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Original Research Article

EVALUATION OF BIOCHEMICAL AND IMMUNOHISTOCHEMICAL PLACENTAL ANTIOXIDANT STATUS IN NORMAL AND GESTATIONAL DIABETES MELLITUS MOTHERS

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ABSTRACT

Background and objectives: Gestational Diabetes Mellitus is associated with derangement of hormones, metabolites, and growth factors in maternal and fetal circulation which may influence the development and function of the placenta. The aim of this study were to compare oxidative stress and antioxidant system markers in GDM patients and normal subjects, and to determine the relationship between oxidative stress and GDM

Materials and Methods: the study comprised 96 placentas of which 48 from normal and 48 from gestational diabetes. All the placental samples were evaluated for the antioxidant status using biochemical and immunohistochemical markers, MDA, SOD and GPX. The data were analysed statistically using unpaired Student T-test.

Results: The present study showed the significant increase in lipid peroxidation with MDA ($p<0.0001$) and GPX activity ($p<0.0001$) placental tissues of GDM than control confirms the oxidative stress in GDM. However SOD was found to be significantly reduced in GDM than control showed the overproduction of superoxide radicals and the compensatory mechanism taken by the placenta. The immunohistochemical stained slides showed low positivity (score 1+) with SOD and positivity (score 2+) in placental villi with GPX in GDM placental tissues. But both the markers expressed a stronger DAB reactivity in blood cells of fetal blood vessels and blood cells of intervillous (IV) or maternal space (score 2+). In control it was placid in placental villi (either score 1+ or 0) positivity in intervillous space blood cells and negative in fetal blood cells (score 0).

Conclusion: From the present study, we conclude that oxidative stress occurs in Gestational Diabetes and antioxidant defense mechanisms are inadequate. Biochemical and immunohistochemical analysis of levels of MDA, SOD and GPX are higher in GDM compared to normal pregnancies and may be useful markers in GDM in controlling the diabetic milieu.

KEY WORDS: Gestational diabetes, GDM, Placenta, Antioxidants, Oxidative stress, SOD, GPX, MDA.

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INTRODUCTION

Gestational diabetes mellitus (GDM) is a metabolic complication of pregnancy, and is associated with increased rates of perinatal complications. It is a heterogeneous disorder involving a combination of factors responsible for decreased insulin sensitivity and inadequate insulin secretion. Additionally, studies have shown that both women diagnosed as GDM and their offspring are at an increased risk for developing type-2 diabetes mellitus (DM) in later life [1-3].

Women with GDM develop an increased severity of insulin resistance which can disrupt the intrauterine milieu, leading to abnormal foetal growth [4]. In GDM, glucose tolerance and metabolism as well as insulin resistance are altered, and the pathophysiologic mechanisms underlying these changes are not completely understood. However, underlying pathophysiology of GDM in most instances is similar to that of type-2 DM, where the increased insulin resistance is converted into maternal hyperglycemia.

The two main pathological mechanisms known to induce GDM involve the biochemical pathways leading to insulin resistance and chronic subclinical inflammation [5]. It has been suggested that prolonged stimulation of acute and chronic inflammation may be involved in the pathogenesis of insulin resistance [7].

Oxidative stress (OS) is a universal phenomenon in pregnancy and do occur the same in GDM [8,9]. It is an imbalance between total free radical production or reactive oxygen species (ROS) and the body's ability to remove them via its antioxidant systems [10,11]. In normal pregnancies, ROS are removed periodically by antioxidants and therefore the standard levels are maintained throughout the pregnancy [12,13]. While in GDM due to hyperglycemia, production of ROS are increased and in there arise the imbalance of free radical and antioxidant system of the body thereby resulting in OS [14,15]. In normal state, oxygen free radicals damaging the normal cells are controlled by the enzymes such as superoxide dismutase (SOD), glutathione peroxidise (GPx), Catalase (CAT), vitamins C and E. In GDM, the levels of these enzymes are reduced enabling OS and damage to the membrane lipids resulting in lipid peroxidation (LP). Under hyperglycemia as in GDM there is an increased concentration of LP products and insulin resistance leading to endothelial dysfunction, characteristic feature of GDM. Recent evidence suggests the involvement of OS biomarkers such as superoxide dismutase (SOD) activity, glutathione peroxidase (GPx) and catalase (CAT) expressions makes a confirmation of the OS in the cellular level [16]. So far GDM cases are been studied more enough with the blood samples. Biochemical and immunohistochemical (IHC) study of GDM placental tissue is seemed to be less in literature though normal fetal growth and development depend largely on placental function. Generally the terminal villi (TV) of placenta are the final branches which establish the fetal contact for maternal circulation [17]. Morphologically and histologically, GDM placenta shows a variety of cellular changes, which when added with the OS shows conspicuous alterations in TV and fetal blood vessels [18].

