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Evaluating the effects of insulin, metformin and glibenclamide on the pups' prefrontal cortex and oxidative stress markers of streptozotocin-induced diabetic pregnant rats

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Abstract: There is an upsurge in gestational diabetes mellitus with many devastating consequences for the mother and developing fetus. Insulin therapy remains a mainstay. However, insulin is expensive and comes with the pain of multiple injections. Therefore, there is a need to explore commonly administered oral hypoglycemic agents to cater for the increasing gestational diabetes mellitus-associated neurological complications. This study assesses the effects of glibenclamide, metformin and insulin on the pups' prefrontal cortex in diabetic pregnant rats. 35 sexually matured adult female rats weighing between 120 g and 160 g were used and assigned into five groups (A to E) of seven rats each group. Diabetes was induced by streptozotocin (45 mg/kg and 35 mg/kg; ip). Hyperglycemic rats were treated with insulin (1.0 UI daily), metformin (200 mg/kg/day) and glibenclamide (0.6 mg/kg/day). Body weight and blood glucose levels were evaluated. Rats were sacrificed at 18-day gestation, the pups were harvested, and their brains were processed for tissue oxidative stress markers and various histological examinations. Glibenclamide and metformin caused a significant blood glucose reduction at 37.9% and 40.7%, respectively, compared to the insulin group (33.09%). There was no significant difference in the body-organ ratio in rats treated with metformin when compared to rats treated with insulin. Metformin and glibenclamide had a significant increase in tissue glutathione reductase and a decrease in malondialdehyde compared with insulin and diabetic control groups. The pups' prefrontal cortex showed degenerated neuronal cells in the diabetic control animals. The diabetic rats treated with metformin and glibenclamide showed improved pyramidal neurons compared with diabetic and insulin groups. This study suggests that metformin and glibenclamide glycemic control may prevent and improve antioxidant enzymes and reverse some neurotoxic effects caused by streptozotocin-induced diabetes in rats.

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Introduction

Diabetes mellitus (DM) is a severe metabolic disorder associated with cerebral alterations [1, 2]. DM is classified into type I or insulin-dependent, type II or insulin-independent, and gestational diabetes [3]. Gestational diabetes mellitus (GDM) is abnormal blood glucose levels that commence during pregnancy [4]. GDM complicates a considerable number of pregnancies, and the prevalence is one in every six births [5, 6]. So, GDM has become a public health issue of grave concern, with more devastating complications for the mother and the child [5, 6]. Several studies have reported various alterations to the brain structure, impairment of normal brain development and neurological deficits, especially in the cerebral cortex of GDM-affected mothers and offspring [7-9]. The mechanism for these neurological disorders was associated with free radical-induced oxidative stress, inflammatory responses, and dysfunction in the insulin-signaling pathway [7, 9]. With dire attempts and efforts to aggressively manage the GDM and its complications [10], it is necessary to ascertain the safety and efficacy of commonly administered oral hypoglycemic agents. The goal of managing GDM is to ensure proper glycemic control through dietary precautions and the administration of insulin or metformin [11, 12]. Though, most pregnant women have trypanophobia and that comes with multiple insulin injections. There are issues of possible risks of transplacental passage and subsequent congenital malformations with the use of oral hypoglycemic agents in pregnancy [13, 14]. Imperatively, a randomized control showed that metformin administration in pregnant women was safer and more tolerable than insulin without any complications [15]. Hence, these oral hypoglycemic agents are now considered better alternatives to insulin therapy during pregnancy due to their convenient administration, cost-effective, and pain-free [16, 17]. Glibenclamide, a second-generation sulfonylurea, represents one of the most widely administered oral hypoglycemic agents [18]. It stimulates insulin secretion and reduces hepatic glucose production, reducing blood glucose [19]. But, the use of glibenclamide is limited due to prolonged hypoglycemia, high secondary failure rate and other adverse events [20, 21]. Moreso, metformin is a commonly used oral anti-hypoglycemic agent, both as monotherapy and in combination with other agents, which reduces elevated blood glucose levels by reducing hepatic glucose output and by improving insulin resistance [22, 23]. Metformin has a lower risk of hypoglycemia than sulfonylureas [24, 25]. Against this backdrop, caution is needed as there is inadequate information to evaluate the risk of these agents in pregnancy [18, 26]. It is also well known to the researcher that there is still little information on the effects of metformin and glibenclamide on the cerebral cortex in pregnancy. Thus, this study assessed the comparative effects of metformin, glibenclamide and insulin on the cerebral cortex of dams and pups of streptozotocin-induced diabetic pregnant rats.

