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Lesões no DNA e capacidade de resposta celular de gestantes e recém-nascidos em regime de hiperglicemia de intensidade variada

Tese apresentada à Faculdade de Medicina, Universidade Estadual Paulista "Júlio de Mesquita Filho", Câmpus de Botucatu, para obtenção do título de Doutora em Ginecologia, Obstetrícia e Mastologia.

Orientadora: Profa. Dra. Iracema de Mattos Paranhos Calderon.
Coorientador: Prof. Dr. Carlos Frederico Martins Menck.

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Dedicatória

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Dizer e Sentir
Diga uma palavra e as flores se abrirão,
Diga apenas o seu nome e os gestos sairão,
Dê um sorriso e mil pessoas olharão,
Dê um passo e você estará perto da multidão.
Um caminho cheio de obstáculos,
Mas todos eles são um grande passo para a reconciliação.
Isabela Vilela Moreli (10 anos)

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"Cada pessoa que passa em nossa vida, passa sozinha, é porque cada pessoa é única e nenhuma substitui a outra! Cada pessoa que passa em nossa vida passa sozinha e não nos deixa só porque deixa um pouco de si e leva um pouquinho de nós. Essa é a mais bela responsabilidade da vida e a prova de que as pessoas não se encontram por acaso."

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Sumário

Apresentação

O Grupo de Pesquisa Diabete e Gravidez – Investigação Clínica e Experimental vem desenvolvendo pesquisas clínicas e experimentais nesta linha há mais de 30 anos. Os primeiros resultados, associando o teste de tolerância à glicose (TTG) e o perfil glicêmico no diagnóstico do diabete melito gestacional (DMG), caracterizaram um grupo de gestantes que, apesar do TTG normal, apresentam picos de hiperglicemia ao longo do dia evidenciados por alterações no perfil glicêmico. Essas gestantes foram consideradas como portadoras de hiperglicemia gestacional leve (HGL) e classificadas no grupo IB de Rudge. Além de outros resultados perinatais adversos (RPA), característicos dos filhos de mães diabéticas, as gestações complicadas por HGL tem risco atribuível de morte perinatal comparável ao observado no grupo de gestantes diabéticas e 10 vezes maior que aquelas com resposta normal aos dois testes diagnósticos. A partir desses resultados, as gestantes com HGL são tratadas com o mesmo protocolo das diabéticas acompanhadas no Serviço Especializado de Diabete e Gravidez da Faculdade de Medicina de Botucatu/Unesp [1-3].

A literatura mais recente reconhece que a hiperglicemia materna, de qualquer intensidade e independente do diagnóstico de DMG, deve ser controlada pelo risco de RPA [4-5]. Tal constatação validou a identificação e o controle da hiperglicemia das gestantes do grupo IB de Rudge, protocolo que vem sendo praticado há mais de 30 anos em nosso serviço.

Na busca pelos fatores envolvidos no desfecho adverso dessas gestações complicadas por hiperglicemia, os resultados do nosso grupo de pesquisa associam a hipóxia intrauterina e a hiperglicemia materna de intensidade variada. Entre outras, esta associação leva a alterações morfológicas e funcionais da placenta,

caracterizadas por comprometimento da vascularização da superfície de trocas materno-fetal, relacionadas a aumento e/ou diminuição dos marcadores da proliferação vascular [6-8], incremento da apoptose [9] e alterações no perfil de citocinas placentárias [10-12]. Paralelamente, nossos estudos translacionais, utilizando modelo experimental em ratas wistar prenhes, com diabete induzido por streptozotocin, relacionaram a intensidade da hiperglicemia materna e o nível de estresse oxidativo, e consequente aumento de danos do DNA, com a ocorrência de RPA nessas gestações [13-17].

Outros estudos da literatura apontam aumento na incidência de câncer em pacientes diabéticos, decorrente da associação estresse oxidativo e hiperglicemia. Nestas condições, mecanismos de reparo de DNA são ativados, na tentativa de garantir a sobrevivência e manter a integridade do genoma. Entretanto, a falha desses mecanismos de reparo pode levar a acúmulo de danos no DNA e favorecer tanto a apoptose das células como o desenvolvimento de câncer [18-20].

Em dezembro de 2009, os resultados do consenso entre a Associação Americana de Diabete e a Sociedade Americana de Câncer destacaram que, em ambos os sexos, o diabete melito tipo 2 (DM2) está associado com risco aumentado de câncer no fígado, pâncreas, cólon e bexiga. Individualizando os sexos, mulheres diabéticas apresentam aumento no risco de câncer de mama e de endométrio e, os homens, redução no risco de câncer de próstata [21].

Alguns estudos destacam, também, a associação entre DMG e câncer. Uma coorte de 37926 mulheres com histórico de DMG identificou risco relativo de 7,1 para desenvolver câncer pancreático [22]. Na Nova Zelândia, a população de mulheres

com DMG tem maior risco de câncer de mama, o que não foi confirmado na população americana [23,24]. Recentemente, o DMG também foi associado a linfoma não-Hodgkin e leucemia mieloide aguda [25]. Apesar do ponto comum, associação entre hiperglicemia e risco de câncer, os resultados da literatura ainda são controversos e os fatores e vias envolvidos ainda não estão totalmente esclarecidos.

Nesse contexto, o interesse em contribuir para minimizar essa lacuna definiu o presente projeto, objeto dessa tese de doutorado. Inicialmente, a análise crítica da literatura relacionada ao tema resultou em um artigo de revisão, “DNA damage and its cellular response in mother and fetus exposed to hyperglycemic environment”, já publicado [26]. Posteriormente, o desenvolvimento do projeto, em si, resultou em um segundo artigo, agora original, “Hyperglycemia differentially affects maternal and fetal DNA integrity and DNA damage response”. A elaboração de dois artigos definiu a forma de apresentação dessa tese, intitulada “Lesões no DNA e capacidade de resposta celular de gestantes e recém-nascidos em regime de hiperglicemia de intensidade variada”.

Referências

01. Rudge MVC, Peraçoli JC, Berezowski AT, Calderon IMP, Brasil MAM. The oral glucose tolerance test is a poor predictor of hyperglycemia during the pregnancy. Braz J Med Biol Res. 1990; 23:1079 - 89.

02. Rudge MV, Calderon IM, Ramos MD, Abbade JF, Rugolo LM. Perinatal outcome of pregnancies complicated by diabetes and by maternal daily hyperglycemia not related to diabetes. A retrospective 10-year analysis. Gynecol Obstet Invest. 2000; 50:108-12.

03. Rudge MVC, Calderon IMP, Ramos MD, Brasil MAM, Rugolo LMSS, Bossolan G, et al. Hiperglicemia materna diária diagnosticada pelo perfil glicêmico: um problema de saúde pública materno e perinatal. RBGO. 2005; 27(11):691-7.

04. Metzger BE, Lowe LP, Dyer AR, Trimble ER, Chaovarindr U, Coustan DR, et al. Hyperglycemia and adverse pregnancy outcomes. N Engl J Med. 2008; 358:1991-2002.

05. Weinert LS. International Association of Diabetes and Pregnancy Study Groups recommendations on the diagnosis and classification of hyperglycemia in pregnancy: comment to the International Association of Diabetes and Pregnancy Study Groups Consensus Panel. Diabetes Care. 2010; 33:675-82.

06. Calderon IMP, Damasceno DC, Amorin RL, Costa RAA, Brasil MAM, Rudge MVC. Morphometric study of placental villous and vessels in maternal hyperglycemia, gestational and overt diabetic pregnancies. Diabetes Res Clin Pract. 2007; 78:65-71.

07. Carvalho-Silva, SAL. Dopplervelocimetria da artéria umbilical e controle

glicêmico materno como marcadores de alterações vasculares e apoptóticas placentárias [tese]. Botucatu: Faculdade de medicina, Universidade Estadual Paulista; 2010.

08. Pietro L, Daher S, Rudge MV, Calderon IM, Damasceno DC, Sinzato YK, et al. Vascular endothelial growth factor (VEGF) and VEGF-receptor expression in placenta of hyperglycemic pregnant women. Placenta. 2010; 31:770-80

09. Sgarbosa F, Barbisan LF, Brasil MA, Costa E, Calderon IM, Goncalves CR, et al. Changes in apoptosis and Bcl-2 expression in human hyperglycemic, term placental trophoblast. Diabetes Res Clin Pract. 2006; 73:143-9.

10. Brogin Moreli J, Cirino Ruocco AM, Vernini JM, Rudge MV, Calderon IM. Interleukin 10 and tumor necrosis factor-alpha in pregnancy: aspects of interest in clinical obstetrics. ISRN Obstet Gynecol. 2012;230742.

11. Moreli JB, Morceli G, De Luca AK, Magalhães CG, Costa RA, Damasceno DC, et al. Influence of maternal hyperglycemia on IL-10 and TNF-α production: the relationship with perinatal outcomes. J Clin Immunol. 2012; 32:604-10.

12. Moreli JB, Corrêa-Silva S, Damasceno DC, Sinzato YK, Lorenzon-Ojea AR, Borbely AU, et al. Dynamics changes in the TNF-alpha/IL-10 ratio in hyperglycemic-associated pregnancies. 2015: *In press*.

13. Damasceno DC, Volpato GT, de Mattos Paranhos Calderon I, Cunha Rudge MV. Oxidative stress and diabetes in pregnant rats. Anim Reprod Sci. 2002; 72:235-44.

14. Spada AP, Damasceno DC, Sinzato YK, Campos KE, Faria PA, Dallaqua B, et al. Oxidative stress in maternal blood and placenta from mild diabetic rats. Reprod Sci.

2014; 21:973-77.

15. Lima PH, Damasceno DC, Sinzato YK, de Souza Mda S, Salvadori DM, Calderon Ide M, et al. Levels of DNA damage in blood leukocyte samples from non-diabetic and diabetic female rats and their fetuses exposed to air or cigarette smoke. Mutat Res. 2008; 653:44-9.

16. Lima PH, Sinzato YK, de Souza Mda S, Braz MG, Rudge MV, Damasceno DC. Evaluation of level of DNA damage in blood leukocytes of non-diabetic and diabetic rat exposed to cigarette smoke. Mutat Res. 2007; 628:117-22.

17. Lima PH, Sinzato YK, Gelaleti RB, Calderon IMP, Rudge MVC, Damasceno DC. Genotoxicity evaluation in severe or mild diabetic pregnancy in laboratory animals. Exp Clin Endocrinol Diabetes. 2012; 120:303–07.

18. Friedberg EC. DNA damage and repair. Nature. 2003; 421:436-40.

19. Berra CM, Menck CF, Di Mascio P. Oxidative stress, genome lesions and signaling pathways in cell cycle control. Quimica Nova. 2006; 29:1340-44.

20. Kryston TB, Georgiev AB, Pissis P, Georgakilas AG. Role of oxidative stress and DNA damage in human carcinogenesis. Mutat Res. 2011; 711:193-01.

21. Giovannucci E, Harlan DM, Archer MC, Bergenstal RM, Gapstur SM, Habel LA, et al. Diabetes and cancer: a consensus report. Diabetes Care. 2010; 33:1674-85.

22. Perrin MC, Terry MB, Kleinhaus K, Deutsch L, Yanetz R, Tiram E, et al. Gestational diabetes as a risk factor for pancreatic cancer: a prospective cohort study. BMC Med. 2007; 5:25.

23. Troise R, Weiss H, Hoover R. Pregnancy characteristics and maternal risk of

breast câncer. Epidemiology. 1998; 9:641-47.

24. Dawson SI. Long-term risk of malignant neoplasm associated with gestational glucose intolerance. Cancer. 2004; 100:149-55.

25. Sella T, Chodick G, Barchana M, Heymann AD, Porath A, Kokia E, et al. Gestational diabetes and risk of incident primary cancer: a large historical cohort study in Israel. Cancer Causes Control. 2011; 22:1513-20.

26. Moreli JB, Santos JH, Rocha CR, Damasceno DC, Morceli G, Rudge MV, et al. DNA damage and its cellular response in mother and fetus exposed to hyperglycemic environment. Biomed Res Int. 2014; doi: 10.1155/2014/676758.

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Review Article

DNA Damage and Its Cellular Response in Mother and Fetus Exposed to Hyperglycemic Environment

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The increased production of reactive oxygen species (ROS) plays a key role in pathogenesis of diabetic complications. ROS are generated by exogenous and endogenous factors such as during hyperglycemia. When ROS production exceeds the detoxification and scavenging capacity of the cell, oxidative stress ensues. Oxidative stress induces DNA damage and when DNA damage exceeds the cellular capacity to repair it, the accumulation of errors can overwhelm the cell resulting in cell death or fixation of genome mutations that can be transmitted to future cell generations. These mutations can lead to and/or play a role in cancer development. This review aims at (i) understanding the types and consequences of DNA damage during hyperglycemic pregnancy; (ii) identifying the biological role of DNA repair during pregnancy, and (iii) proposing clinical interventions to maintain genome integrity. While hyperglycemia can damage the maternal genetic material, the impact of hyperglycemia on fetal cells is still unclear. DNA repair mechanisms may be important to prevent the deleterious effects of hyperglycemia both in mother and in fetus DNA and, as such, prevent the development of diseases in adulthood. Hence, in clinical practice, maternal glycemic control may represent an important point of intervention to prevent the deleterious effects of maternal hyperglycemia to DNA.

1. Introduction

Diabetes mellitus (DM) is a metabolic disease characterized by hyperglycemia resulting from a defect in insulin action and/or production [1]. In pregnancy, hyperglycemia poses a risk to maternal, fetal, and perinatal health [2–4]. Perinatal complications of a diabetic pregnancy include malformations, macrosomia, hypoxia, hypoglycemia, cardiomyopathy, hyperbilirubinemia, and hyperinsulinemia [3, 5–9]. The current literature acknowledges this adverse environment as associated with increased long-term risk for the development of diabetes, obesity, cardiovascular, and malignant diseases (Figure 1) [9–14].

Previous findings by our group have shown that maternal hyperglycemia is also adversely involved in fetal development by changing the placental production of proinflammatory cytokines, that is, TNF-α (tumor necrosis factor alpha) [15, 16]. The cellular redox status may be an important connection between inflammation and adverse perinatal outcomes in hyperglycemic pregnancies [17]. There is considerable evidence that hyperglycemia and inflammation results in the generation of reactive oxygen species (ROS), ultimately leading to increased oxidative stress. In the absence of an appropriate antioxidant response, the system becomes overwhelmed leading to production of reactive molecules that can cause cellular damage and are responsible for the

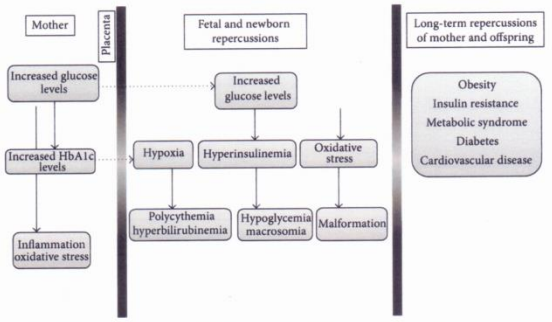

FIGURE 1: Schematic representation of outcomes classically associated with hyperglycemic pregnancies. The representation does not show all possible relationships between the characteristics that are depicted. Adapted from Metzger et al. [75], Negrato et al. [11], and Fraser and Lawlor [52].

late complications of diabetes [17, 18]. During pregnancy the placenta is an additional source of ROS generation, contributing to oxidative stress even in normal pregnancies. This is increased in pregnancies complicated by preeclampsia, intrauterine growth restriction, and pregestational diabetes where oxidative and nitrative stress have been clearly documented [19, 20].

