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The effects of different luteal support hormones on endometrial alkaline phosphatase activity and endometrial thickness in superovulated mice

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Abstract

Background: There are some controversial data on application of progesterone and progesterone plus estrogen at luteal phase.

Objective: To investigate the effects of different luteal support hormones on the Alkaline Phosphates (ALP) activity in the endometrial epithelium and endometrial thickness during superovulation process for obtaining the optimized endometrial receptivity in animal model.

Materials and Methods: Pseudopregnant female Balb/c mice were induced for pseudopregnany through superovulation then the mice were divided into two groups. Experimental group included five groups: the pseudopregnant mice were given four consecutive, daily injections of progesterone (P group), estrogen (E group), estrogen + progesterone (E+P group), antiprogesterone + estrogen (RU 486 + E), and sham group. In the control group, pseudopregnancy was induced in the natural cycle. The uterus was collected after day 4.5 of pseudopregnancy. The samples were prepared for the morphological and morphometrical evaluation of the endometrial ALP activity and endometrial thickness.

Results: ALP activity was observed in all groups except P group. ALP activity of P + E group was similar to E and RU 486 + E groups. Sham group showed high ALP activity compared to the P group. The endometrial thickness was low in the P group and high in the sham group in comparison with other groups.

Conclusion: In conclusion, super ovulation decreased the ALP activity. Estrogen along with progesterone at the luteal phase increased the enzyme activity and the endometrial thickness, compared with the progesterone administration, and thus, progesterone plus estrogen could improve embryo receptivity.

Key words: Implantation, Ovarian hyperstimulation, Alkaline phosphatase enzyme, Endometrium.

Introduction

Implantation process comprises complex series of interaction between embryo and endometrium, which begins with the attachment of blastocyst to the luminal epithelium and ends with the formation of placenta (1). Implantation window is a limited period of endometrium receptivity, when the uterine environment is capable of blastocyst reception. Before and after this period, the uterus cannot receive the blastocyst (1). Several factors, including cytokines, growth factors, etc., are involved in the endometrial receptivity through autocrine and paracrine pathways (2). In response to the ovarian hormones, the luminal epithelium undergoes molecular, morphological, and ultrastructural changes at the endometrial receptivity state (3).

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The maximal endometrial receptivity in the mouse is on day 4 of pregnancy and the duration of window implantation is 24 h. The endometrial receptivity in the mouse and rat is under the control of ovarian hormones (4).

The uterus and oviduct have a high metabolic activity and undergoes the morphological and functional changes during estrous cycle (5). Alkaline phosphatase (ALP) is a nonspecific metalloenzyme that hydrolyzes esterase phosphate in the presence of zinc and magnesium ions (6); and is linked to the plasma membrane via a glycosylphosphatidylinositol and is released in certain physiological and pathological phenomena, including inflammation, remodeling, and cell proliferation. There is a direct correlation between the ALP activity and cell proliferation as well as growth-factor concentrations. ALP activity is affected by the cell proliferation and cell membrane damage (7).

In the pregnant rat, the ALP activity is highly expressed on the day 6 of pregnancy, when compared with day 1 (8). The increase of estrogen concentration after ovarian hyperstimulation leads to changes in the ALP activity in the endometrium (9). The estrogen increases the ALP activity, while the progesterone decreases it in the ovariectomized mice (10, 11).

Maximal endometrial receptivity is characterized by the high ALP activity in the luminal and glandular epithelium, and the ALP activity is dependent on the ovarian hormones (4).

The fluctuation of estrogen concentration in the serum affects the implantation rate in the *in vitro* fertilization (IVF) protocols (2). Furthermore, the increase in estrogen/progesterone (E/P) ratio in the serum alters the pregnancy rate in IVF protocols (13). Moreover, there are reports that progesterone and human chorionic gonadotropin (HCG) administration at luteal phase cannot increase the implantation rate in IVF cases (14). Histomorphometrical studies of endometrium showed that the endometrial receptivity is improved by estrogen + progestron as luteal support hormones rather than progestron solely (15). Although the progesterone is routinely administrated as luteal phase supplementation hormone in the IVF cycle, the addition of estrogen to progesterone is controversial with respect to the implantation rate (16, 18).

