

Increased expression of immune-related genes in leukocytes of patients with diagnosed gestational diabetes mellitus (GDM)

Marzena Wojcik¹, Andrzej Zieleniak¹, Monika Zurawska-Klis^{2,3}, Katarzyna Cypryk^{2,3} and Lucyna Alicja Wozniak¹

¹Department of Structural Biology, Faculty of Biomedical Sciences and Postgraduate Education, Medical University of Lodz, 90-752 Lodz, Poland; ²Diabetology and Metabolic Diseases Department, Medical University of Lodz, 92-213 Lodz, Poland; ³Diabetological Medical Center “OmniMed”, 93-338 Lodz, Poland

Corresponding author: Marzena Wojcik. Email: marzena.wojcik@umed.lodz.pl

Abstract

Compelling evidence indicates that the immune system is linked to metabolism in gestational diabetes mellitus (GDM), but factors participating in these processes still are awaiting identification. Inducible nitric oxide synthase, encoded by the *NOS2* gene, and surfactant protein D, encoded by the *SFTPD* gene, have been implicated in diabetes. We investigated *NOS2* and *SFTPD* mRNA levels in leukocytes obtained from 125 pregnant women with ($n=87$) or without (control group; $n=38$) GDM, and, in turn, correlated their expression with clinical parameters of subjects. Leukocytes were isolated from the blood of pregnant women and *NOS2* and *SFTPD* expression in these cells was determined by quantitative real time PCR (qRT-PCR). Univariate correlation analyses were performed to assess an association between leukocyte *NOS2* and *SFTPD* expression and clinical characteristics of patients. qRT-PCR experiments disclosed significantly increased leukocyte *NOS2* and *SFTPD* mRNA levels in hyperglycemic GDM patients ($P < 0.05$). In the entire study group, there were significant positive associations of leukocyte *NOS2* and *SFTPD* mRNAs with C-reactive protein. Additionally, transcript level of *SFTPD* also correlated positively with fasting glycemia and insulin resistance. This study demonstrates that an impaired glucose metabolism in GDM may be predominant predictor of leukocyte *NOS2* and *SFTPD* overexpression in diabetic patients. Furthermore, alterations in the expression of these genes are associated with glucose metabolism dysfunction and/or inflammation during pregnancy. In addition, these findings support the utilization of leukocytes as good experimental model to study a relationship between immune-related genes and metabolic changes in women with GDM, as well as to assess the potential mechanisms underlying these alterations.

Keywords: Gestational diabetes mellitus (GDM), immune-related genes, leukocytes, *NOS2*, *SFTPD*

Experimental Biology and Medicine 2016; 241: 457–465. DOI: 10.1177/1535370215615699

Introduction

Gestational diabetes mellitus (GDM), defined as glucose intolerance with onset or first recognition during pregnancy, is the most prevalent metabolic disorder occurring during pregnancy that affects from 3% to 17% of all pregnancies, depending on racial and ethnic group, as well as the diagnostic and screening criteria.¹ GDM is associated with increased risk of adverse maternal and perinatal outcomes, including preeclampsia, preterm delivery, cesarean section, macrosomia, and respiratory distress syndrome, among other.² There is also an increased risk of complications after pregnancy such as the development of type 2 diabetes mellitus (T2DM) and cardiovascular disease in the mothers^{3,4} and developing obesity, metabolic syndrome,

and T2DM in the children during childhood and adolescence.⁵ Although the precise mechanisms underlying GDM are still not completely understood, a close immune-metabolic relationship with inflammatory cytokines regulating metabolic homeostasis has been found.^{6,7} Additionally, recent performed placental and blood transcriptome analysis in diabetic Chinese patients has shown GDM-dependent alterations in the expression of numerous immune-related genes, supporting the hypothesis of a linkage of immune system to GDM.⁸ Among a panel of known immune-related genes, inducible nitric oxide (NO) synthase (iNOS), also termed as *NOS2*, and surfactant protein D (SP-D), a collagenous calcium-dependent lectin (or collectin), have drawn much attention as molecules not only related to inflammation but also with glucose and lipid metabolism.

NOS2, encoded by the *NOS2* gene, is one of three different isoforms of the enzyme NO synthase (EC 1.14.13.39) that catalyzes the conversion of L-arginine to NO and L-citrulline with the use of several cofactors, including reduced nicotinamide-adenine-dinucleotide phosphate (NADPH), flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), and tetrahydrobiopterin (BH₄).⁹ NO is a free radical implicated in modulating numerous physiological functions such as vasodilation, learning, memory, platelet aggregation, immune function, and angiogenesis.⁹ However, excessive NO production leads to peroxynitrite (ONOO⁻) formation as a result of its reaction with superoxide anion (O₂^{-•}). Peroxynitrite can affect cell signaling by mediating the oxidation and nitration of various biomolecules.^{10,11} Since iNOS is responsible for NO overproduction in response to inflammatory stimuli in metabolic tissues, it is considered as an important pathogenic factor in the development of disorders associated with a low grade chronic state of inflammation, including obesity-linked insulin resistance and β -cell failure.^{12–14} Indeed, it has been shown that mice lacking iNOS are protected from developing obesity-induced insulin resistance and exhibit improved glucose tolerance.¹² Furthermore, selective iNOS expression in liver results in hepatic insulin resistance, hyperglycemia, and hyperinsulinemia.¹³ Additionally, NOS2 transgenic mice develop type 1 diabetes mellitus (T1DM) with β -cell DNA damages by NO produced in these cells.¹⁴

SP-D, encoded by the *SFTPD* gene, is an important regulator of the innate immunity that mediates clearance of pathogens and modulates the inflammatory response.¹⁵ Moreover, SP-D exhibits anti-inflammatory and antioxidant properties through a decrease of the expression of some pro-inflammatory cytokines and a reduction of lipid peroxidation, respectively.^{16,17} More recently, SP-D has emerged as a factor that appears to link to metabolic disorders. In fact, decreased serum SP-D concentration has been reported in subjects with obesity and T2DM and it negatively correlates with fasting and post-load serum glucose.¹⁸ Additionally, *SFTPD* gene polymorphisms have been found to associate with insulin resistance and T2DM.¹⁹

Although a great deal of information concerning the relationship between immune-related genes such as *NOS2* and *SFTPD*, and diabetes including T1DM and T2DM has been gained in recent years, no progress has been made toward understanding their significance in leukocytes obtained from GDM patients. Therefore, the objective of the present study was to investigate leukocyte *NOS2* and *SFTPD* gene expression in patients with GDM and normal glucose tolerant (NGT) pregnant women in the third trimester of gestation. Subsequently, correlational analyses were used to dissect whether and how any variability in the expression of the aforementioned genes could be explained by the variability in clinical parameters of pregnant women. We focused on leukocytes as an experimental cellular model because these cells are well-known to be involved in regulating inflammatory processes and their use allows to circumvent the invasive and non-ethical procedures involved in taking metabolic tissue samples from pregnant women.

