Original Article

Evaluation of Diabetes Effects on the Expression of Leukemia Inhibitory Factor and Vascular Endothelial Growth Factor A Genes and Proteins at the Time of Endometrial Receptivity after Superovulation in Rat Model

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Abstract

Background: Diabetes, a major metabolic disorder, seems to affect the fertility rates of women in various ways. Due to the uncertainty of the effects of diabetes along with superovulation treatment on the infertility, we investigate the effects of ovulation induction treatment as therapeutic approach on the expression of leukemia inhibitory factor (LIF) and vascular endothelial growth factor A (VEGFA) as two main factors which are involved in the implantation in the streptozotocin (STZ)-induced type 1 diabetic rats. **Materials and Methods:** Type 1 diabetes was induced by injections of STZ in Wistar rats. The animals were kept in diabetic conditions for 4 weeks, while some were treated with insulin for treatment. After treatment, the ovulation was induced by human menopausal gonadotropin (hMG) and human chorionic gonadotropin (hCG). The rats were then sacrificed and the expression of LIF and VEGFA was checked by immunohistochemistry staining method, and the relative expression of LIF and VEGFA was measured by quantitative reverse transcription polymerase chain reaction (RT-PCR) and Western blotting methods. **Results:** It was observed that diabetes and insulin treatment for diabetes altered the expression of Lif and VEGFA in both mRNA and protein levels. However, superovulation treatment seems to ameliorate this alternation for both factors. **Conclusion:** According to our results, diabetes and insulin therapy could alter the expression of Lif and VEGFA genes and proteins that are effective in endometrial receptivity and implantation process. It seems in diabetic cases, the effect of hCG and hMG therapy by itself could regulate the level of expression and presence of these two genes and proteins.

Keywords: *Embryo implantation, insulin, leukemia inhibitory factor, superovulation, vascular endothelial growth factor A*

Introduction

Diabetes mellitus (DM) is a class of metabolic disorders, which is mainly characterized by high blood glucose level.[1] Recently, diabetes could be assumed a vascular disease, and it is one of the strongest risk factors for any form of vascular disease.[2] The prevalence of DM is increasing as there were about 451 million people with diabetes in 2017, and it is estimated that 693 million will be affected by DM in 2045.[3] Diabetes is mainly subclassified into two types: type 1 DM (T1DM) and type 2 DM. T1DM or juvenile onset of diabetes is caused by insulin production deficiency, which mainly occurs due to some autoimmune responses against pancreatic β-cells.[1,4] According to the International Diabetes Federation, 10% of diabetic patients suffer from T1DM. T1DM patients' life is dependent on daily dosage of insulin injection.[5] It has been declared that T1DM increases the risk of fertility disorders in women during their fertile ages.[6,7] Codner *et al*. indicated that high blood glucose level and insulin deficiency, two main characteristics of T1DM, can increase the risk of hypogonadism, puberty delay, and menstrual irregularities such as oligomenorrhea.[8] In addition, several molecules that are involved in the pregnancy process including cadherins and mucin 1; various cytokines including interleukin-6 (IL6), IL10, and leukemia inhibitory factor (Lif); and growth factors such as insulin-like growth factor 1, vascular cell adhesion molecule 1, and vascular endothelial growth factor A (VEGFA) can be affected by diabetic

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conditions that in the current study among them Lif and VEGFA were, because VEGF is an essential factor for uterine permeability and angiogenesis during implantation, and Lif is involved in the implantation process in both the uterine preparation and the attachment reaction.[9-13]

Lif and VEGFA are two of the main factors which are involved in the implantation process and pregnancy achievement.[14,15] Lif, as a class of IL 6 cytokine, is produced by the endometrial gland. Its activity is essential for endometrial epithelium receptivity and stromal decasualization.[16] Studies have mentioned that Lif expression alters during diabetic conditions,[17] and superovulation also may decrease Lif expression.[18] On the other hand, VEGF, which is expressed by endometrial cells, is a crucial growth factor for the endometrial angiogenesis.[15] Insulin therapy increases the expression of VEGF in both mRNA and protein levels,[19] and high level of VEGF might result in abnormal angiogenesis which may lead to pregnancy loss.[20] Moreover, studies have declared that VEGF expression is increased as a result of some of the ovulation induction treatments.[21]

There are three main treatment approaches for infertile women including medical treatments, surgical treatments, and assisted reproductive technology (ART).[22] Among the subfertility therapeutic approaches, ovulation stimulation or superovulation, which is classified as an approach of ART, is the most common.[23]

There is no enough evidence to clarify if T1DM directly affects the embryo implantation process through the alternation of the expression of related genes and proteins. Therefore, the aim of this study was an evaluation of the effects of T1DM condition and its main therapeutic approach (insulin therapy) on the alternation of the Lif and VEGFA expression in subfertility problems and the effect of superovulation on them at the time of embryo implantation.

