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Histopathological and Immunohistochemical Changes in the Amniotic Membrane of Gestational Diabetic Mothers

Cambios Histopatológicos e Inmunohistoquímicos de la Membrana Amniótica de Madres Diabéticas Gestacionales

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SUMMARY: The aim of this study is to examine the changes in the amniotic membrane diagnosed with gestational diabetes mellitus. In this study, as a control group human amnion membrane from normotensive pregnancies was collected from diabetic women at 28–35 weeks of gestation. Gestational diabetes (n= 6) and normal amnion membrane (n= 6) for a total of 12 units were received. Amniotic membrane thickness was measured ($p<0.0001$) and it was significantly higher in GDM groups compared to control group. The diameter of the amniotic epithelial cell nuclei was measured ($p=0.0022$). Gestational diabetes results show that there was weakening between amniotic epithelial cell-cell junction. This study showed that structural changes in epithelial cells of amniotic membrane were formed due to diabetes. The membrane thickness has led to structural changes in diameter and in diabetes group cause extracellular matrix to increase, thus leading to MMP-9 expression increase eventually disrupting matrix balance. In addition, with cd44 increase angiogenesis has been induced and thought to influence material pass between fetus and mother.

KEY WORDS: Amniotic membrane; Gestational diabetic mothers; Immunohistochemical changes.

INTRODUCTION

Gestational diabetes (GDM) is known as carbohydrate intolerance arising at various levels in pregnancy. GDM is one of the most important complications emerging out at pregnancy. Hyperglycemia is characterized by various degrees of fetal (macrosomia, intrauterine growth restriction, premature birth, neonatal morbidity, perinatal mortality) and maternal (cesarean section, maternal death, future diabetes) complications. GDM occurs in 7 % of all pregnancies (Shaat & Groop, 2007; Wier *et al*., 2010). Gestational diabetes (GDM) is associated with increased oxidative stress and overexpression of inflammatory cytokines, both of which might lead to endothelial dysfunction and vascular disease (Di Fulvio *et al*., 2014). The intra-uterine environment with hyperglycemia, hyperinsulinemia and insulin resistance are associated with endothelial dysfunction in their offspring (Leiva *et al*., 2011). Amniotic membrane (AM) is a component of the placenta that originates from the extraembryonic tissue and functions to protect the fetus during pregnancy via supplemental nutrients. AM is composed of three major layers (Sippel *et al*., 2001); a single epithelial layer, a thick basement membrane, and an avascular mesenchyme. E-cadherin is the prototype of the cadherin family that links to catenins to form E-cadherin/catenin complex which is further linked to the actin cytoskeleton (Goodwin & Yap, 2004). MMP-2 and MMP-9 (also known as gelatinase A and B), are capable of digesting collagen IV, a major component of basement membrane. These two MMPs have been identified in human fetal membranes and amniotic fluid. An increase in MMP-9 levels in the fetal membranes and amniotic fluid has been associated with term labor (Vadillo-Ortega *et al*., 1995; McLaren *et al*., 2000; Maymon *et al*., 2000), indicating a role for MMP-9. In human parturition is a transmembrane glycoprotein that has significant roles in cell growth, survival, differentiation (Ponta *et al*., 2003), cell adhesion, motility, matrix degradation and proliferation (Marhaba & Zöller, 2004). In this study, we aimed to investigate changes occurring on the development of amniotic membrane in

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diabetic mothers. the different histologically and immunohistochemically consisting of amniotic membrane in diabetic mother.

MATERIAL AND METHOD

The study protocol was approved by the Medical Committee of Diyarbakir Hospital Maternity and Child Health Hospital and informed consent was obtained from all subjects involved in the study. In this study, amnion membranes were obtained from a total of 12 pregnant women applied to the department of obstetrics and gynecology clinic of Diyarbakir Maternity and Children Hospital. Amnion membrane of babies born at 28–38 weeks of pregnancy were removed. Gestational diabetes (n= 6) and normal umbilical cords (n=6) for a total of 12 units were received. Venous blood samples were taken 15 minutes after patients completed a 12 hour fast (between 08:00 and 10:30 am). Gestational diabetes mellitus was diagnosed by 50 g OGTT (oral glucose tolerance test) venous plasma / serum threshold values as follows: 50 g OGTT <140 mg/dL is normal; 50 g OGTT is a 3-h 100 g OGTT. 50 g OGTT ≥200 mg/dL in patients GDM is diagnosed directly and treatment begins. Fasting blood glucose ≥140 mg/dL are considered diabetes in this case

In all cases, 1–12 cm-long sections of umbilical cord were cut and each amnion membrane was immediately clamped at delivery. The specimens were immersed in 10 % buffered formaldehyde. They were dehydrated in ascending ethanol series, cleaned in xylene and embedded in paraffin, then 4 μm sections were cut and mounted onto slides. They were stained with Hematoxylin and Eosin.

