Original Article

Comparison Study of the Histopathology and Immunohistochemistry of the Amniotic Membrane and its Stem Cells in Normal, Gestational, and Pregestational Diabetes

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ABSTRACT

Introduction: The amniotic membrane (AM), a placental component that feeds embryos throughout pregnancy, has the potential to be used in tissue engineering because it contains stem cells (SCs). Diabetes during pregnancy can interfere with the development of the extraembryonic membrane, the SC, and the embryo, which can cause tissue degeneration.
Objective: This study was aimed at comparing pregnant women with gestational diabetes mellitus (GDM), pre-gestational diabetes mellitus (PGDM), and non-diabetic normal pregnant women in terms of histological features and immunohistochemistry expression of E-cadherin and SC marker molecules.
Materials and Methods: Thirty AM specimens were collected from pregnant women with GDM, PGDM, and non-diabetic normal pregnant women (n = 10 for each group). AM segments undergo a number of histological procedures, then are stained with hematoxylin and eosin for histological examination, Periodic Acid-Schiff (PAS) to measure the thickness of the basement membrane (BM), immunohistochemistry to assess the expression of E-cadherin and SC markers (anti-Nanog and anti-Oct-4).
Results: Both diabetes groups produced a wide range of damage to AM, including vacuole formation, epithelial cell degeneration, hypertrophy and hyperplasia, and AM degeneration. The lining epithelium, BM, and underlying connective tissues in the PAS reaction responded moderately in the PGDM group but severely in the GDM group. AM epithelial cells in the control and GDM groups show a significant expression of E-cadherin. However, in the PGDM group, this expression was barley. The AM of the control group without diabetes had considerably higher anti-Nanog and anti-Oct-4 SC marker expression than did the diabetes groups. The GDM and PGDM groups had significantly decreased expression of the SC markers.
Conclusions: The AM and SCs have suffered severe damage from the GDM and PGDM, which may have an impact on embryonic development and delivery as well as their ability to be employed in regenerative medicine.

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Key Words: Amniotic membrane, anti-nanog stem cell marker, anti-Oct- 4 stem cell marker, diabetic mellitus, e-cadherin.

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INTRODUCTION

Due to its distinct structure and amniotic fluid content (AF), the amniotic membrane (AM), a protective covering of the foetal placenta, is essential in regenerative medicine[1]. In experiments on tissue transplantation, this membrane has showed promise[2]. However, significant improvements have been made to improve AM's qualities, handling, and durability, making it a relatively fresh topic in terms of its applications[3].

Amniotic membrane epithelial (AME), basement membrane (BM), and stroma layer are the three primary layers that make up the amniotic membrane (AM). Human amniotic epithelial cells (hAECs) that can develop into germ layers, exhibit stem cell (SC) markers, and maintain the pluripotency of the undifferentiated epiblast make up the simple epithelial layer known as the AME layer[4]. During gestation, the embryo is supported by the BM. The thick, fibroblastic, spongy, and collagen-rich stroma layer is a mesenchymal layer. The fibroblastic layer may contain rare macrophages with a loose fibroblast network and human amniotic mesenchymal stromal cells (hAMSC), which resemble fibroblasts. The size and thickness of these layers are influenced by the location of hAMSC, which deliver nutrients and oxygen through diffusion processes[4,5,6]. The hAECs and hAMSCs are produced from the amnion's epiblast and hypoblast layers, respectively, eight days after fertilization. They use embryonic stem cells (ESCs) and adult pluripotency to produce a heterogeneous population of pluripotent, multipotent, progenitor, and adult cells[7,8]. Gestational diabetic mellitus (GDM), a form of carbohydrate intolerance, leads to maternal and foetal problems like macrosomia, early delivery, neonatal morbidity, and perinatal death[9,10]. The placental tissues, umbilical cord, and AM are altered and damaged by GDM and pregestational diabetic mellitus (PGDM)[11,12,13]. Human amniotic epithelial cells phenotypic and biological characteristics are affected by maternal metabolic abnormalities in GDM-complicated pregnancies, which may have an effect on the nutritional and metabolic condition of the foetus[2].

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This study compared pregnant women with gestational diabetes mellitus (GDM), pre-gestational diabetes mellitus (PGDM), and non-diabetic normal pregnant women in terms of histological features and immunohistochemistry expression of E-cadherin and SC marker molecules.