The aim of the present work is to investigate amount of OS in the placental tissues of GDM and control pregnancies through biochemical and IHC using OS markers.

MATERIALS AND METHODS

Placental tissue samples were obtained from 96 pregnant women (48 normal and 48 with GDM) who delivered full term (age group between 21-39), singleton live births at the time of term caesarean section. GDM women were identified if they had two or more blood glucose values greater than or equal to the defined threshold levels (fasting, e"95 mg/dL; 1 hour, e"180 mg/dL; 2 hours, e"155 mg/dL; and 3 hours, e"140 mg/dL) on a 100-g oral glucose tolerance test (OGTT) [19]. The exclusion criteria were T1DM, combined DM and hypertensions, positive venereal disease, severe anaemia were excluded from the study. The study protocol was reviewed and approved by the Institutional Ethical Committee and all the participants completed informed written consent to encompass this study.

Preparation of placental extracts and protein estimation: All the placental samples (1gram

each) were weighed and homogenized in 10ml of 0.01M Tris-HCl buffer (pH 7.4) using tissue homogeniser. Tissue homogenates were then centrifuged for 30 minutes at 4000g and the supernatants collected and stored in ice for immediate assay. If the supernatant was not assayed on the same day, it was stored at -80°C but no more than fifteen days. The snap frozen tissue extracts were thawed and centrifuged for 15 minutes at 14500g prior to analysis.

Estimation of placental tissue protein: The supernatant achieved above was used for the determination of total placental protein, estimated by using Lowry et al. method for calculation of specific enzyme (SOD and GPX) activity i.e. enzyme activity/mg protein [20].

Estimation of lipid peroxidation: The extent of LP in tissues was assessed by measuring the level of malondialdehyde (MDA) according to the method of Ohkawa et al. [21]. Briefly, the reaction mixture containing 1 ml of trichloroacetic acid (15%) and 2 ml of thiobarbituric acid (0.38%) were added to 1ml (10%) tissue homogenate. The reaction mixtures were heated for 50 min at 90°C, cooled and centrifuged at 6500 rpm for 20 min. The absorbance of supernatant was spectrophotometrically measured at 532 nm against blank, which contained all reagents except placental tissue homogenate. MDA results were expressed as µmol/l.

Estimation of SOD: The SOD activity was measured spectrophotometrically (420nm) using the method designed by Ahmed et al [22]. According to this assay, the activity of cytosolic SOD was determined by monitoring the auto-oxidation of pyrogallol (0.2mmol/L). One unit of SOD activity is defined as the amount of enzyme required to inhibit the rate of pyrogallol auto-oxidation by 50%. SOD activity in placental tissues was measured from the time dependent inhibition of pyrogallol and expressed as units per milligram of protein (U/mg).

Estimation of GPX: Glutathione peroxidase (GPX) activity was measured by the method of Paglia and Valentine [23]. The enzymati reaction was initiated by adding H_2O_2 to the reaction mixture containing reduced glutathione, reduced nicotinamide adenine dinucleotide phosphate and glutathione reductase. The change in the absorbance at 340nm was monitored by spectrophotometer. One unit of GPX is defined as micromoles of NADPH oxidized per minute. Activity was given in units per milligram of protein (U/mg).

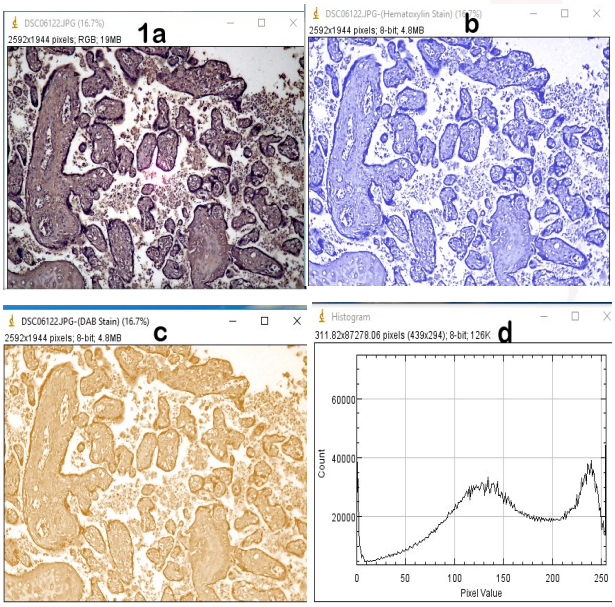
In each case, the activity of SOD and GPx was determined in 3 independent samples, simultaneously conducted trials. The variation of the results of the study obtained for each sample examined did not exceed 10%. The mean values of SOD and GPx activity was calculated based on 3 determinations performed for each sample.