Immunohistochemical Technique. The tissues were fixed in formalin solution and embedded in paraffin wax. Paraffin blocks were cut 4–6 mm thick-sections and mounted on positively charged microscope slides. Sections were deparaffinized with xylene and dehydrated by immersion in graded alcohol solutions. Fixed slides were incubated in ethylenediaminetetraacetic acid (pH 8.0; Merck, Germany) for 3–5 min in a microwave oven (750 W) for antigen retrieval. The sections were subsequently incubated for 20 min in 3 % hydrogen peroxide (H_2O_2)/methanol to block endogenous peroxidase activity, and then rinsed in phosphate-buffered saline (PBS) three times (5 min per wash). Next, the sections were incubated in a blocking solution (Goat serum; Invitrogen, Carlsbad, California, USA). The slides were incubated overnight with a primary antibody E-cadherin (1/100, mouse monoclonal; Santa Cruz Biotechnology, Inc., Santa Cruz, California, USA), and CD44 (1/100, mouse monoclonal; Santa Cruz Biotechnology, Inc., Santa Cruz, California, USA) After washing in PBS, the sections were incubated with horseradish peroxidase-linked streptavidin (Invitrogen). The reaction was visualized by incubating the sections in a solution of 0.1 % 3,30 diaminobenzidine (DAB) and 0.02 % H_2O_2 for 7 min (DAB substrate kit; Invitrogen). Finally, the sections were counterstained with Hematoxylin-Eosin (Sigma, St Louis, Missouri, USA) and coverslipped. Positive immunostaining was defined as the presence of brown chromogen (DAB) at the edge of the hematoxylin-stained cell nucleus and in the cytoplasm or plasma membrane of the cells, as assessed by light microscopy.

Statistical analysis. Statistical analysis was performed with the Statistical Package for the Social Sciences for Windows (version 15.0, SPSS Inc., Chicago, IL, USA). The Mann–Whitney U test was used for the statistics as indicated, and results were expressed as mean ± SD. P values below 0.05 were considered to indicate statistical significance.

RESULTS

In amniotic sections of the control group, cells located on the basement membrane were observed having flat nucleus. Under basal layer of the fibrous structure, mesenchymal and fibroblast cells which are parallel to each other were observed in the fusiform shape. In gestational diabetes group, some cells were observed to have had degenerative changes. In addition, changes in the amniotic membrane thickness were observed (Table I).

Table I. Amniotic membrane thickness was measured. The median values of the two groups showed statistically significant differences in the extent.

Groups	n	Mean	Std. Deviation	Std. Error Mean
1	6	18.7378	1.63246	0.66645
2	6	8.6100	0.64623	0.26382

(Mann-Whitney-U=57.00; p<0.0001).

Both hyperplasia and hypertrophy was observed in nuclei of amniotic epithelial cells were observed (Table II). Amniotic epithelium showed the correct external projections. The diameters of the amniotic epithelial cell nuclei were measured.

Table II. The median values of the two groups showed statistically significant differences in the extent.

Groups	n	Mean	S. D.	Std. Error Mean
1	6	4.1406	0.37456	0.15291
2	6	7.6211	0.53671	0.21911

(Mann-Whitney-U=21.00; p=0.0022)

Control group: Weak CD44 expression was observed in the amniotic epithelial cells, positive CD44 expression in endothelial cells and fibroblasts were apparent (Fig. 1c). Immunohistochemical analysis showed that epithelial cells of amnion membrane, endothelial cells of blood vessels and fibroblast cells of the GDM group expressed high levels of CD44 (Fig. 1d). Normotensive patients showed amniotic membrane expression of E-cadherin positively (Fig. 1e). In the GDM group, amniotic membrane expression of E-cadherin was weak in some areas (Fig.1f).