MATERIALS AND METHODS

With the patients' permission and in accordance with the recommendations of the Medical Ethics Committee, which was approved by the Duhok Directorate of General Health, the Directorate of Planning, and the Scientific Research Division, Kurdistan Region, Iraq, with reference number 08032023-2-10, placenta samples were collected from the Maternity Hospital in Zakho and the Duhok Obstetrics and Gynecology Hospital. The Department of Biology, Zoology Laboratory, Faculty of Science, University of Zakho, conducted this study.

Amniotic membrane samples

In the current study, which took place between October 4, 2021, and June 20, 2022, thirty pregnant women between the ages of 18–40 and full-term (37–40 weeks) gestation were involved and divided into three groups, each with n = 10 pregnant women: Group (1) consists of healthy, non-diabetic women as a control group; Group (2) includes pregnant women with GDM; and Group (3) includes pregnant women with PGDM. All the placental samples included in the present study were taken from pregnant women's with normal deliveries.

In accordance with[14,15], the placenta of these groups was quickly transported to the lab, cleaned in phosphate buffered saline (PBS) with penicillin and streptomycin (200 U/ml penicillin, 200 g/ml streptomycin), and then used. The AM is taken out of the chorion and were washed in PBS.

The step of tissue preparation

1. Fixation with formaldehyde buffered 10%.
2. Dehydrated in rising ethanol series.
3. Then cleaned in xylene.
4. Embedding in paraffin.
5. Cut into 4 µm slices and put on slides.

Some of these slides that included AM slices were prepared for a light microscopic investigation[16] and stained with H&E dye. While additional prepared slides were employed for PAS staining to distinguish the BM[17]. Due to the reactivity of free aldehyde groups found within carbohydrates, the Schiff reagent produces an end product that is an intense scarlet magenta.

Immunohistochemical study

The molecule E-cadherin is essential for maintaining epithelial integrity. The E-Cadherin (Concentrate) kit (M361229-2; Agilent Dako, Santa Clara, California) was prepared in accordance with the manufacturer's instructions E- Cadherin is a calcium-dependent cell–cell adhesion molecule (proteins) in the plasma membrane in epithelial cells. The positive cells showed brown colour. A positive control will be shown by a brown precipitate at the antigen position that has been assessed for immunoreactivity after being counter-stained with hematoxylin[15].

In order to detect AM SCs markers, both the polyclonal antibodies (Abs) for OCT4 (Orb 11184) and NANOG (Orb D69128) were obtained from Biorbyt (Cambridge, United Kingdom). Out of the total number of positive and negative cells, all results are expressed as a relative percentage of positive cells stained dark brown (OCT4 and NANOG were expressed in both the nucleus and the cytoplasm). A brown precipitate at the antigen location that has been counter-stained with hematoxylin and evaluated for immunoreactivity will indicate a positive reaction[14]. This analysis was conducted using a methodology that was consistent with how the kits were created. Then photos were taken using the Dino-Eye: Microscopic Eye-Piece Camera, a digital camera.

Statistical Analysis

The acquired data was statistically analyzed using the SPSS computer program[18]. However, Duncan's multiple-range test[19] was used to partition the means in the ANOVA (both one-two-way ways).

RESULTS

Histological study

Hematoxylin and eosin-stained slides revealed that the non-diabetic control group's AM was composed of three layers: epithelium, thick BM, and vascular connective tissue. One layer of cuboid cells with rounded apexes, vacuolated acidophilic cytoplasm, and round or oval nuclei makes up the epithelium (Figures 1A,B). A compact layer and a fibroblast layer make up the stroma's two layers. Single collagen fibrils aligned and felt-like in parallel layers make up the compact layer of amnion connective tissue (stroma). Fibroblast and mesenchymal cells were developed between the fibrils of the fibroblast layers, which were mostly arranged in bundles and created a network.

(Figures 1 C,D,E,F) show the histopathological study of AM sections from GDM and PGDM groups, respectively, revealed a variety of changes, including epithelial cell hypertrophy and hyperplasia; formation of vacuoles between them; and the degeneration of epithelial cells that had detached from the lining epithelium. Aside from the BM looking thicker in comparison to the control group. Diabetic also resulted in noteworthy modifications to the nucleus and morphology of epithelia cells, in addition to the BM which a appeared thicker in comparison to the control group.

Histochemical Results

Results of the Periodic Acid Schiff (PAS) Reagent

In the control group's PAS-stained AM sections, showed that the lining epithelium, BM, and underlying connective

tissues exhibit normal thickness in AM, which showed a typical reaction to the PAS (Figure 2A). While the AM in the group of PGDM displayed a minimal response to PAS, the BM thickness was increased in comparison to the control (Figures 2 B,C). In contrast, GDM showed a thicker response to PSA, as shown by an increasing thickness of the BM (Figures 2 D,E).