Materials and Methods

Animal model and sample collections

Forty-two Wistar rats (Pasteur Institute, Iran) which were 8–10 weeks' old were used for the experiment. The animals were housed in standard conditions: 20°C–22°C in a temperature-controlled room and 40%–70% humidity, and they were exposed to 12/12-h light/dark cycle with free access to standard water and food. They were kept in the central animal house laboratory of Isfahan University of Medical Sciences. Experimental procedures were approved by the Ethics Committee of Isfahan University of Medical Sciences (IR.MUI.REC.1396.3.366).

The rats were randomly placed in six groups (n = 7 for each group). The groups include no treated control groups, streptozotocin (STZ)-induced type 1 diabetic rats, STZ-induced type 1 diabetic rats treated by insulin, healthy rats superovulated by human menopausal gonadotropin (hMG) and human chorionic gonadotropin (hCG), STZ-induced type 1 diabetic rats superovulated by hMG and hCG, and STZ-induced type 1 diabetic rats treated by insulin and then superovulated by hMG and hCG.

Type 1 diabetes was induced by 60 mg/Kg of STZ (Sigma, Germany) in two intraperitoneal injections within 15 min. In order to verify diabetic condition, fasting blood sugar (FBS) was measured by glucometer (HemoCue Glucose 201+, Sweden), and the rats with FBS higher than 250 mg/dl were chosen for further investigations. In order to stabilize the diabetic condition and to investigate the effects of diabetes itself, these rats were kept diabetic for the following 4 weeks (one sexual cycle).

Insulin treatment for diabetic was as follows: three international units (IUs) of regular insulin (Sobhan Co., Iran) intraperitoneal injection per day for 4 weeks.

Superovulation was induced by 7.5 IU hMG hormone (N. V. Organon, Netherland) intraperitoneal injection, followed by 7.5 IU hCG hormone (N. V. Organon, Netherland) after 48 h.

Every two female rats were then placed in a cage with a male rat for mating, and vaginal plug was checked the following morning. Animals were sacrificed 96 h after hMG injection, and their uterus was rinsed with culture medium for further investigation. The right horn of the uterus was plunged in formalin for histological analyzes and the left horn was snap frozen for molecular experiments.

Immunohistochemical staining

In order to fix endometrial samples, the median part of the uterus of each sample was embedded in 10% buffered formalin (Sigma, USA) (24 h), and the samples were exposed to tissue processing with ascending grade of alcohol and transparency in Xylene (Sigma, USA). Then, the samples were embedded in paraffin, and 4-μm tissue sections from every 500 μm along the uterus were prepared by a microtome. The slices were then removed and placed on a poly-L-lysine-covered slide. They were finally stained by an anti-Lif (Cat. no. STJ29846, St John's Laboratory, United Kingdom) and an anti-VEGFA (Cat no. ABS82, Merck, US) antibody, followed by treatment with horseradish peroxidase (HRP)-conjugated secondary antibody (Cat. No. sc-2357, Santa Cruz, Germany) according to manufacturers' protocol.

Total RNA isolation and cDNA synthesis

Total RNA was extracted from endometrial tissues by RNX-plus (Sinaclon, AryoGen Biopharma Complex, Iran) according to the manufacturer's protocol. The integrity of the extracted RNA was verified by 1% agarose gel electrophoresis. The concentration of total RNA was measured by NanoDrop instrument (Nanolytik, Germany)

for each sample at 260 nm OD (Optical density), and they were stored at −80°C for further analysis. Genomic DNA was eliminated by DNase I treatment using DNase I set (Fermentas, Lithuania). One microgram of total RNA was used for cDNA synthesis by PrimeScript™ RT reagent Kit (Takara, Kusatsu, Japan).

