

The Effect of Ethanolic Extract of *Salvia officinalis* on the Uterine Natural Killer Cells Population at Day 7 of Pregnancy

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Received: 27 July 2014

Accepted: 18 Feb. 2015

Abstract

Background: Pregnancy is one of the changer agents for defensive cells, however it is unknown the effect of the ethanolic extract of the *Salvia officinalis* on the uterine Natural Killer cells population in the pregnant mice that needs to be investigated.

Objective: The aim of this study was to determine the effect of the ethanolic extract of the *Salvia officinalis* on the uterine Natural Killer cells population.

Methods: 10 female mice divided into two groups (control and test group). Mice in the control group mated with fertile male mice and mice in the test group received 100 mg/kg of the ethanolic extract of the *Salvia officinalis* once a day for 14 days, then mated with the fertile male mice. Mice in each group were examined at day 7 of pregnancy for ovarian hormones and evaluation of uterine Natural Killer cells.

Results: Uterine Natural Killer cells population significantly decreased in the test group compare to the control group ($p < 0.05$). Ovarian hormone levels (17- β estradiol and progesterone) were significantly increased in the test group comparison with the control group ($p < 0.05$).

Conclusion: It is concluded that decline in uterine Natural Killer cells population after treatment with the ethanolic extract of the *Salvia officinalis* could be due to hormonal changes that may be affect the implantation and embryo development.

Keywords: *Salvia officinalis*, Ethanolic extract, Pregnancy, Uterine Natural Killer cell

Introduction

Salvia officinalis is a plant species native to the Mediterranean area but it is found in some parts of Iran and Europe [1-3]. It is well-known for phytoestrogenic and anti-inflammatory activities to be caused by flavonoids and phenolic diterpenes, respectively [4 - 8]. *Medicago sativa* and *Salvia officinalis* contain steroid and isoflavonoid such as coumestrol, daidzein and genistein [35, 37] which are recognized as phytoestrogens. The metabolites of phytoestrogens act an estrogenic effect on central nervous system which stimulates cell division and growth of the genital tract of female animals [36]. Another phytoestrogen, *Glycinemax* acton estrogen receptors by isoflavonoid [38]. Clinical. Effect of treatment with *Salvia officinalis* were observed in postmenopausal women [39]. Anti inflammatory activity of the *Salvia officinalis* act through inhibition of prostaglandins synthesis [6-8] as like as anti-inflammatory drugs such as aspirin [9-12] that low dose of aspirin resulted in maintenance of corpus luteum activity [11, 12]. Uterine Natural Killer cells appear during decidualization and then become granulated cells. Uterine Natural Killer cells granules are distinctive with periodic acid Schiff staining [13]. Activation and differentiation of these cells directly or indirectly regulated by ovarian hormones [14, 15] Estrogen and progesterone receptors detected on the surface of the uterine Natural Killer cells but there is some dissertation about their presence according to the type of receptors [16, 17]. These cells have main role in stabilization and maintenance of the pregnancy [18]. Pregnancy is one of the changer agents for defensive cells and it is unknown the effect of ethanolic extract of *Salvia officinalis* on the uterine Natural Killer cells in the pregnant mice. There in, it seems that the phytoestrogenic components of the *Salvia officinalis* could be

effective on the uterine Natural Killer cells population through enhancement of the ovarian hormones level. This report designed to determine the effect of ethanolic extract of *Salvia officinalis* on the uterine Natural Killer cells population at day 7 of pregnancy.

Materials and Methods

Reagents, Standards and HPLC analysis

The reagent was pure HPLC grade. Methanol (Merck) was used. Standards were carnosol and carnosic acid were purchased from Sigma Aldrich. HPLC analysis was done using Shimadzu, Scl-10A model. The system included C₈ column (25 cm) (4.6 mm) (Wakosil 5 (18RS) model, quaternary pump, Aven, UV detector at 230 nm and preservative column 1 cm with class-VP V.R.6.1 software. Mobile phase consisted of methanol (isocratic HPLC). Tempratue was 25°C and flow rate was 0.8 ml/min. The 10 µl loop was used.

Plant Material

Leaves of the *Salvia officinalis* (*Herbarium Code: MPH-826*) were cultured in state of Fars and purchased from giah gostar company of Isfahan identified by botanist. Sample collection was at September 2013. Samples were dried at 25°C then the dried plant powder was obtained. Leaves (20 g) of *Salvia officinalis* were extracted five times for 30 min with 127 ml of ethanol 96 °in an ultrasonic bath at room temperature. Finally concentration was done by rotary evaporator.