Materials and methods

Study population

A total of 125 Caucasian pregnant women (87 with GDM and 38 with normal glucose tolerance, NGT) were recruited and studied. All women underwent a 75-g, 2 h oral glucose tolerance test (OGTT) at 24–28 weeks' gestation or later if it was not possible during this period at the Outpatient Diabetological Clinic in Lodz, Poland. GDM was diagnosed if one or more of plasma glucose levels were elevated during OGTT, according to the criteria set by the Polish Diabetes Association (the modified World Health Organization [WHO] criteria).²⁰

None of the patients or the controls had family history of diabetes in first-degree relatives, GDM in a previous pregnancy, diabetes diagnosed prior pregnancy, systemic infectious, and took any drugs known to affect carbohydrate metabolism.

All clinical investigations were conducted in accordance with the guidelines in The Declaration of Helsinki and were approved by the Bioethics Committee for Research on Humans at the Medical University in Lodz (No. RNN/154/09/KB from 21 April 2009). All participants were comprehensively instructed about the aims of the study and written informed consent.

Anthropometric and biochemical measurements

The information on maternal age and pre-pregnancy weight were collected from medical records, and the pre-pregnancy body mass index (BMI; kg/m²) was calculated.

Serum triglycerides (TGs), HDL-, and LDL-cholesterol (HDL-C and LDL-C) levels were determined by enzymatic colorimetric methods using TG glycerol-3-phosphate-phenol + aminophenazone (GPO-PAP) and the Total Cholesterol CHOD-PAP kits (Roche Diagnostics GmbH, Mannheim, Germany). Glycated hemoglobin (HbA_{1c}) was measured by a latex enhanced turbidimetric immunoassay using specific monoclonal antibodies. The C-reactive protein (CRP) concentration was determined by turbidimetric assay with the use of the cassette COBAS INTEGRA C-Reactive Protein (Latex) according to the manufacturer's instructions (Roche Diagnostics GmbH).

The biochemical assays were carried out with a COBAS INTEGRA analyzer (Roche, SA). Plasma insulin level was quantified using Elecsys insulin assay (Roche Diagnostics GmbH). The homeostasis model assessment (HOMA) index was used to calculate insulin resistance (HOMA-IR) and beta-cell function (HOMA-B) as follow:²¹

$$\text{HOMA-IR} = (\text{fasting insulin } [\mu\text{U/mL}] \times \text{fasting glucose } [\text{mg/dL}]) / 405 \text{ and,}$$

$$\text{HOMA-B} = (360 \times \text{fasting insulin } [\mu\text{U/mL}]) / (\text{fasting glucose } [\text{mg/dL}] - 63)$$

Leukocyte RNA extraction

Leukocytes were isolated from the heparinized venous blood of the subjects (10 mL), as previously described.²²

In brief, after erythrocytes lysis with the use of red blood cell lysis buffer (NH_4Cl , KHCO_3 , EDTA), aliquots were centrifuged and the pellets containing leukocytes were washed twice with the phosphate-buffered saline (PBS). Total RNA was extracted from leukocytes using commercially available acid-phenol reagent (TriReagent[®] Solution, Ambion, United States) according to the manufacturer's procedure. RNA concentration and its purity were assessed by a LAMBDA 25 UV spectrophotometer (PerkinElmer, UK) at UV_{260} and $\text{UV}_{260}/_{280}$, respectively. Samples were stored at -80°C until use.

Gene expression assay

High-quality total RNA ($4\text{ }\mu\text{g}$) was reverse-transcribed with the use of a $(\text{dT})_{18}$ primer and RevertAidTM H Minus M-MuLV reverse transcriptase (Fermentas, Lithuania), according to the manufacturer's recommendations. The levels of *NOS2* and *SFTPD* transcripts were analyzed by quantitative real time PCR (qRT-PCR) with the use of MaximaTM SYBR Green/ROX qPCR Master Mix (2x) (Thermo Scientific, United States) and specific primers for *NOS2*, *SFTPD*, and glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) as a housekeeping gene. The primers were designed based on the GenBank database sequences and are listed in Table 1. Amplification was carried out on 7500 Real Time PCR System (Applied Biosystems, United States) with initial denaturation at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, 60°C for 60 s. All samples were run in duplicate. Amplification of specific transcripts was confirmed by melting curve profiles at the end of each PCR. The specificity of the PCR was further verified by subjecting the amplification products to agarose gel electrophoresis.

The threshold cycle (C_t) of each target product was determined, and ΔC_t between target and endogenous *GAPDH* control was calculated as: $\Delta C_t = C_t(\text{target gene}) - C_t(\text{GAPDH})$. The expression changes between the GDM and the NGT groups were expressed as $\Delta\Delta C_t$ and calculated in the following manner: $\Delta\Delta C_t = \Delta C_t^{(\text{GDM})} - \Delta C_t^{(\text{NGT})}$. The fold change value between the two groups was determined as $2^{-\Delta\Delta C_t}$.²³

Ingenuity pathway analysis (IPA)

To build a highly interconnected gene network and to determine pathways regulated by *NOS2* and *SFTPD* in the context of regulatory networks and pathways reported in the literature, the IPA program (Ingenuity H Systems, Redwood City, CA; <http://www.ingenuity.com>) was

used. In IPA, differentially expressed genes are mapped to genetic networks available in the Ingenuity Pathway Knowledge Base (IPKB) by Ingenuity Systems Inc. In networks, biomolecules are represented as nodes, whereas the biological relationship between two nodes is represented as an edge (line). All edges are supported by at least one reference from the literature stored in the IPKB.

Statistical analysis

The data are presented as the mean $\pm$ standard deviation (SD). The distribution of analyzed biochemical and expression data was checked by the Shapiro-Wilk test. Differences between variables were calculated using the Student's *t*-test. The Pearson's correlations were computed to assess the relationship between variables. *P* value < 0.05 was considered significant. Statistical analyses were carried out using a commercially available statistical software package (Statistica version 8.0, StatSoft, Poland, license no AXAP911E504325AR-K).

