

Histological changes in the amniotic membrane structure of gestational diabetic women's' in comparison with pregestational diabetic and non-diabetic women

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Abstract

The main objective of this study was to compare the demographic characteristics and histological changes in the amniotic membrane (AM) of gestational diabetes mellitus (GDM), pregestational diabetes mellitus (PGDM), and non-diabetic women. A total of thirty AM samples (n = 10 for each group) were obtained from full-term pregnant women without any complications. These samples were processed for paraffin blocks, sectioned, and stained with H&E. The results of demographic characteristics showed the mean maternal age of the PGDM and GDM groups was significantly higher compared with the control. Neonatal weight decreased significantly in the PGDM group, but both diabetic groups showed no significant differences between them in terms of maternal age and neonatal weight. A random blood sugar (RBS) test and WBC count showed a highly significant increase in GDM and a significant increase in PGDM. The highest significant value of RBC was recorded for PGDM. The thickness of the epithelial layer plus the basement membrane (BM) was significantly increased due to the PGDM. while the compact layer plus the fibroblast layer and the total thickness of AM showed a significant increase in GDM. The nucleus diameter of AM epithelial cells was significantly decreased in diabetic groups. The histological examination revealed that both diabetic groups caused several changes and damage in AM, including: hypertrophy and hyperplasia in epithelial cells associated with the formation of the vacuole between them; degeneration of some of these cells that pinched off from the lining epithelium; breakdown of the compact and fibroblast layers and caused damage to the stromal collagen fibril; epithelial cells and their nuclei became elongated and resembled columnar epithelium; and the basement membrane appeared thicker in comparison to the control group. In conclusion, GDM and PGDM induced various alterations and damage to the AM, which in turn delayed embryonic development and the delivery.

Keywords: Amniotic membrane; Gestational diabetes mellitus; Pregestational diabetes mellitus; Histology; Epithelial cells

Introduction

The amniotic membrane (AM) or amnion is a thin membrane that surrounds the embryo and forms a sac (the amniotic cavity) that is filled with amniotic fluid (Mamede *et al.*, 2012; Hilmy *et al.*, 2017). This membrane is a placental component that develops from extraembryonic tissue and serves to protect the fetus during pregnancy by providing supplemental nutrients (Sippel *et al.*, 2001). The thickness of the AM ranges from 0.02 to 0.5 mm, and it is made up of three layers: (i) an epithelial monolayer composed of epithelial cells, (ii) an acellular intermediate basement layer, and (iii) an outer mesenchymal cell layer rich in mesenchymal stem cells and located close to the chorion (Ilancheran *et al.*, 2007; Pappa and Anagnou, 2009; Ferenczy, 2020).

Amniotic membrane is a translucent biological structure that lacks vascular tissue and contains no nerves, muscles, or lymph veins. Its nutrients and oxygen are given by diffusion from the chorionic fluid, amniotic fluid, and fetal surface vessels (Toda *et al.*, 2007). As a result, this membrane is more than just an avascular structure; it performs a variety of metabolic tasks, including the transfer of water and soluble materials and the generation of bioactive substances such as vasoactive peptides, growth factors, and cytokines (Cunningham 2001).

The structure and function of the amnion have been studied, especially the pluripotent qualities of AM cells, which represent a promising source of tissue transplantation (Toda *et al.*, 2007; Mamede *et al.*, 2012; Algaba-Chueca *et al.*, 2020). The application of AM as a wound dressing material for surgical defects of the oral mucosa, ocular surface reconstruction (Hao *et al.*, 2000), and corneal perforations (Toda *et al.*, 2007) has been investigated. AM has anti-inflammatory, anti-bacterial, anti-viral, immunological, anti-angiogenic, and proapoptotic properties.

Diabetes mellitus (DM) is a collection of metabolic illnesses characterized by excessive blood sugar levels, either because the pancreas does not create enough insulin or because cells do not respond to the insulin produced (Manuel *et al.*, 2011). Diabetes is the most prevalent metabolic condition that develops during pregnancy, and it has consequences for both the mother and the fetus. (Salem *et al.*, 2019).

Gestational diabetes mellitus (GDM) is connected with both short-term obstetric and neonatal problems as well as long-term metabolic health implications for kids (Mitanchez *et al.*, 2015). In this context, fetal programming has been proposed as a key mechanism underlying the link between intrauterine diabetes exposure and an increased risk of metabolic dysfunction in adulthood, which leads to type 2 diabetes, obesity, and cardiovascular disease (Hanson and Gluckman, 2014). GDM is thought to cause thickness and width changes in the amniotic membrane, according to Togrul *et al.*, (2017). GDM prevalence is contested because it varies across the globe, depending on population, human race, and diagnostic criteria specified by each country (Joshy and Simmons, 2006). This prevalence is expected to rise as risk factors such as advanced maternal age, obesity, and lifestyle (Bortolon *et al.*, 2016) become more prevalent. Females using corticosteroid medication (Collier *et al.*, 2017), pregnant with gestational diabetes in a previous pregnancy, or pre diabetics are also considered high-risk groups (Song *et al.*, 2018). The primary goal of this study was to examine the demographic characteristics and histological alterations in the amniotic membrane (AM) of women with gestational diabetes, pregestational diabetes, and non-diabetic women.

Material and methods

This study was approved by the Medical Ethics Committee of the Duhok Directorate of General Health, the Directorate of Planning, and the Scientific Research Division, Kurdistan Region, Iraq, with reference number 08032023-2-10.