Preparation of standards and sample solution

Standards solution

Standard solution was prepared by dissolving 5 mg of carnosol (sigma) and carnosic acid (sigma) in 5 ml methanol and

diluting this solution with methanol HPLC grade. The concentration was 1000 and 100 µg/ml, respectively for the carnosol and the carnosic acid.

Sample solution

First, 10 µl of the sample solution was injected to the HPLC grade and the chromatogram was recorded at 230 nm. Next, amount of the carnosol and the carnosic acid calculated in the sample with the comparison of the peaks in the standard and the sample chromatograms.

Animals

Adult female (6-8 weeks old) and male (8-10 weeks old) mice were housed under a 12h light: 12h dark cycle. The adult female mice divided in two groups (control and test group). In the control group, the female mice were mated with a fertile male. The presence of vaginal plug was taken as day 1 of the pregnancy. In the test group, the adult female mice received daily 100 mg/kg of the ethanolic extract of the *Salvia officinalis* once a day for 14 days. Next, these female mice of the test group mated with a fertile male.

Blood sampling and hormonal assay

The blood samples were obtained from pregnant mice at day 7 of the pregnancy. The concentration of the progesterone and 17-β estradiol in sera was measured with ELISA (sandwich method).

Tissue preparation

Five pregnant mice were sacrificed by cervical dislocation in each group of study at day 7 of pregnancy. Uterine horn samples removed from each mice. After preparation of tissue sections, to evaluate the morphology of the tissue and evaluation of uterine Natural

Killer cells population, Sections were stained by hematoxylin and eosin and Periodic Acid Schiff Staining, respectively.

Cell counting

For cell counting, at least 5 sections in each sample were selected randomly and five microscopic field in each section were analyzed. The average of uterine Natural Killer cells was calculated in each group.

Statistical analysis

The frequency of uterine Natural Killer cells within the stroma of uterine horns in two groups was compared using SPSS software and Mann-Whitney test, $p < 0.05$ was considered statistically significant.

Results

HPLC analysis

In this study, calibration curve of standards, chromatograms of the standards and analysis of the leaves of *Salvia officinalis* extract are shown in (Figures 1, 2 and 3), respectively. The retention time of carnosol and carnosic acid were approximately 4.2 and 5.6 min, respectively. The total run time of determination was 40 min. Also, the percent of carnosol and carnosic acid in the extraction of the *Salvia officinalis* is 0.48% and 0.29%, respectively.

Effect of the ethanolic extract of the *Salvia officinalis* on the sera of estradiol-17β and progesterone concentration

The level of 17-βestradiol was significantly different ($p < 0.05$) between control (72.15 ± 8.3) pg/ml and test group (101.51 ± 8.55) pg/ml. Also, the level of progesterone was significantly different ($p < 0.05$) between control (44.29 ± 8.3) ng/ml and test group (113.76 ± 19.52) ng/ml in the control and the test group, respectively.



