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Ovarian Stimulation by Exogenous Gonadotropin Decreases the Implantation Rate and Expression of Mouse Blastocysts Integrins

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ABSTRACT

Background: Integrins are heterodimeric glycoprotein receptors that regulate the interaction of cells with extracellular matrix and may have a critical role in implantation. The aim of this study was to investigate the effect of ovulation induction on the expression of *α4*, *αv*, *β1*, and *β3* integrins in mouse blastocyst at the time of implantation. **Methods:** The ovarian stimulated and non-stimulated pregnant mice were sacrificed on the morning of 5th day of pregnancy. The blastocysts were collected, and the expression of *αv*, *α4*, *β1*, and *β3* integrins was examined using real-time RT-PCR and immunocytochemical techniques, then their ovarian hormones were analyzed at the same time. The implantation sites in uterine horns of other pregnant mice in both groups were determined under a stereomicroscope on the 7th day of pregnancy. **Results:** The results showed that the expression of *αv*, *β1*, and *β3* integrins in both mRNA and protein levels was significantly lower in the ovarian stimulated group than the control group, and the maximum ratio of expression was belonged to *β1* molecule ($P<0.05$). **Conclusion:** The implantation rate in superovulated mice was significantly lower than control mice. It was suggested that ovulation induction decreased the expression of *αv*, *β1*, and *β3* integrins of mouse blastocysts. *Iran. Biomed. J. 18 (1): 8-15, 2014*

Keywords: Blastocyst, Integrins, Implantation

INTRODUCTION

During implantation, the embryonic trophoblast attaches to the uterine epithelial cells via receptors called cell-cell adhesion molecules, most often of the integrin family [1-5].

Integrins are heterodimeric glycoproteins, which have critical roles at the feto-maternal interface [6-9]. Both the blastocyst and uterine epithelium express integrins on their extracellular side. The integrins are the receptors for matrix proteins, such as collagen, fibronectin, laminin, and vitronectin. The interaction between these molecules results in the adhesion of the embryo to the uterine endometrium at the implantation time [6-11].

The integrin subunits express differently during the implantation window not only on the embryonic surface but also on the endometrium [6, 8, 10, 11].

Failure to express integrins and matrix molecules in sufficient amounts may not result in implantation [12-14]. It is well-known that integrin expression is controlled by ovarian steroid hormones [15].

Despite some improvements in assisted reproduction technology in humans, the pregnancy rates remain low due to imbalance in the embryo and uterus dialogue after ovarian stimulation.

The effects of ovarian induction on the embryo and endometrial parameters have been shown by several investigators [16-20].

The embryo quality is an important predictor for successful implantation and pregnancy [21]. The morphological aspects of the embryo can be used as a marker for selection of good quality embryos [22]. Moreover, some alterations in biomarkers and gene expression could not be shown by morphological assessment.

Some studies have demonstrated that the ovarian stimulation may decrease the expression of endometrial integrons, which impair the endometrial receptivity [11, 23-25]. However, all of these studies did not provide detailed information about the changes in embryonic expression of integrins after ovarian stimulation protocol. Therefore the present study, for the first time, shows the effects of ovarian stimulation

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protocol on the mouse embryonic expression of integrins at the mRNA and protein levels. Our hypothesis was that ovarian stimulation would reduce the expression profiles of different integron molecules of blastocysts in comparison with non-stimulated blastocysts.

MATERIALS AND METHODS

Animals. Adult female (8-10 weeks old, weight means: 20 g, n = 80) and male (8-12 weeks old, n = 10) NMRI mice were obtained from Pasture Institute of Iran (Tehran, Iran). The animals were cared for and used according to the guide for the care and use of laboratory animals of Tarbiat Modares University (Tehran, Iran) and housed under a 12 h light:12 h dark at 20-25°C with enough humidity, water and food.

Reagents and culture media. All reagents were purchased from Sigma Aldrich (Germany) except pregnant mare serum gonadotropin (PMSG, Folligon, Intervent, Australia) and human chorionic gonadotropin hormone (HCG, Sereno, Switzerland). For isolation and culture of embryos, T6 medium supplemented with 5 mg/ml BSA was used.

Experimental design. The adult female NMRI mice (n = 80) were randomly divided into two groups including ovarian stimulated and non-stimulated groups. In ovarian stimulated group, the adult mice were superovulated with an i.p. injection of 7.5 IU PMSG and followed by an i.p. injection of 7.5 IU HCG 48 hours later. Then the stimulated and non-stimulated (control) mice were individually mated with fertile males (n = 10). The presence of a vaginal plug at the next morning was designated as day one of pregnancy.

