Oxidative stress and antioxidant defenses in pregnant women

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Objectives: Oxidative stress (OS) is defined as an imbalance in the production of reactive oxygen species and the capacity of antioxidant defenses. The objective of this work was to investigate OS and antioxidant capacity in pregnant women.

Methods: Parameters of the oxidative status and antioxidant capacity in serum and whole blood were evaluated in thirty-nine women with normal pregnancy.

Results: The assessment of antioxidants indicated an increase in superoxide dismutase and catalase activities ($P < 0.05$ and $P < 0.01$) and a decrease in ascorbic acid levels and the total content of sulfhydryl ($P < 0.05$ and $P < 0.001$). Additionally, when the pro-oxidant system was investigated we found an increase ($P < 0.01$) in malondialdehyde and no significant change ($P > 0.05$) in protein carbonylation.

Discussion: This study demonstrates that there is a change in the pro-oxidant and antioxidant defenses associated with body and circulation changes that are inherent to the pregnancy process.

Keywords: Oxidative stress, Antioxidants, Normal pregnancy, Whole blood, Serum

Introduction

The principal causes of oxidative stress (OS) are reactive oxygen species (ROS), which may be broadly defined as derivatives of molecular oxygen.¹ Small amounts of ROS, including hydroxyl radicals ($\bullet\text{OH}$), superoxide anions (O_2^-), and hydrogen peroxide (H_2O_2), are constantly generated in aerobic organisms in response to both external and internal stimuli.² They are very transient due to their high chemical reactivity, which also leads to lipid peroxidation, oxidation of some enzymes, and massive protein oxidation and degradation.³

ROS attack the phospholipids of cell membranes and react with polyunsaturated fatty acids to form lipid peroxides, resulting in cellular injury, and they have been proposed to be a promoter of lipid peroxidation and the endothelial cell dysfunction that is commonly associated with disorders of pregnancy.⁴ The prevention of lipid peroxidation is an essential

process in all aerobic organisms as products from this process can cause DNA damage. To complicate the situation further, an increase in lipid peroxidation and decreased antioxidant protection frequently occurs. As a result, epoxides may spontaneously react with nucleophilic centers in the cell and covalently bind to DNA, RNA, and protein.⁵

Low levels of ROS are indispensable in many biochemical processes, including intracellular messaging to control cell differentiation, cell progression, cell growth, apoptosis,⁶ immunity, and defense against microorganisms.⁷ In contrast, high doses and/or inadequate removal of ROS result in OS, which may cause severe metabolic malfunctions and damage to biological macromolecules.⁸

The removal of these ROS is performed by antioxidant enzymes, such as catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase, glutathione transferase, glutathione reductase, and antioxidant molecules, such as glutathione, that work in concert within the cell to metabolize these noxious forms of oxygen into innocuous substances.⁹ We can define OS as an imbalance between the

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production of ROS and reactive nitrogen species and the ability of antioxidant defenses to scavenge them. It can arise from increased production of ROS and/or a decrease in antioxidant capacity. Among the ROS, there are several free radicals that can induce a great deal of cellular damage by acting on proteins and lipids.

Many studies have shown that there is increased OS during pregnancy, and oxidative damage is increased in pregnancies complicated by disorders such as pre-eclampsia.¹⁰ Currently, there is only scattered information regarding baseline levels of OS and antioxidant status in women with uncomplicated pregnancies.¹¹ The cause of increased maternal OS during pregnancy is unknown; nevertheless, accumulating evidence suggests that the placenta plays an important role in the production of OS.^{12,13}

Recent evidence suggests that OS might have an influence during the entire reproductive span of women's life and even thereafter. It might play a significant role during pregnancy, normal parturition, and initiation of preterm labor. Oocyte maturation, ovarian steroidogenesis, ovulation, implantation, formation blastocyst, luteolysis, and luteal maintenance in pregnancy have been shown to be influenced by OS. Pregnancy is characterized by dynamic changes in multiple body systems resulting in increased basal oxygen consumption.⁹

Studies have also reported the idea that even normal pregnancy (NP) may induce a state of mild OS and lead to peroxidation of lipids, namely arachidonic acid, because plasma F₂-isoprostane levels were found to be higher in severe pre-eclampsia, mild pre-eclampsia, normotensive pregnancy, and non-pregnancy. Pregnant women showed higher levels of isoprostanes, which is important because the free radical-mediated arachidonic acid products might have a significant role in the maintenance of pregnancy.¹⁴

