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Expression of ER-α and ER-β during peri-implantation period in uterus is essential for implantation and decidualization in golden hamster

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ABSTRACT

Aims: The role of estrogen in embryo implantation in golden hamster (*Mesocricetus auratus*) is still ambiguous. In order to clarify it, we investigated the spatial distribution and expression of estrogen receptors, ER-α and ER-β in the uterus of pregnant hamster during peri-implantation period and identified the effect of estrogen receptor antagonist ICI-182,780 on the embryo implantation.
Main methods: We performed in vivo experiments on early pregnant hamsters involving treatment with ICI-182,780, an estrogen receptor antagonist. Immunohistochemistry, western blot analysis and quantitative PCR were employed to evaluate the spatio-temporal distribution and expression of ER-α and ER-β in the uterus of normal early pregnant and treated hamsters.
Key findings: Results showed that embryo implantation was completely absent in ICI-182,780 treated uterine horn while, normal implantation occurred in control and vehicle treated horns. Both the receptors were differentially expressed in the uterus of hamster from day 1 (D1) to day7 (D7). In contrast, treated horns without any implantation site showed no trace of any receptors. Protein and mRNA expression of both the receptors were high around the day of implantation while, ER-β expression was up-regulated on D7 of embryo implantation. P value < 0.05 is considered significant.
Significance: Spatio-temporal expression of ERs in the uterus during peri-implantation period have crucial role for endometrium receptivity and implantation in hamster. Recurrent implantation failure is the devastating problem among the desirable couple and is mainly due to defect in endometrium receptivity. This study may provide a new insight to manage the problem of idiopathic infertility.

1. Introduction

Embryo implantation is the most critical step of pregnancy in mammals. It is a unique biological phenomenon that requires a competent blastocyst and receptive endometrium that cross talk between each other to complete this crucial step of pregnancy. Receptivity of endometrium is determined by highly coordinated molecular and cellular events by estrogen and progesterone [1,2,3]. Ovarian estrogen is responsible for the proliferation of uterine epithelial cell in day 1 (D1) pregnant mice. From D2 onward, the level of progesterone increases due to its secretion from newly formed corpora lutea which is responsible for the proliferation of stromal cells. The stromal cell proliferation is further potentiated by a small estrogen surge during D4 of pregnancy which facilitates embryo implantation [4].

Uterine endometrium is the primary target organ of estrogen that mediates their function via its two classical receptors ER-α and ER-β and also reported to function via G-protein –coupled receptor (GPCR) [5,6]. The role of both receptor subtypes are well characterized in embryo implantation and decidualization in laboratory rodents [7]. Both receptor subtypes belongs to the superfamily of nuclear receptors which generally acts as a ligand inducible transcriptional factor [8,9,10,11]. Interaction of ER with its ligand forms ER homodimer or heterodimer [12,13] that modulates the transcription of their target genes [14,15]. ER-α and ER-β have highly conserved DNA binding and ligand binding domain [16]. The transcriptional activities of ER-α is high in most of the milieu than ER-β [17]. Presence of estrogen receptor has been reported from unfertilized oocyte to various organs of the body with only exception in the morula stage of embryo [18,19,20,21]. During D12 or D13 of gestation, estrogen receptors get accumulated in reproductive organ of developing fetus [20]. The tissue specific distribution

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of ER-α and ER-β suggests that these receptors have dissimilar role in their target tissue [22,23]. There are several published reports suggesting for the existence of ER-α and ER-β in the uterus during early pregnancy in mice [24] and rat [25]. Uterus of human, rhesus monkey, rabbit and baboons are also known to express ER-α and ER-β throughout pregnancy [26,27,28,29]. But till date no report are available regarding the localization and expression of estrogen receptor subtypes in the uterus of golden hamster, *Mesocricetus auratus*, during early and late stage of pregnancy.

Unlike rat and mice, golden hamster does not depend on ovarian estrogen. But, we cannot nullify the requirement of estrogen in the process of embryo implantation in hamster because treatment with anti-estrogen receptor prior to the implantation adversely affects the process. This prompts us to examine whether the uterus of pregnant hamster expresses estrogen receptors to mediate the action of estrogen to complete the process of embryo implantation and decidualization. Hence, in this study we have explored the spatio-temporal localization of ER-α and ER-β and their expression during the peri-implantation period in hamster, using immunohistochemistry, western blot analysis and real time PCR.

2. Materials and methods

All the experiments were conducted according to the institutional guideline and strictly followed the rules of experimental animals (Scientific Procedure) Act 2007, of the Committee for the purpose of Supervision and Control on experiments on Animals (CPSCEA), Government of India, on animal welfare. All experiments were performed in minimum three independent biological and technical replicates.

2.1. Animals and tissue preparation

Golden hamsters were kept in a controlled environment at 14 h light and 10 h dark cycles in an animal house facility of Department of Zoology, Banaras Hindu University, Varanasi, India. They were provided unlimited access of food and water. 10-12 week old hamsters were considered for mating. Adult female hamsters with four consecutive estrous cycles were kept with proven male on the evening of proestrus cycle in 1:1 ratio to get dated pregnancy. Confirmation of sperm positive in vaginal smear in the next morning was considered as day (D1) of pregnancy. Implantation sites were visualized by using intravenous injection of pontamine blue dye (Sigma) in 0.15 M saline. Appearance of blue band along the uterine horn indicate implantation site. The uterine horn between two consecutive implantation sites was considered as inter-implantation site. Pregnant female hamsters of different days were killed at 1600 h and implantation and inter-implantation sites were collected using pontamine blue dye. All the parts of uteri were immediately snap frozen in liquid nitrogen and kept at -80°C until protein and RNA isolation. Different days of tissues for immunohistochemistry were immediately fixed in Bouin's solution for 24 h.

