ISSN 1009 - 3095 *Journal of Zhejiang University(SCIENCE)* V.1, No.3, P.342 - 346, July - Sep., 2000
http://www.chinainfo.gov.cn/periodical; http://www.zju.edu.cn;
http://lib.zju.edu.cn/English; http://www.zjupress.com

EFFECT OF OVARIAN STIMULATION WITH OR WITHOUT GnRH AGONIST ON HAMSTER ENDOMETRIAL SECRETION*

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Received Jan.1, 1999; revision accepted Oct.29, 1999

Abstract: The present study was undertaken to evaluate the endometrial secretion and synthesis of proteins in hamsters that were subjected to ovarian stimulation with or without GnRH agonist (Leuprolide Acetate, LA). The results of protein resolution revealed that the molecular weight of the proteins increased from 31D to 66D, while the intensities of photographed protein bands in the hamsters treated with LA and pregnant mare's serum gonadotropin (PMSG) were less than that in those treated with PMSG alone. The amounts of low and medium weights (31kD and 45kD) proteins newly synthesized in LA + PMSG group and saline group (control) were significantly higher than that in PMSG group. The synthesized and secreted proteins in groups LA and control were similar. The data suggested that a physiologic endometrial protein secretory and synthetic change with GnRH agonist regarcded as a possible cause of the higher pregnancy rate in in vitro fertilization. The degree of endometrial secretion and synthesis varied considerably with the type of ovarian stimulation used.

Key words: GnRHa. protein. endometrium. hamster
Document code: A **CLC number:** R321.33

INTRODUCTION

The pregnancy rate after in vitro fertilization (IVF) still remains at a disappointingly low level despite a number of remarkable improvements in embryo culture, oocyte retrieval, fertilization and embryotransfer (Tan et al., 1992). Some ovarian stimulation methods were improved to improve the outcome of IVF. More recently, the use of gonadotropin with gonadotropin releasing hormone agonist (GnRHa) for ovarian stimulation in IVF has gained widespread popularity. Many IVF programs have used the technique as the predominant method for ovarian stimulation. Some studies suggested that the pregnancy rate with the use of GnRHa is increased significantly in IVF (Oyesanya et al., 1995; Daya et al., 1997), but its mechanisms of action are still unknown.

The synchronous development of the endometrium and embryo is a crucial factor for pregnancy occurrence. The protein patterns in uterus secretions can provide the most reliable information for implantation (Biljan et al., 1997). The development of endometrium is controlled by hormonal secretion that may be other than natural ovarian stimulation (Parinaud et al., 1992). Some authors hypothesized that the improvement in results with GnRHa treatment was mainly mediated by improved endometrial quality (Speirs et al., 1996). However, the effect of LA, a GnRHa, on secretion and synthesis of endometrium in IVF has not been demonstrated.

For these reasons, we designed an animal experiment to compare endometrial secretion and synthesis of proteins during the IVF cycle after treatment with PMSG plus LA, and with PMSG alone.

MATERIALS AND METHODS

Animals: Female 8 - 10 weeks old virgin golden hamsters with average body weight of 80 - 100 g were used in this study. They were given food and water ad librium and kept in a 14 hours light: 10 hours dark regime (light on from

* Project supported by China Medical Board of New York Inc., USA

1:00 h to 15:00 h). The hamster's estrous cycle was determined by daily observation of the vaginal discharge and smear. Only hamsters that had exhibited estrous for more than 2 consecutive regular 4-days were employed in this study.

Pituitary desensitization: LA (Leuprolide Acetate, TAP pharmaceuticals, North Chicago IL, USA) was administered (at a dose of 40 μg) intraperitoneally at 9:00 a.m., beginning on day 3 of the estrous cycle. Vaginal discharge and smear were examined each morning to detect the daily period of estrous. When the hamster showed no typical ovulation discharge and smear pattern for 4 days, it was considered continuous suppression of estrous. In addition, blood was taken from the hamster's dorsal aorta for serum estradiol (E_2) determination by radioimmunoassay. The day when E_2 decreased significantly was defined as the starting time of the pituitary desensitization.