Blood sampling and hormonal assay. To monitor the effect of PMSG and HCG treatment on serum 17-β estradiol and progesterone concentrations, the blood samples were obtained directly from the left ventricle of heart of stimulated and control pregnant mice under anesthesia using ketamin (100 mg/kg) and xylazine (10 mg/kg) (n = 8 in each group of study) on day 5^{th} of pregnancy. Then their sera were collected and stored at -20ºC to perform hormonal assay. The concentration of progesterone and 17-β estradiol in sera was measured using enzyme-linked immunosorbent assay (ELISA) method (Diaplus, USA).

Blastocyst collection. For blastocyst collection, the pregnant mice in stimulated and non-stimulated groups (n = 28 in each group) were sacrificed by cervical dislocation on the morning of 5^{th} day of pregnancy. The uterine horns were dissected and flushed with T6 medium supplemented with 5 mg/ml BSA. The numbers of surviving and degenerated embryos were recorded in each group of study using an inverted microscope. The expanded blastocyst with normal morphology was collected and considered for molecular and immunohistochemistry analyses.

Evaluation of implantation sites. Stimulated and non-stimulated (control) pregnant mice (n = 12 in each group) were sacrificed on day 7 of pregnancy. Their uterine horns were separated and observed under a stereomicroscope. The implantation sites appeared as prominent, dark red spots on the uterine horn. The number of implantation sites was recorded for each group. The ratio of implantation sites (animals sacrificed on day 7) to the number of surviving embryos (animals sacrificed on day 5) was used to calculate the implantation rates.

Evaluation of integrin expression by real-time RT-PCR:

RNA isolation and reverse transcription reaction. The expanded blastocysts derived from the control and stimulated groups (n = 75) were collected and pooled separately (25 embryos for each replicate of experiments). The RNA was isolated from the mouse blastocysts using the RNeasy Mini Kit (Qiagen, Germany). The RNA samples were treated with DNase to eliminate any genomic DNA contamination just prior to proceed with cDNA synthesis. The RNA concentration was determined by spectrophotometery, and the RNA samples were stored at -80°C until use. The cDNA was synthesized in a total volume of 20 µl containing 5 µg total RNA either with reverse transcriptase (+RT cDNA) or without the enzyme (-RT control) using the cDNA kit (Fermentas, EU) and stored at -20°C until use. All experiments were carried out in triplicate.

Quantitative real-time RT-PCR assays. The primers for real-time RT-PCR were newly designed using GenBank (http://www.ncbi.nlm.nih.gov) and Allele ID software. As shown in Table 1, newly designed primers were ordered and synthesized at CinnaGen Co. (Iran, Tehran). The housekeeping gene, *β*-actin, was used as an internal control [26, 27]. The PCR reactions were carried out in a 48-well plate with 20 µl reaction volume consisting of 10 µl 2× SYBR Green RT-PCR Master Mix (Applied Biosystems, UK) with optimized concentrations of forward and reverse primers and diluted RT products.

The mRNA expression of *αv*, *α4*, *β1*, and *β3* integrins in late blastocysts was quantified using the ABI 7500 Sequence Detector (Applied Biosystems, UK) according to the manufacturer's instructions. The PCR protocol included an initial denaturation at 95°C

Table 1. The characteristic of primers used for real-time RT-PCR assays

Target gene	Primer pair sequence (5'-3')	Accession number	Position in sequence	Fragment size (bp)	Tm (°C)
β-actin	5' GGAAAAGAGCCTCAGGGCAT 3' 5' CTGCCTGACGGCCAGG 3'	NM_007393	848-866 803-818	64	66.6
β1 integrin	5' TGCCTACAACTCTCTTTCTTC 3' 5' TGGTTTCAGACTCCTTATTTG 3'	NM_010578	1298-1318 1506-1526	209	58
β3 integrin	5' TGGAAGAGCCTGAGTGTC 3' 5' CGGTAGGTGATATTGGTGAAG 3'	NM_016780	2367-2387 2283-2300	235	60
αv integrin	5' GGAACAACGAAGCCTTAG 3' 5' GTATCCATCTCTGACTGC 3'	NM_008402	2419-2436	154	55
α4 integrin	5' GAATCTCCTCCACCTACTCACAG 3' 5' CCAACGGCTACATCAACATATCC 3'	NM_010576	1461-1483 1565-1587	127	64

for 5 min, followed by 40 cycles consisting of denaturation at 95°C for 15 s. Annealing was carried out at 58°C for αv and α4, 59°C for β1 and β3 integrin subunits in 30 s, then extension at 72°C for 30 s. At the end of amplification cycles, melting temperature analysis was carried out at 95°C for 15 s, 60°C for 1min and 95°C for 15 s. The testicular tissue was used for positive control. Negative samples were processed by the presence of target genes with no-template samples.