Oxidative stress induces protein oxidation, lipid peroxidation, and DNA damage both in mitochondrial and nuclear DNA. Degradation processes can remove lipids and proteins but not DNA, which needs conversely to be repaired. When DNA damage exceeds the cellular capacity to repair it, the accumulation of errors can overwhelm the cell and result in cell death or the incorporation of genome mutations that can be transmitted to future cell generations if they occur in germ cells (Figure 2). In addition, mutations in somatic cells can promote genome instability and directly lead to various human diseases including cancer, neurological abnormalities, immunodeficiency, and premature aging [21–25].

Considering that hyperglycemia may alter genomic integrity and the consequences of this relationship to maternal and fetus genome is unclear, this review aims at (i) assessing the types and consequences of DNA damage during hyperglycemic pregnancy and lifelong risks, (ii) identifying the biological role of DNA repair during pregnancy, and (iii) proposing clinical interventions to maintain genome integrity.

2. Hyperglycemia-Induced Oxidative Stress and Its Effects on DNA Structure

Hyperglycemia causes many of the major complications of diabetes including nephropathy, retinopathy, neuropathy, and macro- and microvascular damage [1]. To date, there is emerging evidence that oxidative stress significantly contributes to the progression of diabetes and its complications and induces alterations in embryonic and fetal development during pregnancy [18, 26]. Li and collaborators [27] found that mothers with GDM and their newborns had higher levels of 8-Isoprostaglandin F2α (an oxidative stress marker) than control group. Hyperglycemia induces ROS production during such processes as nonenzymatic glycosylation, increased generation of superoxide anion radical by the mitochondrial respiratory chain and the overactivation of NADPH oxidase (nicotinamide adenine dinucleotide phosphate-oxidase) [28, 29].

Overproduction of ROS is capable of altering the structure and function of all types of molecules including proteins, membrane lipids, and nucleic acids with serious consequences to cell viability [21, 30]. Different degradation processes can remove oxidized lipids and proteins. DNA, however, has to be repaired or in the case of mitochondrial DNA may even be removed. The latter is intrinsic to the various copies of mitochondrial genome present in each mitochondrion and the fact that many mitochondria populate a cell [21, 31]. ROS are able to induce DNA lesions as abasic sites (AP sites), single strand breaks, and double strand breaks and oxidize DNA bases. All four bases are susceptible to oxidative damage by ROS. However, due to the lower redox potential of guanine this base is more susceptible to oxidation [23, 30, 32]. The oxidized guanine (8-oxodG) has great biological importance as this is a mutagenic lesion that induces G-T transversions. It may also impair DNA replication and transcription and may be an intermediate for other types of lesions in DNA [23, 33].

Substantial evidence suggests that mitochondrial DNA may be more vulnerable than nuclear DNA to certain kinds of damage, in particular, ROS-mediated lesions [31, 34, 35].

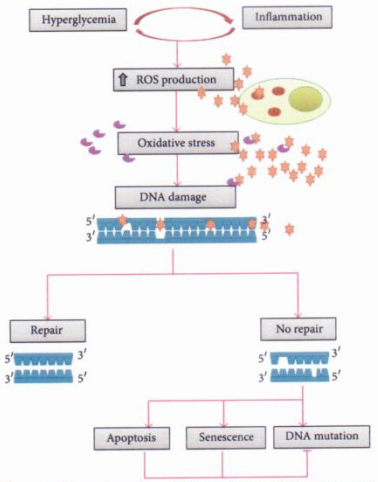

FIGURE 2: Hyperglycemia and inflammation are able to increased ROS production. When ROS production exceeds the detoxification and scavenging capacity of the cell, oxidative stress ensues. Oxidative stress induces DNA damage and when DNA damage exceeds the cellular capacity to repair it, the accumulation of errors can overwhelm the cell resulting in apoptosis, cell senescence, or fixation of genome mutations that will be transmitted to future cell generations. These mutations can lead to and/or play a role in cancer development.

Several reasons may underline this affirmation, including the immediate proximity of mitochondrial DNA to the electron transport chain in the inner mitochondrial membrane, which is the main source of endogenous ROS production. In addition, the repair of mitochondrial DNA lesions occurs only via base excision repair and unlike the nuclear genome, the mitochondrial DNA is not protected by histones [31, 34, 35].

It is important to remember that the genomes of all organisms are constantly being modified by reactive molecules that are produced endogenously, primarily via mitochondrial respiration or by environmental/exogenous physical, chemical, and biological agents including ultraviolet light, ionizing radiation, heavy metals, air pollutants, chemotherapeutic drugs, and inflammatory responses [25, 36].

3. Hyperglycemia, DNA Damage, and Pregnancy: Results of Experimental and Clinical Studies

In nonpregnant context, the relationship between type 1 diabetes mellitus (T1DM), type 2 diabetes mellitus (T2DM), and DNA damage is well established [42–48]. Little is known about DNA damage in pregnancy, especially in pregnancy complicated by pregestational (T1DM or T2DM) or gestational diabetes mellitus (GDM) [7, 37–39, 41, 49].

Experimental studies conducted in our laboratory with streptozotocin-induced diabetic rats showed that the levels of basal DNA damage in leukocyte of mothers with severe diabetes (blood glucose ≥ 300 mg/dL) and their respective fetus was higher when compared with the control group [37, 38]. Subsequently, Lima et al. [7] demonstrated that rats with severe diabetes and their offspring showed higher oxidatively generated DNA damage in leukocyte detected by Fpg (formamidopyrimidine-DNA glycosylase) and endonuclease III-sensitive sites when compared to mild diabetes group (blood glucose levels between 120 and 299 mg/dL). Taken together, these experimental results suggest that the intensity of diabetes is related to the levels of oxidative DNA damage. Thus, hyperglycemia may have repercussions at the DNA level that go beyond the pregnant mother.

In a pilot study, Qiu and collaborators [39] evaluated, in early pregnancy, levels of urinary 8-oxodG trying to determine an association with the risk of GDM development. They observed that the risk for GDM was higher in overweight women with urine 8-oxodG concentrations ≥8.01 ng/creatinine mg (OR = 5.36; 95% CI 1.33–21.55) when compared with lean women who had 8-oxodG concentrations <8.01 ng/creatinine mg. Interestingly, levels of 8-oxodG in umbilical vein plasma in pregestational and control groups were reported to be similar [40].

Evaluation of telomere length is another way to estimate the stability of the genetic material. Telomeric length and telomerase activity (a reverse transcriptase that limits telomere attrition) were studied in mononuclear cells isolated from umbilical cord blood of pregnant women with pregestational diabetes (T1DM and T2DM) and GDM. No difference was found in cord blood telomere length in pregnancies of women with diabetes compared with control subjects, but higher telomerase activity was observed in Type 1 and GDM groups. The upregulation of telomerase may be a compensatory response to *in utero* oxidatively generated DNA and telomere damage [41].

Previous study demonstrated that telomerase is found in nuclei and mitochondria. Telomerase is able to decrease mitochondrial levels of ROS, especially in mitochondria [34, 50]. Recently, Li and collaborators [27] evaluated the mitochondrial translocation of human telomerase reverse transcriptase (hTERT) in mononuclear cells isolated from umbilical cord blood during pregnancies complicated by GDM with confirmed oxidative stress. They found that the ratio of mitochondrial/nuclei hTERT was increased significantly in the newborn of GDM mothers, suggesting that mitochondrial hTERT in cord blood mononuclear cells may have a protective effect on neonatal mitochondrial DNA in GDM pregnancies. The authors concluded that this dynamic translocation could be an *in utero* adaptive response of a fetus that is suffering from elevated oxidative stress and could help our understanding of the roles of oxidative stress in fetal programming.

Table 1: Maternal and fetal DNA integrity in hyperglycemic environment.

Reference	Study type	Type of diabetes	Sample	Evaluation	Main results
[37]	Experimental	Severe	Maternal leukocytes	Comet assay	Basal DNA damage in severe diabetes
[38]	Experimental	Severe	Fetal leukocytes	Comet assay	Basal DNA damage in severe diabetes
[7]	Experimental	Mild and severe	Maternal and fetal leukocytes	Comet Assay with Fpg and Endo III enzymes*	Oxidative DNA damage in severe diabetes
[39]	Clinical	GDM	Maternal urine	8-oxodG levels	Elevated in early pregnancy that results in GDM
[40]	Clinical	Pregestational	Umbilical vein plasma	8-oxodG levels	No difference
[41]	Clinical	Pregestational and GDM	Cord blood mononuclear cell	Telomere length and telomerase activity	Telomerase activity higher in cord blood from TIDM and GDM
[27]	Clinical	GDM	Cord blood mononuclear cells	Mitochondrial translocation of hTERT	Increased mitochondrial hTERT levels in GDM

GDM: gestational diabetes mellitus; hTERT: human telomerase reverse transcriptase. *The endonuclease III (Endo III) and formamidopyrimidine-DNA glycosylase (FPG) are enzymes used to detect oxidative DNA damage.

A few years ago, epigenetic processes have been suggested as a link between maternal pregnancy diabetes and long-term offspring outcomes. Epigenetic modifications, such as DNA methylation, regulate gene expression without altering the DNA sequence. These alterations occur in response to environmental stimuli [51–54]. Recent studies compared the levels of global methylation in the placenta and umbilical cord blood among women with and without gestational diabetes, preeclampsia, and obesity. They found that the mother's metabolic problems during pregnancy may influence the epigenome in the offspring [51]. del Rosario et al. [54] found that epigenetic changes (DNA methylation) may increase the risk of type 2 diabetes; studies support this association but research in this area is still inconclusive [52].

In summary, the results found in the literature indicate that maternal and fetal cells, especially mononuclear cells of blood, respond differently to the hyperglycemic environment (Table 1). While it is clear that hyperglycemia can damage the maternal genetic material, the results in umbilical cord blood (fetal cells) remain unclear. It seems that umbilical cord blood cells have more efficient mechanisms working to protect the genome. Future investigations on the mechanisms involved in genome protection in fetal cells as well as the role of epigenetic changes may shed new light on the outcome on offspring born from women with gestational diabetes.

4. DNA Repair Mechanisms Are Important to Maintain the Genetic Stability

To maintain genetic stability organisms possess cellular mechanisms collectively termed the DNA damage response (DDR) to detect DNA lesions and signal their presence and promote their repair. Cells with DDR defects display higher sensitivity toward DNA damaging agents and many such defects lead to mutagenesis, cytotoxicity, cell death, and disease. In fact, genomic instability and defects in DDR are known to play a role in disease processes such as carcinogenesis, neurodegenerative disorders, immune deficiencies, infertility, aging, cardiovascular disease, and metabolic syndrome [30, 55]. In this session we will focus on DNA repair.

To repair different types of DNA lesions the cell counts on a variety of proteins that presumably undergo crosstalk to form a network for protection of the cellular genome. [25, 56–59].

Nucleotide excision repair (NER), mismatch repair (MMR), and base excision repair (BER) have been implicated in the repair of ROS-induced lesions in DNA. However, BER is the main mechanism involved in the removal of these lesions in nuclear DNA and is the unique mechanism demonstrated for mitochondria damaged DNA [31, 34, 35, 60]. BER predominantly repairs oxidized bases, AP sites, and single strand breaks. In general, BER initiates with the action of a DNA glycosylase that is able to remove the damaged base resulting in an AP site. The AP site is then cleaved by the AP-endonuclease, allowing the DNA polymerase (β in the nucleus or gamma in the mitochondria) to synthesize the repair patch. The latter is relegated based on DNA ligase III activity [60].

5. The Possible Role of DNA Repair during Pregnancy and Diabetes Disease

Studies have demonstrated the importance of DNA repair genes in pregnancy and perinatal development. Patients with mutations in XPD (Xeroderma pigmentosum D) and GTF2H5 (general transcription factor IIH, polypeptide 5), genes involved in the NER pathway and in transcription-couple repair, have the DNA repair diseases: trichothiodystrophy (TTD), xeroderma pigmentosum (XP), Cockayne syndrome (CS), cerebro-ocular facial syndrome (COFS), or a combination [24, 61, 62]. The pregnancies in which the fetus had TTD were at significantly increased risk of preeclampsia, HELLP (hemolysis, elevated liver enzymes, and low platelet count) syndrome, and elevated mid-trimester maternal serum human chorionic gonadotropin levels. The

affected fetus had decreased fetal movement and preterm delivery with higher index of small for gestational age fetus [63]. The authors hypothesized that mutations observed in TTD patients affect placental development. Two years later, the same group revealed that only a specific subset of XPD mutations, which lead to TTD but are unrelated to XP, results in higher risk to develop preeclampsia and other gestational complications [64]. A functional polymorphism (199 Arg-399Gln) in XRCC1 (X-ray repair complementing defective repair in Chinese hamster cells 1), a gene involved in the BER pathway, showed higher frequency among patients with preeclampsia (OR 1.65; 95% CI 1.23–2.19) in an Iranian population [65]. However, this polymorphism was not associated with T2DM in a Polish population [66].

DNA repair was evaluated in lymphocytes of nonpregnant patients with T1DM and T2DM [45, 46]. The results of Blasiak et al. [45] suggest that T2DM may be associated not only with elevated levels of oxidative DNA damage but also with decreased efficacy of DNA repair. In an elegant study Pácal et al. [46] compared DNA damage and repair in lymphocytes of T1DM children, T1DM adults, and T2DM adults. The T2DM diabetics exhibited a significant increase in DNA damage and decreased DNA repair capacity when compared with T1DM (both children and adults). T1DM children displayed a significant decrease of DNA damage and increase in DNA repair when compared with diabetic adults (both T2DM and T1DM). These findings indicate significant age- and DM type-related changes of DNA damage and repair capacity in diabetic subjects.

In summary, the data available suggest that DNA repair mechanisms are involved in the long-term consequences of diabetes in T1DM and T2DM subjects. In pregnancy, DNA repair genes may affect the harmony of maternal-fetal interface resulting in adverse perinatal results.

6. Diabetes and Cancer

Epidemiologic evidence suggests that diabetic patients are at significantly higher risk for many types of cancer. T2DM, GDM, and cancer share many risks factors but potential biological links between the two diseases are unclear [67, 68]. Meta-analyses have reported an increased risk of liver, pancreatic, renal, endometrial, colorectal, bladder, and breast cancer as well as an increase in the incidence of non-Hodgkin lymphoma in T2DM subjects [68]. For those with T2DM compared with those without diabetes, the greatest increase in risk is for hepatocellular carcinoma (RR 2.5; 95% CI 1.8–3.5), with the relative risk for cancer at other sites being between 1.18 (95% CI 1.05–1.32) for breast cancer and 2.22 (95% CI 1.8–2.74) for endometrial cancer in those with diabetes [68, 69]. A prospective cohort study with 37.926 women in Jerusalem observed no cases of pancreatic cancer in the women with T1DM; however, women with a history of GDM showed a relative risk of pancreatic cancer of 7.1 (95% confidence interval 2.8–18.0) [70]. Similar results were observed with a late cohort in Israel [71]. In addition to the relationship between GDM and pancreatic cancer, the authors observed an increased risk of hematologic malignancies like non-Hodgkin's lymphoma, Hodgkin's lymphoma, and acute myeloid leukemia in the same population [71]. A relationship between GDM and breast cancer was found in a New Zealand population, but when studying the U.S. population this association was not observed [72, 73].