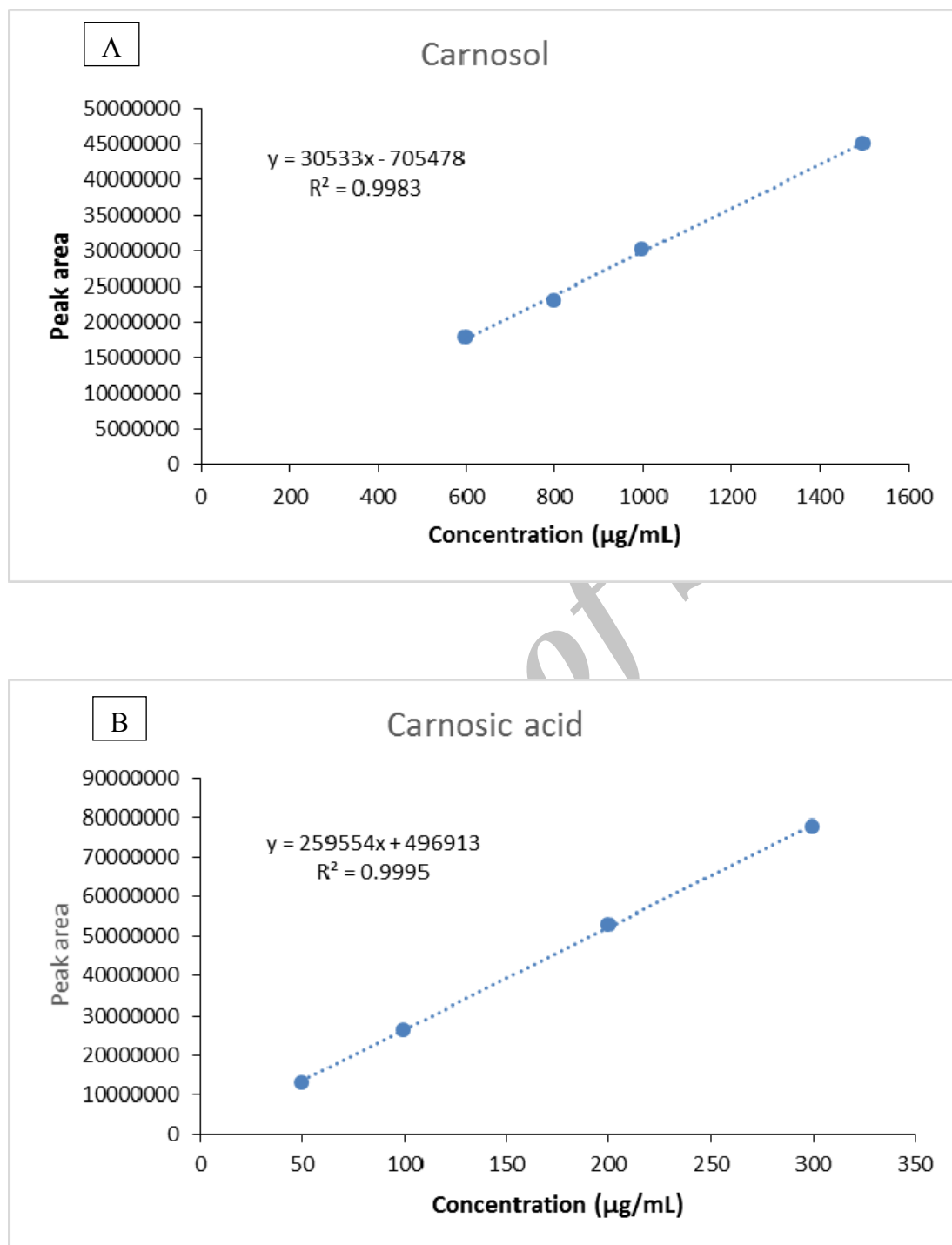


Figure 1- Calibration curve of standards. carnosol (A) and carnosic acid (B). Calibration curve of carnosol depicted in concentration range of 600- 1500 $\mu\text{g/mL}$ with $R^2 = 0.9983$ a and calibration curve of carnosic acid depicted in concentration range of 50-300 $\mu\text{g/mL}$ with $R^2 = 0.9995$

