

Original Article

Changes in reactive oxygen species, superoxide dismutase, and hypoxia-inducible factor-1 α levels in missed abortion

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Abstract: This study aimed to investigate changes in the expression levels of reactive oxygen species (ROS), superoxide dismutase (SOD), and hypoxia-inducible factor-1 α (HIF-1 α) in the trophoblasts of patients who had experienced missed abortions. The missed abortion group included 28 patients with missed abortions. The control group was comprised of 35 women who had elected to undergo surgically induced abortion in their first trimester, and whose embryos were confirmed to be alive before surgery. No woman in either group had any known causative factor for missed or spontaneous abortion. As soon as the diagnosis of "missed abortion" was definitively made, the chorionic trophoblast was obtained by induced abortion operation. The same method was used for individuals in the control group, who were at 7-10 weeks of pregnancy. Levels of ROS, SOD, and HIF-1 α in the chorionic trophoblasts from women in both groups were examined within 1 hour by fluorescent staining, chemiluminometry, and enzyme immunoassay methods. The SOD and HIF-1 α levels were lower and the ROS level was higher in the trophoblasts from women in the missed abortion group compared to levels in the control group ($P < 0.05$). ROS, SOD and HIF-1 α levels in the chorionic trophoblasts from patients with missed abortion are altered compared to levels in control patients. Changes in these factors should be evaluated further for their potential role in missed abortion.

Keywords: Hypoxia-inducible factor-1 α (HIF-1 α), reactive oxygen species (ROS), super oxide dismutase (SOD), missed abortion

Introduction

Missed abortion, also known as delayed abortion, is a common gestational pathology that affects between approximately 15% and 20% of women of childbearing age [1]. Despite the high prevalence of the condition, the explicit pathogenesis of missed abortion is not yet known. Recent studies have suggested roles for hypoxia-inducible factor (HIF) -1 α in embryo implantation, ectopic pregnancy, gestational trophoblastic disease, and other conditions [2, 3]. HIF-1 α is a nuclear transcription factor involved in tissue hypoxia and oxygen-sensing [4] that is widely expressed in histocytes and triggers downstream gene transcription by combining with hypoxia-response element (HRE) sites [5].

Oxidation by reactive oxygen species (ROS) is a basic cause of cell damage. The production

and accumulation of ROS lead to cell aging and apoptosis [12, 13]. ROS include non-radical intermediates and free radicals, the most common of which is the strongly oxidizing superoxide anion [6, 7]. To counteract the effects of ROS, cells have antioxidant-producing systems. For example, superoxide dismutase (SOD) is an important metalloenzyme and antioxidant that catalyzes the dismutation of superoxide into hydrogen peroxide and oxygen molecules, thereby scavenging free radicals and preventing oxygen toxicity [8, 9]. Changes in the concentrations of ROS and SOD have been closely related to many conditions [9], including spontaneous abortion [9].

We hypothesized that missed abortion might be related to the expression of HIF-1 α and an imbalanced oxidation system in embryonic cells. In the present study, we compared the levels of ROS, SOD, and HIF-1 α in chorionic tro-

Table 1. Clinical summary of missed abortion and normal pregnancy control groups

Group	Mean age (years)	Mean gravidity (orders)	Mean period of menolip-sis (days)	Mean max radial line of fertilized egg (mm)
Missed abortion	25.93 \pm 2.83	2.25 \pm 0.89	59.07 \pm 11.91	32.46 \pm 10.04
Early pregnancy	26.60 \pm 3.86	2.06 \pm 1.00	55.74 \pm 6.90	32.49 \pm 9.99

phoblasts from patients who suffered missed abortions to levels in women who underwent surgery-induced abortions in early pregnancy.

Materials and methods

This study was approved by the Fifth Hospital of Shanghai Affiliated to Fudan University and the Minhang District Science and Technology Commission. All subjects gave written and verbal consent to participate. All patients who were treated at a Gynecology and Obstetrics Department with a documented intrauterine pregnancy loss between January 2010 and December 2011 were considered eligible for inclusion in the study. Patients were offered enrollment if they were available to give chorionic trophoblast samples and consented to the necessary trophoblast testing of the miscarried tissue.