Amplification was repeated on cDNA at least three independent isolates of blastocyst mRNA to insure reliability of the data.

For target sequence amplifications, 500 ng RNA was used per 25 μL reaction volume. After completing the PCR run, melt curve analysis was used to confirm the amplified product. The real-time PCR products were further verified by 1% agarose gel electrophoresis. For each sample, the reference gene (β-actin) and the target genes (*αv*, *α4*, *β1*, and *β3*) were amplified in the same run. Standard curves were obtained by using the logarithmic dilution series of total RNA. Then relative quantization of target genes was determined using the Pfaffl method [28].

Evaluation of integrin molecules by immuno-histochemistry. The blastocysts from control and hyperstimulated groups were put on poly-l-lysin coated slides and treated with acid Tyrode's solution (Sigma Aldrich, Germany) for removing the zona pellucida. They were then fixed in acetone for 20 min and permeabilized by 0.3% Triton-X100 for 30 min. Specific binding was blocked with 10% normal goat serum in PBS. Then the fixed samples were incubated with primary antibodies for αv, α4, β1, and β3 integrin diluted in PBS at 4ºC overnight. The FITC-conjugated secondary antibodies diluted in PBS were applied for 2 hours. For nuclear staining, 5 μg/ml propidium iodide was used. Subsequently, the stained blastocysts were studied under a fluorescence microscope with appropriate filters. All experiments were replicated at least three times for each group.

Statistical analysis. Statistical analysis was carried out with SPSS software. The results of sera estradiol and progesterone were compared by independent-*t* test between the groups. The implantation rate between the groups was compared with the Kruskal-Wallis test. The data of real-time PCR between the groups were analyzed by one-way ANOVA and post-hoc Tukey's tests and within the groups by the Kruskal-Wallis and Post-hoc Mann-Whitney tests. A *P* value of less than 0.05 was considered to be statistically significant.

RESULTS

The 17-β estradiol and progesterone concentrations in sera. The 17-β estradiol and progesterone concentrations in sera of pregnant mice on the 5th day of pregnancy were determined prior to cervical dislocation. The content of 17-β estradiol in sera of control and stimulated groups was 8.54 ± 1.4 and 82.66 ± 4.75 pg/ml and that of progesterone level was 18.38 ± 2.6 and 37.88 ± 5.55 ng/ml, respectively. These levels increased significantly in ovarian stimulated mice compared to the control mice ($P<0.05$).

Implantation rates of embryos in stimulated and control mice. The implantation rates in both groups (80.99% in the control and 39.72% in stimulated groups) of study have been summarized in Table 2. Implantation rate in ovarian stimulated group was significantly lower than that in the non-stimulated group ($P<0.001$).

Table 2. The implantation rates of embryos in stimulated and control mice on 7th day of pregnancy

Pregnant mice (n)	Total embryos on day 5 of pregnancy mean ± SD	Surviving embryos on day 5 of pregnancy mean ± SD	Implantation site mean ± SD	Implantation rate
Control (12)	14.2 ± 2.6	13.1 ± 1.9	10.61 ± 0.01	80.99
Stimulated (12)	21.3 ± 3.6	18.15 ± 2.9	7.21 ± 0.06	39.72 *

The ratio of implantation sites to the number of surviving embryos in each group (on day 5 of pregnancy) was considered as the implantation rate. * shows significant difference with the control group ($P \leq 0.001$)

Blastocyst integrin gene expression. The expression of *αv* gene in blastocyst in stimulated group (0.00116 ± 0.00044) was significantly lower than that in the control group (0.0353 ± 0.002; $P<0.05$). There was no statistically difference between the ratio expression of α4 to housekeeping gene between stimulated (0.00099 ± 0.00047) and the control groups (0.00215 ± 0.00011; Fig. 1). The level of blastocysts *β1* mRNA for *β*-actin gene in control and stimulated groups were 0.10353 ± 0.012 and 0.02335 ± 0.00442 and for *β3* was 0.07972 ± 0.012 and 0.00237 ± 0.00089, respectively. These levels in stimulated group were significantly lower than the control groups ($P<0.05$, Fig. 1). The maximum level of gene expression was related to the *β1* integrin and the minimum of that was related to the α4 integrin in both groups of study ($P<0.05$; Fig. 1).

