

# Molecular and biochemical investigations of key antioxidant/oxidant molecules in Saudi patients with recurrent miscarriage

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**Abstract.** The present study was undertaken to: i) Determine the levels of oxidative stress (OS) markers, malondialdehyde (MDA), superoxide anions (SOA) and hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), in both plasma and placental tissues of recurrent miscarriage (RM) patients in comparison with those of healthy pregnant (HP) and non-pregnant (NP) women; ii) determine the levels of enzymatic antioxidants [glutathione peroxidase (GPx), glutathione reductase (GSR), superoxide dismutase (SOD) and catalase (CAT)], and non-enzymatic antioxidant micronutrients [selenium (Se), zinc (Zn), copper (Cu) and manganese (Mn)] in both plasma and placental tissues of RM patients, in comparison with those of HP and NP women; iii) profile differential expression levels of selected antioxidant and apoptosis-related genes in the placental tissues of RM cases, in relation to those of HP women of matched gestational age, using reverse transcription-quantitative polymerase chain reaction (RT-qPCR). The results revealed highly significant increases of all investigated OS markers in plasma and placental tissues of RM patients compared with those of HP women. Moderate, but significant, increases of OS markers were observed in the plasma of HP patients in relation to those of NP women. The activities of antioxidant enzymes exhibited statistically significant decreases in both plasma and placental tissues of RM patients compared with those of HP women. The significantly reduced level of antioxidant enzymes was also evident in the plasma of HP women as compared with those of NP women. Results of RT-qPCR assays clearly indicated

that the expression level of apoptosis-related genes [tumor necrosis factor-related apoptosis-inducing ligand (*TRAIL*) and *SI00A8*], and pro-inflammatory cytokine genes [tumor necrosis factor- $\alpha$  (*TNF- $\alpha$* ), interleukin (*IL*)-6 and *IL*-8] were significantly upregulated in placental tissue of RM cases in relation to those of HP subjects. By contrast, mRNA transcriptional levels of key antioxidant genes (*GPx*, *SOD*, *GSR* and *CAT*) were found to be significantly reduced in placental tissue of RM patients in comparison to those of HP women. In conclusion, our data highlight a plausible cause-effect association between the observed increase in placental OS level and depletion of the activity of antioxidant enzymes. This suggests that OS is a contributing factor in the pathogenesis of idiopathic RM.

## Introduction

Recurrent miscarriage (RM) is a common gestational complication that affects 2-5% of couples trying to conceive (1,2), and previous successful live birth does not preclude its development (3). RM is considered to be a multifactorial disorder that may result from either acquired or inherited factors (4). Several studies have been conducted to examine and identify the molecular mechanism underlying the pathophysiology of RM and yet 50-60% of the cases are of unknown cause (4,5).

Although oxygen is essential for normal cell function, it poses a risk by producing some toxic derivatives through extensive metabolism (5). Several studies have suggested that the oxidative stress (OS) generated during angiogenesis and placental development might be the cause of idiopathic RM (2,5-10). OS is known to result when there is an increased production of reactive oxygen species (ROS) that cannot be balanced by the produced antioxidants. However, ROS are naturally produced during oxygen reduction and within limited levels can act as essential regulators for several cell functions, in addition to playing an important role in several reproductive processes (11). Increased ROS levels are common during placental development and are normally balanced by higher levels of antioxidants (9).

Superoxide anions (SOA), hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), and hydroxyl radicals ( $\cdot$ OH) are the most commonly generated

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ROS in an electron-rich environment, which mainly occurs within the mitochondrial inner membrane and the respiratory chain (6,12). The conversion of oxygen to SOA mainly results after the leakage of 1-3% of electrons through respiratory chain complex I and III (5,12,13). Aerobic cells balance SOA by its dismutation into  $H_2O_2$  through antioxidant superoxide dismutase (SOD) (6). Other antioxidants that can convert SOA into  $H_2O_2$  include cytosolic copper (Cu) and zinc (Zn) dismutase and mitochondrial manganese (Mn) SOD (7). Glutathione peroxidase (GPx) and catalase (CAT) antioxidant enzymes can then transform  $H_2O_2$  into water. In addition, GPx can also reduce lipid hydroperoxides into water by transferring the energy of those reactive peroxides to reduced glutathione (GSH), and thus, resulting in oxidised glutathione (GSSG). This can then be reduced back to GSH by the enzyme glutathione reductase (GSR) via donated electron by NADPH (7,14). However, excessive unbalanced ROS can induce cytotoxicity by alteration in gene expression including genes related to apoptosis (7).

Although, several studies have suggested that OS may be a strong factor causing RM, there is a lack of consensus in the literature. Therefore, the present study was conducted to gain further knowledge regarding the impact of OS and the efficiency of antioxidants in RM cases by examining free radicals and OS markers in addition to measurements of antioxidant levels in placental tissue and plasma from RM patients with reference to healthy pregnant (HP) women. Moreover, these assays are paralleled by profiling the differential expression level of selected antioxidant, pro-inflammatory and apoptosis-related genes in the placental tissues of RM cases in relation to HP women.

## Patients and methods

All materials and solutions were purchased from Sigma-Aldrich (Merck KGaA), unless otherwise specified.

**Samples, collection and preparation.** The study included consenting patients who attended the Department of Obstetrics and Gynaecology at the King Khalid University Hospital (Riyadh, Kingdom of Saudi Arabia) from September 2014 to January 2015, who signed the consent forms provided. Ethics approval was obtained from the Ethics Review Committee of the College of Applied Medical Sciences of King Saud University (CAMS 27-34/35) and the study was conducted according to the Declaration of Helsinki.

Placental tissue and blood plasma of idiopathic RM women of at least 3 consecutive miscarriages ( $n=28$ ),  $35.8 \pm 5.3$  years of age, and of HP women ( $n=28$ ), with no miscarriage history,  $29.3 \pm 2.7$  years of age, were examined. Blood plasma samples were also collected from non-pregnant women (NP) ( $n=28$ ),  $28.6 \pm 4.91$  years of age. Patients with RM were excluded from the study if there were risk factors, such as abnormal menstrual cycle ( $<21$  and  $>35$  days), genital infections, antiphospholipid syndrome (positive anticardiolipin and/or b2 glycoprotein 1 antibodies), and uterus anomalies (observed by ultrasound examination or hysterosonography). No anticoagulant or immunological treatment was applied during the pregnancy. Gestational age of RM patients at the time of miscarriage was  $12.6 \pm 2.8$  weeks, with an average of  $3.5 \pm 0.8$  miscarriages.