In order to obtain the AM, after patient consent, placenta samples were collected from the Maternity Hospital in Zakho and from Duhok Obstruction and Gynecology Hospital. The experimental work for this study was carried out in the Laboratory of Zoology, Department of Biology, Faculty of Science, University of Zakho.

Amniotic membrane samples collection

The present study was including 30 pregnant women with full-term (37-40 weeks) gestation periods, with ages ranging from (18-40) years old during the period from 4th October 2021 to 20 June 2022. These pregnant women were divided into three groups as follows:

Group (1): The control group (normal- non diabetic) was includes (10) pregnant women with normal blood glucose levels

Group (2): GDM group, was includes (10) pregnant women with GDM).

Group (3): PGDM Group, was includes (10) pregnant women with pre-existing diabetes).

Before starting the research, Pre and Gestational diabetes mellitus were diagnosed.

According to AL-Yahya1 and Makhlof (2013) and Togrul et al. (2017), with some modification, the placenta of these groups was rapidly transferred to the laboratory, rinsed in phosphate buffer saline (PBS) containing penicillin and streptomycin (200 U/ml penicillin, 200 g/ml streptomycin), and immediately used.

The AM is separated from the chorion through blunt dissection. The AMs were taken, rinsed in PBS, fixed in 10% buffered formalin, and processed for a light microscopic study.

Maternal and newborn information

From each maternal and newborn, medical records, demographic, and clinical information were gathered. These include maternal age, neonatal weight, random blood sugar (RBS), number of white blood cells (WBCs) and red blood cells (RBCs), drug use, smoking, and alcohol use.

Exclusion criteria

Exclusion criteria include all abnormal conditions during pregnancy, such as Rh-immunization, cardiac disease, renal disorders, Rhesus incompatibility, smoking, corticosteroid therapy, pre-eclampsia, and pregnancy-induced hypertension.

Light microscopy (Histological studies)

For light microscopic studies, small pieces from the AMs were obtained and fixed in 10% buffered formalin. After fixation, these pieces were dehydrated by using a series of ascending concentrations of alcohols (70, 90, 95, and absolute), then cleared in xylol, and finally impregnated and embedded in paraffin. Using a rotary microtome, AMs sections were cut at 3-

5 µm thick, stained by hematoxylin and eosin (H&E) for general histological structure, and mounted on a clean glass slide using Canada balsam (Drury and Wallington, 1980).

Then, with the aid of a light microscope, the total thicknesses of AM, the thicknesses of the AM epithelial layer plus BM and compact layer plus fibroblast layer, and the diameter of the AM epithelial cell nuclei were estimated using an ocular micrometer. In addition to the histological and histopathological examination of the AM in all groups, they were recorded and photographed using a digital camera (Dino-Eye: Microscopic Eye-Piece Camera).

Statistical analysis

The collected data was submitted to the SPSS program (SPSS, 2019) in order to analyze it statistically. However, the means within ANOVA (both one-two-way ways) were separated using Duncan's multiple-range test (Duncan, 1955).

Result:

Demographic characteristics of maternal and neonatal

As indicated in table (1), the mean maternal age in groups of PGDM and GDM was significantly increased ($P < 0.05$) compared with control (normal). While both diabetic groups showed no significant differences ($P > 0.05$) between them in term of maternal age (Fig.1).

Regarding to the neonatal weight, this weight was decreased significantly ($P < 0.05$) in PGDM group in comparison with control. While, GDM resulted in no significant differences in the neonatal weight compared with PGDM and control (Fig.2).

The same table also indicated that the estimation of RBS was highly significant increased due to the GDM and reach (213.631 ± 15.702) than in the PGDM (127.50 ± 4.831) and control (79.46 ± 3.556). PGDM also resulted in significant increase ($P < 0.05$) in RBS than in the control (Fig.3).

In respect to WBC and RBC count, table (1) and figure (4&5), the statistical analysis showed that the mean number of WBC was highly significant elevated in GDM group (15.30 ± 0.399) compared with PGDM and control (8.81 ± 0.534 ; 7.05 ± 0.264) respectively. While PGDM resulted in significant increase in the number of WBC compared with control. But in case of RBC, highest significant ($P < 0.05$) value was recorded for PGDM (4.51 ± 0.109) which significantly increased compared with control and GDM (3.990 ± 0.025 and 3.56 ± 0.079) respectively.

Table (1): Demographic characteristics of maternal and neonatal

Groups	Mother's age (Year)	Infant weight (Kg)	RBS mg\dl	WBC	RBC
Control	24.30 ± 1.19 ^b	3.61 ±0.106 ^a	79.46 ±3.559 ^c	7.05 ±0.264 ^c	3.99 ±0.025 ^b
PGDM	32.00 ± 1.32 ^a	3.27 ±0.046 ^b	127.50 ±4.831 ^b	8.81 ±0.534 ^b	4.51 ±0.109 ^a
GDM	33.30 ± 1.16 ^a	3.45±0.117 ^{ab}	213.63 ±15.702 ^a	15.30 ±0.399 ^a	3.56 ±0.079 ^c
Sig.	*	*	*	*	*

Means with different letters within each parameter are differed significantly. (NS= non-Significant; *= Significant at level (P<0.05). PGDM=Pre gestational Diabetes Mellitus, GDM=Gestational Diabetes Mellitus.

