# Cinnamaldehyde mitigates placental vascular dysfunction of gestational diabetes and protects from the associated fetal hypoxia by modulating placental angiogenesis, metabolic activity and oxidative stress

**Title page**

Original Research Article

# Cinnamaldehyde mitigates placental vascular dysfunction of Gestational Diabetes and protects from the associated fetal hypoxia by modulating placental angiogenesis, metabolic activity and oxidative stress

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ABSTRACT

Gestational diabetes mellitus (GDM) is a major pregnancy-related disorder with an increasing prevalence worldwide. GDM is associated with altered placental vascular functions and has severe consequences for fetal growth. There is no commonly accepted medication for GDM due to safety considerations. Actions of the currently limited therapeutic options focus exclusively on lowering the blood glucose level without paying attention to the altered placental vascular reactivity and remodelling. We used the fat-sucrose diet/streptozotocin (FSD/STZ) rat model of GDM to explore the efficacy of cinnamaldehyde (Ci; 20 mg/kg/day), a promising antidiabetic agent for GDM, and glyburide/metformin-HCl (Gly/Met; 0.6+100 mg/kg/day), as a reference drug for treatment of GDM, on the placenta structure and function at term pregnancy after their oral intake one week before mating onward. Through genome-wide transcriptome, biochemical, metabolome, metal analysis and histopathology we obtained an integrated understanding of their effects. GDM resulted in maternal and fetal hyperglycemia, fetal hyperinsulinemia and placental dysfunction with subsequent fetal anemia, hepatic iron deficiency and high serum erythropoietin level, reflecting fetal hypoxia. Differentially-regulated genes were overrepresented for pathways of angiogenesis, metabolic transporters and oxidative stress. Despite Ci and Gly/Met effectively alleviated the maternal and fetal glycemia, only Ci offered substantial protection from GDM-associated placental vasculopathy and prevented the fetal hypoxia. This was explained by Ci's impact on the molecular regulation of placental angiogenesis, metabolic activity and redox signaling. In conclusion, Ci provides a dual impact for the treatment of GDM at both maternal and fetal levels through its antidiabetic effect and the direct placental vasoprotective action. Lack of Gly/Met effectiveness to restore it's impaired functionality demonstrates the vital role of the placenta in developing efficient medications for GDM.

*Key words:* Gestational diabetes, Placental dysfunction, Fetal hypoxia, Cinnamaldehyde, Vascular protection

*Chemical compounds studied in this article:*

Cinnamaldehyde (PubChem CID: 637511); Glyburide (PubChem CID: 3488); Metformin HCl (PubChem CID: 14219)

## 1. Introduction

Gestational diabetes mellitus (GDM) is a serious pregnancy-related health issue and its prevalence is increasing. According to the International Diabetes Federation's latest report, GDM affects about 16% of pregnancies worldwide [1]. GDM is characterized by maternal glucose intolerance that is first recognized during pregnancy and results in fetal hyperglycemia/hyperinsulinemia and aberrant placental function that has severe consequences for fetal growth [2].

Being the interface between maternal and fetal circulations, the placenta plays a crucial role in pregnancy by executing endocrine, metabolic and nutritional activities that are essential for the development and survival of the fetus. Because of these complex biological features, the placenta forms an important target organ to develop potential therapeutics for pregnancy complications [3].

Maternal (and fetal) hyperglycemia directly affects the placenta vascular reactivity [4]. Hyperglycemia acts as a pro-angiogenic, pro-constrictor, pro-coagulatory, pro-inflammatory and pro-permeability agent [4]. High glucose causes *de-novo* synthesis of diacylglycerol (DAG), which in turn causes an increase in the production of protein kinase C [5], that leads to an increase in the release of the vascular endothelial growth factor (VEGF) [6]; the dominant placental angiogenic factor. Increasingly, fetal hyperinsulinemia is recognized as a cofactor causing placental

hypervascularization in GDM. Hyperinsulinemia enhances the VEGF and endothelial nitric oxide synthase (*eNOS*) gene expression, exerting additional pro-angiogenic effects [7].

Placental angiogenesis is the process of new blood vessels formation in the vascular labyrinth zone; the major compartment of placentae at late gestation [8]. VEGF induces angiogenesis through binding to its receptor (vascular endothelial growth factor receptor 2; VEGFR2) that is expressed in endothelial cells (ECs). This provokes a multistep process within the preexisting blood vessel to form a capillary sprout that creates a new tube-like structure that stabilizes by the recruitment of vascular smooth muscle cells (VSMCs) and pericytes as a mature vessel. In normal ECs, VEGF is produced at a basal rate that stimulates the production of nitric oxide (NO) by eNOS. NO mainly acts as an endothelium-derived relaxing factor to ensure sufficient transfer of nutrients and gases supporting the growth of the fetus. NO also acts as a feed-back inhibitor to prevent further VEGF production [8, 9]. Thus, VEGF/NO balance maintains the placental vascular homeostasis. In GDM, this balance is perturbed as a result of placental metabolic disturbances and oxidative stress [10, 11]. Hyperglycemia turns the normally glycolytic EC metabolism into an oxidative metabolism that elevates the mitochondrial respiration, resulting in excessive production of reactive oxygen species (ROS) [12, 13]. ROS is implicated directly in oxidation of NO to the peroxynitrite radical ($ONOO^-$) which reduces NO bioavailability, rendering vessels prone to constriction [10]. This leads to vascular pathophysiological changes that pose potential concerns to the fetus as the fetal hypoxia. Moreover, experimental and human studies revealed that GDM can cause fetal hypoxia, where fetal hyperglycemia exacerbates fetal oxygen consumption through the aerobic metabolism stimulated by fetal hyperinsulinemia [14, 15].

Subcutaneous insulin injection is the traditional treatment of gestational diabetic women when exercise and diet control are not sufficient. However, insulin has several disadvantages including

potential maternal hypoglycemia, increased appetite and body weight gain, placental transmission and resulting fetal hyperinsulinism and macrosomia, and it does not protect offspring from the long-term metabolic disorders [16-18]. Therefore, it is desirable to find effective alternatives. Synthetic oral hypoglycemic drugs (glyburide, metformin and their combination) are commonly prescribed as medication for type 2 diabetes mellitus and are considered the only available therapeutic option for GDM [19, 20]. Glyburide acts by increasing insulin secretion from the pancreatic β-cells, while metformin acts as an insulin sensitizer by inhibiting the liver glucose output and gluconeogenesis, and increasing the muscle uptake of glucose [19, 21]. Even so, there is a paucity of data about their impact on the placenta function, and recent studies highlighted their adverse effects on the mother and the fetus including maternal gastrointestinal and dermatological issues, neonatal hypoglycemia and electrolytes imbalance [22-24].

Cinnamaldehyde (Ci), a well-know flavorant derived from the dried bark of Cinnamon trees, has attracted increasing interest due to its potential therapeutic benefits. It has been extensively studied as antidiabetic therapy. Zhu and his colleagues reviewed these studies and highlighted its capacity to treat several non-vascular complications of diabetes, across different Type 1 and type 2-rodent models of diabetes mellitus, through its insulin-mimetic, hypolipidemic, anti-inflammatory and anti-oxidative properties [25]. Our group has previously identified the potent antidiabetic effect of Ci on gestationally diabetic rats using the fat-sucrose diet/streptozotocin (FSD/STZ) rat model of GDM that simulates many clinical features of human GDM [26, 27]. Ci increased the insulin secretion and sensitivity, inhibited the induction of oxidative stress and inflammation, and restored the maternal reproductive performance. With regard to diabetes-related macrovascular and microvascular complications, Ci reduced vascular resistance and stenosis, and protected from hypertension in diabetic rats and mice [28-30]. *In vivo* and *in vitro*, Ci induced

relaxation of rat aorta [31], mice mesenteric arteries [32], porcine coronary arteries [33], and human and rat corpus cavernosum [34]. However, there are no previous reports concerning the vasoprotective effect of Ci on the placenta. Therefore, this study aimed to explore the efficacy of the antidiabetic action of Ci on placental dysfunction induced by GDM and to unravel its mechanism of action using genome-wide transcriptional analysis and targeted biochemical and histopathological studies. For comparison, we used the established glyburide/metformin-HCl combined therapy (Gly/Met), the impact on placental vascular reactivity of which is so far been little studied.

## 2. Material and methods

Sections on placental quantification of glycolysis and TCA cycle metabolites, placental oxidative stress and antioxidant markers, and placental transcriptome analysis are described in Appendix I of the supplementary file.

### *2.1. Experimental animals*

Female virgin albino Wistar rats (*Rattus norvegicus*) weighing 100-120 g were received from VACSERA Co., Helwan City, Egypt. The rats were individually housed in conventional polypropylene cages (48 cm long, 33 cm wide and 21 cm high) fitted with stainless steel food hoppers and wood shavings as bedding material. Cages were cleaned twice a week with new bedding material. The room was kept in an air conditioning atmosphere set at 25°C with 12 h dark/light cycles, starting a week before the experiment beginning for acclimatization. Rats were fed with regular diet (RD: starch 60%, corn oil 5% and casein 20%) or fat-sucrose diet (FSD: beef tallow 40%, sucrose 25% and casein 20%) [27], and allowed to drink water *ad libitum*. Significant efforts were done to minimize both animal suffering and the numbers used. The Institutional Animal Care and Use Committee (IACUC), Beni-Suef University, Egypt approved all animal

experiments (BSU / FS/2015/11), in compliance with the guidelines of the National Institute of Health (NIH) for the use of laboratory animals.