Quantitative real-time polymerase chain reaction

Lif and *VEGFA* genes' relative expression level was measured by quantitative real-time reverse transcription polymerase chain reaction (RT-PCR). Beta-actin was used as the reference gene. All primers were designed by Gene Runner software (version 4.0; Hastings Software. Inc., Hastings, USA). The specificity of the primers was verified by BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The sequences of the designed primers are as follow:

- Beta-actin: F (5×-GCCTTCCTTCCTGGGTATG-3×(and R (5×-AGGAGCCAGGGCAGTAATC-3×) with an amplicon size of 178 bp and annealing temperature 60°C
- *Lif*: F (5×-GTCTTGGCCACAGGGATTG-3×) and R (5×-CGTTGAGTTGAGCCAGTTGAC-3×) with an amplicon size of 163 bp and annealing temperature 61.4°C
- VEGFA: F (5×-ACCTCACCAAAGCCAGCAC-3×) and R (5×-CTTGCAACGCGAGTCTGTG-3×) with an amplicon size of 190 bp and annealing temperature 54.4°C.

Applied Biosystems StepOnePlus™ instrument was used for real-time PCR using RealQ Plus 2x Master Mix Green (high ROX) (Ampliqon, Denmark) as described elsewhere.[24] Real-time PCR was performed by standard cycling protocol. The amplification condition was as follows: 10 min at 95°C for denaturation, 15 s at 95°C for denaturation, 60 s for annealing at the specific temperature for each gene, followed by 15 s at 72°C for extension, and the cycles were repeated forty times. In order to minimize experimental error, all of the samples were run duplicated. Gene expression assessment was performed based on the $2^{-\Delta\Delta CT}$ method.[25]

Protein extraction and Western blot analysis

An equal amount of 0.1 g of uterus tissue was homogenized in 1 ml of radio-immune precipitation assay buffer, cold buffer (Cyto Matin Gene, Iran), as mentioned in manufacturer's protocol. Thirty-minute incubation on ice was followed by centrifugation at 27000 g for 20 min. According to manufacturer's instruction, total proteins are presented in the supernatant.

The concentration of protein was quantified by Bradford assay. Ten percent sodium dodecyl sulfate polyacrylamide gel electrophoresis was performed. The samples were then transferred to nitrocellulose membranes (Bio-Rad, US). Skimmed milk solution (5%) was used as the membrane blocking agent. The membranes were blotted by rabbit anti-Lif (Cat. no. STJ29846, St John's Laboratory, United Kingdom) and rabbit anti-VEGFA (Cat no. ABS82, Merck, US) primary antibodies (dilution of 1:250) and incubated overnight at 4°C. PBS (phosphate buffered saline) with 0.05% Tween 20 solution was used as the washing solution. The membranes were washed three times for 15 min. They were then incubated with mouse anti-rabbit immunoglobulin – HRP secondary antibody (Cat. No. sc-2357, Santa Cruz, Alemania) at a dilution of 1:1,000 at room temperature for 90 min. Clarity Western ECL Substrate Detection Reagent (Cat. no. Bio-Rad, 170-5060, US) was used to visualize the blots under SABZ biomedical chemiluminescent system (SABZ Biomedicals, Iran). The bands densitometry was normalized to β-actin (Cat. no. ab8226; Abcam, US) by ImageJ software, version 1.8 (http://rsb.info.nih.gov/ij/).

Statistical analysis

SPSS (Statistical Package for the Social Sciences) software, version 20.0 (SPSS Inc., USA) was used for all statistical analyses. In order to test the normality of the data, Kolmogorov–Smirnov test was performed before further statistical tests. Real-time PCR and Western blot were repeated two or three times, and the final results were expressed as means ± standard error of the mean. Analysis of variance was used to detect statistical significance. $P < 0.05$ was considered as statistically significant.

Results

Immunohistochemistry staining for leukemia inhibitory factor and vascular endothelial growth factor A

The immunohistochemistry staining was performed, and the expression of Lif and VEGFA proteins in the luminal epithelium, glandular epithelium, and stromal cells was detected in all of the groups. Brown color indicates the expression of these proteins in cells (DAB+) [Figure 1].

Relative expression of leukemia inhibitory factor in the mRNA and protein levels

We observed a decrease in the expression of Lif mRNA for the diabetic rats compared to the control groups, although it was not significant. However, the decrease of the Lif protein was statistically significant in the diabetic groups in comparison with the normal control rats. The relative expression of Lif mRNA for insulin-treated diabetic rats was significantly higher than that in control groups, but this increase was not significant at the protein level. Moreover, a slight insignificant decrease was observed in the superovulated diabetic rats and the control groups in the transcript level of Lif, although this decrease was statistically significant at the protein level. No significant alteration was observed between other treatments and untreated control rats, not in the transcript nor at the protein level [Figures 2 and 3].