Missed abortions were diagnosed by transvaginal ultrasound and dynamic decreased human chorionic gonadotropin (hCG) levels in the blood. The diagnoses were confirmed by repeat ultrasound prior to the dilation and curettage (D&C) procedure [10]. Suction curettage was performed according to standard procedures [11]. The chorionic trophoblast was separated from the maternal decidua via a standard technique [12]. Once the chorionic trophoblast had been separated and cleaned, the specimen was divided into equal samples and sent for fluorescence staining, chemiluminometry, and enzyme immunoassay testing, which were performed in parallel.

Samples from 28 patients with missed abortions were collected. In all 28 patients with missed abortion, the time that the embryos remained in the uterus after death was less than 4 weeks. In addition, 35 women in their first trimester of pregnancy who underwent induced abortion surgery were used as a control group. Embryos were confirmed to be living before each surgery.

The 28 missed abortion patients showed no obvious cause for the missed abortion. The mean age of the missed abortion group was 25.93 years (range 29–41 years), the mean time since last menstrual period (TMP) was 59.07 days (range 47–70 days), and the mean gestational age at time of D&C was 8.45 weeks (range 7–10 weeks). The mean gravidity of women in the missed abortion group was 2.25, and the mean maximum radial line of the zygote was 32.46 mm. Most patients sought medical care within the first 1–7 days of vaginal bleeding. In some cases, embryo stasimorphy was identified by ultrasound on the first visit for obstetrical testing in the first trimester.

Table 1 includes all of the demographic data from the two groups. No significant differences were seen between the missed abortion and early pregnancy control groups for age, gravidity, TMP, or maximum radial line of the fertilized egg ($P > 0.05$). No woman in either group had a medical history of pregnancy complications, medical or surgical complications, infection, spontaneous abortion, premature delivery, still-born fetus, threatened abortion, or medication use. Tests for blood coagulation, routine blood analysis, hepatorenal function, and immunological function were performed in all women. No abnormal results were obtained.

Embryonic tissue homogenate

Weighed embryonic tissue was put into a glass homogenizer. A 9-fold volume of normal saline was added, and the tissue was homogenized on ice. Specimens were centrifuged for 5 minutes at 2000 rpm, and the liquid supernatant was used as a 10% tissue homogenate.

Single-cell suspension

A biopsy of trophoblast tissue measuring 1 cm³ was rinsed with ice-cold normal saline. The blood was wiped off, and the sample was dried. Then, 450 μ l of normal saline were added to 0.05 g of the trophoblast sample. The tissue

HIF-1 α is restricted by ROS and SOD

Table 2. Mean with standard deviation ROS, SOD, and HIF-1 α levels in missed abortion and early pregnancy control groups

Group	n	ROS fluorescence intensity	SOD enzyme activity unit (U/mg prot)	HIF-1 α (mg/ml)
Missed abortion	28	758.41 \pm 86.48	0.43 \pm 0.22	0.38 \pm 0.05
Early pregnancy	35	445.84 \pm 70.12	1.39 \pm 0.49	1.62 \pm 0.25

was homogenized and removed from the glass homogenizer by suction with a pipettor. Specimens were filtered through a nylon net screen (100- μ m opening) in vitro, and centrifuged for 4 to 5 minutes at 1000 rpm. To remove cell debris, the sample was centrifuged 3 times with normal saline, for 1 minute each time, at 500 to 800 rpm. Specimens were filtered through a nylon net screen (300- μ m opening) to remove unit cells, and a single-cell suspension was obtained. Samples were inspected by a flow cytometry instrument (BD FACS Calibur Flow cytometer). Trophoblast samples were sent to a cytogenetic laboratory (Shanghai Jiao Tong University Molecular Genetics Laboratory) within 1 hour of sampling, to determine the ROS levels.

ROS, SOD, and HIF-1 α assays

ROS, SOD, and HIF-1 α levels in trophoblasts were measured using fluorescent staining, chemiluminometry, and enzyme immunoassay methods. Factors were tested as specified by the manufacturers of the kits employed: namely, an ROS assay kit (Beyotime, Product No. S0033), SOD assay kit (Nanjing Jiancheng Bioengineering Institute Product No. A001-1), and HIF-1 α ELISA kit (Biovolue, Product No. 50R-E.1465). An American Varian Cary-Eclipse 500 fluorospectrophotometer and a BD FACS Calibur flow cytometer were used. 2',7'-dichlorofluorescein diacetate (DCF) is a stain that freely penetrates the cell membrane. Once oxidized, it produces fluorescent DCF, allowing the ROS concentration in the cell to be assayed. A strong DCF fluorescence intensity signifies a high ROS concentration. Flow cytometry with an argon ion laser at 488 nm was used to observe more than 50,000 cells. Results are expressed as one-parameter histograms. A right-shifted wave crest signified a high ROS concentration.