Results

Subject's characteristics

Clinical characteristics of 87 GDM and 38 NGT patients are given in Table 2. Fasting and post-load glucose concentrations as well as the HbA_{1c} levels were significantly higher in the GDM women compared with the NGT controls ($P < 0.05$), as expected. No significant differences existed in maternal age and the variables of maternal adiposity (i.e. pre- and pregnancy BMI, body weight gain), lipid metabolism (i.e. TGs, LDL-C, HDL-C, and total cholesterol-TC), inflammation (CRP), insulin resistance and secretion (i.e. insulin, HOMA-IR, and HOMA-B), between the two groups ($P > 0.05$).

Leukocyte gene expression and correlations

qRT-PCR analyses indicated that leukocyte *NOS2* and *SFTPD* mRNA levels were significantly higher in the GDM group compared to the control group ($P < 0.05$ as assessed by the Student's *t*-test) with a 9.34- and 2.53-fold up-regulation, respectively (Figure 1).

To examine whether leukocyte *NOS2* and *SFTPD* expression is associated with clinical characteristics of the patients given in Table 2, linear correlation analyses were performed in the entire study group with the use of the Pearson correlation coefficient (*r*). As shown in Table 3, there were significant positive correlations of the expression of *NOS2*

Table 1 Real-time quantitative PCR primer sets

Gene primer	Accession number	Primer sequence 5'→3'	Amplicon size (bp)
<i>NOS2</i>	NM_000625	Forward: CCCAAATACGAGTGGTTTCG Reverse: TCTCTGTGCCCATGTACCA	133
<i>SFTPD</i>	NM_003019	Forward: TGCTTTCCTGAGCATGACTG Reverse: AAGCCCTGTCTATTCCACTTG	164
<i>GAPDH</i>	NM_002046	Forward: GGTGGTCTCCTCTGACTTCAACA Reverse: GTTGCTGTAGCCAAATTCGTTGT	127

Table 2 Clinical characteristics of subjects

Variables	NGT group (n = 38)	GDM group (n = 87)	P
Age (years)	28.6 ± 4.6	29.9 ± 4.6	0.151
Pre-pregnancy BMI (kg/m ²)	24.2 ± 4.5	25.0 ± 6.5	0.449
Body weight gain (kg)	9.9 ± 5.4	10.6 ± 8.2	0.559
TGs (mg/dL)	224.1 ± 63.2	245.1 ± 79.6	0.242
TC (mg/dL)	259.7 ± 41.8	265.5 ± 52.8	0.628
HDL-C (mg/dL)	79.2 ± 15.2	71.6 ± 21.6	0.101
LDL-C (mg/dL)	145.0 ± 63.4	152.5 ± 56.1	0.625
HbA _{1c} (%)	5.2 ± 0.4	5.4 ± 0.4	0.021*
Fasting plasma glucose (mg/dL)	77.5 ± 7.8	89.9 ± 18.6	<0.001*
1 h plasma glucose (mg/dL)	152.3 ± 36.1	187.3 ± 30.0	<0.001*
2 h plasma glucose (mg/dL)	113.9 ± 21.3	163.3 ± 22.3	<0.001*
CRP (mg/L)	5.2 ± 4.9	4.4 ± 3.9	0.448
Insulin (μU/mL)	8.1 ± 9.4	9.0 ± 10.7	0.645
HOMA-IR	1.6 ± 1.8	2.3 ± 2.9	0.164
HOMA-B	242.5 ± 315.7	142.2 ± 195.9	0.110

BMI, body mass index; CRP, C-reactive protein; HDL, high-density lipoprotein; HOMA-B, homeostasis model assessment of β -cell function; HOMA-IR, homeostasis model assessment of insulin resistance; LDL, low-density lipoprotein; TC, total cholesterol; TGs, triglycerides.

Data are presented as mean $\pm$ standard deviation.

* $P < 0.05$ GDM vs. NGT as assessed by the Student's t -test.

($r = 0.23$, $P = 0.022$) and *SFTPD* ($r = 0.34$, $P = <0.001$) with CRP in pregnancy. Additionally, *SFTPD* mRNA positively associated with the fasting plasma glucose concentration ($r = 0.19$, $P = 0.041$) and indicator of insulin resistance, i.e. HOMA-IR ($r = 0.21$, $P = 0.040$). To investigate whether there are any correlations between the expression changes of the aforementioned genes, the Pearson correlation coefficient (r) and P values were obtained. As shown in Table 3, there was strong positive correlation between the expression of both genes ($r = 0.77$, $P = <0.001$).

Bioinformatics analysis of molecular pathways

To establish biological relationships of *NOS2* and *SFTPD* with well-known in the literature genes and/or gene products, functional analyses were made using the IPA database. The 12 different molecular and cellular pathways linked to *NOS2* were found, whereas no gene and/or gene product related with *SFTPD* was detected (Figure 2). The genes/proteins engaged in these pathways were related in particular with immune or inflammatory functions as well as angiogenesis, and they belonged to the following functional categories:

- (i) cytokines/growth factors: interleukin-1 beta (IL-1 β) encoded by the *IL1B* gene; interleukin-6 (IL-6) encoded by *IL6* gene; interleukin-10 (IL-10) encoded by the *IL10* gene; tumor necrosis factor-alpha (TNF- α) encoded by the *TNFA* gene; interferon-gamma (IFN- γ) encoded by the *IFNG* gene/