Experts assembled jointly by the American Diabetes Association (ADA) and the American Cancer Society (ACS) reviewed the possible biological links between diabetes and cancer risk. They suggested that diabetes may influence the neoplastic process by several mechanisms, including hyperinsulinemia, hyperglycemia, or chronic inflammation without reference to DNA damage and repair [67]. However, the increase in DNA damage and decrease in DNA repair observed in T2DM subjects may provide a new link between diabetes and cancer [45, 60, 74].

7. Proposed Clinical Intervention Strategy for Maintenance of Genomic Integrity

7.1. Control of Maternal Hyperglycemia. Maternal hyperglycemia is able to induce fetal hyperglycemia [1, 4] (Figure 1), increase the release of proinflammatory cytokines [15, 16], and ROS production [17, 18] (Figure 2). Thus, it appears that maternal glycemic control during hyperglycemic pregnancies is an old and safe strategy to assure maintenance of genomic integrity. Clinical studies have already demonstrated the benefits of maternal glycemic control during pregnancy and how to maintain optimal glucose levels without gestational risk [75, 76].

Nonpregnant adults with diabetes and pregnant women with GDM or pregestational diabetes (T1DM or T2DM) presented different glycemic recommendations [1]. During pregnancy, the glycemic limits are stricter than in nonpregnant state to prevent alteration in both maternal and fetal health [1, 75, 76]. Based on recommendations from the Fifth International Workshop-Conference on Gestational Diabetes Mellitus [77] and ADA's statement [1] it is important to maintain maternal capillary glucose concentrations at <95 mg/dL (<5.3 mmol/L) in the fasting state, <140 mg/dL (<7.8 mmol/L) at 1 h, and <120 mg/dL (<6.7 mmol/L) 2 h after starting the meal. For women with overt diabetes who become pregnant, the optimal glycemic goals are premeal, bedtime, and overnight glucose between 60 and 99 mg/dL (3.3–5.4 mmol/L) and peak postprandial glucose between 100 and 129 mg/dL (5.4–7.1 mmol/L) and HbA1C of 6.0% [78].

Diet therapy, control of weight gain, and increasing physical activity are the standard treatment of GDM [77]. Insulin administration is only performed for pregnant women who fail to maintain glycemic goals as well as to the ones who show signs of excessive fetal growth or overt diabetes. It is recommended that insulin administration be individualized to achieve the glycemic goals stated [77]. During the last decade, there was an increased interest in the use of oral antihyperglycemic agents as an alternative to insulin in achieving good glycemic control. However, the results are inconclusive [79, 80].

7.2. Antioxidant Supplementation during Pregnancy. Antioxidant supplementation is a questionable strategy during

pregnancy. The effects of vitamin C supplementation, alone or in combination with other supplements, have been evaluated on pregnancy outcomes. No difference was seen in the risk of stillbirth, perinatal death, birth weight, or intrauterine growth restriction between women supplemented with vitamin C alone or in combination with other supplements and placebo. In fact, women supplemented with vitamin C alone or combined with other supplements were at increased risk of giving preterm birth [81]. The same researchers also determined the effectiveness and safety of any vitamin supplementation on the risk of spontaneous miscarriage, maternal adverse outcomes, and fetal and infant adverse outcomes. The results indicated that vitamin supplements, alone or in combination, prior to pregnancy or in early pregnancy, did not prevent miscarriage or stillbirth. However, it was found that women taking vitamin supplements were less likely to develop preeclampsia while more likely to have multiple pregnancies [82, 83]. Mothers that have taken antioxidant supplementation during pregnancy had decreased frequency of micronucleus (a test used to quantify chromosomal damage) in peripheral blood mononuclear cells prior to and after hydrogen peroxide exposure. The additional antioxidants intake during pregnancy was also beneficial to reduce the frequency of micronucleus after hydrogen peroxide exposure in cord blood cells. The data demonstrated a positive effect of antioxidant supplementation on micronucleus frequency [84]. Experimental results in a model of diabetic pregnancy indicate that high doses of dietary antioxidants were need to normalize the development of offspring. However, treatment with such high doses may also have adverse effects in nondiabetic pregnancy [85].

It is clear based on the above findings that results about antioxidant supplementation during pregnancy are still inconclusive, and little is known about their impact at the DNA level. Despite this fact, taken together the data support the notion that maternal glycemic control is a good and safe plan to reduce the factors associated to genomic instability in hyperglycemic pregnancy.

8. Conclusions

Although it is clear that hyperglycemia can damage the maternal genetic material, the results obtained for cord blood are not yet clear. The data seem to support the hypothesis that umbilical cord blood cells have more efficient mechanisms to protect the genome than the mother's cells. DNA repair may be thus considered an important mechanism to prevent the deleterious effects of hyperglycemia in the genetic material. However, functional studies demonstrating the ability of DNA repair mechanisms in dealing with insults resulting from hyperglycemia during pregnancy need to be developed. For the time being, the control of maternal hyperglycemia seems a safe and important strategy to prevent the deleterious effects of hyperglycemia on maternal and potentially fetal DNA.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

All authors equally participated in the development of this paper. All authors also read and approved the final paper.

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References

[1] American Diabetes Association (ADA), "Standards of medical care in Diabetes," *Diabetes Care*, vol. 37, pp. S14–S80, 2014.

[2] U. J. Eriksson, "Congenital anomalies in diabetic pregnancy," *Seminars in Fetal and Neonatal Medicine*, vol. 14, no. 2, pp. 85–93, 2009.

[3] M. V. C. Rudge, I. M. P. Calderon, M. D. Ramos et al., "Hiperglicemia materna diária diagnosticada pelo perfil glicêmico: um problema de saúde pública materno e perinatal," *Revista Brasileira de Ginecologia e Obstetrícia*, vol. 27, no. 11, pp. 691–697, 2005.

[4] J. Yang, E. A. Cummings, C. O'Connell, and K. Jangaard, "Fetal and neonatal outcomes of diabetic pregnancies," *Obstetrics & Gynecology*, vol. 108, no. 3, part 1, pp. 644–650, 2006.

[5] J. G. Eriksson, T. J. Forsen, C. Osmond, and D. J. P. Barker, "Pathways of infant and childhood growth that lead to type 2 diabetes," *Diabetes Care*, vol. 26, no. 11, pp. 3006–3010, 2003.

[6] I. M. P. Calderon, L. T. R. L. Kerche, D. C. Damasceno, and M. V. C. Rudge, "Diabetes and pregnancy: an update of the problem," *Annual Review of Biomedical Sciences*, vol. 9, pp. 1–11, 2007.

[7] P. H. O. Lima, Y. K. Sinzato, R. B. Gelaleti, I. M. P. Calderon, M. V. C. Rudge, and D. C. Damasceno, "Genotoxicity evaluation in severe or mild diabetic pregnancy in laboratory animals," *Experimental and Clinical Endocrinology and Diabetes*, vol. 120, no. 5, pp. 303–307, 2012.

[8] E. M. Wendland, M. R. Torloni, M. Falavigna et al., "Gestational diabetes and pregnancy outcomes—a systematic review of the World Health Organization (WHO) and the International Association of Diabetes in Pregnancy Study Groups (IADPSG) diagnostic criteria," *BMC Pregnancy and Childbirth*, vol. 12, article 23, 2012.

[9] H. Lehnen, U. Zechner, and T. Haaf, "Epigenetics of gestational diabetes mellitus and offspring health: the time for action is in early stages of life," *Molecular Human Reproduction*, vol. 19, no. 7, pp. 415–422, 2013.

[10] A. Ornoy, "Prenatal origin of obesity and their complications: gestational diabetes, maternal overweight and the paradoxical effects of fetal growth restriction and macrosomia," *Reproductive Toxicology*, vol. 32, no. 2, pp. 205–212, 2011.

[11] C. A. Negrato, R. Mattar, and M. B. Gomes, "Adverse pregnancy outcomes in women with diabetes," *Diabetology and Metabolic Syndrome*, vol. 4, article 41, 2012.

[12] C. S. Wu, E. A. Nohr, B. H. Bech, M. Vestergaard, and J. Olsen, "Long-term health outcomes in children born to mothers with diabetes: a population-based cohort study," *PLoS ONE*, vol. 7, no. 5, Article ID e36727, 2012.

[13] S. Brewster, B. Zinman, R. Retnakaran, and J. S. Floras, "Cardiometabolic consequences of gestational dysglycemia," *Journal*

of the American College of Cardiology, vol. 62, no. 8, pp. 677–684, 2013.

[14] N. A. West, K. Kechris, and D. Dabelea, "Exposure to maternal diabetes in Utero and DNA methylation patterns in the offspring," *Immunometabolism*, vol. 1, pp. 1–9, 2013.

[15] J. B. Moreli, G. Morceli, A. K. C. De Luca et al., "Influence of maternal hyperglycemia on IL-10 and TNF-α production: the relationship with perinatal outcomes," *Journal of Clinical Immunology*, vol. 32, no. 3, pp. 604–610, 2012.

[16] J. B. Moreli, S. Corrêa-Silva, D. C. Damasceno et al., "Dynamics changes in the TNF-alpha/IL-10 ratio in hyperglycemic-associated pregnancies," Under review.

[17] J. L. Evans, I. D. Goldfine, B. A. Maddux, and G. M. Grodsky, "Oxidative stress and stress-activated signaling pathways: a unifying hypothesis of type 2 diabetes," *Endocrine Reviews*, vol. 23, no. 5, pp. 599–622, 2002.

[18] D. C. Damasceno, G. T. Volpato, I. de Mattos Paranhos Calderon, and M. V. Cunha Rudge, "Oxidative stress and diabetes in pregnant rats," *Animal Reproduction Science*, vol. 72, no. 3-4, pp. 235–244, 2002.

[19] A. Agarwal, S. Gupta, and S. Sikka, "The role of free radicals and antioxidants in reproduction," *Current Opinion in Obstetrics and Gynecology*, vol. 18, no. 3, pp. 325–332, 2006.

[20] L. Myatt, "Reactive oxygen and nitrogen species and functional adaptation of the placenta," *Placenta*, vol. 31, pp. S66–S69, 2010.

[21] C. M. Berra, C. F. M. Menck, and P. Di Mascio, "Oxidative stress, genome lesions and signaling pathways in cell cycle control," *Quimica Nova*, vol. 29, no. 6, pp. 1340–1344, 2006.

[22] S. Lagerwerf, M. G. Vrouwe, R. M. Overmeer, M. I. Fousteri, and L. H. F. Mullenders, "DNA damage response and transcription," *DNA Repair*, vol. 10, no. 7, pp. 743–750, 2011.

[23] L. F. Agnez-Lima, J. T. A. Melo, A. E. Silva et al., "DNA damage by singlet oxygen and cellular protective mechanisms," *Mutation Research*, vol. 751, no. 1, pp. 15–28, 2012.

[24] M. C. S. Moraes, J. B. C. Neto, and C. F. M. Menck, "DNA repair mechanisms protect our genome from carcinogenesis," *Frontiers in Bioscience*, vol. 17, no. 4, pp. 1362–1388, 2012.

[25] T. Iyama and D. M. Wilson III, "DNA repair mechanisms in dividing and non-dividing cells," *DNA Repair*, vol. 12, no. 8, pp. 620–636, 2013.

[26] P. Rösen, P. P. Nawroth, G. King, W. Möller, H. J. Tritschler, and L. Packer, "The role of oxidative stress in the onset and progression of diabetes and its complications: a summary of a congress series sponsored by UNESCO-MCBN, the American diabetes association and the German diabetes society," *Diabetes/Metabolism Research and Reviews*, vol. 17, no. 3, pp. 189–212, 2001.

[27] P. Li, Y. Tong, H. Yang et al., "Mitochondrial translocation of human telomerase reverse transcriptase in cord blood mononuclear cells of newborns with gestational diabetes mellitus mothers," *Diabetes Research and Clinical Practice*, vol. 103, pp. 310–318, 2014.

[28] M. Brownlee, "Biochemistry and molecular cell biology of diabetic complications," *Nature*, vol. 414, no. 6865, pp. 813–820, 2001.

[29] D. H. Endemann and E. L. Schiffrin, "Nitric oxide, oxidative excess, and vascular complications of diabetes mellitus," *Current Hypertension Reports*, vol. 6, no. 2, pp. 85–89, 2004.

[30] S. P. Jackson and J. Bartek, "The DNA-damage response in human biology and disease," *Nature*, vol. 461, no. 7267, pp. 1071–1078, 2009.

[31] O. A. Kovalenko and J. H. Santos, "Analysis of oxidative damage by gene-specific quantitative PCR," in *Current Protocols in Human Genetics*, chapter 19, unit 19.1, 2009.

[32] S. Steenken, "Electron transfer in DNA? Competition by ultrafast proton transfer?" *Biological Chemistry*, vol. 378, no. 11, pp. 1293–1297, 1997.

[33] M. Dizdaroglu, "Oxidatively induced DNA damage: mechanisms, repair and disease," *Cancer Letters*, vol. 327, no. 1-2, pp. 26–47, 2012.

[34] J. H. Santos, J. N. Meyer, B. S. Mandavilli, and B. van Houten, "Quantitative PCR-based measurement of nuclear and mitochondrial DNA damage and repair in mammalian cells," *Methods in Molecular Biology*, vol. 314, pp. 183–199, 2006.

[35] B. van Houten, V. Woshner, and J. H. Santos, "Role of mitochondrial DNA in toxic responses to oxidative stress," *DNA Repair*, vol. 5, no. 2, pp. 145–152, 2006.

[36] T. Lindahl, "Instability and decay of the primary structure of DNA," *Nature*, vol. 362, no. 6422, pp. 709–715, 1993.

[37] P. H. O. Lima, Y. K. Sinzato, M. D. S. S. de Souza, M. G. Braz, M. V. C. Rudge, and D. C. Damasceno, "Evaluation of level of DNA damage in blood leukocytes of non-diabetic and diabetic rat exposed to cigarette smoke," *Mutation Research*, vol. 628, no. 2, pp. 117–122, 2007.

[38] P. H. O. Lima, D. C. Damasceno, Y. K. Sinzato et al., "Levels of DNA damage in blood leukocyte samples from non-diabetic and diabetic female rats and their fetuses exposed to air or cigarette smoke," *Mutation Research/Genetic Toxicology and Environmental Mutagenesis*, vol. 653, no. 1-2, pp. 44–49, 2008.

[39] C. Qiu, K. Hevner, D. Abetew, D. A. Enquobahrie, and M. A. Williams, "Oxidative DNA damage in early pregnancy and risk of gestational diabetes mellitus: a pilot study," *Clinical Biochemistry*, vol. 44, no. 10-11, pp. 804–808, 2011.

