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Mitochondrial manganese superoxide dismutase mRNA expression in human chorioamniotic membranes and its association with labor, inflammation, and infection

NANDOR GABOR THAN[1], ROBERTO ROMERO[1,2,3], ADI L. TARCA[1,3,4], SORIN DRAGHICI[4], OFFER EREZ[1,2], TINNAKORN CHAIWORAPONGSA[1,2], YEON MEE KIM[1], SUN KWON KIM[1], EDI VAISBUCH[1,2], & GERARD TROMP[3]

[1]*Perinatology Research Branch, NICHD, NIH, DHHS, Detroit, Michigan, USA,* [2]*Department of Obstetrics and Gynecology,* [3]*Center for Molecular Medicine and Genetics, and* [4]*Department of Computer Science, School of Medicine, Wayne State University, Detroit, Michigan, USA*

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Abstract

Objective. Human parturition is characterized by the activation of genes involved in acute inflammatory responses in the fetal membranes. Manganese superoxide dismutase (Mn SOD) is a mitochondrial enzyme that scavenges reactive oxygen species (ROS). Mn SOD is up-regulated in sites of inflammation and has an important role in the down-regulation of acute inflammatory processes. Therefore, the aim of this study was to determine the differences in Mn SOD mRNA expression in the fetal membranes in patients with term and preterm labor (PTL) as well as in acute chorioamnionitis.

Study design. Fetal membranes were obtained from patients in the following groups: (1) term not in labor ($n = 29$); (2) term in labor ($n = 29$); (3) spontaneous PTL with intact mebranes ($n = 16$); (4) PTL with histological chorioamnionitis ($n = 12$); (5) preterm prelabor rupture of the membranes (PPROM; $n = 17$); and (6) PPROM with histological chorioamnionitis ($n = 21$). Mn SOD mRNA expression in the membranes was determined by quantitative real-time reverse transcription-polymerase chain reaction.

Results. (1) Mn SOD mRNA expression was higher in the fetal membranes of patients at term in labor than those not in labor (2.4-fold; $p = 0.02$); (2) the amount of Mn SOD mRNA in the fetal membranes was higher in PTL than in term labor or in PPROM (7.2-fold, $p = 0.03$; 3.2-fold, $p = 0.03$, respectively); (3) Mn SOD mRNA expression was higher when histological chorioamnionitis was present both among patients with PPROM (3.8-fold, $p = 0.02$) and with PTL (5.4-fold, $p = 0.02$) than in patients with these conditions without histological chorioamnionitis; (4) expression of Mn SOD mRNA was higher in PTL with chorioamnionitis than in PPROM with chorioamnionitis (4.3-fold, $p = 0.03$).

Conclusion. The increase in Mn SOD mRNA expression by fetal membranes in term labor and in histological chorioamnionitis in PTL and PPROM suggests that the fetus deploys anti-oxidant mechanisms to constrain the inflammatory processes in the chorioamniotic membranes.

Keywords: *Fetal gender, gene expression, preterm delivery, preterm labor, preterm prelabor rupture of the membranes, reactive oxygen species, scavenger*

Introduction

Human parturition involves a 'common pathway' that is activated by physiological signals in term labor and by pathological processes in preterm labor [1]. This common pathway is clinically manifested in the increased contractility of the myometrium, the ripening and remodeling of the cervix and the activation of the maternal decidua and the chorioamniotic membranes [1–3]. The chorioamniotic membranes undergo complex morphological and biochemical changes [4] that are associated with the activation of genes involved in acute inflammatory responses [5–7]. Indeed, our group has reported

Correspondence: Roberto Romero, MD, Perinatology Research Branch, NICHD/NIH/DHHS, Wayne State University/Hutzel Women's Hospital, 3990 John R, Box 4, Detroit, Michigan 48201, USA. Tel: +1-313-993-2700. Fax: +1-313-993-2694. E-mail: prbchiefstaff@med.wayne.edu

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microarray experiments which have revealed that human term labor is characterized by an acute inflammation gene expression signature in the fetal membranes in the absence of evidence of clinical and histological chorioamnionitis [8].

In addition to a pro-inflammatory response in the chorioamniotic membranes, our microarray experiments also revealed that labor at term is associated with the 2.6-fold up-regulation of *SOD2*, which encodes manganese superoxide dismutase (Mn SOD) [8]. These results were consistent with a prior microarray experiment performed by our group, in which we found differential expression of *SOD2* in the fetal membranes in spontaneous preterm labor (PTL) with intact membranes when compared with preterm prelabor rupture of the membranes (PPROM) [9]. Moreover, this gene was differentially up-regulated in the fetal membranes of patients with histological chorioamnionitis [9].

Mn SOD is a member of an evolutionarily conserved iron/Mn superoxide dismutase family, which localizes to the mitochondria and scavenges reactive oxygen species (ROS) generated by oxidative phosphorylation [10–13]. ROS represent a double-edged sword; in low concentrations, they participate in physiological intracellular signaling pathways and defense mechanisms against infections [14–16]. However, in higher concentrations, ROS may lead to enhanced oxidative stress and play an important role in aging and the pathophysiology of various diseases, such as atherosclerosis, cardiovascular diseases, cancer, spontaneous abortion, preeclampsia, or PTL [16–44]. In addition, ROS activate nuclear factor (NF)-κB and enhance the subsequent expression of cyclooxygenase-2 (COX-2), which leads to the orchestration of the inflammatory pathway [39,45] and the propogation of parturition [46]. Inflammatory processes then may lead to the increased generation of ROS and oxidative stress [47]. On the contrary, inflammatory processes up-regulate Mn SOD that in turn down-regulates inflammation and oxidative stress by suppressing NF-κB, activator protein (AP)-1, and mitogen activated protein kinase (MAPK) pathways. Thus, Mn SOD has been proposed to have an important function in protection against cell injury mediated by inflammation and oxidative stress [48–52]. Remarkably, Mn SOD has been implicated as a longevity-associated gene, which has a higher expression in females than in males [28,43,53].

