A Cellular Study of Inflammatory Responses in Women with Polycystic Ovary Syndrome as: "An In vitro Model of Anti Breast Tumor Response"A Case Control Study

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Abstract

Background: Although Polycystic Ovary syndrome (PCOS) is a common endocrine disorder among women of reproductive age; is unclear whether PCOS increases the risk of subsequent development of, Gynecologic cancers namely breast cancer. The present study we aimed to compare the antitumoral ability of peripheral blood mononuclear cells (PBMCs) of women with PCOS with that of healthy controls using the co-culture system between effector cells and target tumor cell lines.

Materials & Methods: PBMCs were isolated from 25 women with PCOS and 25 non hirsute eumenorrheic healthy controls by density gradient centrifugation ficoll. Breast tumor cell lines (MDA-468, MCF-7) were incubated as the two target cells and were cultured adjacent to PBMCs in the transwell co-culture system. Proliferation rate of the effectors cells evaluated by BrdU cell proliferation assay after 48 and 72 hours and T CD3+ lymphocytes were assessed using flow cytometry. TNF-α cytokine production was evaluated in cell culture supernatant by sandwich ELISA technique.

Results: After 48 hours incubation with MDA-468 and MCF-7, the mean proliferation score of PBMCs was significantly higher in women with PCOS compared to that of healthy controls (921.04; P=0.021 vs 287.6; P=0.002, respectively). In PCOS women, after 72 hours of incubation, TNF-α concentration was significantly reduced compared to 48-hour cultures (921.04 ± 271.4 pg/dl vs 545.6 ± 151.1 pg/dl at 48 h and 72 h intervals respectively, P<0.05); it was increased in healthy controls. There was no significant difference in CD3+ CD8+ cells between the PCOS group and healthy controls.

Conclusion: The ability of PBMCs to produce of TNF-α in women with PCOS decreased gradually; as a result of which they may lack the ability required to form an in vitro efficient antitumor response to breast tumor cell lines. It is assumed that threshold activation of mononuclear cells is reduced in women with PCOS and a low-grade inflammatory condition may provide a positive background for arising myeloid derived suppressor cells (MDSCs).

Background

Polycystic ovary syndrome (PCOS) is one of the most common endocrinopathies among reproductive aged women with an estimated prevalence of about 10% (1, 2). Clinically, it is characterized by hyperandrogenism and chronic anovulation leading to menstrual irregularities and sub-fertility with polycystic morphology of ovaries (3-6). Although the etiology of PCOS is not fully understood, the role of hyperandroginemia and obesity are known to be major factors involved in PCOS (7). A chronic low-grade inflammation is observed in women with PCOS that may make them susceptible to other associated comorbidities of PCOS including cardiovascular disease (CVD) and cancers (8).

Breast cancer is the most common cancer and the leading cause of death in females worldwide , accounting for 23% of all new cancer cases and 14% of cancer deaths (9). In addition, it is also the most common cancer diagnosed among women in Iran (10). It is a complex and multi-factorial disease (11). Some studies have shown that serum levels of some cytokines such as interleukin 18 (IL-18), interleukin

6 (IL-6) and tumor necrosis factor (TNF)-α are remarkably increased in obesity, PCOS and breast cancer (12-14). IL-18 a proinflammatory cytokine that induces the production of TNF-α (15),has been shown to be associated with body mass index (BMI), total testosterone levels, insulin resistance and breast cancer development (16-18). Moreover, IL-6 is involved in the host immune defense mechanism as well as the modulation of growth and differentiation in breast tumor cells (14).

It has been shown that several markers of systemic inflammation, such as C-reactive protein (CRP), leukocytes number and pro-inflammatory cytokines (such as TNF-α) are increased in women with PCOS. Thus the altered metabolic and hormonal environment along with low-grade chronic inflammation among women with PCOS could increases their risk of some types of cancer including gynecologic ones (19,20). In this respect, some epidemiological studies report a positive association between high endogenous androgens level in PCOS and the risk of breast cancer, while data from experimental studies are insufficient and controversial (21, 22). In the present study, we aimed to compare the anti-tumoral activity of women with PCOS to that of healthy controls in terms of cell proliferation, cytokine production and expression of CD8 marker in peripheral blood lymphocytes using incubation with breast tumor cell lines.

Methods

The study protocol was approved by the medical ethics committee of the Research Institute for Endocrine Sciences (RIES) of I.R.Iran. Written informed consent was obtained from all participants.

