ORIGINAL ARTICLE

EFFECTS OF METFORMIN AND INSULIN ON MORPHOLOGY, STEREOLOGY AND MEAN MORPHOMETRIC DIFFUSION CAPACITY IN DIABETIC PLACENTA

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Background: Insulin given for the treatment of GDM is associated with hypoxic changes whereas Metformin has beneficial micro-vascular effects on the placenta. This comparative drug study was conducted with objectives to compare the effects of Metformin and Insulin on the morphology and stereology in gestational diabetes. And to calculate and compare the mean morphometric diffusion capacity for oxygen in Metformin and Insulin treated gestational diabetics. **Methods:** This clinical trial was conducted from Jan 2018 to Feb 2019 in the Department of Pharmacology, Basic Medical Sciences Institute, in collaboration with the Department of Obstetrics and Gynaecology, Jinnah Postgraduate Medical Centre, Karachi. Out of 136 high-risk females, 83 confirmed gestational diabetics were enrolled in second trimester and were further randomised into Group A (oral Metformin therapy) and Group B (subcutaneous Insulin therapy). They were followed till term and after delivery. Collected placentae (35 in each group) were subjected to gross, microscopic and detailed stereological study. **Results:** Placental weight on gross, hypoxic changes such as immature villi, chorangiosis and syncytial knots on light microscopic morphology, placental volume, the total volume of placental villi and total volume of foetal connective tissue on stereology were significantly more in Insulin-treated diabetic placentae whereas the mean villi diameter and mean morphometric diffusion capacity was significantly more in Metformin-treated placentae. **Conclusion:** Metformin-treated placentae were significantly different as compared to the insulin-treated placenta. Metformin treated placentae showed better morphometric diffusion capacity for oxygen than insulin treated placentae.

Keywords: Gestational diabetes mellitus, Insulin, Metformin, Placenta, Stereology

Pak J Physiol 2022;18(2):22–7

INTRODUCTION

Stereology is the study of tissues that impart practical lengths, along with densities for obtaining numbers of important structures in microscopic sections.[1] This system works with an applicable approach, usually composed of a grid of points or lines, applied to the area required to estimate numerical evaluation and area densities of biological structures.[2,3]

Gestational diabetes is the raised sugar level in maternal blood (FBS >100 mg/dl, RBS >126 mg/dl) as gestational hormones decrease insulin effectiveness, causing multiple maternal and foetal complications.[4] Placenta performs many important functions compulsory for the growth of the foetus. High maternal glucose level causes histological alterations in the placenta such as augmented number of vessels in villous (chorangiosis), distorted development of villi, syncytial knots, areas of fibrosis and ischemia and also on stereological details, increased surface area, total volume, the volume of inter-villous space and terminal villi.[5] These modifications affect placental physiology.[6] Furthermore, these placental morphological changes are responsible for the decrease in the oxygen delivery to the foetus due to a relative increase in size and amount of villous and vascular sections, with the increase in thickness of the basal membrane and multiple cellular depositions.[7]

Thus, Gestational diabetes mellitus demands early diagnosis and suitable treatment to reduce the feto-maternal problems coupled with it.[8] Inject-able Insulin was considered to be the best but; due to anabolic effects of insulin, multiple changes in placental morphology are documented with it with unfavourable gestational outcomes. Another option is the oral drug Metformin, which provides better compliance with added valuable effects as indicated by improved gestational consequences. However, whether Metformin compared to Insulin produces less hypoxic changes in the placenta on stereology was still left to be addressed. This study was conducted to evaluate and compare the stereological details of the placenta of mothers given Metformin *vs* Insulin for GDM.

METHODOLOGY

This clinical trial was carried out from Jan 2018 to Feb 2019 at Basic Medical Sciences Institute, in collaboration with the Department of Obs/Gyn, Jinnah Postgraduate Medical Centre (JPMC) after approval from the Institutional Review Board, JPMC, Karachi and BASR, University of Karachi. The study was further registered with clinicaltrial.gov (NCT number: NCT04907708).

