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Evaluation of Hormonal Effects on Peripheral Blood Lymphocyte Apoptosis in Normal Menstruating Females

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Abstract

Background Apoptosis is a physiological type of cell death plays an important role in the regulation and maintenance of cell populations in tissues upon physiological and some pathological conditions.

Objective To evaluate the hormonal status and peripheral blood lymphocyte apoptosis in normal menstruating female during normal menstrual cycle.

Methods 50 healthy menstruating females with regular cycles were studied. Two samples of peripheral blood were aspirated; the first during the ovulation day and the second at the first day of the menstrual cycle. Lymphocyte separation was done; by Trypan blue exclusion test, and the morphological features of lymphocyte apoptosis by the DNA binding dye (Hoechst stains). Hormonal assessments of FSH, LH, Estradiol, and progesterone were also done.

Results Lymphocyte apoptosis in the first day of cycle was (9.31%±1.7) using Trypan blue exclusion test and (9.76%±1.36) using Hoechst stain while at the day of ovulation the percentages were (2.33%±0.7) and (1.38%±0.84) respectively with P value < 0.00001. Serum level of hormones were FSH (5.76±1.77; 9.14±3.34), LH(3.65±1.4; 28.35±18.94), Estradiol (40.05±14.73; 206.38±70.3) and Progesterone (0.35±0.13; 1.41±0.98) in the first day of the menstrual cycle and during ovulation respectively which showed a highly significant difference with P value <0.00001.

Conclusion The increment in the lymphocyte apoptosis in the first day of the menstrual cycle compared with ovulation day is mostly due to low ovarian steroid hormones and gonadotrophins. This might confirm the hypothesis that FSH, LH, estrogen and progesterone act as survival hormones for different tissues including peripheral blood lymphocytes.

Key words Lymphocyte apoptosis, FSH, LH, estrogen, progesterone, Hoechst stains

Introduction

Apoptosis is a physiological type of cell death that plays a key role in normal development and is critical for cellular homeostasis including immune cellular homeostasis and homeostasis in a variety of tissues [(1)]. The maintenance of tissue homeostasis is finely tuned between cell proliferation and programmed cell death (apoptosis). The maintenance of this balance is crucial to any multicellular organism. Too much proliferation leads to hyperplasia and to anatomical and physiological problems that are associated with it. The worst-case is a total loss of homeostatic control and development of cancer. If apoptosis supersedes proliferation, the result is a reduction of the tissue mass. If the process of apoptotic cell death is abnormal, it eventually reaches a point where physiological function is no longer possible [(2)]. Apoptosis represents a normal function to eliminate excess, old, injured or dysfunctional cells. Many evidences suggest that apoptosis helps to maintain cellular homeostasis during the menstrual cycle by eliminating senescent cells from the functional layer of the uterine endometrium during the late secretory and menstrual phase of the cycle [(3)].

Apoptosis has a key role in the regulation of the T-cell repertoire, both in the development of T-cells in the thymus, and in the elimination of activated T-cells in the periphery. Circulating T cells become active that is, they proliferate and produce proteins that promote inflammation when their receptors bind tightly to foreign antigens. Such activity is valuable when an infectious agent is still present, but when the infection is gone, the cells must die. Otherwise they might accumulate, giving rise to chronic inflammation and possibly to autoimmunity [4, 5].

Apoptosis of unwanted cells is induced by different mechanisms among which is by deprivation of survival factors as cytokines or interleukines and hormones as sex hormones which depend on Fas- Fas ligand interaction [4].

Hormones such as estradiol regulate function directly related to cellular event such as apoptosis. There are accumulating evidences suggesting that steroid hormones regulate apoptosis in hormone responsive tissues. This depends on hormone for survival and proliferation extends to neoplasm arising from these tissues. These reports also show that one mechanism by which estrogens may affect apoptosis is through the increased expression of bcl-2 gene, a member of a family of apoptosis regulating proteins whose expression has been shown to suppress apoptosis. These studies provide evidence that estrogens may play a role in both tumor genesis and drug resistance through suppression of apoptosis. From these studies, it was found that estrogen withdrawal induces apoptosis [6, 7].