A total of 25 cases of PCOS, based on the Rotterdam criteria (23, 24) and aged 18-45 years, who referred to Reproductive Endocrinology Research Center diagnosis, were recruited for the purpose of the present study. These women had at least two of the following criteria: i: / oligo/anovulation (defined as either regular or irregular menstruation ≥ 34 days or history of eight or fewer menstrual cycles in a year); ii: / clinical symptoms of hyperandrogenism (including hirsutism diagnosed based on a standardized scoring system of modified Ferriman-Gallwey ≥ 8, acne and androgenic alopecia) or biochemical hyperandrogenism ,defined as the increase of one or more serum level of androgens (testosterone or androstenedione) to> 95th percentile, presented in the selected healthy non-hirsute eumenorrheic women of the study population) (25); iii:/ polycystic ovaries (polycystic ovaries with >12 follicles /ovary, 2−9 mm in diameter and/or increased ovarian volume (10 cm^3)) and exclusion of other etiologies. A total of 25 non hirsute eumenorrheic healthy age and BMI-matched women, aged 18-45 years, were selected as controls.

All women with histories of cancers, primary or acquired immunodeficiency, suffering from infectious diseases and systemic disorders were exclude. We also excluded women with a family history of cancer, those with early menopause (occurring at or before age 45 years) (26), and substance dependents (addiction). Characteristics of the study groups are presented in table 1.There were no statistically significant differences in baseline characteristics of the participants.

In this study we evaluated the proliferative response of lymphocytes as the mean proliferation score (presented through absorbance), measured of TNF-α concentration using a standard graph and determined percentage of the CD3+ CD8+ lymphocytes as the main effectors cell population.

Tumor cell line culture

Two human breast cancer cell lines (MCF-7, MDA-MB-468) were purchased from the Cell Bank of Institute Pasteur Karaj, Iran. Cell lines were cultured in RPMI-1640 (Gibco, Invitrogen, USA) and DMEM (Gibco, Invitrogen, USA) media supplemented with 10% fetal bovine serum (FBS), 100 u/ml penicillin/streptomycin and (Atocel, Austria) in humidified 5% CO2 incubator at 37°C. After reaching the log phase of growth and 80% confluency in the T25 tissue culture flask, growing cells were detached from the culture bed using trypsin-EDTA solution and divided into 24 well culture plates or maintained at -80°C for future use or as backup data.

Isolation of Peripheral Blood Mononuclear Cells

Heparinized blood samples were taken and diluted with RPMI medium at a ratio of 1:1. Peripheral blood mononuclear cells (PBMCs) were separated by density gradient centrifugation (Ficoll hypauque 1077 Sigma, USA). The inter phase layer including mononuclear cells was collected and washed 3 times with RPMI medium PBMCs were then suspended in Complete Tissue Medium (CTM) and cell suspension was stained with trypan blue and viability was determined.

Co-culture system

Tumor cell lines ($5×10^5$cell/well) were pre-seeded in the lower chamber at Transwell 24-well plates (SPL, Korea) in RPMI-1640/DMEM medium supplemented with 10% FBS and incubated overnight in humidified CO_2 incubator at 37°C. Subsequently, PBMCs ($2×10^6$ cells) were added into the upper insert chamber with a pore size of 0.4 μm (SPL, Korea) at a ratio of E:T (1:4). For an incubation period of 72 h. As a negative control, PBMCs were cultured in the upper chamber of the plates in the absence of tumor cell lines. The samples for cytokines assay were separately created from the supernatants of cancer cell lines and PBMCs cultures in Transwell chambers at two time intervals of 48 and 72 h incubation following co-culture.

Evaluation of proliferative response of Peripheral Blood Mononuclear Cells

The non-radioactive Cell Proliferation assay (colorimetric) ELISA kit, BrdU (Roche Diagnostic, Germany; Ref:11647229001) was used to evaluate PBMCs proliferation rate during co-culture with tumor cell lines as well as in the absence of tumor cell lines as controls at two time intervals. The BrdU cell proliferation assay was performed according kit instructions. Absorbance was measured at 450 nm with a reference wave length at 690 nm.

Immunophenotyping of Peripheral Blood Mononuclear Cells

To determine the percentages of TCD3+ CD8+ lymphocytes in cultured cells, we used a combination of phycoerythrin-conjugated anti-CD8 (PE, clone SK1), and fluorescein isothiocyanate-conjugated anti-CD3 (FITC; Leu 4 FITC, clone SK7) mAbs (BD Biosciences, Cat No: 340044); Percentages of total mononuclear cells, as well as TCD3+ CD8+ lymphocytes, were evaluated by flow cytometry (FACSCalibur) at two different time intervals.