Materials and methods

Chemicals and hyperglycemic agents: Streptozotocin (STZ) was purchased from Sigma Aldrich Chemical, Lagos, Nigeria. Metformin and glibenclamide insulin were purchased over the counter at Zayo Pharmaceuticals, Lagos, Nigeria. Glibenclamide and metformin were ground into a fine powder and dissolved in distilled water before administering to rats. The doses of glibenclamide (0.6 mg/kg) and metformin (200 mg/kg) were chosen based on previously published study [27].

Animals: 35 sexually matured female Wistar rats obtained from the College of Medicine, University of Lagos's animal house Lagos, Nigeria were used. The rats weighing between 120 g and 160 g were housed in a standard well-ventilated metal cage in the rat control room of the Department of Anatomy, University of Lagos. They were maintained with 12-hour light and 12-hour dark cycles. The rats had access to food (rat chow, Livestock Feeds Plc.) and tap-water *ad libitum* except during overnight fasting before blood collection for fasting glucose estimate. Rats were acclimatized for two weeks and were divided into five groups (n=7). Thus, group A (non-diabetic

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control), B (diabetic control), C (insulin), D (metformin) and E (glibenclamide). Groups A, B, D & E were administered orally through metal oropharyngeal cannula, while group C was administered intraperitoneally to enhance insulin activity. All the laboratory protocols were done according to the standard guidelines of the University of Lagos, College of Medicine, Research Grants and Experimentation Ethical Committee (RGEEC).

Induction of experimental diabetes: DM was induced in groups B, C, D and E with STZ. STZ was dissolved in 0.1 mol/L of sodium citrate buffer (pH 4.5) [28], and injected intraperitoneally using a high dose of 45 mg/kg on the first day and a low dose of 35 mg/kg on the second day [29]. Only rats with blood glucose levels greater than 150 mg/dl were used in the study [30]. Glucometer (Accu-Chek Active, Roche Diagnosis, Germany) was used to determine the blood glucose level of the rats. Blood samples were obtained from the cut-tail tip of conscious rats.

Mating period and pregnancy: The female rats were mated with non-diabetes males of proven fertility. The mating occurred on the proestrus stage of the oestrous cycle, and the presence of sperms in the vaginal smear on the following morning was denoted as day 0 of the pregnancy [31].

Experimental procedure: Group A, non-diabetes control rats, received water throughout the experiment; group B, diabetes control rats, received water (0.5 ml); group C, diabetes rats, were treated with insulin (1.0 I.U./kg/day), and group D diabetes rats received 200 mg/kg/day of metformin period. All rats had their body weights and blood glucose measured and recorded daily throughout the experiment.

Euthanasia and sample collection: On the 18^{th} day of pregnancy, the rats were anaesthetized with pentane, followed by total body perfusion with 0.1 M phosphate buffer saline through transcardial perfusion and euthanised by cervical dislocation. The pups (foetal) were collected and examined for any morphological deformity, and after that, they were carefully dissected, and their brain was excised for various analyses.

Biochemical analysis: After the pup's brains were harvested, they were rinsed in PBL, and 0.5 g of the cerebral cortex was homogenized in 5.0 ml sodium phosphate with 01.0% triton with 01.0% Triton X-100 (50 mM; pH 7.5). The homogenates were centrifuged for 10 min at 20 000 g (4 ^{o}C). The supernatants were decanted into Eppendorf tubes, labelled, and used for oxidative stress analysis.