Ovarian stimulation: Thirty hamsters were randomly divided into three groups (10 hamsters per group). Hamsters in the PMSG group were intraperitomeally injected with 40 i.u. PMSG at 9:00 a.m. of day 1, and with 100 i.u. human chorionic gonadotropin (HCG, Sigma Chemical Company, USA) at 1:00 p.m. of day 3. After pituitary desensitization with LA, the hamsters in the LA + PMSG group were intraperitoneally injected with 40 i.u. PMSG, and 100 i.u HCG was injected into hamsters in the PMSG. group. Controls were given normal saline equal in volume that of the PMSG and LA + PMSG given these two groups at the same time.

Uterus flushing preparation for SDS-polyacrylamide gel electrophoresis (SDS-PAGE): Experiments were performed 48 hours after HCG injection or ovulation. Hamsters were anesthetized with diethyl ether. The uterus was rapidly exposed and clamped at the uterotubal junctions by using sterile technique. The luminal contents of the uterus were immediately collected in situ by flushing the uterus cavity with 2 ml of sterile 0.9% saline. Uterus flushing was centrifuged at 800g for 10 min to remove cellular debris. The supernatant was filtered and lyophilized, and stored at −20 ℃. The lyophilized uterus flushing was dissolved with 20 μl of sample buffer (6.25 mmol/L Tris, pH 6.8, 1% SDS, 2.5% β-mercaptoethanol, 10% glycerol, and 0.001% bromphenol blue) and heated at 90 ℃ for 5 min. The sample analyzed by 10% SDS-PAGE and stained with sliver stain and photographed following destaining. The molecular weight of the separated protein bands was determined by comparison with commercially available molecular weight marks.

The culture of endometrial explant: The endometrial tissue was torn from the uterus using sterile forceps after the endometrium was exposed by a longitudinal incision along the uterus horn. The endometrium was then rinsed in Hank's solution and cut into 1 mm3 pieces. The minced tissue was incubated with Dulbecco's minimum essential medium (DMEM, Gibco Lab, Grand Island, NY, USA) in the presence of L-[4, 5-^{3}H]-leucine (specific activity: 85 Ci/mmol) (Amersham Corp., Arlington Heights, IL, USA) for 24 hours in a humidified chamber at 37 ℃ in an atmosphere of 5% CO_2 in air. The culture medium was collected, cleared of cellular debris by centrifugation at 1200g for 20 min at 4 ℃. The supernatant was filtered and lyophilized and stored at −20 ℃.

Aliquots of the lyophilized endometrial incubation medium were dissolved in 20 μl of sample buffer by 10% SDS-PAGE as decribed above. Different molecular weight bands were cut out from three gels and incubated in DMSO and PPO. Each band cut from the gel was counted by liquid scintillation in radioisotope counter.

Data analysis: Statistical analysis was performed with one-way AVOVA (analysis of variance), Newman-Keul's test, and Student's t-test. Differences with a P value of less than 0.05 were considered significant.

RESULTS

Analysis of pituitary desensitization:

A normal adult female hamster has a regular 4-day estrous cycle. In this study, the time of ovulation occurred approximately seven hours after the onset of darkness on day 1. The postovulatory discharge was found on day 2 morning and the smear consisted almost exclusively of abundant oval epithelial cells. The typical vaginal discharge disappeared and no periodic changes of vaginal cells were observed under microscope. E_2 decreased when the hamsters were injected

with LA for 4 days beginning on day 3 of the estrous cycle. The serum E_2 levels in the control hamster increased on day 4 and day 1 of the next cycle. An E_2 peak appeared before and after ovulation following pituitary desensitization with LA (Fig.1). In group PMSG, the E_2 level was elevated on the day of HCG injection (1621.3 ± 225.5 pg/ml), then reduced two days later (166.3 ± 65.0 pg/ml). But in group LA + PMSG, the serum E_2 was at low level on the day of HCG injection (123.0 ± 28.3 pg/ml), ($P < 0.01$ compared with group PMSG), E_2 concentration increased two days later (889.4 ± 374.9 pg/m), ($p < 0.05$ compared with group PMSG).

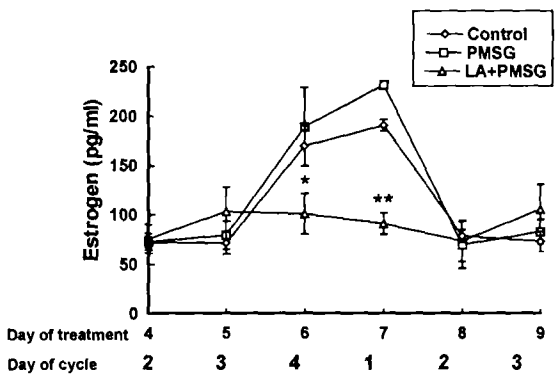

Fig.1 Serum estradiol (E_2) concentration in the hamsters treated with leuprolide acetate (LA) and saline

* $P < 0.05$, ** $P < 0.01$ compared with control group and PMSP group.