[40] J. Verhaeghe, R. van Bree, and E. van Herck, "Oxidant balance markers at birth in relation to glycemicand acid-base parameters," *Metabolism: Clinical and Experimental*, vol. 60, no. 1, pp. 71–77, 2011.

[41] J. A. Cross, R. C. Temple, J. C. Hughes et al., "Cord blood telomere length, telomerase activity and inflammatory markers in pregnancies in women with diabetes or gestational diabetes," *Diabetic Medicine*, vol. 27, no. 11, pp. 1264–1270, 2010.

[42] A. R. Collins, K. Raslová, M. Somorovská et al., "DNA damage in diabetes: correlations with a clinical marker," *Free Radical Biology and Medicine*, vol. 25, no. 3, pp. 373–377, 1998.

[43] Y. Dinçer, T. Akçay, H. Ilkova, Z. Alademir, and G. Özbay, "DNA damage and antioxidant defense in peripheral leukocytes of patients with Type I diabetes mellitus," *Mutation Research*, vol. 527, no. 1-2, pp. 49–55, 2003.

[44] V. Pitozzi, L. Giovannelli, G. Bardini, C. M. Rotella, and P. Dolara, "Oxidative DNA damage in peripheral blood cells in type 2 diabetes mellitus: higher vulnerability of polymorphonuclear leukocytes," *Mutation Research*, vol. 529, no. 1-2, pp. 129–133, 2003.

[45] J. Blasiak, M. Arabski, R. Krupa et al., "DNA damage and repair in type 2 diabetes mellitus," *Mutation Research*, vol. 554, no. 1-2, pp. 297–304, 2004.

[46] L. Pácal, J. Varvařovská, Z. Rušavý et al., "Parameters of oxidative stress, DNA damage and DNA repair in type 1 and type 2 diabetes mellitus," *Archives of Physiology and Biochemistry*, vol. 117, pp. 222–230, 2011.

[47] E. Tatsch, G. V. Bochi, S. J. Piva et al., "Association between DNA strand breakage and oxidative, inflammatory and endothelial

biomarkers in type 2 diabetes," *Mutation Research*, vol. 732, no. 1-2, pp. 16–20, 2012.

[48] M. Witczak, T. Ferenc, E. Gulczynska, D. Nowakowska, D. Lopaczynska, and J. Wilczynski, "Elevated frequencies of micronuclei in pregnant women with type 1 diabetes mellitus and in their newborns," *Mutat Res*, pp. S1383–5718, 2014.

[49] M. T. Goodarzi, A. A. Navidi, M. Rezaei, and H. Babahmadi-Rezaei, "Oxidative damage to DNA and lipids: correlation with protein glycation in patients with type 1 diabetes," *Journal of Clinical Laboratory Analysis*, vol. 24, no. 2, pp. 72–76, 2010.

[50] N. K. Sharma, M. Lebedeva, T. Thomas et al., "Intrinsic mitochondrial DNA repair defects in Ataxia Telangiectasia," *DNA Repair*, vol. 13, pp. 22–31, 2014.

[51] Y. Nomura, L. Lambertini, A. Rialdi et al., "Global methylation in the placenta and umbilical cord blood from pregnancies with maternal gestational diabetes, preeclampsia, and obesity," *Reproductive Sciences*, vol. 21, no. 1, pp. 131–137, 2014.

[52] A. Fraser and D. A. Lawlor, "Long-term health outcomes in offspring born to women with diabetes in pregnancy," *Current Diabetes Reports*, vol. 14, article 489, 2014.

[53] S. M. Ruchat, M. F. Hivert, and L. Bouchard, "Epigenetic programming of obesity and diabetes by in utero exposure to gestational diabetes mellitus," *Nutrition Reviews*, vol. 71, supplement 1, pp. S88–S94, 2013.

[54] M. C. del Rosario, V. Ossowski, W. C. Knowler, C. Bogardus, L. J. Baier, and R. L. Hanson, "Potential epigenetic dysregulation of genes associated with MODY and type 2 diabetes in humans exposed to a diabetic intrauterine environment: an analysis of genome-wide DNA methylation," *Metabolism*, vol. 63, pp. 654–660, 2014.

[55] J. W. Harper and S. J. Elledge, "The DNA damage response: ten years after," *Molecular Cell*, vol. 28, no. 5, pp. 739–745, 2007.

[56] C. R. R. Rocha, L. K. Lerner, O. K. Okamoto, M. C. Marchetto, and C. F. M. Menck, "The role of DNA repair in the pluripotency and differentiation of human stem cells," *Mutation Research*, vol. 752, no. 1, pp. 25–35, 2013.

[57] J. H. Hoeijmakers, "Genome maintenance mechanisms for preventing cancer," *Nature*, vol. 411, no. 6835, pp. 366–374, 2001.

[58] E. C. Friedberg, "A brief history of the DNA repair field," *Cell Research*, vol. 18, no. 1, pp. 3–7, 2008.

[59] E. C. Friedberg, "DNA damage and repair," *Nature*, vol. 421, no. 6921, pp. 436–440, 2003.

[60] S. Mitra, I. Boldogh, T. Izumi, and T. K. Hazra, "Complexities of the DNA base excision repair pathway for repair of oxidative DNA damage," *Environmental and Molecular Mutagenesis*, vol. 38, pp. 180–190, 2001.

[61] J. M. Graham Jr., K. Anyane-Yeboa, A. Raams et al., "Cerebro-oculo-facio-skeletal syndrome with a nucleotide excision-repair defect and a mutated XPD gene, with prenatal diagnosis in a triplet pregnancy," *The American Journal of Human Genetics*, vol. 69, no. 2, pp. 291–300, 2001.

[62] A. R. Lehmann, "DNA repair-deficient diseases, xeroderma pigmentosum, Cockayne syndrome and trichothiodystrophy," *Biochimie*, vol. 85, no. 11, pp. 1101–1111, 2003.

[63] R. Moslehi, C. Signore, D. Tamura et al., "Adverse effects of trichothiodystrophy DNA repair and transcription gene disorder on human fetal development," *Clinical Genetics*, vol. 77, no. 4, pp. 365–373, 2010.

[64] R. Moslehi, A. Kumar, J. L. Mills, X. Ambroggio, C. Signore, and A. Dzutsev, "Phenotype-specific adverse effects of XPD mutations on human prenatal development implicate impairment of TFIIH-mediated functions in placenta," *European Journal of Human Genetics*, vol. 20, no. 6, pp. 626–631, 2012.

[65] I. Saadat, Z. Beyzaei, F. Aghaei, S. Kamrani, and M. Saadat, "Association between polymorphisms in DNA repair genes (XRCC1 and XRCC7) and risk of preeclampsia.," *Archives of gynecology and obstetrics*, vol. 286, no. 6, pp. 1459–1462, 2012.

[66] J. Kasznicki, R. Krupa, J. Blasiak, and J. Drzewoski, "Association between polymorphisms of the DNA repair genes XRCC1 and hOGG1 and type 2 diabetes mellitus in the polish population," *Polskie Archiwum Medycyny Wewnetrznej*, vol. 119, no. 3, pp. 122–128, 2009.

[67] E. Giovannucci, D. M. Harlan, M. C. Archer et al., "Diabetes and cancer: a consensus report," *Diabetes Care*, vol. 33, no. 7, pp. 1674–1685, 2010.

[68] Z. Zelenko and E. J. Gallagher, "Diabetes and cancer," *Endocrinology Metabolism Clinics of North America*, vol. 43, pp. 167–185, 2014.

[69] P. Vigneri, F. Frasca, L. Sciacca, G. Pandini, and R. Vigneri, "Diabetes and cancer," *Endocrine-Related Cancer*, vol. 16, no. 4, pp. 1103–1123, 2009.

[70] M. C. Perrin, M. B. Terry, K. Kleinhaus et al., "Gestational diabetes as a risk factor for pancreatic cancer: a prospective cohort study," *BMC Medicine*, vol. 5, article 25, 2007.

[71] T. Sella, G. Chodick, M. Barchana et al., "Gestational diabetes and risk of incident primary cancer: a large historical cohort study in israel," *Cancer Causes and Control*, vol. 22, no. 11, pp. 1513–1520, 2011.

[72] R. Troisi, H. A. Weiss, R. N. Hoover et al., "Pregnancy characteristics and maternal risk of breast cancer," *Epidemiology*, vol. 9, no. 6, pp. 641–647, 1998.

[73] S. I. Dawson, "Long-term risk of malignant neoplasm associated with gestational glucose intolerance," *Cancer*, vol. 100, no. 1, pp. 149–155, 2004.

[74] T. B. Kryston, A. B. Georgiev, P. Pissis, and A. G. Georgakilas, "Role of oxidative stress and DNA damage in human carcinogenesis," *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis*, vol. 711, no. 1-2, pp. 193–201, 2011.

[75] B. E. Metzger, L. P. Lowe, A. R. Dyer et al., "Hyperglycemia and adverse pregnancy outcomes. The HAPO Study Cooperative Research Group," *New England Journal of Medicine*, vol. 358, pp. 1991–2002, 2008.

[76] American Diabetes Association (ADA), "International Association of Diabetes and Pregnancy Study Groups recommendations on the diagnosis and classification of hyperglycemia in pregnancy (consensus panel)," *Diabetes Care*, vol. 33, no. 3, pp. 676–682, 2010.

[77] B. E. Metzger, T. A. Buchanan, D. R. Coustan et al., "Summary and recommendations of the Fifth international workshop—conference on gestational diabetes mellitus," *Diabetes Care*, vol. 30, supplement 2, pp. S251–S260, 2007.

[78] J. L. Kitzmiller, J. M. Block, F. M. Brown et al., "Managing preexisting diabetes for pregnancy: summary of evidence and consensus recommendations for care," *Diabetes Care*, vol. 31, no. 5, pp. 1060–1079, 2008.

[79] P. Merlob, O. Levitt, and B. Stahl, "Oral antihyperglycemic agents during pregnancy and lactation: a review," *Pediatric Drugs*, vol. 4, no. 11, pp. 755–760, 2002.

[80] K. Castorino and L. Jovanovič, "Pregnancy and diabetes management: advances and controversies," *Clinical Chemistry*, vol. 57, no. 2, pp. 221–230, 2011.

[81] A. Rumbold and C. A. Crowther, "Vitamin C supplementation in pregnancy," *Cochrane Database of Systematic Reviews*, Article ID CD004072, 2005.

[82] A. R. Rumbold, F. H. E. Maats, and C. A. Crowther, "Dietary intake of vitamin C and vitamin E and the development of hypertensive disorders of pregnancy," *European Journal of Obstetrics Gynecology and Reproductive Biology*, vol. 119, no. 1, pp. 67–71, 2005.

[83] J. A. Spinnato II, S. Freire, J. L. Pinto F. Silva et al., "Antioxidant therapy to prevent preeclampsia: a randomized controlled trial," *Obstetrics and Gynecology*, vol. 110, no. 6, pp. 1311–1318, 2007.

[84] I. Decordier, K. de Bont, K. de Bock et al., "Genetic susceptibility of newborn daughters to oxidative stress," *Toxicology Letters*, vol. 172, no. 1-2, pp. 68–84, 2007.

[85] J. Cederberg and U. J. Eriksson, "Antioxidative treatment of pregnant diabetic rats diminishes embryonic dysmorphogenesis," *Birth Defects Research A: Clinical and Molecular Teratology*, vol. 73, no. 7, pp. 498–505, 2005.

Artigo Original

Hyperglycemia Differentially Affects Maternal and Fetal DNA Integrity and DNA Damage Response[1]

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Abstract

Objective: The purpose of this study was to assess markers of oxidative stress, DNA damage and the cellular response in maternal and umbilical cord blood of pregnancies complicated by hyperglycemia. **Methods:** One hundred forty-four pregnant women, classified in normoglycemics (ND), mild gestational hyperglycemia (MGH), gestational diabetes mellitus (GDM) and type 2 diabetes mellitus (DM2), were included. The nuclear and mitochondrial DNA damage were evaluated by gene-specific quantitative PCR and the expression of genes and proteins involved in base excision repair (BER) pathway were assessed by real time PCR and western blot, respectively. The apoptosis was evaluated *in vitro* experiment. These analyses were performed in samples from maternal and umbilical cord blood. **Results:** The mothers with GDM and DM2 were characterized by oxidative stress, increase of nuclear and mitochondrial DNA damage and decrease expression of genes and proteins involved in BER. In addition, the levels of hyperglycemia were associated to *in vitro* cellular apoptosis. The newborns of diabetic mothers presented increase of BER genes and proteins expression, and the hyperglycemia environment *in vitro* was not able to induce apoptosis. Blood levels of DNA damage in umbilical cord were similar among groups. **Conclusions:** In this study, maternal hyperglycemia observed in GDM and DM2 groups was associated to oxidative stress and, consequently with nuclear and mitochondrial DNA damage. However, integrity of DNA from umbilical cord blood cells was preserved, suggesting the better involvement of DNA repair mechanisms in these fetuses.

Key words: Diabetes, Pregnancy, DNA damage, DNA repair

Introduction

Diabetes mellitus (DM) is a metabolic disease characterized by hyperglycemia resulting from a defect in insulin action and/or production [1]. In pregnancy, hyperglycemia poses both short-term and long-term risks to the health of women and their offspring [2]. Newborns (NB) of hyperglycemic mothers exhibit an increased risk for malformations, macrosomia, hypoxia, and perinatal death, which are associated with hypoglycemia, hyperbilirubinemia, hyperinsulinemia and hyperleptinemia [3-5]. During adult life, these metabolic alterations increased risk of metabolic, cardiovascular and malignant diseases [2,5].

Oxidative stress has been widely studied as an important link between hyperglycemia and its complications, including alterations in embryonic and fetal development during pregnancy [6-8]. The basal explanation is that hyperglycemia leads to mitochondrial reactive oxygen species (ROS) overproduction and thus can induce protein oxidation, lipid peroxidation and DNA damage in both mitochondrial (mtDNA) and nuclear DNA (nDNA) [6,9-11]. Different degradation processes can remove oxidized lipids and proteins; however, DNA has to be repaired or even be removed in the case of mtDNA damage.

The mtDNA is more vulnerable than nDNA to ROS-mediated lesions [10-12]. One of several reasons that underlie this observation is that only base

excision repair (BER) removes mtDNA lesions [13]. BER is the main mechanism involved in the removal ROS-induced lesions i.e., oxidized bases, AP sites, and single strand breaks. BER is initiated by DNA glycosylases (i.e., human 8-oxoguanine DNA glycosylase - hOGG1), which recognize and remove specific damaged or inappropriate bases, forming AP sites. These are then cleaved by an AP endonuclease, such as human AP Endonuclease (APE-1), forming a single strand break. The resulting break can then be processed by either short-patch or long-patch BER. In the short-patch, APE-1 interacts with DNA polymerase beta (POLβ) leading to single-nucleotide gap synthesis. In the long-patch, APE-1 similarly interacts with Flap endonuclease 1 (FEN-1) for synthesis of 2-10 new nucleotides [14].