Determination of oxidative stress markers: The cerebral homogenates were used to measure the concentrations of reduced glutathione (GSH) [32], antioxidant enzymes superoxide dismutase (SOD) and malondialdehyde (MDA) were determined [33, 34].

Tissue processing for histology: The cerebral tissue collected and was carefully dissected out, trimmed of fats, and fixed in 10% formal saline. The fixed tissues were transferred to 70%, 90% and 100% ethanol for dehydration and then cleared in xylene. Once cleared, the tissues were infiltrated in molten paraffin wax in the oven at 60 ^{o}C. The tissues were subsequently embedded, and serial sections of 3 µm were obtained from a solid block of tissue using a rotary microtome. The sections were transferred into warm water at 40 ^{o}C. The floating sections were picked up with albumenized slides and allowed to dry on the hotplate. The slides were labelled, dewaxed, cleared with xylene and passed through two changes of 100%, 70%, 50% ethanol and water. The slides were stained with haematoxylin and eosin stains, and photomicrographs were taken at x100 magnification [35].

Statistical analysis: The data obtained were analyzed by one-way ANOVA to compare and find out the difference between the control and the experimental groups followed by post-hoc test to determine the statistical significance within groups. All analyses were done using Graph Pad Prism 8 (Graph pad, soft San Diego, CA 92108, USA). The P-value of less than 0.05 was considered significant and the data were expressed as the mean±SEM.

ISSN: 2789-1895 online
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Results

In **Table 1**, streptozotocin-induced diabetic rats (group A) showed a progressive rise in fasting blood glucose levels compared to the non-diabetic rats, who received water throughout the experiment. After 18 days of treatment, there was a significant reduction in the blood glucose level in the insulin-treated group C (78.5±9.63, 33.09%), while metformin-treated group D had a significant reduction in blood glucose of (114±35.13, 40.73%) when compared with insulin and glibenclamide. The glibenclamide-treated group had a reduced blood glucose of 82.07±24.53 (37.85%) with no statistically significant difference when compared with insulin-treated group C.

Also, as shown in **Table 1**, at the end of the treatment period, the diabetic rats treated with insulin gained a mean weight of 4.35±0.14 g (3.49%). The diabetic control rat (group B) lost a mean weight of 4.90±0.57 (4.20%), while the diabetic rats treated with glibenclamide (group E) lost a mean weight of 0.68±0.97 (0.53%). Interestingly, the treated group D had a moderate weight gain of 0.90 (3.17%) before and after the treatment period.

Table 1: Body weight and blood glucose levels of maternal diabetic rats

Group	Before treatment	Before treatment	After treatment	After treatment
n = 7	Blood glucose (mg/dL)	Body weight (g)	Blood glucose (mg/dL)	Body weight (g)
Control (A)	091.20±1.69	136.00±11.26	086.10±2.89	152.12±14.30
Diabetic (B)	164.00±13.44	136.04±6.89	247.00±8.09a	132.78±7.33[a]
Insulin (C)	237.20± 8.63	120.20±6.19	158.70±9.00[a,b]	124.55±6.05[b]
Metformin (D)	280.60±51.08	116.62±7.00	166.30±15.82[a,b]	120.52±6.57[b]
Glibenclamide (E)	216.80±38.93	126.08±10.00	134.73±14.40[a,b,c]	125.40±16.97[b]

Data presented in MEAN ±SEM, at P< 0.05 level of significance (ANOVA). [a]significant difference compared with control group, [b]significant difference from the diabetic group, [c]significant difference from the insulin group.