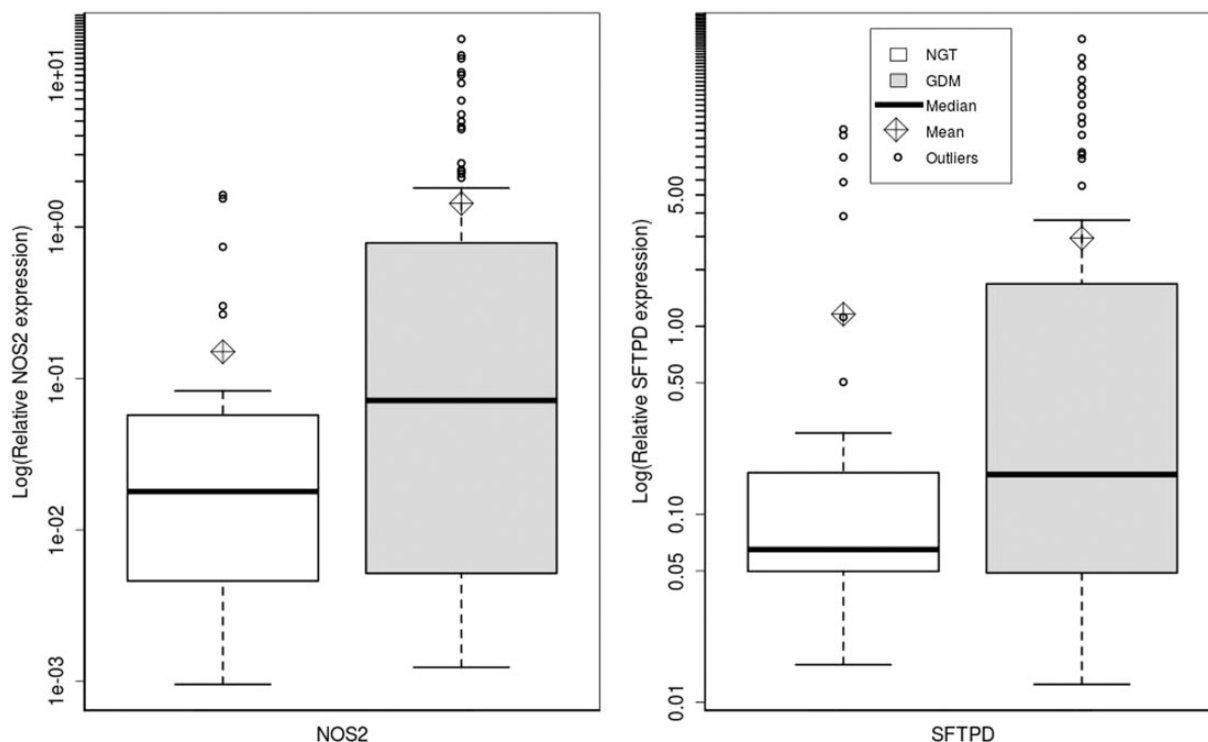


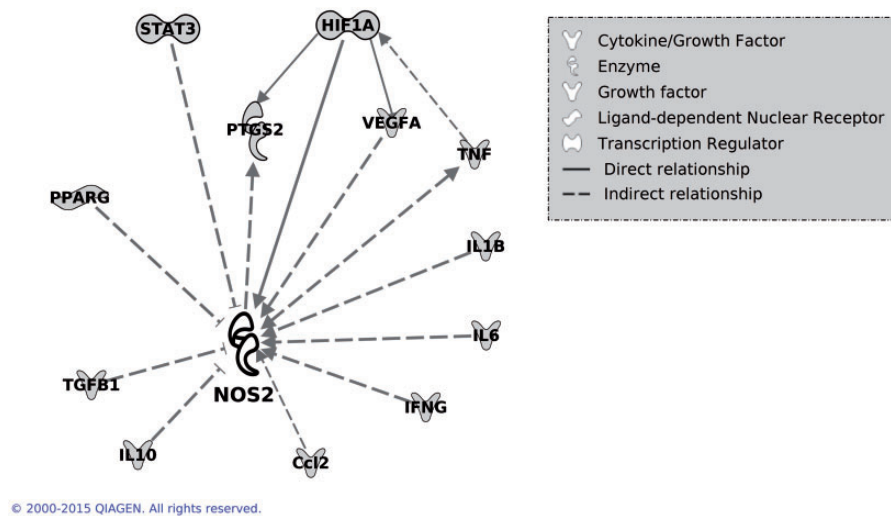
Figure 1 Boxplots of leukocyte gene expression of *NOS2* and *SFTPD* in the NGT and GDM groups. Each point represents an individual sample. $P < 0.05$ GDM vs. NGT as assessed by the Student's t -test

Table 3 Univariate correlations between gene expression of *NOS2* and *SFTPD*, and clinical variables of the subjects in the entire study group ($n = 125$)

Variables	<i>NOS2</i>		<i>SFTPD</i>	
	<i>r</i>	<i>P</i>	<i>r</i>	<i>P</i>
Age (years)	0.16	0.073	0.02	0.812
Pre-pregnancy BMI (kg/m ²)	0.07	0.453	−0.001	0.990
Body weight gain (kg)	0.06	0.529	0.13	0.160
TGs (mg/dL)	−0.14	0.274	−0.12	0.328
TC (mg/dL)	−0.21	0.096	−0.12	0.322
HDL-C (mg/dL)	−0.24	0.067	−0.17	0.166
LDL-C (mg/dL)	−0.10	0.445	−0.06	0.636
HbA _{1c} (%)	0.12	0.208	0.13	0.156
Fasting plasma glucose (mg/dL)	0.17	0.070	0.19	0.041 ^a
1 h plasma glucose (mg/dL)	0.14	0.181	0.09	0.361
2 h plasma glucose (mg/dL)	0.08	0.393	0.07	0.450
CRP (mg/L)	0.23	0.022 ^a	0.34	<0.001 ^a
Insulin (μIU/mL)	0.05	0.627	0.19	0.059
HOMA-IR	0.07	0.486	0.21	0.040 ^a
HOMA-B	−0.08	0.442	−0.04	0.743
<i>NOS2</i>			0.77	<0.001 ^a

BMI, body mass index; CRP, C-reactive protein; HDL, high-density lipoprotein; HOMA-B, homeostasis model assessment of β -cell function; HOMA-IR, homeostasis model assessment of insulin resistance; LDL, low-density lipoprotein; TC, total cholesterol; TGs, triglycerides. *r*- and *P* values are given.

^aSignificant correlation as assessed by Pearson's coefficient method.

**Figure 2** Schematic diagram of molecular connections between *NOS2* and identified genes/biomolecules created with the use of the Ingenuity Pathway Analysis (IPA) software

vascular endothelial growth factor A (VEGF-A) encoded by the *VEGFA* gene, and transforming growth factor beta 1 (TGF- β 1) encoded by the *TGFB* gene,

- (ii) chemokine: monocyte chemoattractant protein-1 (MCP-1/CCL2) encoded by the *Ccl2* gene,
- (iii) enzyme: cyclooxygenase (COX)-2 encoded by the *PTGS2* gene,
- (iv) transcription factors: hypoxia-inducible factor-1 α (HIF-1 α) encoded by the *HIF1A* gene; STAT3,

encoded by the *STAT3* gene, and peroxisome proliferator-activated receptor gamma (PPAR- γ) encoded by the *PPARG* gene.

Discussion

To date, no published reports are currently accessible on the relationship of the expression of the immune-related *NOS2* and *SFTPD* genes in leukocytes with metabolic changes

occurring during diabetic pregnancy. Hence, we conducted the present study using leukocytes as an important and well-known source of numerous inflammatory mediators that are engaged in various inflammatory events.