One of the major F₂-isoprostanes, 8-iso-prostaglandin F_{2α} (8-iso-PGF_{2α}), is increased in several syndromes associated with oxidant injury, and estimation of isoprostanes is now regarded as a biomarker for *in vivo* measurement of OS. In relation to antioxidants, the lipid-adjusted alpha and gamma tocopherol levels showed a significant decrease in mid- to late-stage pregnancy compared to non-pregnancy.¹⁵

Because OS can play both physiological and pathological roles in the progression and outcome of pregnancy, we attempted to characterize the profiles of antioxidant response of normal pregnant women at the Federal University of Santa Maria Hospital, Brazil, by determining enzymic antioxidants (SOD, CAT), non-enzymic antioxidants (ascorbic acid and total sulfhydryl content) as well as lipid peroxidation, and protein oxidation profile in the third trimester of pregnancy.

Materials and methods

Patients and controls

Thirty-nine women from the Federal University of Santa Maria Hospital (UFSM, Santa Maria, RS, Brazil) were included in the study. None of these women had a history of other relevant disease states. Women with unfavorable outcome of pregnancy were excluded from this investigation. Each woman was carefully selected by a clinical evaluation, and participants were separated into two patient groups. The control group (CG) consisted of total $n = 9$ non-pregnant, healthy female volunteers who did not present any disease and had not been submitted to any pharmacological therapy during the previous month. The second group, designated NP, consisted of 30 pregnant women with a singlet pregnancy, normal diastolic and systolic blood pressure (mmHg), and ages ranging from 27 to 45 years. A detailed description of the groups is given in Table 1.

None of the subjects in the study were smokers or were using antioxidant supplementation. All women of the NP group were being treated with ferrous sulfate and were in the third-gestational trimester (Table 1). Women in the CG were not using drugs of any kind. The Human Ethics Committee of UFSM approved the project under number 86-2006, and subjects gave informed consent.

Collection of blood samples

Eighteen milliliters of peripheral blood was collected by venous puncture from the ante-cubital region. Blood was collected into three vacutainer plastic tubes containing anticoagulant sodium citrate 3.2%, EDTA (K3E), and an additional tube with serum clot activator without anticoagulant for the separation of blood fractions.

Hematological parameters

Tests were run to determine the erythrocyte count, hemoglobin, and hematocrit of both control individuals

Table 1 Clinical characteristics of the study population

	Control group	Normal pregnancy group
Age (years)	35.16 ± 1.52	35.80 ± 1.32
Gestational age (weeks/range)	–	29.8 ± 0.71
Systolic blood pressure (mmHg)	112.5 ± 3.16	110.0 ± 2.07
Diastolic blood pressure (mmHg)	72.90 ± 2.49	70.66 ± 2.17
Erythrocytes (RBC) (10 ⁶ /mm ³)	4.38 ± 0.07	3.89 ± 0.11**
Hemoglobin (HGB) (g/dl)	13.26 ± 0.25	11.98 ± 0.29**
Hematocrit (HCT) (%)	40.0 ± 0.76	35.0 ± 0.87***
<i>n</i>	8–9	14–30

Results are expressed as the mean value ± SEM ($n = 39$). Data were analyzed by unpaired *t*-test. ** $P < 0.01$; *** $P < 0.001$.

and patients using an ABX Micros 60 automatic cell counter (ABX Horiba, Kyoto, Japan) Measurements were taken using the impedance and photometry method and provided 18 reading parameters.

Determination of lipid peroxidation

We used the protocol previously described by Jentzsch that uses the formation of thiobarbituric acid reactive substances (TBARS) during an acid-heating reaction to determine an index of lipid peroxidation.¹⁶ Briefly, 200 μ l of serum samples was mixed with 1.5 ml of 0.2 M orthophosphoric acid, 250 μ l of 0.1 M of thiobarbituric acid, and 550 μ l of distilled water. The samples were then heated in a boiling water bath for 45 minutes. TBARS were determined by the absorbance at 532 nm and were expressed as malondialdehyde (MDA) equivalents (nmol MDA/ml of serum).