Measurement of Tumor Necrosis Factor-alpha concentration

TNF-α concentration in the supernatants collected from PBMCs co-cultured with and without tumor cell lines was determined and reported in pg/dL using the sandwich ELISA kit (Quantikine, R&D Systems, USA), for human TNF-α, according to the manufacturer's instruction, Results were calculated using a standard curve.

Statistical analysis

All continuous variables were checked for normality using the one-sample Kolmogorov−Smirnoff test and expressed as mean (standard deviation) if variables had a normal distribution, or median with inter-quartile range (IQ25-75) for variables with skewed distribution. Characteristics of study participants were compared between the PCOS and control groups using two independent-sample t-test or the equivalent nonparametric Mann-Whitney U test.; categorical variables, expressed as percentages, were compared using Pearson's   test. Statistical analysis was performed using software package SPSS (version 15) and GraphPad Prism. Significance level was set at $P<0.05$, and confidence interval (CI) as 95%.

Results

Lymphocyte proliferation

As presented in Figures 1(A and B), significant differences we observed in the mean lymphocyte proliferation score in the presence of each cell line ,compared to PBMCs alone cultures. After 72 hours of incubation, these differences increased in the PCOS (0.718 ± 0.167 vs 0.331 ± 0.066 in PCOS group,

P=0.03 and 0.409 ± 0.079 vs 0.190 ± 0.03 in controls after 72 hours co-cultivation with MDA-468, P=0.018) (Figure 1B). There were no significant differences in the mean lymphocyte proliferation score of PCOS and control groups in co-cultures of MCF-7 and MDA-468 cell lines (0.401 ± 0.14 versus 0.484 ± 0.153 after 48 hours and 0.718 ± 0.167 versus 0.628 ± 0.13 after 72 hours as the mean of proliferation score in co-culture of MDA-468 and MCF-7, respectively). The mean lymphocyte proliferation score in PCOS group was higher than the healthy group (0.225 ± 0.126 versus 0.028 ± 0.009, P=0.04 and 0.417 ± 0.138 versus 0.23 ± 0.06, P>0.05 in 48 h and 72 h co-cultivation, respectively) (Figure 1C).The impact of time was significant in both cell lines and the mean lymphocyte proliferation scores of both cell lines after 72 h was higher than that of 48 h interval (0.417 ± 0.128 vs 0.225 ± 0.126 in PCOS group and 0.23 ± 0.06 vs 0.028 ± 0.009 in healthy controls, P<0.01) (Figure 1D). The optical density (OD) value in respect to the rate of proliferation in the control group culture was subtracted from the absorbance or OD reading of co-cultures as the baseline (Optical density and absorbance both measure the amount of light "absorbed" when passing through an optical component).

TNF-α concentration measurement

The study revealed significant differences in the mean TNF-α concentration scores in PCOS and Healthy groups after 48 h of incubation. (930.4 ± 313.8 pg/dl in PCOS group versus 89.18 ± 25.44 pg/dl in healthy controls, P=0.007) (Figure2A). The mean TNF-α concentration score after 72 h was increased in healthy group compared to PCOS group (489.7 ± 153.5 pg/dl versus 167.8 ± 85.09 pg/dl respectively, p<0.05) (Figure 2B). The impact of time was significant in both cell lines, as presented in Figure 3, TNF-α production was decreased in PCOS group (921.04 ± 271.4 pg/dl versus 545.6 ± 151.1 pg/dl in 48 h and 72 h intervals respectively, P<0.05).

Cytotoxic cellular population

Figure 4 presents the flow cytometry diagrams associated with the percentages of cytotoxic lymphocytes culture in healthy and PCOS groups. The percentage of CD3+CD8+ T lymphocytes (CTLs) was determined at three stages: 0: (before cell culture, A), 2: 48 (B) and 3: 72 (C) h intervals in PCOS group (Figure 4). Although the percentage of these lymphocytes was increased after co-culture, as presented in Figure 5, no significant differences were observed in the mean percentage of cytotoxic cells between the two groups (16.8 ± 1.14 versus 13.33 ± 1.84 in the PCOS and healthy groups respectively, P>0.05). The impact of time was not significant, but the number of these cells increased after 72 h of co-cultivation (16.8 ± 1.14 in 72 h vs 12.19 ± 1.7 in 48 h intervals) (Figure 6). This means that in both study groups, the cytotoxic cells proliferated after exposure to the tumor cells.