On the other hand, insulin treatment leads to a statistically significant increase in the expression of Lif both at the mRNA and protein levels in comparison with diabetic rats.

The superovulated healthy groups showed no significant difference compared to the diabetic groups in the transcript level; however, at the protein level, a significant upregulation was observed in the superovulated groups in comparison with the diabetic rats. The superovulated diabetic rats showed no significant difference compared to the diabetic rats both at the transcript and protein levels. In addition, insulin treatment along with superovulation leads to an increased expression of Lif at both transcript and protein levels compared to the diabetic rats; however, statistical significance was only observed at the protein level. Moreover, diabetes seems to reduce the expression of Lif so that the relative amount of Lif was lower in the superovulated diabetic rats compared to the superovulated rats, although statistical significance was just observed at the protein level. Finally, insulin treatment seems to increase the amount of Lif expression as insulin-treated superovulated diabetic rats had a significantly higher amount of Lif mRNA and protein compared to the diabetic superovulated rats [Figures 2 and 3].

Relative expression of VEGFA in the mRNA and protein levels

The relative expression of VEGFA was increased in all of the groups in comparison with the untreated control group in the transcript level; however, the statistical significance was only observed in the insulin-treated diabetic and superovulated insulin-treated diabetic rats. The same trend of increase was observed at the protein level, although the insulin-treated diabetic group was the only significantly increased group compared to the untreated control rats [Figures 4 and 5].

The insulin treatment significantly increased the amount of VEGFA level both in the transcript and protein levels compared to the diabetic rats. In addition, the relative mRNA level of VEGFA was significantly higher in the superovulated insulin-treated rats in comparison with the diabetic rats, but this trend was not observed at the protein level. Moreover, a significant reduction was occurred in both transcript and protein levels of VEGFA for insulin-treated superovulated diabetic rats in comparison with insulin-treated diabetic rats. And finally, it seems that insulin treatment significantly upregulated the relative amount of VEGFA mRNA in the insulin-treated superovulated diabetic rats at comparison with superovulated diabetic rats, although this upregulation was not statistically significant at the protein level [Figures 4 and 5].

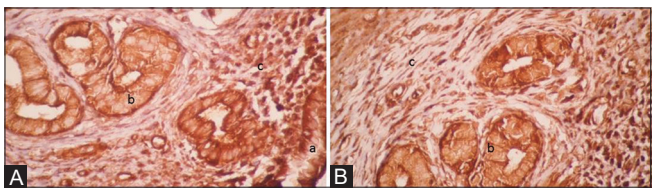

Figure 1: The immunohistochemistry staining of the endometrium with antileukemia inhibitory factor (A) and antivascular endothelial growth factor A (B) antibody by light microscopy. Expression of leukemia inhibitory factor and vascular endothelial growth factor A proteins in the luminal epithelium (a), glandular epithelium (b), and stromal cells (c) was detected in all of the groups. The leukemia inhibitory factor + and vascular endothelial growth factor A + cells are shown in a brown color (×40)

Discussion

Type 1 diabetes is a severe metabolic disorder caused by insulin production insufficiency, which can affect the quality of life in various aspects. Studies have shown that T1DM can affect the rate of female fertility.[8] On the other hand, it has been mentioned that some ovulation induction treatments may attribute to the risk of T1DM in the offspring of diabetic patients.[26] Moreover, as the main therapeutic approach for T1DM is continual daily insulin injection, it might be necessary to consider the effects of superovulation, the main therapeutic approach for infertility problems, for T1DM patients. Therefore, the focus of this study was to investigate the effects of superovulation as a treatment for insulin-dependent diabetes-related infertility problem on Lif and VEGFA expression in the rat endometrium at the time of endometrial receptivity.