Determination of the total sulfhydryl content

Total-plasma sulfhydryl groups were determined as described by Ellman.¹⁷ In short, an aliquot of plasma was reacted with 250 μ M DTNB (5,5'-dithiobis-(2-nitrobenzoic acid) in a final volume of 2 ml of potassium phosphate at 500 mM, and the absorbance was read at 412 nm. A standard curve with cysteine (0.01–0.5 μ mol SH/ml) was constructed to calculate the total sulfhydryl concentration in the samples.

Assay of protein carbonyls

The concentration of protein carbonyls was measured by reaction with 2,4-dinitrophenyl hydrazine (DNPH) following the method of Levine *et al.*¹⁸ Assays were performed in duplicate for both the DNPH-treated samples and blanks. A serum aliquot containing approximately 5 mg of protein was diluted in 20 mM HEPES buffer at pH 7.2 and then placed in four tubes. A half-volume of 10% trichloroacetic acid (TCA) was added to precipitate the protein. Tubes were centrifuged at 3700 rpm for 5 minutes in a tabletop centrifuge, and the supernatant was discarded. Two tubes then received 250 μ l of 10 mM DNPH in 2 M HCl, and the same volume of 2 M HCl was added to the other two tubes. The pellets were resuspended using a pipette tip and vigorous vortexing. The reaction was left in the dark at room temperature for 30 minutes and vortexed after 15 minutes. The protein was precipitated by addition of 250 μ l of 10% TCA followed by centrifugation for 5 minutes at 3700 rpm. After discarding the supernatant, the pelleted protein was washed two times with 1.0 ml of 1:1 ethyl acetate and ethanol at room temperature. Pellets were broken up with a pipette tip and vortexing and then centrifuged at 3700 rpm for 5 minutes before discarding the supernatant. After the third wash, the pellets were incubated at 37°C for 10 minutes in 1.5 ml of 80 mM phosphate-buffered saline buffer at pH 8.0 with 2% sodium dodecyl sulfate, and 0.5%

EDTA, and the sample was then analyzed at 370 nm. The results are expressed as nmol of protein carbonyl per mg of protein.

SOD and CAT activities

Citrated whole blood was used to determine SOD and CAT activities. SOD activity was assayed by measuring the inhibition of 1 mM adrenaline auto-oxidation by absorbance at 480 nm using a glycine buffer (50 mM, pH 10.2) as described by Bannister and Calabrese.¹⁹ CAT activity was determined with the method of Aebi by measuring the rate of catalysis of 30 mM H₂O₂ at 240 nm in 50 mM potassium phosphate buffer at pH 7.0.²⁰

Ascorbic acid (vitamin C) quantification

Vitamin C analysis was performed using the method described by Lloyd *et al.*²¹ with the following modifications. The serum samples were de-proteinized with 15% TCA, using 400 μ l of sample and 800 μ l of 15% TCA. The sample was then mixed by vortexing for 15 seconds and centrifuged at 1800 $\times g$ for 15 minutes. Then 400 μ l of supernatant was transferred to another tube, and 120 μ l of 2,4-dinitrophenyl hydrazine-thiourea-CuSO₄, DTC (a solution of 5 ml of 2,4-dinitrophenylhydrazine at 0.1 mol/l in 4.5 M H₂SO₄ with 0.25 ml of 0.66 mol/l thiourea and 0.25 ml of 0.027 mol/l cupric sulfate) was added. Samples were vortex mixed for 10 seconds, closed with filmed paper, and incubated at 60°C for 60 minutes. After incubation, samples were transferred to an ice bath for 10 minutes and 600 μ l of 12 M H₂SO₄ was added. Samples were vortex mixed for 10 seconds and analyzed at 520 nm. All samples were measured in duplicate. Calibration curves were prepared with L(+)-ascorbic acid (Vetec Quimica Fina Ltda, Rio de Janeiro, Brazil) following the same procedure as used with the samples and were used to determine the concentration of ascorbic acid in the samples.

Protein determination

Protein was determined with the Coomassie blue method according to Bradford²² with bovine serum albumin as the standard.

Statistical analysis

Data were analyzed by parametric unpaired *t*-test with a level of significance of 5%. All results were statistically analyzed and were found to follow a normal distribution when analyzed by the Kolmogorov-Smirnov (KS) normality test. Values of *P* < 0.05 were regarded as statistically significant. Correlation was evaluated by Pearson's test, and *P* < 0.05 was considered significant.