The effects of diabetes, insulin, metformin, and glibenclamide on pup's prefrontal cortex are shown in **Figure 1**. There was a significant decrease in GSH of diabetic control rats (group B) compared with the non-diabetic control (group A). Interestingly, the diabetic rats treated with metformin (group D) and glibenclamide (group E) had a significant improvement in GSH activity compared with diabetic control and diabetic rats treated with insulin (group C). Further investigation on MDA activity showed a significant increase in diabetic rats compared to the non-diabetic control group. Notably, the diabetic rats that received metformin and glibenclamide (groups D and E) had a significant decrease in the MDA levels compared with diabetic rats that received insulin and diabetic control rats.

Figure 2 shows pups' prefrontal cortex hematoxylin and eosin staining. The section of the pups' prefrontal cortex of the control group showed well-developed prefrontal pyramidal cells (**Figure 2A**). The diabetic group showed degenerated pyramidal cells (**Figure 2B**). The prefrontal cortex section of the group treated with insulin showed poorly stained pyramidal cells (**Figure 2C**). The diabetic group treated with metformin showed normal developing pyramidal cells compared with control (**Figure 2D**). The diabetic group treated with glibenclamide showed mildly congested pyramidal cells and vacuolation (**Figure 2E**).

ISSN: 2789-1895 online
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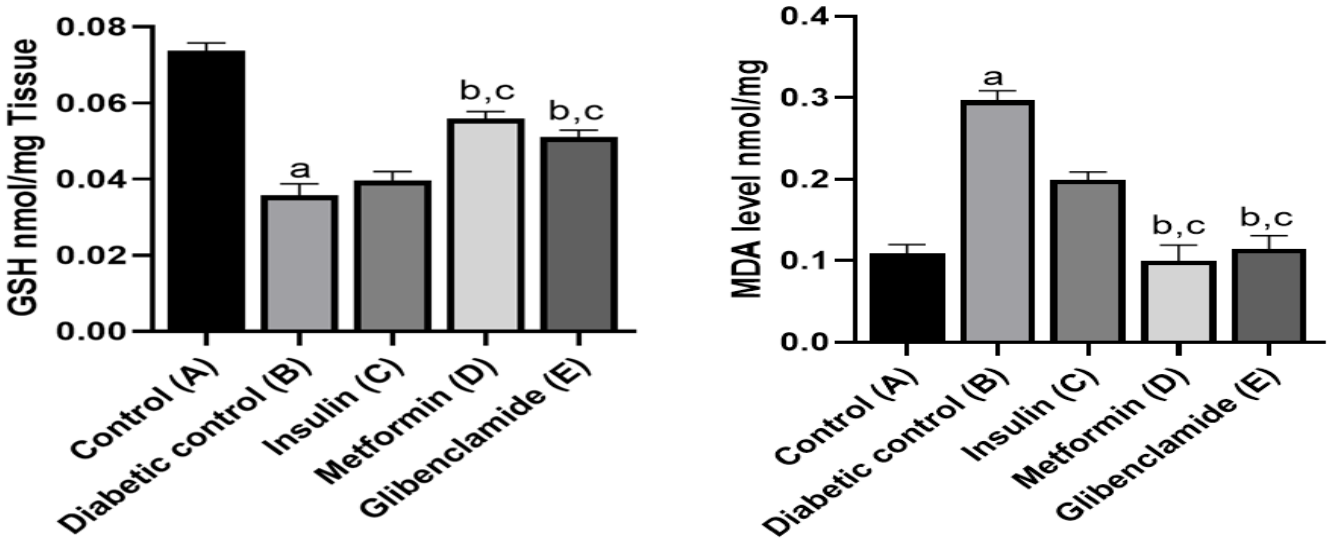
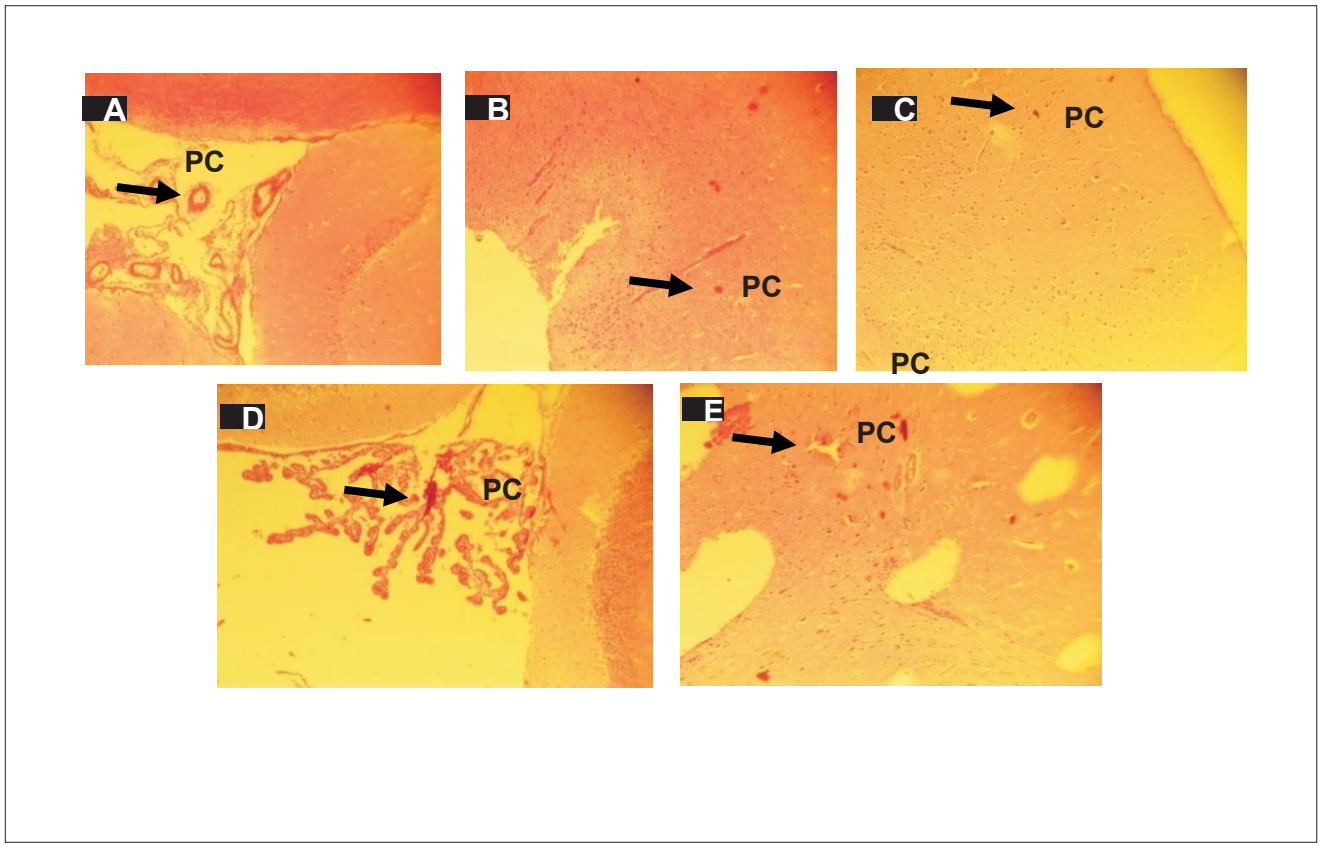
Figure 1: Effects of insulin, metformin and insulin on pups' prefrontal cortex oxidative stress markers



[a]P < 0.05 vs control (A), [b]P < 0.05 vs diabetic group (B) and [c]P < 0.05 vs insulin (C).