Maternal blood and placental tissue samples were collected at the time of delivery from HP women or at the time of miscarriage from RM patients. Full depth placental tissue was collected, in which the *Decidua basalis* and the chorionic plate was trimmed off, leaving trophoblastic tissue. Tissue was then washed in 0.1 M phosphate-buffered saline (PBS) and dissected into 1.5 g pieces and placed into two Corning® cryogenic vials (Corning, Inc.). One contained RNAlater® (Thermo Fisher Scientific, Inc.) (4°C) for immediate RNA stabilization and protection, and thus, reliable gene expression profiling, while the other contained PBS for biochemical assays. All collected tubes were kept at 4°C for 24 h. Subsequently, cryovials were immediately snap-frozen in liquid nitrogen prior to storage at -80°C until further use. Venous blood samples (6 ml) were collected from all participants into cold BD Vacutainer™ Plastic Blood Collection Tubes (BD Biosciences) with  $K_2EDTA$  for the measurement of biochemical OS markers (SOA,  $H_2O_2$  and lipid peroxides), activities of antioxidant enzymes (SOD, GPx, GSR and CAT), in addition to the non-enzymatic antioxidants GSH, Zn, selenium (Se), and Cu. All centrifugation steps were conducted at room temperature. Plasma was obtained by centrifugation at  $3,000 \times g$  for 20 min and then transferred into the Eppendorf tubes within 1 h and stored at -80°C. For GSH and GSSG analysis, whole blood aliquot samples (30  $\mu$ l) were centrifuged and 33.3  $\mu$ l of 5-sulphosalicylic acid (1 g/ml) were added for protein precipitation and cellular disruption to release GSH. Samples were then diluted with 936.7  $\mu$ l sodium phosphate buffer (pH 7.5) and then centrifuged for 5 min at  $12,000 \times g$ , and the supernatant was kept at -80°C until the time of analysis.

## Measurement of OS markers

**$H_2O_2$ .** Levels of  $H_2O_2$  were measured as described previously (7). Briefly, reaction mix (horseradish peroxidase dissolved in Kreb's Ringer buffer 10  $\mu$ g/ml, 100  $\mu$ l; sodium phosphate reaction buffer 50 mM; pH 7.4) was added to 50  $\mu$ l diluted samples and standards and incubation followed for 30 min at room temperature. A total of 50  $\mu$ l of 10 mM Amplex Red Reagent (ARR; 10-acetyl-3,7-dihydrophenoxazine; Thermo Fisher Scientific, Inc.) was added to commence reaction, and fluorescence was measured at 590 nm. ARR reacts with  $H_2O_2$  in the presence of peroxide resulting in red fluorescent oxidation resorufin products.

**SOA.** Samples (0.1 ml) were incubated for 5 min at 37°C with 1 ml of PBS (2 g glucose, 2 g of fatty acid-free bovine serum albumin/l) with and without 30  $\mu$ g SOD following previous publication (6,15), and were mixed with 0.1 ml reaction solution of ferricytochrome-c (1.2 mM). Tube containing only buffer and ferricytochrome-c was used as blank control. A spectrophotometer, equipped with a thermostated cuvet, was used for measuring absorbance at 550 nm. Results were converted to nM of reduced ferricytochrome-c by using an absorptivity value of  $1.96 \times 10^4 \text{ l} \cdot \text{mol}^{-1}$ . SOA levels were determined by calculating the difference between the samples without SOD and the samples with added SOD.

**Lipid peroxidation (LPO).** The level of the end product of LPO was determined, malondialdehyde (MDA), using thiobarbituric acid (TBA) which reacts with MDA producing a fluorescence product that can be measured by spectrophotometry (7,16,17). Briefly, plasma (150  $\mu$ l) or placental tissue

supernatant (1 ml) was mixed with 1 ml trichloroacetic acid (17.5%) and 1 ml TBA (0.6%), followed by incubation in hot water bath (100°C) for 15 min, and then left to cool. After that, 1 ml trichloroacetic acid (70%) was added to the mixture, incubated for 20 min at room temperature, and then centrifuged at 2,000 x g for 15 min. The supernatant was decanted and the absorbance was measured at 535 nm. Applying  $1.56 \times 10^5 \text{ M}^{-1} \cdot \text{cm}^{-1}$  as an extinction coefficient, MDA levels were calculated.

**Enzymatic assays for enzymatic antioxidant measurements.** Frozen tissue was thawed in ice, homogenized in 0.1 M potassium chloride buffer, and centrifuged at 10,000 x g, 4°C for 10 min. Supernatants were used for different assays.

**Measurement of SOD activity.** Serum samples (250  $\mu\text{l}$ ) were mixed with xanthine (25  $\mu\text{l}$ ,  $1.142 \text{ mg} \cdot \text{ml}^{-1}$ ), hydroxyl ammonium chloride (25  $\mu\text{l}$ ), water (125  $\mu\text{l}$ ), and xanthine oxidase (75  $\mu\text{l}$ ,  $0.1 \text{ U} \cdot \text{ml}^{-1}$ ). The mixture was incubated at 25°C for 20 min, and sulphonic acid (0.5 ml,  $3.3 \text{ mg} \cdot \text{ml}^{-1}$ ) and  $\alpha$ -naphthylamine (0.5 ml,  $1 \text{ ng} \cdot \text{ml}^{-1}$ ) were added, and further incubated at room temperature for 20 min. Absorbance was measured at 530 nm.

**Measurement of CAT activity.** CAT activity was measured as previously described (18), using  $\text{H}_2\text{O}_2$  as the substrate. The decomposition of  $\text{H}_2\text{O}_2$  was observed at 240 nm by measuring the decrease in absorbance. The activity was expressed as  $\mu\text{M}/\text{min}/\text{mg}$  protein using a coefficient of  $0.0436 \text{ mM}^{-1} \cdot \text{mg}^{-1}$ .

**Measurement of GPx activity.** GPx activity was measured as described previously (19). Briefly, assay solution (800  $\mu\text{l}$ ) of 50 mM Tris-HCl buffer (pH 7.6), 1 mM EDTA, 1 IU GSR, 0.25 mM GSH and 0.2 mM NADPH were added to diluted plasma (1:10; 50  $\mu\text{l}$ ) or placental tissue supernatant (50  $\mu\text{l}$ ). After 5 min, 100  $\mu\text{l}$  of  $\text{H}_2\text{O}_2$  (15 mM) were added to the sample mix and the absorbance change was examined at 340 nm for 30 min. GPx activity was determined by calculating the enzyme catalysing the oxidation of 1  $\mu\text{M}$  or 1 nM GSH per minute.