Discussion

Most of the studies on the prevalence of cancer in women with polycystic ovary syndrome have been epidemiological. One of the novelties in our study was the use of experimental methods to evaluate the probability of these women having breast cancer. In this study, we evaluated the response of peripheral blood cells of women with PCOS to two of the most well-known breast cancer cell lines (MDA-468 and MCF-7). MDA-468 is commonly used in breast cancer research and was extracted from a pleural effusion of mammary gland and breast tissues, and has proven useful for the study of breast cancer proliferation. MCF-7 has a proliferative response to estrogens and express progesterone receptors. This cell line retained several characteristics of differentiated mammary epithelium, such as the ability to process estradiol via cytoplasmic estrogen receptors. TNF-α inhibits the growth of MCF-7 breast cancer cells. Due to the attributes of these cell lines, they are suitable options to investigate the proliferative response and also study of the effect of TNF-α and estrogen present in the peripheral blood of women with PCOS on the development of antitumor responses.

In this study, we found that the proliferation score of PBMCs was higher in women with PCOS, contrary to results observed in healthy controls, whom mean TNF-α concentration was reduced during incubation in the PCOS group, indicating reduced ability of PBMCs for production of TNF-α in women with PCOS.

The role of inflammation in development of cancer was first described in 1863 by Rudolf Virchow (27, 28), by observing inflammatory cells that led to the hypothesis "*Tumors rise in inflammatory sites*" (27, 28). The dual role of TNF-α as a pro-inflammatory cytokine, in creation of antitumor response and progression of tumor is complicated (29, 30).

PCOS is considered as a low-grade inflammatory state, with increasing serum levels of pro-inflammatory cytokine TNF-α especially in obese women with PCOS compared to their obese counterparts without PCOS (31-35). Obesity is one of the proved risk factors for breast cancer (36) and on the other hand, the chronic inflammation caused by TNF-α secretion in women with PCOS also increases the risk of cancer (30, 34). So there is also the possibility of cancer in these women. While obesity, highly common in PCOS, is associated with an increase in rate of breast cancer (37), anovulation as the main manifestation of PCOS, has been shown to decrease breast cancer risk (38). A meta-analysis including two case–control and one cohort study reported no elevation in breast cancer risk in PCOS women (OR = 1.0; 95% CI = 0.6–1.4) (39).

In the present study we observed a higher secretion of TNF-α secretion in women with PCOS, compared to healthy controls following co-culture with breast tumor cell lines after 48 hours, although that this secretion ceased after 72-hour co-culture, vice versa, in healthy controls, the increasing production of TNF-α continued for up to 72 hours. It seems that despite the exaggerated response early in the incubation period, PBMCs of PCOS women could not maintain their powerful anti-tumor response.

In vitro, CD3^{+}CD8^{+} T lymphocytes are considered as the two best indicators for assessment of the anti-tumor responses (40), they produce interferon-γ following interactions with their tumor targets, which subsequently lead to cell cycle inhibition, apoptosis, angiostasis and induction of macrophage

tumoricidal activity (41). When the immune system fails to eliminate all tumor cells, tumors with reduced immunogenicity may emerge with the ability to avoid immune attacks. This combination of host-protective and tumor-promoting functions of inflammatory/immune cells has led to the concept of 'cancer immuno-editing' (41).

In previous studies conducted on the immune status of women with PCOS, it has been reported that the number of CD8^{+} T lymphocytes is reduced in the peripheral blood these women, compared to healthy women (42), on the other hand, contrary to finding of the present study, results of the Mahmoud et al. study on infiltration of CD8^{+} T lymphocytes to breast tumor tissue show that the number of these lymphocytes is increased in tumor tissues (40). while it was not observed in present study.

Results of immunophenotyping analysis showed that percentage of CD3^{+}CD8^{+} T lymphocytes was increased following co-culture with breast tumor cell lines in both healthy and PCOS groups with no significant differences between these two groups. Also CD3^{+}CD8^{+} T lymphocytes did not increase in number in many cases. Data shows that PBMCs isolated from women with PCOS had stronger proliferative response to breast tumor antigenic stimulation compared to healthy controls, this proliferative response continued for up to 72 hours of co-culture in vitro, possibly indicating that this was an antigenic stimulation of CD3^{+}CD8^{+} T lymphocytes as effector cells in tumor response, and that just the threshold of PBMCs activation was reduced in women with PCOS.