Figure 2: Photomicrograph of sections in the pup's prefrontal cortex

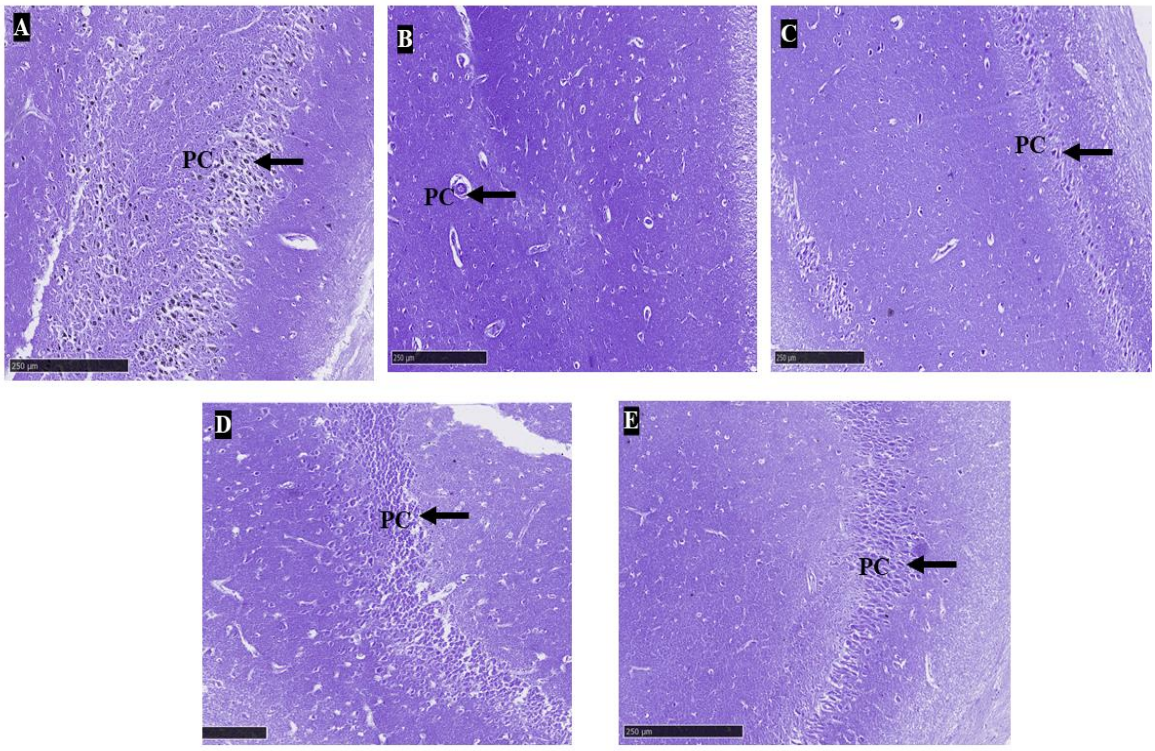


Group A demonstrates normal and well-developed pyramidal cell (arrow) (H & E; x100), group B showed a degenerated pyramidal cell (Arrow) (H & E; x100), group C demonstrates a mildly degenerated pyramidal cell (H & E; x100), group D demonstrates normal pyramidal cell (Arrow) (H & E; x100), group E showed mild congested pyramidal cells and vacuolated glial cell (GC) (H & E; x100).

ISSN: 2789-1895 online
ISSN: 2958-3101 print

Figure 3 shows pups' prefrontal cortex with Nissl staining of pyramidal neurons. The control group showed a cluster of pyramidal cells with a deep Nissl stain, as indicated by well-formed neurons (**Figure 3A**). The diabetic group showed degenerated pyramidal cells and vacuolated cytoplasm (**Figure 3B**). The prefrontal cortex section of the group treated with insulin showed a mild Nissl stain of the pyramidal nuclei, improved stained nuclei compared with diabetic control (**Figure 3C**). The diabetic group treated with metformin (**Figure 3D**) and glibenclamide (**Figure 3E**) showed some clusters of developing pyramidal neurons with deeply stained Nissl bodies.

Figure 3: Effects of metformin, glibenclamide and insulin on prefrontal cortex neuronal cells



Control (A), diabetic (B), insulin (C), metformin (D), glibenclamide (E)
Black arrow = pointing at pyramidal neuron, PC= Pyramidal cell.