**Measurement of GSR activity.** GSR activity was determined following a previously described protocol (7). Briefly, 100  $\mu\text{l}$  serum samples were added to 2 ml reaction mix (100 mM potassium phosphate buffer pH 7.4, 50  $\mu\text{l}$  of 80 mM EDTA, 100  $\mu\text{l}$  2 mM NADPH, and 100  $\mu\text{l}$  of 0.3 mM flavine adenine dinucleotide) and incubated for 2 min followed by addition of 100  $\mu\text{l}$  GSSG (7.5 mM). Using a spectrophotometer, reaction was examined at 340 nm for 2 min. One unit of GSR is equal to the amount of enzyme to reduce 1  $\mu\text{M}$  of NADPH/min at 25°C.

#### Non-enzymatic assays

**Measurement of GSH and GSSG.** Following a previous described protocol (20,21), glutathione reductase-DTNB (5,5-dithiobis-2-nitrobenzoic acid) was used to determine the GSH levels. Twenty-five microliters of samples and standards (ranging from 20 to 80  $\mu\text{M}$ ) were incubated separately for 3 min at 37°C with the reaction mix (150  $\mu\text{l}$  of 100 mM sodium phosphate buffer pH 7.4, 50  $\mu\text{l}$  of 8 mM EDTA, 50  $\mu\text{l}$  DNTB solution, and 100  $\mu\text{l}$  of 2 mM NADPH). Then, 25  $\mu\text{l}$  glutathione reductase were added and absorbance was measured at 410 nm. GSSG was measured similarly, but the reaction mix also contained triethanolamine, to prevent pH increase and oxidation, and 2-vinyl pyridine for GSH derivatization.

GSSG standards ranged from 0 to 10  $\mu\text{M}$  and were examined simultaneously.

**Measurement of Se, Zn, Cu and Mn antioxidants.** Both plasma and placental tissue were freeze dried at -45°C and ground into powder. Ground plasma (100 mg) and 20 mg of ground placental tissue were used, in which samples were digested with ultra-pure nitric acid (0.5 ml, 68%) and  $\text{H}_2\text{O}_2$  (0.2 ml, 35%). The product was diluted using ultra-pure water and then measured for Se, Cu, Zn and Mn using inductively coupled plasma mass spectrometry (ICP-MS) (HP 4500; Yokokawa Electric Co.), as described previously (22).

**Gene expression profiling.** Placental tissue samples previously stored in RNeasy lysis buffer at -80°C were allowed to thaw on ice, and were subsequently used for total RNA extraction and relative gene expression quantification using reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Approximately 100 mg of placental tissues were homogenized in 1 ml TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) by the use of TissueLyser LT (Qiagen, Inc.), and total RNA was subsequently extracted by standard procedure. Genomic DNA was then eliminated and cDNA was synthesized from total RNA (1  $\mu\text{g}$ ) in a final reaction volume of 20  $\mu\text{l}$  using the QuantiTect Reverse Transcription kit (QuantiTect; Qiagen, Inc.), according to the manufacturer's instructions. The RT reaction was carried out on Veriti 96-Well Thermal Cycler (Applied Biosystems) at 42°C for 45 min and then inactivated at 95°C for 15 min. The resultant diluted cDNA (1:10, 5  $\mu\text{l}$ ) was then used to perform RT-qPCR using the QuantiTect SYBR-Green PCR kit (Qiagen, Inc.). A total of 100 nM of the following gene primers were used: CAT (Hs\_CAT\_1\_SG QuantiTect Primer Assay, QT00079674); GPx (Hs\_GP\_x\_1\_SG QuantiTect Primer Assay, QT00203392); GSR (Hs-GSR\_1\_SG QuantiTect Primer Assay, QT00038325); SOD (Hs\_SOS\_1\_SG QuantiTect Primer Assay, QT01664327); tumor necrosis factor- $\alpha$  (*TNF- $\alpha$* ) (Hs\_TNF\_3\_SG QuantiTect Primer Assay, QT01079561); interleukin (IL)-6 (Hs\_IL6\_1\_SG QuantiTect Primer Assay, QT00083720); IL-8 (Hs\_IL8\_1\_SG QuantiTect Primer Assay, QT00000322); and 18S (Hs\_RRN18S\_1\_SG QuantiTect Primer Assay, QT00199367), in a final reaction volume of 25  $\mu\text{l}$ , containing the cDNA sample (5  $\mu\text{l}$ ), SYBR-Green PCR Master mix (12.5  $\mu\text{l}$ ), QuantiTect Primer Assay (10X, 2.5  $\mu\text{l}$ ), and RNase-free water (2.5  $\mu\text{l}$ ). A two-step cycling reaction was conducted; following an initial polymerase activation at 95°C for 10 min, samples were subjected to 40 cycles of i) denaturation at 95°C for 15 sec, followed by ii) annealing and elongation at 60°C for 1 min. TaqMan gene expression assays (Applied Biosystems) were performed with the following gene primers: tumor necrosis factor-related apoptosis-inducing ligand (*TRAIL*) (Hs00921974\_ml TaqMan Gene Expression); calcium binding protein A8 (*S100A8*) (Ha00374263\_ml TaqMan Gene Expression); and TaqMan endogenous housekeeping gene, hypoxanthine-guanine phosphoribosyltransferase (*HPRT1*). The amplification program and PCR amplicon specificity were performed and assessed as documented by us previously (6). A three-step cycling reaction was carried out; following an initial polymerase activation at 95°C for 10 min, samples were subjected to 40 cycles of i) denaturation at 95°C for 15 sec, followed by ii) annealing at 55°C

Table I. Levels of H<sub>2</sub>O<sub>2</sub>, SOA, and MDA, which is used as an index of LPO, in plasma and placental tissue of NP, HP and RM women.