The Sample size was calculated using 'Comparing of Two Means' on www.Openepi.com by a reference article with a mean difference of surface size of placental morphology, at the power of 80% and confidence level of 95%, the minimum sample size came out to be 23 in each group (raised to 35).[9]

After written informed consent, induction of diagnosed GDM patients was done as per WHO criteria (FBS >100 mg/dl, RBS >126 mg/dl) and out of 136 high-risk females, 83 confirmed GDM patients were enrolled. They were further randomised to Group A, 42 GDM patients were prescribed oral Metformin therapy (500 mg, TDS with dose escalation therapy) along with adjuvant diet control Whilst 41 patients in Group B were prescribed subcutaneous Insulin (0.8–0.9 IU/kg/day) with a strict nutritional check. They were monitored till the term and, after delivery, a comprehensive stereological study was done on saved placentae.[10]

Data were evaluated for 35 placentae in each group who were able to complete the study. After gross examination, histological slides were made for light microscopy. These slides were analysed on a light microscope for villous immaturity, chorangiosis, infarction, ischemia, villous fibroid necrosis, nucleated foetal RBCs, calcification and syncytial knots.

For stereology, placentae were assessed for volumes of components using a Nikon microscope (Eclipse 50i) attached to the DS-camera control unit DSL2 (M380E). Ten randomly selected microscopic fields were taken from two selected slides either from 12 O'clock, 6 O'clock and Centre positions from each placenta. The microscope stage was blindly and randomly displaced for the stereological assessment using a point-counting method to reveal the volume of placental components.[11]

From the slide, five sections were studied in detail (a total of 350 sections in each group). A 100 squared grid with 1 Cm of each square, was overlaid on the microscopic fields and for density of placental structures, intersecting points of the grid lying on these placental structures were counted. Volume density of each component (villi, inter-villous space, foetal capillaries and foetal connective tissue) was obtained by: Volume Density of placental component (Vd)= Number of intersecting points of grid observed on the object of focus/Total number of points used in the relevant grid section[11]. (Figure-1, 2).

The placental volume was estimated (V=W/D)[10] with the density of 1.05−3 gm and total volume for all placental components (villi, inter-villous space, foetal capillaries and foetal connective tissue) was calculated. (Vd×V)

The diameter of the villi and the foetal capillary was measured through the measuring scale available inside the computer and microscope. The Surface area of placental villi (SA villi) and capillaries (SA cap) was calculated by the intersection counting method for which a horizontal line grid was used.

SA villi=2×No. of Intersections of horizontal lines made by villi (I_V)/Lt)
SA cap=2×No. of Intersections of horizontal lines made by foetal capillaries (Ic/Lt)

where 'I' is the mean of villi and capillary intersections with horizontal lines whereas 'Lt' is the total length of grid horizontal lines.[11] (Figure-3).

Orthogonal intercepts are the horizontal or vertical lines perpendicular to the villous membranes, were also determined using a similar square grid. Arithmetic mean and the harmonic mean was calculated and for random plane sectioning, the harmonic mean was finally multiplied with 0.848.[12] (Figure-4).

The mean of diffusion capacity for oxygen across the villous was calculated as:

Mean diffusion capacity (villi)=MDC (villi)=Surface area of exchange (S.A v+S.A cap)×Krogh's constant for O_2 (2.3×10^{-8})/2×Harmonic mean thickness (VM)

This represents almost 90% of the diffusion capacity of oxygen across the placental membranes.[13]

Collected data were analysed and statistical tests were applied accordingly for categorical and numerical values, and $p<0.05$ was considered significant.

RESULTS

The patients enrolled were similar except for random blood sugar levels ($p=0.00$) (Table-1).

The gross examination revealed that the size, length, width and breadth of the placenta across the two groups was non-significant and the only mean weight of the placenta was statistically different between the groups. ($p=0.00$) (Table-2).

