

ORIGINAL ARTICLE

THE METABOLIC EFFECTS OF METFORMIN ON HUMAN UMBILICAL VEIN ENDOTHELIAL CELLS ISOLATED FROM THE UMBILICAL CORD STUMPS OF NEWBORNS

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ABSTRACT:

Introduction: Metformin has been a mainstay of type 2 diabetes treatment for decades. The medicine makes the usage of cell energy better; reduces the cellular oxidative stress and alters the way how placental cells produce energy at a basic level.

Objective: This study focused to determine the impact of metformin on the metabolism and mitochondrial function on human umbilical vein endothelial cells obtained from umbilical cord stump of newborn.

Methods: The study was conducted at Saidu Medical College, Swat from Jan 2025 to March 2025. Human Umbilical Vein Endothelial Cells (HUVECs) from the stump of umbilical cord were collected from 50 normal women who underwent elective caesarean sections at full term or had a normal spontaneous vaginal delivery. For all experiments in the lab the Primary HUVECs collected were separated and isolated by conducting a method involving DNase enzyme treatment and centrifugation which is a specialized process to isolate specific cells. The isolated cells were then placed in a special culture medium at different cell densities where they were allowed to develop into Cultured HUVECs. These cells were grown in an environment having 5% carbon dioxide and normal oxygen levels at 37 °C for four days before the treatment with metformin. After which cultured cells were treated with metformin for one day.

Results: RNA from Cultured HUVECs extracted and quantified. Confirmation of all samples was done by Agarose gel electrophoresis. It was found that 18S and 28S rRNA bands showed no signs of degradation and were sleek and uncluttered. Then cDNA was generated by reverse transcription of RNA and assayed by RT-PCR. Number of copies of gene were measured from known amounts of human DNA as reference.

Conclusion: Metformin administered at prescribed doses during gestational period has no statistically significant effect on the fetus.

Keywords: Metformin, Newborns, Pregnancy, Umbilical vein endothelial cells,

INTRODUCTION:

Women with Type 2 Diabetes in pregnancy who are taking metformin, show two important advantages. Firstly, it controls blood glucose levels and secondly it decreases the expected maternal gain in weight.¹ It is further being studied whether Metformin has any role in treating pre-eclampsia during gestation because the disease can threaten both the mother and child. Metformin is also used as a drug of choice in polycystic ovarian disorders.²

It has been seen that the rate of metformin prescription for gestational diabetes is climbing worldwide,

and its therapeutic advice for treating PCOS and related conditions is also rising globally.^{3,4} More pregnancies treated means more fetuses are likely to be exposed to this medicine.⁵

Many studies show that metformin does not only stay in maternal blood but also crosses placenta and reaches the blood of fetus in uterus.⁶

Placenta is highly metabolically active organ and the role of metformin is still not fully understood and has yet to be studied and explored.⁷

Metformin has been a mainstay of type 2 diabetes treatment for decades. It has multiple mechanisms of actions. The medicine makes the usage of cell energy better; reduces the cellular oxidative stress and alters the way how endothelial cells produce en-

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Received: 02 Apr 2025; Revised: 25 May 2025; Accepted: 04 Oct 2025

ergy better; reduces the cellular oxidative stress and alters the way how endothelial cells produce energy at a basic level 8. It may increase the activity of GLP-1 which improves glucose control.⁹ As far as the effects on maternal hormones is concerned, metformin increases sensitivity of insulin at cellular level and increases adiponectin hormone by adipose tissues which in turn improves the sensitivity of insulin. Women who have insulin resistance or PCOS, metformin may reduce androgen levels. The medicine tends to suppress the levels of glucagon on hepatic glucose production.¹⁰

The placenta continuously regulates and controls whatever reaches the fetus, and the role of metformin on the fetus still remains incompletely established.¹¹

Studies show that the mothers who took metformin during their gestational period and had well controlled blood glucose throughout pregnancy delivered babies with low birth weight. This establishes the fact that the drug may be the cause of slightly smaller than the average baby in utero rather than uncontrolled diabetes mellitus.¹²

Further studies proved that after birth these babies grew unusually faster than the others. These babies then were found to have higher rate of accumulation of body fat and become overweight in childhood.¹³ There is evidence, though, that assures that not all fetuses who are exposed to metformin in utero will become obese later in life because the life style and food also is considered.^{10,13} It is suggested that because metformin reduces the levels of ATP via its metabolites, placenta cannot transfer the required nutrients to the fetus and this slow nutrient delivery over the gestation period leads to low birth weight of the newborn.¹⁴ Studies are being done to determine the effects of metformin on the overall devel-

opment.

Aim of study: This study focused to determine the impact of metformin on the metabolism and mitochondrial function on human umbilical vein endothelial cells obtained from umbilical cord stump of newborn.