Assayed parameters	Subjects		
	NP women (n=28)	HP women (n=28)	RM women (n=28)
Plasma H <sub>2</sub> O <sub>2</sub> (nmol/ml)	7.04±1.12	8.11±1.14 <sup>a</sup>	10.2±1.47 <sup>b,c</sup>
Placental tissue H <sub>2</sub> O <sub>2</sub> (nmol/min/mg)	-	2.41±0.35	3.38±0.46 <sup>d</sup>
Plasma SOA (nmol/ml)	30.1±4.50	35.3±5.45 <sup>a</sup>	45.2±6.10 <sup>b,c</sup>
Placental tissue SOA (μmol/min/mg)	-	4.67±0.62	5.93±0.78 <sup>d</sup>
Plasma MDA (nmol/ml)	5.03±0.70	5.73±0.76 <sup>a</sup>	7.87±0.96 <sup>b,c</sup>
Placental tissue MDA (nmol/g wet weight)	-	258±35.7	334±45.8 <sup>d</sup>

<sup>a</sup>P<0.05, compared with plasma of NP women; <sup>b</sup>P<0.0001, compared with plasma of NP women; <sup>c</sup>P<0.001, compared with plasma of HP women; <sup>d</sup>P<0.0001, compared with placental tissue of HP women. H<sub>2</sub>O<sub>2</sub>, hydrogen peroxide; SOA, superoxide anions; MDA, malondialdehyde; LPO, lipid peroxidation; NP, non-pregnant; HP, healthy pregnant; RM, recurrent miscarriage.

Table II. GPx, GSR, CAT and SOD enzymatic activities in plasma and placental tissue of NP, HP and RM women.

Assayed parameters	Subjects		
	NP women (n=28)	HP women (n=28)	RM women (n=28)
Plasma GPx (nmol/min/ml)	1.19±0.17	1.06±0.13 <sup>a</sup>	0.86±0.10 <sup>b,c</sup>
Placental tissue GPx (μmol/min/mg protein)	-	0.36±0.04	0.28±0.04 <sup>d</sup>
Plasma GSR (nmol/min/ml)	1.86±0.26	1.60±0.23 <sup>a</sup>	1.29±0.17 <sup>b,c</sup>
Placental tissue GSR (μmol/min/mg protein)	-	0.61±0.08	0.48±0.06 <sup>d</sup>
Plasma CAT (nmol/min/ml)	3.10±0.36	2.78±0.38 <sup>a</sup>	2.25±0.30 <sup>b,c</sup>
Placental tissue CAT (μmol/min/mg protein)	-	0.91±0.12	0.70±0.10 <sup>d</sup>
Plasma SOD (nmol/min/ml)	2.08±0.27	1.83±0.23 <sup>a</sup>	1.48±0.20 <sup>b,c</sup>
Placental tissue SOD (μmol/min/mg protein)	-	1.88±0.20	1.52±0.19 <sup>d</sup>

<sup>a</sup>P<0.05, compared with plasma of NP women; <sup>b</sup>P<0.0001, compared with plasma of NP women; <sup>c</sup>P<0.001, compared with plasma of HP women; <sup>d</sup>P<0.0001, compared with placental tissue of HP women. GPx, glutathione peroxidase; GSR, glutathione reductase; CAT, catalase; SOD, superoxide dismutase; NP, non-pregnant; HP, healthy pregnant; RM, recurrent miscarriage.

for 30 sec, and iii) elongation at 72°C for 30 sec. Each tissue sample was represented by two biological replicas and three technical replicas, with the inclusion of a no-template control. Raw data were analyzed using the Rotor-Gene Q software version 2.3 (Qiagen, Inc.) to calculate the threshold cycle (Cq) using the second derivative maximum method. The relative gene expression level (fold-change) was determined after normalization to the expression levels of *18S* and *HPRT1* as a housekeeping gene for SYBR-Green and TaqMan<sup>®</sup> assays, respectively, with the 2<sup>-ΔΔCq</sup> method as previously described (6). The relative gene expression data were subjected to Student's t-test in order to identify significant differences between RM samples compared with uncomplicated HP controls. Expression levels in terms of fold change were considered statistically significant at P<0.05.

**Statistical analysis.** Statistical analysis was performed using the computer-based package of Minitab software (v.13.1, 2001; Minitab Ltd.). Sample analysis was run in duplicate for all investigated parameters and results are presented as means ± SD. Values of the activities and concentrations of

individual parameters were compared between different groups of the study subjects using one-way analysis of variance (ANOVA) followed by the post-hoc Tukey-HSD test for multiple comparisons. P<0.05 was considered to indicate a statistically significant difference.

## Results

**Levels of OS markers in plasma and placenta.** Plasma H<sub>2</sub>O<sub>2</sub> levels were significantly higher (10.2±1.47 nmol/ml) in RM patients in relation to both HP (8.11±1.14 nmol/ml; P<0.001) and NP (7.04±1.12 nmol/ml; P<0.0001) subjects. HP subjects, however, showed moderately increased (P<0.05) plasma H<sub>2</sub>O<sub>2</sub> levels compared with NP samples (Table I). Placental tissue H<sub>2</sub>O<sub>2</sub> generation rates showed highly significantly elevated levels in RM patients compared with HP women (3.38±0.46 vs. 2.41±0.35 nmol/min/mg protein, respectively, P<0.0001; Table I). Similar increases of SOA (Table I) and MDA (Table I) were observed in both plasma and placental tissue of RM patients compared with both HP (P<0.001

Table III. GSH, GSSG and GSH/GSSG ratio in plasma and placental tissue of NP, HP and RM women.

Assayed parameters	Subjects		
	NP women (n=28)	HP women (n=28)	RM women (n=28)
Plasma GSH ( $\mu\text{mol/l}$ )	2.86 $\pm$ 0.30	2.51 $\pm$ 0.36 <sup>a</sup>	1.97 $\pm$ 0.29 <sup>b,c</sup>
Placental tissue GSH (nmol/mg tissue)	-	6.86 $\pm$ 0.95	5.40 $\pm$ 0.77 <sup>d</sup>
Plasma GSSG ( $\mu\text{mol/l}$ )	0.049 $\pm$ 0.007	0.056 $\pm$ 0.008 <sup>a</sup>	0.071 $\pm$ 0.010 <sup>b,c</sup>
Placental tissue GSSG (nmol/mg tissue)	-	0.14 $\pm$ 0.018	0.18 $\pm$ 0.025 <sup>d</sup>
Plasma GSH/GSSG	55.6 $\pm$ 6.88	48.5 $\pm$ 6.61 <sup>a</sup>	28.8 $\pm$ 3.88 <sup>b,c</sup>
Placental tissue GSH/GSSG	-	49.6 $\pm$ 6.75	31.0 $\pm$ 4.19 <sup>d</sup>

<sup>a</sup>P<0.05, compared with plasma of NP women; <sup>b</sup>P<0.0001, compared with plasma of NP women; <sup>c</sup>P<0.001, compared with plasma of HP women; <sup>d</sup>P<0.0001, compared with placental tissue of HP women. GSH, reduced glutathione; GSSG, oxidised glutathione; NP, non-pregnant; HP, healthy pregnant; RM, recurrent miscarriage.