Recent studies showed that estrogens may function as endocrine disrupters both in wildlife and humans, leading to developmental defects, disease and, potentially, cancer. The potential exists that these compounds, acting through the ER (Estrogen Receptors), can affect the apoptotic pathways of estrogen-responsive cells. With mounting evidence for the role of estrogen in the regulation of apoptosis, different studies had shown that estrogen can inhibit tumor necrosis factor (TNF)-induced apoptosis [7].

Studies had shown that estrogen may modulate the immune actions of leptin. Leptin induces the release of pro-inflammatory cytokines, including TNF-alpha, from human Peripheral Blood Mononuclear Cells (PBMCs). Estrogen can inhibit the signaling and immune actions of leptin. This could explain the sexual dimorphism observed in the immune response [8].

In vitro studies showed that estrogen decreases apoptosis of peripheral blood mononuclear cells from women with normal menstrual cycles and decreases TNF-alpha production in SLE patients but not in normal subjects[9].

Aim of the study

To evaluate the hormonal status, and peripheral blood lymphocyte apoptosis in normal menstruating females during normal menstrual cycle.

Methods

This study included 50 healthy menstruating females with regular cycles, their age ranged between 20 to 35 years with a mean of (23.96±3.31). Two blood samples were aspirated from each volunteer; the first was taken at first day of the menstrual cycle and the second at the day of ovulation.

7 ml of blood were taken and divided in 2 parts; 2ml was mixed with EDTA as anticoagulant for lymphocytes separation and study. The remaining 5ml of blood were examined for hormonal assessment using VIDAS assay kits for FSH, LH, Estradiol, and Progesterone hormones using mini VIDAS apparatus.

Trypan blue exclusion test was used to determine cell count and cell viability by using Heamocytometer Neubauer counting chamber and light microscope according to a method described by Doyle and Griffiths, 2000[10].

Hoechst stain (DNA binding) was used to study cell morphology using fluorescent microscope

using a method described by Harley and Prescott, 1996 [11].

The data were presented as mean ± standard deviation. Paired T-test was used to compare between ovulation day and the first day of the menstrual cycle. Difference was considered significant statistically when P< 0.05.

Results

The hormonal estimation including Estradiol, Progesterone, FSH, and LH in venous blood of fifty normal healthy female during ovulation day and the first day of the menstrual cycle were listed in table 1.

Table 1: The values of hormones during ovulation day and the first day of the menstrual cycle

Hormones	Ovulation day Mean± SD	First day of menstrual cycle Mean± SD	t test p value
Estradiol E2 (pg/ml)	206.38±70.3	40.05±14.73	<0.00001
Progesterone (ng/ml)	1.41±0.98	0.35±0.13	<0.00001
FSH (miu/ml)	9.14±3.34	5.76±1.77	<0.00001
LH (miu/ml)	28.35±18.94	3.65±1.4	<0.00001

While table 2 shows the percentage of apoptotic lymphocytes stained by Hoechst stain and also Trypan blue exclusion test for lymphocytes viability at the ovulation day and at the first day of the menstrual cycle.

Table 2: The percentage of apoptotic lymphocytes stained by Hoechst stain and Trypan blue exclusion test at the ovulation day and at the first day of the menstrual cycle.

Dead cells percentage	Ovulation day Mean± SD	First day of menstrual cycle Mean± SD	t test p value
Trypan blue	2.33±0.7	9.31±1.17	P<0.00001
Hoechst stain	1.38±0.84	9.76±1.36	P<0.00001

Figure 1 shows trypan blue exclusion test of peripheral blood lymphocyte in normal menstruating females, were the dead cells stained blue while normal cells exclude the dye.

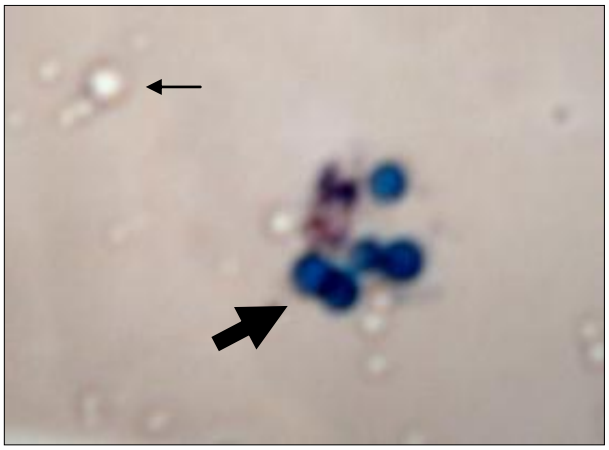

Figure 1:Trypan blue exclusion test ;thin arrow normal lymphocyte , thick arrow dead cell stained blue.