ALP activity as an endometrial receptivity marker has not been demonstrated after using estrogen and estrogen + progesterone at the luteal phase in the hyperstimulated cases during endometrial receptivity. The hormones can change the ALP activity, which subsequently changes the implantation window.

Therefore, it is necessary to characterize the ALP activity in the animal models by the administration of estrogen + progesterone combination as the luteal-phase support hormones that mimic hyperstimulation at the implantation window. In this study, the quantitative and qualitative assessment of ALP in the luminal and glandular epithelium as well as the endometrial thickness following the administration of estrogen, progesterone, estrogen + progesterone, and RU 486 + estrogen as luteal-phase support hormones, were carried out at the implantation-window period to determine the best condition for implantation.

Materials and methods

Animals

The animals were obtained from the animal house of Tabirz University of Medical Science. Adult male and female mice (8–10 weeks) were housed under temperature and light-controlled conditions with free access to food and water.

Preparation of animals

Male mice were vasectomized and after recovery were used for induction of pseudopregnancy. Female mice were kept separately, until the estrous cycles of the mice became similar. The female mice, based on superovulation, were divided into two groups: control and experimental. Five mice were in each group.

The female mice in the experimental groups were superovulated by the administration of a single dose of 10 IU of PMSG (pregnant mare serum gonadotrophin, Tehran Jehad), and after 48 h, 10 IU of HCG (Daru Pakhsh), the injections were done intraperitoanally. The mice were mated with the vasectomized mice to produce pseudopregnancy.

In the control group, pseudopregnancy was induced in the natural cycle without any superovulation. Female mice of the control as well as the experimental groups were housed overnight with vasectomized males and the presence of vaginal plaque was checked in the following morning; a successful mating was considered as the first day of pseudopregnancy. The experimental group, based on hormone therapy at the luteal phase, was divided into five groups:

1) Sham group: The superovulated mice that were induced for pseudopregnancy without administration of any hormones for the luteal

phase. This group received only the vehicle (olive oil).
2) E group: The pseudopregnant mice that were superovulated, which received consecutive daily estrogen (10ng in vehicle /mouse, Iran Hormone) injection (I.P.), until day 4.
3) P group: The pseudopregnant mice that were superovulated, which received consecutive daily progesterone (1 mg in vehicle /mouse, Iran Hormone) injection (I.P.), until day 4.
4) E + P group: The pseudopregnant mice that were superovulated, which received consecutive daily estrogen + progesterone (10ng + 1 mg) injection (I.P.), until day 4.
5) RU 486 + E group: The pseudopregnant mice that were superovulated, which received consecutive daily antiprogesterone (Sigma) + estrogen (1mg + 10ng) injection (I.P.), until day 4.

Tissue preparation

The mice in all the groups were scarified by cervical dislocation after 4.5 days of pseudopregnancy. The samples were obtained from the 1/3 middle part of the uterine horns for carrying out the ALP-activity and endometrial-thickness studies. The samples immediately were fixed in formaldehyde and then were embedded in paraffin wax, and after preparation of 5-µm section, the sections were prepared for the study of ALP activity and quantitative evaluation of endometrial thickness. The evaluation of enzyme ALP was carried out by Gomori technique (17). The samples were incubated in β glycerophosphate as substrate, at 37°C for 6 h. After washing of samples with water, they were incubated in 2% nitrate cobalt for 2 min, then washed with water, and were incubated in 1% ammonium sulfide. The counter staining was done by safranin. Intensity of ALP activity was measured by graded eye piece for all the groups. Assessment of endometrial thickness was performed using H&E staining.

Morphometrical study

After preparation of samples with Gomori technique, the ALP activity in the luminal and glandular epithelium was measured in the four directions of each slide, and then the data were converted to micron (µm) by slide measurement.
For the assessment of endometrial thickness, the extracted uterus was divided into four pieces. The pieces were separately embedded in paraffin wax in a defined direction. Five sections were provided for each piece. Each section was stained with H&E and was subsequently measured in all the four directions for endometrial thickness. Then, the data were changed to micron by slide measurement and were analyzed by statistical method.

Statistical analysis

The collected data from each group were analyzed by SPSS software with one-way ANOVA method.

Results

The results of this study are presented in two parts: morphology and morphometry.