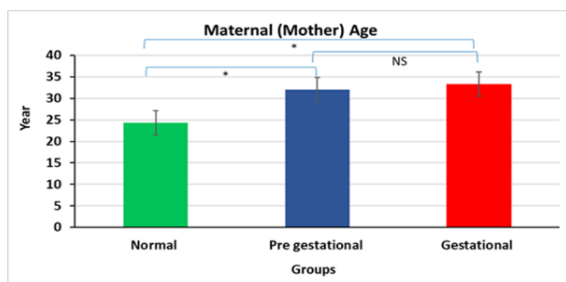


Figure (1): Means of the maternal age as affected by the studied groups.

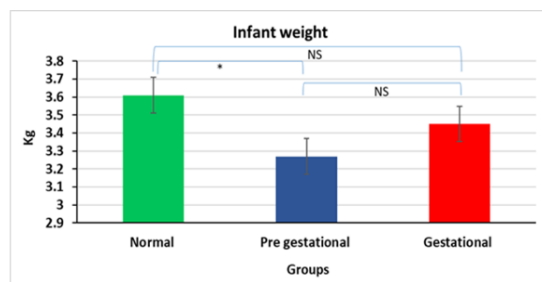


Figure (2): Means of the Infant weight as affected by the studied groups.

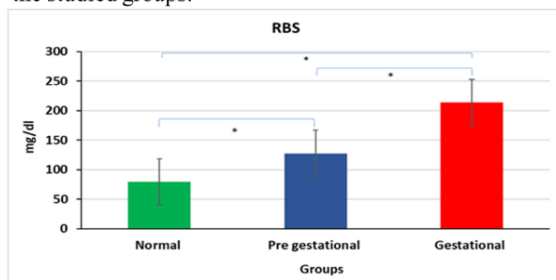


Figure (3): Means of the RBS as affected by the studied groups.

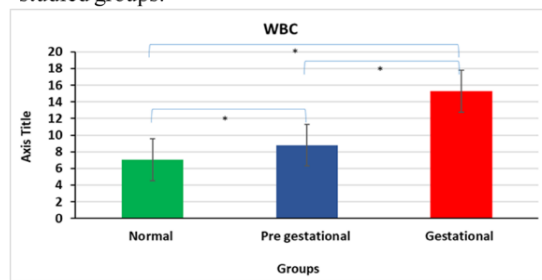


Figure (4): Means of the WBC as affected by the studied groups.

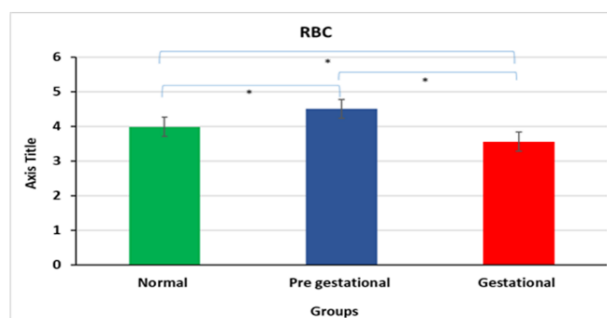


Figure (5): Means of the RBC as affected by the studied groups.

Thickness of amniotic membrane layers

Table (2), showed the statistical analysis of the AM layers thickness. The results revealed that the thickness of epithelial layer plus BM was highly significant increase in group of PGDM

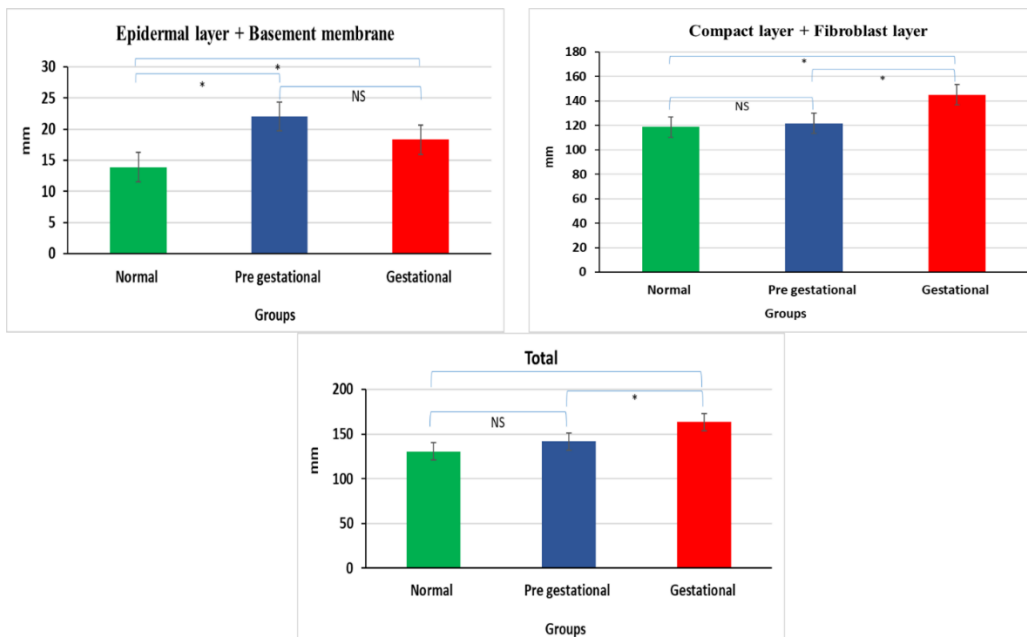
(22.04±0.72) compared with control (13.88±2.14), however, when comparing this thickness with GDH group, it is observed there was no significant differences between them (Fig.6)

For compact layer and fibroblast layer, the GDM resulted in high significant increase in the thickness of this layer and reached to the (144.98± 10.68) compared with PGDM and control (121.65±4.41;118.70±6.27) respectably. While PGDM and control were didn't differ significantly (P>0.05) between each other (Fig.7). Table (2), also showed that the total thickness of AM was highly significant increase in GDM group than other groups. While this total thickness of AM in PGDM and control group didn't differ significantly (P>0.05) between each other (Fig.8).