*2.2. Experimental setup*

Fig. 1 summarizes the experimental setup. In total, 32 female albino rats were divided randomly into four groups (eight rats / group):

▪ Normally pregnants (NP): Fed on RD. After four weeks of dietary intake, and daily up to the end of the experiment, these rats orally administered 0.5% dimethyl sulfoxide (DMSO) as a vehicle.

▪ Gestationally diabetics (GD): Fed on FSD. After four weeks of dietary intake, and daily up to the end of the experiment, these rats orally administered the DMSO vehicle.

▪ Gestationally diabetics with cinnamaldehyde treatment (GD+Ci): Fed on FSD. After four weeks of dietary intake, and daily up to the end of the experiment, these rats orally administered Ci (20 mg/kg/day in the DMSO vehicle [35]).

▪ Gestationally diabetics with glyburide/metformin-HCl treatment (GD+Gly/Met): Fed on FSD. After four weeks of dietary intake, and daily up to the end of the experiment, these rats orally administered Gly/Met (0.6+100 mg/kg/day in the DMSO vehicle [21]).

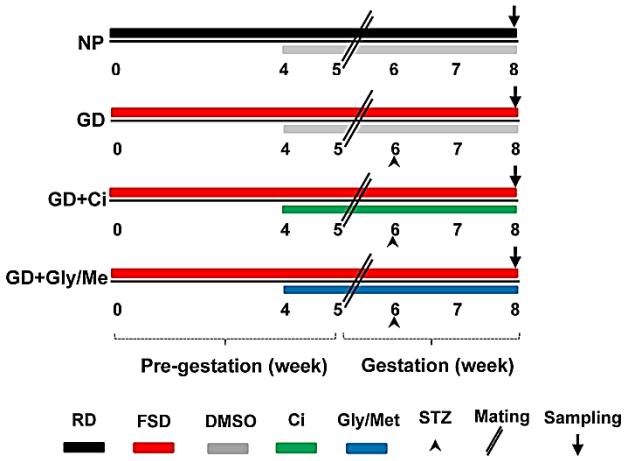
At the fifth experimental week, all rats were mated with males overnight. In the morning, the positive vaginal smear revealed the zero gestational day (rats with negative vaginal smears were omitted in order to keep the FSD/STZ-model criteria of feeding FSD for six weeks before STZ injection at, exactly, the seventh day of gestation; the end of the implantation phase of the embryo [36]). The pre-gestational period is the time before mating, while that after mating is the gestational period.

At the seventh day of gestation (end of the sixth experimental week), dams of all treatments were fasted for 16 h and those of GD, GD+Ci and GD+Gly/Met were intraperitoneally injected

156 with STZ (25 mg/kg body weight in citrate buffer, pH 4.5) [27]. NP-group was injected with the
157 buffer only.

158 At the 20$^{th}$ day of gestation (the eighth experimental week), a blood sample from each dam was
159 collected from the lateral tail vein to estimate the 2 h postprandial glucose and insulin levels
160 following an oral intake of glucose solution (3 g/kg b.wt.) to assess GDM development [27].

161 At the 21$^{st}$ day of gestation, dams fasted overnight ($n$= 4-6) were sacrificed under diethyl ether
162 anesthesia for dissection. Placentae were immediately excised and weighed. Randomly, one
163 placenta from each dam was kept in RNA*later*® (Sigma-Aldrich Co., USA) and stored at -80°C
164 for the transcriptome analysis, another was fixed in 10% neutral-buffered formalin for paraffin
165 section preparation and histopathological examination, and the remaining (4 to 6) placentae were
166 kept freshly at -80°C for the biochemical analyses. Fetuses of each dam were delivered, weighed
167 and two samples of blood were collected from the jugular vein. One was collected on the
168 anticoagulant EDTA for the hematocrit test (Hct%), while the serum was separated from the second
169 sample and stored at -80°C for detection of glucose, insulin and erythropoietin (EPO) levels. In
170 addition, fetal liver samples were dissected and kept at -80°C for iron content measurement.



171

172 **Fig. 1.** Experimental setup. NP: normally pregnants, GD: gestationally diabetics, GD+Ci: gestationally
173 diabetics with cinnamaldehyde treatment, GD+Gly/Met: gestationally diabetics with glyburide/metformin-

HCl treatment, RD: regular diet, FSD: fat-sucrose diet, DMSO: dimethyl sulfoxide, Ci: cinnamaldehyde, Gly/Met: glyburide/metformin-HCl, STZ: streptozotocin. *(Single column fitting image)*

### *2.3. Biochemical measurements*

#### *2.3.1. Blood glucose, insulin, EPO and Hct% detection*

Blood glucose levels were measured using a commercial blood glucometer (Accu-chek Active, Roche Diagnostics, Germany). Insulin and EPO levels were determined using rat ELISA kits (BioSource Europe S.A. and Cusabio Biotech Co., Belgium; respectively) following the manufacturer's instructions. Fetal Hct% was detected using the microcentrifuge method [37].

#### *2.3.2. Fetal liver iron content analysis*

Iron was measured in the fetal liver tissue of all experimental groups. About 100 mg from each sample ($n$= 4-6/ group) was weighed, dried in oven at 60°C for minimal 48 h, digested in a mixture of 37% $H_2O_2$ and 69% $HNO_3$ using a microwave [38], and diluted with the ultra-pure MilliQ water (Bedford, USA). The analysis was performed using the Inductive Coupled Plasma-Mass Spectrometer (ICP-MS, 7700x, Agilent Technologies, Santa Clara, CA, USA). For quality control, blanks and certified reference material (Mussle tissue "SRM 2976"; Institute for Reference Materials and Measurements, Geel, Belgium) were processed the same way as the experimental samples. The recovery rate was within 10% of the certified values. Iron content was expressed on a dry tissue weight basis (µg/g dry weight).

### *2.4. Placental histological and histochemical staining*

Placentae ($n$=3/ group) were fixed overnight in 10% neutral buffered formalin to harden. Using a stainless-steel razor blade, placentae were cut horizontally at their half thickness [39, 40] to expose the full face of the placenta for better examining the histopathological changes across a wide surface area of the different placental layers. Samples were dehydrated, cleared, and paraffin

197 embedded. The flat cut edges were positioned in contact with the bottom of the cassette to be
198 sectioned (at 5 μm thick) and then loaded on glass slides. Slides were stained with hematoxylin
199 and eosin (H&E) [41] and periodic acid-Schiff (PAS) [42], upon deparaffinization and rehydration,
200 for the detection of the histopathological changes and glycogen content, respectively. Length of
201 the Labyrinth placental layer was measured using ImageJ software. Briefly, H&E stained placental
202 sections were imaged by Nikon AZ100 macroscope using 0.5x objective lens. The length of the
203 labyrinth layer was measured at the longest axis of the placenta starting from the lower boundary
204 of the basal layer (B) to the outer boundary of the centered umbilical cord (UC) as indicated in
205 Figure 4A using the public domain image analysis software ImageJ [43]. Data were presented as
206 means ± SEM ($n$=3). PAS-staining was semi-quantitatively assessed using the histochemical score
207 (H-Score) as described previously [44]. In brief, intensity of the staining was categorized into four
208 levels: 0 (none), 1 (low), 2 (moderate) and 3 (heavy). Percentage of the positively-stained cells
209 was graded as follow: (0) < 5%, (1) 6-25%, (2) 26-50%, (3) 51-75% and (4) > 75%. The final
210 scoring was demonstrated by multiplying the percentage of the positively stained cells and the
211 staining intensity. Histopathological scoring was estimated from five random fields of each sample
212 slide. Lesion severity was divided into four grades: (-) not present, (-/+) few "<10%", (+) moderate,
213 and (++) extensive. Images were scored by two independent experienced assessors blinded to the
214 corresponding treatment.

215 *2.5. Statistical analysis*

216 For all biochemical measurements, feto-placental parameters and H-scores from the
217 histopathological analysis, one-way analysis of variance (ANOVA) followed by Tukey Kramer's
218 post hoc test was carried out by the statistical SPSS software (v22, Chicago, USA). Regarding
219 placental/fetal weight ratio, for each dam/group, every individual placenta and related fetus were

weighed, divided by each other to get their placental/fetal weight ratio. Then, average weights of placentae, fetuses and their corresponding ratios were calculated for each dam for ANOVA. Data are shown as means ± SEM. For biochemical and feto-placental parameters $n$= 4-6, for the histopathological analysis $n$=3. Effects were significant at $p < 0.05$. Transcriptome data analysis is presented in details in Appendix I of the supplementary file.

## 3. Results

### *3.1. Effect of GDM and its treatment with Ci and Gly/Met on maternal and fetal biomarkers, and feto-placental characteristics*

To confirm the anti-hyperglycemic effect of Ci and Gly/Met treatments, maternal and fetal glucose and insulin levels were assessed at term pregnancy. As expected, gestational diabetes induced by FSD-feeding/low dose STZ-injection caused a significant increase in maternal and fetal blood glucose levels along with a decrease in maternal insulin, but fetal hyperinsulinemia compared to normal pregnant controls (Table 1). Consistent with previous studies [21, 26], Ci and Gly/Met treatments significantly alleviated the impact of GDM on both maternal and fetal glucose and insulin levels. While Gly/Met showed an obvious improvement of these parameters, it appeared to induce a decrease in fetal blood glucose, although the difference with the NP-group was not statistically different.