E-Cadherin Expression in Amniotic Epithelial Cells

E-cadherin was substantially expressed positively in the AM epithelial cells of the control group (Figures 3 A,B) and the GDM group (Figures 3 C,D), according to the immunohistochemistry analysis. However, the PGDM group's cells only showed weak E-cadherin expression (Figures 3E,F).

Detection of Amniotic Membrane Stem Cells markers [Anti-Nanog (H-155) and Anti-Oct-3/4, Antibodies]

The result of the present study indicated that, in comparison to PGDM and GDM, the AM in the control group (non-diabetic) displayed a highly substantial increase ($P \leq 0.001$) in the expression of the anti-Nanog (H-155) SC marker. At the same time, there was no significant difference ($P \geq 0.05$). between the PGDM and GDM groups (Table 1, Figure 4).

When compared to PGDM and GDM, the anti-Oct-3/4 stem cell marker expression in the AM of the control group (non-diabetics) was significantly higher ($P \leq 0.001$) than that in the diabetic groups. When compared to GDM, PGD's expression of this SC marker did not differ significantly ($P \geq 0.05$) (Table 2, Figure 5). This increase in the expression of these SC markers in the control (normal) group shows that the mesenchymal and epithelial stromal cells of AM contain a significant number of SCs that are functionally active. DM in both groups caused damage to the AM tissue and its cells, which significantly reduced the expression of these markers.

In the AM epithelial and underlying layer, the immunohistochemical analysis of the histological AM sections revealed that the control group had significantly higher expression of the SC marker molecules anti-Nanog (H-155) (Figure 6) and anti-Oct-3/4 (Figure 7) than the GDM and PGDM groups, which showed significantly lower expression of these markers. The information shown in the tables and figures above is supported by the histological study result.

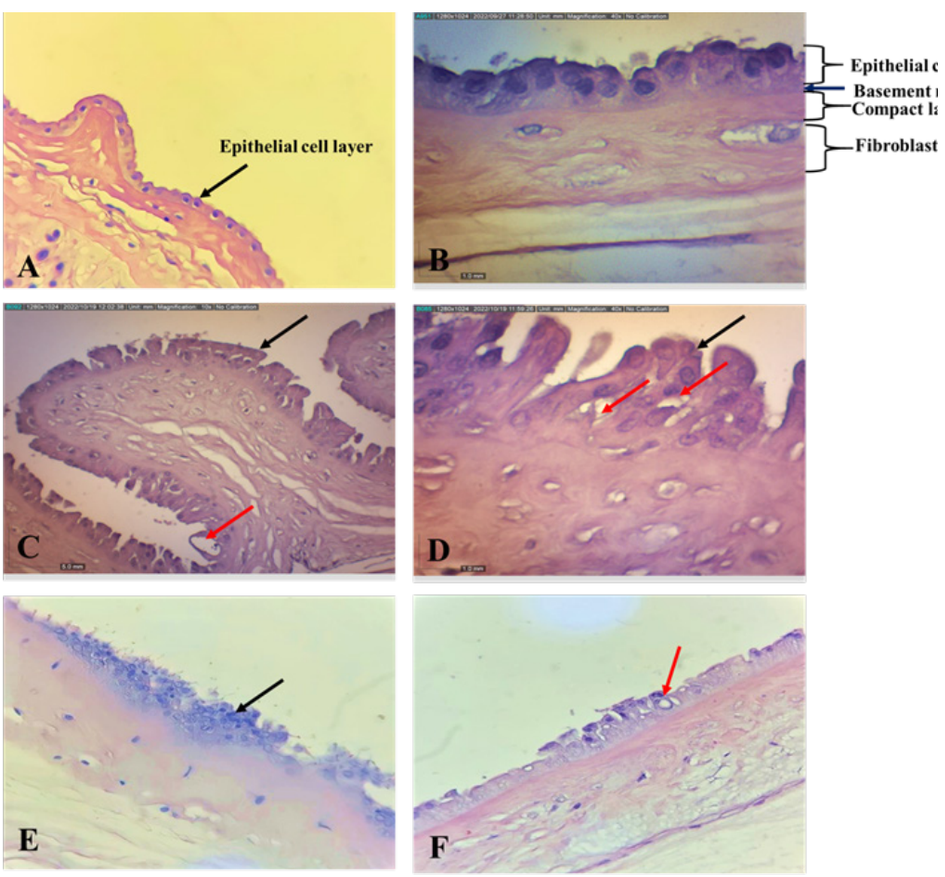

Fig. 1: Amniotic membrane histological sections: (A&B) from the control group (women without diabetes) demonstrates the amniotic membrane's normal structure and their layers. (C&D): in the GDM group showed degenerated cells pinched off from the lining epithelium and hyperplasia and hypertrophy of the epithelial cells (black arrow) and (E&F) in the PGDM group, (E) showing hyperplasia and hypertrophy of epithelial cells (black arrow). (C&F): showing the formation of a vacuole between epithelial cells (red arrows). (A, C, E, and F: 100X; B and D: 400X.)

