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کارگاه آنلاین کاربرد نرم افزار SPSS در پژوهش
A study on Stress, Depression and NK Cytotoxic Potential in Women with Recurrent Spontaneous Abortion

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ABSTRACT

It has been recognized that acute and chronic stress has an impact on the immune system. Acute stress may have a stimulating effect on the immune system, while in the case of chronic stress specially depression, the immune system could be down-regulated. However, an association between depression and a higher number of circulating white blood cells with increased activity has been reported. Elevation in immune cell numbers and alteration in cytokine profiles are documented for women suffering sporadic spontaneous abortion with a high stress score. In spite of these contradictory results and to make a new approach in immunological (NK activity) as well as psychological parameters (stress/depression) in women suffering from recurrent spontaneous abortion (RSA) the present study was planned.

Forty-five women with a history of RSA and a matched control group were participated in this study. A questionnaire for life events known as life change units (LCU) and the Beck Depression Inventory (BDI) outlines were used and the socio-psychological events were recorded after visiting and interview. Fresh peripheral blood lymphocytes were taken as a source of NK activity and K562 cell line were used as NK sensitive target. The experiments were performed and the cells were analyzed with a flow-cytometer.

The stress and the depression scores were determined 245±83.6 and 27.6±8.8 for women with RSA and 224±79.6 and 19.4±7.1 for non-RSA group respectively. There was an association between life stress scores and depression scores with r=0.65 and P=0.000 for RSA women. A correlation with r = -0.34 and P = 0.02 was found between depression scores and NK cytotoxicity. The Pearson correlation test showed a lack of relationship between high stress score and NK activity with the r=0.011 and P=0.95, but r= -0.30 and P=0.072 was obtained for high depression scores and NK cytotoxicity. Therefore, it could be suggested that in the case of women with a history of recurrent spontaneous abortion, modulation for immunological parameters (i.e immunotherapy) concurrently with managing psychological aspects (stress/depression) could be modified for the benefit of the patients.

Key words: Cytotoxicity, Immunologic; NK cells; Flow cytometry; Spontaneous abortion; Stress; Suppression

INTRODUCTION

The psycho-neuro-immuno-endocrine network has
be involved in abortion. It has been reported that some patients suffering from recurrent abortion may present with psychological disorder. Some investigators tried to explain the influence of psychological stress on pregnancy outcome by elevated stress hormones (catecholamines, cortisol) that reduce fetal vascularization and oxygen supply which may possibly induce abortion. Furthermore, with regard to immunological parameters Arck et al. demonstrated significant increase in the numbers of CD8+ T cells, as well as TNFα and Tryptase+ mast cells in the deciduas of women suffering sporadic spontaneous abortion with a high stress score compared with control group. Chronic stress can lead to fewer circulating B, T and NK cells, as well as decreasing proliferation responses of lymphocytes and reducing NK cell activity. In contrast to the down regulation with the longer lasting chronic stress, acute stress such as examination seems rather to have an activating effect on immune parameter. An increase in IL-1β, IL-2 and soluble ICAM-1 plasma concentrations was seen following experiencing acute stress. Depression is also associated with a higher number of circulating white blood cells, primarily neutrophils and total number of lymphocyte (NK, B and T cells) and their activities. In addition, depression was shown to be associated with alteration in cytokine profiles. Immune system cells, in particular lymphocytes and macrophages have specific, high-affinity and saturable binding sites for most neuro-endocrine peptides. However, studies of isolated immune cells in culture show that hormones have a variety of effects on these cells. For example, estrogen decreases cell-mediated immunity and suppressor cells which permit enhanced B lymphocyte activation and antibody production. In animal models, hormone effects in immune system have been shown to be complex with different effects depending on the strain and the specific disease. NK cells are element of innate immune system and account for 5-15% of peripheral blood lymphocytes population. These cells have the ability to both lyse target cells and provide an early immune regulation cytokines without prior immunization. New insight into NK biology has suggested major role of NK cells in infection control and uterine function in pregnancy. Recent studies show that NK cells play important role in the control of migration and differentiation of trophoblasts and implantation of embryo through secreting various cytokine. Several recent studies have indicated that RSA is associated with and alteration in endometrial and peripheral leukocyte population. In addition, significant higher number of activated leukocytes have been shown in deciduas and specially increased in NK cell population in relation with pregnancy failure. Therefore, the present study has focused on immunological and psychological aspects to have a new approach for looking at these aspects simultaneously, it is hoped that medical laboratory available tests as well as psychological care in clinic would benefit patients who suffer socio-medical problems.