Fetuses of GD-group also revealed 15% drop of Hct%, a 4-fold increase in serum EPO and 28% reduction of their liver iron content compared to NP-controls, indicating fetal hypoxia (Table 1). In the Ci-treated group, Hct% and liver iron content were similar to controls, whereas EPO levels were intermediate between NP and GD. Compared with GD-group, serum EPO and liver iron content were almost unaffected by Gly/Met-treatment while Hct% was even lower.

242 Concerning the feto-placental unit, no change was observed in fetal weight between NP, GD
243 and GD+Ci groups, while the weight of Gly/Met-treated fetuses was 35% lower (Table 1).
244 Compared to NP, placental weight increased in response to diabetes (0.58 ± 0.03 g vs. 0.39 ± 0.01
245 g, $p < 0.05$), while it was fully or partly restored by Ci and Gly/Met treatments (0.37 ± 0.01 g and
246 0.49 ± 0.02 g), respectively. Moreover, GDM increased placental insufficiency, as assessed by the
247 placental-to-fetal ratio, which was prevented by Ci, but exacerbated by Gly/Met (Table 1).
248 Taken together, these observations demonstrate that GDM caused impairment of placental
249 function and disturbed the fetal parameters. Moreover, Ci and GD+Gly/Met treatments exerted a
250 different placental and fetal responses, irrespective of their hypoglycemic action, suggesting a
251 direct effect on the placental development and functioning.
252
253 **Table 1.** Effect of gestational diabetes and its treatment with cinnamaldehyde and
254 glyburide/metformin-HCl on maternal and fetal physiological characteristics at term pregnancy.

| Parameter | Group | | | |
|---|---|---|---|---|
| | NP | GD | GD+Ci | GD+Gly/Met |
| *Maternal* | | | | |
| Glucose (mg/dl) | 103.25 ± 4.42[a] | 325.08 ± 43.53[b] | 139.71 ± 13.25[a] | 101.17 ± 7.76[a] |
| Insulin (µIU/ml) | 41.95 ± 3.43[b] | 30.57 ± 1.34[a] | 39.52 ± 1.32[b] | 44.33 ± 2.25[b] |
| *Fetal* | | | | |
| Glucose (mg/dl) | 88.63 ± 11.40[a] | 200.22 ± 32.68[b] | 91.00 ± 9.77[a] | 57.67 ± 10.84[a] |
| Insulin (µIU/ml) | 4.90 ± 0.70[a] | 10.30 ± 0.74[b] | 6.11 ± 0.76[a] | 4.11 ± 0.70[a] |
| Hct (%) | 54.52 ± 0.76[c] | 46.54 ± 1.33[b] | 51.70 ± 1.85[c] | 40.53 ± 2.04[a] |
| EPO (pg/ml) | 186.31 ± 22.99[a] | 910.96 ± 97.43[c] | 541.35 ± 22.39[b] | 879.75 ± 118.33[c] |
| Liver iron content (µg/ g dry tissue) | 1155.63±93.35[b] | 788.96 ± 30.49[a] | 1019.13 ± 7.97[b] | 935.84 ± 53.01[ab] |
| Body weight (g) | 3.81 ± 0.09[b] | 3.79 ± 0.16[b] | 3.42 ± 0.25[b] | 2.47 ± 0.08[a] |
| Placenta weight (g) | 0.39 ± 0.01[a] | 0.58 ± 0.03[c] | 0.37 ± 0.01[a] | 0.49 ± 0.02[b] |
| Placental/fetal weight | 0.11 ± 0.004[a] | 0.15 ± 0.009[b] | 0.11 ± 0.008[a] | 0.20 ± 0.01[c] |

Data presented as mean ± SEM (*n*=4-6). Different letters indicate significant differences ($p < 0.05$). NP: normally pregnants, GD: gestationally diabetics, GD+Ci: gestationally diabetics with cinnamaldehyde treatment, GD+Gly/Met: gestationally diabetics with glyburide/metformin-HCl treatment.

### *3.2. Effect of Ci and Gly/Met on GDM-induced placental transcriptional responses*

To unravel the molecular basis of the effect of the treatments on the GD-placenta, we performed a genome-wide transcriptome analysis using Next Generation Sequencing (NGS). Quality-control report of RNA sequence (RNAseq)-reads was provided in Supplemental Table S1. Overall, 1351 genes were differentially expressed across all experimental groups (FDR < 0.05). 463 genes were differentially expressed in GD-group compared to NP. Gly/Met reduced the number of differentially expressed genes (DEGs) by about 50%, whereas Ci increased it by about 40%; compared to those of GD vs. NP (Fig. 2A). This suggests that Ci induced a specific set of genes rather than only reducing the effect of diabetes on placental gene expression. Consistently, only 8 genes were differentially expressed in GD+Gly/Met-group compared to GD, while GD+Ci vs. GD showed 199 DEGs (Fig. 2A).

To better understand this global pattern, we performed Quality Threshold Clustering (QTC) on the 1351 DEGs. This resulted in eight clusters with contrasting gene expression profiles (Fig. 2B). The two largest clusters contained 288 (cluster 1) and 283 (cluster 2) transcripts, whose expression was down- or up-regulated, respectively, in response to diabetes with little or no effect of Ci or Gly/Met. Genes in cluster 1 were overrepresented for the process of localization establishment, while those in cluster 2 were overrepresented for gene ontologies related to protein localization, cellular component assembly and regulation of metabolic processes (Supplemental Table S2A and B). Genes in cluster 3 (235 genes) and 4 (106 genes) also showed opposite patterns, up- and down-regulated in the diabetic group, where both treatments, but particularly the Ci-treatment, reduced this response. Interestingly, cluster 3 was overrepresented for gene ontologies related to

279 angiogenesis and blood vessel remodelling, axon guidance and extension, ROS biosynthesis,
280 platelets-derived growth factor (PDGF)-pathway, response to hyperoxia, carboxylic acid
281 transmembrane transport and regulation of cell proliferation, and organ developmental processes
282 (Fig. 3A and Supplemental Table S2C). Cluster 4 did not yield a significant gene ontology
283 enrichment. Another 86 transcripts (cluster 5) displaying a pattern where Ci reduces the effect of
284 GD but without any related gene ontologies. Clusters 6, 7 and 8 represented genes that are not
285 affected by GD, but specifically up- or down-regulated by Ci (86, 52 and 47 genes, respectively).
286 These transcripts, that represent functions of Ci not directly related to GD, were not
287 overrepresented for any gene ontology category. In summary, the transcript expression patterns
288 revealed that GDM affects placental angiogenesis, metabolic transporters and oxidative stress and
289 these effects are alleviated by Ci, but not Gly/Met. A detailed list of DEGs of these three enriched
290 pathways with the full names, biological functions, FDR, p-values, log2fold changes are shown in
291 Supplemental Table S3.