In this study, 87 women with diagnosed GDM and 38 pregnant women with NGT at the third trimester of gestation, selected based on rigorous inclusion criteria, were enrolled. Compared to the NGT subjects, the GDM patients were hyperglycemic and had significantly higher HbA_{1c} levels.

In our gene expression experiments, significantly higher leukocyte *NOS2* and *SFTPD* mRNA levels were detected in the GDM group compared to those in the NGT group, suggesting that hyperglycemic conditions in GDM women might affect leukocyte overexpression of these genes. Hence, both genes appear to be pathophysiologically important in the context of diabetic pregnancy. It is not surprising that high-glucose conditions can modulate the expression of various genes at their transcriptional level since complex alterations in the expression of genes involved in insulin action, glucose and lipid metabolism, as well as oxidative stress, and inflammation have been observed in leukocytes of hyperglycemic GDM patients.²²

In regard to iNOS, dysregulation of its expression resulting in NO overproduction has been found to associate with diabetes and its complications.^{24,25} In the present study, we observed nearly 10-fold up-regulation of leukocyte *NOS2* mRNA in hyperglycemic GDM women. This finding is, at least in part, compatible with the hypothesis that hyperglycemia is linked to increased iNOS gene expression and nitrosative stress since hyperglycemia-induced oxidative stress results in excessive production of reactive oxygen species (ROS) which, in turn, reacting with NO give reactive nitrogen species (RNS) and further nitrosative stress.⁹ Indeed, it has been shown that hyperglycemia stimulates iNOS gene expression and nitrosative stress in diabetic embryopathy and activation of c-Jun N-terminal kinases 1 and 2 (JNK1/2) appears to play a key role in this process.²⁶ However, further studies are warranted to establish causality between hyperglycemia and leukocyte *NOS2* overexpression in GDM women in the context of nitrosative stress. It is of note that several studies have reported iNOS overexpression in metabolic tissues of both dietary and genetic models of obesity.^{27,28} However, since no significant differences in BMI and body weight gain occur between the GDM and NGT groups in our study, we can exclude the possibility lipid-induced alterations in iNOS expression *in vivo*.

In the current study, we also observed the positive correlation between leukocyte *NOS2* mRNA level and plasma CRP in pregnant women. CRP is an acute-phase plasma protein produced primarily by liver that is used as a marker of the immune system activation. Our findings stay in agreement with the published data showing CRP-mediated iNOS induction in macrophages^{29,30} and in IL-1 β -stimulated cardiac myocytes.³¹ Additionally, Hattori et al.³² reported that CRP can stimulate not only iNOS gene expression but also the expression of IL-6 and MCP-1 in vascular smooth muscle cells (VSMC) through the nuclear factor (NF)- κ B-dependent mechanism, suggesting a direct role of

CRP in the pathogenesis of inflammation/atherosclerosis. Considering these findings, we cannot rule out the possibility that pregnant women with elevated plasma CRP concentration accompanied by leukocyte *NOS2* overexpression may be at risk of atherosclerosis development since inflammation and oxidative stress, both processes occurring during gestation, as well as different leukocyte subpopulations are involved in the pathology of this disease.³³

The SP-D is a lung-specific protein that enhances clearance of pathogens and modulates inflammatory responses. There is growing evidence suggesting that SP-D can be an important factor at the cross-roads of inflammation, obesity, and insulin resistance.^{18,34} In fact, serum SP-D concentration is reduced in subjects with both obesity and T2DM and, moreover, it inversely associates with fasting and post-load serum glucose and positively links to insulin sensitivity.¹⁸ In the light of these findings, our study showing significantly increased leukocyte *SFTPD* expression in hyperglycemic GDM patients, as well as its positive correlations with fasting plasma glucose, HOMA-IR, and CRP in the entire study group is rather surprising. This divergence in results may partly result from differences in several anthropometric and biochemical parameters of study subjects (i.e. age, BMI, or insulin sensitivity), as well as various measurements of SP-D content (protein versus mRNA) performed in different types of samples (serum versus leukocytes). Nevertheless, we cannot exclude the possibility that SP-D upregulation at the gene expression level in hyperglycemic GDM women observed in our research could be an effective compensation for enhanced oxidative stress and inflammation, the two interconnected processes induced by high glucose concentration, in diabetic patients, since SP-D has been recognized as a molecule with antioxidant and anti-inflammatory properties.^{16,17}

We also observed the existence of clinical associations between leukocyte *SFTPD* expression and fasting glycemia, insulin resistance, and inflammation among pregnant women, which reflect functional and biological importance of this biomolecule during gestation. The reasons for these associations are currently unknown. However, previous findings showing increased SP-D expression in the presence of pro-inflammatory cytokines such as IL-1 β i TNF- α in pancreatic β -cells of pregnant mice³⁵ could support, at least partially, our observation regarding the relationship between leukocyte SP-D expression and inflammation during pregnancy.

Another novel issue in our study was the strong positive correlation between leukocyte *NOS2* and *SFTPD* expression in the entire study group, which suggests the existence of a close relationship between these genes at their transcriptional level during normal pregnancy.

To determine potential molecular up- and downstream targets of *NOS2* and SP-D biomolecules, a highly interconnected gene/protein network was built based on regulatory networks and pathways reported in the literature by using the IPA program, which is currently a powerful and convenient bioinformatics tool for finding a set genes/molecules that can be looked up in greater detail by building a custom pathway. As shown in Figure 2, 12 individual genes/proteins related with *NOS2* were identified, whereas

no molecular pathways were detected for SFTPD. The candidate genes for NOS2 regulation were categorized into four functional groups, including (i) cytokines/growth factors (*IL1B*, *IL6*, *IL10*, *TNFA*, *IFNG*, *VEGFA*, *TGFB*), (ii) chemokine (*Ccl2*), (iii) enzyme (*PTGS2*), and (iv) transcription factors (*HIF1A*, *STAT3*, *PPARG*).

Accumulating evidence has shown that NOS2 induction may be engaged in pathological states linked to increased concentrations of pro-inflammatory cytokines. To date, several pro-inflammatory cytokines, such as IL-1 β , IFN- γ , TNF- α , IL-6, and MCP-1/CCL2 have been recognized to stimulate NOS2 production in different cell types, including pancreatic β -cells,^{36,37} myocytes,³⁸ adipocytes,³⁹ and macrophages⁴⁰ among other. Notable relationships between NOS2 and cytokines with anti-inflammatory properties also occur in the literature. In this regard, elevated IL-10 expression has been reported to associate with significant decrease in iNOS expression and cell death in atherosclerotic plaques.⁴¹ In the case of TGF- β , a pleiotropic polypeptide engaged in cell growth, differentiation, and immune modulation, its anti-inflammatory effect on macrophages and VSMCs through inhibition of cytokine-mediated iNOS induction has been demonstrated.^{42,43} Since GDM is associated with low-grade systematic inflammation and plasma levels of the above-mentioned cytokines are changed in diabetic patients,⁴⁴ it is reasonable to assume that they could play a relevant role in NOS2 regulation in leukocytes of GDM patients.