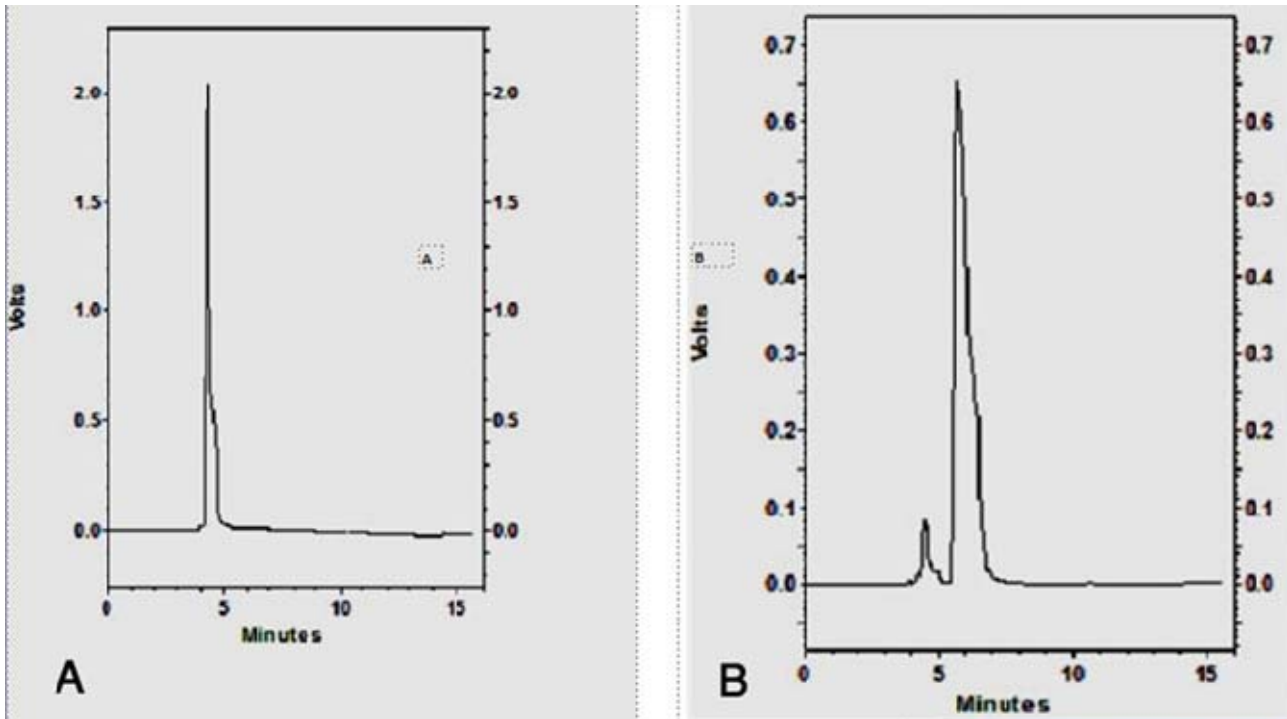


Figure 2- HPLC analysis of standards. Chromatogram of carnosol (A) and carnosic acid (B)

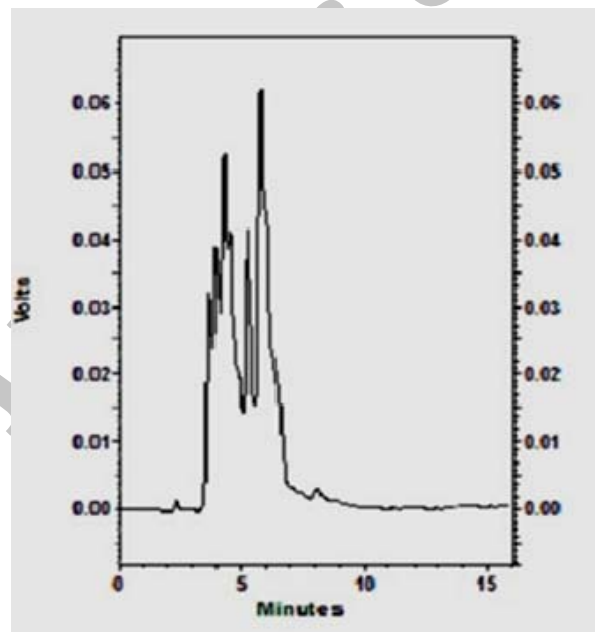


Figure 3- HPLC analysis of carnosol and carnosic acid in the extract of *Salvia officinalis*

Effect of the extract of the *Salvia officinalis* on the morphology of the uterus

The morphology of the uterine tissue that

were stained with hematoxylin and eosin was not shown remarkable changes between the control group and the test group (Figure 4).

Effect of the ethanolic extract of the *Salvia officinalis* on the uterine Natural Killer cells number

To compare the number of uterine Natural Killer cell in each group, we evaluated 5 slides in each group with random selection of 5 region in each section. These slides were observed with 1000x magnification (Figure 5).

Furthermore the number of uterine Natural Killer cell per section in the test group was significantly ($p < 0.05$) lesser than control group. The mean percentage of uterine Natural Killer cells was 10.12 ± 3.06 and 4.84 ± 1.34 in the control and in the test group, respectively.