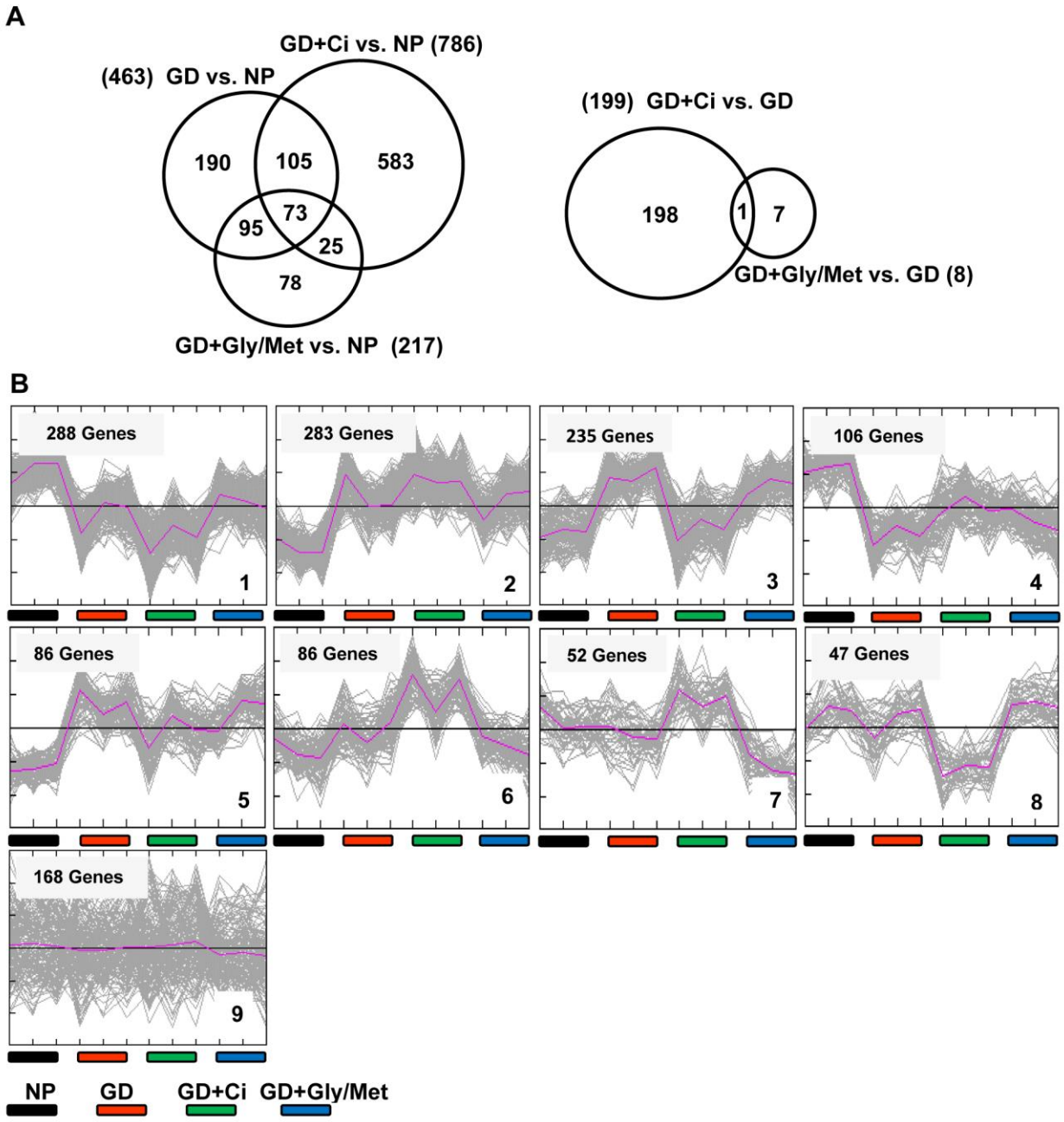



**Fig. 2.** Effect of gestational diabetes and its treatment with cinnamaldehyde and glyburide/metformin-HCl on genome-wide transcriptional responses of the placenta at term pregnancy. **(A)** Venn diagram showing an overview of the 1351 differentially expressed genes between all experimental groups (FDR < 0.05). **(B)** QTC clustering of the gene expression (Pearson correlation, cluster diameter = 0.5, minimum cluster size = 40). Clusters number and size are indicated. The x-axis shows three biological replicates for each group. The y-axis shows normalized expression levels. NP: normally pregnants, GD: gestationally diabetics, GD+Ci: gestationally diabetics with cinnamaldehyde treatment, GD+Gly/Met: gestationally diabetics with glyburide/metformin-HCl treatment. *(Double column fitting image)*

### *3.3. Effect of Ci and Gly/Met on GDM-induced placental angiogenesis and hypervascularization*

Excessive placental angiogenesis (hypervascularization) is the most common placental pathology in GDM. It reflects an adaptive response to the circulatory disturbances in the labyrinth zone that leads to major impacts on fetal growth [11]. Upregulation of genes involved in the angiogenesis was apparent in GD-placenta. Our transcriptome data showed GDM induced elevated expression levels of VEGF-signaling pathway molecules' including *Vegfr2*, members of the conserved neuronal axon-guidance cue family that regulate the vascular sprout formation and integrity including semaphorin/plexin/neuropilin (*Sema/Plx/Nrp*) [45], *Slit/Robo* [46], *ephrin/Eph* [47] and *Notch/Dll4* [48], as well as the major mediators of vascular cell adhesion and migration through the extracellular matrix; integrins [49], vasodilator-stimulated phosphoprotein (*Vasp*) [50], and ADAM-metallopeptidase (*Adamts*) [51], along with the secreted modular calcium-binding protein 1 (*Smoc1*) that provokes sprout growth [52] and the homeobox gene *Esx1* that is linked to labyrinth layer overgrowth [53] (GD vs NP; Fig. 3B, Supplemental Table S3). The impact of GDM on the expression of most of these genes (in addition to *Vegfa*) was strongly reduced by Ci-administration, but largely unaffected by Gly/Met, suggesting fine tuning of placental sprouting angiogenesis under Ci-treatment. Our results are consistent with earlier studies that showed the Ci role in reducing VEGF and NO production, regulating their downstream signaling and controlling angiogenesis [54-56]. To verify the functional relevance of these transcriptional changes, we performed a histological analysis of the placental labyrinth layer length (LLL). Gestational diabetes increased LLL by 32% compared to NP. Ci reduced this increase to only 11%, whereas the Gly/Met treatment had little effect (Fig. 4A and B).

### *3.4. Effect of Ci and Gly/Met on GDM-induced placental vascular remodelling*

Although there was a significant upregulation of phospholipase C delta 1 (*Plcd1*), *eNOS,* solute carrier 7a2 (*Slc7a2*; L-arginine transporter) and soluble guanylyl cyclase (*Guc1*) genes which reflect the vasorelaxant effect of NO on the GD-placenta neovascularization [8, 57], we found an overexpression of five isoform transcripts of phosphodiesterases (*Pdes*) in the GD-group compared to NP-controls (Fig. 3B and Supplemental Table S3). PDEs are the only enzymes that degrade the cyclic guanosine monophosphate (cGMP) and, thus, block NO-stimulating vasodilatation leading to hypertension and congestion [58]. In addition, our transcriptome data indicated a substantial upregulation of two transient receptor potential cation channel genes (*Trpv2* and *Pkd2l1*), Ras-related glycolysis inhibitor and calcium channel regulator (*Rrad*), and downregulation of store-operated calcium entry-associated regulatory factor (*Saraf*) in the diabetic placenta, which suggests increased $Ca^{2+}$ influx and the subsequent vasoconstriction [59-65]. Moreover, the reported increase in *Vegfr2* expression might induce the phosphorylation of the transmembrane junctional adhesion molecules, resulting in increased ECs paracellular permeability, which causes vasogenic edema [4]. Besides, GD-placenta overexpressed the platelets-derived growth factor C (*Pdgfc*) that might promotes vascular smooth muscle cells (VSMCs) growth, proliferation and the inward migration into the intimal layer of blood vessels resulting in the thickening of its walls and decreasing the lumen space [30]. Expression of *Pdes*, *Trpv2*, *Pkd2l1*, *Saraf*, *Rrad* and *Pdgfc* were modulated by Ci administration, while Gly/Met didn't change any of these transcripts significantly compared to GD-group (Fig. 3B and Supplemental Table S3). Further, prostaglandin-endoperoxide synthase 2 (*Ptgs2*) and GTP-cyclohydrolase 1 (*Gch1*) transcripts were exclusively upregulated in Ci-treated placenta, while acetylcholinesterase (*Ache*) was downregulated, suggesting a potent vasodilatory effect of cinnamaldehyde [31, 66, 67].

In order to evaluate the functional relevance of these transcriptional changes, we performed a microscopical analysis of the structure of the labyrinth vasculature. Vasoconstriction and thickening of the interhemal membranes were a prominent feature in GD-placenta compared to NP-controls (Fig. 7N, and Table 2). As well, main fetal vessels showed stenosis, hyperplasia, edema and widening of the surrounding space. On the other hand, Ci-placenta displayed an opposite phenotype, while Gly/Met treatment caused a mild response (Fig. 7O and 7P, and Table 2). Effects of the treatments on the vascular architecture suggest that in addition to the antihyperglycemic effect it shares with Gly/Met, the action of Ci also involve a direct vascular protection.