When the rate of DNA damage exceeds the cellular capacity to repair it, the accumulation of errors can overwhelm the cell and result in cell death or in the fixation of mutations that may be transmitted to future generations if they occur in germ cells. Mutations that occur in somatic cells can lead to changes in cellular functions, which can contribute to cancer development [15,16].

The literature acknowledges that the repair capacity of DNA damage plays a critical role in the maintenance of genome stability [17]. Specifically, in DM type 2, it has been shown that patients have increased levels of nuclear DNA damage, high rate of lymphocyte apoptosis, and decreased repair capacity

of hydrogen peroxide- and doxorubicin-induced DNA damage [18,19]. In human diabetic pregnancies, data related to genome stability are still limited. A pilot study in health pregnant women associated DNA damage and risk of GDM [20] but no studies involving diabetic pregnant women and DNA damage and/or repair are to our knowledge available. Data from animal models support the correlation between diabetes and DNA damage with two studies in rats with streptozotocin-induced diabetes reporting high levels of nuclear DNA damage in maternal and NB leukocytes [21-23].

The objective of this study was to assess DNA damage and its cellular response in maternal and umbilical cord blood of pregnancies complicated by hyperglycemia. Our hypothesis was that, in these pregnancies, hyperglycemia and oxidative stress may affect both maternal and fetal DNA integrity and cellular response.

Materials and Methods

Ethics statement

This study was conducted in the Diabetes and Pregnancy Service of the Botucatu Medical School/UNESP, Brazil, and was approved by the local Research Ethics Committee (protocol #507/2012). Written informed consent was obtained from all subjects according to the principles of the Declaration of Helsinki.

Study design and subjects selection

One hundred forty-four pregnant women with several degrees of hyperglycemia were enrolled to this cross-sectional study. To calculate the sample size, it was considered a completely randomized experiment with four groups. Supposing that 20 degrees of liberty would be sufficient to reduce the residual variance and to reveal the effect of groups, the minimum subject for each group of mothers and newborns would be six.

Pregnant women with Diabetes mellitus type 2 (DM2; n = 23) were referred to our service with a confirmed diagnosis. The research subjects without pre-gestational diabetes underwent 75 g-GTT test, recommended by ADA [1], and the glucose profile (GP) test, recommended by Rudge [24], between 24th and 28th gestational weeks. According to the 75 g-GTT and GP results, pregnant women were classified into the following study groups: non-diabetic (ND; normal 75-g GTT and GP; n = 46), mild gestational hyperglycemia (MGH; normal 75-g GTT and abnormal GP; n = 30) or gestational diabetes mellitus (GDM; abnormal 75-g GTT first reported during the pregnancy; n = 45) (Figure 1).

The inclusion criteria were as follows: (a) hyperglycemia defined at the maximum gestational age of 28-30 weeks for women with MGH and GDM, and 20 weeks for DM2 pregnant women; (b) prenatal and delivery care at the Service; (c) absence of clinically diagnosed infections and negative

serology for HIV and syphilis, multiple pregnancies, DM1, fetal malformations, fetal death, alcohol or illicit drugs habits; (d) deliveries before the 36th week of gestation.

In MGH or GDM pregnant women the hyperglycemia was controlled through a combination of lifestyle changes, individualized diet and exercise; insulin therapy was used when necessary. Patients with DM2 followed the same protocol but received insulin therapy since the start of treatment to replace oral hypoglycemic agents. The goals of maternal glycemic control were fasting glucose ≤ 95 mg/dL, 1 hour postmeal ≤ 140 mg/dL, and 2 hours postmeal ≤ 120 mg/dL, resulting in a daily glycemic mean (GM) ≤ 120 mg/dL [1].

Sample collection

Maternal blood samples were collected from 36th week of pregnancy and just prior to the beginning of labor. The umbilical cord blood was collected intradelivery, shortly after clamping. The samples of maternal or umbilical cord blood were collected in Vacutainer tubes (Becton Dickinson, USA) treated with EDTA, serum or heparin tubes.

Characterization of the population

The population of pregnant women was characterized by age, body mass index (BMI) in pre-pregnancy and third trimester of pregnancy, weight gain during pregnancy, gestational age at delivery, presence of hypertension

(gestational or chronic hypertension), glycemic mean (GM) and glycated hemoglobin (HbA1c) levels. The GM was calculated by arithmetic mean of plasma glucose levels evaluated in all GPs during treatment. Plasma glucose levels were evaluated by glucose oxidase method (Glucose Analyzer II Beckman, Fullerton, CA, USA). The HbA1c levels were evaluated by chromatography (high-performance liquid chromatography—D10™ Hemoglobin Testing System, Bio-Rad Laboratories, Hercules, CA, USA).

The perinatal results were evaluated by the glucose, insulin, leptin (predictor of NB weight), hematocrit, hemoglobin, pH and total bilirubin in umbilical cord blood. The corporal weight, ponderal index (the weight/length^3 X 100 ratio), NB classification in small (SGA), adequate (AGA), or large (LGA) for the gestational age (the weight/gestational age ratio) and 1st and 5th min Apgar scores were evaluated at delivery.

Hematological parameters (i.e., hematocrit and hemoglobin) were determined in total blood samples with a Sysmex KX-21N (Roche). After evaluation of the hematological parameters, total blood aliquots were centrifuged (4°C for 15 min at 1.000×g) for plasma collection and remaining analyses. Bilirubin concentrations were evaluated by a colorimetric method using BuBc slides (VITROS Chemistry products, Johnson & Johnson), insulin levels were measured by a chemiluminescent immunoassay using microparticles (ARCHITECT insulin, Abbott Laboratories, São Paulo, SP, Brazil)

and leptin by ELISA kit (R&D system, MN, USA).

Evaluation of oxidative stress

Since imbalance between antioxidant and oxidants generates the condition of oxidative stress, estimation of total protein reduced thiols, 8-iso-PGF2α and the antioxidant capacity are useful in the prediction of this condition.

- *Total protein reduced thiols – indirect marker of protein oxidation*

Total reduced thiols were determined in a spectrophotometer (Spectra Max PLUS 384, Molecular Devices) using 5,5-dithionitrobenzoic acid (DTNB-Sigma Aldrich, St Louis, MO, USA). Thiol residues react with DTNB, cleaving the disulfide bond to give 2-nitro-5-thiobenzoate (NTB2), which ionizes to the NTB22 di-anion in water at neutral and alkaline pH. The NTB22 was quantified in a spectrophotometer by measuring the absorbance at 412 nm, and was expressed as mM of reduced thiols /ml of serum.

- *8-Isoprostaglandin F2α levels (8-iso-PGF$_{2\alpha}$) – lipid peroxidation marker*

The 8-iso-PGF2α was detected using a commercially available Direct 8-iso-PGF2α EIA kit (Enzo Life Sciences, Farmingdale, USA). All procedures were carried out following the manufacturer recommendations.

- *Antioxidant capacity*

Antioxidant capacity in serum samples was measured using Amplex Red/horseradish peroxidase fluorescence assay (Invitrogen, Paisley, UK).

Serum samples were incubated with 20μM hydrogen peroxide for 30 minutes at 37 °C. After this period, samples were incubated with Amplex Red (50 mM) and horseradish peroxidase type II (0.1 U/ml) in order to quantify the remaining hydrogen peroxide in each sample. Fluorescence readings were determined in GloMax®-Multi+ Microplate Multimode Reader (Promega) with Ex/Em = 530/580 nm.

DNA damage analysis

Gene-specific quantitative PCR (QPCR) was used to assay nDNA and mtDNA damage [10,11]. Briefly, total genomic DNA was isolated using QIAGEN Genomic Tip and Genomic DNA Buffer Set Kit (QIAGEN). The quantitation of the purified genomic DNA was performed fluorimetrically using PicoGreen dsDNA quantitation reagent (Molecular Probes). Lambda(λ)/HindDIII DNA (Gibco) was used to generated a standard curve and to adjust the final DNA concentration to 3 ng/μL. The "hot start" PCR were performed using the Gene Amp XL PCR Kit (Applied Biosystems) with 15 ng de DNA, 1X Buffer, 100ng/μL of BSA, 200μM of dNTPs, 20 pmol of each primer (Table 1), 1.3 mM final concentration of Mg^{++} and water to a total volume of 45μL. The reaction was brought to 75°C prior to addition of 1U/reaction of enzyme (0.5μL of the polymerase in 4.5 μL of sterile water). We quantitatively amplify an 8.9-kb and 221-bp fragment of the mitochondrial genome and 13.5-Kb of nuclear genome. Amplification of hyperglycemic samples (MGH, GDM and DM2

groups) was compared to non hyperglycemic samples (ND group) and relative amplifications were calculated. These measurements were used to estimate the lesion frequency present in the DNA based on a Poisson distribution.

Isolation of Peripheral Blood Mononuclear Cells (PBMC)

Blood samples were collected in tubes containing heparin as anticoagulant. The samples were diluted in phosphate-buffered saline (PBS), layered onto Ficoll-Pacque (Pharmacia Biotech, Uppsala, Sweden), and centrifuged at 40 minutes at 1.800 rpm. The intermediate phase with the PBMC was collected and washed two times in Dulbecco's modified eagle medium DMEM low glucose (12320-032-Gibco Invitrogen, Paisley, UK) supplemented with 10% of fetal bovine serum (FBS, Gibco Invitrogen, Paisley, UK). The $2.0x10^6$ cells aliquots were frozen (liquid nitrogen) in FBS with 10% of dimethyl sulfoxide (Sigma Aldrich, St Louis, MO, USA). The samples were thawed in the same medium with 10% FBS. In order to verify the viability of PBMC after isolation and thawing, 1% Trypan blue (Gibco, Invitrogen, Paisley, UK) solution was added at 1:1 volume ratio. The number of dead PBMC in a sample of 100 cells was counted using a light microscope. Only samples with viability > 95% were used in the experiments.

RNA extraction and cDNA synthesis

RNA extraction and cDNA synthesis was performed in PBMC using power

SYBR® Green Cells-to-CT™ Kit (Ambion, Carlsbad, CA, USA) as recommended by the manufacturer.

Real-time PCR

Real-time PCR was used to evaluate the expression of genes involved in different steps of BER. This technique was performed using Power SYBR® Green PCR Master Mix in an Applied Biosystems 7500 Fast Real-Time PCR System (both from Applied Biosystems, Foster City, CA, USA). Real-time PCR was carried out using specific primers for hOGG1, APE-1, FEN-1, POLβ and GAPDH (Table 2). For negative controls, we used a complete DNA amplification mix in which the target cDNA template was replaced with water. The 2^ΔΔCT method of analysis was used with the GAPDH gene for normalization. All samples were run in triplicate. Amplifications were performed using the default cycling conditions: enzyme activation at 95°C for 10 min, 40 cycles of denaturation at 95°C for 15 s, and annealing/extension at 60°C for 60 s. GeneAmp software (Applied Biosystems, Foster City, CA, USA) was used to quantify the expression levels (Quantitative PCR).

Western blot

Western blot was performed to quantify the expression of proteins involved in BER. Initially, PBMC from maternal and umbilical cord blood were lysed with a syringe in ice-cold RIPA buffer (1% NP-40, 0.25% Na-deoxycholate, 150

mM NaCl, 1 mM EDTA, 1 mM PMSF, 1 mM Na3VO4, 50 mM Tris-HCl, pH 7.4), supplemented with a cocktail of protease inhibitors (Sigma Chemical Co, St Louis, MO, USA). Proteins were separated electrophoretically using 15% SDS-PAGE, and proteins on the gel were transferred to a 0.45 µm to nitrocellulose membrane (Millipore, Bedford, MA, USA). The transfer of proteins was confirmed by staining the membranes with a 10% Ponceau S solution (Sigma Aldrich, St Louis, MO, USA). The blotted membranes were blocked with TBS-T-milk 3% (140 mM NaCl, 20 mM Tris–HCl pH 7.4, 0.1% Tween-20, 3% powdered milk) for 1 h and washed three times with TBS buffer. Subsequently, membranes were incubated at 4°C with anti-hOGG1 (at 1:1000, Novus Biologicals, Littleton, CO, USA), anti-APE-1 (at 1:1000, Novus Biologicals, Littleton, CO, USA), anti- FEN-1 (at 1:1000, Novus Biologicals, Littleton, CO, USA) or anti-Polβ (at 1:500, Santa Cruz, CA, USA) in TBS-T-milk 3% overnight and washed three times with TBS buffer. The membranes were then exposed to horseradish peroxidase-conjugated antibody (1:1000, Jackson Immuno Research, USA) in TBS-T-milk 3% for 1 h and washed three times with TBS buffer. Immunoreactive bands (peroxidase activity) were detected by the Enhanced chemiluminescence method (ECL). The quantitative analysis of all DNA repair protein expression levels was performed by densitometry using Image J software (v. 1.43 u, NIH, Bethesda, MD, USA). β-actin levels estimated in the same membrane was used as loading control.

PBMC cultures

Considering that prolonged hyperglycemia with consequent oxidative stress and DNA damage are some of the triggers of apoptosis [9], the PBMC isolated from Non-diabetic mothers and their newborns were exposed to different glucose concentrations *in vitro*.

Initially, PBMC were suspended in Dulbecco's modified eagle medium DMEM low glucose (12320-032; Gibco Invitrogen, Paisley, UK), with 2% FBS, and 1% of gentamicin (Gibco Invitrogen, Paisley, UK). The cells were placed in 96 wells plate in the presence of 5 mmol/L (90 mg/dL, control sample – physiological concentration of glucose – equivalent of normoglycemia in the case of patients), 17.5 mmol/L (315 mg/dL, moderated concentration of glucose) and 30 mmol/L (540 mg/dL high glucose concentration – equivalent of severe hyperglycemia in the case of patients) of D-glucose (Sigma Aldrich, St Louis, MO, USA). Glucose concentrations in incubating medium followed values observed in patients and literature [9,25]. Cells were incubated in 5% CO_2 incubator at 37°C for 48 h. Caspase 3/7 activity (Caspase-Glo® 3/7 Assay, Promega) and ATP levels (CellTiter-Glo® Luminescent Cell Viability Assay, Promega) were determined by luminescence in GloMax®-Multi+ Microplate Multimode Reader (Promega). Staurosporine 1mM (abcam, Cambridge, UK) was used to positive control of caspase and negative control of ATP analysis.

Statistical Analysis

Analysis of variance and Tukey's multiple comparison test were used for quantitative variables with normal distribution. For quantitative variables with an abnormal distribution, the generalized linear model with gamma distribution and the log-link function and the LSMeans test were utilized for multiple comparisons. Analyses were performed using SAS software, version 9.1 and Prism taking into account a 95% level of statistical significance ($p < 0.05$).

Results

Maternal clinical background and perinatal outcomes evaluation

GDM and DM2 patients were older ($p < 0.0001$) and had the highest BMI in the third trimester of pregnancy ($p = 0.0003$). In addition, the diabetics groups and MGH group had the highest pre-gestational BMI ($p < 0.0001$). The GM was progressively higher in relation to the severity of clinical conditions of the mother ($p < 0.0001$) and the HbA1c levels in the third trimester ($p < 0.0001$) confirmed the GM levels; therefore, the values of GM did not exceed the glycemic goals recommended by ADA [1] (Table 3).