Results

Hematological parameters

A significant difference between the two groups of women was evident with regard to hematological

parameters measured in peripheral blood. The erythrocyte count, hemoglobin, and hematocrit all were significantly decreased in the NP group compared with the CG (Table 1).

Determination of lipid peroxidation

To verify the oxidative damage in membrane lipids, MDA content was measured, and it was found to be significantly enhanced in the NP group compared with the CG as shown in Fig. 2.

Determination of total sulfhydryl content

A significant difference in levels of the total sulfhydryl content of pregnant women was found. The plasma total sulfhydryl content was decreased in the NP group ($0.36 \pm 0.02 \mu\text{mol/ml}$, SEM) with $P < 0.001$ when compared with the CG (0.67 ± 0.04 , SEM) (Table 2).

Determination of carbonylation of serum proteins

To assess the oxidative damage to proteins, the carbonyl groups were evaluated. It was found that proteins in the NP group had a larger carbonyl content than in the CG, but this difference was not statistically significant ($P > 0.05$) (Fig. 1).

Table 2 Values of antioxidant substances in blood from NP and controls

	Control group	Normal pregnancy group
Superoxide dismutase (U SOD/mg protein)	38.0 ± 2.09	$43.5 \pm 1.47^*$
Catalase (units/mg of protein)	13.75 ± 1.43	$17.15 \pm 0.33^{**}$
Ascorbic acid ($\mu\text{mol/l}$)	4.25 ± 0.75	$2.77 \pm 0.25^*$
Total sulfhydryl content ($\mu\text{mol SH/ml}$ of plasma)	0.67 ± 0.04	$0.36 \pm 0.02^{***}$
<i>n</i>	7–9	11–30

Results are expressed as the mean value $\pm$ SEM for $n = 7$ –30. Independent experiments performed in triplicate.

Data were analyzed by unpaired *t*-test. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

Figure 1 Box-whisker plot of protein carbonyl content in serum obtained from the control group (C) and the normal pregnant group (NP). Data represent the mean value $\pm$ standard error of the mean for 23 individuals. $P > 0.05$ for NP when compared with the C.

Figure 2 Box-Whisker plot of MDA content in serum obtained from the control group (C) and the normal pregnant group (NP). Data represent the mean value $\pm$ standard error of the mean for 27 individuals. $^{**}P < 0.01$ for NP when compared with the C.

SOD and CAT activities

The levels of SOD and CAT activity are presented in Table 2. SOD activity was increased in the NP group (43.5 ± 1.47 U/mg protein, SEM) with $P < 0.05$ when compared with the CG (38.0 ± 2.09 , SEM). The CAT activity was also increased in the NP group (17.15 ± 0.33 U/mg protein, SEM) with $P < 0.01$ when compared with the CG (13.75 ± 1.43 , SEM) (Fig. 2). SOD and CAT activities demonstrated a positive correlation as illustrated in Fig. 3 ($P < 0.05$).

Plasma vitamin C quantification

This exogenous antioxidant showed a statistically significant difference between groups. The plasma vitamin C was decreased in the NP group ($2.77 \pm 0.25 \mu\text{mol/l}$, SEM) with $P < 0.05$ when compared with the CG (4.25 ± 0.75 , SEM) (Table 2).

Discussion

Our results showed that the amounts of MDA, a pro-oxidant, were increased in women during the third trimester of gestation. In parallel with this change, there was an increase in the amounts of the antioxidants SOD and CAT during the same period. As previously reported,^{9,12,13} these changes might be related to the process of pregnancy itself and the placental circulation that plays an important role in OS during this period.