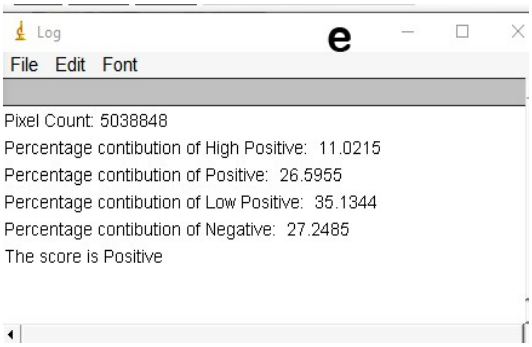
Immunohistochemistry for antioxidant enzymes: IHC is a more advanced staining technique, which makes use of antibodies to highlight specific antigens in the tissue. IHC was carried out with the primary antibodies SOD-1 and GPx-1 procured from Santa Cruz Biotechnology, US (SC-271014 and SC-22145, respectively), in order to observe antioxidants changes in placental tissue of both control and GDM. For the estimation of antioxidant status of SOD and GPx in placental sections, the secondary antibody kit (ImmunoCruz mouse ABC staining system, SC-2017, Santa Cruz Biotechnology, US) was employed. According to Manufacturer's protocol [24], in brief, after 3 micron placental tissue sections, the slides were incubated with 0.1-1% hydrogen peroxide in phosphate buffered solution (PBS) for 15 minutes; wash in PBS twice for 5 minutes each; incubating sections for 1 hour in 1.5% blocking serum in PBS; incubate sections with primary antibody SOD and GPx (5.0µg/ml, diluted in 1.5% blocking serum in PBS) for 30 minutes at room temperature; wash with three changes of PBS for 5 minutes each; incubate sections for 30 minutes in biotinylated secondary antibody (1µg/ml in PBS); wash three changes of PBS for 5 minutes each; incubate sections for 30 minutes with equal mixture of avidin and biotinylated horseradish peroxidase (AB reagents); wash with 3 changes of PBS for 5 minutes each; incubate sections in 2-3 drops of peroxidase substrate (mixture of 1.6ml of distilled water, 5 drops of 10X substrate buffer, 1 drop of 50X 3,3'-diaminobenzidine (DAB) chromogen and 1 drop of 50X peroxidase substrate) for 30 seconds or until desired stain intensity develops; the section may be checked

for staining by rinsing with H_20 and viewing under a microscope; finally counterstained with Harris hematoxylin for 5-10 seconds; immediately wash with several changes of double distilled water; destain with acid alcohol and bluing reagent; wash with tap water; dehydrate with 2X 95% ethanol for 10 seconds each, 2X 100% ethanol for 10 seconds each, 3X xylene for 10 seconds each, and wipe off the excess xylene; at last, the IHC stained sections were mounted with 1-2 drops of permanent mounting medium and cover with a glass coverslip and the slides were ready for light microscopic observations using trinocular microscope (CX31, Olympus, Tokyo, Japan).

Image analysis: IHC microphotographs of placental villi of both control and GDM were taken with Sony DCR W530 digital camera (Tokyo, Japan) fitted on trinocular head. A total of 236 images (118 each) were analyzed independently for DAB reactivity and were assigned a score as high positive (3+), positive (2+), low positive (1+) and negative (0) using ImageJ software [25] with IHC Profiler plugin [26] for Windows (Fig.1).

Fig.1a-e: Screen-shot images of ImageJ software with IHC profiler plugin for Windows was used to measure or score the DAB chromogen reactivity in IHC stained placental sections according to the optical density of stains used (here DAB and Hematoxylin). a: original microphotograph of IHC-DAB stained placental image; b: abstracted image of hematoxylin from original; c: abstracted image of DAB from original; d: histogram profile of DAB reactivity; e: final scoring of DAB reactivity from the original image.





Statistical analysis: Data was fed in computer program SPSS ver.15 for Windows (SPSS Inc, Chicago, IL, USA). The statistical significance of difference between the two groups was evaluated by using Student unpaired t-test. Data were presented as mean±SD. P-value less than 0.05 were considered statistically significant.

RESULTS

96 Placental samples (48 each from control and GDM) were analysed to access the oxidative status and compare the levels of SOD and GPX. The oxidative status of control and GDM placental tissues were measured via lipid peroxide and protein concentrations.

