

# Comparison of Expression of Chemokine Receptor 4 in Maternal Decidua and Chorionic Villi in Women with Spontaneous Miscarriages and Women Opting for Termination of Viable Pregnancies

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## ABSTRACT

**Background:** Early pregnancy losses can be a distressing experience both for the parents and the treating clinician. We aim to explore the role of chemokine receptor 4 (CXCR4) in early pregnancy losses by comparing its expression among patients with spontaneous miscarriages and patients undergoing termination of viable pregnancies for unwanted pregnancies. **Aim:** The aim of the study was to investigate the expression of CXCR4 in early pregnancy losses and correlate the various clinical parameters with differential expression of the above receptor in the chorionic villi and maternal decidua. **Study and Setting:** The present study is a case-control study done in a tertiary care center. **Methodology:** Fifty patients attending outdoor and antenatal clinic of the hospital aged 18–40 years with spontaneous miscarriage under 20 weeks of gestational age were included as cases and compared with fifty females of comparable age group (18–40 years) seeking medical termination of pregnancy as controls. Chorionic villi and decidua obtained from the cases and controls were analyzed for CXCR4 expression. **Statistical Analysis:** The results were analyzed using mean  $\pm$  standard deviation, percentiles values, Chi-square test, and *P* value to determine the association of CXCR4 expression in decidua and chorionic villi of cases versus controls. **Results:** CXCR4 expression was significantly downregulated in cases as compared to the controls with *P* < 0.001. The mean normalized ratio of CXCR4 expression to housekeeping gene ( $\beta$  Actin) expression in the case group was  $1.607 \pm 1.108$  and in the control group, it was  $2.506 \pm 1.457$ . There was a strong correlation between the expression of CXCR4 and maternal age. With increasing age, the expression of CXCR4 was more downregulated in both the cases and control groups (*P* < 0.001). The expression of CXCR4 was elevated in controls as compared to cases in <30 years age group (*P* = 0.009). CXCR4 expression was higher in primigravida than in multigravida (*P* = 0.001), and as the number of previous miscarriages increased, the expression of CXCR4 was found to be decreased (*P* = 0.021). **Conclusion:** CXCR4 expression is significantly reduced in women with spontaneous miscarriages in comparison with viable pregnancies. and possibly, therapies targeted at increasing the expression of CXCR4 can be used as a treatment modality for management of spontaneous miscarriages.

**KEYWORDS:** Chemokines, chemokine receptor 4, miscarriage, trophoblast

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## INTRODUCTION

Spontaneous miscarriage is a common and distressing complication of early pregnancy. Early abortions are defined as those that occur before the 12<sup>th</sup> week of gestation, with late abortions being those that occur from 12 to 20 weeks of pregnancy, and 500 g or less. The etiology of miscarriage is multifactorial having been attributed to hematologic, anatomical, hormonal, immunological, and genetic pathologies, however, remains unknown in most even after extensive evaluation.<sup>[1,2]</sup> The vast majority of spontaneous miscarriage results from chromosomal abnormalities, particularly trisomies. Patients with idiopathic recurrent miscarriage and repeated euploid pregnancy losses pose a difficult therapeutic challenge.

The chorionic villi are the functional unit of the placenta. They provide oxygen and nourishment to the fetus and also serve as an excretory unit. Communication between fetal trophoblast cells and maternal immune cells dictates placental development and vasculogenesis during early pregnancy.<sup>[3]</sup>

Chemokines are a family of small (8–10 kD) proteins that act primarily as chemoattractants for special types of leukocytes and with their receptors (seven-transmembrane G protein-coupled receptors) play a pivotal role in implantation and vascularization of the placenta. About 40 different chemokines and 20 different receptors for chemokines have been identified. Chemokine receptor 4 (CXCR4) is an extraordinary chemokine receptor, in which it has only one recognized ligand, CXCL12. It plays a key role in developmental processes including organogenesis, angiogenesis, and embryogenesis.<sup>[4]</sup> It is specifically upregulated in the human endometrium during the implantation window and increased CXCR4 immunostaining has been observed in the cultured endometrial epithelium only when a blastocyst is present.<sup>[5,6]</sup>