Chronic inflammation and obesity can cause deviation of cell differentiation to myeloid derived suppressor cells (MDSCs) (43, 44). Low-grade inflammatory environments in women with PCOS and the presence of obesity as well in these patients may stimulate peripheral differentiation of MDSCs that these cells may suppress efficient tumor response to breast cancer (45).

The main strength of the present study is its design, which matches at unlikely that our results have been affected by differences in age and BMI, two main factors influencing inflammatory status; this method provides a different approach to study the association of malignancies with gynecological disease. The results of this study represent a new approach to further investigate the risk of breast cancer in women with PCOS.

Our study does have some limitations. We lacked sufficient power to run subgroup analysis according to the various phenotypes of PCOS, while PCOS has a broad spectrum of manifestations that maybe highly influence the patient's inflammatory status. Although we tried to lower the probability of confusing the results by considering precise inclusion and exclusion criteria, we did not adjust for potential influencing factors, including life styles. The inability to isolate purified lymphocyte populations to detect the main source of TNF-α release was a limitation to our research.

Generalization of our findings requires replication of data in larger populations with different ethnic backgrounds. A larger study considering PCOS phenotypes, life styles, co-morbid conditions, as well as

previous medical treatments and running various comprehensive methods for assessment of antitumor responses is highly recommended.

Conclusions

In conclusion, we found that the proliferation score of PBMCs was higher in women with PCOS, contrary to results observed in healthy women, whom mean TNF-α concentration was reduced during incubation in the PCOS group, indicating reduced ability of PBMCs for production of TNF-α in women with PCOS. The ability of PBMCs to produce of TNF-α in women with PCOS decreased gradually; as a result of which they may lack the ability required to form an in vitro efficient antitumor response to breast tumor cell lines. It is assumed that threshold activation of mononuclear cells is reduced in women with PCOS and a low-grade inflammatory condition may provide a positive background for arising myeloid derived suppressor cells (MDSCs).

The main strength of the present study is its design, which matches at unlikely that our results have been affected by differences in age and BMI, two main factors influencing inflammatory status; this method provides a different approach to study the association of malignancies with gynecological disease. The results of this study represent a new approach to further investigate the risk of breast cancer in women with PCOS.

Abbreviations

PCOS: Polycystic Ovary Syndrome

PBMC: Peripheral Blood Mononuclear Cell

MCF-7: Michigan Cancer Foundation

MDSC: Myeloid Derived Suppressor Cell

RPMI: Roswell Park Memorial Institute

DMEM: Dulbecco's Modified Eagle's Medium

TNF-α: Tumor Necrosis Factor Alpha

CVD: Cardiovascular Disease

BMI: Body Mass Index

Declarations

Ethics approval and consent to participate:

The study protocol was approved by the medical ethics committee of the Research Institute for Endocrine Sciences (RIES) of I.R.Iran. Written informed consent was obtained from all participants.

Consent to publish:

Written informed consent was obtained from all participants.

Availability of data and materials:

This data is sent as an attachment to the journal. The specifications of the materials used in the manuscript are listed.

Competing interests:

The authors declare that they have no competing interests.

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Authors' Contributions:

Supervision, Project administration and funding acquisition: NM; performing design and contributing to the design of the in-vitro and in-vivo experiments and interpreting of results: All authors; designing, performing and interpretation of Flow cytometry data: by MM; Collecting the samples and interpretation of data analysis: FRT; Directing the study and writing the paper: FR and NM; Writing and critically reviewing the manuscript: All authors. All authors contributed in this study have read and approved the manuscript.

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Table

Table 1. Baseline characteristics of the study participants

	PCOS (n = 25)	Control (n = 25)	P- value
Age (years)	30.2± 6.1	30.8± 5.8	0.5
BMI (kg/m^2)	22.8± 6.4	23.09± 2.8	0.8
WC (cm)	79.8± 8.1	78.7± 8.5	0.4
HC (cm)	100.1± 6.8	99.1± 6.5	0.1
SBP (mmHg)	101.1± 10.7	101.1± 9.3	0.2
DBP (mmHg)	62.8± 11.7	65.4± 8.1	0.1
Parity	1.4± 0.6	1.5± 0.7	0.5