From the analysis of lipid peroxide concentrations, significantly increased level of LP was observed in GDM placenta with mean value of 6.81±0.87µmol/l compared to normal placenta with a mean value of 2.76±0.27µmol/l ($p<0.0001$) (Table 1).

Superoxide dismutase (SOD) activity was shown to be significantly reduced ($p<0.0001$) in GDM placenta with a mean of 407.04±25.04U/mg of protein when compared to the mean activity of 597.25±16.02U/mg in control group (Table 1).

Table 1: Biochemical activities of SOD, GPX and MDA in placental tissues of control and GDM groups.

Parameter	Control (n=48)	GDM (n=48)	t-value	P-value
SOD (U/mg of protein)	597.25±16.02	407.04±25.04	44.33	p<0.0001
GPX (U/mg of protein)	26.1±2.02	32.94±2.78	13.76	p<0.0001
MDA (µmol/mg)	2.76±0.27	6.81±0.8751	48.51	p<0.0001

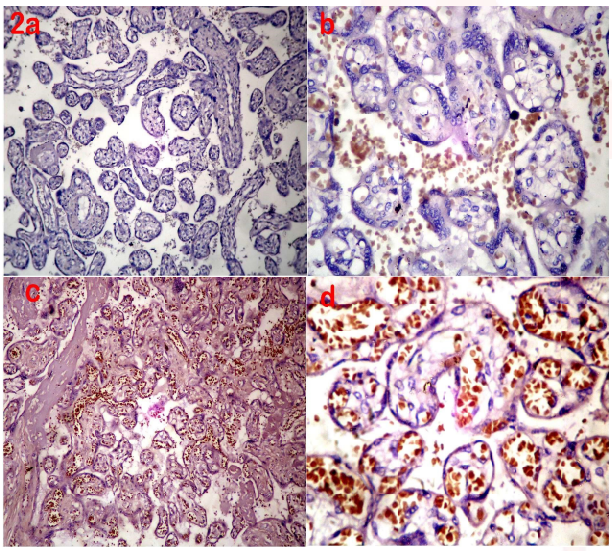
Values are presented as Mean±SD or number.

The placental activity of glutathione peroxidase (GPX) significantly increased ($p<0.0001$) in GDM placenta with a mean of 32.94±2.78U/mg of protein when compared to the mean activity of

26.1±2.02U/mg of protein for control group (Tab.1).

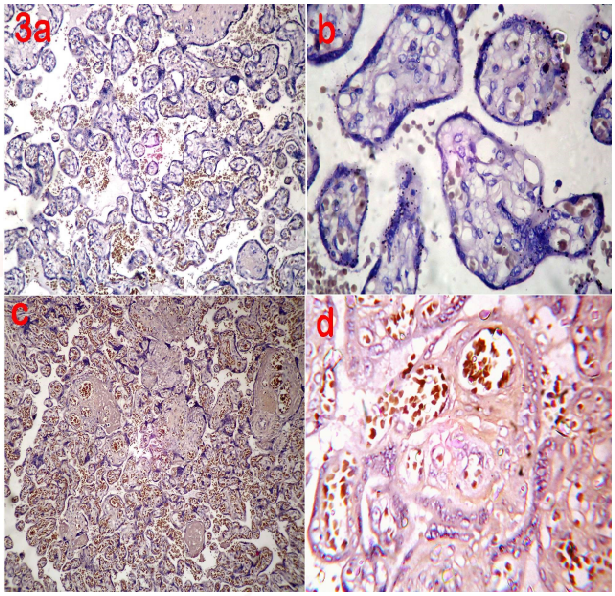
IHC placental tissue activity: GDM placental villi (PV) stained with SOD-1 showed the low positivity (score 1+). Mesenchyme of terminal villi showed less DAB reactivity but was greatly increased in blood cells of fetal blood vessels and blood cells of intervillous (IV) or maternal space (score 2+). Here and there the larger stem villi showed low positivity (1+). The control PV showed mild DAB reactivity (either score 1+ or 0) but positivity (score 1+) was seen in blood cells of IV space and mesenchyme of stem villous (score 1+) but not in fetal vessel or cells (score 0) (Fig.2)

Fig.2a-d: Immunoperoxidase staining of formalin fixed, paraffin embedded human placental tissues showing the localization of SOD-1. The staining was developed using HRP-DAB detection method and the sections were further counterstained with hematoxylin. a&b: control placental sections (100x&400x respectively) showing the weak expression in terminal villi but strong in blood cells of both fetal and maternal origin. c&d: GDM placental sections (100x&400x respectively) showing the strong expression in all types of villi especially more in blood cells of both fetal and maternal origin. 100x Scale bar=137µ and 400x Scale bar=35µ



The control placental sections stained with anti-GPX shows the weak positivity in placental villi but it was stronger DAB reactivity was in blood cells present in both the villi and maternal or intervillous space. In contrast, the GDM placental villi sections showed strong DAB reactivity in villous mesenchyme and blood vessels (score 2+). In addition the blood cells in PV and IV space also showed the strong DAB positivity (score 2+) (Fig.3).