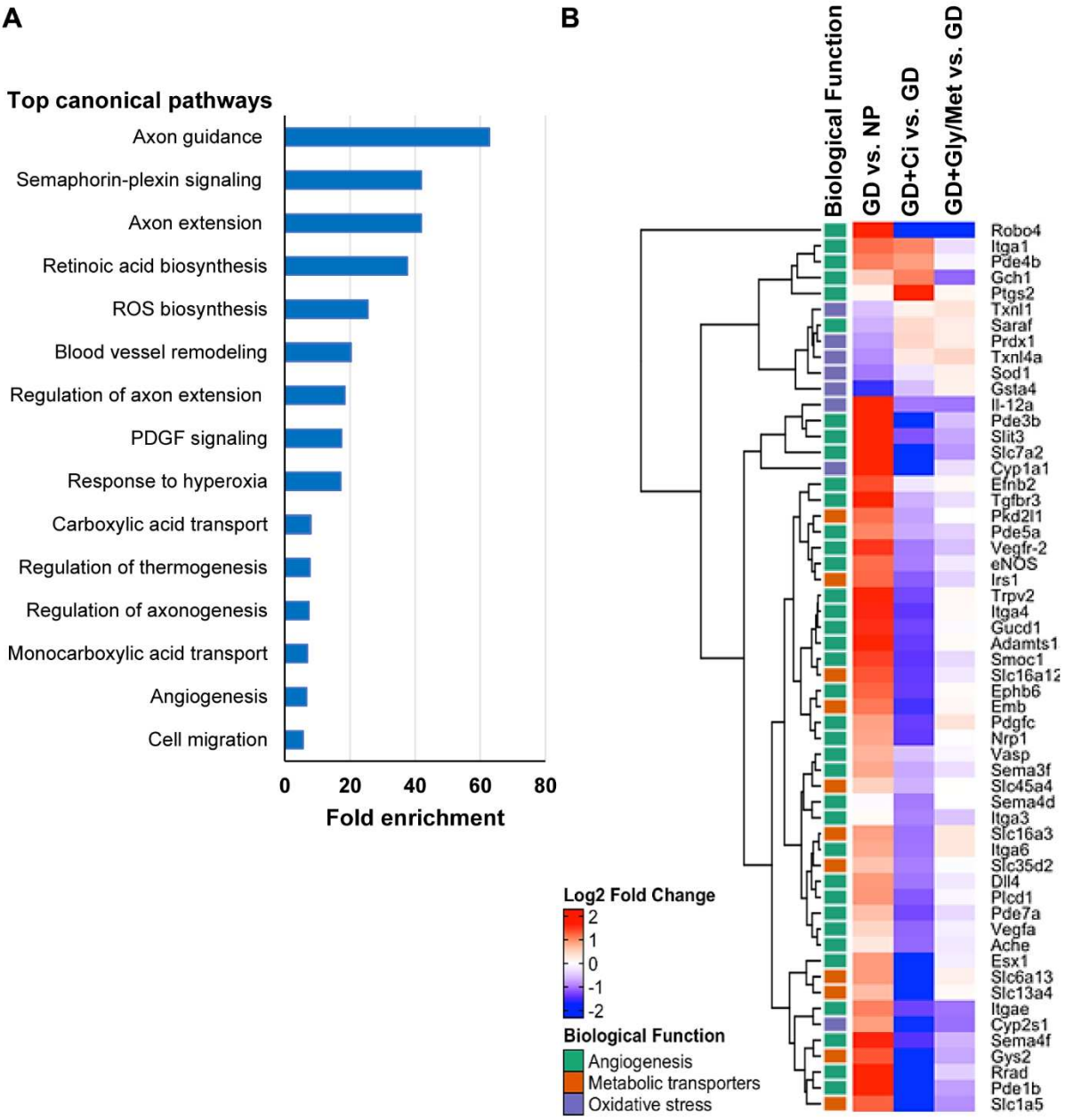



**Fig. 3.** Functional overrepresentation analysis of differentially expressed genes in placental tissue responding to gestational diabetes and its treatment with cinnamaldehyde and glyburide/metformin-HCl. **(A)** Overrepresented pathways of cluster 3 (FDR < 0.05) generated by PANTHER classification system (full list in Supplemental Table S2C). **(B)** Comparison analysis for the effect of Ci and Gly/Met on DEGs significantly affected by GDM in the three major ontologically enriched biological functions (angiogenesis, metabolic transporters and oxidative stress) with adjusted p-value < 0.05 and $+0.5 \leq \log2FC \leq -0.5$ at least in one of the comparisons (full names, adjusted p-values, p-values, log2fold changes and biological functions of each gene in Supplemental Table S3). NP: normally pregnants, GD: gestationally diabetics, GD+Ci: gestationally diabetics with cinnamaldehyde treatment, GD+Gly/Met: gestationally diabetics with glyburide/metformin-HCl treatment. *(Double column fitting image)*

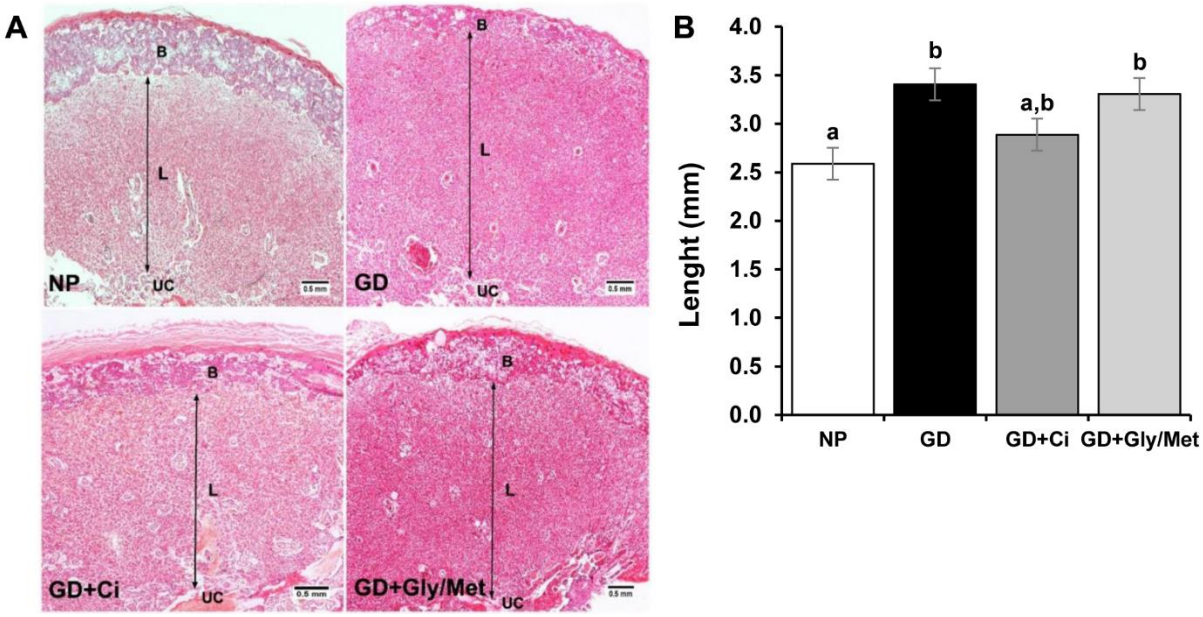



**Fig. 4. (A & B)** Effect of gestational diabetes and its treatment with cinnamaldehyde and glyburide/metformin-HCl on the placenta labyrinth layer length of the different groups; scale bar= 500 µm. NP: normally pregnants, GD: gestationally diabetics, GD+Ci: gestationally diabetics with cinnamaldehyde treatment, GD+Gly/Met: gestationally diabetics with glyburide/metformin-HCl treatment, B: basal layer, L: labyrinth layer, UC: umbilical cord. Data presented as mean ± SEM (*n*=3). Different letters indicate significant differences ($p < 0.05$). *(Double column fitting image)*

### *3.5. Effect of Ci and Gly/Met on GDM-induced placental metabolic disturbances*

#### *3.5.1. Glucose oxidation*

Transcripts encoding carboxylic acid transporters related to glucose oxidation, including solute carrier (S*lc*) *45a4*, *13a4*, *1a5*, *6a13*, 1*6a3* and embigin were enriched among the upregulated genes in the diabetic placenta (GD vs NP; Fig. 3B and Supplemental Table S3), suggesting a higher placental metabolic rate. *Slc45* encodes a sugar transporter that can transport both the monosaccharides (glucose, fructose and galactose) and the disaccharide sucrose in an $H^+$-dependent manner [68]. Slc13a4 is a member of the citrate transporter family; Slc13 [69]. Citrate is the first molecule that forms during the tri-carboxylic acid (TCA)-cycle's reactions. Slc1a5 (also

known as ASCT2) is a high-affinity glutamate transporter [70]. Glutamate enters TCA-cycle in the form of α-ketoglutarate causing an increase in TCA-cycle intermediates. Slc6a13 is the γ-aminobutyric acid transporter [71]. Succinate may derive from the γ-aminobutyric acid shunt pathway in correlation with the expression level of *slc6a13*. Slc16a3 (also known as monocarboxylate transporter 4; MCT4) specifically export lactic acid derived from glycolysis [72], while embigin is a glycosylated ancillary protein that closely associated with MCT2 to maintain its lactate-importing activity [72]. We propose that as GD-placenta become highly metabolically active, due to the maternal hyperglycemic flux, overproduction of glycolysis and TCA-cycle metabolites leads to the observed overexpression of the related transporters. To validate this idea, we performed a quantification of organic acids (OAs) from glycolysis and TCA-cycle using the Ultra-Performance Liquid Chromatography-Mass Spectrometry (UPLC-MS). Consistent with our hypothesis, GD-placenta showed a remarkable increase in the concentration of pyruvic, citric, glutamic, succinic, fumaric, malic and lactic acids compared to the NP-controls. Succinic acid was the most frequent followed by fumaric and malic acids. Citric and lactic acids showed a 2-fold increase, while pyruvic and glutamic acids displayed about 40% and 60% increases, respectively (Fig. 5A, and absolute values in Supplemental Table S5). Moreover, upregulation of *Slc16a12* (the creatine transporter) in GD-placenta might explain an elevation of the energy demand for fetal growth besides glycolysis (the main source of energy) (Fig. 3B and Supplemental Table S3). Creatine is the main energy buffer that rapidly provides ATP for the high energy-consuming processes [73, 74]. Both Ci and Gly/Met induced a marked decrease in the glycolysis and TCA-cycle's OAs levels. At the transcript level, expression of the related transporters showed significant downregulation only in response to Ci, suggesting an alternative mechanism for Gly/Met action (Fig. 3B and Supplemental Table S3).

### *3.5.2. Glycogen storage*

Upregulated expression of *Slc35d2*, glycogen synthase 2 (*Gys2*), insulin receptor substrate1 (*Irs1*) and transforming growth factor-beta receptor 3 (*Tgfbr3*) in the GD-placenta provided an evidence for the increase in cellular glycogen storage. We assume that *Slc35d2* mediated UDP-glucose cellular uptake, where *Gys* converted it to glycogen through *Irs-1* activation [75]. Histochemical detection of the placenta glycogen content showed several-folds increase in GD-placenta compared to NP-group (Fig. 5B and C). Placental glycogen is mostly stored in the glycogen storage cells (GlyCs) and in the spongiotrophoblast cells which are mainly located at the basal layer of the placenta [76]. The significant upregulation of *Tgfbr3* might explain the numerous glycogen storage cells observed in the GD-placental labyrinth layer (Fig. 7F and Table 2) that are considered an adaptive mechanism to replace the damaged GlyCs in the basal layer in order to protect the fetus from the maternal hyperglycemic flux. TGF-β is an important regulator of tissue morphogenesis and remodelling and its pathway is strongly involved in endothelial-to-mesenchymal transition (EndMT) [77], where the labyrinth endothelial cells might undergo a series of molecular events that lead to changes in their phenotype toward mesenchymal cells that then possibly differentiated into glycogen storage cells. Ci more strongly reduced glycogen increment in the placenta than Gly/Met (Fig. 5B and C), possibly through the potent downregulation of the previously mentioned related glycogen synthetic genes.