In the GDM group, there was a thickening in the vessel wall of amniotic epithelial basement membrane, and also membrane showed positive immunoreaction for MMP-9 expression. Additionally, loose connective tissue and fibroblast positively reacted with MMP9 associated with increased hyaluronic acid density. (Fig. 1h).

DISCUSSION

In gestational diabetes mellitus, the expression and structure of glucose amino glycan chain with covalent bonds to proteoglycans could change placental membrane Angiogenesis modulation is responsible for the installation of the cellular barriers, like the key members of the basal membrane (Kirn-Safran *et al*., 2009). In GDM, relation with abnormal placental morphology and accordingly with fetal morbidity was demonstrated (Giachini *et al*., 2008).

E-cadherin is an important molecule in the maintenance of epithelial integrity (Takeichi, 1990). Intracellular junctions are major contributors to the epithelial barrier, and are composed of E-cadherin-mediated intercellular attachments. Damage to the epithelium may result in the loss of E-cadherin expression in the cell membrane and intercellular junction (Symington *et al*., 1993; Hirai *et al*., 1989; Carter *et al*., 1990). E-cadherin expression differed significantly between the GDM and control groups. The importance of tight junction proteins such as E-cadherin in amnion membrane barrier function is widely accepted. Loss of E-cadherin expression may result in the loss of epithelial cells. CD44 is a receptor for hyaluronic acid (HA), which mediates cell-to-cell and cell-to-matrix interactions through its affinity for HA. Adhesion with HA plays an important role in cell migration, tumor growth and progression (Vikesaa *et al*., 2006). In our study, amniotic