Table IV. Se, Cu, Zn and Mn levels in plasma and placental tissue of NP, HP and RM women.

Assayed parameters	Subjects		
	NP women (n=28)	HP women (n=28)	RM women (n=28)
Plasma Se ( $\mu\text{mol/l}$ )	1.09 $\pm$ 0.14	0.95 $\pm$ 0.13 <sup>a</sup>	0.78 $\pm$ 0.09 <sup>b,c</sup>
Placental tissue Se (nmol/g)	-	16.8 $\pm$ 2.26	12.0 $\pm$ 1.73 <sup>d</sup>
Plasma Cu ( $\mu\text{mol/l}$ )	28.3 $\pm$ 3.81	24.5 $\pm$ 3.41 <sup>a</sup>	19.6 $\pm$ 2.75 <sup>b,c</sup>
Placental tissue Cu (nmol/g)	-	98.1 $\pm$ 14.7	75.1 $\pm$ 11.1 <sup>d</sup>
Plasma Zn ( $\mu\text{mol/l}$ )	4.03 $\pm$ 0.58	3.55 $\pm$ 0.49 <sup>a</sup>	2.84 $\pm$ 0.36 <sup>b,c</sup>
Placental tissue Zn (nmol/g)	-	621 $\pm$ 87.8	490 $\pm$ 67.1 <sup>d</sup>
Plasma Mn (nmol/l)	48.6 $\pm$ 7.30	42.2 $\pm$ 7.12 <sup>a</sup>	32.2 $\pm$ 4.73 <sup>b,c</sup>
Placental tissue Mn (nmol/g)	-	6.75 $\pm$ 0.81	5.45 $\pm$ 0.75 <sup>d</sup>

<sup>a</sup>P<0.05, compared with plasma of NP women; <sup>b</sup>P<0.0001, compared with plasma of NP women; <sup>c</sup>P<0.001, compared with plasma of HP women; <sup>d</sup>P<0.0001, compared with placental tissue of HP women. Se, selenium; Cu, copper; Zn, zinc; Mn, manganese; NP, non-pregnant; HP, healthy pregnant; RM, recurrent miscarriage.

and P<0.0001, respectively) and NP (P<0.0001) samples. An increase (P<0.05) of plasma SOA and MDA levels was observed in HP in relation to NP subjects, as well.

**Levels of GPx, GSR, CAT and SOD enzymatic antioxidants.** All examined plasma enzymatic antioxidants showed similar patterns of highly significant decreases in RM patients compared with both HP (P<0.001) and NP (P<0.0001) samples (Table II). However, the antioxidant plasma levels of HP participants were slightly, however significantly, decreased compared with those of NP samples (P<0.05). Examination of GPx, GSR, CAT and SOD in placental tissue of RM patients also revealed highly significant decreases (P<0.0001) compared with the levels in HP placental tissues (Table II).

#### Non-enzymatic antioxidants

**Measurement of GSH and GSSG.** GSH showed highly significant decreases in RM plasma in relation to HP (P<0.001) and NP (P<0.001) subjects (Table III). It was also observed that HP subjects had moderately lower GSH levels (P<0.05) compared with those in NP subjects. Similar findings were

observed in placental tissue samples of RM patients which had very significantly decreased GSH levels compared with those of HP women. However, GSSG plasma levels were very significantly higher in RM patients compared with both HP (P<0.001) and NP (P<0.0001) subjects, and also in HP compared with NP subjects (P<0.05; Table III).

**GSH/GSSG ratio.** As GSH is considered an important scavenger for ROS, its ratio with GSSG is used as an OS marker (23). The results (Table III) revealed that RM plasma samples had very significantly lower GSH/GSSG ratios compared with those in plasma of both HP (P<0.001) and NP (P<0.0001) subjects, and HP plasma samples had significantly lower ratio than NP (P<0.05) plasma. Similarly, GSH/GSSG ratios in placental tissue of RM patients were highly significantly decreased when compared with those found in HP women (P<0.0001).

**Se, Cu, Zn and Mn.** The examined non-enzymatic micronutrient antioxidants (Table IV) showed very similar patterns of highly significant decreases in plasma of RM patients in relation to both HP (P<0.001) and NP (P<0.0001) subjects.

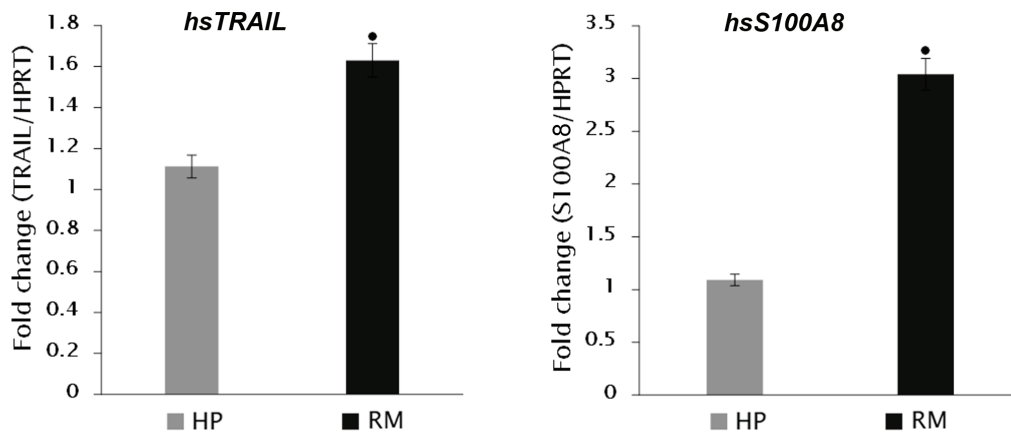


Figure 1. Relative gene expression of *TRAIL* and *S100A8* in placental tissues of Saudi women with RM. \* $P < 0.001$ . *TRAIL*, tumor necrosis factor-related apoptosis-inducing ligand; RM, recurrent miscarriage.