On the basis of our previous microarray experiments [8,9], *SOD2* seems to be involved in the processes leading to term and preterm parturition in the fetal membranes. In order to confirm these microarray results and to reveal the differences in *SOD2* expression in the chorioamniotic membranes between term and preterm parturition and with acute inflammation, we performed quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR) on samples taken from normal pregnant women at term with or without labor and from patients presenting with PTL or PPROM, with or without histological signs of chorioamnionitis. Indeed, our study confirmed increased Mn SOD mRNA expression in the fetal membranes in term and preterm parturition, and chorioamnionitis. Furthermore, we report gender differences in the differential expression of Mn SOD in chorioamnionitis that may influence the redox balance and the inflammatory processes in the fetal membranes.

Materials and methods

Study design and population

This cross-sectional study was designed to examine the differential expression of *SOD2* in the fetal membranes of patients in the following groups: (1) term not in labor ($n = 29$); (2) term in labor ($n = 29$); (3) PTL ($n = 16$); (4) PTL with histological chorioamnionitis ($n = 12$); (5) PPROM ($n = 17$); and (6) PPROM with histological chorioamnionitis ($n = 21$). Patients presenting with medical complications, multiple pregnancies, fetal congenital or chromosomal abnormalities were excluded. All patients provided written informed consent prior to the collection of samples. The collection and utilization of samples for research purposes were approved by the Institutional Review Boards of the *Eunice Kennedy Shriver* National Institute of Child Health and Human Development (NIH/DHHS) and Wayne State University. Many of these samples have been employed to study the biology of inflammation and labor in both normal pregnant women, and those with complicated pregnancies.

Definitions

Pregnancies were considered normal when there was no evidence of medical, obstetrical, or surgical complications, resulting in a term delivery ($\geq$37 gestational weeks) of a healthy neonate whose birthweight was above the 10th percentile for gestational age [54]. Normal pregnant women in the term not in labor group underwent elective Cesarean section. Labor was defined as the presence of regular uterine contractions that occurred at a frequency of at least 2 in every 10 min associated with cervical changes which led to either preterm ($<$37 weeks of gestation) or term ($\geq$37 weeks of gestation) delivery. PPROM was diagnosed $<$37 weeks of gestation in the presence of vaginal pooling and a positive nitrazine or ferning test documented by a sterile speculum examination at admission [55]. Trans-abdominal

amniocentesis was performed under ultrasonographic guidance at the discretion of the treating physician in a subset of patients presenting with PTL or PPROM for the determination of the microbiologic state of the amniotic cavity. Amniotic fluid was transported to the laboratory in a capped plastic sterile syringe and cultured for aerobic and anaerobic bacteria as well as for genital mycoplasmas. White blood cell count, glucose concentration and Gram-stain for microorganisms were performed in amniotic fluid shortly after collection.

Placental histopathological examinations

Chorioamniotic membranes containing maternal decidua were obtained from placentas delivered by spontaneous labor or Cesarean section at the Hutzel Women's Hospital (Wayne State University, Detroit, MI). Tissue samples were fixed in 10% neutral buffered formalin overnight and embedded in paraffin. Five micrometer paraffin sections were stained with hematoxylin and eosin and examined using bright-field light microscopy. Histopathological examinations were performed by pathologists blinded to the clinical information based on the diagnostic criteria previously described [56]. Histological chorioamnionitis was diagnosed in the presence of acute inflammation in the fetal membranes, chorionic plate of the placenta, or umbilical cord using previously described criteria [57,58].

Total RNA extraction

Fetal membranes were dissected from placentas, rinsed thoroughly with a sterile ice-cold phosphate buffered saline solution (Sigma Chemical Company, St Louis, MO), cut into small pieces, placed in RNAlater solution (Ambion, Austin, TX), and stored at 4°C for no longer than 2 weeks. Total RNA was isolated with a modification of the standard guanidinium isothiocyanate–cesium chloride method [9,59]. Briefly, tissues were homogenized with a PRO200 rotor-stator homogenizer (Pro Scientific, Monroe, CT) in the presence of 4 mol/l guanidinium isothiocyanate, 0.1 mol/l mercaptoethanol, 0.5% sarkosyl, and 5 mmol/l sodium citrate (pH 7). Solid CsCl was added to the samples in a final concentration of 0.25 g/ml, and then the samples were pelleted by ultracentrifugation according to the protocol. RNA pellets were resuspended and extracted with chloroform: isoamylalcohol, and the RNA was precipitated with ethanol and glycogen (Roche Molecular Biochemicals, Indianapolis, IN) as a carrier. Before the first use, the RNA was pelleted and resuspended in water that contained RNasin (Promega Corp, Madison, WI).