The morphological changes of lymphocyte apoptosis was studied by the DNA binding dye (Hoechst stain) which included: membrane blebbing, kidney shaped nucleus, lobulation of nucleus and lastly distruction of cell into apoptotic bodies. As shown in figure 2.

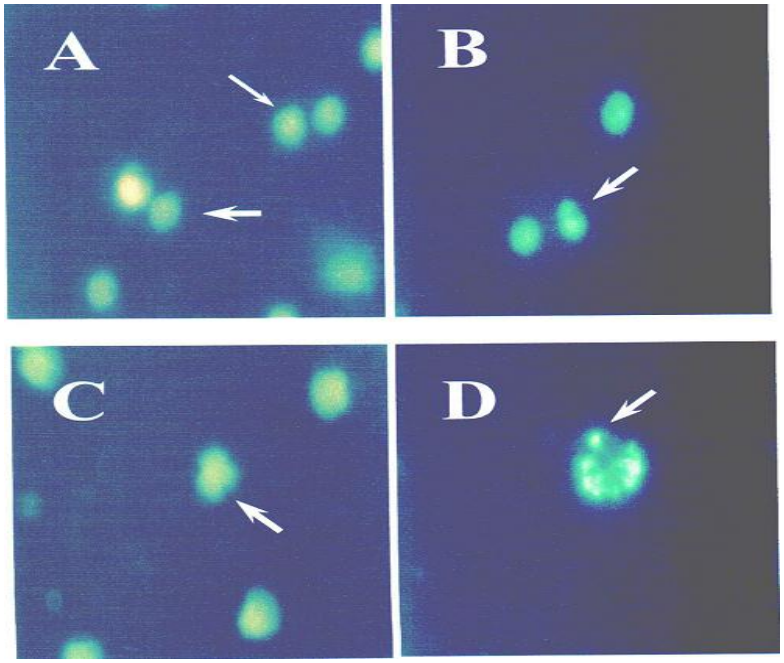

Figure 2: Fluorescent staining (1000X magnification) of peripheral blood lymphocytes of normal menstruating female by Hoechst stain (DNA binding stain) shows: *A*. normal lymphocytes. *B*. kidney shaped nucleus. *C*. lobulated nucleus. *D*. destruction of cell into apoptotic bodies.

Discussion

Female menstrual cycle is normally associated with many hormonal and physiological changes not only in the reproductive system but throughout the whole body. This cycle sometimes associated with pain, hyperpyrexia, psychological changes, and many other sign and symptoms that felt by some females during that period.

In this study peripheral blood lymphocyte apoptosis was studied in normal menstruating females. The results showed that the percentage of apoptotic lymphocytes were found to be significantly higher with values of (9.31 % ± 1.17) by Trypan blue exclusion test and (9.76 % ±1.36) by Hoechst stain during the first day of the menstrual cycle than during ovulation with values of (2.33 % ± 0.7) by Trypan blue exclusion test and (1.38 % ± 0.84) by Hoechst stain. This was with the association of significantly higher estradiol (E2) level during ovulation with a value of (206.38 ± 70.3) than during the first day of the menstrual cycle with a value of (40.05 ± 14.73). The decreased estrogen level during the first day of the menstrual cycle may have played a role in increasing lymphocyte apoptosis. These findings were similar to what was found by Yuan and Giudice in 1997[12], Kaipia and Hsueh in 1997[13] they claimed that the process of apoptosis is hormonally controlled process and that estrogens inhibit this process.

The results simulate those of Conlon and Raff in 1999[14]; they found that hormones act as survival factors to escape cell death and that failure to supply adequate levels of survival factors lead to activation of apoptosis.

This can explain the low lymphocyte apoptosis percentage during ovulation. This can also explain the high lymphocyte apoptosis percentage during the first day of the menstrual cycle; this result was found by Burow and coworkers in 1999[7] that hormones withdrawal induces apoptosis.