MATERIALS AND METHODS

Forty-five women with history of three or more RSA were included, women with anatomical, genetical, hormonal or infection causes were excluded after visiting and interview by specialist. In addition, a non-RSA control group was included in the present study consisting of forty-five healthy multiparous women with no history of abortion. After clinical consideration, the specialist has interviewed selected cases, regarding stress as well as depression. The assistant co-worker in this project performed interviews and filled in all the valid questionnaire forms.

Stress Score Assessment

Holmes and Rahe social re-adjustment rating scale was used for a total amount of stress that an individual might have experienced. Stress in women was recorded as a valid approach to assess non-biased stress scores based on life stress questionnaire. Therefore, by adding up the relative stress values, known as Life Change Units (LCU), for various events the total score determined. Persons with a low stress tolerance may find themselves overstressed with a score of 150. A score of 250 or more is considered high. The test is used to determine disease susceptibility. With a score of 150 or less an individual has a 37% chance of becoming seriously ill. Between 150 to 300, it jumps to 51%, with over 300 scores; there is an 80% chance of a serious illness in the next 2 years. The scale of scores for stress assessment stands for 150; which means an individual would be glad and/or normal, but there might be a slight risk of illness (but still needs to take care of his/herself). The scores between 150 and 250 would be considered as low stress value, and the shift to 300 scores would be supposed for mild to moderate condition. The score 300+ means
extreme care and/or at a greatly increased risk of serious illness. Questionnaire life events have been considered based on the Arck et al 2001.1,17

**Depression Evaluation**

The Beck Depression Inventory (BDI) is a 21 item self-report rating inventory measures characteristic attitudes and symptoms of depression. This inventory is a self-administered 21 item self-report scale measuring supposed manifestations of depression. The BDI demonstrates high interval consistency, with alpha coefficient of 0.88 and 0.81 for psychiatric and non-psychiatric populations, respectively. Clinical rating for psychiatric patients are reported as high to moderate ranging from 0.55 to 0.96 with \( r = 0.72 \). The highest score on each of the twenty-one questions is three, the highest possible total for the whole test is sixty-three, and the lowest possible score for the whole test is zero. Total score levels for depression assessment are as follows: 5-9 would be considered normal, 10-18 is mild to moderate depression, 19-29 stands for moderate to severe depression and 30-63 considered for severe depression.17-21

**Natural Killer Potential Assessment**

Peripheral blood was collected in EDTA and then peripheral blood lymphocytes (PBL) were separated using a Ficoll-Hypaque gradient technique (lymphoprep; Norway). Lymphocytes were isolated, washed and brought to a concentration of 5x 10^5 cells/ml in RPMI 1640 + 10% FCS (Gibco, Germany). The K562 tumor cell line (obtained from pasteur Institute-Iran), maintained in continuous suspension culture in RPMI 1640+10% FCS, supplemented with L-glutamine, 100 µg/ml streptomycine, 100U/ml penicillin (Jaber-Ebn-Hayyan, Iran). The K562 is an NK-sensitive human erythromyelocytic leukemia cell line.22 These cells were used as sensitive target cells for evaluation natural killer cell cytotoxicity in vitro assay.

The non-radioactive method assessment for NK cell cytotoxicity was modified from the procedure of Vital et al (1989) and Gilman (1999)(23;24). A working solution was prepared by adding 0.5ug/ml of propidium iodide (PI, Sigma) in RPMI 1640+10% FCS.

![Figure 1. The NK cytotoxicity assessment in PBL samples obtained from the women with RSA and non-RSA. The kinetic of NK cytotoxicity is summarized, the representative NK cytotoxicity from four different samples with the ratio of 6:1, 12:1, 25:1 and 50:1 has been illustrated. The sample numbers 1 and 3 were taken from non RSA group, and the others were from RSA women. These show variation cytotoxicity potential in different individuals. The outcome results in the groups were achieved from the data analysis and it is indicated in table 1.](image-url)
The lymphocytes (as effectors) and K562 (as target) cell lines were mixed and cultured in the same tube with Effector: Target ratios of 50:1, 25:1, 12:1 and 6:1 respectively. Briefly: the tubes containing the mixed cells were centrifuged for 3 min at 300g at room temperature, then kept in 37°C for 150 minutes in a humidified 5% CO₂ incubator, then the tubes were resuspended. In the working solution, a concentration of 1x10⁵ cell/ml was prepared to avoid recycling of NK cells. The samples were then incubated for 1 h at 37°C, 5% CO₂, then the cell concentration brought to 1x10⁶ cell/ml and was ready for running by flow cytometry. In order to monitor the spontaneous death rate, the only target cells were incubated accompanied with the processing. The final concentrations of 1x10⁵ cells/ml have been running as control. The cells were analyzed with a FACSscalibour flow cytometry (Becton-Dickinson, USA). A representative dot plot is shown for cell analysis by flow-cytometre (Figure 1). 1x10⁵ cells were run in each event and the data obtained were analysed using a cell Quest software installed in the flow system. The percentage of dead target cell was calculated as: the percentage of dead target cell in the test tube including effector cells subtracted the percentage of dead target cells in control tube with absence of effector cells (Figure 2).