### *3.6. Effect of Ci and Gly/Met on GDM-induced placental histopathological alterations*

The observed elevation of lactic and succinic acids levels along with the overexpressed proinflammatory cytokine interleukin-12a (*Il12a*) (Fig. 3B and Supplemental Table S3) in GD-placenta might reflect a local inflammatory status causing micro- and macrovascular placental degenerative lesions [71, 78, 79]. In order to validate this assumption, we performed a

430 microscopical examination of the placenta structural layers (Fig. 7 and Table 2). Congestion of the
431 maternal arterial canals and the infiltration of leukocytes were apparent in GD-placenta (Fig. 7J).
432 In addition, we observed degeneration of basal layer's glycogen cells (Fig. 7B), necrosis of
433 spongiotrophoblastic cells (Fig. 7F), cytolysis of giant cell nuclei (Fig. 7J) and disruption of
434 labyrinth trophoblastic septa (Fig. 7F). These observations were similar to placental structural
435 abnormalities found by Furukawa *et al.* [76], Gül *et al.* [80] and Bhattacharjee *et al.* [81] in
436 response to the diabetic hyperglycemia.

437 Thus, we conclude that GD induced different compensatory mechanisms in the placenta aimed
438 to minimize the effects of the maternal hyperglycemic status on the fetus, including enhanced
439 glucose metabolism, glycogen storage and rapid supply of ATP through creatine. Both Ci and
440 Gly/Met modulated this situation as a result of their hypoglycemic actions and largely protected
441 the placenta from the observed structural abnormalities.

442

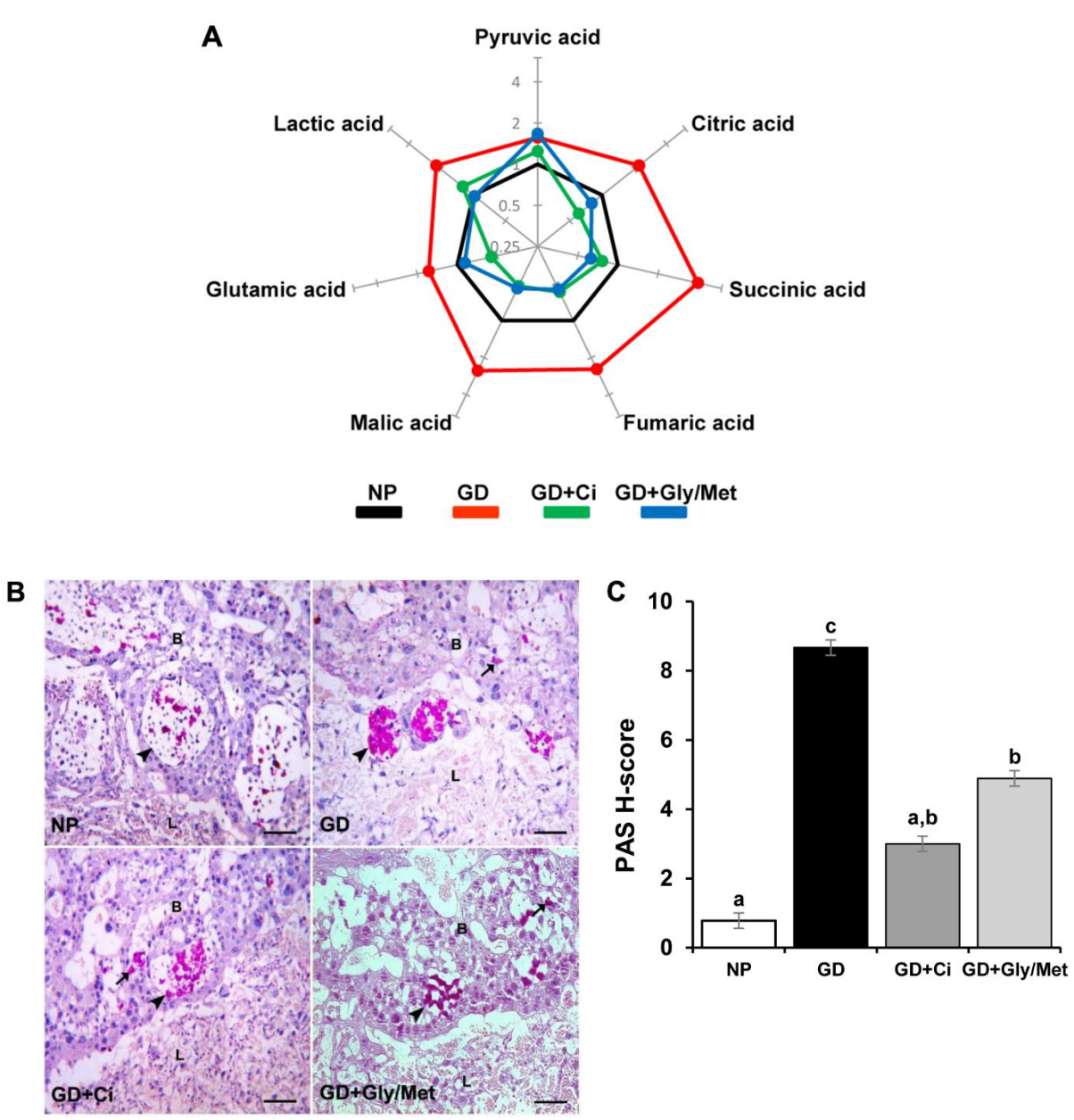



**Fig. 5.** Effect of gestational diabetes and its treatment with cinnamaldehyde and glyburide/metformin-HCl on glucose oxidation and the glycogen storage. **(A)** Showing placental glycolysis end products and TCA-cycle metabolites concentrations at term pregnancy. Data were normalized corresponding to the mean values of the normal pregnant controls ($n$=4-5). Absolute values in Supplemental Table S5. **(B and C)** Showing placental PAS-staining of the different groups and its quantification using H-score; scale bar= 100 µm. B: basal layer, L: labyrinth layer, arrow heads: PAS-positive glycogen storage cells, arrows: PAS-positive spongiotrophoblast cells. Data presented as mean ± SEM ($n$=3). Different letters indicate significant differences ($p < 0.05$). NP: normally pregnants, GD: gestationally diabetics, GD+Ci: gestationally diabetics with cinnamaldehyde treatment, GD+Gly/Met: gestationally diabetics with glyburide/metformin-HCl treatment. *(Double column fitting image)*

473 *3.7. Effect of Ci and Gly/Met on GDM-induced placental oxidative stress*

474 Observed placental metabolic disturbances in GD-placenta could render the endothelial cells
475 vulnerable to oxidative stress and vascular dysfunction [10, 11]. The transcriptome data revealed
476 overexpression of cytochrome P450 (*Cyp*)-*1a1* and *2s1* in GD-placenta (Fig. 3B and Supplemental
477 Table S3); enzymes catalyzing malondialdehyde (MDA) production [82, 83]. MDA is a metabolite
478 produced from lipid peroxidation in cells and reflects oxidative membrane damage. It can be
479 generated by enzymatic (mostly cytochrome P450) and nonenzymatic (ROS) means [10]. Thus,
480 we set out to characterize the placental redox status. For that end, level of MDA was measured, in
481 addition to the total non-enzymatic antioxidant power (FRAP), antioxidant metabolites
482 (glutathione, ascorbate, polyphenols, flavonoids and tocopherols) and enzymatic antioxidants
483 (superoxide dismutase "SOD", catalase "CAT", peroxidases "POX", peroxiredoxin "Prx",
484 thioredoxin "Trx", glutaredoxin "Grx", glutathione peroxidase "GPX", glutathione reductase
485 "GR", glutathione S-transferase "GST" and dehydroascorbate reductase "DHAR").

486 We found a marked increase in GD-placental MDA level compared to NP group, suggesting
487 oxidative damage to the membranes (Fig. 6 and Supplemental Table S6). Diabetic placenta showed
488 an increase in FRAP, presumably in response to this stress condition. However, levels of the
489 antioxidant metabolites tocopherols, polyphenols and flavonoids were unchanged. Placental levels
490 of ASC and GSH decreased remarkably in response to diabetes, while tASC and tGSH showed an
491 opposite pattern (Fig. 6 and Supplemental Table S6). This clearly indicates a strong oxidation of
492 ASC and GSH to dehydroascorbate (DHA) and oxidized glutathione (GSSG), respectively, owing
493 to a high $H_2O_2$ content. Glutathione-ascorbate system (GSH/ASC) scavenges $H_2O_2$ through the
494 mutual action of glutathione, ascorbate and GSH/ASC-regenerating enzymes; GR and DHAR [10,
495 84]. DHAR activity was suppressed in response to diabetes while GR activity was relatively

unchanged compared to NP group (Supplemental Table S6). Enzymatic antioxidant activities of SOD, CAT, GPX and Trx are strongly induced in GD-placenta (Fig. 6 and Supplemental Table S6), whereas POX and Prx remain largely unchanged (Supplemental Table S6). SOD catalyzes superoxide dismutation to oxygen and $H_2O_2$, while CAT, GPX, Trx, POX and Prx are $H_2O_2$-detoxifying enzymes. No significant changes were recorded for Grx and GST activities (Supplemental Table S6). Grx catalyzes the general disulphide reductions of oxidized proteins, while GST detoxifies the electrophilic compounds as the oxidized lipids.