On light microscopic examination, significantly more villi were immature, more chorangiosis and Syncytial knots in group B placentae were observed. (Table-3)

On stereological comparison, group B placentae were significantly more in total volume ($p=0.02$), the total volume of foetal connective tissue ($p=0.04$) and the mean volume of villi ($p=0.05$). The mean diameter of villi was more in Group A placentae ($p=0.05$) and the MMDC was significantly different between the groups with better values in Group A ($p=0.04$) (Table-4).

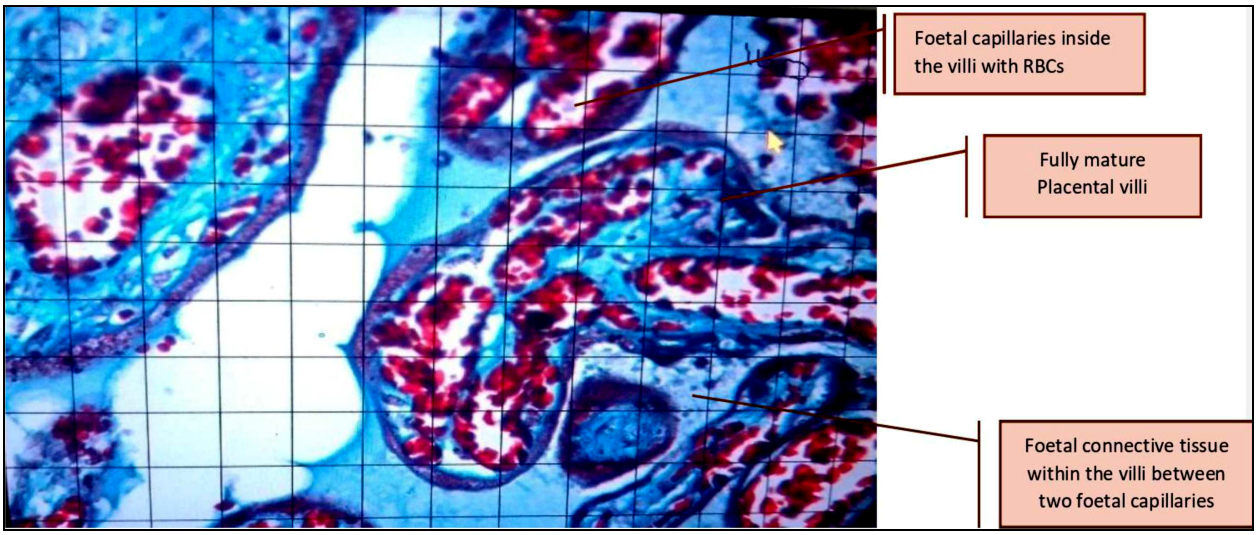

Figure-1: Grid imposed on a histological section of Metformin treated placenta. Intersecting points are counted on different components of placenta. ×40