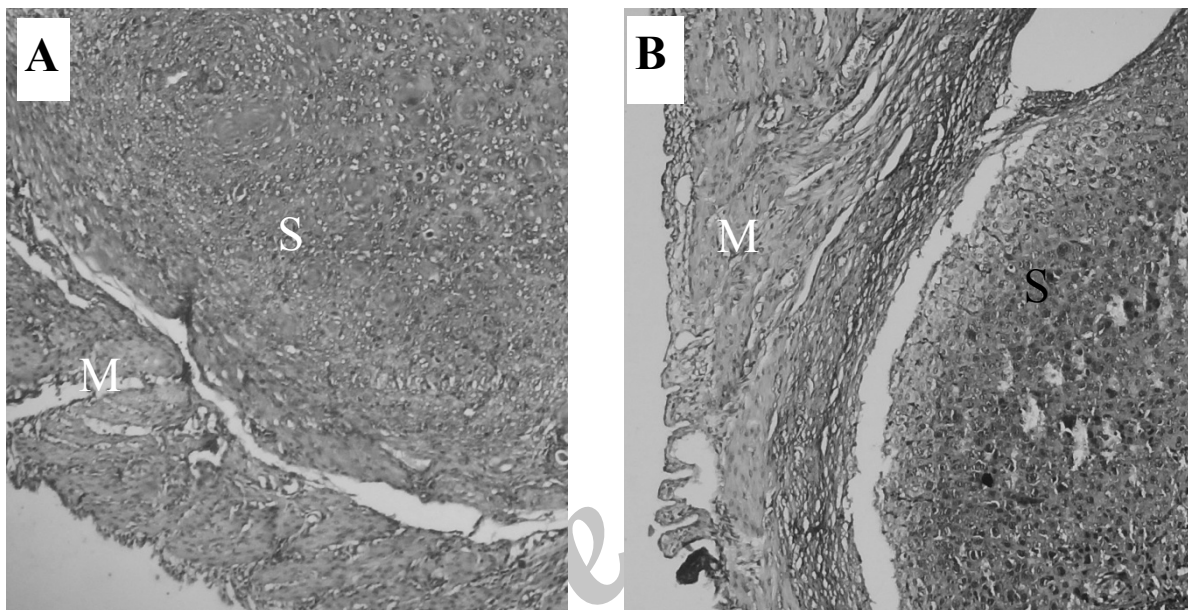


Figure 4- Representative histology of uterine tissue at day 7 of pregnancy stained with hematoxylin and eosin in the control group (A) and the test group (B). There was not remarkable changes between the structure of uterine horns in the control and test group. Slides were observed with 100x magnification. S is representative of stroma and M is representative of myometrium

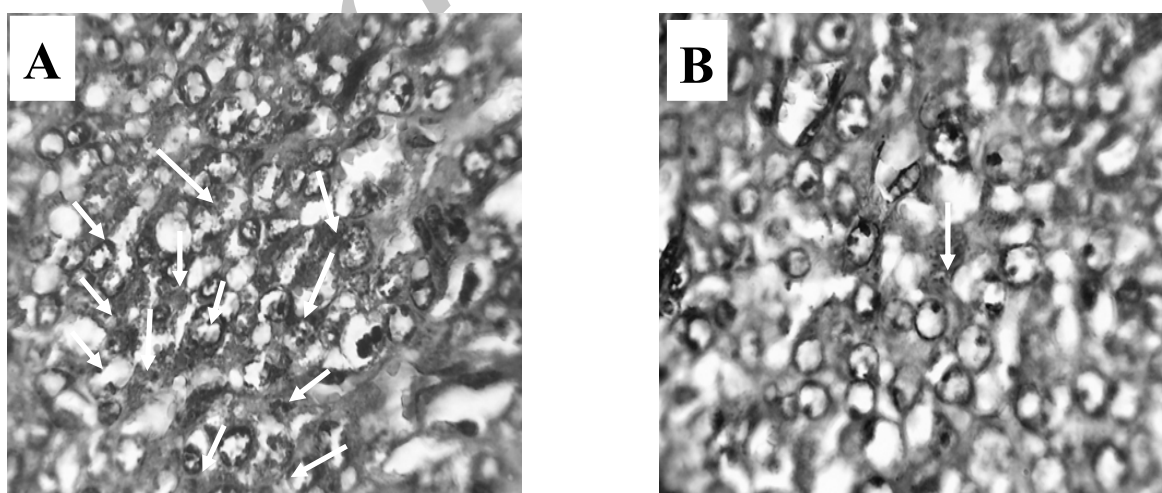


Figure 5- Representative comparison of uterine Natural Killer cells population at day 7 of pregnancy between the control group (A) and the test group (B), stained with Periodic Acid Schiff. 5 Slides in each group and 5 field in each slide were analyzed. The data are expressed as the mean \pm S.D, $p < 0.05$ significantly different from control group. Arrows show uterine Natural Killer cells with periodic acid schiff positive granules. Slides were observed with 1000x magnification