Table (2): Thickness of amniotic membrane layers

Groups	Epithelial layer plus basement membrane (µm)	Compact layer plus fibroblast layer (µm)	Total (µm)
Control	13.88 ±2.14 ^b	118.70 ±6.27 ^b	130.52 ±6.17 ^b
PGDM	22.04 ±0.72 ^a	121.65 ±4.41 ^b	141.84 ± 5.71 ^b
GDM	18.30 ±2.24 ^{ab}	144.98 ±10.68 ^a	163.27 ±10.88 ^a
Sig.	*	*	*

Means with different letters within each parameter are differed significantly. (NS= non-Significant; *= Significant at level (P<0.05). PGDM=Pre gestational Diabetes Mellitus, GDM=Gestational Diabetes Mellitus.



Diameter of amniotic membrane epithelial cell nuclei

The nucleus diameter of AM epithelial cells was highly significant decreased in group of PGD (2.330±239) than control group (7.04±0.435), and significantly deferred in comparison

with GDM group (4.52 ± 0.302). GDM resulted in significant decreased in the nucleus diameter compared with control (Table 3) and (Fig.9).

Table (3): Diameter of nucleus as affected by the studied groups.

Groups	Diameter of nucleus (μm)
Control	7.04 ± 0.435^a
PGDM	2.33 ± 0.239^c
GDM	4.52 ± 0.302^b
Sig.	*

Means with different letters within each parameter are differed significantly. (NS= non-Significant; *= Significant at level ($P < 0.05$). PGDM=Pre gestational Diabetes Mellitus, GDM=Gestational Diabetes Mellitus.

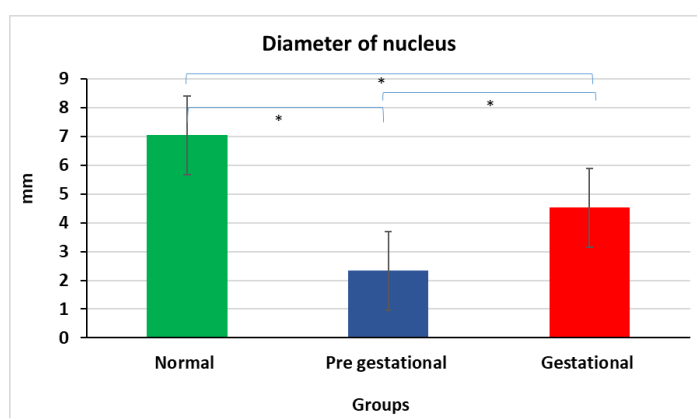


Figure (9): Means of the diameter of nucleus as affected by the studied groups

Thickness of placenta

Table (4) and figure (10) indicated that the placenta thickness was not differ significantly ($P > 0.05$) among study groups (control, GDM and PGDM).

Table (4): Thickness of placenta as affected by the studied groups.

Thickness of placenta (cm)	
Normal	2.26 ± 0.197^a
Pre gestational	2.75 ± 0.271^a
Gestational	2.64 ± 0.226^a
Sig.	NS

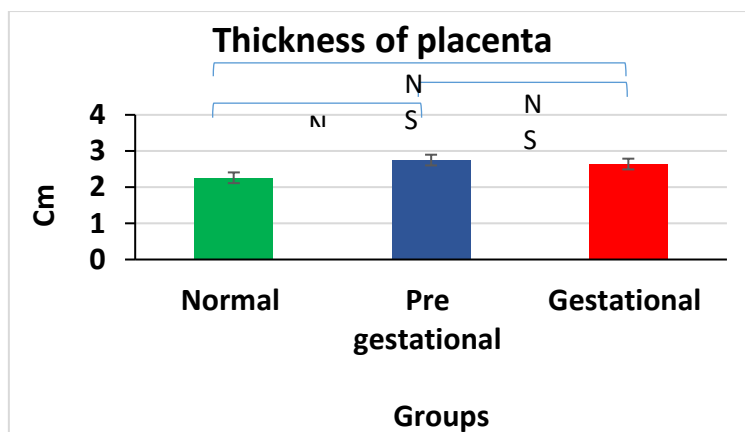


Figure (10): Means of the thickness of placenta as affected by the studied groups

Histological studies

Figures (11&12), of the control (non-diabetic) group indicated that the AM is an avascular membrane, which means it contains no blood vessels or nerves and consists of three different layers: the epithelium, basement membrane, and connective tissue (stroma).

The epithelial layer is the innermost layer adjacent to the fetus, and it is also called the amniotic epithelium. This layer was formed by one layer of cuboidal cells with a rounded apex, vacuolated acidophilic cytoplasm, and rounded or oval centrally located nuclei. These cells were resting on the thick BM and avascular connective tissue (stroma). The stroma consists of a compact layer and a fibroblast layer; the compact layer of amnion connective tissue (stroma) consists of single collagen fibrils that are arranged felt-like in parallel layers. While the fibrils of fibroblast layers were mostly arranged in bundles, leading to the formation of a network, between them the fibroblast and mesenchymal cells were founded.