Quantitative real-time reverse transcription-polymerase chain reaction

Total RNA (2.5 μg) from each sample and a positive control sample were reverse transcribed using Superscript II reverse transcriptase, random hexamer primers and oligo(dT) primers (Invitrogen Life Technologies, Rockville, MD). The standard curve was run with Mn SOD mRNA and 18S ribosomal RNA to determine the quantity of cDNA needed for an approximate cycle threshold (C_t) of 25. Subsequently, cDNA derived from an equivalent of 75 ng RNA from each sample were run in triplicate on 96-well plates to obtain technical replicates for both the target and reference assays. A 'calibrator' sample was run in triplicate in all plates to account for plate effects. In addition, a negative control containing no RNA and 12.5 ng of human genomic DNA were also tested in duplicates. Samples from the study groups were randomly allocated on the plates; the Mn SOD and 18S rRNA assays were run with the same allocation on the parallel plates. The qPCR reactions were assembled based on the TaqMan Universal PCR Master Mix protocol (Applied Biosystems), using the 18S rRNA TaqMan gene expression assay (Hs99999901_s1; Applied Biosystems, Foster City, CA) for the quantification of the housekeeping gene and self-designed primers and probe (forward primer: 5′-TTCTGGACAAACCTCAGCCC-3′; reverse primer: 5′-CGTTTGATGGCTTCCAGCA-3′; probe: 5′-CCCTTTGGGTTCTCCACCACC GTT-3′) for Mn SOD mRNA. Data were collected by the ABI Prism 7700 Sequence Detection System (Applied Biosystems).

Statistical analysis

Demographic and clinical characteristics of the study groups were compared using the Pearson's chi-square test and the Fisher's exact test for proportions, and the Mann–Whitney *U* test for non-normally distributed continuous variables using SPSS version 12.0 (SPSS, Chicago, IL). Quantitative RT-PCR data were analyzed using the R statistical software [60].

Gene expression levels were profiled in multiple sample groups by qRT-PCR experiments, using between 12 and 29 samples per group. The RT reactions were run on 96-well plates. Samples from the study groups were randomly allocated on the plates, and the target gene and the 18S reference assay were run in parallel on each given plate. Each reaction was repeated either two or three times to obtain technical replicates for both the target assay and the reference assay. A 'calibrator' patient sample was placed on all plates to account for eventual plate effects. Briefly, the $\Delta\Delta$ method [61,62] was used to

generate an outcome variable, *Y*, which is a surrogate of the $\log_2$ concentration of the target gene in each patient sample, corrected already for potential plate effects.

A linear model was employed in which *Y* values were fitted using the *Group* variable and the gestational age as predictors without including the interaction term between these two variables. The coefficients of the two predictors in the linear model were estimated together with their significance *p*-values.

The outcome variable, *Y*, included also a positive constant to render the *Y* values positive so that large values correspond to high expression. A false discovery rate adjustment [63] of resulting *p*-values was performed to account for all parallel tests. For each pair-wise comparison, the *Group* effect was considered significant, if the adjusted *p*-values were <0.05 and the magnitude of change was at least two-fold (one C_t unit difference). For the gestational age effect, adjusted *p*-values <0.05 were considered significant.

Results

Demographic, clinical, and histopathologic data

Demographic and clinical characteristics of the study groups are displayed in Table I. The diagnosis of chorioamnionitis was stratified based on the presence of either maternal or fetal inflammatory response. Among patients with PPROM and histological chorioamnionitis, seven had a marked maternal inflammatory response, two had fetal inflammatory response, and 12 had both. Among patients with PTL and chorioamnionitis, a maternal inflammatory response was diagnosed in one case, whereas 11 patients had both maternal and fetal inflammatory responses. Amniocentesis was performed in case of 14 patients with PPROM and 10 patients with PTL. Among these, positive amniotic fluid culture was detected in 64.2% (9/14) of patients with PPROM and in 40% (4/10) of patients with PTL. Microorganisms detected in amniotic fluid cultures are presented in Table II.

Manganese superoxide dismutase mRNA expression is increased in term and preterm labor

Mn SOD mRNA expression did not change with gestational age in any of the groups. Mn SOD mRNA expression in the fetal membranes was 2.4-fold higher in women with spontaneous labor than in those who were not in labor at term ($p=0.02$; Figure 1).

Patients with PTL without histological chorioamnionitis had a higher Mn SOD mRNA expression in the fetal membranes than that of women in spontaneous labor at term (7.2-fold, $p=0.03$; Figure 2A) and those with PPROM without histological chorioamnionitis (3.2-fold, $p=0.03$; Figure 2B).

Increased manganese superoxide dismutase mRNA expression with the presence of histological chorioamnionitis and fetal female gender

Histological chorioamnionitis was associated with higher Mn SOD mRNA expression among patients with PPROM (3.8-fold, $p=0.02$; Figure 3A) as well as those with PTL (5.4-fold, $p=0.02$; Figure 3B) when compared with those without histological chorioamnionitis. Among patients with histological chorioamnionitis, Mn SOD mRNA expression in the fetal membranes was higher in patients with PTL than in those with PPROM (4.3-fold, $p=0.03$; Figure 4).

There was a significant interaction between fetal gender and the presence of histological chorioamnionitis in patients with PPROM and PTL overall ($p=0.033$). The presence of histological chorioamnionitis resulted in a 13.4-fold increase in Mn SOD mRNA expression in patients with PPROM ($p=0.047$, Figure 5) and in a 12-fold increase in patients with PTL ($p=0.047$, Figure 6) who delivered a female neonate. A similar pattern in the changes of Mn SOD mRNA levels was observed in patients with PTL and PPROM who delivered a male neonate; however, these differences were not significant (PPROM: 2.3-fold, $p=0.2$, Figure 5; PTL: 3.5-fold, $p=0.2$, Figure 6).