Statistical analysis

To determine the expression levels of ROS, SOD, and HIF-1 α in trophoblasts, data were

confirmed using an unpaired *t*-test and Q-Q plots. A *P*-value < 0.05 was considered statistically significant. Statistical analysis and graphs were produced using SPSS v.16 (SPSS Inc., Chicago, IL, USA).

Results

The levels of ROS, SOD, and HIF-1 α in trophoblasts differed significantly between women in the missed abortion group and in the early pregnancy control group. As shown in **Figure 1**, the fluorescence intensity of ROS in chorionic trophoblasts from the missed abortion patients was significantly higher than that in the control group (*P* < 0.05). Trophoblast extracts from the missed abortion group had a lower SOD enzyme activity level (*P* < 0.01) and lower mean concentration of HIF-1 α (*P* < 0.001) compared to the control group (**Table 2**). The fluorescent labeling experiments (**Figure 1**) revealed a clear rightward shift of the wave crest of the missed abortion group, indicating elevated ROS concentrations. The ROS and HIF-1 α levels were inversely correlated with each other both in the missed abortion group (*r* = -0.512, *P* < 0.01, **Figure 2A**) and in the early pregnancy control group (*r* = -0.621, *P* < 0.01, **Figure 2B**).

Discussion

A recent study showed that pregnancy is an oxidative stress state associated with increased ROS levels in the placenta. At the same time, antioxidants such as SOD are elevated so that an oxidant-antioxidant equilibrium can be maintained at a new level, and pregnancy can continue [6]. In the present study, we observed higher ROS levels in trophoblast samples from patients who had experienced missed abortions compared to those in the early pregnancy control group. The SOD levels were lower in the missed abortion group than those in the early pregnancy control group. These results suggest that the ROS-producing and antioxidant defense systems may have been in disequilibrium in the trophoblasts of embryos from missed abortion patients. Such changes in the cellular redox

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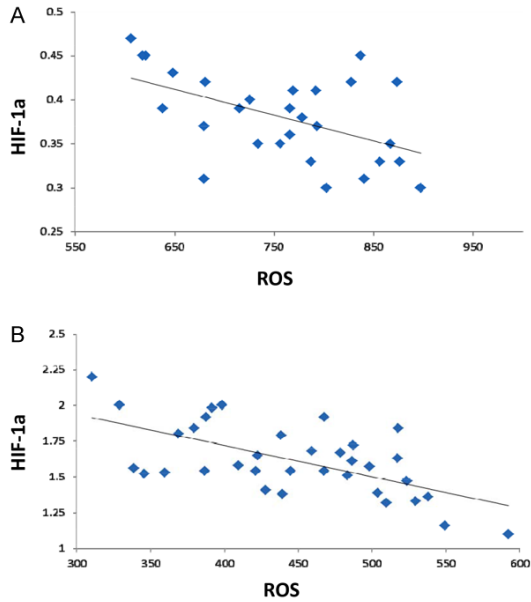


Figure 1. Correlation analysis of ROS levels in trophoblasts in each group. ROS were measured using a fluorescent staining method. The wave crest for the missed abortion group (A) showed a clear rightward shift, indicating a high ROS concentration relative to the control group (B).

state may have affected trophocyte growth and, thereby, caused embryonic development to cease, resulting in the missed abortions.

Letta et al. found that the effect of hypoxia on HIF-1 α expression peaked at 7 to 9 weeks of pregnancy, and anoxia was important for adjusting trophocyte invasion and differentiation [13, 14]. Consistent with that study, we found that embryos in the missed abortion group stopped developing at 7 to 10 weeks of pregnancy. During this time period, the embryo is under its lowest oxygen tension and HIF-1 α is normally expressed at high levels [15]. The HIF-1 α levels in trophoblasts from the missed abortion group were significantly lower than levels in the control group. Thus, low expression of HIF-1 α at 7 to 10 weeks of gestation could have left the embryo unable to tolerate its hypoxic environment, resulting in disruptions of trophocyte growth and embryonic development, and ultimately resulting in miscarriage.