In comparison to the GD-group, Ci administration markedly increased GSH and ASC placental content, while those of MDA, FRAP, flavonoids and tASC were decreased. Also, enzymatic activities of SOD, CAT, Trx and GPX were normalized. These results confirm the potent antioxidant impact of Ci found by Subash-Babu *et al.* [85] and Zhu *et al.* [25]. On the other hand, Gly/Met-treated group showed a significant increase in placental MDA content. This, might be a consequence to the decreased membrane-embedded lipophilic antioxidants; tocopherols. However, Gly/Met showed comparable effects to Ci on the other antioxidant markers (Fig. 6 and Supplemental Table S6).

At the transcript level, there was no significant differential expression for *Cat*, *Pox*, *Gpx*, *Gr*, *Grx* and *Dhar* between the experimental groups, while the expression of *Sod*, *Trx*, *Prx* and *Gst* was markedly downregulated in GD-placenta compared to NP-group and slightly modulated with both treatments (Fig. 3B and Supplemental Table S3); which is not following their activity profile. In line with Lappas *et al.* [10], discrepancies between the expression and activity of the antioxidant enzymes may be due to the preconditioned programming to oxidative stress during the course of pregnancy in GDM that increase the ability of placenta to accommodate the stress at term pregnancy.

Overall, we concluded that the maternal hyperglycemia caused disturbances of the placenta metabolic activities that lead, with the observed overexpression of *Cyp1a1*, *Cyp2s1*, *eNOS* and *Slc7a2*, to accumulation of ROS that initiated the placenta defense system to protect the fetuses from the hyperglycemia-associated oxidative damage through buildup of antioxidant molecules and activation of redox-regulating enzymes. Owing to its hypoglycemic and antioxidant activity, Ci attenuated this oxidative state effectively more than Gly/Met.

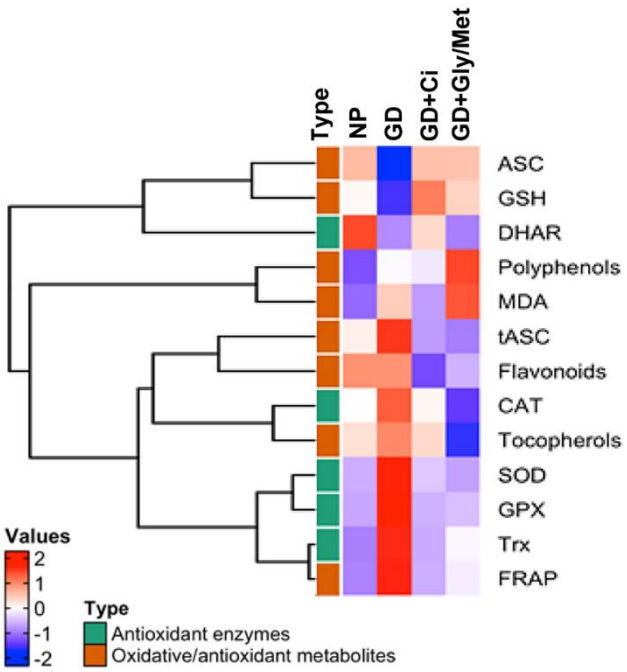



**Fig. 6.** Effect of gestational diabetes and its treatment with cinnamaldehyde and glyburide/metformin-HCl on placental oxidative stress and anti-oxidant markers at term pregnancy. NP: normally pregnants, GD: gestationally diabetics, GD+Ci: gestationally diabetic with cinnamaldehyde treatment, GD+Gly/Met: gestationally diabetics with glyburide/metformin-HCl treatment. ASC: reduced ascorbate, CAT: catalase, DHAR: dehydroascorbate reductase, FRAP: ferric reducing antioxidant power, GSH: reduced glutathione, GPX: glutathione peroxidase, MDA: malondialdehyde, SOD: superoxide dismutase, tASC: total ascorbate, Trx: thioredoxin. Results are presented as an average of four biological replicates in each experimental group. Data were normalized to remove variations in absolute values (Supplemental Table S6) and clustered hierarchically using Euclidean distance matrix on R to show the pattern across the different groups. *(Single column fitting image)*

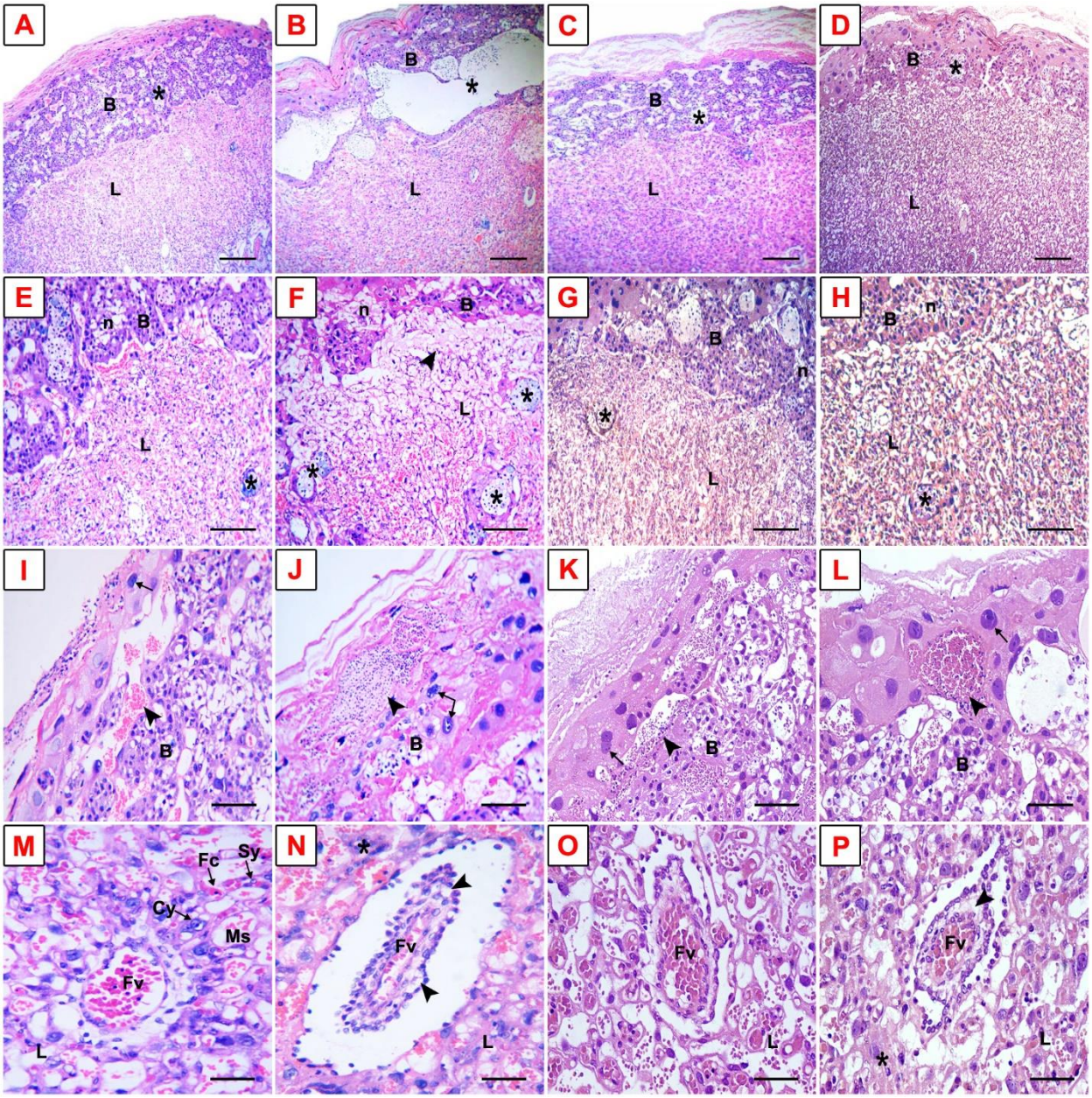



**Fig. 7.** Placental histopathological alterations induced by gestational diabetes at term pregnancy and the effect of cinnamaldehyde and glyburide/metformin-HCl treatments. NP: normally pregnants, GD: gestationally diabetics, GD+Ci: gestationally diabetics with cinnamaldehyde treatment, GD+Gly/Met: gestationally diabetics with glyburide/metformin-HCl treatment. **(A-D)** Cystic degeneration of the glycogen cells (*) at the basal layer; scale bar = 400 µm. **(E-H)** Contact area between basal and labyrinth layers with disruption of the labyrinth trophoblastic cells and deposition of homogeneous substances of damaged erythrocytes and cell debris (arrow head), necrosis of the spongiotrophoblast cells (n), and endothelial to mesenchymal transition "glycogen cells formation" (*); scale bar= 200 µm. **(I-L)** Congestion of the maternal arterial canals and the inflammatory infiltration of leukocytes (arrow heads), and cytolysis of giant cells (arrows); scale bar= 100 µm. **(M-P)** Constriction of fetal vessels (Fv), thickening of the interhemal membranes (*) and vasogenic edema (arrow heads); scale bar= 50 µm. B: basal layer, L: Labyrinth layer, Fc fetal capillary, Ms: maternal sinusoid, Cy: cytotrophoblast cell, Sy: syncytiotrophoblast cell. Histopathological scores in Table 2. *(Double column fitting image)*