CXCR4 is responsible for directing CD56brightCD16-NK cells into the decidua, which, in turn, modulates the immune milieu at the maternal–fetal interface for a smooth pregnancy. Despite the established role of CXCR4 signaling in the regulation of angiogenesis and trophoblast survival, the expression of CXCR4 in extravillous trophoblast has received little attention.<sup>[7]</sup> The placental bed, as we all know, plays an important role in successful placentation during gestation,<sup>[8,9]</sup> and hence, a better understanding of the placental vasculature can help to elucidate the mechanism of placental insufficiency.<sup>[10]</sup> Various studies have already demonstrated the important dynamic role

that CXCR4 plays at the maternal–fetal interface during early pregnancy. The importance of CXCR4 expression in uterine cells in preventing embryo loss has already been demonstrated in mice studies.<sup>[11,12]</sup> Therefore, it was interesting to speculate that the alteration of CXCR4 expression in the placental bed might be associated with pregnancy disorders like early human maternal spontaneous miscarriages. To verify this hypothesis, we aimed to investigate the expression of CXCR4 in early pregnancy losses and correlate the various clinical parameters with differential expression of the above receptor in the chorionic villi and maternal decidua.

## METHODOLOGY

Before initiating the study, approval of the University's Ethical Committee was obtained. Informed consent was obtained from every patient. Patients were allocated as per inclusion and exclusion criteria. The necessary investigations done in the study were done in the institute and funded by the government.

### Inclusion criteria

Fifty patients attending outdoor and antenatal clinic of the hospital aged 18–40 years with spontaneous miscarriage under 20 weeks of gestational age were included as cases. After a detailed history, examination, and urinary pregnancy test, ultrasonography was done to confirm intrauterine pregnancy. Fifty females of comparable age group (18–40 years) from the same geographical locality who had a history of amenorrhea and positive urinary pregnancy test and sonographic evidence of intrauterine pregnancy but wanted medical termination of pregnancy under family planning program were grouped as controls.

### Exclusion criteria

Patients with diabetes, hypertension, thyroid disorder, TORCH infection, APLA syndrome, and chromosomal abnormalities for whom requisite tests of blood sugar, TORCH, thyroid function test, abnormal fetal karyotype, lupus anticoagulant, anticardiolipin, and beta-2 glycoprotein were done were excluded from the study.

Chorionic villi and decidual samples were collected by dilatation and curettage and expression analysis of CXCR4 was done in all patients.

### Processing of tissue samples

Chorionic villi and decidua samples were collected in Roswell Park Memorial Institute media, washed with PBS-1X to remove maternal blood contamination, and stored at –20°C. The tissue samples were then proceeded for RNA isolation which was done by the method developed by Chomczynski and Sacchi in 1987.<sup>[13]</sup> This was followed by DNase treatment and

cDNA preparation. The cDNA synthesized from RNA of the chorionic villi and decidua samples extracted from both group of patients were then subjected to quantitative polymerase chain reaction (PCR) or real-time PCR amplifications with primers of  $\beta$ -Actin (housekeeping gene as an internal control) and CXCR4.

### Statistical analysis

Since there were no studies available in relation to the primary outcome that has been assessed in this study, it was planned as a pilot-scale study with a sample size of 50 cases and 50 controls which was adequate for any comparative control of the pilot study. The results were analyzed using mean  $\pm$  standard deviation, percentiles values, Chi-square test, and *P* value to determine the association of CXCR4 expression in decidua and chorionic villi of cases versus controls.

## RESULTS

The study population was stratified on the basis of maternal age, number of previous abortions, gestational age at which loss occurred, gravid status, parity, anemia, and mental stress factor. Mental stress was measured by Hamilton's Depression Rating Scale (0–7 being normal) and Hamilton's Anxiety Rating Scale (0–13 being normal). The mean age of abortion among cases and controls was  $27.48 \pm 4.147$  years and  $25.94 \pm 4.012$  years, respectively, which was statistically insignificant (*P* = 0.062). The cases and controls in our study were mostly housewives with no statistical difference in maternal occupation in the groups (*P* = 0.99). The socioeconomic status distribution among cases and controls in the study group was also insignificant (*P* = 0.339). As per Table 1, mental stress was not seen to have any significant causal effect on early miscarriages (*P* = 0.99). Anemia was present only in 12% of cases (*P* = 0.118). Most patients with spontaneous miscarriages were multigravidae. When cases were considered, 9 out of 50 cases had no previous abortion or 1 previous abortion and 41 cases had more than 1 abortion, whereas in the controls, none of them had any previous abortion as control consisted of patients who wanted their abortion willingly on completion of their family. This difference of distribution in the cases and controls was statistically significant (*P* < 0.001). Maximum abortions in both cases and controls were in the first trimester (*P* = 0.002). The mean normalized ratio of CXCR4 expression to the house keeping gene ( $\beta$  Actin) expression in the case group was  $1.607 \pm 1.108$ , while in the control group, it was  $2.506 \pm 1.457$ . Thus, CXCR4 expression was significantly downregulated in cases as compared to the controls (*P* < 0.001).