Histopathological examination of AM sections taken from both diabetic groups (GDM and PGDM) revealed several changes (Figs. 13, 14, 15 and 16), including: hypertrophy and hyperplasia in epithelial cells, which appeared in greater number and size than in normal (non-diabetic) groups; formation of the vacuole between these cells; degeneration of epithelial cells that pinched off from the lining epithelium; and degeneration of AM in both groups. As shown in the figures above, diabetes also resulted in the breakdown of the compact and fibroblast layers and damage of the stromal collagen fibril.

Diabetes mellitus (GDM and PGDM) caused obvious changes in the morphological structure of epithelia cells and their nuclei, as shown in figures (14A and 16 A&B), where these cells with nuclei become elongated and resemble columnar epithelium, and the basement membrane appeared thicker in comparison to the control group

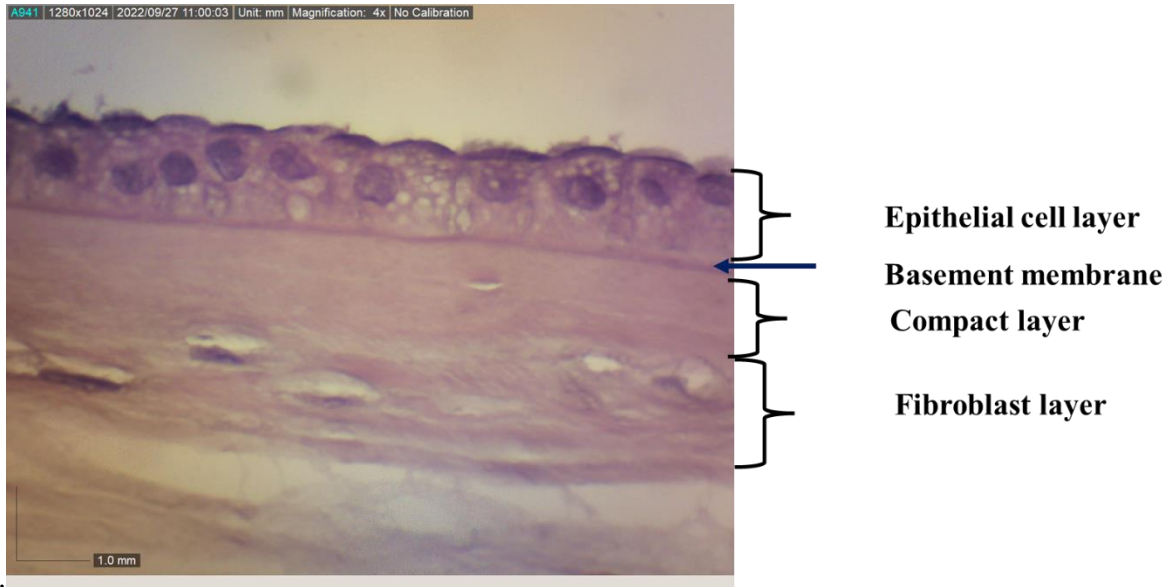


Figure (11): A section of amniotic membrane in control (non-diabetic women) showing normal structure of amniotic membrane layers. (H&E, 400x).

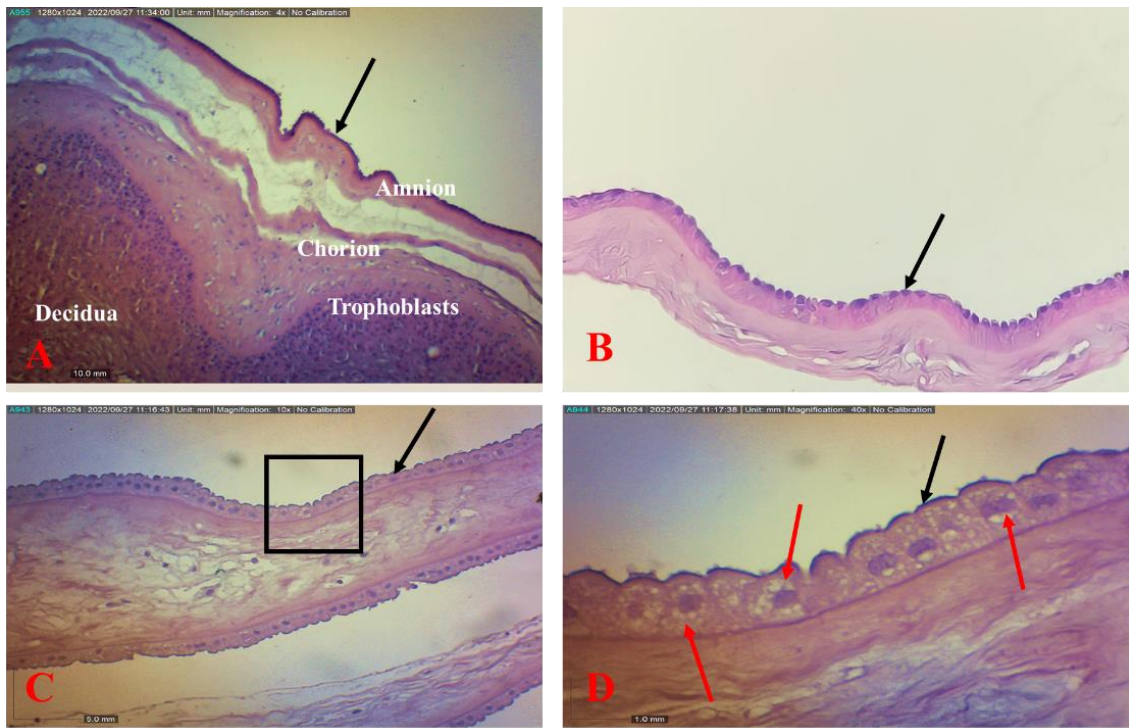


Figure (12): A section of amniotic membrane in control (non-diabetic women) shows the normal structure of the amniotic membrane, showing the lining epithelium, which consists of cuboidal cells with a rounded apex (black arrow) covering the basement membrane and underlying connective tissues. Note in Fig. (D) the higher magnification of part of Fig. (C); the red arrows indicate the vacuoles. (H&E, A:40x; B&C:100x; D:400x).