Discussion

Principal findings of this study

(1) Mn SOD mRNA expression in the fetal membranes was higher in women in labor than in those not in labor at term; (2) Patients with PTL had a higher Mn SOD mRNA expression in the fetal membranes than women at term in labor or patients with PPROM; (3) Histological chorioamnionitis was associated with increased Mn SOD mRNA expression in the fetal membranes among patients with PTL or PPROM; (4) Among patients with histological chorioamnionitis, Mn SOD mRNA expression was higher in those with PTL than in those with PPROM; (5) Among patients with PPROM or PTL, female fetuses had a greater increase in Mn SOD mRNA expression in the chorioamniotic membranes in chorioamnionitis than males.

Reactive oxygen species, oxidative stress, and mitochondrial manganese superoxide dismutase

Oxygen is consumed for the generation of adenosine triphosphate (ATP) in the mitochondria of aerobic

Table I. Demographic and clinical characteristics of the study population.

	Term not in labor ($n=29$)	Term in labor ($n=29$)	p-value	PPROM ($n=17$)	PPROM with chorioamnionitis ($n=21$)	p-value	PTL ($n=16$)	PTL with chorioamnionitis ($n=12$)	p-value
Maternal age (years)*	29 [22–33]	22 [19–25]	0.001	27 [22–31]	27 [22–34]	NS	24 [18–28]	21 [18–30]	NS
Gestational age at diagnosis (weeks)*	–	–		31 [30–32.9]	30 [25.6–31.4]	NS	26 [23.1–31.8]	28.9 [25.5–32]	NS
Gestational age at delivery (week)*	39.1 [38.7–39.4]	40 [39.6–40.4]	<0.001	31.7 [30.1–33.1]	31 [29.1–32.3]	NS	29.6 [24.2–33.1]	29.1 [25.6–32]	NS
Diagnosis to delivery interval (days)*	–	–		1 [0–5]	3 [1–10]	NS	3 [0.3–5]	1 [0–6.5]	NS
Gravidity*	3 [2.5–4.5]	2 [1–4]	NS	3 [1.5–6]	4 [2.5–5.5]	NS	3 [1–4]	3 [1.3–3]	NS
Parity*	2 [1–2]	1 [0–2]	<0.05	2 [0–3.5]	2 [1–4]	NS	1 [1–2]	0.5 [0–1]	NS
Birth-weight (g)*	3370 [3160–3765]	3220 [3100–3580]	NS	1530 [1365–1850]	1700 [995–1920]	NS	1040 [705–1870]	1045 [670–1683]	NS
Female fetus (%)†	41.4 (12/29)	65.5 (19/29)	NS	29.4 (5/17)	33.3 (7/21)	NS	25 (4/16)	58.3 (7/12)	NS
Positive amniotic fluid culture (%)†,‡	–	–		0 (0/8)	46.2 (6/14)	0.051	0 (0/11)	30 (3/10)	NS
Ethnic origin (%)§			NS			NS			NS
African-American	65.5 (19/29)	93.1 (27/29)		82.4 (14/17)	90.5 (19/21)		87.5 (14/16)	83.3 (10/12)	
Caucasian	13.8 (4/29)	3.4 (1/29)		17.6 (3/17)	9.5 (2/21)		12.5 (2/16)	16.7 (2/12)	
Other	20.7 (6/29)	3.4 (1/29)		0 (0/17)	0 (0/21)		0 (0/16)	0 (0/12)	

Values are presented as median [interquartile range] or percentage.
NS, Statistically not significant.
Comparisons between two groups were performed with the *Mann–Whitney test, †Fisher's exact test and §Pearson's chi-square test.
‡PPROM ($n=8$); PPROM with chorioamnionitis ($n=14$); PTL ($n=11$); PTL with chorioamnionitis ($n=10$).

Table II. Microorganisms detected in positive amniotic fluid cultures.

	PPROM with chorioamnionitis ($n=14$)	PTL with chorioamnionitis ($n=10$)
Ureoplasma ureolyticum	3	1
Mycoplasma hominis	1	2
Gardnerella vaginalis	2	–
Lactobacillus species	–	1
Peptostreptococcus species	1	–
Prevotella species	1	–
Candida albicans	1	–

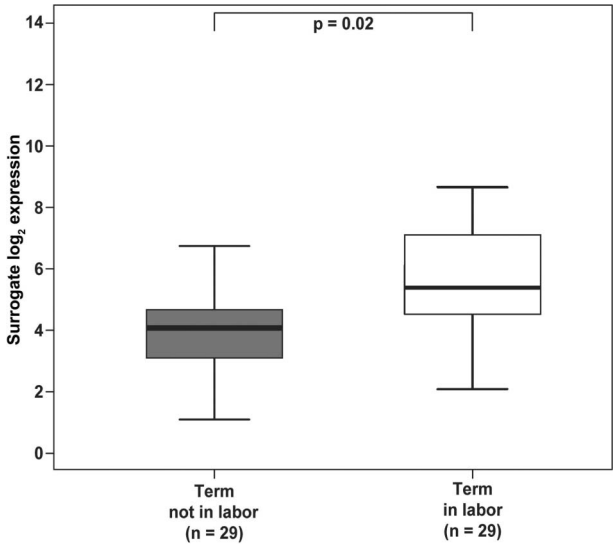

Figure 1. Mn SOD mRNA expression was 2.4-fold higher in the fetal membranes of women with spontaneous labor than in women who were not in labor at term ($p=0.02$). Groups are represented by different colors. *Y*-axis represents units of $-\Delta C_t$ with an arbitrary zero point, so that each unit measures a two-fold change.