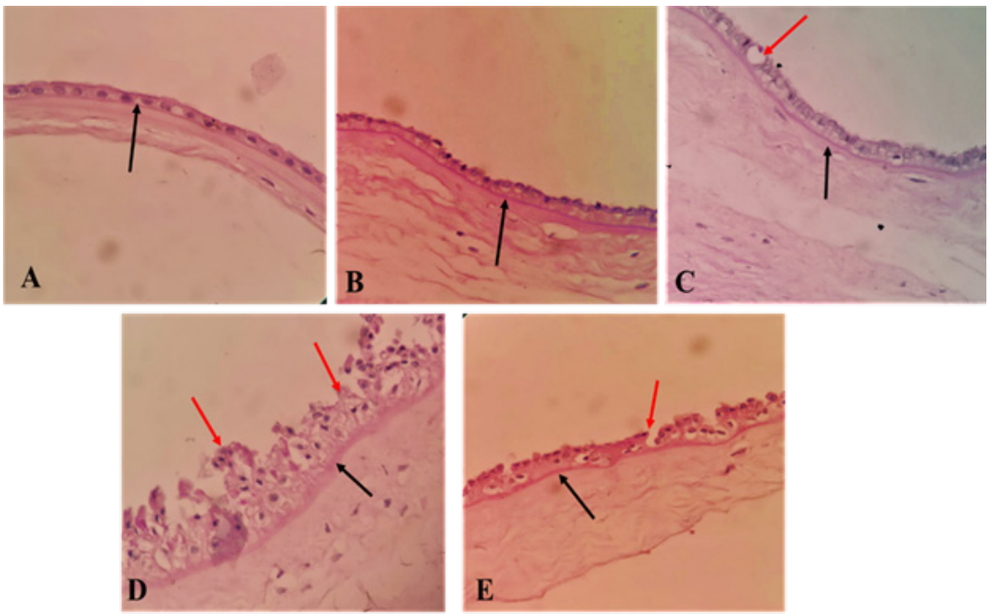

Fig. 2: The lining epithelium, basement membrane (BM), and underlying connective tissues in the control group (A) exhibit normal thickness in an amnion membrane (AM) segment stained with PAS. (B&C) PGDM displayed a minimal response to PAS. Be aware of the rise in BM thickness when compared to the control. Vacuoles between epithelial cells and their degeneration are indicated by the red arrow. (D&E) GDM demonstrated a thicker response to PSA, as shown by an increase in BM thickness. The red arrow denotes the pinching off of epithelial cells from the lining epithelium as well as their hyperplasia, hypertrophy, and degeneration (PAS, A, B, C, and E: 100x; D: 400x).