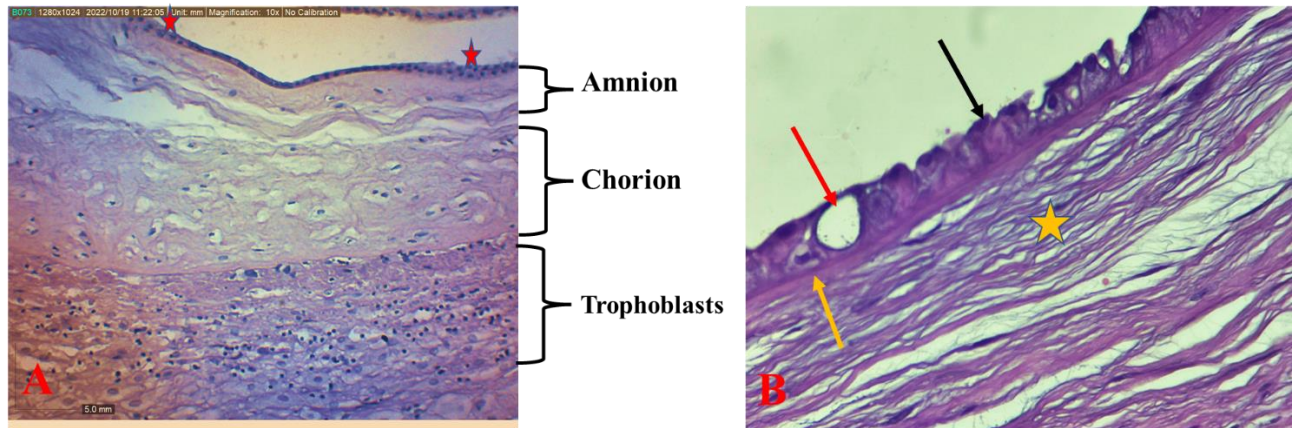


Figure (13): A section of amniotic membrane in the GDM group showing: (A): amniotic membrane layers, hyperplasia in epithelial cells (red star), and degeneration change were observed in this membrane. (B): hyperplasia and hypertrophy of epithelial cells which become elongated and resemble columnar epithelium (black arrow); formation of a vacuole between epithelial cells (red arrow); increase in thickness of basement membrane (yellow arrow); and damage of the underling stromal collagen fibril (yellow star). (H&E,40x).

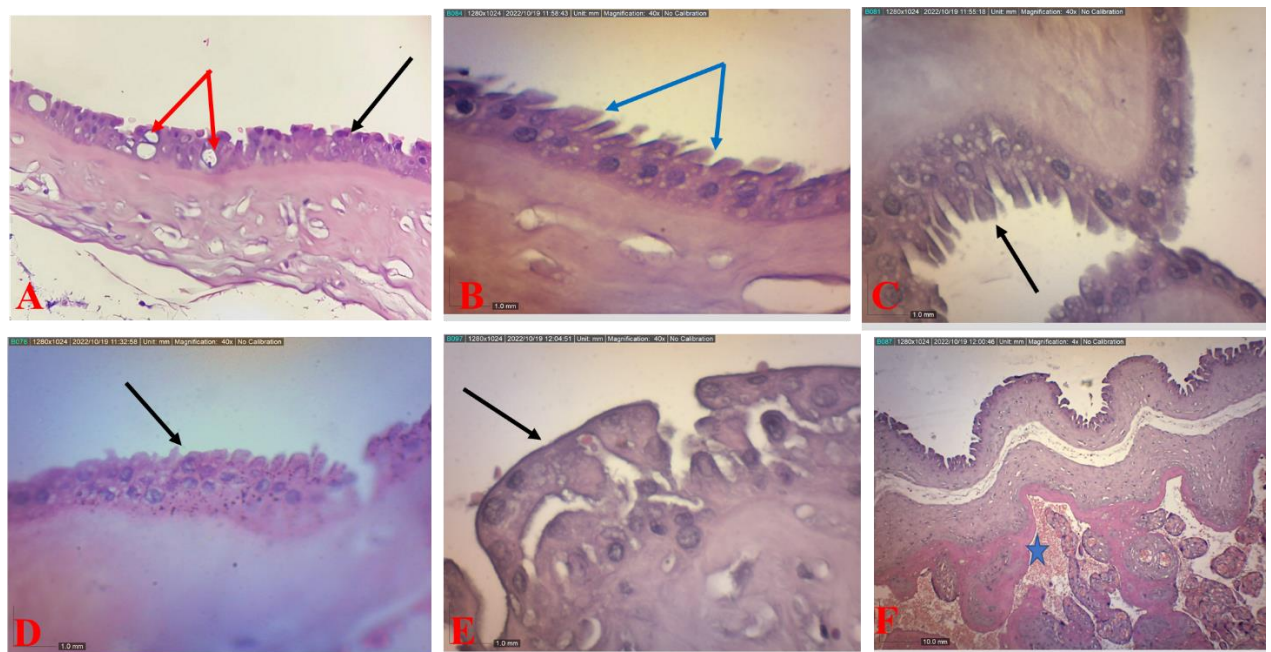


Figure (14): A section of amniotic membrane in the GDM group shows: (A): hypertrophy of epithelial cells (black arrow) and formation of vacuole between these cells (red arrows). (B & C): degenerated cells pinched off from the lining epithelium (blue arrow). (C, D, E, and F): hyperplasia and hypertrophy of the epithelial cells (black arrow), in addition to the degeneration of the amniotic membrane and change in its thickness with projection in some cells. Note: chorangiosis in (E) (blue star). (H&E, A: 100x; B, C, D, & E: 400x; F: 40x).