Newborns from MGH, GDM and DM2 groups had the highest leptin ($p = 0.0162$) and hematocrit levels ($p = 0.0026$). Glycemia and insulin in cord blood were not different among groups; the NB weight ($p < 0.0001$) and percentage of LGA ($p < 0.0001$) were increased only in MGH groups

(Table 04).

Oxidative stress

Levels of reduced thiols were significantly decreased in serum of mothers with GDM (p = 0.0043) and DM2 when compared with ND group (p = 0.027) (Figure 2-A1), and were not significantly different in the umbilical cord blood serum (Figure 2-B1).

The 8-iso-PGF2α serum levels were higher in DM2 mothers than other groups (p = 0.0061) (Figure 2-A2), whereas the GDM group showed relevant augmentation in serum of umbilical cord blood when compared with ND group (p = 0.0217) (Figure 2-B2).

No significant differences on the antioxidant capacity were observed in maternal serum (Figure 2-A3) or in NB (Figure 2-B3).

Nuclear and mitochondrial DNA damage

The levels of nDNA and mtDNA damage in maternal leukocytes (Figure 3-A1; Figure 3-A2, respectively) were higher in both GDM (p = 0.02) and DM2 groups (p = 0.0007) when compared to ND group. In contrast, no significant differences were found in nDNA or mtDNA damage in umbilical cord blood leukocytes (Figure 3-B1 and Figure 3-B2).

mRNA expression of APE-1, FEN-1 and POLβ

The mRNA hOGG1 expression was not detectable with the methodologies

employed in maternal or NB PBMC. The mRNA of APE-1, FEN-1 and POLβ were expressed in all study groups; in hyperglycemic groups were observed opposite results between mothers and NB (Figure 4).

mRNA APE-1 expression was lower in mothers of DM2 group than others groups (p = 0.0008) (Figure 4-A1), and it was higher in NB of GDM group than others groups (p = 0.0032) (Figure 4-B1). mRNA FEN-1 expression was lower in GDM mothers than ND and MGH groups, and it was lower in mothers with DM2 compared to MGH group (p = 0.0006) (Figure 4-A2). In addition, the highest mRNA FEN-1 expression was found in NB of GDM and DM2 groups compared to ND group (p = 0.0016) (Figure 4-B2). mRNA POLβ expression was lower in mothers of GDM and DM2 groups compared to ND group (p = 0.0044) (Figure 4-A3), and it was higher in NB of DM2 group compared to ND group (p = 0.0036) (Figure 4-B3). The summary of these results are in Table 1S (supplementary archive).

Protein expression levels of hOGG1, APE-1, FEN-1 and POLβ

The BER proteins evaluated were identified in all groups. The results are similar to observed in mRNA analysis and confirm the opposite response observed between mothers and NB of diabetic groups (Figure 5; Figure 6).

The levels of hOGG1 expression were lower in mothers with GDM only when compared to DM2 group (p = 0.0105) (Figure 5-A1; Figure 5-a1), and it was higher in NB of DM2 group than ND and MGH groups (p = 0.0039) (Figure 5-B1; Figure 5-b1). APE-1 expression was lower in mothers of GDM and DM2 groups when compared to ND group (p = 0.0145) (Figure 5-A2; Figure 5-a2), and it was higher in NB of GDM group than others groups (p = 0.0164) (Figure 5-B2; Figure 5-b2). Maternal FEN-1 levels were similar in all groups (Figure 6-A1; Figure 6-a1); and increased in NB from GDM group when compared to ND and MGH groups (p = 0.0099) (Figure 6-B1; Figure 6-b1). POLβ expression was lower in mothers of MGH compared to ND group (p = 0.0363) (Figure 6-A2; Figure 6-a2), and it was elevated in NB of DM2 group when compared to others groups (p = 0.0077) (Figure 6-B2; Figure 6-b2). The summary of these results are in Table 1S (supplementary archive).

Apoptosis and ATP production analysis

The activity of effector caspases-3 and caspase-7 was elevated in PBMC isolated of mothers without diabetes and exposed to 30 mmol/L (540 mg/dL) of glucose compared with 95 mmol/L (90 mg/dL) concentrations (p < 0.001)

(Figure 7-A1). To confirm these results, we evaluated ATP levels in the same samples and observed ATP reduction in 30 mmol/L glucose concentration (p = 0.0480) (Figure 7-A2). Activation of caspases-3 our caspase-7 was not observed in PBMC of NB (Figure 7-B1); despite this, the ATP levels were reduced in 30 mmol/dL (540 mg/dL) glucose concentration (p = 0.0439) (Figure 7-B2).

Discussion

Almost all of the complications associated with diabetes in pregnancy are linked to maternal hyperglycemia [1]. In this study, pregnant women of MGH, GDM and DM2 groups had different hyperglycemia levels, which became progressively higher in relation to the severity of clinical conditions, and that impacted the results obtained solely for the mothers. The groups of mothers with GDM and DM2 were characterized by oxidative stress, increase of nuclear and mitochondrial DNA damage as well as decrease expression of genes and proteins involved in BER. In addition, the levels of hyperglycemia were associated to *in vitro* cellular apoptosis in maternal PBMC.

It is widely accepted that hyperglycemia leads to oxidative stress. Important studies showed increased biomarkers of oxygen radical damage and abnormalities in the antioxidant defenses of diabetic patients [26]. Plasma and urinary concentrations of 8-iso-PGF2α were associated to DM2 in non-pregnant women [27,28], and to GDM in pregnant mothers [29]. Our

maternal results reinforce these data.

In the present study, thiols in their reduced form were lower in GDM and DM2 groups, suggesting the presence of oxidized proteins. Levels of8-iso-PGF2α, a lipid peroxidation marker, was high only in the DM2 group. The differences among hyperglicemic levels overweight and obesity, with correspondent insulin resistance, may have influenced ROS production [30,31] and contributed to our results. Overweight, insulin resistance and hyperglycemia have been associated to differences in vascular disorders, cytokines production and apoptosis in placentas of diabetic mothers [32-36].

No difference was found in maternal antioxidant capacity after H_2O_2 treatment. Probably, antioxidant defenses were not enough to control the elevated oxidative stress in GDM and DM2 groups, as demonstrated by increased serum protein oxidation or lipid peroxidation in these diabetic mothers. This observation is consistent with previous studies in rats, which demonstrated higher levels of lipid peroxidation as gauged by malondialdehyde levels, even in presence of increased antioxidants enzymes in maternal blood of streptozotocin-induced diabetic pregnant rats [7]. Human studies report increased production of ROS and decrease in antioxidant defenses of diabetic pregnant women [37,38]. Thus, the results of our study reinforce that antioxidant defense in pregnancy complicated by diabetes or hyperglycemia is not enough to defending against the

exacerbated oxidative stress condition.

Besides hyperglycemia and oxidative stress, our results showed that pregnant women with GDM or DM2 exhibited a significant increase in nDNA and mtDNA damage. Some studies have suggested that mtDNA is more susceptible than nDNA to genotoxic agents, most notably ROS [11,12]. Damage in DNA, if not repaired, could lead to mutations, which are associated to many different diseases including cancers, both in mother [39] and NB [40]. MtDNA damage can also lead to mitochondrial dysfunction, promote and maintain increased ROS production, which could leak out the mitochondria, affecting the nuclear genome.

The maternal glucose can readily cross the placenta and it has always been associated with adverse perinatal outcomes [41-43]. In this study, NB of hyperglycemic mothers were characterized by elevated levels of leptin, increase in body weight, higher hematocrit levels and high rate of LGA. Furthermore, the NB of diabetic mothers presented increase of BER genes and proteins expression, and the hyperglycemia environment *in vitro* was not able to induce apoptosis in NB cells. Interesting, only NB from GDM mothers show increase in lipid peroxidation without association with DNA damage. Irrespective of the groups, blood levels of DNA damage in umbilical cord were similar.

According to some studies, there is a compensatory response in umbilical

cord blood cells of NB exposed to hyperglycemia, relative to the higher telomerase activity, which is able to maintain telomere structure in nDNA [44]. Another explanation is related to the increase ratio of mitochondrial/nuclei hTERT, suggesting a protective effect on fetal mtDNA [29]. In contrast, our previous experimental studies showed higher levels of oxidative DNA damage in both, streptozotocin-induced diabetic pregnant rats and their NB, when glucose levels were ≥ 300mg/dL [21-23]. In this study, the maternal glucose was maintained at lower levels than those observed in diabetic rats, and this may explain the differences in offspring DNA damage between STZ-diabetic rats and diabetic mothers.

The most intriguing result in the present study was that, in hyperglycemic conditions, the maternal and fetal compartment respond differently to DNA insults. The GDM and DM2 mothers exhibited nDNA and mtDNA damage, which were not observed in their newborns. In addition, these mothers had lowest expression of BER genes (APE-1, FEN-1, POLβ) and protein (hOGG1, APE-1), associated to apoptosis in high glucose concentrations (*in vitro* experiment). Conversely their NB showed increase of these genes and proteins expression and no *in vitro* induction of apoptosis in hyperglycemic concentrations. Overall, these results indicate that while damage in the mother genome was evident, the fetal genome was well protected.

The umbilical cord contains at least three populations of stem cells, each

with unique features and properties. These stem cells possess highly efficient DNA repair network that becomes less efficient upon differentiation, and also have an anaerobic metabolism, which reduces mitochondrial number and oxidative stress [45,46]. The higher telomerase activity [44] and the increased mitochondrial/nuclei ratio of human translocation telomerase reverse transcriptase (hTERT) [29] have been also considered as a possible protective factor for DNA umbilical blood cells in hyperglycemic pregnancies. These data suggest that umbilical cord blood cells have potentially several mechanisms at play to protect the fetal DNA against genomic insults in hyperglycemic and oxidative stress conditions.

The results of our study open a wide window for future researches. However, it is important to recognize its limitations, especially in respect to the difference between the number of mothers and NB evaluated. This was due to problems of inadequacy of some samples and difficulty to get and to process samples at night and weekend births. Although limited, our results highlight that protective mechanism for fetal DNA-damage may be dependent of glycemic levels, reinforcing yet again the importance of adequate maternal glycemic control in pregnancies complicated by diabetes.

In conclusion, this study has demonstrated that maternal hyperglycemia observed in GDM and DM2 groups was associated to oxidative stress and, consequently with nDNA and mtDNA damage. However, integrity of DNA

from umbilical cord blood cells was preserved, suggesting the better involvement of DNA repair mechanisms in these fetuses.

Supplementary Material

Table 1S. Summary of results of gene and protein expression of BER

Acknowledgments

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Competing Interests

The authors have declared that no competing interest exists.

References

1. American Diabetes Association (ADA). Standards of medical care in Diabetes - 2014. Diabetes Care. 2014;37:S14-S80.

2. Ornoy A. Prenatal origin of obesity and their complications: Gestational diabetes, maternal overweight and the paradoxical effects of fetal growth restriction and macrosomia. Reprod Toxicol. 2011; 32:205-12.

3. Calderon IMP, Kerche LTRL, Damasceno DC, Rudge MVC. Diabetes and pregnancy: an update of the problem. ARBS. 2007; 9:1–11.

4. Yang J, Cummings EA, O'connell C, Jangaard K. Fetal and neonatal outcomes of diabetic pregnancies. Obstet Gynecol. 2006; 10:644-50.

5. Lehnen H, Zechner U, Haaf T. Epigenetics of gestational diabetes mellitus and offspring health: the time for action is in early stages of life. Mol Hum Reprod. 2013; 19:415-22.

6. Brownlee M. Biochemistry and molecular cell biology of diabetic complications. Nature. 2001; 414:813-20.

7. Spada AP, Damasceno DC, Sinzato YK, Campos KE, Faria PA, Dallaqua B, et al. Oxidative stress in maternal blood and placenta from mild diabetic rats. Reprod Sci. 2014; 21:973-77.

8. Damasceno DC, Netto AO, Iessi IL, Gallego FQ, Corvino SB, Dallaqua B, et al. Streptozotocin-induced diabetes models: pathophysiological mechanisms

and fetal outcomes. Biomed Res Int. doi: 10.1155/2014/819065.

9. Arya AK, Pokharia D, Tripathi K. Relationship between oxidative stress and apoptotic markers in lymphocytes of diabetic patients with chronic non healing wound. Diabetes Res Clin Pract. 2011; 94:377-84.

10. Kovalenko OA, Santos JH. Analysis of oxidative damage by gene-specific quantitative PCR. Curr Protoc Hum Genet. 2009; Chapter 19:Unit 19.1.

11. Santos JH, Meyer JN, Mandavilli BS, Van Houten B. Quantitative PCR-based measurement of nuclear and mitochondrial DNA damage and repair in mammalian cells. Methods Mol Biol. 2006; 314:183-99.

12. Yakes FM, Van Houten B. Mitochondrial DNA damage is more extensive and persists longer than nuclear DNA damage in human cells following oxidative stress. Proc Natl Acad Sci U S A. 1997; 94:514-9.

13. Mandavilli BS, Santos JH, Van Houten B. Mitochondrial DNA repair and aging. Mutat Res. 2002; 509:127-51.

14. Mitra S, Boldogh I, Izumi T, Hazra TK. Complexities of the DNA base excision repair pathway for repair of oxidative DNA damage. Environ Mol Mutagen. 2001; 38:180-90.

15. Pácal L, Varvařovská J, Rušavý Z, Lacigová S, Stětina R, Racek J, et al. Parameters of oxidative stress, DNA damage and DNA repair in type 1 and type 2 diabetes mellitus. Arch Physiol Biochem. 2011; 117:222-30.

16. Agnez-Lima LF, Melo JT, Silva AE, Oliveira AH, Timoteo AR, Lima-Bessa KM, et al. DNA damage by singlet oxygen and cellular protective mechanisms. Mutat Res. 2012; 15-28.

17. Jackson SP, Bartek J. The DNA-damage response in human biology and disease. Nature. 2009; 461:1071-8.

18. Blasiak J, Arabski M, Krupa R, Wozniak K, Zadrozny M, Kasznicki J, et al. DNA damage and repair in type 2 diabetes mellitus. Mutat Res. 2004; 554:297-304.

19. Tatsch E, Bochi GV, Piva SJ, De Carvalho JA, Kober H, Torbitz VD, et al. Association between DNA strand breakage and oxidative, inflammatory and endothelial biomarkers in type 2 diabetes. Mutat Res. 2012; 732:16-20.

20. Qiu C, Hevner K, Abetew D, Enquobahrie DA, Williams MA. Oxidative DNA damage in early pregnancy and risk of gestational diabetes mellitus: A pilot study. Clin Biochem. 2011; 44:804-8.

21. Lima PH, Sinzato YK, de Souza Mda S, Braz MG, Rudge MV, Damasceno DC. Evaluation of level of DNA damage in blood leukocytes of non-diabetic and diabetic rat exposed to cigarette smoke. Mutat Res. 2007; 628:117-22.

22. Lima PH, Damasceno DC, Sinzato YK, de Souza Mda S, Salvadori DM, Calderon I de M, et al. Levels of DNA damage in blood leukocyte samples from non-diabetic and diabetic female rats and their fetuses exposed to air or cigarette smoke. Mutat Res. 2008; 653:44-9.