Morphological assessment

The data revealed that the ALP activity in the luminal and glandular epithelium mainly was localized on the apical border of the cells (Figure 1). The expression of enzyme reaction in the luminal epithelium of E + P group was observed to be more than the other groups (Figure 1a, e). The luminal epithelium in the P group showed absence of ALP activity (Figure 1c). Although the enzyme reaction in the glandular epithelium of all groups was expressed, it was not high in comparison with the luminal epithelium-excluded p group. Ablation of progesterone and estrogen injection (RU 486 + E) caused higher expression of ALP activity in the endometrium (Figure 1f).

The data obtained for endometrial thickness demonstrated that the thickness in the P group was noted to be markedly reduced in comparison with that in the control group. Furthermore, the thickness was observed to be greatest in the sham group, when compared with the other groups (Figure 2).

Morphometrical assessment

The morphometrical data obtained from this research showed the absence of enzyme activity in the luminal epithelium of P group, while no significant differences were observed between the control and sham groups.

The ALP activity increased significantly in the E group, when compared with the control and sham groups. Also, the ALP activity in the E + P group was higher than the other groups. Furthermore, the RU 486 + E group demonstrated higher enzyme reaction than the control group. However, there were no significant differences observed in the ALP activity among E, E + P, and RU 486 + E groups (Table I).

Our results with respect to the glandular epithelium of all the groups showed a significant reduction in the enzyme activity in the P group when compared with the control group. There were

no significant differences in the ALP activity in the glandular epithelium between E + P and E groups, but it was significant in comparison with the control and the sham groups. Also, the ALP activity in the glandular epithelium in the RU 486 + E group was the highest in comparison with the other groups. Comparison of the ALP activity among E, E + P, and RU 486 + E groups showed that there were no significant differences among these groups (Table I).

Comparison of endometrial thickness in all groups showed that in the endometrial thickness was the lowest in the P group, while in the sham group, the thickness was the highest in comparison with the other groups. There was no significant increase in the thickness in the E and E + P groups in comparison with that in the control group, whereas in the RU 486 + E group, the endometrial thickness was lower than the control group (Table I).