Analysis of uterus flushing:

A band of proteins was clearly present in the uterus flushing analyzed by 10% SDS-PAGE. A representative photograph prepared from samples of three groups stained with sliver stain is shown in Fig.2. There were three protein bands mainly in three groups respectively, which were similar in electrophoretic properties. The molecular weights of the three kinds of proteins were 31 kD, 45kD and 66kD, respectively. The intensities of the three bands in group LA + PMSG were not different from those in hamsters treated with saline, but in group PMSG, the intensities of the three bands were significantly increased.

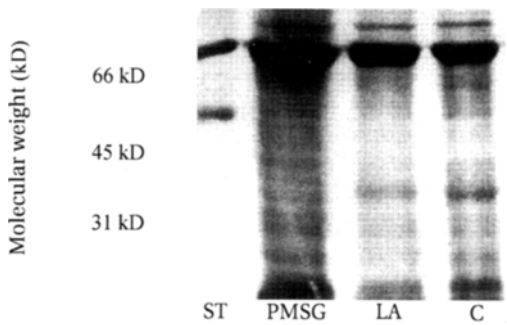

Fig.2 Polyacrylamide gel electrophoresis of uterine flushings obtained from hamsters treated with LA + PMSG and PMSG alone, stained with silver. Lane ST = molecular weight standards, lane PMSG = hamster treated with PMSG alone; Lane LA = hamster treated with LA + PMSG; Lane C = control hamster treated with saline. (kD = kilo-dalton)

Separation and analysis of media proteins:

A photograph of 10% SDS-PAGE of [^{3}H]-leucine-labelled media proteins revealed the presence of three bands of proteins per lane. The molecular weights of the three bands were > 66 kD, 45-31 kD and < 31 kD, respectively. Separation of media proteins from incubation of hamster endometrial tissue was monitored for estimation of newly synthesized [^{3}H]-leucine-labelled proteins by liquid scintillation counting (Table 1). The contents of low molecular (MW < 31 kD) weight and medium molecular (MW 45-31 kD) weight proteins in the PMSG group were significantly less than those in the LA +

Table 1 Amounts of newly synthesized [^{3}H]-leucine-labelled proteins in hamster endometrial explant cultures (mean ± SD, $n = 3$)

Groups		CPM values		
	MW	< 31 kD	31 ~ 45 kD	> 66 kD
Group	control	340.00 ± 60.09	117.35 ± 9.93	151.42 ± 37.36
Group	PMSG	191.92 ± 44.59*	97.90 ± 21.73**	175.00 ± 12.31
Group	LA + PMSG	282.96 ± 1.54#	181.54 ± 21.16##	18.56 ± 19.78

* $P < 0.05$, ** $P < 0.01$ compared with control group, # $P < 0.05$, ## $P < 0.01$ compared with PMSG group

PMSG group and saline group, and there was no difference between the protein contents in the latter two groups. Statistical analysis of high molecular weight (MW > 66kD) proteins showed no significant differences among the three groups.

DISCUSSION

The live birth rate following in vitro fertilization and embryo transfer (IVF-ET) treatment still does not exceed 20%. Most attempts to establish human pregnancy by IVF-ET failed after embryo transfer. In view of the observation that 65-90% of apparently normal embryos fail to implant after embryo transfer, it is considered that the low pregnancy rate in IVF-ET is mainly due to the low implantation rate in the uterus cavity (Biljan et al., 1997). There are two factors influencing implantation: the quality of the embryo and the uterus environment. To establish a pregnancy, a viable embryo needs an adequate endometrium for attachment and implantation. An adequate endometrial transformation can yield some characteristic proteins in the uterine secretions. These proteins deserve further investigation as possible components of an intrauterine environment favorable for implantation (Lopata, 1996). Therefore, our questions are: what is the role of the endometrial function in the establishment of pregnancy? Can endometrial quality be accurately assessed with current techniques of ovarian stimulation? What are the factors influencing the quality of the endometrium and how can quality be improved?