Immunohistochemical staining. The immunohistochemical staining of different subunits of integrin in control and stimulated groups were shown in Figures 2 and 3, respectively. As demonstrated in the Figures, the intense staining was belonged to *αv*, *β1* and *β3* integrins; however, α4 showed a relative week staining in both groups of study.

DISCUSSION

Integrins are heterodimeric glycoproteins that express on the endometrial, decidual, and trophoblast cells. Their distribution in the endometrium and embryonic surface is cyclically changed during the menstrual and estrus cycles and implantation window [7, 8, 10, 11].

In the present study, for the first time, our findings showed a significant decrease in the expression of *αv*, *β1*, and *β3* integrins at the mRNA levels of blastocysts derived from the ovarian stimulated mice compared to the control group ($P<0.05$).

The main explanation for these differences is the imbalance between the serum levels of estradiol and progesterone in the superovulated subjects and non-stimulated control group.

As demonstrated in some investigations during ovarian stimulation, the steroid hormones are produced at the supraphysiological levels, and they have an impact on some parameters of embryo quality including the gene expression [19, 29].

Our data confirmed that the concentration of the 17-β estradiol and progesterone in stimulated mice was 10 and 2 folds more than the control mice, respectively.

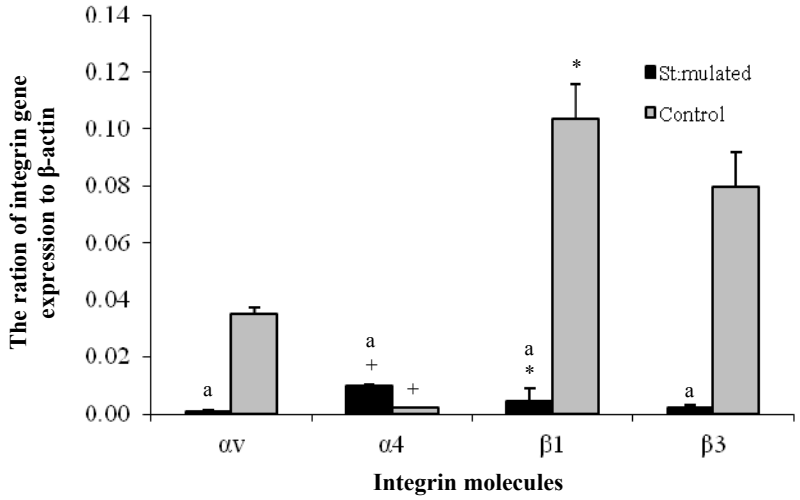

Fig. 1. The ratio of *αv*, *α4*, *β1*, and *β3* integrins to *β*-actin gene expression in blastocysts derived from the control and ovarian stimulated mice. Values are means ± SE. (a) indicates differences with control group ($P \leq 0.05$), * and + show the maximum and the minimum levels of expression, respectively.