Another important biomolecule related with iNOS is the growth factor VEGF-A, well-known endothelial cell mitogen that promotes angiogenesis in embryonic development, wound healing, and inflammatory fibrosis,^{45–47} which has been reported to up-regulate iNOS and eNOS production in human umbilical vein endothelial cells (HUVEC) via the stimulation of VEGF receptor-2.⁴⁸ Given that VEGF-A is thought to control angiogenesis and endothelial cell permeability during the physiology and pathophysiology of pregnancy,⁴⁹ this biomolecule could be involved in NOS2 dysregulation in leukocytes during gestational diabetes.

As found in our bioinformatics analysis, NOS2 appears to be also an important target for some transcription factors, including STAT3, PPAR- γ , and HIF-1 α . Several lines of evidences have revealed an inhibitory effect of both STAT3 and PPAR- γ on iNOS gene expression in VSMCs,⁵⁰ mesangial cells,⁵¹ and pancreatic β -cells.⁵² Interestingly, PPAR- γ , a member of the ligand-activated nuclear receptor superfamily that controls the expression of many genes involved in glucose and lipid metabolism, as well as adipocyte differentiation, and inflammation, has been proposed to protect pancreatic β -cells against IL-1 β -induced dysfunction of β -cells through a decrease of NF- κ B activity and, in turn, inhibition of iNOS expression and NO formation.⁵² Contrary to STAT3 and PPAR- γ , HIF-1 α has been demonstrated to up-regulate NOS2 expression, as well as other genes engaged in blood flow and inflammation such as VEGFA and PTGS2.^{53,54} The latter encodes prostaglandin-endoperoxide synthase (PTGS), also known as cyclooxygenase-2 (COX-2), that plays a key role in prostaglandin biosynthesis and, moreover, it is therapeutic target for non-steroidal anti-inflammatory drugs. Given that NO

and prostaglandin (PG) E2 produced by NOS2 and COX-2, respectively, are believed to be important mediators in inflammation, several studies have suggested the existence of a relationship between them. Indeed, iNOS by NO production has been found to induce COX-2 expression.^{55,56} Taken together, because the involvement of the aforementioned transcription factors and COX-2 in the pathological events associated with inflammation during GDM has been observed,^{57,58} deepen the study of each of these genes in the context its linkage to NOS2 in leukocytes of GDM women appears to be warranted.

In conclusion, this study provides several new findings. First, we demonstrated increased leukocyte NOS2 and SFTPD gene expression in hyperglycemic women with gestational diabetes, suggesting that a high glucose level may affect transcriptional regulation of these immunologically relevant genes. Second, using correlation analysis, we found positive correlations between (i) NOS2 mRNAs and CRP, and (ii) SFTPD mRNA and CRP, fasting glycemia, and insulin resistance in the entire study group, implying that alterations in the expression of these genes may represent the response to glucose metabolism dysfunction and/or inflammation during pregnancy. Third, by the use of the IPA program, we identified 12 potential molecular up- and downstream targets of NOS2 belonging to cytokines, enzymes, and transcription factors that are mainly engaged in the regulation of inflammatory events. However, further studies on validation of the identified genes and their functionality in leukocytes of women with and without GDM are required to clarify the mechanisms by which NOS2 work in these cells. Such studies are currently under way in our group.

Author contributions: MW designed and performed the research study and wrote the article. AZ performed the research study and statistical analysis. LAW managed the project. KC and MZK were responsible for clinical aspect of the project.

ACKNOWLEDGMENTS

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: in part by the Healthy Ageing Research Centre project (REGPOT-2012-2013-1, 7 FP).

DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

REFERENCES

1. Coustan DR, Lowe LP, Metzger BE, Dyer AR; International Association of Diabetes and Pregnancy Study Groups. The Hyperglycemia and Adverse Pregnancy Outcome (HAPO) study: paving the way for new diagnostic criteria for gestational diabetes mellitus. *Am J Obstet Gynecol* 2010;**202**:654.e1–6
2. Forsbach-Sánchez G, Tamez-Peréz HE, Vazquez-Lara J. Diabetes and pregnancy. *Arch Med Res* 2005;**36**:291–9

