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The Effects of Ovarian Hyper Stimulation on Mouse Uterine Dendritic Cells at Early Pregnancy

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Abstract

Ovarian induction is widely used in IVF clinics. Following hyper-stimulation, the increase in secretion of estradiol and progesterone by the ovary, can affect the frequency and functions of endometrial immune cells, particularly dendritic cells (DCs). Considering the important role of these cells in endometrial receptivity and implantation as well as fetal protection, we studied the post hyperstimulation alterations of uterine DCs in early pregnancy of mouse.

The frequency, distribution, maturation state and phenotype of decidual and splenic DCs were investigated in ovarian stimulated mice by immunohistochemical methods. The serum estradiol and progesterone were also measured by ELISA method.

Our results showed a remarkable difference in frequency, maturation status and subpopulations of DCs as well as hormones concentrations in hyper-stimulated group compared with control mice.

Regarding to the importance of fine-regulation of DCs frequency and functions for endometrial receptivity and pregnancy establishment and maintenance, it seems that their alternation following hyperstimulation could affect the efficacy of Artificial Reproduction Technology (ART).

Introduction

Successful pregnancy is characterized by the adaptation of maternal immune system to the semi allograft fetus [1]. To explain this immunological tolerance, several molecular and cellular immunoregulatory mechanisms has been proposed [2]. A prominent explanation in this area is the presence of an immunosuppressive microenvironment at the feto-maternal interface that limits innate and adaptive maternal responses to the fetal allo-antigens and help to maintain tolerance. There are available evidences that such exceptional immunological environment is related to a unique cocktail of cytokines and hormones resulting to generation of immunoregulatory cells [3].

Uterine dendritic cells (uDCs) are defined as one of the major regulatory cells that play a crucial function in constructing a favorable immunological environment for embryo implantation and establishment of pregnancy [4,5].

In early pregnancy, uDCs secrete critical cytokines which help decidualization and embryo implantation by regulating stromal cell differentiation and vascular maturation [6]. uDCs are also important for establishment of the suppressive microenvironment of the feto-maternal interface through regulation of T cell mediated immunity and induction of regulatory T cells, etc. which collectively causes a tolerogenic response toward semi-allogeneic embryo [7,8].

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Some important changes in number, maturation and phenotype of peripheral and uterine dendritic cells are reported during normal pregnancy, which seems to be hormonally controlled [9,10,11,3,12].

Several studies demonstrated that DCs express receptors for ovarian hormones such as progesterone and estradiol [13] which profoundly regulate their recruitment to the endometrium especially in early pregnancy. It also is well established that ovarian hormones exert immunomodulatory effects on dendritic cells differentiation, maturation and function resulting in tolerogenic DCs which are indispensable for maintaining a successful pregnancy as well. Thus, ovarian hormones play their protective roles throughout pregnancy at least in part by modulating the DCs function [14,9,13,15].

Ovarian hyper-stimulation is widely used in IVF clinics. The main purpose of this method is stimulating folliculogenesis and increasing the number of mature oocytes in one menstrual cycle [16]. Following ovarian hyper-stimulation, a dramatic increment in hormonal secretion of ovary, particularly estradiol and progesterone takes place [17,18].

Many studies have shown that the up-regulated levels of ovarian hormones insert unfavorable effects on reproductive organs and embryo development; reduced fetal growth, prolonged gestation period and increased post implantation mortality has been reported in such conditions [19]. However, the exact effect of ovarian hyper-stimulation on the uterine immune cells' frequencies and functions is poorly understood.

Since dendritic cells are crucial for special immunological state required for appropriate implantation and development of successful pregnancy [6,5], it is hypothesed that the elevated estradiol and progesterone levels after hyper-stimulation can affect their normal function which in turn may alter the pregnancy outcome following ART procedures.

Therefore, the aim of this study was to find out the effect of ovarian stimulation on frequency, phenotype and maturation state of uterine dendritic cells in early mouse pregnancy. To investigate the systemic effects of hormonal changes on dendritic cells the same variables were measured in spleen of pregnant mice as well. We report the alternation in uDCs population following hyperstimulation for the first time. Our results can improve the knowledge of immunological mechanisms related to IVF failures and consequent abortion.