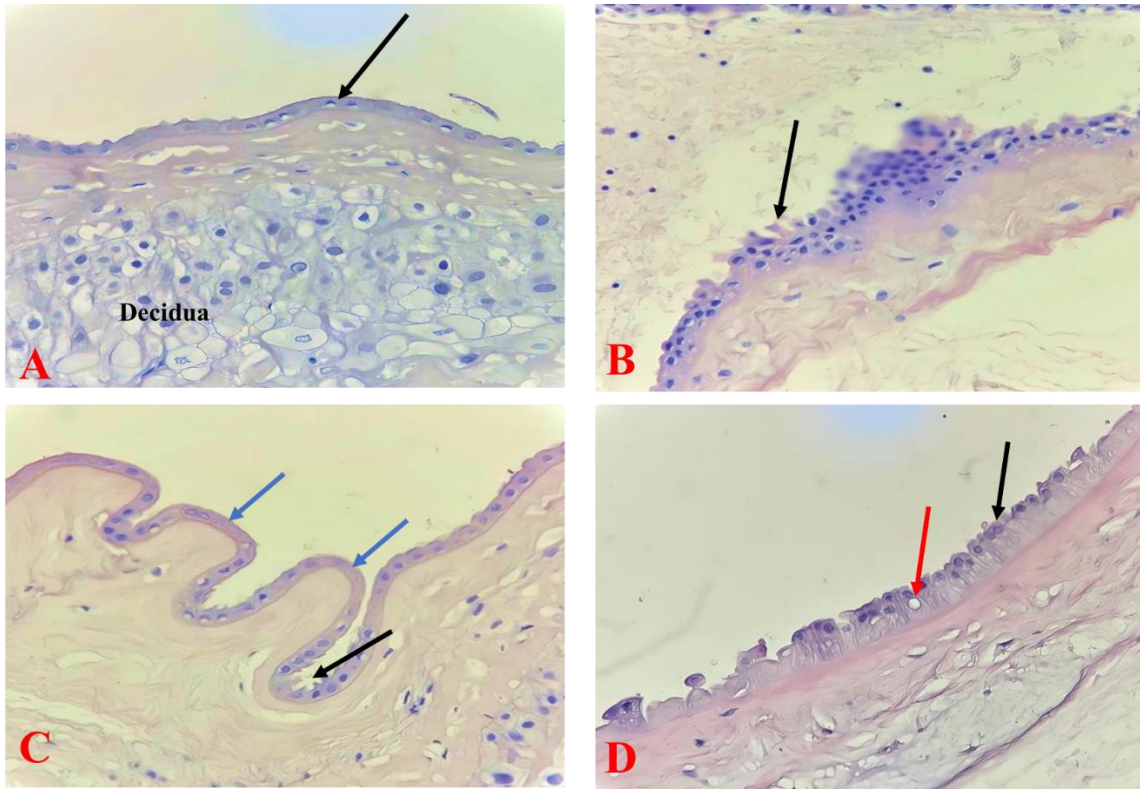


Figure (15): A section of amniotic membrane in the PGDM group shows degeneration of the amniotic membrane. (A): The presence of epithelial areas with dead epithelial cells (black arrow). (B): hyperplasia in epithelial cells and degenerated cells pinched off from the lining epithelium (black arrow). (C): Degenerated cells pinched off from the lining epithelium (black arrow); the blue arrow indicates dead epithelial cells; and some areas of epithelium appeared without cells. (D): hyperplasia and hypertrophy of the epithelial cells (black arrow); the red arrow indicates the formation of a vacuole between these cells. (H&E, A, B, C, and D: 100x).