Discussion

The result of the present study indicated that ethanolic extract of *Salvia officinalis* resulted in reduction of the uterine Natural Killer cells population in the test group compare to the control group. Furthermore the ovarian hormones level was significantly increased in the test group compare to the control group. Uterine Natural Killer cells population belong to innate immune system with diverse function such as killing the tumor cells, virally infected cells and cytokine production. Uterine Natural Killer cells function during pregnancy regulated by interaction of stimulatory and inhibitory signaling pathways [13, 18]. During the early pregnancy, uterine Natural Killer cells are the most lymphocyte population in the uterus of primate, human and rodents [19]. These cells secrete some cytokines and growth factors that help implantation, trophoblast cell proliferation, differentiation and maintenance of the pregnancy [13, 18]. Beside, increased uterine Natural Killer cells resulted in more loss of pregnancy through secretion of IFN- γ [20, 21]. Uterine Natural Killer cells number change during murine sexual cycle and found at decidua after implantation then activated at day 7 of the pregnancy [22]. Considering to the cyclic changes of these cells, contribution, migration, implantation and function of these cells regulated by the ovarian hormones [14, 15]. In fact, it is proved the presence of estrogen and progesterone receptors on the surface of the uterine Natural Killer cells [23 – 25]. Elevated estrogen synthesis after treatment with the ethanolic extract of the *Salvia officinalis* can be due to phytoestrogenic effect of the extract lead in enhancement in the number of follicles, corpus luteum and also increase in luteinizing hormone level. Although part of the estrogen

can be induced by autocrine and paracrine growth factors synthesis, perhaps this enhancement resulted in the progesterone production [26]. The findings in our study illustrated that increased ovarian hormones level in the test group comparison to the control group is compatible with recent study. Researchers have also reported the effect of anti-inflammatory activity of the *Salvia officinalis* extract. Additionally anti-inflammatory mechanism of *Salvia officinalis* is related to inhibition of prostaglandins synthesis by phenolic diterpenes, carnosol and carnosic acid components [6, 7]. Evaluation of these components in our study displayed the presence of these metabolites. Inhibitory effect of carnosol and carnosic acid on prostaglandins components is well-known [27, 28]. Furthermore, inhibition of prostaglandins synthesis can be followed by the maintenance of the corpus luteum activity and increase in the progesterone synthesis [11, 12]. Present findings in our study demonstrated that enhancement in the progesterone level after treatment with the extract of *Salvia officinalis* can be compatible with the presence of phenolic diterpene, carnosol and carnosic acid components as anti-inflammatory ingredient in the ethanolic extract of *Salvia officinalis* which have inhibitory activity for prostaglandins. Furthermore increase in the ovarian hormones level directly or indirectly affect on the distribution and the number of uterine Natural Killer cells. It seems that estrogen is one of the key factor in suppressing uterine Natural Killer cells proliferation, thus elevated estrogen level resulted in decline in the uterine Natural Killer cells number at implantation site [29]. Also, increase in the progesterone level associated with binding the progesterone with its receptor on the surface of the uterine Natural Killer cells lead in apoptosis induction and reduction

of the uterine Natural Killer cells number [25, 30, 31]. Based on the findings in our research, uterine Natural Killer cells number decreased in the test group compare to the control group. Other studies have been declared ovarian hyperstimulation resulted in decrease in the uterine Natural Killer cells numbers [32, 33]. Whereas another study pointed the increase in the uterine Natural Killer cells number after supra physiologic treatment doses of estrogen [22]. Considering to the decline in the uterine Natural Killer cells number which affected by hormonal changes, it is inferred that induced immune responses in the uterus is different from other tissues that could be due to impressible by the ovarian hormones [34]. In attention to hormonal changes in the pregnant mice after 2 weeks treatment with ethanolic extract of *Salvia officinalis* in our study, it is inferred that some

signals, local interaction and local growth factors derived from implantation site are helpful point for precise understanding of the mechanisms involved in reduction of the uterine Natural Killer cells number. It is inferred that the ethanolic extract of *Salvia officinalis* could reduce the number of uterine Natural Killer cells at day 7 of pregnancy. Considering the important role of these cells in angiogenesis and establishment of a successful pregnancy, it may affect the development of embryo and implantation that needs to be more investigated peri-implantation through exact molecular assessment in future study.

Acknowledgement

This work supported by Razi herbal medicine research center of Lorestan University of medical sciences.

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