Materials and Methods

Animals

Adult female (6-10 weeks old) and male (8-10 weeks old) NMRI mice were obtained from the Pasteur Institute of Iran, housed in an animal facility under optimal condition of temperature, humidity and 12-h light/ dark cycle. All animals were handled under procedures approved by the ethical committee of Tarbiat Modares University (TMU.5065844)

Experimental design

Ten female NMRI mice were randomly divided into two groups:

Group 1: served as a control, received no treatment.

Group 2: hyper stimulated mice, all mice in hyper stimulated group were given an i.p. injection of 10 IU pregnant mare serum gonadotropin (PMSG, folligon, intervent, Australia). After 48h 10 IU human chronic gonadotropin hormone (HCG, sereno, Switzerland) was injected.

The Female mice in both groups were allowed to mate with a fertile male and checked for vaginal plug every morning. The day of vaginal plug detection was designed to be the day 0.5 of pregnancy.

Blood sampling and hormonal assay

To study the effect of PMSG and HCG treatment on plasma estradiol-17β and progesterone concentrations, blood samples were taken from pregnant mice of both groups on 7th day of gestation. The sera were then collected and preserved at -20ċ until the hormones assay. The concentration of serum progesterone and estradiol-17β were measured according to manufacturer protocol, using an enzyme-linked Immunosorbent assay (ELISA kit) (Diaplus, USA).

Tissue specimens

Pregnant mice were sacrificed by cervical dislocation on 7th day of gestation. Spleen and uterus tissues were removed and frozen sections were prepared at 5 µm thickness and fixed in ice-cold acetone (Merck, Germany) for 2 min. The tissue sections were preserved at -70ċ until staining.

Immunohistochemical staining

For double Immunohistochemical staining, Acetone-fixed cryostat sections were heated for 10 min at Room Temperature (RT) and washed three times with 0.15 M TBS (Tris buffered saline, pH 7.4). To block the non-specific binding, tissue sections were treated sequentially with protein block serum-free solution (Dako, Denmark) for 10 min and 2% normal goat serum solution (Dako, Denmark) for 15 min. After that, the slides were incubated for 75 min at RT with appropriate dilution of the hamster anti mouse CD11c (Abcam, UK). In the next step, to neutralize the endogenous biotin, one drop streptavidin solution (Dako, Denmark) was added on each slide for 15 min followed by one drop of biotin (Endogenous Biotin Blocking Kit, Dako, Denmark) for 15 min. The endogenous alkaline phosphatase was also blocked using the levamisole as its specific inhibitor. The sections were then treated with biotin-conjugated goat anti hamster IgG (Abcam, UK) for 45 min at RT followed by alkaline phosphatase conjugated Streptavidin (Roch, Germany) diluted in a ratio 1:3000 in TBS for 40 min. The blue alkaline phosphatase substrate (Abcam, UK) was then added on each slides for 10 min. After washing the specimens with tap water, tissue sections were incubated serially with one of the rat anti-mouse monoclonal antibodies CD11b, CD8α, CD86, MHC-II or CD40 (Abcam, UK), o.3 % H2O2 as the inhibitor of endogenous peroxidase, biotin-conjugated goat anti rat antibody (Abcam, UK), and Horse Radish Peroxides (HRP) conjugated Streptavidin (Abcam, UK). To develop the second stain, diaminobenzidin substrate (DAB) (Abcam, UK) were added to the slides for 10 min. Finally, the specimens were rinsed in tap water and treated with nuclear fast red as counter staining (Abcam, UK). The sections were dehydrated in gradient concentrations of ethanol, cleared with Histoclear (Casa Alvarez, Spain) and mounted in Vecta mount (vector, Laboratories, USA). For single immunohistochemical staining of the cells with anti-CD11c antibody, the counter staining, dehydration and mounting were done after development of blue stain of alkaline phosphatase substrate.

The samples were washed with TBS three times after each step of immunostaining. Negative controls were stained using

the same procedure but substituting the primary antibody or secondary antibody with TBS. And the result was appeared to be always negative.

In these immunostaining, the alkaline phosphatase activity evoke a blue reaction, whereas peroxides activity shown brown.