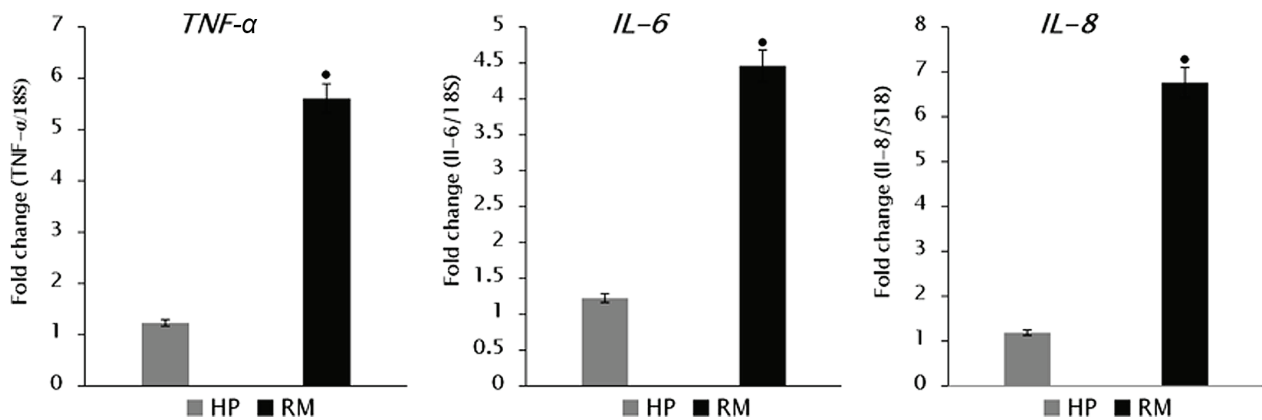


Figure 2. Relative gene expression of *TNF-α*, *IL-6* and *IL-8* in placental tissues of Saudi women with RM. \* $P < 0.001$ . *TNF-α*, tumor necrosis factor- $\alpha$ ; IL, interleukin; RM, recurrent miscarriage.

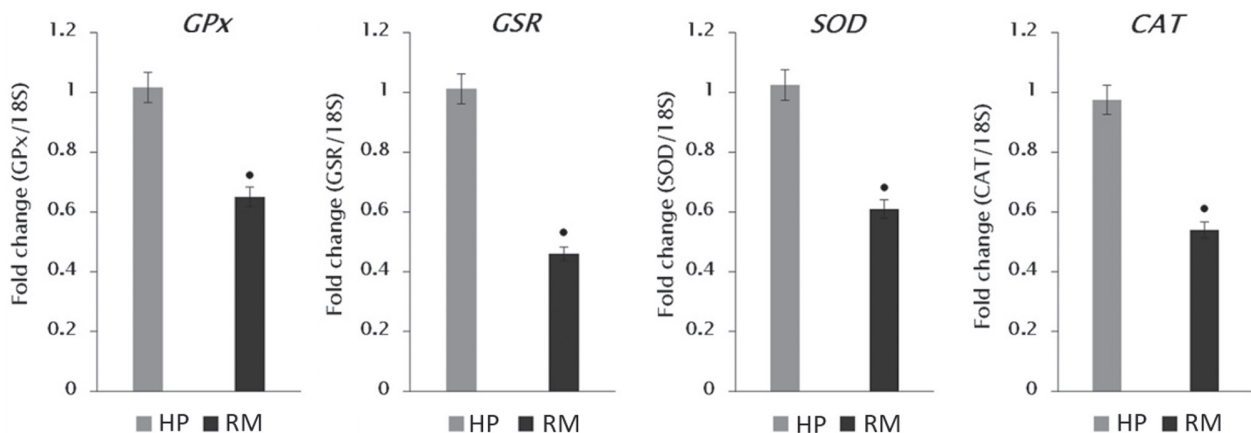


Figure 3. Relative gene expression of *GPx*, *GSR*, *SOD* and *CAT* in placental tissues of Saudi women with RM. \* $P < 0.001$ . *GPx*, glutathione peroxidase; *GSR*, glutathione reductase; *SOD*, superoxide dismutase; *CAT*, catalase; RM, recurrent miscarriage.

Data also indicated that plasma micronutrient levels were moderately, however significantly, lower ( $P < 0.05$ ) in HP subjects compared with the NP ones. Furthermore, the levels of Se, Cu, Zn and Mn were very significantly decreased in placental samples of RM patients compared with those of HP subjects ( $P < 0.0001$ ).

#### Gene expression profiling

*Proinflammatory TNF-α and cytokines as markers of OS and LPO.* All pro-inflammatory cytokines and apoptosis-related genes that were selected for the expression level examination exhibited highly significant increases ( $P < 0.001$ ) in RM placenta samples; *TRAIL* ( $1.63 \pm 0.27$  fold-change), *S100A8*

( $3.04 \pm 0.25$  fold-change), *TNF- $\alpha$*  ( $5.61 \pm 0.26$  fold-change), *IL-6* ( $4.46 \pm 0.348$  fold-change), and *IL-8* ( $6.76 \pm 0.428$  fold-change), compared with HP women (Figs. 1 and 2).

**Expression of antioxidant genes.** The mRNA expression levels (Fig. 3) showed highly significant decreases ( $P < 0.001$ ) of all examined antioxidant genes; *GPx* (fold-change  $0.65 \pm 0.17$ ), *GSR* (fold-change  $0.46 \pm 0.09$ ), *SOD* (fold-change  $0.61 \pm 0.15$ ), and *CAT* (fold-change  $0.55 \pm 0.087$ ), compared with the expression levels observed in the HP placental samples.

## Discussion

The underlying molecular and subcellular mechanism of RM pathogenesis is not fully understood, and thus, its management is difficult and challenging (3). It was previously suggested that RM may be a result of the action of ROS generated during the first trimester of pregnancy. ROS are reactive chemical molecules involved in cell signalling and homeostasis and can be increasingly released in cell processes that involve high demand of oxygen production or consumption (24), such as placenta development. With all the evidence in the literature suggesting the association of OS in RM patients (2,5,10), there is no consensus regarding its negative impact and the underlying mechanism. Therefore, in addition to measuring levels of some OS markers, the current study examined the levels of a comprehensive list of key antioxidants in plasma and placental tissue of RM patients in relation to those of HP and NP participants in order to test the effect of OS. Moreover, the molecular basis of RM remains unclear, thus the present study also attempted to quantify the gene expression of some antioxidant enzymes and selected pro-inflammatory and apoptosis-related genes in placental tissue of RM patients compared with those of HP subjects.