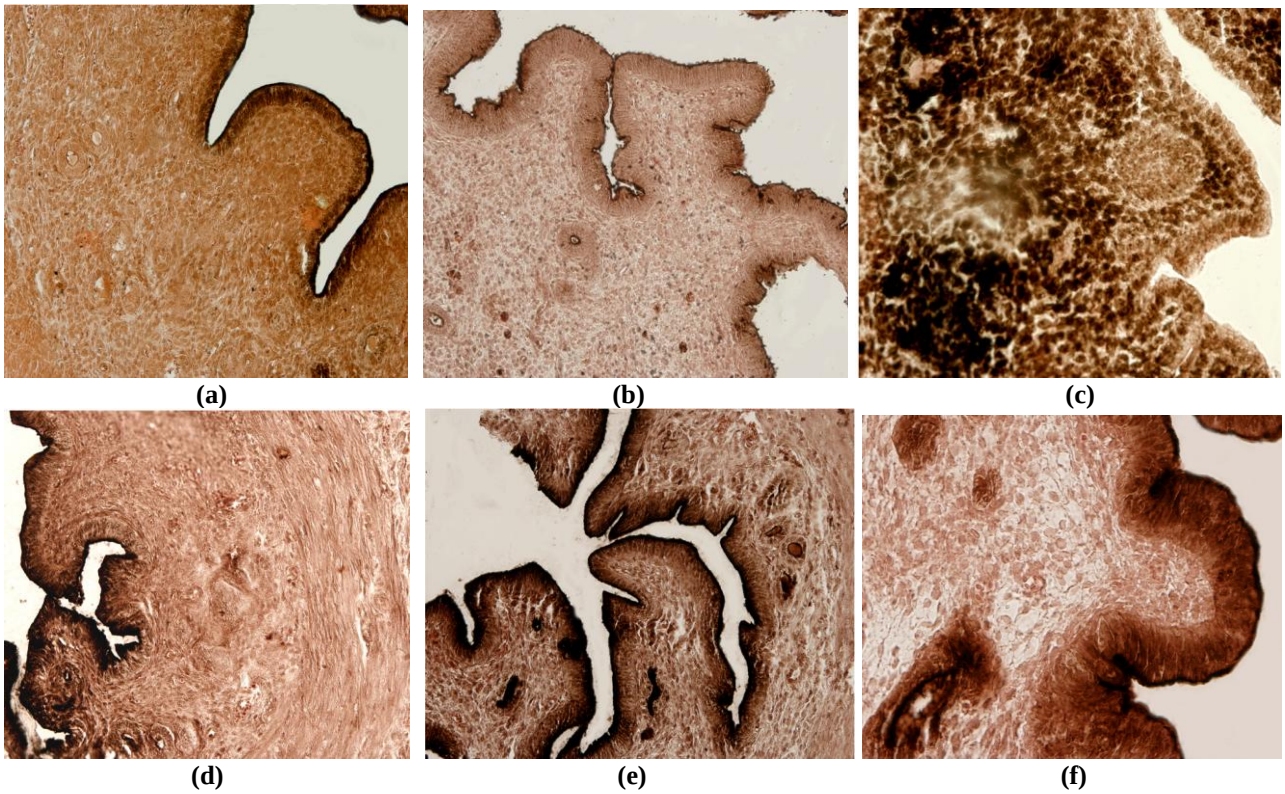

Figure 1. ALP activity in the luminal and glandular epithelium of control and experimental groups. control (a), sham (b), progesterone (c), estrogen (d), estrogen + progesterone (e), RU 486 + estrogen (f). (Gomori technique; Mag: (a),(b),(d),(e),(f) X100, (c) X400).

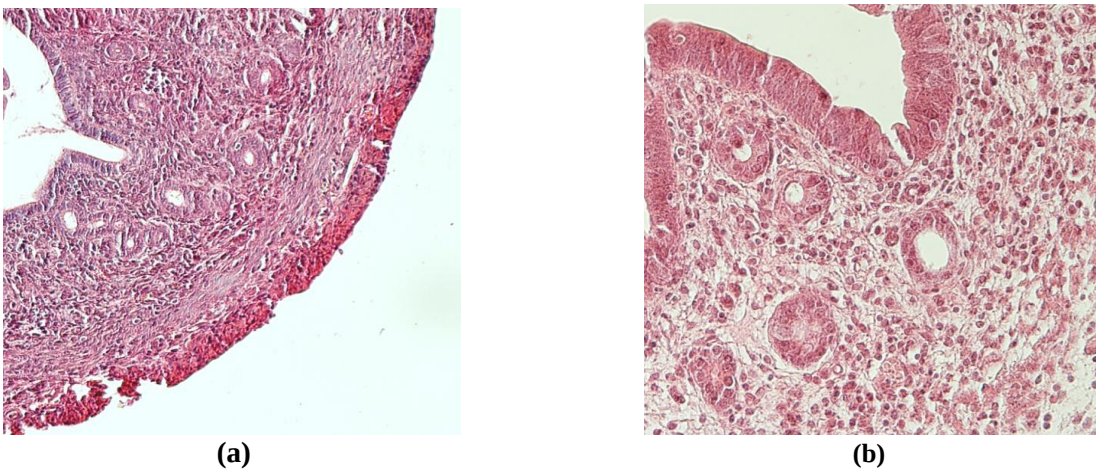

Figure 2. Endometrial micrographs with H and E staining: progesterone (a), estrogen (b). (H&E: Mag: (a) X100, (b) X400).

Table I. Morphometrical assessment of the mouse endometrial thickness and intensity of ALP activity in the luminal and glandular epithelium at the 4.5 day of pseudopregnancy. Values are Means ±SD. (p- value=0.00005).

Morphmetrical parameter (µm)	Groups					
	Control	Sham	Estrogen	Progesterone	E+P	RU486+E
Hight of ALP in the luminal epithelium	5.3 ± 0.85	3.8 ± 0.00	7.3 ± 0.8	0	8.4 ± 1.0	7.2 ± 0.96
Hight of ALP in the glandular epithelium	3.4 ± 0.85	2.1 ± 0.20	5.3 ± 0.85	1.9 ± 0.16	5.7 ± 0.0	6.7 ± 1.1
Endometrial thickness	404 ± 17.54	471 ± 15.2	446 ± 24.6	324 ± 6.9	452 ± 0	345 ± 8

Discussion

Fundamental implantation process is necessary for the structural and biochemical alterations in the uterus. These alterations in the uterus are under the control of ovarian hormones. During the preparation of endometrium for receptivity, the morphological and biochemical changes mainly occur in the luminal epithelium during the implantation time (12).