For DCs counting, 3 sections in each sample were selected randomly and in each section 15 individual high power fields (HPF, x40 objective) were analyzed using Image J software. Dendritic cells (blue stained) were counted and calculated as a percentage of the total number of nucleated cells (nuclear fast red stained).

To measure the relative percentage of DCs expressing one of the CD8α, MHC-II, CD11b, and CD86 markers, double positive cells (mixture of blue and brown colors) were also counted in the same manner and identified as a percentage of the total number of CD11c+ cells (blue stained.)

Statistical analysis

Five animals were examined in each experimental group. The normal distribution of the obtained data approved by the Kolmogorov-Smirnov test. The differences between groups were evaluated using a standard parametric test (T-test). Results were considered statically significant if the p-value was less than 0.05.

Results

Estrogen and progesterone measurement

Serum estradiol-17β and progesterone concentrations in control and hyper-stimulated groups were measured by sandwich ELISA method. The level of estradiol-17β in control and hyper-stimulated groups were respectively 94.4±7.6 and 163±8.6 pg/ml and the level of progesterone in the same groups were 28±5.1 and 188.8±14.5 ng/ml. The level of both hormones was significantly higher in hyper-stimulated group than in control mice ($p< 0.01$).

Distribution of dendritic cells in the Spleen

In both groups (control and hyper-stimulated) DCs were dispersed around the lymphoid follicles of the white pulps with a less accumulation in the red pulp. No statistical difference was found in distribution of dendritic cells in the control group compared with ovarian stimulated mice **(Figure 1)**. The average density of splenic DCs (SDCs) in control and ovarian stimulated group was 4.4±0.4% and 3.6 ± 0.5%. The relative percentage of CD11b+ DCs and CD8α+DCs in control group was not different when compared with the ovarian stimulated group. On an average, 39.5±3.7% and 34.6±7.2% of splenic DCs expressed CD11b in control and hyper-stimulated groups respectively, while the relative percentage of CD8α+ DCs in control and hyper-stimulated groups was 65.3±5.1% and 61.6 ± 4.6%.

In addition, we found that the expression of CD86, CD40 and MHC-II markers on the SDCs was similar in both groups. In the ovarian stimulated group, 89.4 ± 3.6%, 36.2 ± 3.3% and 87.9 ± 5.4% of SDCs express CD86, CD40 and MHC-II respectively. While, in the control group the mean percentage of SDCs expressing these markers was 92.8 ± 3.3%, 41.1 ± 4.02% and 91.3 ± 2.6%.

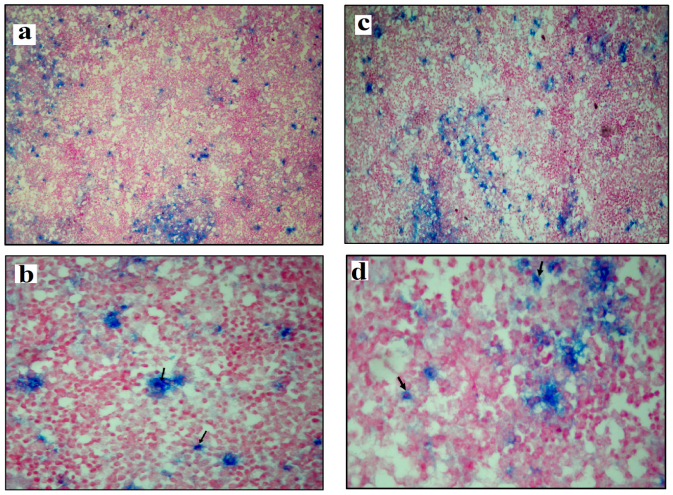

Figure 1: Distribution of dendritic cells in the spleen of control **(a, b)** and hyper stimulated **(c, d)** pregnant mice. Cryosections were prepared from spleen and stained for CD11c as specific marker of dendritic cells. In these immunostaining, the alkaline phosphatase were used which evoke a blue reaction. The CD11c positive cells (blue) are dendritic cells that some of them are marked with arrow (a and c, 100X; b and d, 400X). There was no difference in frequency and distribution of splenic DCs between ovarian induced and control mice.