23. Lima PH, Sinzato YK, Gelaleti RB, Calderon IM, Rudge MV, Damasceno DC. Genotoxicity evaluation in severe or mild diabetic pregnancy in laboratory animals. Exp Clin Endocrinol Diabetes. 2012; 120:303-7.

24. Rudge MVC, Peraçoli JC, Berezowski AT, Calderon IMP, Brasil MAM. The oral glucose tolerance test is a poor predictor of hyperglycemia during the pregnancy. Braz J Med Biol Res. 1990; 23:1079-89.

25. Oleszczak B, Szablewski L, Pliszka M. The effect of hyperglycemia and hypoglycemia on glucose transport and expression of glucose transporters in human lymphocytes B and T: an in vitro study. Diabetes Res Clin Pract. 2012; 96:170-8.

26. West IC. Radicals and oxidative stress in diabetes. Diabet Med. 2000; 17:171-80.

27. Davì G, Ciabattoni G, Consoli A, Mezzetti A, Falco A, Santarone S, et al. In vivo formation of 8-iso-prostaglandin f2alpha and platelet activation in diabetes mellitus: effects of improved metabolic control and vitamin E supplementation. Circulation. 1999; 99:224-9.

28. Devaraj S, Hirany SV, Burk RF, Jialal I. Divergence between LDL oxidative susceptibility and urinary F(2)-isoprostanes as measures of oxidative stress in type 2 diabetes. Clin Chem. 2001; 47:1974-9.

29. Li P, Tong Y, Yang H, Zhou S, Xiong F, Huo T, et al. Mitochondrial translocation of human telomerase reverse transcriptase in cord blood

mononuclear cells of newborns with gestational diabetes mellitus mothers. Diabetes Res Clin Pract. 2014; 103:310-8.

30. Collins AR, Raslová K, Somorovská M, Petrovská H, Ondrusová A, Vohnout B, et al. DNA damage in diabetes: correlation with a clinical marker. Free Radic Biol Med. 1998; 25:373-7.

31. Cerdá C, Sánchez C, Climent B, Vázquez A, Iradi A, El Amrani, et al. Oxidative stress and DNA damage in obesity-related tumorigenesis. Adv Exp Med Biol. 2014; 824:5-17.

32. Sgarbosa F, Barbisan LF, Brasil MAM, Costa E, Calderon IMP, Gonçalves CR, et al. Changes in apoptosis and Bcl-2 expression in human hyperglycemic, term placental trophoblast. Diabetes Res Clin Pract. 2006; 73:143-9.

33. Calderon IM, Damasceno DC, Amorin RL, Costa RA, Brasil MA, Rudge MV. Morphometric study of placental villi and vessels in women with mild hyperglycemia or gestational or overt diabetes. Diabetes Res Clin Pract. 2007; 78:65-71.

34. Pietro L, Daher S, RudgeMV, Calderon IM, Damasceno DC, Sinzato YK, et al. Vascular endothelial growth factor (VEGF) and VEGFreceptor expression in placenta of hyperglycemic pregnant women. Placenta. 2010; 3:7707-80.

35. Moreli JB, Morceli G, De Luca AK, Magalhães CG, Costa RA, Damasceno DC, et al. Influence of maternal hyperglycemia on IL-10 and TNF-α production: the relationship with perinatal outcomes. J Clin Immunol. 2012;

32:604-10.

36. Moreli JB, Corrêa-Silva S, Damasceno DC, Sinzato YK, Lorenzon-Ojea AR, Borbely AU, et al. Dynamics changes in the TNF-alpha/IL-10 ratio in hyperglycemic-associated pregnancies. 2015: *In press*: Diabetes Research and Clinical Practice. doi:10.1016/j.diabres.2015.01.005

37. Djordjevic A, Spasic S, Jovanovic-Galovic A, Djordjevic R, Grubor-Lajsic G. Oxidative stress in diabetic pregnancy: SOD, CAT and GSH-Px activity and lipid peroxidation products. J Matern Fetal Neonatal Med. 2004; 16:367-72.

38. Grissa O, Atègbo JM, Yessoufou A, Tabka Z, Miled A, Jerbi M, et al. Antioxidant status and circulating lipids are altered in human gestational diabetes and macrosomia. Transl Res. 2007; 150:164-71.

39. Polyak K, Li Y, Zhu H, Lengauer C, Willson JK, Markowitz SD, et al. Somatic mutations of the mitochondrial genome in human colorectal tumours. Nat Genet. 1998; 20:291-3.

40. Wu CS, Nohr EA, Bech BH, Vestergaard M, Olsen J. Long-term health outcomes in children born to mothers with diabetes: a population-based cohort study. PLoS One. 2012; 7:e36727.

41. Pedersen J, Bojsen-Moller B, Paulsen H. Blood sugar in newborn infants of diabetic mothers. Acta Endocrinol (Copenh). 1954; 15:33-52.

42. Jansson T, Powell TL. Role of the placenta in fetal programming:

underlying mechanisms and potential interventional approaches. Clin Sci (Lond). 2007; 113:1-13.

43. Fraser A, Lawlor DA. Long-term health outcomes in offspring born to women with diabetes in pregnancy. Curr Diab Rep. 2014; 14:489.

44. Cross JA, Temple RC, Hughes JC, Dozio NC, Brennan C, Stanley K, et al. Cord blood telomere length, telomerase activity and inflammatory markers in pregnancies in women with diabetes or gestational diabetes. Diabet Med. 2010; 27:1264-70.

45. Ali H, Mulla FA. Defining umbilical cord blood stem cells. Stem Cell Discovery. 2012; 2:15-23.

46. Rocha CR, Lerner LK, Okamoto OK, Marchetto MC, Menck CF. The role of DNA repair in the pluripotency and differentiation of human stem cells. Mutat Res. 2013; 752:25-35.

Table 1. Gene targets, primers pairs and cycles for QPCR

Targets Genes	Fragment length	Primers Pairs	Cycles
Nuclear fragment Region near the β Globin gene	13.5 kb	F: 5'-CGA GTA AGA GAC CAT TGT GGC AG-3' (GI 48510) R: 5'-GCA CTG GCT TAG GAG TTG GAC T-3' (GI 62007)	75°C – 2 min 94°C - 1 min 94°C – 15 seg 64°C – 12 min 72°C – 10 min 21 cycles
Mitochodrial fragment	8.9 Kb	F: 5'-TCT AAG CCT CCT TAT TCG AGC CGA-3' (GI5999) R: 5'-TTT CAT CAT GCG GAG ATG TTG GAT GG-3' (GI14841)	75°C – 2 min 94°C - 1 min 94°C – 15 seg 64°C – 12 min 72°C – 10 min 17 cycles
Normalize Mitochodrial small fragment	221bp	F: 5'-CCC CAC AAA CCC CAT TAC TAA ACC CA-3' (GI14620) R: 5'-TTT CAT CAT GCG GAG ATG TTG GAT GG-3' (GI14841)	75°C – 2 min 94°C - 1 min 60°C – 45 seg 72°C – 45 seg 72°C – 10 min 17 cycles

F: foward; R: reverse.

Table 2. Primers used for real time PCR

Primer	Forward 5'-3'	Reverse 5'-3'	TM (°C)
hOGG1 (A)	GTGGACTCCCACTTCCAAGA	CGATGTTGTTGTTGGAGGAA	55
hOGG1 (B)	GTTCTGCCTTCTGGACAATCT	CCATACTTGATCCGCTAGTACAC	55
APE-1	CTGCCTGGACTCTCTCATCAATAC	CCTCATCGCCTATGCCGTAAG	57
FEN-1	CGGGCTGTGGACCTCATC	CAAGTCGCCGCACGAT	58
POLB	GTGCAGAGTCCAGTGGTGACA	CAGTTTTGGCTGTTTGGTTGATT	57
GAPDH	CAAGAGCACAAGAGGAAGAGAG	CTACATGGCAACTGTGAGGAG	55

In hOGG1 analysis was tested two pairs of primers (A and B) and HS01114116_G1 OGG1 Taqman Assay.(Applied Biosystems, Foster City, CA, USA). In all experimentes hOGG1 expresion was not detectable.

Table 3. Maternal clinical background

	ND (n = 46)	MGH (n = 30)	GDM (n = 45)	DM2 (n = 23)	*p*
Age (years)	26.1±7.9a0	28.4±6.4a0	30.9±4.9b0	33.2±7.1b0	**<0.0001**
BMI 1 (kg/m^2)	26.4±5.0a1	31.6±7.9b1	33.3±7.1b1	34.8±5.9b1	**< 0.0001**
BMI 2 (kg/m^2)	31.1±5.5a2	35.0±7.3a2	37.4±6.5b2	37.2±9.9b2	**0.0003**
Weight gain (kg)	12.3±5.1	9.9±6.1	10.3±8.0	10.2±6.5	ns
GA (weeks)	39.5±1.7a3	39.3±1.1a3	38.9±1.3a3	37.5±0.8c3	**<0.0001**
Hypertension	03 (6.52)	12 (40.00)	07 (15.55)	11 (47.82)	ns
GM (mg/dL)	83.1±7.8a5	91.3±11.7b5	101.9±14.2c5	109.6±13.8d5	**< 0.0001**
HbA1c (%)	5.3±0.4a6	5.5±0.5b6	5.6±0.5b6	6.5±1.1c6	**< 0.0001**

Clinical data are presented as means ± standard deviation or n (%). 1: Evaluated in pre-pregnancy; 2: Evaluated in the third trimester of pregnancy; BMI: body mass index; GA: Gestational age at delivery; GM: Glycemic mean; HbA1c: Glycated hemoglobin. Values followed by different letters and same index significantly differ ($p < 0.05$). ns: not significant ($p > 0.05$).

Table 4. Perinatal results

	ND (n = 27)	MGH (n = 20)	GDM (n = 21)	DM2 (n = 19)	*p*
Glycemia (mg/dL)	65.5±19.3	61.50±15.9	73.5±30.9	68.9±22.6	ns
Insulin (μU/mL)	5.3±5.4	9.3±13.7	8.9±9.1	8.0±5.5	ns
Leptin (pg/mL)	52.1±63.5a0	219.4±311.9b0	147.3±107.2b0	253.8±361.5b0	**0.0162**
Weight (g)	3198.8±421.2a1	3637.2±579.2b1	3317.2±559.7a1	3070.2±488.5a1	**<0.0001**
PI (g/cm^3)	0.028±0.002	0.028±0.003	0.029±0.003	0.030±0.003	ns
SGA	03 (11.1)	03 (15.0)	0.0 (0.0)	0.0 (0.0)	
AGA	22 (81.5)	13 (65.0)	20 (95.2)	16 (84.2)	**<0.0001**
LGA	02 (7.4)	04 (20.0)	01 (4.8)	03 (15.8)	
Ht (%)	48.0±5.5a2	49.4±4.7b2	51.8±5.5b2	53.4±7.5c2	**0.0026**
Hb (g/dL)	15.9±2.2	16.1±1.5	16.6±1.8	17.0±2.3	ns
pH	7.26±0.12	7.28±0.10	7.27±0.09	7.30±0.07	ns
Bilirubin (mg/dL)	2.02±0.57	2.10±0.73	2.03±0.74	2.22±0.63	ns
Apgar 1 < 7	05 (18.52)	05 (25.00)	03 (14.29)	02 (10.53)	ns
Apgar 5 < 7	01 (3.70)	02 (10.00)	0 (0)	0 (0)	ns

Data are presented as means ± standard deviation or n (%). PI: ponderal index (weight/length^3 x100); weight/gestational age classification: small (SGA), adequate (AGA), and large (LGA) for gestational age; Ht: hematocrit; Hb: hemoglobin. Values followed by different letters and same index significantly differ ($p < 0.05$). ns: not significant ($p > 0.05$).

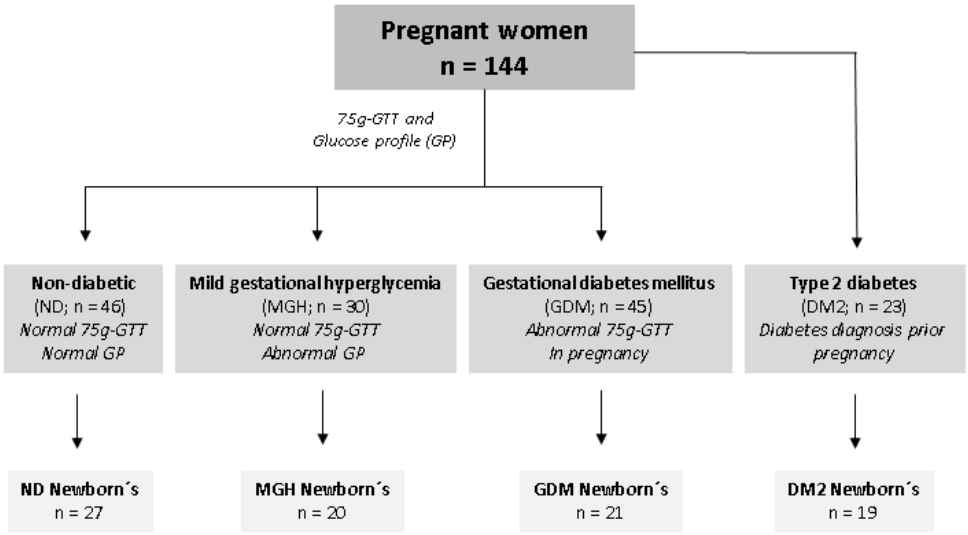

Figure 01. Definition of study groups and sample size.The MGH and GDM diagnosis was established between 24th and 28th gestational weeks according the 75 g-GTT and glucose profile results. The DM2 were referred to the Diabetes and Pregnancy Service with a confirmed diagnosis.