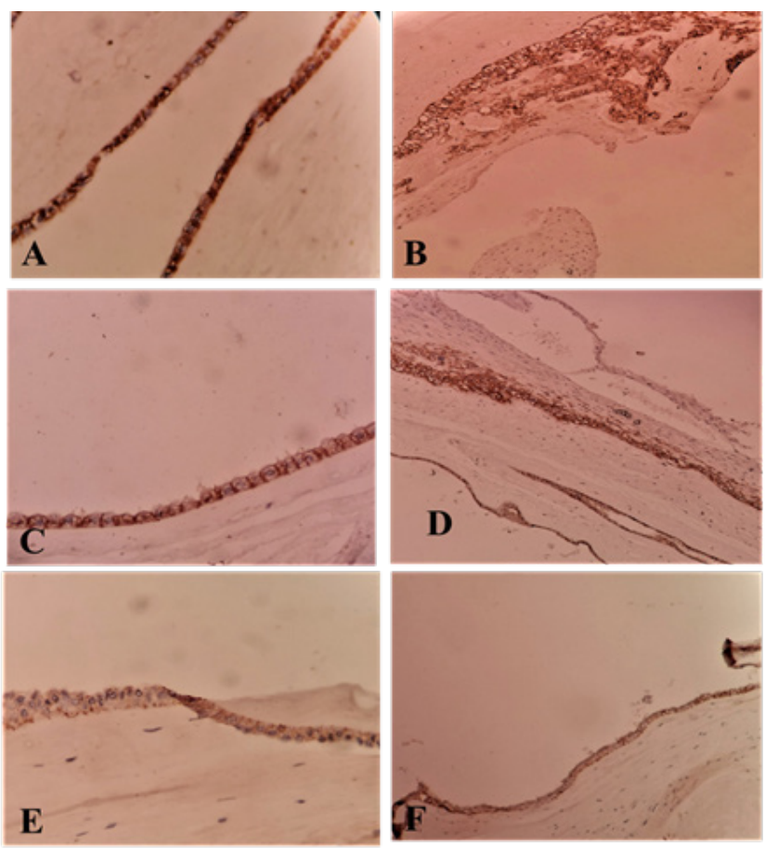

Fig. 3: E-Cadherin immunostaining of amnion membrane (AM) showed that both the control group (A&B) and the GDM (C&D) groups had highly positive E-Cadherin expression in amniotic epithelial cells. E-Cadherin expression was minimal in the amniotic epithelial cells of the PGDM group (E&F). (A, C, and E: 100x; B, D, and F: 40x).

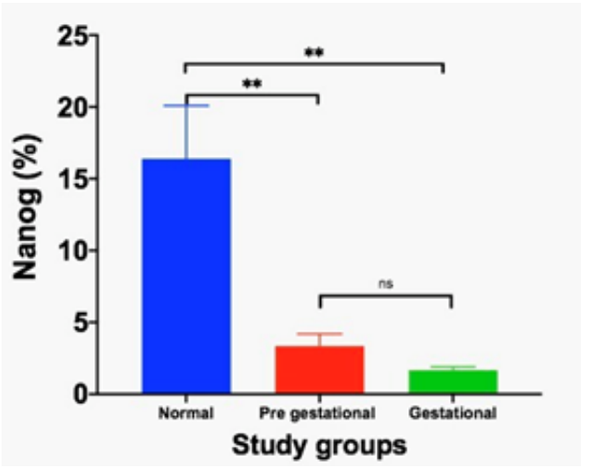

Fig. 4: Shows the anti-Nanog (H-155) stem cells marker expressed in the AM of the groups under study (Mean ± SE).

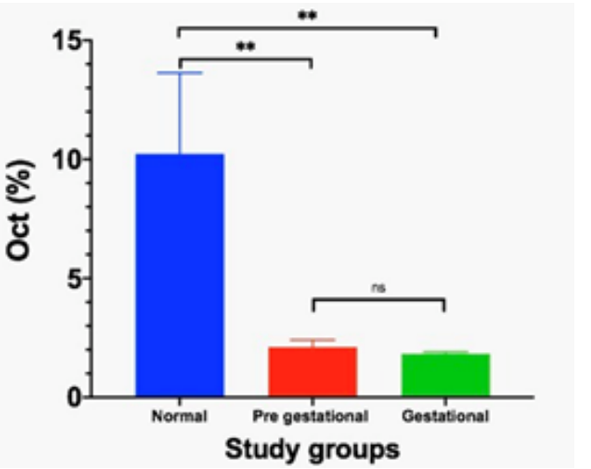

Fig. 5: Shows the Anti-Oct-3/4, stem cells marker expressed in the AM of the groups under study (Mean ± SE).