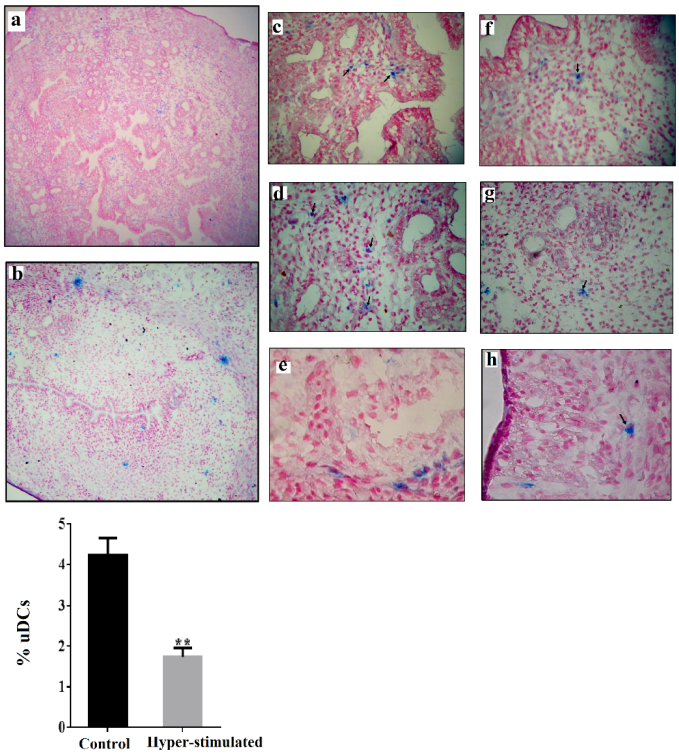

Figure 2: Distribution of dendritic cells in the uterus of control **(a, c-e)** and hyper stimulated **(b, f-h)** pregnant mice. Cryosections were prepared from uterus and stained for CD11c. In these immunostaining, the alkaline phosphatase activity evoke a blue reaction. The CD11c positive cells (blue) are dendritic cells that some of them are marked with arrow. (a and b, 100X) (c-h, 200X). Hyper-stimulation caused down regulation of DCs frequency in all areas of pregnant mice uterus including near the lumen (f compared to c), glandular epithelium (g and d) and myometrium (h and e). The graph shows the Mean ± SD of uDCs percentage. ** means $p<0.01$.

Distribution of dendritic cells in the uterus

Immunochemical staining showed that dendritic cells were present in all areas of the uterus, particularly scattered in the

stroma, around glandular and luminal epithelial layer and in the myometrium. The comparative analysis of uDCs in control and ovarian stimulated groups demonstrated that the average density of uDCs was significantly lower in the ovarian stimulated group than in control mice. The mean percentage of uDCs in control and hyper-stimulated groups was 4.2±0.4% and 1.7±0.2% ($p<0.01$; **Figure 2**).The relative percentage of CD8α+DCs was significantly higher in ovarian stimulated group when compared with control mice. The average density of CD8α+DCs in control and ovarian-stimulated group was 34.9±7.7% and 49.05±3.1% ($p<0.01$). Conversely, the relative percentage of CD11b+ DCs was 66.4±8.1 % and 52.6±2.8 % in control and ovarian stimulated group ($p<0.01$). This data showed that the CD8α+DCs were the dominant subpopulation in hyper-stimulated group compared with controls where CD11b+ DCs were more frequent. Moreover, we found that the expression of CD86, CD40 and MHC-II markers on the uDCs in ovarian-stimulated group was significantly lower than control mice. In the ovarian- stimulated group, 80.8±2.9%, 26.8±5.4% and 56.87±2.9% of uDCs express CD86, CD40 and MHC-II respectively. While, in the control group the mean percentage of uDCs that expressing these markers was 93.1±2.6%, 38.4±2.6% and 88.8±3.9 % respectively, ($p< 0.01$), ($p< 0.01$), ($p< 0.01$) ; **Figure 3 and Figure 4**).