LPO, resulting from ROS, can cause disruption in the membrane lipid bilayer that may inactivate membrane-bound receptors and enzymes, increasing the tissue permeability (25). Current results showed highly significantly increased LPO levels in plasma and placental tissues of RM patients compared with those of HP and NP women. High LPO, as gestation progresses, has been previously reported in placenta and umbilical cord (17). Similar results have also been obtained by others reporting elevated MDA in the serum of RM women and increase has also been observed in the villous decidual tissues of women with early pregnancy loss (26). This has also been supported by other studies that showed increase of MDA levels in serum of failed pregnancy (27) and in RM women (28). The consensus between current findings and previous studies supports the suggestion that increased plasma and placental LPO is a high risk factor causing oxidative cell damage that might result in miscarriage. SOA are the most common oxygen-free radicals created from molecular oxygen by an electron addition. SOA cannot penetrate the lipid membrane and is mostly present within its production compartment (29). The present study demonstrated that the levels of SOA and  $H_2O_2$  generation rates in plasma and placental tissue of RM patients are highly significantly increased compared with those of HP and NP women, and are moderately increased in plasma of HP women compared with those of NP women. SOA generation within and out of the cell is an initial step in ROS formation. Therefore, increased

levels in plasma or placental tissue will result in OS, and may have a direct effect on causing RM. Dismutation of SOA and production of  $H_2O_2$  are catalysed by SOD, which is regarded as the first line of defence.  $H_2O_2$  is not a free radical, however due to its high ability to penetrate biological membranes is considered important by being an intermediate molecule in forming more ROS radicals, such as hydroxyl, which can cause increased damage to biological systems, more than any other radical (30).

During normal pregnancy, the placental OS is balanced by the quenching of ROS through the action of antioxidant enzymes, including SOD, CAT, GPx and GSR, that act together with non-enzymatic antioxidants, such as vitamin C and E, Se, and GSH. This antioxidant control system of OS in normal pregnancy is suggested to be a causative factor of RM when insufficiently functioning. Thus, relating RM to OS may not only be due to increased ROS generation in early gestation, but can also result from insufficient levels of antioxidants required to combat the excessive ROS, both of which have been demonstrated in RM subjects of this study. It is likely that the significantly low GPx activity and downregulation of its gene transcripts that was documented in the plasma and placental tissue of RM patients in current study, was due to the decreased levels of glutathione required for the elimination of excessive ROS in RM women. Similar results have been previously obtained showing that serum GPx activities are significantly decreased in RM patients compared with NP women (26,28), and in HP women compared with NP women (30). Similarly, SOD activity levels in the current study showed significant decreases in both plasma and placental tissue of RM patients compared with those of HP women. Moreover, the mRNA expression levels of SOD were found to be significantly downregulated in trophoblast cells isolated from RM placentas. This conforms with previous studies that have reported similar significantly low levels of SOD in RM patients compared with HP women (28,31,32). This suggests that decreased levels of SOD enzyme in placental tissue might be associated with increased generation of SOA, and thus of OS, that may be involved in the pathogenesis of RM.

The control of  $H_2O_2$  production is the second enzymatic step that plays a vital role against ROS propagation, and CAT and GPx catalyse the conversion of  $H_2O_2$  to  $H_2O$ . Indeed, GPx is the only enzyme able to quench  $H_2O_2$  in mitochondria, since CAT is not expressed there, and requires GSH to convert  $H_2O_2$  into  $H_2O$ . In the present study, CAT activity and its gene transcripts underwent highly significant decreases and downregulation in plasma and placental tissue of RM patients compared with those of HP and NP women. Other studies have also reported significant decreases in serum CAT activity in RM patients compared with HP women (28,32). Thus, it is hypothesized that the decreases in CAT activity were due to its consumption in protecting cells from the harmful effect of  $H_2O_2$ . The present study also profiled differential mRNA expression levels of *GPx*, *GSR*, *SOD* and *CAT* genes, in placental tissue of RM patients compared with those of HP women. The observed downregulation in the expression of all investigated antioxidant enzyme levels might be linked to the detrimental impact of OS on trophoblast cells, whereby leading to ROS generation that may inactivate both *de novo* protein synthesis, as well as gene transcription processes.

GSR and NADPH are important to maintain glutathione in its active reduced state via the redox cycle activity. Under the high OS conditions observed in the present study, GSH levels were lowered, and subsequently the GSSG content was significantly higher leading to a very significant drop in the GSH/GSSG ratio in RM patients compared with HP and NP women. The GSH/GSSG ratio represents the major cellular redox buffer, and therefore is a representative indicator of the redox environment of the cell (33). This observed reduction in GSH/GSSG ratio should have been met with a proportional increase in GSR activity in order to reduce the GSSG back to GSH, thereby maintaining the natural balance of those two parameters. However, the present results indicated significantly low GSR activity in plasma and placental tissue of RM patients compared with those of HP women, which is in agreement with previous studies (28,32,34). This decrease in the enzyme activity might be a direct consequence of OS damage caused by increased GSSG levels, leading to downregulation of GSR gene expression levels presently noted.

Pregnancy comes with physiological changes that can cause reduced bioavailability of some dietary components in addition to the increased demand for various nutrients and micronutrients for developing the foetus (35). Se is known to be significantly low during pregnancy and decreased levels in the blood of HP women have been reported during delivery compared with those of NP women (33,36), pre-eclamptic patients (37), as well as recurrent loss and pre-term delivery patients (38) relative to HP women. Results of the current study indicated a very significant reduction in the plasma and placental tissue of RM patients compared with HP subjects. Changes in Se homeostasis during pregnancy are probably caused by increased oxygen demand in the mother's body and developing foetus. A potential reason behind the increased demand for Se during pregnancy may be the increased mass of erythrocytes in the foetus (38). It has been suggested that the loss of antioxidant capacity might be attributed to low Se, leading to damage to biological membranes and DNA (39), which may cause RM. Cu, Zn and Mn are important cofactors for a number of antioxidant enzymes, including Cu/Zn-SOD and Mn-SOD that may protect the placenta from generating SOA and initiation of OS. In the present study, the levels of Cu, Zn and Mn in plasma and placental tissues of RM patients were significantly lower compared with those of HP women. This is in agreement with previous studies that have reported significant decreases in Cu and Zn levels in serum of women with unexplained RM compared with normal HP women (26). Results of the present study also indicated significantly decreased plasma Cu, Zn and Mn in HP subjects compared with NP women. To this end, several studies have reported that, whereas plasma Zn concentrations decline as the pregnancy progresses (16,40,41), Cu plasma levels increase before returning to normal NP values after delivery (40,41). The increased levels with the progression of pregnancy could be partly related to synthesis of ceruloplasmin, a major Cu-binding protein, due to altered levels of oestrogen (41).