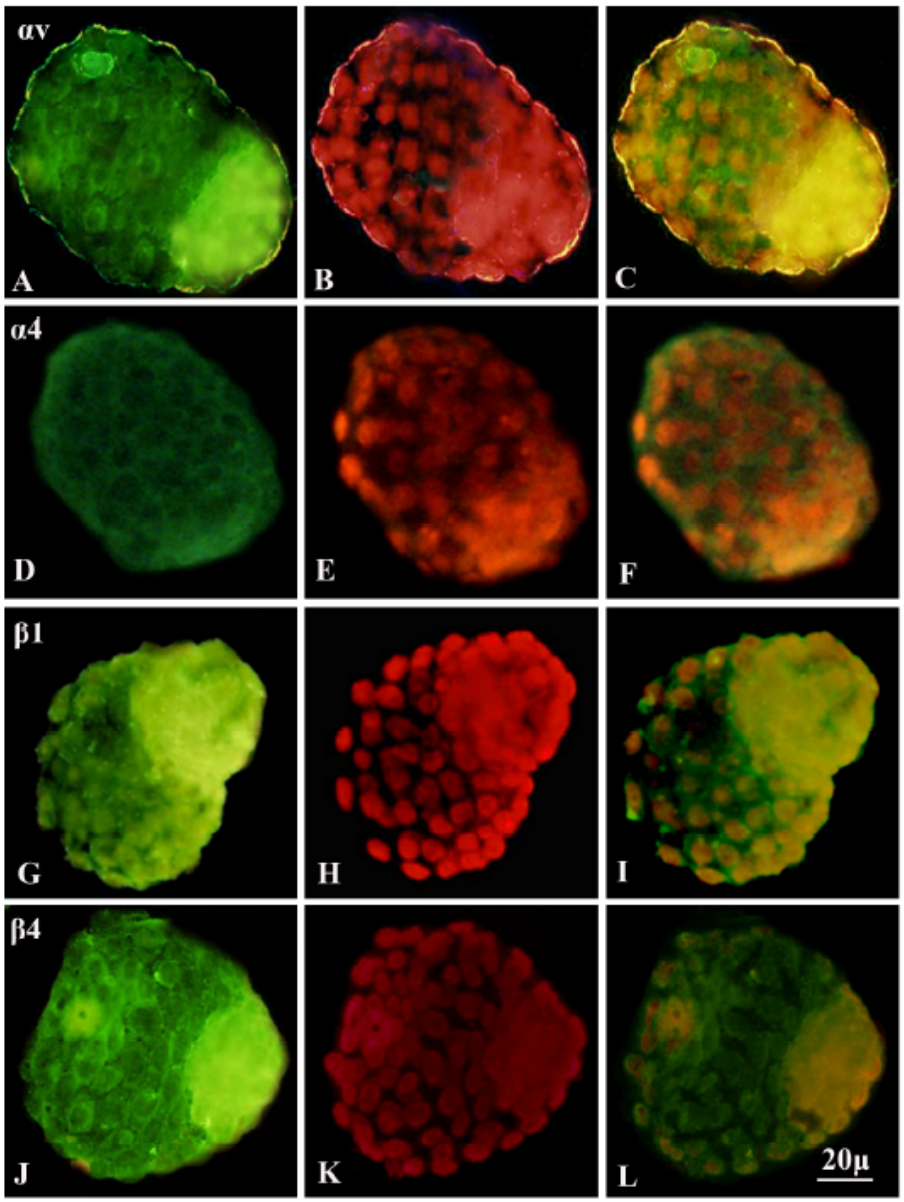

Fig. 2. Immunohistochemical staining of different integrin molecules of blastocysts derived from non-stimulated control pregnant mice. The first column from left shows the positive reaction without nuclear counter-staining, the second column shows the blastocyst with nuclear staining using propidium iodide, and the merge of the same Figures is shown in the third column. **A-C** relates to *αv*, **D-F** to *α4*; **G-I** to *β1*, and **J-L** to *β3* integrin molecules, respectively.

Furthermore, the expression of the integrin molecules was controlled by ovarian hormones, thus their imbalance in stimulated subjects affected to their integrin molecule expression.

In agreement with our explanation, it has been shown that in human and other experimental studies, the ovarian stimulation causes a reduction in the expression of endometrial integrins [11, 23-25, 30].

Meyer *et al.* [30] showed a reduction in the expression of the αvβ3 integrin in endometrial tissue of women who underwent controlled ovarian stimulation.

Chen *et al.* [24] demonstrated that the endometrial integrin β3and leukemia inhibitory factor expressions in the peri-implantation phase were significantly lower in stimulated cycles compared to the natural cycle controls.

Ruan *et al.* [23] reported that the superovulation of mice with different protocols caused a significant decrease not only on the expression of endometrial integrin *β3* subunit during the implantation window but also in their implantation rates.

Because of the integrin expression on the external surface of expanded blastocyst has a critical role to initiate the adhesion of embryo to endometrium [31], thus its down expression and deleterious effect on their implantation.