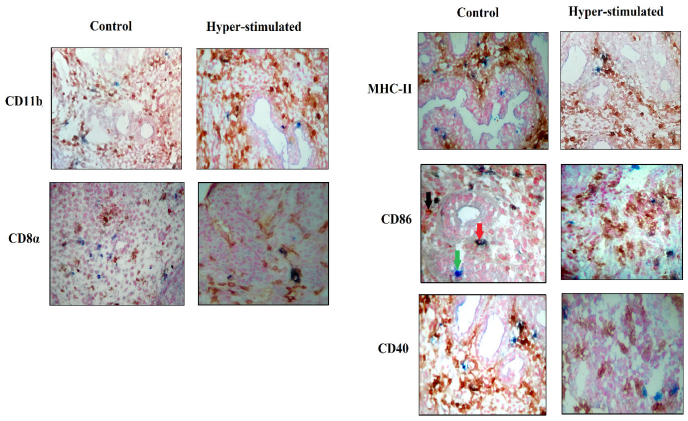

Figure 3: Double Immunostaining of dendritic cells in the uterus of control and hyper stimulated pregnant mice. Cryosections were prepared from uterus of pregnant mice in the both group and stained for CD11c and one of the antibodies anti- CD11b, CD8α, MHC-II, CD86 and CD40. Arrows show a sample of staining pattern. Green arrow (blue color, single positive cells: $CD11c^+$), black arrow (brown color, single positive cells: $CD86^+$) and red arrow (dark blue color, double positive cells: $CD11c^+$ $CD86^+$).

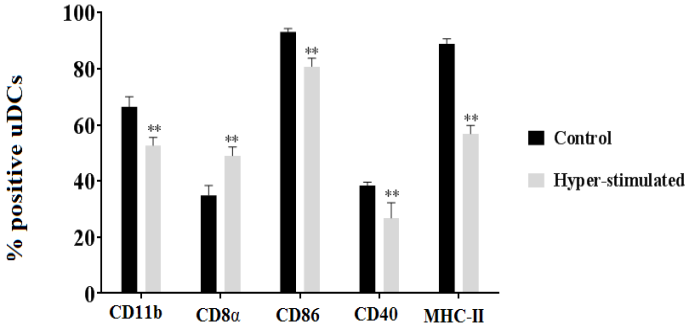

Figure 4: Effect of ovarian hyper-stimulation on surface phenotype of uterine DCs. The graph indicate that ovarian hyper-stimulation significantly decreased the expression of MHC-II and co-stimulatory molecules (CD86, CD40) on uDCs meanwhile the expression of CD8α was up regulated. The frequency of CD11b, CD8α, MHC-II, CD86 and CD40 positive cells is shown in $CD11c^+$ cells (uDCs) population. ** indicates $p<0.01$.

Discussion

In this study, we have evaluated the effect of the ovarian induction on relative percentage, maturation status and immune phenotype (with emphasis on myeloid and lymphoid markers) of decidual and splenic DCs.

Our results revealed that the number, phenotype and maturation stage of decidual dendritic cells obviously changed following ovarian induction. While no significant difference was seen in frequency, distribution and immunophentype of splenic DCs between hyper-stimulated compared to non-stimulated pregnant mice. In the other words, ovarian stimulation in spite of increasing the serum levels of estradiol and progesterone as the main regulators of immune cells recruitment to uterine and decidua during menstrual cycle and pregnancy, proved no considerable systemic effect on DCs distribution such as the splenic dendritic cells. Consistent with this observation, Ho et al. demonstrated that peripheral B cells, NK cells, CD4+ and CD8+T cells and serum level of interleukin-2 (IL-2), interleukin-4 (IL-4), interferon-gamma (INF-γ) have not changed after super-ovulation with gonadotropin [20]. Zarnani et al. also showed that at all stages of mouse estrous cycle, despite the fluctuations in the concentration of estrogen and progesterone hormones, both subsets of lymphoid and myeloid dendritic cells were present equally in the spleen [21].