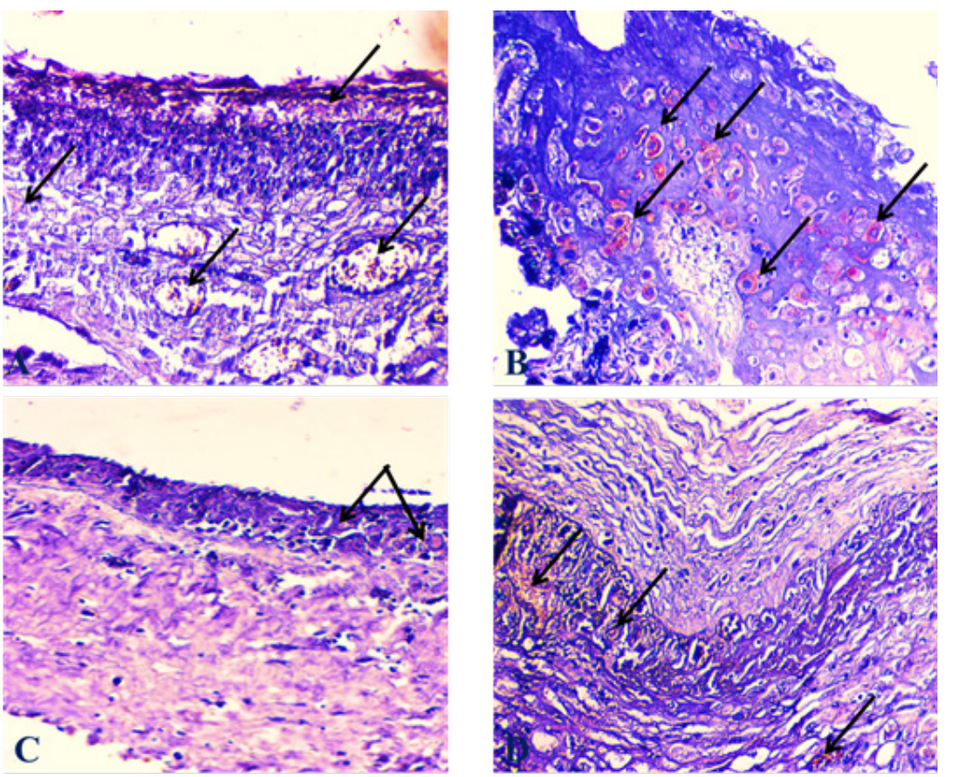

Fig. 6: Immunohistochemical staining of the amniotic membrane (AM) and underlying layers with an anti-Nanog stem cell marker revealed the positive cells stained with a dark brown color (Black arrows). Control group (A&B): the cells of AM showed highly significant increase in the expression of this marker compared with PGDM (C), and GDM (D) groups. While this membrane and their cells in groups of the (PGDM and GDM), showed significant decrease in the expression of this marker. (400X).

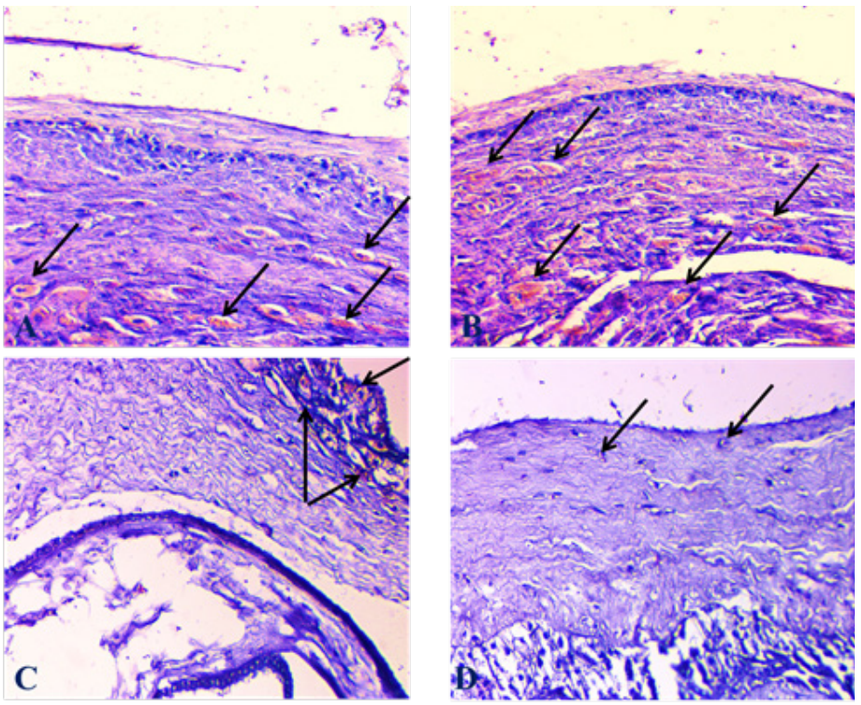

Fig. 7: Anti-Oct-3/4 stem cell marker immunohistochemical staining of the amniotic membrane and underlying layers revealed the positive cells stained with a dark brown color (Black arrows). Control group (A&B), PGDM group (C), and GDM group (D). (400X).

Table 1: Shows the anti-Nanog (H-155) stem cells marker expressed in the AM of the investigated groups (Mean ± SE).

Stem cell marker	Groups			
% of Anti-Nanog (Mean ± SE)	Control	PGD	GDM	Sig.
	16.39±3.7^{a}	3.35±0.85^{b}	1.67±0.23^{bc}	*

Within each parameter, means with alternative letter combinations differed significantly. NS stands for non-Significant, while * means significant at level (P <0.05). GDM stands for gestational diabetes; PGDM stands for pregestational diabetes.

Table 2: Shows the anti-Oct-3/4 stem cells marker expressed in the AM of the investigated groups (Mean ± SE).