With increasing age, the expression of CXCR4 was significantly more downregulated in both the cases and control groups, as shown in Table 2 (*P* < 0.001) demonstrating a strong correlation between expression of CXCR4 and maternal age. When comparing in women 30 years or less, the expression of CXCR4 was significantly more in controls as compared to cases (*P* = 0.009). In women above 30 years of age, although CXCR4 was expressed more in controls, it was found to be statistically insignificant (*P* = 0.247). There was no co-relation of CXCR4 and socioeconomic status (*P* = 0.30) and anemia status (*P* = 0.875). CXCR4 expression was significantly higher in primigravida than in multigravida (*P* = 0.001).

At the period of gestation <10 weeks, CXCR4 expression was more in controls than cases (*P* < 0.001), while at gestation more than 10 weeks, no statistically significant difference was found between the two groups (*P* = 0.745). CXCR4 expression was found to be inversely related to the number of previous miscarriages, with CXCR4 expression significantly decreasing with increasing number of miscarriages (*P* = 0.021).

**Table 1: Baseline parameters**

Parameters	Cases, n (%)	Controls, n (%)	<i>P</i>
Age group (years)			
≤30	37 (74)	44 (88)	0.072
>30	13 (26)	6 (12)	
Mean maternal age at abortion (years)	27.5±4.1	25.94±4.012	0.062
Occupation			
Housewife	47 (94)	48 (96)	0.99
Working	3 (6)	2 (4)	
Socioeconomic status			
Low	13	12	0.339
Middle	35	38	
High	2	0	
Mental stress			
Present	1	0	0.99
Absent	49	50	
Gravida			
Primi	9 (18)	0	0.003
Multi	41 (82)	50	
Maternal anemia			
Present	44	38	0.118
Absent	6	12	
Number of previous miscarriages			
0-1	9	0	<0.001
>1	41	0	
Gestation at miscarriage (weeks)			
≤10	37	46	0.002
>10	13	4	

**Table 2: Expression of chemokine receptor 4 in relation to baseline parameters**

	Cases (P)	Controls (P)	P
Mean normalized ratio of mRNA expression of CXCR4	1.607±1.108	2.506±1.457	<0.001
Age (years)			
≤30	2.09±0.85	2.80±1.32	0.006
>30	0.289±0.45 (0.001)	0.52267±0.173 (0.001)	0.246
Socioeconomic status			
High	2.090±0.00	NA	
Middle	1.980±0.948	2.514±1.551	0.310
Low	1.463±1.163 (0.3)	2.503±1.448 (0.983)	0.300
Mental stress			
No	1.639±1.110	2.506±1.457	0.001
Yes	0.849±0.00 (0.85)	NA	NA
Gravida status			
Primi	2.788±1.274	NA	
Multi	1.367±0.892 (0.001)	2.5243±1.447	
Gestational age of fetus (weeks)			
10	1.544±1.147	2.543±1.432	<0.001
>10	1.846±0.978 (0.403)	2.075±1.911 (0.543)	0.745
Number of previous abortions			
0	2.788±1.27	2.52±1.447	0.611
1	1.892±0.834	NA	
>2	1.174±0.846 (0.021)	NA	

CXCR4=Chemokine receptor 4, mRNA=Messenger RNA, NA=Not available

## DISCUSSION

Spontaneous miscarriage, or miscarriage, is a frustrating and heart-wrenching experience for both the patient and the physician. Ideally, all pregnancy losses should be evaluated, but the high cost of medical care necessitates the implementation of certain selection criteria.