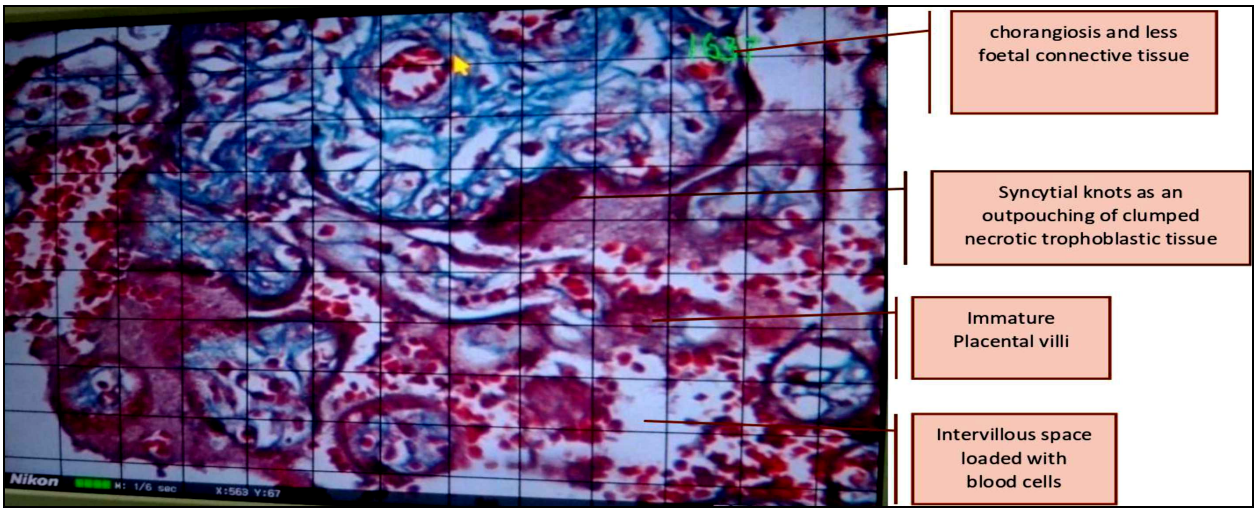

Figure-2: Grid imposed on a histological section of insulin treated placenta. Intersecting points are counted on different components of placenta. ×40

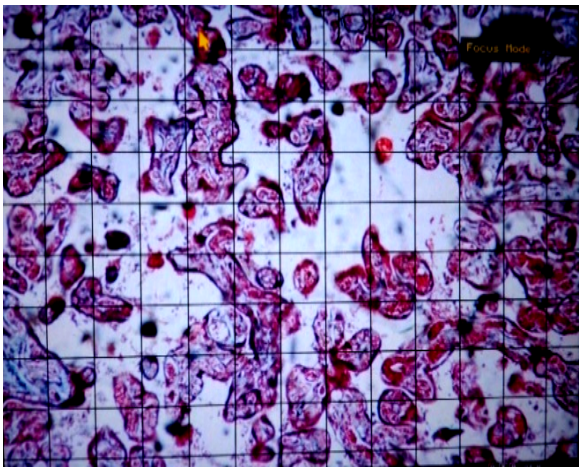

Figure-3: The section of placenta for intersection counting in horizontal lines ×10

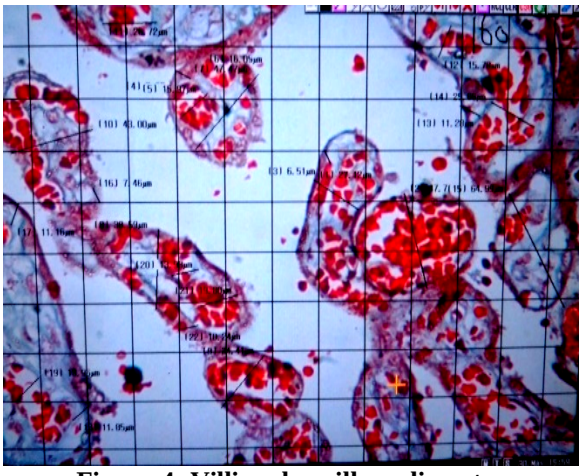

Figure-4: Villi and capillary diameter measurement

Table-1: Maternal characteristics (Mean±SD)

Characteristics	Group A n=35	Group B n=35	*p*
Age (years)	31.05±3.97	31.74±4.00	0.47
Weight (kg)	81.53±10.53	77.5 ±8.43	0.07
Height of the fundus (weeks)	31.31±2.77	30.97±2.85	0.61
FBS-1 (mg/dl) (>100mg/dl)	107.65±13.57	115.25±25.42	0.12
RBS (mg/dl) (>126mg/dl)	163.34±35.29	223.62±68.94	0.00*
HBAIC_1 (5.5–6.5) (%)	5.32±0.43	5.44±0.31	0.17

*Statistically significant (independent *t*-test applied); FBS: fasting blood sugar, RBS: random blood sugar; HBAIC 1: Glycated sugar at enrolment

Table-2: Placental gross morphological features (Mean±SD)