3. Buchanan TA. Pancreatic B-cell defects in gestational diabetes: implications for the pathogenesis and prevention of type 2 diabetes. *J Clin Endocrinol Metab* 2001;**86**:989–93
4. Carr DB, Utzschneider KM, Hull RL, Tong J, Wallace TM, Kodama K, Shofer JB, Heckbert SR, Boyko EJ, Fujimoto WY, Kahn SE. Gestational diabetes mellitus increases the risk of cardiovascular disease in women with a family history of type 2 diabetes. *Diabetes Care* 2006;**29**:2078–83
5. Bellamy L, Casas JP, Hingorani AD, Williams D. Type 2 diabetes mellitus after gestational diabetes: a systematic review and meta-analysis. *Lancet* 2009;**373**:1773–9
6. Winkler G, Cseh K, Baranyi E, Melczer Z, Speer G, Hajós P, Salamon F, Turi Z, Kovács M, Vargha P, Karádi I. Tumor necrosis factor system and insulin resistance in gestational diabetes. *Diabetes Res Clin Pract* 2002;**56**:93–9
7. Morisset AS, Dubé MC, Côté JA, Robitaille J, Weisnagel SJ, Tchernof A. Circulating interleukin-6 concentrations during and after gestational diabetes mellitus. *Acta Obstet Gynecol Scand* 2011;**90**:524–30
8. Zhao YH, Wang DP, Zhang LL, Zhang F, Wang DM, Zhang WY. Genomic expression profiles of blood and placenta reveal significant immune-related pathways and categories in Chinese women with gestational diabetes mellitus. *Diabet Med* 2011;**28**:237–46
9. Förstermann U, Sessa WC. Nitric oxide synthases: regulation and function. *Eur Heart J* 2012;**33**:829–37
10. Hill BG, Dranka BP, Bailey SM, Lancaster JR Jr, Darley-Usmar VM. What part of NO don't you understand? Some answers to the cardinal questions in nitric oxide biology. *J Biol Chem* 2010;**285**:19699–704
11. Nomiya T, Igarashi Y, Taka H, Mineki R, Uchida T, Ogihara T, Choi JB, Uchino H, Tanaka Y, Maegawa H, Kashiwagi A, Murayama K, Kawamori R, Watada H. Reduction of insulin-stimulated glucose uptake by peroxynitrite is concurrent with tyrosine nitration of insulin receptor substrate-1. *Biochem Biophys Res Commun* 2004;**320**:639–47
12. Perreault M, Marette A. Targeted disruption of inducible nitric oxide synthase protects against obesity-linked insulin resistance in muscle. *Nat Med* 2001;**7**:1138–43
13. Shinozaki S, Choi CS, Shimizu N, Yamada M, Kim M, Zhang T, Shiota G, Dong HH, Kim YB, Kaneki M. Liver-specific inducible nitric-oxide synthase expression is sufficient to cause hepatic insulin resistance and mild hyperglycemia in mice. *J Biol Chem* 2011;**286**:34959–75
14. Takamura T, Kato I, Kimura N, Nakazawa T, Yonekura H, Takasawa S, Okamoto H. Transgenic mice overexpressing type 2 nitric-oxide synthase in pancreatic beta cells develop insulin-dependent diabetes without insulinitis. *J Biol Chem* 1998;**273**:2493–6
15. Haagsman HP1, Hogenkamp A, van Eijk M, Veldhuizen EJ. Surfactant collectins and innate immunity. *Neonatology* 2008;**93**:288–94
16. Sin DD, Pahlavan PS, Man SF. Surfactant protein D: a lung specific biomarker in COPD? *Ther Adv Respir Dis* 2008;**2**:65–74
17. Bridges JP, Davis HW, Damodarasamy M, Kuroki Y, Howles G, Hui DY, McCormack FX. Pulmonary surfactant proteins A and D are potent endogenous inhibitors of lipid peroxidation and oxidative cellular injury. *J Biol Chem* 2000;**275**:38848–55
18. Fernández-Real JM1, Valdés S, Manco M, Chico B, Botas P, Campo A, Casamitjana R, Delgado E, Salvador J, Fruhbeck G, Mingrone G, Ricart W. Surfactant protein d, a marker of lung innate immunity, is positively associated with insulin sensitivity. *Diabetes Care* 2010;**33**:847–53
19. Pueyo N1, Ortega FJ, Mercader JM, Moreno-Navarrete JM, Sabater M, Bonàs S, Botas P, Delgado E, Ricart W, Martínez-Larrad MT, Serrano-Ríos M, Torrents D, Fernández-Real JM. Common genetic variants of surfactant protein-D (SP-D) are associated with type 2 diabetes. *PLoS One* 2013;**8**:e60468
20. Definition and diagnosis of diabetes mellitus and intermediate hyperglycemia: Report of WHO/IDF consultation. World Health Organization and International Diabetes Federation. WHO Document Production Services, Geneva, Switzerland, 2006
21. Matthews DR, Hosker JP, Rudenski AS, Naylor BA, Treacher DF, Turner RC. Homeostasis model assessment: insulin resistance and β -cell function from fasting plasma glucose and insulin concentrations in man. *Diabetologia* 1985;**28**:412–9
22. Wojcik M, Zieleniak A, Mac-Marcjanek K, Wozniak LA, Cypryk K. The elevated gene expression level of the A(2B) adenosine receptor is associated with hyperglycemia in women with gestational diabetes mellitus. *Diabetes Metab Res Rev* 2014;**30**:42–53
23. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C(T)}$ method. *Methods* 2001;**25**:402–8
24. Abu El-Asrar AM, Desmet S, Meersschaert A, Dralands L, Missotten L, Geboes K. Expression of the inducible isoform of nitric oxide synthase in the retinas of human subjects with diabetes mellitus. *Am J Ophthalmol* 2001;**132**:551–6
25. Mishra A, Newman EA. Inhibition of inducible nitric oxide synthase reverses the loss of functional hyperemia in diabetic retinopathy. *Glia* 2010;**58**:1996–2004
26. Yang P, Cao Y, Li H. Hyperglycemia induces inducible nitric oxide synthase gene expression and consequent nitrosative stress via c-Jun-N-terminal kinase activation. *Am J Obstet Gynecol* 2010;**203**:185.e5–11
27. Perreault M, Marette A. Targeted disruption of inducible nitric oxide synthase protects against obesity-linked insulin resistance in muscle. *Nat Med* 2001;**7**:1138–43
28. Fujimoto M, Shimizu N, Kunii K, Martyn JA, Ueki K, Kaneki M. A role for iNOS in fasting hyperglycemia and impaired insulin signaling in the liver of obese diabetic mice. *Diabetes* 2005;**54**:1340–8
29. Ratnam S, Mookerjee S. The regulation of superoxide generation and nitric oxide synthesis by C-reactive protein. *Immunology* 1998;**94**:560–8
30. Arcoleo F, Milano S, D'Agostino P, Misiano G, Cappelletti S, Gromo G, Marcucci F, Leoni F, Cillari E. Effect of partially modified retro-inverso analogues derived from C-reactive protein on the induction of nitric oxide synthesis in peritoneal macrophages. *Br J Pharmacol* 1997;**120**:1383–9
31. Ikeda U, Maeda Y, Yamamoto K, Shimada K. C-Reactive protein augments inducible nitric oxide synthase expression in cytokine-stimulated cardiac myocytes. *Cardiovasc Res* 2002;**56**:86–92
32. Hattori Y, Matsumura M, Kasai K. Vascular smooth muscle cell activation by C-reactive protein. *Cardiovasc Res* 2003;**58**:186–95
33. Weber C, Zernecke A, Libby P. The multifaceted contributions of leukocyte subsets to atherosclerosis: lessons from mouse models. *Nat Rev Immunol* 2008;**8**:802–15
34. Stidsen JV, Khorrooshi R, Rahbek MK, Kirketerp-Møller KL, Hansen PB, Bie P, Kejlving K, Mandrup S, Hawgood S, Nielsen O, Nielsen CH, Owens T, Holmskov U, Sørensen GL. Surfactant protein d deficiency in mice is associated with hyperphagia, altered fat deposition, insulin resistance, and increased basal endotoxemia. *PLoS One* 2012;**7**:e35066
35. Layden BT, Durai V, Newman MV, Marinellarena AM, Ahn CW, Feng G, Lin S, Zhang X, Kaufman DB, Jafari N, Sørensen GL, Lowe WL Jr. Regulation of pancreatic islet gene expression in mouse islets by pregnancy. *J Endocrinol* 2010;**207**:265–79
36. Eizirik DL, Flodström M, Karlén AE, Welsh N. The harmony of the spheres: inducible nitric oxide synthase and related genes in pancreatic beta cells. *Diabetologia* 1996;**39**:875–90
37. da Silva Krause M, Bittencourt A, Homem de Bittencourt PI, McClenaghan NH, Platt PR, Murphy C, Newsholme P. Physiological concentrations of interleukin-6 directly promote insulin secretion, signal transduction, nitric oxide release, and redox status in a clonal pancreatic β -cell line and mouse islets. *J Endocrinol* 2012;**214**:301–11
38. Bedard S, Marcotte B, Marette A. Cytokines modulate glucose transport in skeletal muscle by inducing the expression of inducible nitric oxide synthase. *Biochem J* 1997;**325**:487–93
39. Kapur S, Marcotte B, Marette A. Mechanism of adipose tissue iNOS induction in endotoxemia. *Am J Physiol* 1999;**276**:E635–41
40. Gomes RN, Teixeira-Cunha MG, Figueiredo RT, Almeida PE, Alves SC, Bozza PT, Bozza FA, Bozza MT, Zimmerman GA, Castro-Faria-Neto HC. Bacterial clearance in septic mice is modulated by MCP-1/CCL2 and nitric oxide. *Shock* 2013;**39**:63–9
41. Mallat Z, Heymes C, Ohan J, Faggini E, Lesèche G, Tedgui A. Expression of interleukin-10 in advanced human atherosclerotic plaques: relation to inducible nitric oxide synthase expression and cell death. *Arterioscler Thromb Vasc Biol* 1999;**19**:611–6