Statistical Analysis
The data was analysed using SPSS software installed in IUMS computer network. The Student t-test was used to test statistical significances of the mean values for ages, NK cells activity and the scores obtained. Correlations were determined by the Pearson test. The significance level was set at p value < 0.05 for all the tested data.

RESULTS
The analysis results of psychological parameters as well as cell mediated cytotoxicity of women with history of RSA and multiparous control group are summarized in Table 1. The mean age was 29.7±4.5
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years for RSA women and 31.1 ± 3.8 years for the control group. The mean ± SD of gravidity was 3.3 ± 0.7 for control group, and the mean number of spontaneous abortion were 3.4 ± 0.7 in the RSA group.

The life event scores of the stress questionnaire were calculated 254 ± 83.6 for women with RSA and 224 ± 79.6 for women in control group. The comparison between the means does not reach to a statistical significant level (P= 0.086). The depression score means were determined 27.6 ± 8.8 in RSA women and 19.4 ± 7.1 in control group respectively. The difference between the two means is statically significant (P= 0.000). The analyses indicate an association between life stress scores and depression scores in RSA women with r = 0.65, P=0.000 and it was determined with r = 0.45 and P= 0.002 for control group respectively. The NK cell activity in the two groups has been indicated in Table 1. The regression analysis was performed for stress as well as depression scores with NK cytotoxicity in both RSA and non-RSA women. The association between NK cytotoxicity and stress scores were determined with the r = -0.054, P= 0.79 for the ratio 50:1 of Effector: Target cells. In addition, similar data was obtained for the other ratios tested. Therefore, the lack of correlation was concluded for this association speculation. A weak negative correlation between the depression scores and NK cytotoxicity was seen with r = -0.34 and P= 0.02 in RSA women for the ratio of 50:1. Similar data was seen for the other ratios tested. There was no relationship between stress scores and NK cytotoxicity in non-RSA group (data not shown). However, the depression scores and the percentage of NK cytotoxicity show a weak negative correlation (r = -0.36 and P= 0.014 for the ratio 50:1) for non RSA group. The regression analyses for the other ratios show a similar trend as mentioned before.

Moreover, the scores of high depression and high stress in the women were analyzed (Table 1). The patients were divided into two sub-groups, low (≤ 249) and high (>250) for stress scores, along with low (< 19) and high (≥ 19) for depression scores. The mean ± SD was determined 311±57 (N=26) for the high-stress subgroup, and 30±7.6 (N= 37) for high-depression subgroup. The association between the high stress and the high depression scores was significant (r= 0.422, P= 0.045). The Pearson correlation test shows a lack of relationship between high stress score and NK cytotoxicity (r= 0.011, P = 0.95, N= 26). The same analysis for high depression scores and NK cytotoxicity was determined with r = -0.30 and P= 0.072, N= 37. In non-RSA women, there was also no association for either high stress sub-group or high depression sub-group and NK cytotoxicity (P≥ 0.05). Moreover, the analysis performed for looking at the relationship between the stress or depression scores and the ages of the women involved in this study, however, no consistent association was found.

Table 1. NK cytotoxicity potential, depression and stress scores measurements in women with RSA and non-RSA group

<table>
<thead>
<tr>
<th>E:T ratio</th>
<th>RSA</th>
<th>Non-RSA</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>50:1</td>
<td>%27.1±6.5</td>
<td>%19.3±3.9</td>
<td>0.000</td>
</tr>
<tr>
<td>25:1</td>
<td>%21±4.6</td>
<td>%13.97±3.5</td>
<td>0.000</td>
</tr>
<tr>
<td>12:1</td>
<td>%14±5.2</td>
<td>%8.87±2.1</td>
<td>0.000</td>
</tr>
<tr>
<td>Stress</td>
<td>254±83.6</td>
<td>224±79.6</td>
<td>0.086</td>
</tr>
<tr>
<td>Depression</td>
<td>27.6±8.8</td>
<td>19.4±7.1</td>
<td>0.000</td>
</tr>
<tr>
<td>High stress</td>
<td>311±57</td>
<td>292±37</td>
<td>0.007</td>
</tr>
<tr>
<td>High depression</td>
<td>30±7.6</td>
<td>25±6.1</td>
<td>0.010</td>
</tr>
</tbody>
</table>