Ovarian stimulation is one of the most important requirement in IVF-ET procedures, because it can affect the quality of not only the oocytes but also the endometrium (Parinaud et al., 1992). Leuprolide Acetate (LA) is commonly used to improve pregnancy rate and control LH peak in preparation for IVF-ET, and its routine use has been advocated. It was noted that following pituitry regulation, aside from the human menopausal gonadotropin (HMG) naturally present in human beings, additional HMG is required to achieve follicle nativity and normal corpus luteum function during IVF-ET. Some researchers found that ovarian stimulation with GnRHa could advance endometrial maturation and increase implantation rate (Oyesanya et al., 1995). The effect of ovarian stimulation on endometrial secretion and its relationship with alterations of endogenous estrogen have been defined.

GnRHa has been shown to prevent an endogenous LH surge, as judged by E_2 level (Balasch et al., 1991; Pellicer et al., 1992). In the present study, the serum E_2 of hamsters did not increase on day 6 and day 7 after injection, which showed that the pituitary function had been suppressed. On the day of HCG injection, E_2 level in group LA + PMSG was lower than that in group PMSG. Neveu et al. (1987) found that in human beings serum E_2 concentration was significantly decreased on the day of HCG administration (Neveu et al., 1987), which was consistent with our observation. In this study, the suprophysiological levels of E_2 as seen in hamsters of group PMSG may have a negative effect on the endometrial environment, while the lower E_2 levels during GnRHa stimulation may have a beneficial effect on the developing endometrium. On the other hand, forty-eight hours following HCG injection, serum E_2 levels in LA + PMSG group was higher than that in PMSG group. The result of endometrial protein analysis suggested that the higher E_2 levels during the receptive phase of the endometrium may probably improve implantation by causing helpful changes in uterine protein secretion.

In the present study, two approaches were used for the analysis of endometrial secretary proteins. One was to determine the in vivo protein profile of the uterine fluid obtained from flushing the uterine cavity with saline, which comprised of protein from plasma transudation and from secretions of the endometrium. Another was to measure the proteins synthesized by the endometrium in vitro using radioisotope incorporation technique. Similar endometrial protein patterns but dissimilar endometrial protein amounts were observed in the three groups, especially in the PMSG group. On the other hand, the secretion of endometrial proteins in the LA + PMSG group was quite similar to that in the hamster with normal estrous cycle (control group). Therefore, it appears that the endometrial secretion proteins of hamsters by treated with PMSG alone were in unphysiological condition, while they were in physiological condition by treated

with LA + PMSG. This phenomenon of above indicated that the changes of physiologic endometrial protein secretion and synthesis with GnRHa would facilitate implantation of embryo or uterine receptivity in IVF cycles. As shown by SDS-PAGE, in PMSG group, the secretion of endometrium proteins was increased. But newly synthesized proteins, especially the low and medium molecular weight proteins, were inhibited in PMSG group. It should be confirmed that the reduction of low and medium molecular weight proteins affects the endometrium negatively. But the decrease of the low molecular proteins in the endometrium can be cancelled by using GnRHa. From the above discussion, it can be concluded that the abnormal alteration of E_2 induced by PMSG has a negative impact on the endometrium. And the reason for implantation of secretion and/or synthesis of endometrial proteins induced by GnRHa may be related to the fact that GnRHa modifies the endogenous hormone secretions including E_2, and that the changes of endogenous hormones develop the internal environment of the endometrium. Some investigators reported that asynchrony between the embryo and the uterine environment is one of the causes of unsuccessful implantation in IVF (Oliveira et al., 1997; Schwartz et al., 1997). Clinically, it was observed that the pregnancy rate of patients using gonadotropin with GnRHa in the IVF cycle increased. The possible mechanism of this increased pregnancy rate may be related to the improvement of endometrial quality induced by endogenous hormones such as E_2 following administration of GnRHa.

In summary, this study indicated that the quality of uterine secretion and synthetic proteins play a crucial role in the success of an IVF-ET program. The degree of endometrial protein secretion and synthesis varies considerably with the type of ovarian stimulation. Ovarian stimulation with LA, in hamster, facilitates the formation of a physiologic endometrium environment. It can be concluded that LA associated with gonadotropin produces endometrial secretion of better aptitude for development than stimulation treatments without GnRHa. The clinical efficacy of GnRHa in IVF-ET cycles could be the result of an improved uterine receptivity to transferred embryos.

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