Stem cell marker	Groups			
% of Anti-Oct-3/4, (Mean ± SE)	Control	PGD	GDM	Sig.
	10.23±3.4^{a}	2.11±0.3^{b}	1.83±0.08^{b}	*

Within each parameter, means with alternative letter combinations differed significantly. NS stands for non-Significant, while * means significant at level (P <0.05). GDM stands for gestational diabetes; PGDM stands for pregestational diabetes.

DISCUSSION

The embryo is completely encased by the AM, the innermost layer of the fetus's membranes, which also lines the amniotic cavity and protects it from outside threats while a woman is pregnant. This membrane is thought to be a useful treatment tool for numerous diseases. It is utilized because it is a rich source of stem cells with a high proliferation and plasticity ratio that can proliferate and differentiate in *vitro*, and it can be extracted from leftover fetal material[20,21]. But some diseases result in alterations and damage to the AM; one of these diseases is diabetic mellitus, which is the most prevalent metabolic condition that develops during pregnancy and can have negative effects on the mother and the developing fetus[13,15]. These findings are consistent with the current study, which discovered that epithelial cell hypertrophy and hyperplasia, vacuole formation between epithelial amniotic cells, associated with a change in these cells' morphological structure, and obvious degeneration of these cells that pinched from the lining epithelium, were all caused by both GDM and PGDM. As indicated by[15], these changes happened as a result of the amniotic epithelial cell-cell connection being weaker due to diabetes mellitus.

Histochemical and immunohistochemical Study

The results of this investigation demonstrated that the lining epithelium, BM, and underlying connective tissues all became thicker as a result of the DM in the PAS-stained AM sections. While in the normal (control) group, these layers and their thicknesses were typical. Numerous publications, including[17,22,23,24], have discussed this discovery, pointing out that when the diabetic group's AM was stained with PAS, there was a noticeable rise in BM thickness and a strong positive response, which was thicker than in the healthy group. DM caused an accumulation of mucopolysaccharides as a result of restricted intrauterine growth and insufficient uteroplacental circulation, which resulted in this increase in thickness[24,25].

The AM epithelial cells had a substantial positive expression of E-cadherin in both the control group and

the GDM group. While E-cadherin was only moderately expressed by the AM epithelial cells in the PGDM group. According to Togrul *et al*[15], diabetes-related damage to AM cells causes poor E-cadherin identification. E-cadherin is a key molecule in the maintenance of epithelial integrity[26].

The intercellular attachments that make up intracellular junctions, which are mediated by E-cadherin, are crucial in the development of the epithelial barrier. Loss of E-cadherin expression in the cell membrane and intercellular communication may be caused by epithelial damage[24,27]. It is well accepted that tight junction proteins like E-cadherin are necessary for the AM barrier to operate. Therefore, if E-cadherin expression is reduced, the epithelial cells may disappear[28]. According to[27], E-cadherin promotes intercellular adhesion and draws actin filaments and catenin proteins to cell borders to improve barrier function, it makes its use as a sign of a healthy epithelial barrier crucial.

Detection of Amniotic Membrane Stem Cells markers

Anti-Nanog and anti-Oct-3/4 SC markers in the control group dramatically increased in the AM compared to the PGDM and GDM groups, showing that stem cells are prevalent and in good health in AM epithelial and mesenchymal stromal cells. In both groups, diabetes mellitus caused damage to the AM tissue and its cells, which significantly decreased the expression of these markers. The phenotype and biological characteristics of AMSCs are disrupted by maternal metabolic disturbances in pregnancies complicated by GDM and PGD, and these disturbances are, as indicated by[2], ultimately linked to the nutritional and metabolic status of the mother and fetus. This result was in line with[14,29,30], who demonstrated that a number of SC marker indicators, such as anti-smooth muscle actin, anti-vimentin, anti-OCT 34, and anti-Nanog, are produced by both mesenchymal stromal cells and amniotic epithelial cells.

Epithelial and mesenchymal stromal cells exhibit a wide range of cellular and molecular markers, demonstrating the presence of SCs. Due to their immunomodulatory, proliferative, and differentiation capabilities, as well as their distinct advantages over other SC types, Human amniotic epithelial Cells (hAECs), which are derived from placental tissues, offer substantial therapeutic promise in regenerative medicine[31].

Three layers make up the avascular AM, and inside each layer are SCs, extracellular matrix, and collagen, which act as the structural matrix and source of strength. AM, which is abundant in SCs, is very useful for tissue remodeling and benefits from the presence of numerous growth factors, cytokines, and other regulatory chemicals[32,33].