Opposite to spleen tissue, we observed that the density of uDCs significantly decreased in hyper-stimulated group as compared with non-stimulated mice. The reason for differential responses of decidual and splenic DCs to hormonal changes is not completely clarified, but it may be related to the differential expression of ovarian sex hormone receptors on various tissues. In consist with this idea, an immunohistochemical study on mammalian tissues revealed that the spleen is a non-target tissue for progesterone with weak expression of the Progesterone Receptor (PR), while all cell types of endometrium and myometrium extremely express this receptor [22]. This difference may be due to the importance of sex hormones, particularly progesterone, in the female reproductive tract functions and modulations. Progesterone and estradiol participate in divers activities and tissue remodeling of female reproductive organs throughout the menstrual cycle and importantly in pregnancy including the embryo development, uterine receptivity for implantation, the progression of deciduazation, placentation and eventually parturition [23]. Furthermore, ovarian hormones play a crucial protective role throughout pregnancy by modulating the immune cells function [24,14].

There is a substantial body of evidence from in vivo and in vitro studies revealing that the chemokine-mediated trafficking of immune cells especially dendritic cells in to endometrium are strongly controlled by ovarian hormones [25].

Ovarian sex hormones specifically progesterone and estradiol, affect uterine epithelial cells (uECs) which are fruitful producers of cytokines and chemokines in uterine which in turn regulate the recruitment of immune cells as well as dendritic cells to the uterus [26]. Estradiol and progesterone may directly regulate cytokine/ chemokine secretion by uterine epithelial cells or indirectly through modulating the secretion of growth factors such as Keratinocyte Growth Factor (KGF) by uterine stromal fibroblast cell. Accordingly, Haddad et al. showed that estradiol treatment of uterine epithelial cell in presence of KGF regulated the production of CCL20 (MIP-3α) and CXCL-1 by uECs [27].

The local effect of progesterone and estradiol on secretion of chemo-attractant factors is not limited to uterine epithelial cells. Some studies proved a regulatory effect for estradiol and progesterone on the production of Monocyte Chemotactic Protein-1(MCP-1) and IL-8 by endometrial endothelial cells as well [28]. More studies indicated that progesterone also modulate the level of CXCL-14 in human endometrium [29]. Importantly, CXCL-14, CCL-20, MCP-1 and IL-8 are the chemokines responsible for recruitment of immature DCs to the decidua [30,31].

Additionally, it was shown that the influx rate of immune cells into the endometrium by progesterone and estradiol is dose dependent and may be mutually exclusive. Some studies indicated that following progesterone withdrawal in the time of menstruation or parturition, the observed up-regulation of uterine MCP-1 and IL-8, leads to a significant influx of macrophage, monocyte and neutrophil into the uterus [32,33].

These effects could also be related to the regulation of GM-CSF production by uterine epithelial cells. It is well proven that GM-CSF is synthetized by the uterine epithelial cells particularly at the implantation period, and regulates the migration, proliferation and function of uDCs [5]. Comparably, Wira et al. indicated that in ovariectomized rats, the number of endometrial MHC-II+ cells (macrophage, granulocytes, and dendritic cells) has diminished significantly. Treatment of these animals with estradiol but not progesterone retrieved the recruitment of MHC-II+ cells to the endometrium [34]. In support of this suggestion, Robertson et al. showed that the treatment of ovariectomized mice with estradiol, induced the production of GM-CSF by uterine epithelial cells while the co-administration of estradiol with progesterone reduced estradiol induced GM-CSF [35,36]. Also, Kaplan et al. reported that treatment of leprosy with recombinant GM-CSF can directly induce migration of DCs to the skin [37]. Taken together, it seems that estrogen promotes the recruitment of dendritic cells into the endometrium through up-regulation of GM-CSF production, meanwhile progesterone suppresses the migration of DCs via down- regulation of GM-CSF secretion. According to significantly higher levels of progesterone compared with estradiol in hyper-stimulated group, it seems that the suppressing effect of progesterone on the frequency of uDCs is more dominant and leads to the observed decrease in decidual DCs frequency in hyper-stimulated animals compared to non-stimulated group. Xiu et al. also have indicated that high level of progesterone can reverse the positive effect of estradiol on both differentiation and functions of bone marrow-derived dendritic cells [38]. However, the exact molecular mechanisms by which estrogen and progesterone regulate uDCs need further study.