The human placenta is classified as hemochorial, and the establishment of maternal placental circulation is influenced by trophoblastic invasion. Extravillous trophoblastic invasion transforms the low-caliber, high-resistance spiral arteries into high caliber with low resistance.²³ This remodeling of the placental vascular bed causes invasion of the cytotrophoblast cells on the maternal spiral arteries that feed the intervillous space, which involves the endothelium and muscular tunica media. The arteries lose their smooth muscle and become sinusoids that lack any contractility, which results in an oxidative burst.²⁴

The maternal-placental interface is formed by syncytiotrophoblasts (STBs). These cells are the likely

Figure 3 Pearson's correlation between SOD and CAT in citrated whole blood ($P < 0.05$).

origin of the factors that disturb the maternal endothelium. Significant quantities of syncytiotrophoblast microparticles (STBMs), a product of the normal turnover and renewal of the syncytial surface by apoptosis, circulate in maternal blood during pregnancy.²⁵ The STBMs may lead to activation of maternal neutrophils and subsequent production of ROS mediated by nicotinamide adenine dinucleotide phosphate (NADPH) oxidase.²⁶

The NADPH oxidase is an important enzyme that generates superoxide radicals localized in the placenta syncytial microvillous membrane.^{27,28} This enzyme may play a role in placental lipid peroxidation by generating increased amounts of the superoxide radicals, which could explain the increased activity of the enzyme SOD observed in this work. We propose that the STBMs may be contributing to the formation of ROS during pregnancy when they are released into the maternal-placental interface.

Other researchers have reported that pregnancy itself may produce OS as a result of increased metabolic activity in the placental mitochondria and a reduced scavenging power of antioxidants. Increased levels of plasma thiols and placental lipid peroxides in pregnant women have been reported.^{29,30} In another study, researchers showed that $\text{PGF}_{2\alpha}$ levels were significantly higher during the NP period than during non-pregnancy. The researchers suggested that an increased level of $\text{PGF}_{2\alpha}$ might be necessary in the NP process and parturition.³¹

Other reports have demonstrated that plasma levels of lipid hydroperoxides and/or TBARS, CAT, and SOD increase with advancing gestational age.^{9,32} Another cross-sectional study has shown that MDA levels are significantly increased in NP compared to non-pregnancy.³³ In addition, pregnancy was

associated with successively increasing levels of 8-iso- $\text{PGF}_{2\alpha}$ with advancing gestational age, but lipid-adjusted alpha and gamma-tocopherol levels decreased in the same period.¹⁵

This is important because substantial increases in OS have been hypothesized to lead to acute pregnancy complications³⁴ or spontaneous abortion.³⁵ Additionally, under pro-thrombotic conditions related to both thrombophilia and pregnancy, hemostatic responses, and microthrombi are generated, which could lead to ischemic conditions and mechanical stress in blood vessels and the consequent generation of ROS.^{36,37}

Still, there are factors that are associated with endothelium injury and coagulation resulting from inflammation, which increase the activity of leukocytes and result in oxidative bursts. Thus, a cascade of events initiated by OS leads to a series of responses, such as vasoconstriction, membrane oxidation, or further pro-coagulation, each of which includes further generation of ROS.³⁸

Our results are consistent with other reports in the literature^{9,32,33} that suggest that the increase in SOD activity should be followed by increased CAT activity. The increase in SOD activity is an important antioxidant defense system that involves the catalysis of the superoxide anion (O_2^-) by SOD and the formation of peroxide (H_2O_2), and oxygen. The peroxide, in turn, is converted to water by CAT.

Another cause for the increase of pro-oxidants in NP that is reported here could be the iron supplementation these women were undergoing. We could hypothesize that iron supplementation is responsible for stress. Iron supplementation during pregnancy is currently a routine practice for most pregnancy care centers, particularly in developing countries. Reports

indicate that iron is a free radical generator.³⁹ Indeed, the chemical properties of Fe render it a potential hazard within the organism, and ferrous iron (Fe^{2+}) in small, non-protein shielded chelates can catalyze the production of oxygen radicals or ROS, which in turn can lead to peroxidation and radical chain reaction with molecular damage.⁴⁰

Iron is also involved in the Fenton reaction, generating a hydroxyl radical ($\bullet\text{OH}$) that is particularly toxic. In fact, it is one of the most reactive forms of the free radicals. Thus, iron is significantly involved in toxic cellular injury⁴¹ and is probably responsible for a portion of the formation of ROS in pregnant women.

Together, these results and the knowledge of the importance of the balance between the generation of oxygen species and antioxidant systems in our body, both in healthy individuals and those with pathology, suggest that more studies should be conducted. The objective of these future endeavors should be to determine other important enzymes, and their possible mechanisms of action, that could be involved in OS in uncomplicated gestational processes.

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