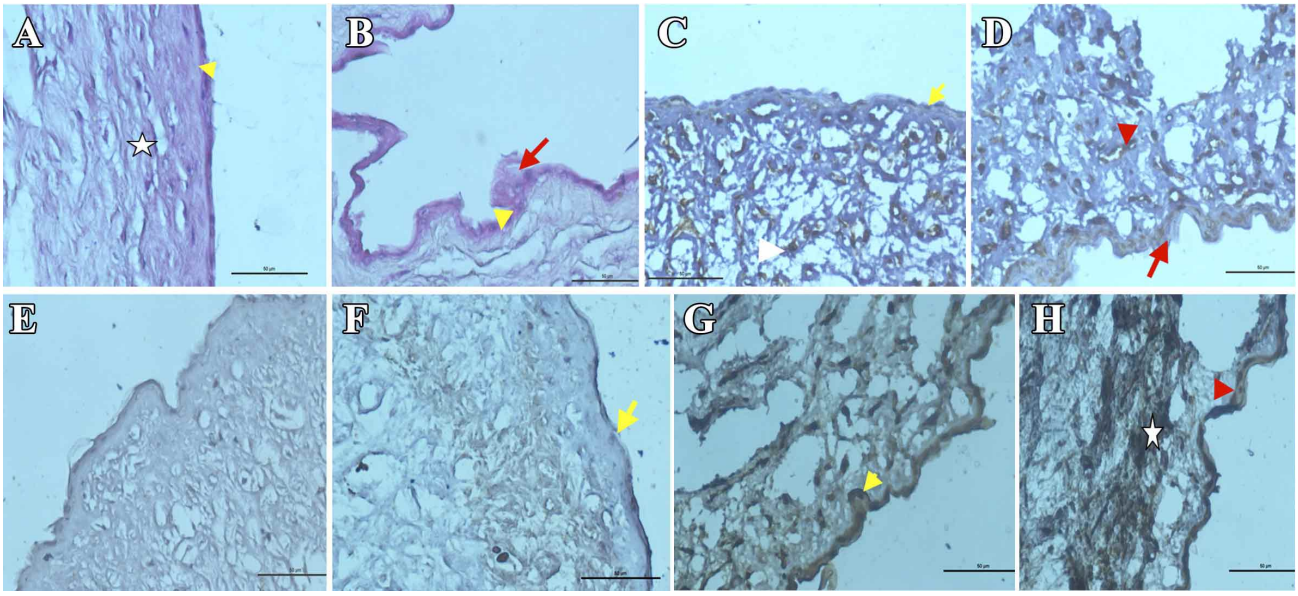

Fig. 1a. Control group; Flat appearance on the basement membrane of the nucleus (arrow), normal distribution of loose connective tissue(star). H-E staining. Bar 50 µm. b. GDM group; hypertrophy and hyperplasia in epithelial cells (yellow arrow), degeneration and projections in some cells (red arrow). H-E staining. Bar 50 µm. c. Control group; Weak CD44 expression in the amniotic epithelial cells (yellow arrow), positive CD44 expression in fibroblast cells (white arrow) CD44 immunohistochemistry staining. Bar 50 µm. d. GDM group; Positive CD44 expression in the amniotic epithelial cells and endothelial cells (red arrow), CD44 immunohistochemistry staining. Bar 50 µm. e. Control group; positive E-Cadherin expression in amniotic epithelial cells. E-Cadherin immunostaining. Bar 50 µm. f. GDM group, Weak E-Cadherin expression in amniotic epithelial cells (yellow arrow), E-Cadherin immunostaining. Bar 50 µm. g. Control group; MMP9 positive expression in thin amniotic membrane and fibroblast cells (arrow) MMP9 immunohistochemistry staining. Bar 50 µm. h. GDM group; Strong expression of MMP9 in basal membrane of vessel wall and amniotic epithelium (arrows). Also MMP9 positive reaction in loose connective tissue (star) MMP9 immunohistochemistry staining. Bar 50 µm.

section of the GDM group showed a significant increase in CD44 expression compared to control group. Cd44 induces the development of epithelial cells and connective tissue. In addition, there was an increase in CD44 expression in endothelial cells by stimulating angiogenic factors.

The role of CD44-hyaluronate interactions in connective tissue may be important for differentiation of endothelial cells during angiogenesis.

The studies showed that MMP-9 activity in the fetal membranes increased markedly at onset of the time of labor. According to Galewska *et al*. (2008), the umbilical cord plasma of pre-eclamptic subjects contained large amounts of MMP9 in the form of complexes with other plasma components, and zymographic analysis demonstrated increased gelatinolytic activity at a position corresponding to MMP9 compared to control samples. In our study, expression of MMP9 in the connective tissue showed a significant increase in the matrix.

Integrity of the extracellular matrix is necessary for the protection function of the amniotic membrane. The balance between MMP9 activity is essential for normal pregnancy. In diabetic group, the increase of MMP9 activity may lead to a disruption of this balance. In this study, structural changes in the field of near in the area of amniotic membrane epithelial cells were formed due to diabetes.

The membrane thickness has led to a change in diameter and cell. In conclusion we state that gestational diabetes histopathologically is thought to cause thickness and diameter changes in amniotic membrane while immunohistochemically due to MMP-9 expression increase in extracellular matrix, HA balance was disrupted and eventually may have impact on fetal and maternal angiogenesis association.

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RESUMEN: El objetivo de este estudio fue examinar los cambios en la membrana amniótica diagnosticada con diabetes mellitus gestacional (DMG). En este estudio, como grupo control, se recogió la membrana amniótica de embarazos normotensos de mujeres diabéticas a las 28 y 35 semanas de gestación. La muestra consistió en 6 casos con diabetes gestacional (n = 6) y 6 casos de membrana amniótica normal (n = 6), para un total de 12 casos. El espesor de la membrana amniótica se midió (p <0,0001) y fue significativamente mayor en los grupos de DMG en comparación con el grupo control. Se midió el diámetro de los núcleos de las células epiteliales amnióticas (p = 0,0022). Los resultados demostraron que en la DMG hubo debilitamiento entre la célula epitelial amniótica-célula de unión. Este estudio mostró que los cambios estructurales en las células epiteliales de la membrana amniótica se presentaron debido a la DMG. El espesor de la membrana ha dado lugar a cambios estructurales en el diámetro y en el grupo de DMG debido a un aumento de la matriz extracelular, lo que condujo al aumento de la expresión de MMP-9, eventualmente interrumpiendo el equilibrio de la matriz. Además, el aumento de cd44 indujo la angiogénesis y se cree que también influye en el material que se comparte entre el feto y la madre.

PALABRAS CLAVE: Membrana amniótica; Madres diabéticas gestacionales; Cambios inmunohostoquímicos.

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