PCOS=polycystic ovary syndrome; BMI=body mass index; WC=waist circumference; HC= hip circumference; SBP=systolic

blood pressure; DBP=diastolic blood pressure

Figures
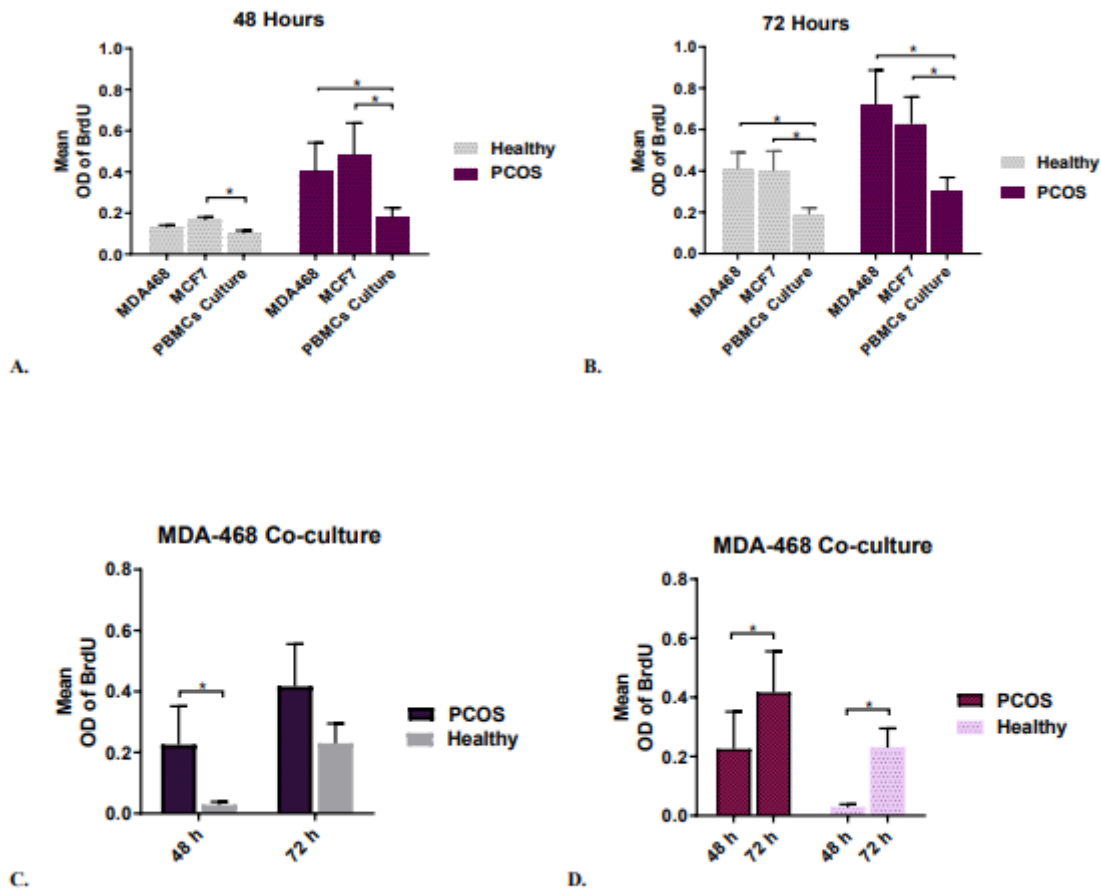
Figure 1

Comparison of mean lymphocyte proliferation score in PCOS and healthy groups. The mean lymphocyte proliferation of MD-468 and MCF-7 cell lines co-culture is compared with PBMCs culture in 48 h (A) and 72 h (B). C: Comparison mean lymphocyte proliferation score in PCOS and healthy groups, and D: Comparison mean lymphocyte proliferation at two time intervals (48 and 72 h) during co-culture with the MDA-468 cell line.
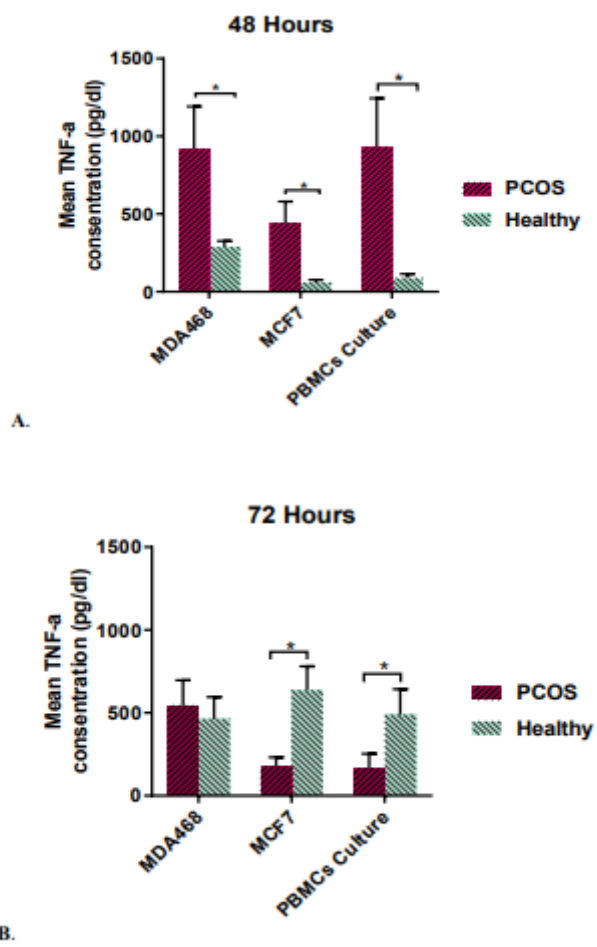
Figure 2

