25-dihydroxyvitamin D3 on cytokine production by human decidual cells, Biol. Reprod. 75 (6) (2006 Dec) 816–822, https://doi.org/10.1095/biolreprod.106.054056.
[38] Y. Chen, Q. Wu, J. Wei, J. Hu, S. Zheng, Effects of aspirin, vitamin D3, and progesterone on pregnancy outcomes in an autoimmune recurrent spontaneous abortion model, Braz. J. Med. Biol. Res. 54 (9) (2021 Jun 14), e9570, https://doi.org/10.1590/1414-431X2020e9570.
[39] C.N. Hsu, Y.L. Tain, Impact of arginine nutrition and metabolism during pregnancy on offspring outcomes, Nutrients 11 (7) (2019 Jun 27) 1452, https://doi.org/10.3390/nu11071452.