METHODS

The study was conducted at Saidu Medical College, Swat from January 2025 to March 2025.

Human Umbilical Vein Endothelial Cells (HUVECs) from the stump of umbilical cord of healthy neonates were collected from 50 normal women who underwent elective caesarean sections at full term or had a normal spontaneous vaginal delivery. The mothers who were participants had normal clinical profiles that broadly represented the general pregnant population. All of the women met the inclusion criteria. Verbal informed consent before the delivery was taken and study was fully explained to the participants. Immediately after delivery, the tissues were washed with sterile saline to remove maternal blood, placed in leak proof biohazard bags and packed in insulated shipping containers with wet or dry ice to maintain temperature. All of the placentas were transferred immediately to the laboratory for processing and stored at -80 degrees Celsius.

Only those women were included in the study who did not have diabetes mellitus throughout the gestational period. Those with anomalies of fetus, multiple pregnancies, severe pre-eclampsia, or any form of diabetes during pregnancy were excluded, as were women who delivered before 37 completed weeks or had taken metformin at any point during the pregnancy. Demographic characteristics of the study population are shown in Table 1

Table 1. Demographic characteristics of the study population

Variable	Mean (IQR) or n (%)
Mother's age, years	35 (31–37)
Mother's BMI, kg/m ²	29.1 (24–31)
Gender of newborn	
Female	31 (53)
Male	26(46)
Mother's BMI category	
BMI ≤ 25 kg/m ²	21 (35)
BMI > 25 kg/m ²	36 (65)
Weight of placenta, g	618.1 (541–679)
Weight of newborn, g	3638.1 (3266–3873)
Weight at birth, centile	73 (51–93)

Abbreviations: BMI, body mass index; IQR, inter-quartile range.

Note: IQR= Continuous variables are presented as median

n (%) =categorical variables

Culture of primary umbilical vein endothelial cells from the stump of umbilical cord.

For all experiments in the lab the Primary HUVECs collected from the stump of newborn were separated and isolated by conducting a method involving DNase enzyme treatment and centrifugation which is a specialized process to isolate specific cells.

The isolated cells were then placed in a special culture medium at different cell densities where they were allowed to develop into Cultured HUVECs. These cells were grown in an environment having 5% carbon dioxide and normal oxygen levels at 37 °C for four days before the treatment with metfor-

min. After which cultured cells were treated with metformin for one day (from day 4 to day 5) in concentrations (metformin at either 0.01 mmol/L or 0.1 mmol/L for 24 hours) which are therapeutically prescribed to diabetic pregnant ladies. 15. The cells were collected after 120 hours of the treatment.

On the other hand, the control cells received only deionized water in the volume (1:1000 vol/vol). Protocol was followed as described in another approved study¹⁵. The culture medium was replaced every 24 hours.

Assessment of Mitochondrial Function

The Cultured HUVECs were detached from the culture dish by using an enzyme, TrypLE. The collected cells were centrifuged and resuspended in the mitochondrial respiration medium (miR-05) to assess the function of mitochondria. The methodology was followed from a previous published study.¹⁶ To measure mitochondrial oxygen consumption, the cell suspension was transferred to an instrument (Oxygraph-2K) which continuously stirred the cell suspension at 37 °C. To make cell membrane more permeable, digitonin (10 µg/ml) was added, which allows the experimental substrates and inhibitors to directly interact with mitochondria.

To monitor oxygen consumption and for the assessment of mitochondrial energy production, a series of chemical reactions were done. To determine basal mitochondrial respiration, pyruvate (25 mol/l) and malate (2 mol/l) were added. In order to start oxidative phosphorylation ADP (10 mol/l) was introduced and thus active ATP production was started. Later, to evaluate the mitochondrial complex I activity, glutamate (10 mol/l), which is an important component of electron transportation, was also added. The integrity of mitochondrial membrane was tested by the addition of Cytochrome c (0.01 mol/l).

If the oxygen consumption did not rise after the addition of Cytochrome c, it indicates that there is no damage of the membrane and there is no leakage. Rotenone (0.5 $\mu\text{mol/l}$) was then introduced to further verify that complex I activity genuinely increased the oxygen consumption. Data generated was managed, processed and analyzed using commercially available software.

Analysis of glucose and lactate concentrations were done. Cultured HUVECs metabolism and the test of mitochondrial stress were followed according to a previously published protocol.¹⁶

Cultured HUVECs Lipid Content Identification

Cultured HUVECs were put in solution containing 4% paraformaldehyde(buffered) to fix them and processed for five minutes with 1 $\mu\text{g/mL}$ solution of Nile Red for staining at 37 °C in dark. Fluorescence was quantified by fluorescence spectroscopy.