cells to produce water in a reaction catalyzed by cytochrome C oxidase [16,23,43]. However, the mitochondrial electron transport chain is also a major generator of ROS, as ≈1–3% of the oxygen forms superoxide anion (O_2^-) by both complexes I and III, which are mainly released into the mitochondrial matrix [16] and then are converted to hydrogen peroxide either spontaneously or by Mn SOD [16,23,43]. Hydrogen peroxide is normally detoxified by glutathione peroxidase to form water, but its increased amounts are also converted to additional free oxygen radicals, such as hydroxyl radical ($^{\bullet}OH$) [16]. Besides in the mitochondria, ROS may also be generated by nicotine adenine dinucleotide phosphate oxidases, cyclooxygenases, or peroxisomes [16,32].

ROS are continuously produced and have beneficial effects in low concentrations, being secondary messengers in intracellular signaling pathways and having an important role in gene transcription, cell proliferation, metabolism, and apoptosis [14,16]. ROS also have a pivotal role in host defense against infectious agents, as being essential for phagocyte killing during the respiratory burst [15,16]. Moreover, ROS have been implicated in the regulation of a variety of physiological processes in human reproduction, such as follicular development, cyclic endometrial changes, fertilization, or embryo development [33].

In contrast, the overproduction of ROS has deleterious effects by causing oxidative damage to proteins, lipids, and DNA [28,43,64]. Consequently, oxidative stress plays a key role in cellular aging and has been implicated in the pathophysiology of a broad range of diseases, such as atherosclerosis, cardiovascular diseases, hypertension, cancer, and diabetes [16–19,23,28,32,43,44]. Oxidative stress may also lead to an altered fate of trophoblastic cells, and is implicated in the pathophysiology of spontaneous abortions, embryopathies, fetal growth restriction, preeclampsia, and PTL [20–22,24–27, 29–31,33–42,65–67]. An inverse relationship between mitochondrial ROS production and longevity was also observed in different species [68–72].

SODs are antioxidant enzymes that protect cells against the harmful effects of ROS and maintain physiological redox balance [10–13,16,73]. There are four known classes of SODs that differ by (1) their protein structure, (2) the redox-active metal ions (Cu/Zn, Fe, Mn or Ni) in their catalytic center and (3) their intra- or extra-cellular localization [11]. Mn and Fe SODs have a similar protein fold, whereas the Cu/Zn and Ni SODs are structurally distinct [11]. Mn SOD was first discovered in *Escherichia coli*, and later it was found to be highly conserved in all species from bacteria to humans [12,74]. In humans, Mn SOD is encoded by *SOD2* which is located on chromosome 6q25.3. The encoded polypeptide subunit is synthesized in the cytosol and imported into the mitochondrial matrix, where its N-terminal mitochondrial targeting sequence is cleaved [10]. In eukaryotes, Mn SOD is a homotetramer with the Mn-binding sites at the interfaces [11,13].

Mn SOD scavenges mitochondrial ROS and has a pivotal role in the first line of protection against oxidative stress in the mitochondria [10,12,13]. It is not surprising that increased Mn SOD expression protects against pro-apoptotic stimuli and ischemic damage and results in an extended life-span in yeast, whereas the knockout of Mn SOD results in severe mitochondrial disease, neonatal lethality in mice, and in a critically reduced life-span in yeast and *Drosophila* [16,44,70,72,75–80]. Deficient activity of Mn SOD is associated with several human diseases, including cancers, aging, progeria, transplant rejection, or asthma [78].

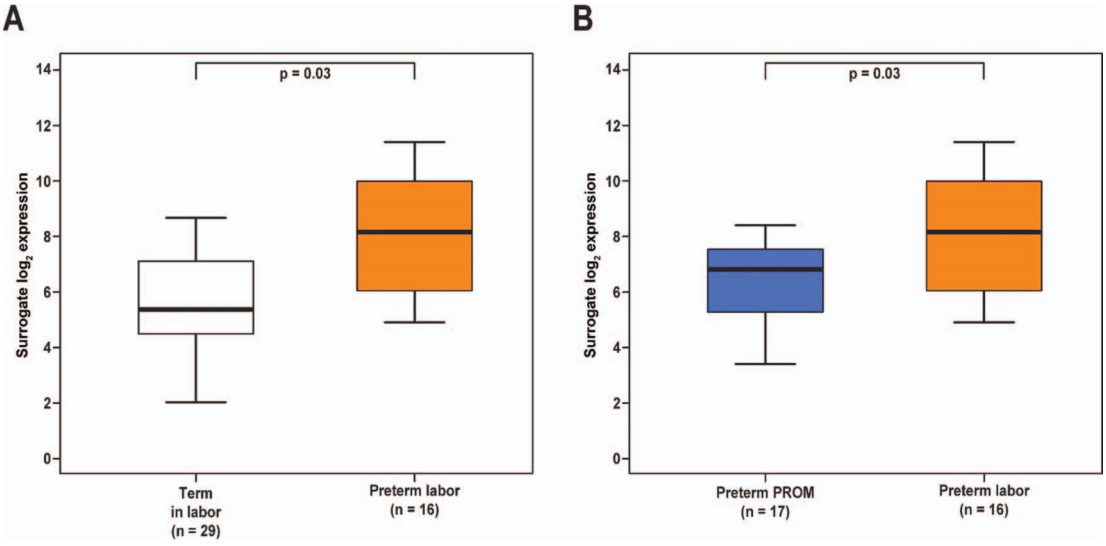

Figure 2. The amount of Mn SOD mRNA was higher in the fetal membranes of patients presenting with spontaneous preterm labor without histological chorioamnionitis than (A) in women with spontaneous labor at term (7.2-fold, $p = 0.03$) or (B) in patients presenting with preterm PROM without histological chorioamnionitis (3.2-fold, $p = 0.03$).