Overall, the current study's findings and those of earlier studies by[15] showed that GDM findings pointed to a weakening of the amniotic epithelial cell–cell interaction and that diabetes-induced structural abnormalities in the epithelial cells of AM. Patients with diabetes have an enlarged extracellular matrix, which causes structural changes in membrane thickness that ultimately throw off the matrix's delicate equilibrium. A boost in CD44 has also resulted in angiogenesis, which is considered to influence the movement of materials between the mother and the foetus. Due to the various compounds the AM epithelium generates that aid in the initiation and maintenance of uterine contractility, the AM is essential for labor and delivery and performs a variety of metabolic functions.

CONCLUSION

In conclusion, the AM and SCs have suffered severe damage from the GDM and PGDM, which may have an impact on embryonic development and delivery as well as their ability to be employed in regenerative medicine.

CONFLICT OF INTERESTS

There are no conflicts of interest.

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الملخص العربى

دراسة مقارنة للانسجة المرضية والكيمياء المناعية النسجية للغشاء السلوي وخلاياه الجذعية وعلاقتها بسكري الحمل وسكري ما قبل الحمل

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المقدمة: الغشاء الأمنيوسي والذي هو أحد مكونات المشيمة والتي تغذي الأجنة طوال فترة الحمل، لديه القدرة على استخدامه في هندسة الأنسجة وذلك لاحتوائه على الخلايا الجذعية. يسبب داء السكري أثناء الحمل بتعطيل نمو الاغشية خارج جنينية وتطور ونمو الخلايا الجذعية والاجنة، مما قد يؤدي إلى تنكس الأنسجة.

الهدف: تهدف هذه الدراسة إلى مقارنة كل من السمات النسجية والتعبير الكيميائي المناعي النسجي لجزيئات الـ E-cadherin- وواسمات الخلايا الجذعية anti Nanog (H 155) and anti Oct 3/4 SC في النساء الحوامل المصابات بداء سكري الحمل، وداء السكري قبل الحمل، والنساء الحوامل الطبيعيات غير المصابات بالسكري

المواد وطرائق العمل: تم الحصول على ثلاثين عينة من الغشاء السلوي (١٠ لكل مجموعة) من النساء الحوامل المصابات بداء سكري الحمل، وداء السكري قبل الحمل، والنساء الحوامل الطبيعيات غير المصابات بالسكري. خضعت هذه العينات الى التقطيع النسجي ولونت بملون الهيماتوكسيلين والايوسين لغرض الدراسة النسجية، وحمض الدوري شيف(PAS Periodic Acid Schiff) للكشف عن سمك الغشاء القاعدي، وكذلك تحليل المناعي النسجي للتعبير عن E-cadherin وواسمات الخلايا الجذعية .(anti-Nanog and anti-Oct-٤)

النتائج: تسببت كلتا المجموعتين من مرضى السكري في العديد من التغييرات والأضرار في الغشاء السلوي ، تتضمن فرط التنسج وفرط التضخم في الخلايا الظهارية وتكون فجوات ؛ مع حدوث تنكس في كل من هذه الخلايا والغشاء السلوي. فيما يتعلق بتفاعل الكاشف PAS، أظهر كل من سمك البطانة الظهارة ، والغشاء القاعدي ، والأنسجة الضامة الكامنة في النساء المصابات بسكري قبل الحمل استجابة ضعيفة لهذا الكاشف بينما سجلت مجموعة سكر الحمل استجابة قوية لهذا الكاشف. اظهر الفحص الكيميائي المناعي للـ E-Cadherin للخلايا الظهارية للغشاء السلوي كشفا موجبا معنويا للـ E-cadherin لمجموعة السيطرة ومجموعة سكري الحمل. بينما اظهرت للخلايا الظهارية للغشاء القاعدي في مجموعة سكري قبل الحمل كشف منخفض للـ E-Cadherin. اظهر الغشاء السلوي في مجموعة السيطرة الغير مصابة بالسكر كشفا موجبا معنويا عالي لواسمات الخلايا الجذعية anti-Nanog (H-155) and anti-Oct-3/4 SC مقارنة بمجموعتي مرضى السكري .أظهرت كل من مجموعة سكر الحمل ومجموعة السكر قبل الحمل استجابة معنوية ضعيفة لواسمات الخلايا الجذعية.

الاستنتاجات: تشير هذه النتائج إلى أن كل من سكر الحمل و السكر قبل الحمل يسبب تأثير ضار معنوي للغشاء السلوي والخلايا الجذعية والذي بدوره يؤثر على نمو الجنين وعملية الولادة, و وربما يعيق استخدامها في الطب التجديدي.