Our data indicated that diabetic condition leads to a downregulation of Lif expression, especially at the protein level. On the other hand, we observed that insulin treatment could significantly increase the amount of Lif level both at transcript and protein levels in comparison with diabetes groups. Similarly, Albaghdadi and Kan indicated that Lif expression is decreased due to the diabetes in the endometrium.[17] Moreover, Wang *et al.* declared that Lif-Stat signaling pathway deficiency due to diabetic condition is responsible for implantation failure.[27] On the another side, based on our findings, it seems that superovulation may decrease the level of Lif expression, although it was not statistically significant; however, the reduction of Lif protein level was significant in the superovulated diabetic groups in comparison with the untreated control rats which might occur due

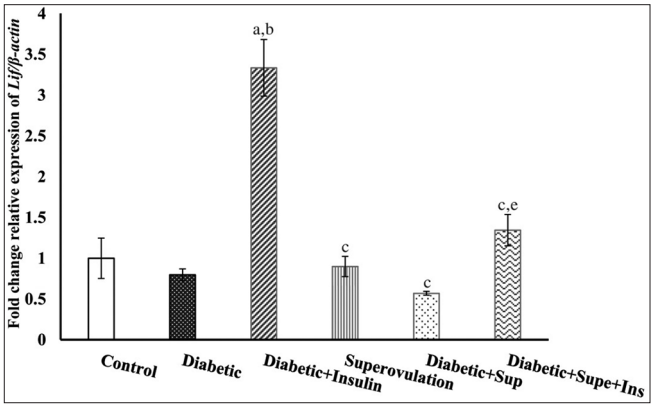

Figure 2: Comparison of leukemia inhibitory factor gene expression in rat endometrium at the time of embryo implantation. a, b, c, and e indicate a statistically significant difference between the groups. a: *P* <0.0001 versus control, b: *P* <0.0001 versus diabetes, c: *P* <0.0001 versus diabetes + insulin (Ins), and e: *P* <0.05 versus diabetes + superovulation

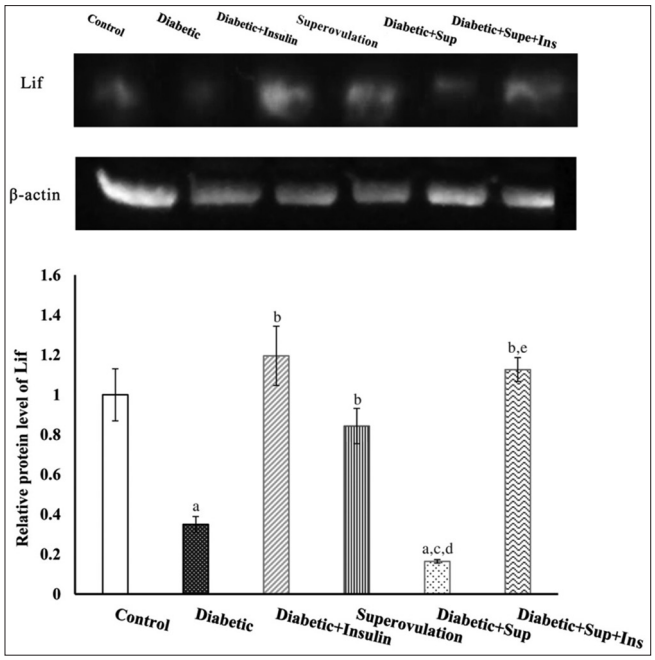

Figure 3: Comparison of leukemia inhibitory factor protein level in rat endometrium at the time of embryo implantation. The Western blotting results and quantitative relative protein expression of leukemia inhibitory factor normalized to β-actin and compared with the control group. a, b, c, d, and e indicate a statistically significant difference between the groups. a: *P* < 0.001 versus control, b: *P* < 0.001 versus diabetes, c: *P* < 0.001 versus diabetes + insulin (Ins), d: *P* < 0.001 versus superovulation (Sup), and e: *P* < 0.001 versus diabetes + superovulation

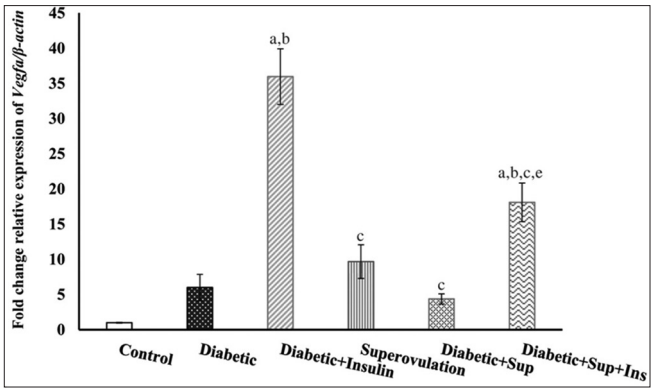

Figure 4: Comparison of vascular endothelial growth factor A gene expression in rat endometrium at the time of embryo implantation. a, b, c, and e indicate a statistically significant difference between the groups. a: *P* < 0.0002 versus control, b: *P* < 0.002 versus diabetes, c: *P* < 0.0001 versus diabetes + insulin (Ins), and e: *P* < 0.0005 versus diabetes + superovulation