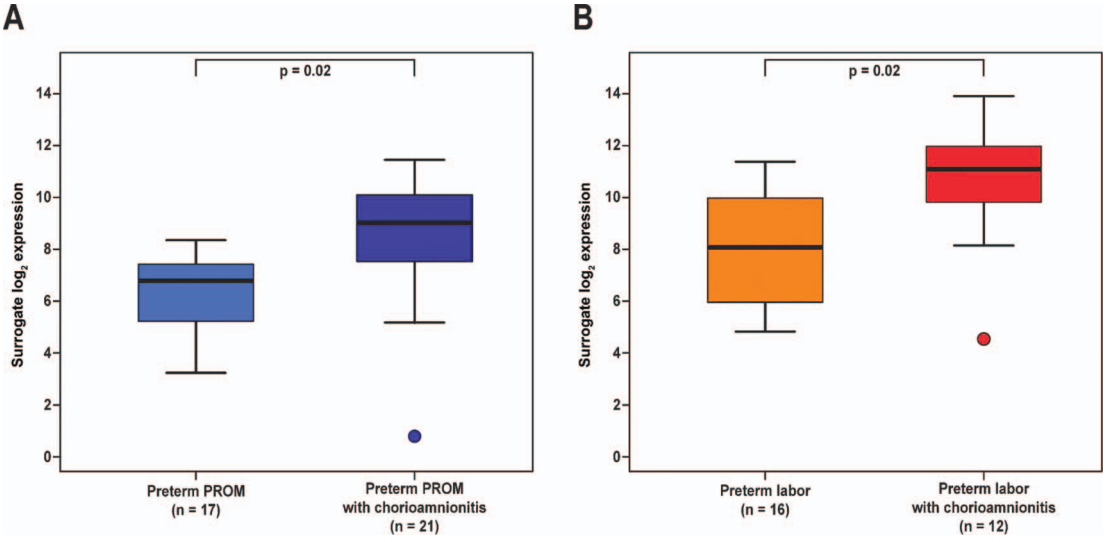

Figure 3. Mn SOD mRNA expression in the fetal membranes was increased when histological chorioamnionitis was present both in patients with (A) preterm PROM (3.8-fold, $p = 0.02$) or (B) spontaneous preterm labor (5.4-fold, $p = 0.02$).

Increased manganese superoxide dismutase expression upon oxidative stress and inflammation

Recent data provided evidence that ROS physiologically regulate immune signaling pathways, and that increased oxidative stress results in the exaggerated activation of the redox-sensitive AP-1, NF-κB, c-Jun N-terminal kinase and MAPK signal transduction pathways, which subsequently leads to an enhanced generation of proinflammatory molecules, such as tumor necrosis factor (TNF)-α, interleukin (IL)-8, or COX-2, which then orchestrate inflammation [39,45,81–89]. Exaggerated inflammatory processes then, in turn, lead to the increased generation of ROS, oxidative stress, and tissue injury [47,90].

Of importance, Mn SOD expression is significantly up-regulated in sites of inflammation by both ROS and pro-inflammatory mediators [48,50,91–94]. ROS may stimulate Mn SOD expression through TNF-α or toll-like receptor (TLR)-4 but NF-κB activation and *N*-acetylcysteine can reliably decrease this effect [87,93]; lipopoly-saccharide (LPS) up-regulates Mn SOD through CD14 and TLR-4 [94]; and TNF-β, IL-1α, and IL-1β may also stimulate the overexpression of Mn SOD mRNA [48]. In fact, the *SOD2* promoter contains consensus binding sites for transcription factors such as AP-1,

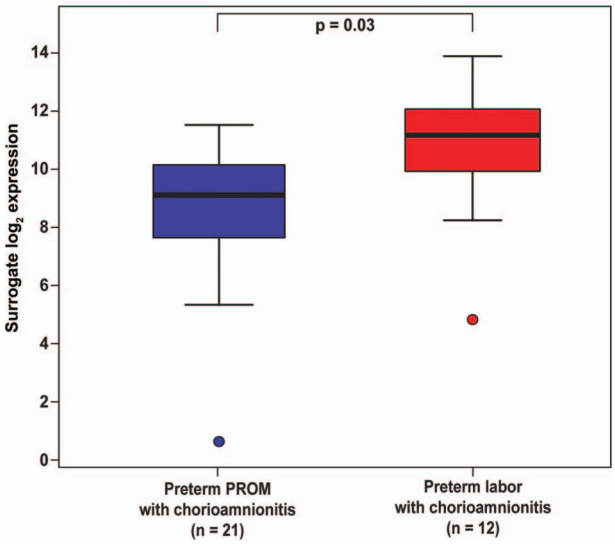

Figure 4. Mn SOD mRNA expression in the fetal membranes was higher in patients with spontaneous preterm labor and chorioamnionitis than in those with preterm PROM and chorioamnionitis (4.3-fold, $p = 0.03$).