Comparison of mean TNF-α concentration in PCOS and healthy groups The mean TNF-α concentration in supernatant of MD-468 and MCF-7 cell lines co-culture is compared with PBMCs culture in 48 h (A) and 72 h (B).
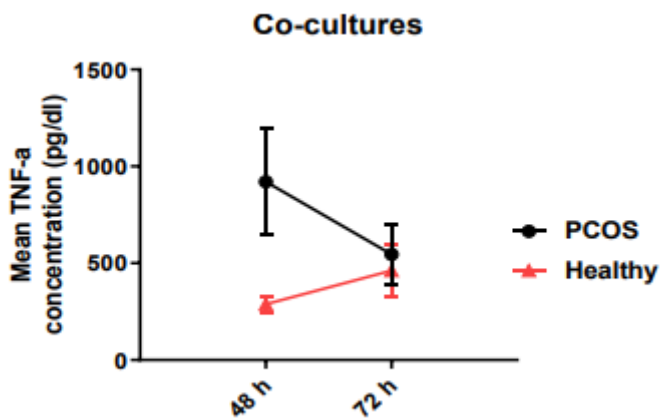
Figure 3

Comparison of mean TNF-α level at two time intervals of 48 and 72 h following co-culture with MDA-468 tumor cell line.
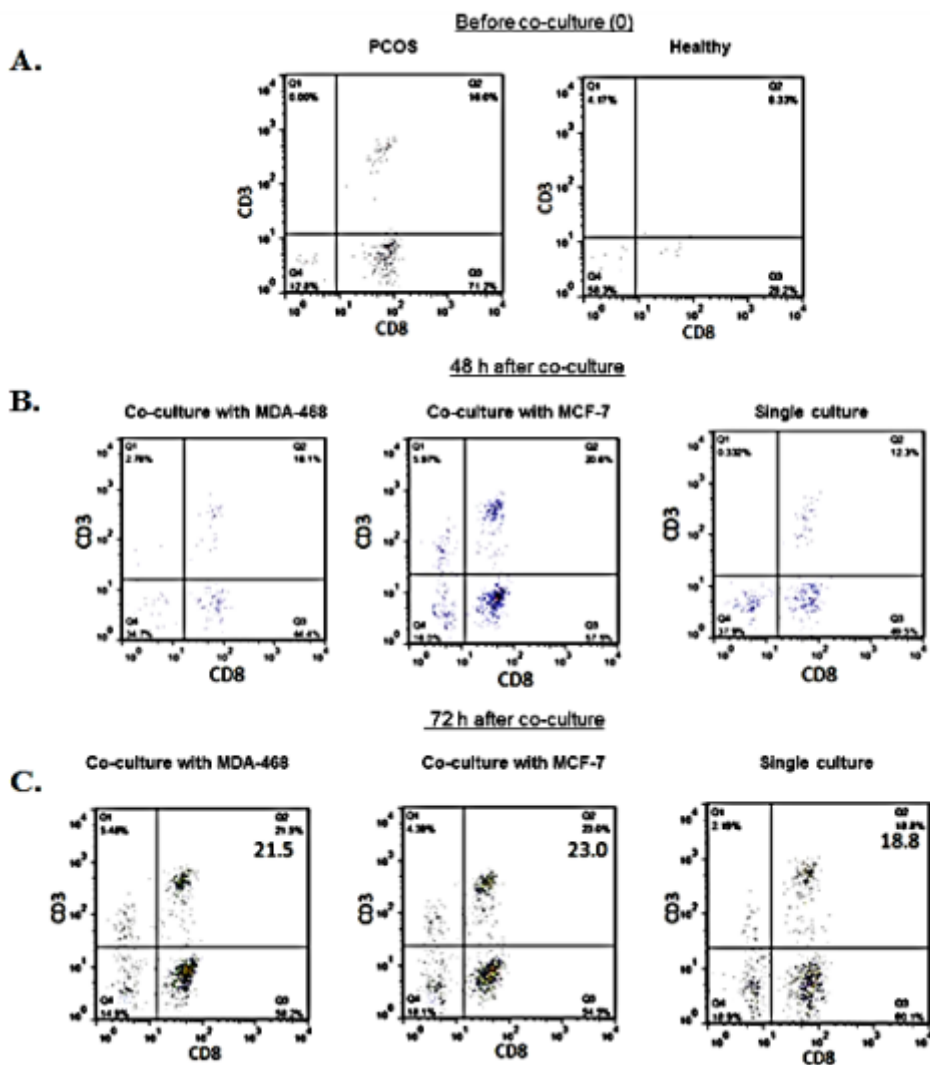
Figure 4