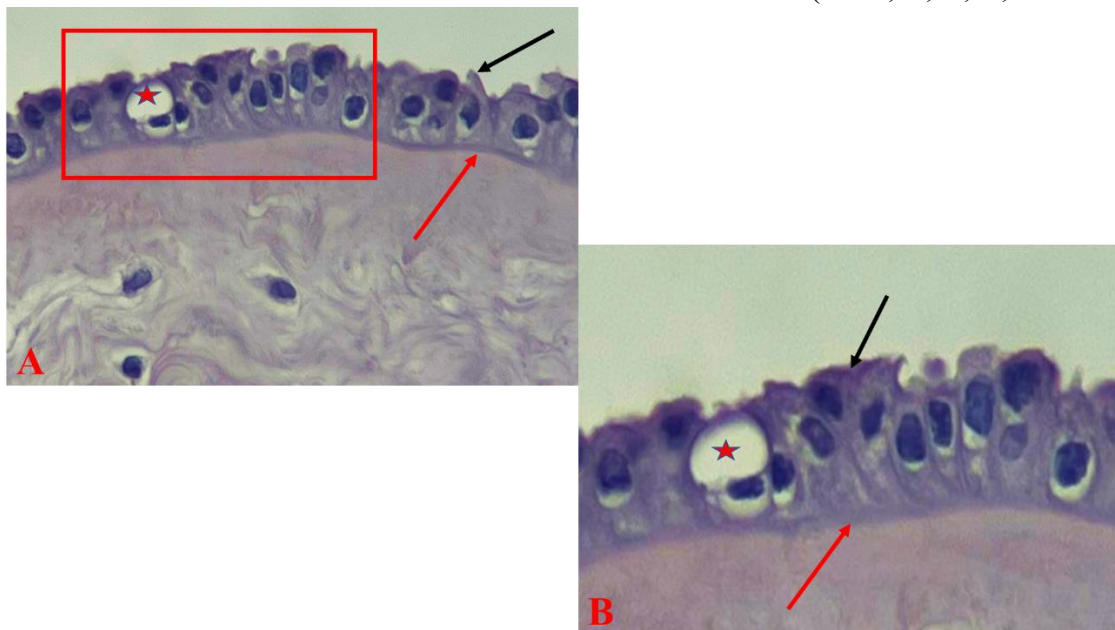


Figure (16): A section of amniotic membrane from the PGDM group exhibits changes in the morphology of the epithelial cells as well as their nucleus, which becomes elongated and

resembles columnar epithelium. The basement membrane appeared thicker (red arrow) than in the control group. The red star represents the creation of vacuoles within the epithelial cell. (H&E, A: 40x and B: 1000x (high magnification of the section of Fig. A).

Discussion

The amniotic membrane, also known as the amnion, is the innermost layer of the fetal membranes that completely surrounds the embryo and delimits the amniotic cavity, which is filled with amniotic fluids. This membrane is thought to be a potential treatment for a variety of diseases (Mamele *et al.*, 2012, Ambrasio *et al.*, 2019).

The current study found that the mean maternal age in the PGDM and GDM groups was considerably higher than in the non-diabetic (control) group. The results also showed that the newborn weight was significantly lower in the PGDM group than in the control group, but there was no significant difference between the effects of PGDM and GDM on this weight. This finding contradicted the findings of Alo khudir, (2017), who found no statistical difference in maternal age and infant weight between the DM and control groups. While Weli, (2021), found a considerable rise in neonatal weight when compared to the control.

The current study found a considerable rise in WBC and RBC count in both diabetic groups when compared to controls. This conclusion was verified by Zhang *et al.*, (2021), who found that the WBC, platelet, and RBC parameters in GDM altered dramatically as gestation progressed, implying that a moderate inflammatory and imbalanced immune response occurs in GDM and may play a role in the etiology of GDM.

According to the thickness of the AM layers, PGDM and GDM both induced a considerable rise in the thickness of the epithelial layer plus BM and compact layer plus fibroblast layer. The results also revealed that GDM greatly increased the total thickness of AM. This conclusion was consistent with the findings of Togrul *et al.*, (2017), Nergiz *et al.*, (2019), and Weli, (2021), who found that the amniotic epithelial BM and total AM thickness were considerably larger in the GDM group as compared to the control group. According to Benirschke *et al.*, (2006), the alterations in BM and AM thicknesses were caused by the formation of mucopolysaccharides, which happens as a result of intrauterine growth retardation.

The present findings indicated that PGDM induced a highly significant decrease in the diameter of the nucleus of AM epithelial cells, whereas GDM caused a significant drop in this diameter. This result was consistent with Togrul *et al.*, (2017), who demonstrated that membrane thickness caused a change in diameter and cell size, and they indicated that GDM is thought to cause thickness and diameter alterations in AM histopathologically.

Histological analysis of the AM in the control group revealed that the AM is made up of one layer of cuboidal cells with rounded apexes and vacuolated cytoplasm. The nuclei are either vesicular, rounded, or oval in shape. These cells were resting on a thick BM, and the CT stroma is made up of collagen fibers, fibroblast cells, and mesenchymal cells. These findings agreed with those of (Niknjad *et al.*, 2008, Al-Yaha & Makhouf, 2013, Ferenczy, 2020, and Weli.2021), who established that the AM is composed of an epithelial monolayer, a thick BM, and an avascular stroma. According to the findings of this study, the AM includes no blood vessels or

nerves, as stated by AL-Yaha and Makhlof (2013). The nutrients required in this situation are given directly by diffusion from the amniotic fluid and/or the underlying decidua.

The histopathological study revealed that both GDM and PGDM caused several changes and damage to the AM, including hypertrophy and hyperplasia in epithelial cells, the formation of a vacuole between epithelial amniotic cells, and a change in the morphological structure of these cells, which became elongated and resembled columnar cells, in addition to the obvious degeneration of these cells that pinched from the lining epithelium. This finding was also documented by Togrul *et al.*, (2017), who demonstrated that both hyperplasia and hypertrophy were detected in the nucleus of amniotic epithelial cells in the GDM group, and that these structure alterations in AM epithelial cells were created as a result of diabetes. Weli (2021) reported the same result, stating that the DM produced various modifications and harm to the AM. According to Togrul *et al.*, (2017), diabetic damage to AM cells results in poor detection of E-cadherin, and E-cadherin is a crucial molecule in the maintenance of epithelial integrity, as stated by Takeichi, (1990).

The AM has multiple metabolic functions and also plays an essential role during delivery through to the many compounds produced by the AM epithelium which enable for the onset and maintenance of uterine contractility, so in conclusion, GDM and PGDM induced various alterations and damage to the AM, which in turn delayed embryonic development and delivery.

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