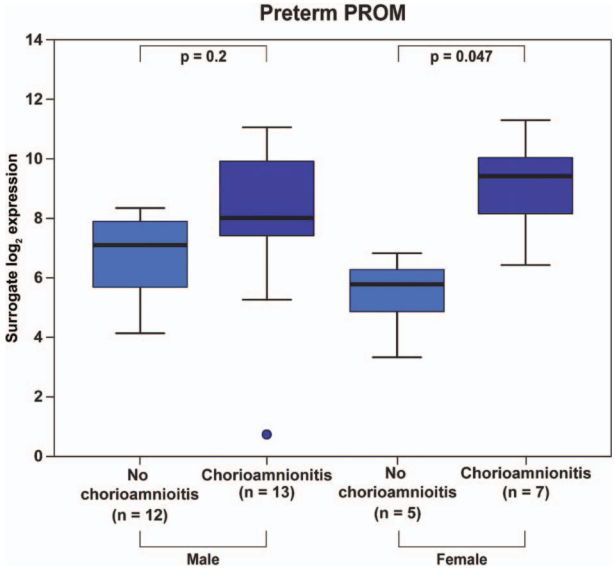

Figure 5. Among patients with preterm PROM, those who delivered a female neonate had a higher increase (13.4-fold, $p = 0.047$) in Mn SOD mRNA expression upon chorioamnionitis than those who delivered a male neonate (2.3-fold, $p = 0.2$).

AP-2, NF-κB, SP-1, which can be responsible for its overexpression upon cellular redox changes and inflammation [95–97].

It has been suggested that increased ROS production triggers cellular anti-oxidant defence mechanisms, and up-regulation of Mn SOD expression may be protective against cell injury caused by inflammation and oxidative stress [48,49]. Indeed, treatment with anti-oxidants can effectively block innate and adaptive immune responses through the following mechanisms: (1) inhibiting LPS-induced TLR-4 and

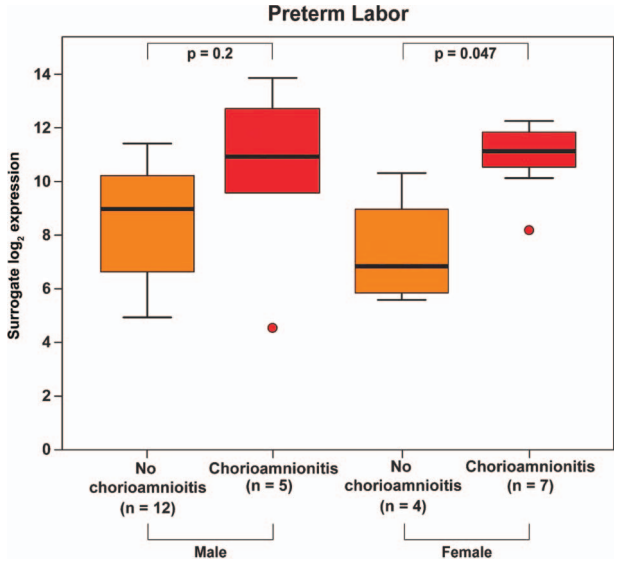

Figure 6. Among patients with spontaneous preterm labor, those who delivered a female neonate had a higher increase (12-fold, $p = 0.047$) in Mn SOD mRNA expression upon chorioamnionitis than those who delivered a male neonate (3.5-fold, $p = 0.2$).

NF-κB signaling and IL-8 transactivation [87]; (2) suppressing LPS-stimulated generation of proinflammatory cytokines (TNF-α, IL-1β) and ROS (NO_2^- and O_2^-) [98]; (3) reducing cytotoxic T-cell response and allograft rejection [99]; or (4) generating antigen-specific hyporesponsiveness of T-cells [89]. In fact, the overexpression of Mn SOD suppresses TNF-induced apoptosis, LPS-induced activation of NF-κB pathway as well as TNF-mediated activation of AP-1, stress-activated c-Jun protein kinase, and MAPK [51,52]. As immunoregulatory proteins, such as galectin-1, indole 2,3-dioxygenase, IL-6, and transforming growth factor-β, contain redox-sensitive cysteine residues [86,100–104] and their activity is highly dependent on the redox status [12,86], Mn SOD may also have important immunoregulatory effects through modification of the activity of these proteins.

Up-regulation of manganese superoxide dismutase mRNA expression in the fetal membranes upon term and preterm labor

Our study has shown that Mn SOD mRNA expression in the fetal membranes was 2.4-fold higher in women in labor than in those not in labor at term. This finding is in agreement with our previous microarray data [8], which showed the 2.6-fold up-regulation of *SOD2* in the fetal membranes from women in labor compared with non-laboring women at term [8]. Mn SOD protein was shown to be ubiquitously produced by the fetal membranes and the myometrium in another study [105], revealing an intense Mn SOD immunostaining of the amnion and the decidua, a moderate immunostaining of

extravillous trophoblasts and a faint immunostaining of the chorion, myocytes, and myometrial endothelial cells [105]. However, the strength of the immunostaining and Mn SOD enzyme activity did not differ between tissue samples collected before or after the onset of labor either in the fetal membranes or in the myometrium [105].

Of importance, Mn SOD is also expressed by the syncytiotrophoblast and endothelial and stromal cells of the human villous placenta [105,106]. Mn SOD protein synthesis was shown to be up-regulated in placentas delivered after a short labor (<5 h) compared with non-laboring controls. In parallel, evidence for oxidative stress, activation of the p38 MAPK and NF-κB pathways, and an increase in COX-2 and proinflammatory cytokines in the same tissues was also presented [107]. Therefore, Cindrova-Davies et al. suggested that this up-regulation of Mn SOD may be a transient compensatory mechanism for coping with the increased oxidative stress during term parturition [107]. Our findings support this concept and suggest that the acute inflammatory process in the fetal membranes during term parturition is associated with the up-regulation of Mn SOD expression [8] to compensate for oxidative stress. However, functional experiments are needed to prove this hypothesis.