In our present study, expression of CXCR4 in deciduas and chorionic villi of early miscarriage cases was found to be significantly downregulated than that of the controls. The mean normalized ratio of CXCR4 expression to housekeeping gene ( $\beta$  Actin) expression in the case group was  $1.607 \pm 1.108$  and in the control group, it was  $2.506 \pm 1.457$  which was statistically significant. This downregulation of CXCR4 expression in decidua and chorionic villi could disturb the maternal–fetal interface immune milieu due to altered recruitment of uterine natural killer cells, reduced angiogenesis, decreased trophoblast invasion, and augmented trophoblasts apoptosis, ultimately resulting in defective implantation as well as placentation causing fetus rejection in early miscarriage patients.

Immunologic tolerance necessary at the maternal–fetal interface for pregnancy maintenance occurs by recruitment of CD56 bright CD16-NK cells in decidua, caused by upregulation of the CXCR4/CXCL12 axis. The utilization of CXCR4 by uNK cells is necessary for trophoblast invasion into spiral arteries. CXCR4-CXCL12 promotes crosstalk between

trophoblasts and DSCs in the first trimester, maintaining a fine balance between trophoblast invasiveness and overinvasiveness,<sup>[14]</sup> hence establishing a unique maternal–fetal immune milieu that contributes to embryo survival and development in the uterus until parturition. CXCR4/CXCL12 interactions play roles in almost all facets of maternal–fetal interaction.<sup>[15]</sup>

CXCR4 is known to be an extraordinary chemokine receptor which plays an important role in hematopoiesis and developmental processes of organogenesis, vascularization, and embryogenesis.<sup>[4]</sup> Its upregulation during implantation in natural as well as hormone replacement therapy cycles in the endometrium, produces, in the presence of a blastocyst, a surface polarization of the CXCR4 receptors suggesting the vital role it plays in healthy communication between trophoblast and endometrium and thus in fetal survival.<sup>[16,17]</sup>

CXCR4 suppresses apoptosis and increases the viability of trophoblasts through MAPK pathway. Hence, lack of CXCR4 results in augmented trophoblastic apoptosis and significant uteroplacental pathology.<sup>[7]</sup> Kumar *et al.*, have shown that greater expression of CXCR4 was present in early compared to term placenta, implying its importance in early placental development.<sup>[18]</sup> Zhou *et al.* concluded that CXCR4/CXCL12 not only enhances trophoblast invasiveness by amplifying MMP-9 and MMP-2 secretion but also limits overinvasiveness by upregulating CD82.<sup>[19]</sup> CXCR4 activation increases the

CXCL12-CXCR4 signaling axis stimulates vascular endothelial growth factor (VEGF) synthesis and this in turn induces CXCR4 and CXCL12 production. This synergistic regulation influences placental vascularization.<sup>[20]</sup>

Another study by Zhou *et al.* demonstrated that embryonic stem cells (ESCs) of the menstrual period did not express CXCR4, however, its expression increased in the proliferative and secretory phase, with the highest intensity of expression in the first trimester. Moreover, E and P significantly upregulated the mRNA and protein expression of CXCR4 in ESCs ( $P < 0.01$ ), reiterating the fact that this could be one of the mechanisms of progesterone supplements playing a role in prevention of miscarriages.<sup>[21]</sup>

Pouysségur and Lenormand in their mice studies demonstrated that disruption of ERK2 locus which is activated by CXCR4 leads to embryonic lethality after implantation stage.<sup>[11]</sup> Similarly, Lin *et al.* found that CXCR4 expression in uterine cells can prevent embryo loss in nonobese diabetic mice.<sup>[12]</sup>

Thus, our study, in accordance with all the available literature, re-inforces the role of CXCR4 in rescuing early pregnancies by its dynamic role at the maternal-fetal interface, further suggesting that downregulation of the same is associated with early spontaneous miscarriages. The salient strength of this study remains the fact that this is the first study in humans to find a correlation between spontaneous miscarriages and CXCR4. Weaknesses include its small sample size. Findings of this study further needs to be assessed in a larger population.

## CONCLUSION

CXCR4 expression is significantly reduced in women with spontaneous miscarriages in comparison with viable pregnancies, and possibly, therapies targeted at increasing the expression of CXCR4 can be used as a treatment modality for the management of spontaneous miscarriages.

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## Conflicts of interest

There are no conflicts of interest.

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