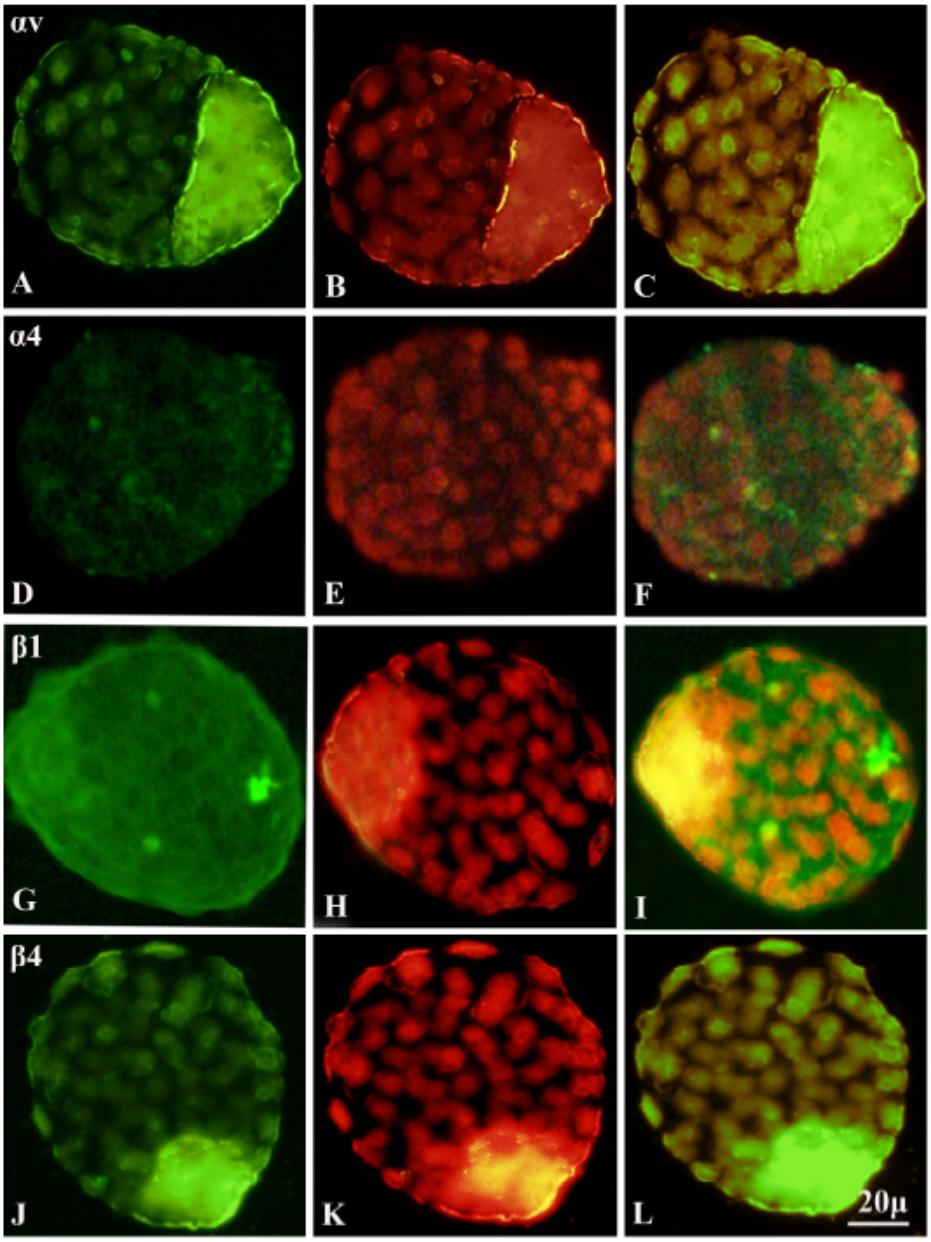

Fig. 3. Immunohistochemical staining of different integrin molecules of blastocysts derived from ovarian stimulated pregnant mice. The first column from left shows the positive reaction without nuclear counter-staining, the second column shows the blastocyst with nuclear staining using propidium iodide, and the merge of the same Figures is shown in the third column. **A-C** relates to *αv*, **D-F** to *α4*, **G-I** to *β1*, and **J-L** to *β3* integrin molecules, respectively.

In agreement with this suggestion in other parts of our study, we showed that the embryo implantation rate in stimulated mice (with the same method) was significantly reduced in comparison with non-stimulated group.

Furthermore, the low implantation rate in stimulated subjects was demonstrated in human and mouse models with different stimulation protocols [16, 32]. In another point of view, the ovulation induction may affect the extracellular matrix ligands on the embryonic surface, and it could be considered for future investigations.

Among different integrin molecules evaluated in this study, the *β1* molecules had higher level and α4 molecules had lower level of expression in both groups of study. It seems that the *β1* subunit is more critical for embryo implantation. In agreement with our suggestion, some investigations showed that the expression of *β1* subunits was prominent at the time of implantation at the embryonic surfaces [33-35].

In conclusion, the ovulation induction by PMSG and HCG causes a decrease in expression of *αv*, *β1*, and *β3* integrins of the blastocyst, and it has deleterious effect on the blastocyst implantation.

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