Our study has also revealed that Mn SOD mRNA expression in the fetal membranes was 7.2-fold higher in spontaneous PTL than in spontaneous labor at term in cases without histological signs of chorioamnionitis. As Mn SOD mRNA expression did not change with gestational age in any of the groups, this finding cannot simply be the consequence of the difference in the gestational ages in the two groups. Our findings also confirm the differences between physiological and pathological processes involved in term and preterm parturition [1] and also suggest that the extent of oxidative stress and inflammatory processes in PTL are greater than that of in term parturition. Evidence in support of this concept was reported by Keelan et al. who have shown a marked 4.5–6- and 2.8–5-fold increase in the concentrations of proinflammatory cytokines (IL-1β, IL-6, IL-8) in the amnion and the choriodecidua in spontaneous PTL compared with spontaneous labor at term [108] and suggested it as evidence for the exaggerated inflammatory activation of the membranes in PTL [1,3,109]. This interpretation is consistent with our previous observations by measuring the concentration of a wide range of inflammatory cytokines and chemokines in amniotic fluid [110–120]. As Mn SOD mRNA expression was higher (3.2-fold) in PTL than in PPROM in our study, it may also imply significant differences in the redox status and the pathological process in the fetal membranes between these two syndromes [1,3,109,121–125].

Up-regulation of manganese superoxide dismutase mRNA expression in the fetal membranes upon chorioamnionitis

This study has shown that the amount of Mn SOD mRNA was significantly higher in the fetal membranes of those patients with PTL or PPROM who had chorioamnionitis. This result is in good agreement with the above discussed phenomenon on the up-regulation of Mn SOD mRNA expression in sites of inflammation [48,50,91–94]. Of importance, our finding that Mn SOD mRNA expression was significantly higher in PTL than in PPROM either with (4.3-fold, $p = 0.03$) or without (3.2-fold, $p = 0.03$) inflammation in the fetal membranes suggests a deficient anti-oxidant production in PPROM compared with PTL, which may lead to a higher oxidative stress and may contribute to the different clinical presentation of these obstetrical syndromes. These observations are consistent with our previous reports that the concentrations of proinflammatory cytokines, such as IL-1β, TNF-α, IL-6 and chemokines such as IL-8 are greater in PTL with intact membranes with intra-amniotic infection/inflammation than in women with PPROM with intra-amniotic infection/inflammation [111,114, 118,126,127].

Although intra-amniotic infection and inflammation are a major cause of spontaneous preterm birth (PTB), and cytokines, chemokines and innate immune molecules play a central role in the mechanisms of disease in cases of PPROM and PTL [1,3,5,9, 108,109,121,124,125,128–134], recent studies have revealed that there are fundamental differences in the pathogenesis of these two syndromes [9,124, 125,132]. As reviewed by Woods [135], ROS formation and/or anti-oxidant depletion during pregnancy may lead to extensive changes in collagen metabolism, consequent tissue damage, and premature rupture of the chorioamniotic membranes [135], and that the supplemental administration of anti-oxidant vitamins C and E may decrease PPROM [135,136]. Interestingly, women who subsequently presented with PPROM had higher first trimester amniotic fluid concentrations of isoprostanes, an oxidative stress biomarker, than those women who delivered at term, suggesting the association between an early oxidative stress and PPROM [137].

A synergistic effect of the presence of histological chorioamnionitis and fetal gender on manganese superoxide dismutase mRNA expression in the fetal membranes

Another important finding of this study is that among patients with PTL and PPROM, female

fetuses had a greater increase in Mn SOD mRNA expression in their chorioamniotic membranes in histological chorioamnionitis than male fetuses. Indeed, there is accumulating evidence in humans and rats that Mn SOD expression is higher in females than in males [28,43]. This observation was attributed to the regulation of Mn SOD expression by estrogens [28,43], which has a critical role in human reproduction. Indeed, Mn SOD expression undergoes cyclic changes during the menstrual cycle in the human endometrium, where it has a pivotal role in protecting decidual stromal cells from ROS during decidualization and in the establishment of pregnancy [43,53,138,139].

SOD2 is one of the most important genes associated with longevity [12], and according to the free radical and mitochondrial theories of aging [17,18], Mn SOD has been implicated in the relatively longer lifespan of females compared with males [28,43]. This may also have obstetrical implications because a higher incidence of PTB and PPROM has been observed among women delivering male newborns compared with female newborns in different populations [140]. In a recent review, Di Renzo et al. reported that male gender is an independent risk factor for adverse pregnancy outcome with a higher incidence of PTB and worse outcome in the perinatal period [141].

Moreover, Clifton et al. have previously shown that fetal gender has a fundamental influence on both endocrine and immune regulation during pregnancies complicated by asthma [142–145]. It is tempting to speculate that the gender difference in the regulation of Mn SOD mRNA expression with inflammation in the fetal membranes may have an impact on pregnancy outcome through the regulation of redox status and inflammatory processes.

Conclusions

Our results suggest a potential compensatory antioxidant mechanism for Mn SOD in the fetal membranes when exposed to oxidative stress and inflammation, and imply that gender differences may influence the redox balance and immunoregulation within the fetal membranes in chorioamnionitis.

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