A liquid-liquid extraction protocol¹⁷ was used for the extraction of lipids from Cultured HUVECs which were then homogenized in chloroform-methanol. Before the second round of mixing and spinning, internal standards (isotopically labeled for lipid and acylcarnitine quantification) were spiked into each sample. The organic phase was then isolated and dried under nitrogen. Reconstitution in propan-2-ol-acetonitrile-water preceded LCMS analysis.

Mass spectrometry was employed to quantify the amounts of target lipid and acylcarnitine. Calibration was performed immediately before every run.

RNA isolation and Gene expression profiling

RNA from Cultured HUVECs extracted and quantified by a specified spectrophotometer as detailed

previously¹⁸. Confirmation regarding the integrity of all samples was done by Agarose gel electrophoresis. It was found that 18S and 28S rRNA bands showed no signs of degradation and were sleek and uncluttered. Then cDNA was generated by reverse transcription of RNA and assayed by RT-PCR used as described previously¹⁸. Number of copies of gene were measured from known amounts of human DNA as reference. In all experiment groups, the reference gene B2M, was found steady. Gene expressions were consistent, thus, confirmed that RNA input was uniform and cDNA synthesis was reliable throughout.

Protein expression profiling

Extraction of proteins from Cultured HUVECs was performed using a previously described method.¹⁹ 20 μg of the extracted protein per lane were loaded onto the 10% polyacrylamide gel and then transferred to PVDF membranes. Incubation of PVDF membranes with target-specific primary antibodies was carried out. Later, incubation was performed with secondary antibodies (anti-rabbit). Positive and negative controls were used to validate the specificity all antibodies which were run in parallel. Chemiluminescence technique was used to detect the bands of proteins. To quantify the expression levels of extracted proteins from Cultured HUVECs a densitometer was used.

Statistical analyses

Data was analyzed by Multilevel mixed-effects linear regression model because the data was grouped and related. The different treatment groups were included as a fixed effect, (measuring and comparing the effects of these treatments) and the cells from which each biological sample was obtained was included as a random effect. This help to contr-

of the natural cell variations that was unrelated to the treatments.

Each biological sample served as its own control when comparing treatments. Since all samples went through same experimental conditions, therefore, random allocation to treatment groups was not needed (repeated -measures study design). Data was analyzed using R software (version 4.2.2). Values <0.05 were considered to be statistically different.²⁰

RESULTS

Effect of Metformin on Cultured HUVECs Respiratory Function. Culture cells which were exposed to metformin showed low oxygen consumption, which suggests reduction in oxidative phosphorylation. However, the oxygen use by the cells in different concentrations of metformin were different (more at 0.1 mmol/L). Similarly, respiration which was linked to complex I also showed a significant decrease in percentage. The outer cell membrane of mitochondria remained intact throughout the experiment. This proved that the low energy in Cultured HUVECs was due to Complex I inhibition.

Glycolytic rate and capacity

When metformin inhibits oxidative phosphorylation, the Cultured HUVECs adapt by increase in glycolysis and thus the levels of glucose and lactate

rise. However, increased glycolysis may lower the energy reserve capacity and the cells do not respond well at high energy demands. It was observed that most of the genes involved in glycolysis remained unaffected. Metformin suppresses the genes involved in pyruvate dehydrogenase activity and so pyruvate converts into lactate and is used for energy production in mitochondria. Thus, Cultured HUVECs rely more on glycolysis rather than on mitochondrial energy production. Table 2

Lipid uptake and oxidation

Metformin suppresses the breakdown of lipids in Cultured HUVECs and increases the storage within the cells. Triglycerides (specifically with, long chain polyunsaturated fatty acids) and all other forms of lipids showed intracellular accumulation and thus helpful in the growth and brain development of the fetus. The oxidation of fatty acids is decreased by metformin. Thus, less fat is burnt and accumulates in the cells. Metformin also upregulates fatty acid transport proteins FATP1, FATP2, and FATP4 at higher concentrations, Table 3

Table 2. Outcome found on gene expression of placental hexokinase isoforms and glucose transporters in human Cultured HUVECs associated with Metformin

Gene	0.1 mmol/L Metformin (Copy number $\pm$ SE)	p value	n	0.01 mmol/L Metformin (Copy number $\pm$ SE)	p value	n
HK1	15,125 $\pm$ 1354	<0.05	29	21,372 $\pm$ 1698	<0.05	31
HK2	4095 $\pm$ 627	<0.05	31	5062 $\pm$ 965	<0.05	29
HK3	11,527 $\pm$ 1897	<0.05	30	12,994 $\pm$ 2507	<0.05	30
HK4	1262 $\pm$ 519	<0.05	31	2370 $\pm$ 997	<0.05	30
GLUT1	191,669 $\pm$ 47,185	<0.05	29	170,660 $\pm$ 34,412	<0.05	30
GLUT3	3489 $\pm$ 557	<0.05	31	3332 $\pm$ 545	<0.05	31
GLUT4	1239 $\pm$ 350	<0.05	29	2338 $\pm$ 739	<0.05	29

Results are shown as copy number $\pm$ standard error (SE).

n expresses the number of biological replicates. p values: A mixed-effects linear regression model was used for all statistical comparisons. Treatment group was entered as a fixed factor; placenta of origin was treated as a random factor to account for inter-cellular variability. The vehicle-treated group served as the reference throughout.