The fresh peripheral lymphocytes were the source of NK cell activity for both RSA and non-RSA women. The tests were prepared 2-3 times for each sample and mean±SD for each ratio tested are presented. The P value shows the comparison between the two mean groups. The stress and depression scores obtained according to a standard questionnaire described in material and method section. E:T stands for Effector lymphocytes used in experiment process and K562 as Target cells according to procedure described in the text.

DISCUSSION

The present study was an attempt to look at an aspect of psychological parameters in relationship with functional immune system in part. According to the literature survey the mechanisms involved in some unexplained miscarriages have speculated to be related with interface of immunological functions. For instance, it has been reported that emotional stress is often followed by increased susceptibility to infections. Natural killer cells in first line of immunological defence play a major role in the immediate immune response controlling this susceptibility.25 In addition, it has been suggested that catecholamines are responsible for altered cellular immunity after stress.26 Moreover, alteration in cytokine profile balances and cellular patterns such as T, B and mast cells were considered for women who had abortion experiences with psychiatric histories.1,27 Our data show an elevated stress score (not statistically significant) as well as
increased depression score (P=0.000) in RSA women in comparison with the non-RSA women. According to the questionnaire used in this study the mean stress scores obtained in non-RSA population were scaled in mild to moderate grade, so the explanation of this high-background may need a social attention and should be discussed in detail, but, it is out of the focus of the present study. This study indicated that both increased NK cytotoxicity and the augmented psychopathological scores were seen in the RSA women. Similar association was concluded partially by Arck and Hori.\textsuperscript{1,2,28} However, there are some contradictory reports which do not support this concept.\textsuperscript{29} The result of present study shows that women with RSA experience have considerable psychological disorder (which could be seen by the high stress/depression scores). One possible reason is that abortion could be a psychological trauma and stressful life event, this chronic stress might cause depression in these groups of women, and the depression may influence the hormonal status as well as immunological factors. There are several reports that show it in spite of that the women who had association between stress and abortion, but there was not correlation with their pregnancy outcome. These reports also considered the physiological markers i.e. hormones concentration. However, our investigation did not cover the women’s hormones, but several investigators have discussed hormonal changes in recurrent miscarriage in recent years.\textsuperscript{1,27} From all the aspects of immune system, although we evaluate the natural killer activity, but not hormonal state, the relationships between lymphocytes and hormonal status have been noted elsewhere.\textsuperscript{26,30} For instance, it has been suggested that the accumulation and survival of uterine mucosal lymphocytes of NK cells are hormonally indirect dependent.\textsuperscript{50,31} At this time, there are enough reports that show TH1/TH2 cytokine imbalance at a feto-maternal interface, and it has been supposed that immunological function could influence of some unexplained miscarriage.\textsuperscript{10,28} Hence, it could be concluded that women with a history of abortion might be at risk of abortion due to influence the immune mediator either indirectly (hormonal alteration) or directly (cytokine profile imbalance TH1/TH2). Furthermore, there are more investigations which indicated increased levels of abortogenic type TH1 cell cytokines and decreased levels of pregnancy protective cytokines (TH2) that could be linked to stress-triggered embryonic loss.\textsuperscript{32,33} In experimental model it has been shown that stress promotes neurotransmitter substance P release in tissues and this substance increase the production of decidual tumor necrosis factor (TNFα) by CD8+ T cells.\textsuperscript{34} However, linkage of immune-hormone system by various mechanisms may protect the pregnancy outcome. For instance progesterone-induced blocking factor (PIBF) induces increased production of TH2-type cytokines by stimulating asymmetric antibody synthesis by B cells which might contribute to protection of the fetus.\textsuperscript{30} As mentioned above, all the aspects discussed could be considered for the RSA and similar cases involved. It is proposed that immunotherapy would be recommended for down-regulated NK activity, and psychotherapy has been reported to result in a successful pregnancy outcome in patients with a history of recurrent spontaneous abortion.\textsuperscript{10} Although we consider two aspects related to RSA women, more aspects should be considered being able to handle an abortion state. Using a helping system for improvement of individual stress managing capabilities may be possible approach for benefit to patients together with using appropriate immunotherapy approaches.

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