This study also profiled the mRNA expression levels of pro-inflammatory cytokines (*TNF- $\alpha$* , *IL-6* and *IL-8*), *S100A8* and *TRAIL* in RM samples compared with HP women. *TRAIL* is also a TNF member that can induce apoptosis via its apoptotic receptors (42). Apoptosis plays an important

role in normal human placental development and any altered balance between proliferation and apoptosis of villous trophoblast is associated with abnormal pregnancies (43). Increased levels of villous trophoblast apoptosis have been identified in several placental pathologies including RM (44). *TRAIL* and its receptors appear to be differentially expressed in villous placenta. Expression of *TRAIL* and its two decoy receptors DcR1 and DcR2 are localized predominantly in the syncytiotrophoblast, whereas cytotrophoblast cells have been shown to express high levels of DR4 and DR5 (45) that trigger apoptotic signalling via activation of the classic caspase-dependent 'death' pathway (46). It has been previously shown (47) that *TRAIL* and *S100A8* are differentially expressed genes in placental tissue in RM compared with normal pregnancy. The study also suggested that *TRAIL* can be used as a potential predictive biomarker in maternal serum for early pregnancy complications (47). This supports our current findings of highly significant expression levels of both *TRAIL* and *S100A8* in placental tissue of RM compared with HP samples. Similar findings have been previously documented (48) demonstrating that serum *TRAIL* is significantly increased in patients of RM. This suggests that *TRAIL* might have a direct effect on RM and could be used as a predictive biomarker in maternal serum in women with history of RM.

A number of cytokines have been reported essential for the reproductive processes, and it has been suggested that a lower index of T-helper1/T-helper2 (Th1/Th2) immune response supports a physiological pregnancy (49). The increased production of Th1 cytokines, such as *TNF- $\alpha$*  and interferon- $\gamma$  (IFN- $\gamma$ ), compared with the Th2 cytokine has been linked to recurrent spontaneous abortions (50). *TNF- $\alpha$*  is a cytokine that is important in inflammation and has been strongly suggested to have a role in cardiovascular disease pathogenesis. *TNF- $\alpha$*  is also found during most inflammatory stages and can trigger the production of the pro-inflammatory cytokine *IL-6* (51). Proinflammatory cytokines have been suggested as markers for OS and LPO (52).

It has been demonstrated that *TNF- $\alpha$*  acts in synergism with other inflammatory cytokines and stimulates the synthesis and release of prostaglandins inducing uterine contractions and the onset of labour (53). It has also been proposed that *TNF- $\alpha$*  plays an important role in apoptosis. Cytokine receptors, such as tumor necrosis factor-receptor 1 (*TNF-R1*), have also been associated with miscarriage. This is probably due to their role as apoptotic mediators through pro-inflammatory cytokines, such as *TNF- $\alpha$*  (54). *TNF- $\alpha$*  can inhibit the proliferation of human trophoblast cells *in vitro* (55) and the administration of *TNF- $\alpha$*  and IFN- $\gamma$  to normal pregnant mice has been shown to lead to abortion (55). Our findings indicated significant increases in the expression levels of *TNF- $\alpha$*  in placental tissue of RM patients compared with those of HP women. These results are supported by previous findings of the expression of *TNF- $\alpha$*  in peripheral blood cells (56) and the similar high significant increase in serum *TNF- $\alpha$*  in RM compared with HP women (28,32). Taken together, these findings suggest that OS and *TNF- $\alpha$*  have a potential detrimental effect on pregnancy and high levels may play an important role in the pathogenesis of RM.

Cytokines are also known to play an important role in implantation and balance of locally produced pro-inflammatory, and

anti-inflammatory cytokines have been previously suggested to be critical for a successful pregnancy (57). Thus, imbalanced cytokine production may occur in early pregnancy loss. *TNF- $\alpha$*  and *IL-6*, as inflammatory factors, may have immune-modulatory and anti-inflammatory effects in addition to maintaining the maternal-foetal immune tolerance. Their overexpression could induce several pathologies including RM (58-61). The current results demonstrated that the expression levels of *TNF- $\alpha$*  and *IL-6* in placental tissues of RM patients were significantly higher than those of HP women. To this end, it has been reported that any disturbance in the balance of *TNF- $\alpha$*  and *IL-6* could eventually lead to miscarriage (62,63). It has been previously shown that *TNF- $\alpha$*  and *IL-6* concentrations in murine trophoblasts with recurrent spontaneous abortion are significantly higher than in control group (63). This suggests that *TNF- $\alpha$*  and *IL-6* might play an important role in the embryonic development as well. *IL-8* is a pro-inflammatory cytokine that is often associated with inflammation and its secretion is increased by OS, thereby causing recruitment of inflammatory cells and inducing further increase in OS mediators (64). The present results showed upregulated mRNA expression of *IL-8* gene in the placental tissue of RM women compared with HP samples. It is thus proposed that the increased mRNA expression levels of pro-inflammatory cytokines in placental tissue could be considered as another causative factor of the pathogenesis of RM.

In conclusion, the current results confirm several previous studies linking OS and RM. Antioxidant and pro-oxidant status might be a useful tool in estimating the risk of OS and associated diseases and they are becoming increasingly important. This is because they may assist in designing strategies for the prevention and management of OS. Such strategies include the use of micronutrient supplements to maintain effective antioxidant defence in women at risk of RM.

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## Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

## Authors' contributions

MAMAS, YAAS and AFA conceived and designed the study. MAMAS, MMA and AFA were responsible for the methodology. MAMAS, FSA, HKG and YAAS were responsible for the data validation. YAAS, HKG and MAMAS performed formal analysis. MAMAS, YAAS, MMA and HKG were involved in the investigative aspects of the study. YAAS and MMA provided resources. MAMAS and FSA wrote and prepared the original draft. MAMAS wrote, reviewed and edited the manuscript. YAAS was responsible for the project

administration and funding acquisition. All authors read and approved the final manuscript.

## Ethics approval and consent to participate

Ethics approval was obtained from the Ethics Review Committee of the College of Applied Medical Sciences of King Saud University (CAMS 27-34/35; Riyadh, Kingdom of Saudi Arabia). Signed consent forms were obtained from all subjects who participated in this research.

## Patient consent for publication

Not applicable.

## Competing interests

The authors declare that they have no competing interests.

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