In the endometrium, estradiol and progesterone are the main regulators of the cyclical transformations and prevent cell death. This is responsible for the cyclical shedding during menstruation [14]. In this study estrogen and progesterone were significantly higher during ovulation than during the first day of the menstrual cycle and they may have played a role in reducing lymphocyte apoptosis percentage while the reverse was true during the first day of the menstrual cycle as both hormones were of a lower value with a highly significant increase in peripheral blood lymphocyte apoptosis.

Gosden and Spears in 1997[16] had found that progesterone emerges as a major survival factor in the reproductive system; this was obvious during ovulation with reduced lymphocyte apoptosis percentage by both Trypan blue exclusion test and Hoechst stain.

Results of this study also showed that the level of FSH in peripheral blood in the first day of the menstrual cycle was significantly lower than during ovulation (5.76 ± 1.77; 9.14 ± 3.34) respectively. The peripheral blood lymphocyte apoptosis was significantly higher in the first day of the menstrual cycle than during ovulation (9.31 % ± 1.17, 2.33% ± 0.7) respectively by Trypan blue exclusion test and (9.76% ± 1.36, 1.38% ± 0.84) respectively by Hoechst stain, this may explain the change in FSH and may reflect the increase in lymphocyte apoptosis in the first day of the menstrual cycle.

In this study, it was found that both estrogen and progesterone were lower in the first day of the menstrual cycle than during ovulation (40.05 ± 14.73; 206.38 ± 70.3); (0.35 ± 0.13; 1.41 ± 0.98) respectively with increased lymphocyte apoptosis due to decreased hormones levels reflecting a relation between estrogen, progesterone and lymphocyte apoptosis, this simulate the results of Lü and coworkers in 2002[17], they found that T cells may have sex hormones receptors. Among human T cells, estrogen receptors are present only in T cells of the $CD8^+$ suppressor/cytotoxic subset. Further, the effect of estrogen and progesterone on rhesus monkey B-cell physiology in vitro is mediated indirectly through $CD8^+$ T cells. Thus, it is likely that B-cell immunity in women is regulated by $CD8^+$ T cells under the influence of ovarian steroid hormones.

Changes at the cellular level *in vivo* and in cell culture *in vitro* showed that sex steroids have a major importance in over all pathogenesis of immune disorders. It is also mentioned that normal females have greatest changes of immune function during menstrual cycle. These changes involve changes in the immunoglobulines and changes in the cytokines and chemokines (down regulation of T helper-2 cells) [6, 18].

Lü and coworkers in 2003[18] found also that endogenous ovarian steroids regulate the immunoglobulin-secreting cell (ISC) frequency and this may explain why women are more resistant to viral infections and tend to have more immune-mediated diseases than men do. They found that Immunoglobulin A (IgA)-secreting cells (IgA-ISC) were fourfold more frequent than IgG-ISC in peripheral blood mononuclear cells (PBMC) and ISC frequency in PBMC was highest during the periovulatory stage of the menstrual cycle. While Poznansky and coworkers in 2002[19] found that LH, FSH, estrogens, androgens, progesterone, and thyroid hormones all decline during infection.

Another study by DA-SILVA in 1999 [20] showed that sex hormones have direct immunological effects that impact a clear gender dimorphism on the immune system. Globally, estrogens depress T cell-dependent immune function and diseases, but enhance antibody production and aggravate B cell-dependent diseases.

These findings might explain the difference between the strength and nature of immune responses between women and men. Hormonal and cellular immune responses in females are stronger than those in males. Immunoglobulin M (IgM), but not IgG, levels and CD4/CD8 T-cell ratios are significantly higher in the blood of women than in that of

men. Women also develop autoimmune diseases at a much higher rate than men do. These observations clearly demonstrate a role for ovarian steroid hormones in mediation of the immune system [21].

After viral exposure females are more likely to develop a T helper- 1 -type response. When T helper-2 responses predominate such as lymphocytic choriomeningitis virus infections, females have a more severe disease. Clearly, ovarian sex hormones affect the nature and effectiveness of antiviral immunity [22] [23].

Conclusion

From this study, we concluded that lymphocytes apoptosis increased in the first day of menstruation compared with the day of ovulation which is mostly due to the changes in the ovarian steroid hormones and gonadotrophins.

Our study also concluded that FSH, estrogen, and progesterone are survival hormones (anti apoptotic) and that they reduce peripheral blood lymphocyte apoptosis.

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