The activity of enzymes in the luminal epithelium of endometrium changes at the receptivity phase. The ALP activity increases in some mammals and rodents, such as rat and mouse, in the duration of endometrial receptivity (4). The ALP activity in natural cycle without any hyperstimulation demonstrated adequate expression during the implantation window. Emadi's data and other studies also confirmed these results (4, 10).

Fossum *et al* demonstrated that ovarian hyperstimulation with PMSG and HCG reduces the implantation rate (19). Earlier investigations similarly demonstrated that ovarian hyperstimulation decreases the implantation rate in the mouse and causes the reduction of permeability vessels and decidulizations in the rat endometrium (20).

Our data showed decreased ALP activity after hyperstimulation when compared with the natural cycle, which was also confirmed by an earlier study (10). Subsequently, it is presumed that the reduction of implantation rate may be related to the ALP activity after hyperstimulation.Progesterone as luteal support hormone is used after hyperstimulation. The ALP activity in the epithelium decreases following progesterone administration (10), which was also confirmed by our data. However, the ALP activity in ovariectomized mice increased following progesterone treatment (8). These differences among the studies might be related to the hyperstimulation and ovariectomy states of the animals. Our results revealed that estrogen and estrogen + progesterone administration after ovarian hyperstimulation caused increase in the ALP activity in the luminal and glandular epithelium, as well as the increase in the endometrial thickness. Comparison of both estrogen and estrogen + progesterone administration revealed no significant differences between them.

Observation of Bucci in the ovariectomized rats showed that the estrogen administration caused increase in the ALP activity, whereas the enzyme activity was decreased following estrogen+ progesterone administration (9). These differences between our results and that of Bucci can be with respect to experimental conditions.

The ALP activity was also observed to increase during cell proliferation (7), since estrogen has the proliferative role and progesterone provides supporting role for the endometrium (21). It is believed that the high activity of ALP after injection of estrogen + progesterone and estrogen may be related to high cell activity of the epithelium.

Moreover, ALP activity in the endometrium increases during pregnancy, which may be owing to the alterations of the hormones in the serum (9, 10). Abolition of progesterone by RU 486 and injection of estrogen in this study caused increase of ALP activity in the epithelium, while the endometrial thickness decreased, which was also indirectly observed in earlier studies (4, 9).

Though there are some controversies about the role of estrogen and progesterone at luteal phase, some clinical data confirm these results; the addition of estrogen to progesterone during luteal phase results in an increase in the implantation and pregnancy rate (7, 16). Thus, increase in the E/P ratios in infertile patients causes better pregnancy outcome (13), However, it has also been shown that there are no advantages in addition of estrogen to progesterone in pregnancy rate at the luteal phase (22, 24). ALP activity was observed to increase at the endometrial receptivity state and its activity depends on the ovarian hormones (4). Yet,

our data agree with the addition of estrogen to progesterone at the luteal phase.

Furthermore, our results showed that abolition of progesterone by RU 486 caused a significant reduction in the endometrial thickness, whereas estrogen + progesterone and estrogen injections increased the endometrial thickness.

The assessment of ALP activity and endometrial thickness revealed that both the endometrial thickness and the ALP activity were low in the P group. This shows that the progesterone administration solely has not supplied the endometrial environment as in the natural cycle. Furthermore, there were direct correlations in the ALP activity and endometrial thickness following estrogen, progesterone and estrogen + progesterone treatment at the luteal phase.

In conclusion, the histochemical and morphometrical results of this study show that progesterone administration could not provide suitable condition of endometrium for implantation in comparison with other hormone administrations. With respect to both ALP activity and endometrial thickness, this study suggests that estrogen addition to progesterone as luteal support hormones is necessary to achieve an appropriate endometrial condition to implantation. Therefore, a combination of estrogen and progesterone at the luteal phase may improve the implantation rate.

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