Table3

Effect of metformin on gene expression of fatty acid

transporters and endothelial lipase

Data: copy number (mean $\pm$ SE).

n: number of Cultured HUVECs in each treatment group, (all samples underwent two independent analysis).

p values Comparison was made in the analysis of each treatment group with the vehicle treated (control) group and assuring that samples came from different Cultured HUVECs

Abbreviations: FATP = fatty acid transport protein; LIPG = endothelial lipase; SE = standard error.

Gene	0.1 mmol/L metformin (Copy number, mean $\pm$ SE)	p value	n	0.01 mmol/L metformin (Copy number, mean $\pm$ SE)	p value	n
FATP1	3820 $\pm$ 563	<0.01	31	3541 $\pm$ 560	<0.01	32
FATP2	3195 $\pm$ 313	<0.01	29	2611 $\pm$ 450	<0.01	28
FATP3	2884 $\pm$ 358	<0.01	29	2789 $\pm$ 361	<0.01	31
FATP4	8367 $\pm$ 866	<0.01	33	11,003 $\pm$ 849	<0.01	31
FATP5	811 $\pm$ 198	<0.01	33	675 $\pm$ 139	<0.01	31
FATP6	29,213 $\pm$ 3548	<0.01	31	34,563 $\pm$ 4671	<0.01	33
LIPG	8353 $\pm$ 725	<0.01	30	8011 $\pm$ 1024	<0.01	30

DISCUSSION

In vitro impact of metformin- treated Cultured HUVECs derived from stump of newborn is being studied since long. Metformin decreases the oxidative phosphorylation of the mitochondria. The genes which are responsible for the glycolysis are not affected by metformin. However, the glycolysis increases to provide energy. The genes which control the pyruvate dehydrogenase enzyme is affected by the doses of metformin given therapeutically. When pyruvate dehydrogenase is low more lactate is formed to

Provide energy, But in the stressful conditions, the supply of energy is compromised due to metformin's affects on the metabolism of trophoblasts. However, the lipids, as triglycerides containing long chain polyunsaturated fatty acids and very long chain polyunsaturated fatty acids show a decrease in oxidation. This reduction of oxidation is because metformin induces a blockade of the chain of electron transport. Thus, the increase in these fatty acids leads to accumulation of lipids in the cells.²¹

Our study also supports the findings. Metformin also causes reduction in Complex I leading to rise in the process of glycolysis.²² The findings of our research work are also consistent with this result. Some studies show that Complex I is inhibited by metformin in other tissues.²² But this study was on intestinal cells rather than on human umbilical endothelial cells from the stump of newborn, thus differs from our study. Another study proved that metformin only inhibits Complex I at very high concentrations of metformin and not on the concentrations which are prescribed in gestational diabetes.²³ This is different from our work where only therapeutic ranges of metformin were used.

A meta-analysis of several studies concluded that the diabetic mothers who received metformin as a treatment for glucose control delivered babies with low birth weight as compared to women who received treatments other than metformin, even when the glucose levels of both groups remained normal and similar.^{24,25,26} Nevertheless, our study was only on the metformin-induced metabolic changes.

Another study supports that the use of metformin in gestational period to control diabetes has direct affect on fetus because it can cross placenta and directly affects the metabolism of mitochondrial metabolism by entering into the blood of fetus. The medicine then decreases the energy production through electron transport chain and disturbs the production of ATP leading to altered growth of fetus and birth weight below the normal.⁴ Whereas, our experiments were in vitro.

The limitations of our study were that it was a short-term study. In the experiments metformin was given to already Cultured HUVECs from the newborn umbilical cord stump. The results could have been

different if the cells were treated with the drug for a much longer time as throughout the whole period of gestation. Long term experiment could have revealed changes which were not found in this study. Additional studies are the required to determine what alterations may be possible to the growth and development of fetus if the drug is prescribed continuously to diabetic pregnant women.

CONCLUSION:

Metformin administered at prescribed doses during gestational period has no statistically significant effect on the fetus

DECLARATION OF INTEREST: The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS:

A.I.: Data collection, data analysis, literature review, write-up and references

A.G.: Conceptualization, proofreading, and final editing of manuscript

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