Fig.3a-d: Immunoperoxidase staining of formalin fixed, paraffin embedded human placental tissues showing the localization of GPX-1. The staining was developed using HRP-DAB detection method and the sections were further counterstained with hematoxylin. a&b: control placental sections (100x&400x respectively) generated a weak expression in placental villi but stronger staining were observed in blood cells of both villi and intervillous space. c&d: GDM placental sections (100x&400x respectively) showed strong staining pattern in mesenchyme, blood vessels and blood cells in villi and intervillous space. 100x Scale bar=137µ and 400x Scale bar=35µ



DISCUSSION

The placenta is a rich source of oxidants and antioxidants [27]. The increased metabolic activity in the mitochondria of the placenta generates ROS and superoxide generation from NADPH oxidase. The placenta is also capable of inducing protective enzymatic and non- enzymatic scavengers against these free radicals. The placenta provides the interface of the maternal and fetal circulations, and it may play a crucial role in protecting the fetus from adverse effects of the maternal diseases, whereas disturbances in placental function may exacerbate this state [28].

OS is the steady state level of oxidative damage in a cell, tissue or organ, caused by ROS. Most ROS come from endogenous sources exist in biological cells and tissues at low but measurable concentrations. Their concentrations are determined by the balance between their rates of production and clearance by various antioxidant compounds and enzymes [29]. In a normal healthy human body, the generations of ROS are

effectively kept in check by the antioxidant defense mechanism.

In GDM there is an excess amount of glucose in maternal blood. This excess amount of maternal blood glucose and depleted antioxidant defense system in GDM [30] creates an imbalance in the pro & anti-oxidant status leading to an increased generation of end products of LP like Malondialdehyde (MDA). Also toxic oxygen free radicals and reactive non-radical species have been implicated to the formation of advanced glycation end products (AGEs), may be a major contributor to the pathological manifestations of DM. The present study revealed the biochemical and IHC indices of oxidative damage and antioxidant status in placenta of GDM and control. This study showed the MDA increased extremely ($p<0.0001$) in GDM than control, resultant due to the increased OS which are in agree with previous findings [29,31-35].

In a study conducted in placental tissues of normal healthy pregnancies MDA levels were reported to be decreased in the second and third trimester [36]. As a physiological phenomenon there is an increased OS during the first trimester which in course of the pregnancy, in the third trimester, is decreased [37]. In complicated pregnancies such as GDM, OS continues till the delivery because of the imbalance of the antioxidant system of the body [38], as reported in the present study.

The present study showed a significant increase in the placental GPX activity in GDM group than the control whereas significant decrease was observed in placental SOD in GDM as compared to the controls. This study revealed a significant fall in SOD levels, which could be due to excessive oxidative stress. Decrease in SOD levels can result not only an increase in the superoxide-free radical but also an elevation of other ROS and intensification of lipid peroxidation processes in diabetes [39,40]. In immunohistochemical staining, the placental erythrocytes or blood cells showed the positivity of DAB reactivity (score 2+) indicated the occurrence of OS in GDM women than the non-diabetic pregnant women [13,40]. Since the placenta, itself as a protective organ for the fetus could act as oxidative barrier against the antioxidant depletion can be cause for the low positivity if SOD in the placental villi [41].

Biochemical and IHC analysis of the present study revealed the severe OS and the poor antioxidant capacity and thrive for the balance in the antioxidant system in GDM placental tissues.

GDM is linked with fetal macrosomia [42], endothelial dysfunction associated with dyslipidemia, also known as hypertriglyceridemia, a positive correlation between maternal triglycerides and neonatal body weight [43-45].

In conclusion, alterations in placental tissue SOD, GPX and MDA in GDM patients indicate the depletion of antioxidant mechanism and prognosis of oxidative stress that may be important in the pathophysiology of GDM. Therefore, estimations of these antioxidant enzymes might be used as marker in the management of glycemic control and reducing diabetic complications. Thus, additional clinical trials with larger populations are needed to replicate the results of this study.

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Conflicts of Interests: None

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