Recent studies have demonstrated that early embryonic and placenta development occur in a relatively hypoxic environment [14, 16]. When the embryonic trophocyte implants in the uter-

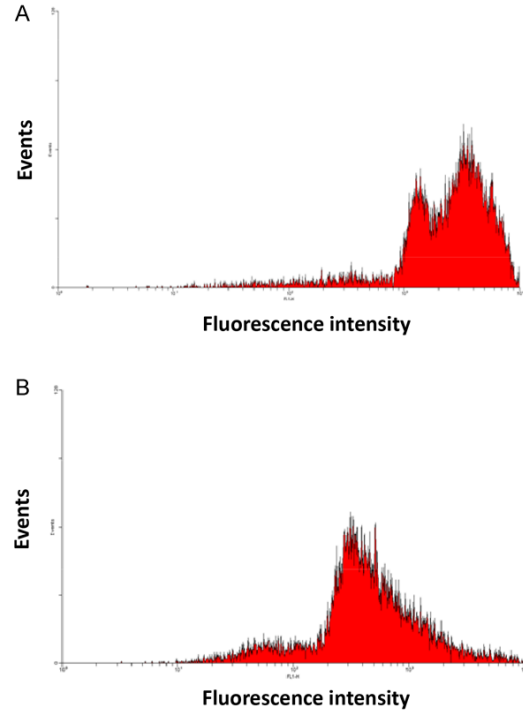


Figure 2. Correlation analysis of scatterplots for the levels of ROS and HIF-1 α by group. ROS and HIF-1 α levels in trophoblasts were measured using a fluorescent staining method and enzyme immunoassay in the missed abortion group (A) and the early pregnancy control group (B). ROS and HIF-1 α levels were inversely correlated in both groups, with $r = -0.512$, $P < 0.01$ for missed abortion group, and $r = -0.621$, $P < 0.01$ for the early pregnancy control group.

us, the uterine blood supply has not yet been established. However, HIF-1 α is expressed at moderate levels, allowing the embryo to tolerate a hypoxic environment and, thus, to proceed with its embryonic development [17]. Under conditions of uterine vascular hyperplasia, the embryo gradually reaches a hyperoxic state, and the HIF-1 α levels decline. In a state of hyperplasia, cytotrophoblasts are transformed into extravillous trophocytes. This transformation facilitates trophocyte infiltration, enabling the cells to reach the uterine spiral arteries and to form gestation-supporting blood vessels [14]. The resultant transition from relative hypoxia to normoxia enables embryos to tolerate ischemic reperfusion. Even when the placenta is functioning well, oxygen availability has an important influence on the trophocytes. Variations in oxygen supply have been closely correlated with some diseases of pregnancy [6].

Our study showed that the ROS and HIF-1 α levels were inversely correlated in both the missed abortion and control groups. This finding suggests that lower HIF-1 α availability is associated with higher ROS levels, and it supports the notion that ROS have an inhibitory action on HIF-1 α . Our results are consistent with a pathogenesis of missed abortion involving three aspects. First, ROS may cause lipid peroxidation damage to embryos. The generated oxygen radicals may attack the combined HIF-1 α and hypoxia response element (HRE) sites, such that HIF-1 α cannot bind HRE to promote the transcription of genes downstream of the HRE elements. Second, increased ROS levels might change the partial pressure of oxygen in embryonic cells. In a hyperoxic environment, HIF-1 α is gradually degraded, resulting in low levels of HIF-1 α . Third, when their development has been incomplete, embryonic trophoblasts enter a cycle of ischemia and reperfusion prematurely, causing ischemic reperfusion damage to the embryo, which then, in a vicious cycle, produces even more ROS.

In the present group of women who had missed abortions, a disequilibrium of ROS and SOD appeared to lead to a redox imbalance, which might have inhibited the expression of HIF-1 α . The resulting accumulation of ROS and the low expression of HIF-1 α might be important contributing factors for the missed abortions. Nevertheless, it is still unclear whether changes in levels of ROS, SOD, and HIF-1 α are the cause or result of missed abortion. Further studies about the causal relationships between these factors and missed abortion are clearly needed.

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Disclosure of conflict of interest

None.

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