Discussion

Prior attempts at elucidating the regulation of blood glucose's entry into neural tissue in pregnancies have taken many approaches. In STZ-induced diabetic rats, insulin deficiency or hyperinsulinemia develops as a consequence of the irreversible destruction of beta cells of the pancreas, resulting in hyperglycemia [36]. Glibenclamide and metformin significantly reduced blood glucose concentration in our study but there was no significant difference in the body weights across all the treated groups, similar to previous studies' findings [27, 37]. The mechanisms of action of glibenclamide and metformin are well documented [38]. We found that glibenclamide and metformin were as effective as Insulin in glycemic control. It is still debatable whether metformin and glibenclamide can be used during pregnancy instead of insulin due to the fear of teratogenic effects on the developing fetus [39]. However, most researchers have been limited to the use of Insulin controlling the blood sugar levels because it has been the only drug deemed safe for use in pregnancy. In this study, we studied the pups' prefrontal cortex cytoarchitecture and biomarkers in streptozotocin-induced pregnant Wistar rats treated with glibenclamide,

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metformin and insulin for possible cognitive deficits associated with the use of oral hypoglycemic agents. At present, there is a growing acceptance of glibenclamide and metformin as the primary therapy for gestational diabetes [40]. This notion supported our findings that rats treated with metformin and glibenclamide showed a rapid and steady decrease in blood glucose levels. Langer et al. [14] in his study compared glibenclamide with Insulin in the treatment of gestational diabetes and found that daily blood glucose level is similar between subjects on glibenclamide and Insulin. This corroborates our findings that glibenclamide and Insulin significantly decreased the blood glucose level during the gestational period. Of relevance is that the undesirable effects induced by streptozotocin were entirely overridden, as observed in the control rats, and enhanced insulin secretion occurred after glucose overload [41].

Dhulkotia et al. [42] did a systematic review and meta-analysis to compare oral hypoglycemic agents versus Insulin in managing diabetes in pregnancy. They concluded there is no difference in glycemic control. This supports our findings that metformin and glibenclamide do have reasonable glycemic control. Furthermore, in the present study, the treatment of streptozotocin-induced diabetic rats with metformin, glibenclamide and insulin, to an extent, prevented the prefrontal cortex lesions compared with diabetic rats without. The neuroprotective effects of glibenclamide, metformin, and insulin result from good glycemic control, which corresponds with improved oxidative stress markers. Data strongly suggest that diabetes may not necessarily cause permanent damage if intervention is initiated on time and glibenclamide and metformin are well-placed to manage hyperglycemia [27, 43]. The preservation of pyramidal neurons in the pups of diabetic rats treated with metformin and metformin showed that they possess neuroprotective effects. Interestingly, Metformin and glibenclamide significantly reduced the oxidative stress caused by people with diabetes by improving the antioxidant (Glutathione) and reducing the malondialdehyde. The current study was supported by other studies, which reported an increase in the level of antioxidant enzymes in diabetic patients or rats who received either metformin or glibenclamide monotherapy [44, 45]. Furthermore, results from the morphology of the prefrontal cortex of the diabetic rats showed that Metformin and glibenclamide alleviated prefrontal neuronal loss. The pyramidal neurons in the prefrontal cortex of the pups treated with metformin and glibenclamide were preserved from oxidative injury through antioxidant properties and positive glycemic control. This makes glibenclamide and metformin an experimentally effective alternative to insulin therapy. However, it remains to be examined whether glibenclamide inhibits conception in experimental rats and whether the litters are at high risk of developing type 2 diabetes. Further studies are required to ascertain their safety during the pregnancy.

Conclusion: This study suggests that metformin and glibenclamide hyperglycemia control may prevent and reserve some neuropathological conditions associated with diabetes in streptozotocin-induced diabetic dams and pups' rats.

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