Characteristics	Group A n=35 Mean±SD	Group B n=35 Mean±SD	*p*
Placental surface size 1cm	15.25±2.58	16.22±3.2	0.16
Placental surface size 2cm	13.71±2.35	13.74±2.3	0.95
Placental width (cm)	2.3±0.59	2.37±0.68	0.64
Placental weight (gm)	626.85±115.0	712.28±110.56	0.00*

*Statistically significant (independent *t*-test applied)

Table-3: Microscopic morphology of placenta [n (%)]

Microscopic Examination	Group A (n=35)	Group B (n=35)	*p*
Villous immaturity			
Present	13 (37.1)	24 (68.5)	0.00*
Not present	22 (62.8)	11 (31.4)	
Chorangiosis			
Present	8 (22.8)	22 (62.8)	0.00*
Not present	27 (77.1)	13 (37.1)	
Infarction			
Present	10 (28.5)	10 (28.5)	>0.9
Not present	25 (71.4)	25 (71.4)	
Villous fibrinoid necrosis			
Present	23 (65.7)	26 (74.2)	0.43
Not present	12 (34.2)	9 (25.7)	
Nucleated RBCs			
Present	0 (0)	1 (2.8)	NA
Not present	35 (100)	34 (97.1)	
Ischemia			
Present	6 (17.14)	5 (14.2)	0.74
Not present	29 (82.8)	30 (85.7)	
Calcification			
Present	15 (42.8%)	8 (22.8%)	0.07
Not present	20 (57.1%)	27 (77.1%)	
Syncytial knots			
Present	10 (28.5)	20 (57.1)	0.01*
Not present	25 (71.4)	15 (42.8)	

*statistically significant (chi-square applied),
NA: Chi-square not applicable due to less cell count

Table-4: Stereological measurements group A *vs* B

Parameters	Group A n=35	Group B n=35	*p*
Villi volume density (gm/Cm^3)	55.59±11.15	55.79±9.75	0.93
Inter-villous space volume density (gm/Cm^3)	48.73±10.66	46.59±11.04	0.41
Foetal capillaries volume density (gm/Cm^3)	15.12±5.7	18.56±22.14	0.37
Foetal connective tissue volume density (gm/ Cm^3)	38.92±7.74	37.00±9.21	0.34
Placental volume (Cm^3)	597.51±108.14	659.72±118.52	0.02*
Total volumes of placental villi (Cm^3)	312.23±88.35	349.09±65.85	0.05*
The total volume of the Inter-villous area (Cm^3)	274.54±84.77	283.52±76.26	0.64
The total volume of foetal capillaries (Cm^3)	82.52±45.25	94.40±22.91	0.17
The total volume of foetal connective tissue (Cm^3)	229.48±62.04	256.79±50.44	0.04*
Mean villi diameter (Cm)	55.05±10.51	49.44±12.88	0.05*
Mean foetal capillary diameter(Cm)	18.49±6.98	20.49±7.58	0.25
Villous surface density (gm/ Cm^3)	9.11±3.38	9.20±2.79	0.90
Capillary Surface density (gm/ Cm^3)	8.32±2.71	8.44±2.44	0.85
Harmonic mean thickness of Villous membrane (Cm)	8.03±2.67	8.42±2.02	0.55
Corrected harmonic mean diameter (Cm)	6.7±2.17	6.98±2.25	0.59
Morphometric diffusing capacity (Cm^2.min-1. kpa1)	3.66±1.43	3.02±1.15	0.04*

Group A: Metformin with diet control group= diabetic pregnancies
Group B: Insulin with diet control group = diabetic pregnancies
*statistically significant (students *t*-test applied)

DISCUSSION

Stereology is an elaborated study to provide the quantitative assessment of densities and volumes of different important elements such as villi, inter-villous space, foetal capillaries and foetal stroma.[14] Various volumes of placental elements indicate a thorough interpretation for the exchange of gases between the foetus and placenta.[15] The malformation of these components, increase in the thickness of the endothelial cells and thickening of basal membranes of the trophoblast can lower the oxygen diffusion across the placental bed.[16]