2.2. Treatment of estrogen receptor antagonist (ICI-182,780)

ICI-182,780 is a pure antiestrogen which has ability to bind with both the receptor subtypes and prevent their dimerization which ultimately leads to its degradation [30]. On D3 of gestation period, one group of (n = 7) hamsters were anaesthetized and injected with 0.1 mg ICI-182,780 (ZENECA pharmaceuticals, USA) diluted in 25 μl normal saline using Hamilton syringe at utero-tubal junction in one uterine horn whereas the contralateral horns were injected with 25 μl normal saline which served as control. Dose of ICI-182,780 was decided on the basis of previous report on golden hamster [31]. On D5 of gestation, all hamsters were injected intravenously pontamine sky blue dye (SIGMA-ALDRICH, USA) before 10 min of killing. After killing, uterine horns were removed out and washed thoroughly in normal saline. Both horns were stripped of fat and connective tissues and each implantation and inter-implantation site was cut precisely by sharp blade. Implantation and inter-implantation sites were fixed in Bouin's solution at room temperature for immunohistochemistry.

2.3. Immunohistochemical staining

Immunohistochemical staining was performed following method of Pakrasi and Jain [32] with slight modification. In brief, the whole uteri/implantation or inter-implantation sites were removed and fixed in Bouin's solution overnight. Further, tissues were dehydrated in ascending grades of ethanol and cleared in xylene and embedded in paraffin wax. A 5 μm tissue sections were mounted on poly-L-lysine coated slides. After deparaffinization in xylene and rehydration in descending grades of ethanol, tissues were incubated with normal horse serum for 2 h to block any nonspecific reaction. The sections were incubated with primary antibody of ER-α (Abcam, UK, Cat # ab45005) and ER-β (Thermo fisher, USA, Cat # PA1-311) for overnight at 4°C. This is followed by incubation with appropriate biotinylated secondary antibody (Abcam, UK) for 1 h at room temperature. Section were treated with 3% H_2O_2 (Sigma, USA) for 2 min to block endogenous peroxidase activity. Slides were washed thoroughly in PBS (pH = 7.4) between incubation. Finally, sections were stained using ABC kit (Vector laboratory, USA) according to the manufacturer's instruction. The signal reaction was performed by DAB (Sigma, USA). Brown deposits indicate the positive signals. Images were captured by Leica DMIRB, Leica Microsystems Wetzlar, GmbH, Germany having external camera of canon (Japan).

2.4. Western blot analyses

Western blot analyses were performed following method of Pakrasi and Jain [32] with slight modification. In brief, whole uteri were taken up to D4 of pregnancy for extraction of protein. D5 to D7 uteri were repeatedly flushed with normal saline to remove any extent of embryo from the implantation site. After that implantation sites and inter-implantation sites were separated with the help blue colour impression of pontamine dye (Sigma, USA). Different tissues were homogenized in lysis buffer containing 150 Mm NaCl, 50 Mm Tris (pH 8), 1% NP-40, 0.1% SDS, 0.5% Sodium deoxycholate and fresh protease inhibitor (1 μg/ml aprotinin, 1 mM EDTA, 0.5 μg/ml leupeptin, 100 μg/ml PMSF and 1 mM sodium orthovanadate). Tissue homogenate were kept on rocker at 4°C for 30 min. Homogenate were centrifuged at 10,000 × *g* at 4°C for 10 min and supernatant were collected and stored at -80°C. Protein concentration was determined using Quick Start Bradford Protein Assay (Bio-Rad, USA). 35 μg of protein were boiled in Laemmli 2× sample buffer (4% SDS, 20% Glycerol, 125 mM Tris (pH 6.8) 0.02% Bromophenol blue and 10% β-mercaptoethanol) for 5 min. Proteins were subjected for 10% SDS-PAGE and transferred to PVDF membrane (Millipore, USA). The membranes were blocked in 5% non-fat milk in TBS-T (10 mM Tris-HCl (pH 8), 150 mM NaCl and 0.05% tween 20) for 2 h at room temperature. Membranes were incubated with primary antibodies for ER-α (Abcam, UK, Cat # ab45005, 1:400) and ER-β (Thermo-Scientific, USA, Cat # PA1-311, 1:600) for overnight at 4°C. After incubation, membranes were washed two times (10 min each) with TBS-T and subjected for secondary (Anti rabbit IgG, 1:3000) incubation in 5% milk for 2 h at room temperature. The membranes were washed and incubated with ECL reagent (Millipore, USA) and exposed to X-ray film for the detection of desired bands. Equal loading of protein was confirmed by β-actin (Thermo-Scientific, USA, 1:600). Density of bands was quantified using image-J software, NIH.

2.5. Gene expression analysis

Total RNA was extracted from the uterine horn of pregnant golden hamster using Trizol-reagent (Invitrogen, USA). RNA was treated with DNase (Ambion, USA) to avoid any DNA contamination. RNA was run on 2% gel to check their integrity and quantified by nanodrop