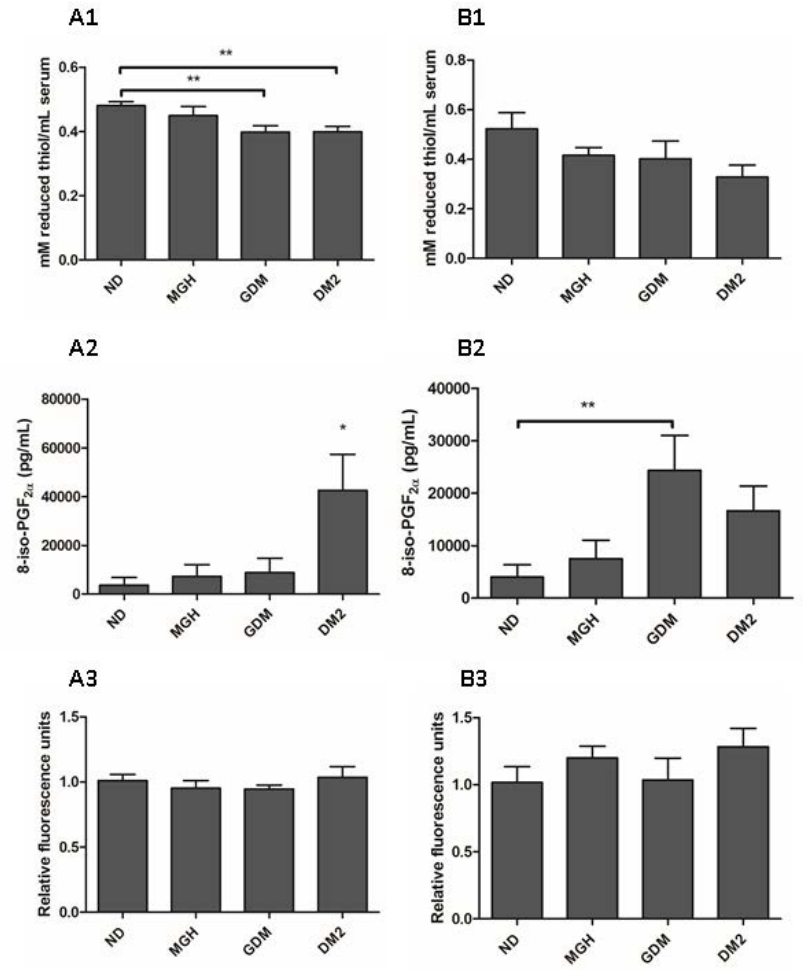

Figure 02. Evaluation of oxidative stress markers in serum from mothers [A] and umbilical cord blood [B]. A1 and B1: Total reduced thiols determined by DTNB method. A2 and B2: 8-iso-PGF2α evaluated by EIA method. A3 and A4: Antioxidant capacity after incubation with 20µM hydrogen peroxide for 30 minutes at 37 °C. Hydrogen peroxide not degraded by samples was determined by Amplex Red/ horseradish peroxidase fluorescence assay. Values as mean ± SEM, *$p < 0.05$ vs others groups; ** $p < 0.05$ vs appointed study group. n=15/group.

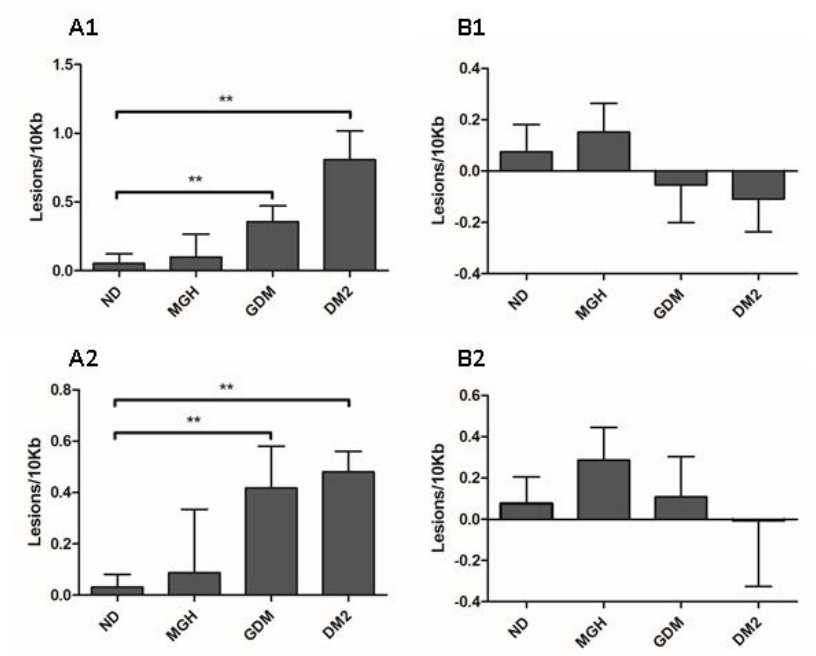

Figure 03. Nuclear [1] and mitochondrial [2] DNA damage from maternal [A] and umbilical cord blood [B]. DNA damage determined by Gene-specific quantitative PCR (QPCR). Values as mean ± SEM, ** $p < 0.05$ vs appointed study group. n=15/group.

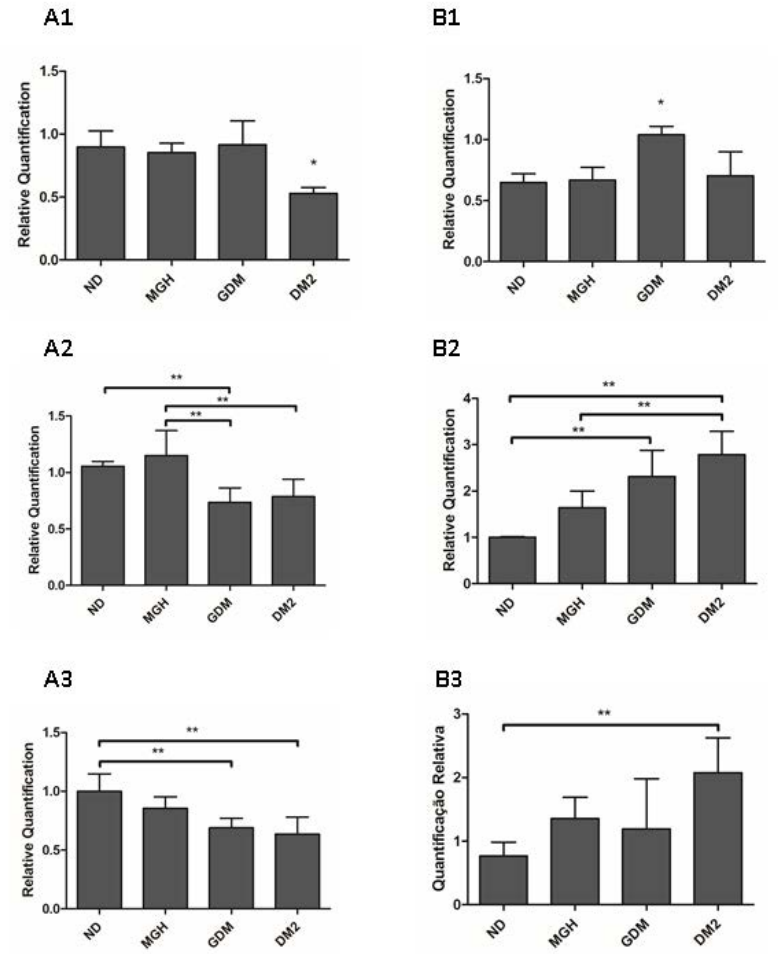

Figure 04. Gene expression of APE-1 [1], FEN-1 [2] and POLβ [3] in PBMC isolated from maternal [A] and umbilical coord blood [B]. Each reaction run in triplicates. All expression was normalized by GAPDH. hOGG1 expression was not detected with the methodologies employed. Values as mean ± SEM; *$p < 0.05$ vs others groups; ** $p < 0.05$ vs appointed study group. n=15/group.

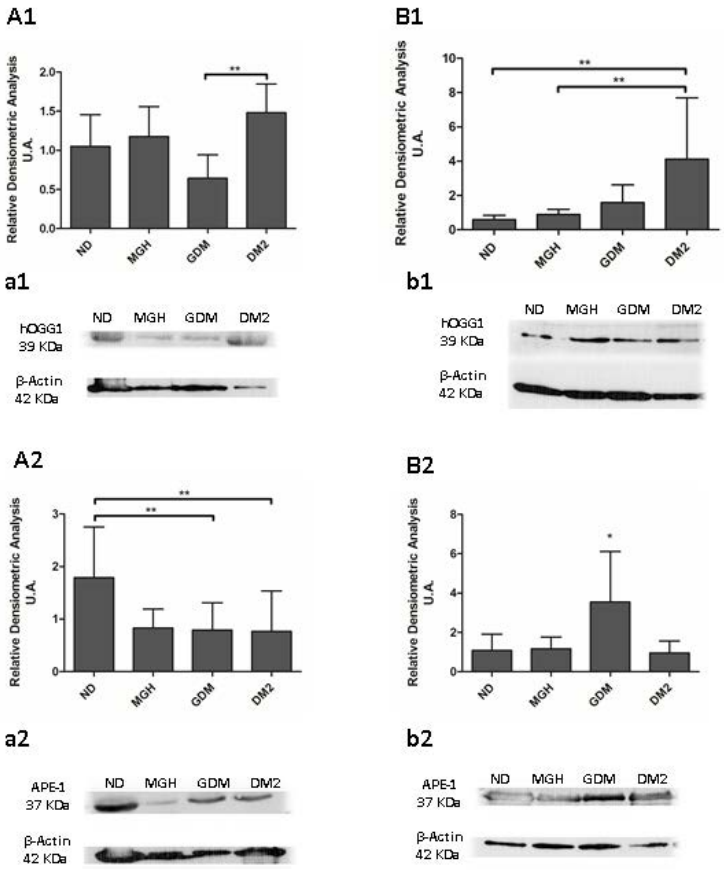

Figure 05. Protein expression of hOGG1 [1] and APE-1 [2] in PBMC isolated from maternal [A,a] and umbilical coord blood [B,b]. The relative band intensities from western blot experiments were normalized to the level of β-actin and analyzed with Image J software [A1, A2, B1, B2]. Values as mean ± SEM; *$p < 0.05$ vs others groups; ** $p < 0.05$ vs appointed study group. n = 08/group.

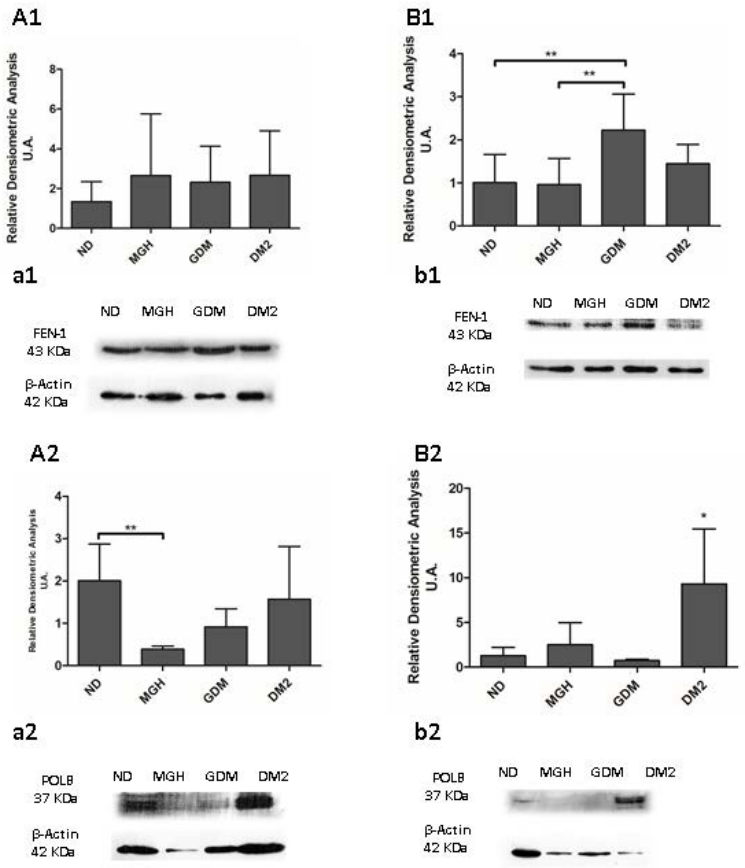

Figure 06. Protein expression of FEN-1 [1] and POLβ [2] in PBMC isolated from maternal [A,a] and umbilical coord blood [B,b]. The relative band intensities from western blot experiments were normalized to the level of β-actin and analyzed with Image J software [A1, A2, B1, B2]. Values as mean ± SEM; *$p < 0.05$ vs others groups; ** $p < 0.05$ vs appointed study group. n = 08/group.

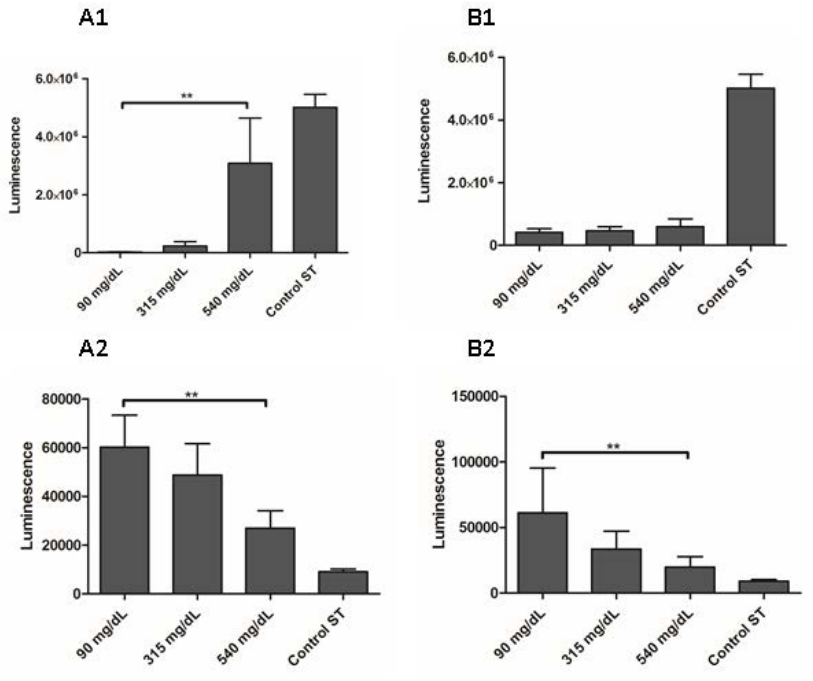

Figure 07. Caspase 3/7 activity [1] and ATP levels [2] in PBMC isolated from maternal [A] and umbilical cord blood [B] of non-diabetic group. PBMC were exposet to 5 mmol/L (90 mg/dL), 17.5 mmol/L (315 mg/dL) and 30 mmol/L (540 mg/dL) of glucose during 48h. Control ST: Staurosporine 1mM. Values as mean ± SEM, ** $p < 0.05$ vs appointed study group. n=06/group.

Table 1S. Summary of results of gene and protein expression of BER

	ND		MGH		GDM		DM2	
	Gene	Protein	Gene	Protein	Gene	Protein	Gene	Protein
Mothers								
hOGG1	nd							
APE-1	nd							↓*
FEN-1	nd			↓	↓	↓**	↓	
POLβ	nd		↓	↓	↓*		↓	
Newborns								
hOGG1	nd							
APE-1	nd							
FEN-1	nd	↑	↑	↑	↑			
POLβ	nd				↑	↑	↑	↑

nd: not detected; ns: not significant; ↑ increase of expression.; ↓ decrease of expression.; * compared only with MGH group; ** compared only with DM2 group.

Anexo

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Ilustríssima Senhora
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Departamento de Ginecologia e Obstetrícia da
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Prezada Drª Iracema,

Informo que o Projeto de Pesquisa (Protocolo CEP 4048-2011) Associação entre estresse oxidativo, lesões no DNA e capacidade de resposta celular em gestantes e recém-nascidos sob regime de hiperglicemia de intensidade variada, que tem a coordenação de Vossa Senhoria, teve autorizada a inclusão do Sub-Projeto: Lesões no DNA e capacidade de resposta celular de gestantes e recém-nascidos em regime de hiperglicemia de intensidade variada, quer será conduzido pela aluna Jusciele Brogin Moreli.

Atenciosamente,

Prof. Dr. Trajano Sardenberg
Coordenador do CEP