42. Förstermann U, Schmidt HH, Kohlhaas KL, Murad F. Induced RAW 264.7 macrophages express soluble and particulate nitric oxide synthase: inhibition by transforming growth factor-beta. *Eur J Pharmacol* 1992;**225**:161-5
43. Feinberg MW, Watanabe M, Lebedeva MA, Depina AS, Hanai J, Mammoto T, Frederick JP, Wang XF, Sukhatme VP, Jain MK. Transforming growth factor-beta1 inhibition of vascular smooth muscle cell activation is mediated via Smad3. *J Biol Chem* 2004;**279**:16388-93
44. Gomes CP, Torloni MR, Gueuvoghlian-Silva BY, Alexandre SM, Mattar R, Daher S. Cytokine levels in gestational diabetes mellitus: a systematic review of the literature. *Am J Reprod Immunol* 2013;**69**:545-57.
45. Flamme I, Frolich T, Risau W. Molecular mechanisms of vasculogenesis and embryonic angiogenesis. *J Cell Physiol* 1997;**173**:206-10
46. Brown LF, Yeo KT, Berse BK, Yeo TK, Senger DR, Dvorak HF, Van de Water L. Expression of vascular permeability factor (vascular endothelial growth factor) by epidermal keratinocytes during wound healing. *J Exp Med* 1992;**176**:1375-9
47. Fehrenbach H, Kasper M, Haase M, Schuh D, Muller M. Differential immunolocalization of VEGF in rat and human adult lung, and in experimental rat lung fibrosis. Light, fluorescence, and electron microscopy. *Anat Rec* 1999;**254**:61-73
48. Kroll J, Waltenberger J. VEGF-A induces expression of eNOS and iNOS in endothelial cells via VEGF receptor-2 (KDR). *Biochem Biophys Res Commun* 1998;**252**:743-6
49. Lygnos MC, Pappa AI, Papadaki HA, Relakis C, Koumantakis E, Anagnostou NP, Eliopoulos GD. Changes in maternal plasma levels of VEGF, bFGF, TGF-beta1, ET-1 and sKL during uncomplicated pregnancy, hypertensive pregnancy and gestational diabetes. *In Vivo* 2006;**20**:157-63
50. Marrero MB, Venema VJ, He H, Caldwell RB, Venema RC. Inhibition by the JAK/STAT pathway of IFNg- and LPS-stimulated nitric oxide synthase induction in vascular smooth muscle cells. *Biochem Biophys Res Commun* 1998;**252**:508-12
51. Yu Z, Zhang W, Kone BC. Signal transducers and activators of transcription 3 (STAT3) inhibits transcription of the inducible nitric oxide synthase gene by interacting with nuclear factor kappaB. *Biochem J* 2002;**367**:97-105
52. Chen F, Zhou X, Lin Y, Jing C, Yang T, Ji Y, Sun Y, Han X. Resveratrol prevents interleukin-1 β -induced dysfunction of pancreatic β -cells. *J Biomed Res* 2010;**24**:381-8
53. Hellwig-Bürgel T, Stiehl DP, Wagner AE, Metzen E, Jelkmann W. Review: hypoxia-inducible factor-1 (HIF-1): a novel transcription factor in immune reactions. *J Interferon Cytokine Res* 2005;**25**:297-310
54. Hu R, Dai A, Tan S. Hypoxia-inducible factor 1 alpha upregulates the expression of inducible nitric oxide synthase gene in pulmonary arteries of hypoxic rat. *Chin Med J (Engl)* 2002;**115**:1833-7
55. Perkins DJ, Kniss DA. Blockade of nitric oxide formation down-regulates cyclooxygenase-2 and decreases PGE2 biosynthesis in macrophages. *J Leukoc Biol* 1999;**65**:792-9
56. Salvemini D, Misko TP, Masferrer JL, Seibert K, Currie MG, Needleman P. Nitric oxide activates cyclooxygenase enzymes. *Proc Natl Acad Sci USA* 1993;**90**:7240-4
57. Di Francesco L, Dovizio M, Trenti A, Marcantoni E, Moore A, O'Gaora P, McCarthy C, Tacconelli S, Bruno A, Alberti S, Gizzo S, Nardelli GB, Orso G, Belton O, Trevisi L, Dixon DA, Patrignani P. Dysregulated post-transcriptional control of COX-2 gene expression in gestational diabetic endothelial cells. *Br J Pharmacol* 2015;**172**:4575-87
58. Dincer UD. Fetal exposure to a diabetic intrauterine environment resulted in a failure of cord blood endothelial progenitor cell adaptation against chronic hypoxia. *Stem Cells Cloning* 2015;**8**:1-14

(Received June 29, 2015, Accepted October 14, 2015)