Flow cytometry diagrams associated with evaluating the percentage of T CD3+ CD8+ lymphocytes in three intervals, before co-culture, 48 h (B) and 72 h (C) culture in PCOS group.
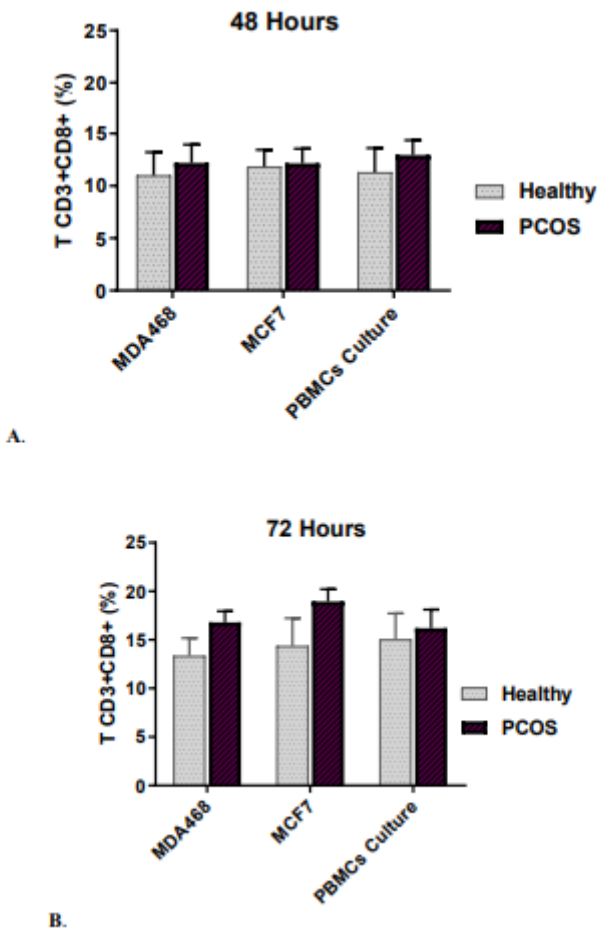
Figure 5

Comparison of mean percentage of TCD3+ CD8+ cytotoxic cells in PCOS and healthy groups Following co-culture with both tumor cell lines and PBMCs culture, the percentage of these cytotoxic cells showed no statistically significant difference between the two healthy and PCOS groups in 48 h (A) and 72 h (B) intervals.
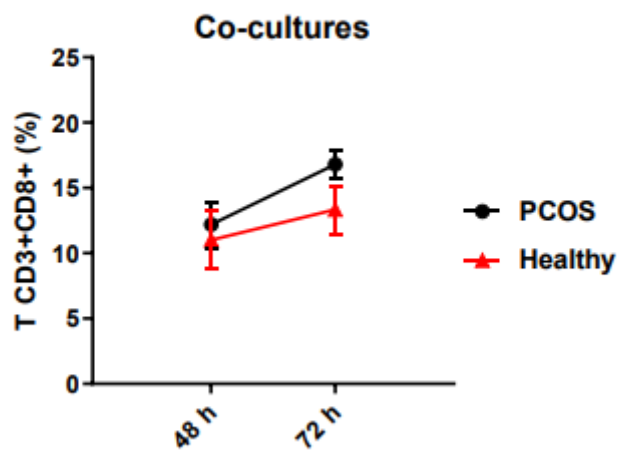
Figure 6

Comparison of mean percentage of TCD3+ CD8+ cytotoxic cells in two intervals of 48 and 72 h following co-culture with the tumor cell lines.