to the accumulation of the effects of both diabetes and superovulation. Likewise, studies have shown that Lif expression might be downregulated in response to superovulation treatments.[18] In contrast, some studies have mentioned that Lif expression can be upregulated after superovulation therapeutic approach.[28] Therefore, it seems that there is a controversial response to different superovulation drugs, and further investigations are needed to find the exact effect of each of these drugs on the expression of Lif. As a conclusion, superovulation approach after insulin treatment seems to have the best results in our study for diabetic condition, and it seems that it can restore the downregulation of Lif that is observed both in diabetic conditions and in superovulation for nondiabetic rats.

Moreover, our data showed that diabetes, especially insulin treatment for diabetic rats, significantly increased the levels of VEGFA. Poulaki *et al.* observed that the levels of VEGFA were significantly upregulated due to insulin therapy.[19] In addition, Shi *et al.* showed that the serum level of VEGF was increased in the STZ-induced type 1 diabetic rats.[29] On the other hand, we observed that the level of VEGFA was upregulated due to the superovulation treatment, although this was not statistically significant. Similarly, studies have indicated that VEGF level might be upregulated by superovulation treatments.[21] In contrast, some other studies have elucidated that VEGFA is downregulated by superovulation drugs.[30] Moreover, Xu *et al.* declared that estrogen treatment can downregulate the increase of VEGFA as the result of metabolic syndrome.[31] Our data showed a downregulation at the protein level of VEGFA in the superovulated diabetic rats in comparison with untreated diabetic groups. This controversy could be due to the type of ovulation induction drug, and it seems that the drugs which are suggested for this reason should be chosen wisely and physicians should consider the other symptoms of each patient. Therefore, we may conclude that although insulin treatment is not avoidable in type 1 diabetic patients, superovulation treatment might be able to ameliorate the upregulation of VEGFA, which is mainly occurred because of insulin treatment.

Conclusion

According to our results, diabetes and insulin therapy which is unavoidable in these patients could alter the expression of Lif and VEGFA genes and proteins that are effective in endometrial receptivity and implantation process. Although further investigations are necessary to completely clarify the effects of superovulation for infertility treatments in case of type 1 diabetes, hCG and hMG might be two more suitable options for follow-ups in human researches in the case of superovulation as the treatment for type 1 diabetic patients who are suffering from fertility problems.

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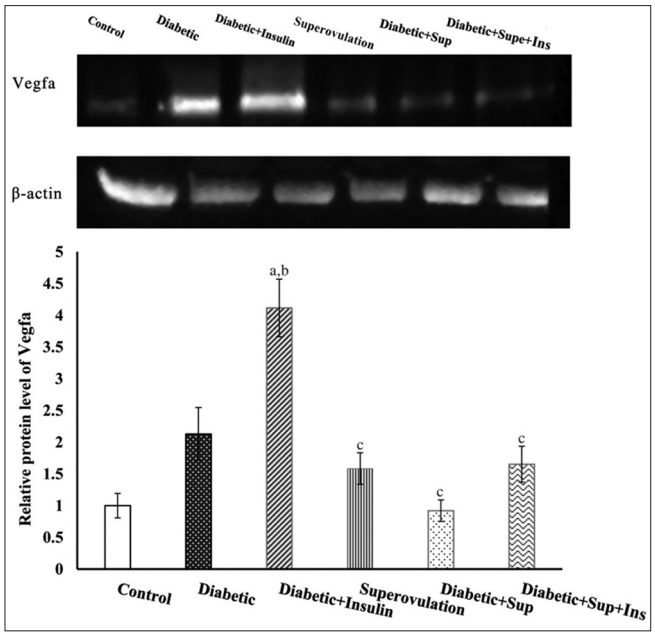

Figure 5: Comparison of vascular endothelial growth factor A protein level in rat endometrium at the time of embryo implantation. The Western blotting results and quantitative relative protein expression of leukemia inhibitory factor normalized to β-actin and compared with the control group. a, b, and c indicate a statistically significant difference between the groups. a: *P* < 0.001 versus control, b: *P* < 0.001 versus diabetes, and c: *P* < 0.001 versus diabetes + insulin (Ins)

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Conflicts of interest

There are no conflicts of interest.

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