Hypoxia leads to development of many free radicals and super-oxides that are responsible for placental cell damage and apoptosis. Later on this leads to serious pregnancy outcomes.[17]

When we compared the two group placentae grossly, it was revealed that the placentae were significantly heavier in the Insulin-treatment group. Different studies have documented that the placenta of patients treated with insulin was significantly heavier compared to normal placenta.[18] Our study results reveal that insulin-treated placentae showed morphology with more hypoxic changes. Another study declared that the villi in the Insulin-treated placenta were significantly showing hypoxic morphological changes with immature villi and syncytial knots formation. Even with more weight and larger sizes of the placenta, the foetal maternal outcomes in the Insulin-treated patients were not satisfied thus confirming the fact that immature villi and more degenerative morphology can lead to adverse outcomes in Insulin-treated patients.[19] It is indicated that the placentae of Insulin-treated mothers had significantly higher volumes, due to the anabolic action of Insulin along with multiple growth mediators released to compensate for the hypoxic environment in these placentae. This fact has already been documented

that the terminal villous volume was significantly more in GDM, compared to the control group.[7] In another study, it was observed that the diabetic placenta has significantly more inter-villous and blood vascular volume when compared to the control group.[20] However, none of these studies has provided stereological comparative differences in details of placenta with metformin *vs* insulin treatment. A study[21] on Insulin-dependent patients showed placental stereology resulting in significantly increased placental weight, placental volume and villi volume and is similar to our results. An increased volume of villi and foetal connective tissue can be related to compensating for the chronic foetal hypoxia. Recently, a suggested hypothetical model for the insulin-treated diabetic placenta showed that intrauterine hypoxia increases surface area for oxygen exchange by increasing the volume of villi. But these villi lack proper maturity and are unable to compensate for the increased oxygen demand of macrosomic babies leading, at times, to unexplained intrauterine deaths.[22]

Our study results indicated that the placentae in cases treated with Metformin had significantly more dilated, fully mature maternal villi. This shows better maternal blood flow due to Metformin's favourable vasculo-protective effects which helps in free and maximum delivery of oxygen to the growing foetus. The less volume of inter-villous space in these placentae also favours the more rapid entry and delivery of oxygen to the baby. This was further proven when MMDC was calculated and was significantly more when compared with placentae of insulin-treated cases. MMDC is a diffusive conductance and can be calculated by evaluating stereological estimates of placental structural quantities. This approach has proved valuable in a variety of comparative studies.[23]

There was a significant increase in placental weight, placental volume, volume of the inter-villous space and the trophoblasts in the diabetic group with a significant reduction in the villous membrane specific diffusing capacity between the diabetic *vs* control groups, though we were unable to find any stereological studies for the metformin-treated placentae alone or with insulin. Jauniaux study results were just similar to our results for insulin-treated diabetics for GDM, indicating that reduction in the diffusing capacity of the villous membrane surely contribute to the foetal hypoxia and increased foetal and neonatal morbidity in insulin-treated diabetics.[20]

A recent meta-analysis on 4,533 women from 23 clinical trials has also confirmed that metformin has shown the most promising results in successfully controlling glycaemia and preventing neonatal complications in GDM patients as compared to other pharmacological interventions, and one of the probable reasons along with good glycaemic control could be its vasculo-protective beneficial effects on the placental tissues.[24]

CONCLUSION

Metformin-treated placentae had mature villi with significantly more mean villi diameter and mean morphometric diffusion capacity for oxygen, making it a better alternative for gestational diabetes.

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Received: 18 Mar 2022 **Reviewed:** 5 Jun 2022 **Accepted:** 8 Jun 2022

Contribution of Authors:
RA: Conceptualization, and designed the study, collected data, and did main write up of manuscript.
FA: Analysed and interpreted data, drafted manuscript reviewed proofread final manuscript.
AS: Reviewed literature, reviewed and proofread final manuscript.

Conflict of Interest: None
Funding: None to declare