Moreover, our finding showed that the expression of CD86, CD40 and MHC-II co-stimulatory molecules on uDCs is dramatically decreased in ovarian stimulated group compared with control mice. It seems that DC maturation is affected by increased level of ovarian hormones. Several studies established that estradiol and progesterone exert immunomodulatory effects on DC maturation [10,39,12].

Butts et al. demonstrated that progesterone treatment of bone marrow-derived dendritic cells decreased the expression of CD80 and MHC class II co-stimulatory molecules by these cells [9]. In addition, Lianga et al showed that the mouse bone marrow cells in presence of progesterone produced more numbers of immature DCs and promoted the maintenance of DCs at an immature differentiation stage [15]. This inhibitory effects of progesterone on DCs maturation may be through down regulation of GM-CSF production. Together with above hypothesis, Robertson et al revealed that, in GM-CSF null mutant mice, the expression of MHC-II in uterine dendritic cells detectably decreased [35,36]. Regarding to the elevated levels of progesterone and estradiol following hyper-stimulation as well as the immunomodulatory effects of these hormones on DCs maturation, our finding can be explained.

In this study, we also observed that the CD8α+, CD11b- dendritic cells were the dominant subpopulation in hyper-stimulated group compared with controls where CD11b+ dendritic cells were more frequent. There is available evidence that point to a main role for ovarian hormones in regulating balance of myeloid and lymphoid DCs. It was shown that prior to the induction of EAE, pre-treatment of mice with estradiol, leads to a significant decrease in the frequency of CD11b+ DCs in the brain [40]. Furthermore, Estradiol promoted the differentiation of DCs which had lower level of CD11b expression [41]. However, there is some controversy about the exact effect of estradiol on DC phenotype. Some reports indicated that estradiol enhanced the differentiation of CD11b+ DCs from bone marrow progenitors [42]. This opposing results might be related to the dose depended manner of hormones effects. Furthermore, these hormonal effects usually were individually examined on DCs while the interfering impact of hormones on each other have been neglected.

There is however poor information about progesterone induced modulation of myeloid and lymphoid DCs balance. The progesterone may act on uterine DCs through down-regulation of GM-CSF production [21]. Some studies also reported that GM-CSF preferentially promotes the differentiation of CD11b+DCs from myeloid progenitors [42].

Previous studies demonstrated that murine CD11b+ DCs had less ability to produce IL-12 and shifts the T cell responses to Th2 type, whereas CD8α+DCs induce the Th1 immunity [43,44]. Now, it is well proven that proper balance of TH1/TH2 responses, is necessary for maintenance of pregnancy while overstimulation of TH1 immunity may be hazardous for successful pregnancy [45]. Regarding the differential capacity of myeloid and lymphoid DCs in induction of T cell cytokines profiles, it seems that any changes in their balance following ovarian induction can affect the pregnancy outcome.

It is well established that DCs as the inducers and regulators of the T cell responses play crucial role in establishment and maintenance of pregnancy [46]. DCs are not only essential for induction of tolerogenic responses at the feto-maternal interface but play an important role in uterine receptivity and embryo implantation [6]. It is shown that during implantation, uDCs are recruited in to the endometrium and secrete the pivotal factors such as TGF-ß and sFLT1 which modulate the tissue remodeling and angiogenesis to promote uterine receptivity [47].

In agreement with this idea, Krey et al. reported that the depletion of uterine DCs before implantation time leads to pregnancy failure through disturbed embryo implantation and decidualization [48,6]. Furthermore, it was shown that DC-based immunotherapy decreases the abortion rate in abortion-prone mice, although the exact mechanism of this action is unknown [43].

All these information indicated that fine balance of dendritic cells frequency and subpopulation is crucial for establishment

of special immunological state which is required for appropriate implantation and development of successful pregnancy; therefore, it is likely that any change in their frequency, maturation state and subpopulation following hyper-stimulation can interfere with normal uterine receptivity in implantation time and eventually the fate of pregnancy.

Since no difference in the rate of successful implantation between fresh and frozen embryos in IVF procedure was reported [49], and considering the undesirable effects of ovarian induction on uterine immune cell frequency and distribution delayed embryo transfer in IVF clinics to eliminate the harmful effects of hyper-stimulation is suggested.

Acknowledgements

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