**Table 2.** Effect of gestational diabetes and its treatment with cinnamaldehyde and glyburide/metformin-HCl on the development of placental lesions at term pregnancy.

| Histopathological changes | Group score | | | |
|---|---|---|---|---|
| | NP | GD | GD+Ci | GD+Gly/Met |
| *Basal layer* | | | | |
| Cystic degeneration of glycogen cells | - | ++ | -/+ | -/+ |
| Necrosis of spongiotrophoblast cells | -/+ | + | -/+ | -/+ |
| Congestion of maternal arterial canals | - | ++ | -/+ | + |
| Inflammatory infiltration of leukocytes | -/+ | ++ | + | + |
| Cytolysis of giant cells | - | + | -/+ | -/+ |
| *Labyrinth layer* | | | | |
| Disruption of trophoblastic cells | - | + | - | - |
| Endothelial to mesenchymal transition (glycogen cells formation) | -/+ | ++ | -/+ | -/+ |
| Constriction of fetal vessels | - | ++ | -/+ | + |
| Vasogenic edema | -/+ | + | -/+ | -/+ |
| Thickening of the interhemal membranes | - | ++ | - | + |

(-) not present, (-/+) few "<10%", (+) moderate and (++) extensive ($n$=3). NP: normally pregnants, GD: gestationally diabetics, GD+Ci: gestationally diabetics with cinnamaldehyde treatment, GD+Gly/Met: gestationally diabetics with glyburide/metformin-HCl treatment.

## 4. Discussion

As expected, the FSD/STZ treated rats model elevated the maternal blood glucose level and reduced the serum insulin concentration; the two hallmarks of GDM [2]. In brief, FSD-intake induced the insulin resistance via the glucose/fatty acid cycle [86]. The high fat consumption activates the oxidation of fatty acids that decreases the insulin-mediated reduction of liver glucose production and blunts the glucose-uptake by the peripheral tissues. In addition, the low dose injection of STZ caused a mild disruption of the maternal pancreatic β-cells (through ROS generation) and lowered the insulin secretion. On the other hand, this model induced fetal

hyperglycemia and hyperinsulinemia. Concisely, maternal hyperglycemia resulted in a transplacental flux of glucose down the concentration gradient into the fetal circulation, and according to Pedersen's hypothesis, this fetal hyperglycemia stimulated the fetal pancreatic β-cell hyperplasia to increase the insulin secretion in order to achieve normoglycemia [87, 88]. Due to the short pregnancy period of rats, and as Ci is effective for a treatment duration of 21 to 60 days [25], Ci was given one week prior to mating. Treatment intervention after the development of GDM (next to STZ injection at the late early gestation), will not be enough for the proper effectiveness of Ci. In human pregnancy, time factor allows Ci administration after GDM diagnosis at the late early- or mid-gestation. Consistent with previous studies [19, 21, 25, 26], Ci and Gly/Met administration effectively alleviated the maternal and fetal glucose and insulin levels through their insulin sensitizers and secretagogues actions.

Unlike human gestational diabetes, we didn't get fetal macrosomia from this GDM-rat model. This could be attributed to the fact that the accumulation of fat in human fetuses occurs intra-uterine, while it takes place in rats after birth due to the short pregnancy period [89]. Although the change in fetal body weight between GD and NP-groups was insignificant, we found a remarkable increase in the placenta weight which, in turn, increased the placental-to-fetal weight ratio that indicates a greater placental insufficiency under the diabetic condition. The increase in placental weight might be induced as an adaptive response to the circulatory disturbances in the labyrinth zone [11]. Ci administration kept the fetal body weight and placental weight close to normal and prevented the placental insufficiency. Conversely, Gly/Met partially alleviated the increase in placental weight, but significantly decreased fetal body weight that exacerbates the placental insufficiency and could reflect fetal growth restriction condition. A meta-analysis by Balsells *et*

*al.,* [90] that compared effects of glyburide, metformin and insulin as treatments for gestational diabetes, described similar effects.

Our results show a significant decrease in Hct% and liver iron content, together with an increase in the serum EPO level of GD-group; which are signs of fetal hypoxia [91]. Oxygenation of the fetal tissues mainly depends on the placental ability to provide sufficient oxygen supply to the fetal circulation. In GDM, shortage of fetal oxygen supply is often a result of placental insufficiency that leads to intrauterine hypoxia [91]. This hypoxia stimulates fetal EPO synthesis to increase fetal erythrocyte production in order to elevate the oxygen-carrying capacity. Effective erythropoiesis is dependent on the adequate availability of fetal iron [92]. GDM induces alterations in placental iron homeostasis at the level of uptake, transport, sensing, and regulation [93]. In our study, GDM induced overexpression of the placental transferrin receptor 2 (*Tfr2*), scavenger receptor class A member 5 (*Scara5*), ferroportin 1 (*Fpn1*) and ceruloplasmin (*Cp*) (p-value $< 0.05$ and $+0.5 \leq \log2FC \leq -0.5$; Supplemental Table S4). *Tfr2* and *Scara5* mediate the iron uptake from the maternal blood, *Fpn1* is the only known transporter regulating iron export towards the fetus, while *Cp* facilitates the export of $Fe^{3+}$ by *Fpn1* and oxidizes the toxic $Fe^{2+}$ to $Fe^{3+}$ to reduce the placental oxidative stress [93]. Upregulation of these genes might be an adaptational mechanism to increase the placental iron transfer to the fetus in order to compensate the observed decrease in the fetal liver iron stores. In a previous investigation, we observed a decrease in the hemoglobin and hematocrit levels of the gestational diabetic rats [94] that could be a sign of maternal iron deficiency. We hypothesize that the impaired iron supply to fetal erythroid precursors caused anemia that does not respond to the elevated EPO and led to hypoxia. As Ci intake restored the fetal liver iron content, placental expression levels of iron homeostasis genes were nearly stabilized and Hct% and serum EPO were markedly improved. On the contrary, fetal

liver iron deficiency observed in the Gly/Met-treated group may explain the overexpressed placental iron homeostasis genes, the drop in fetal Hct% and the related increase in the serum EPO, and support the assumption of the growth-restricted fetuses of that group.

Upregulation of genes involved in the angiogenesis (*Vegfr2*, *Sema*/*Plx*/Nrp, *Slit*/*Robo*, *ephrin*/*Eph*, *Notch*/*Dll4*, integrins, *Vasp*, *Adamts*, *Smoc1* and *Esx1*) was apparent in GD-placenta that might leading to the observed overgrowth and hypervascularization (Fig. 8A). Concerning vascular remodelling (Fig. 8B1), VEGF dependent activation initiates a signal transduction resulting in the tyrosine phosphorylation PLCd1, that causes an increase in the intracellular inositol-1,4,5-trisphosphate (IP3) and release of $Ca^{2+}$ from the endoplasmic reticulum (ER). The elevated intracellular $Ca^{2+}$ stimulates eNOS to produce NO using L-arginine as a substrate [95]. In healthy pregnancies, NO activates Guc1 within ECs and VSMCs to produce cGMP from guanosine triphosphate (GTP). cGMP-activated protein kinase G (PKG), then, inhibits the $Ca^{2+}$ release from ER, reduces the sensitivity of VSMCs contractile elements to $Ca^{2+}$, and induces the vascular dilation to enhance circulation, and nutrient and gas uptake by the fetus [8, 57]. In this study, and as a result of the overexpressed *Pdes* (that block NO vasodilatory effect) in GD-placenta, an intravascular pressure could be generated causing a mechanical stretch on the surface of the VSMCs that triggered the upregulation of the cation channels (*Trpv2* and *Pkd2l1*) [59, 60] which, together with the downregulation of *Saraf* that prevents excess intracellular $Ca^{2+}$ filling, cause accumulation of $Ca^{2+}$ in VSMCs [61] (Fig. 8B lower right section). *Rrad* is a Ras-related GTPase that is selectively overexpressed in muscles of type II diabetic patients [62]. Under elevated intracellular $Ca^{2+}$ concentration, RRAD activate $Ca^{2+}$/calmodulin-dependent protein kinase 2 [63] that phosphorylates the myosin light chain kinase (MLCK) augmenting VSMCs contractility and aggravates vasoconstriction [64, 65]. Moreover, and secondary to the

643 vasoconstrictive remodeling, the GD-placenta overexpressed *Pdgfc* that might promote narrowing
644 of the vascular lumens [30]. It is relevant here to mention that, the sustained vasoconstriction and
645 stenosis observed in GD-placenta may possibly have initiated a positive feedback mechanism that
646 stimulated *eNOS* overexpression to produce more NO to increase vascular dilatation. But in fact,
647 NO might be only inducing placental angiogenesis without initiating vasodilation (due to the
648 previously mentioned factors) creating a vicious cycle that exacerbates placental
649 hypervascularization (Fig. 8). This may explain the altered fetoplacental characteristics and could
650 be, also, a key reason for the observed fetal hypoxia. The detected vascular constriction and
651 stenosis in GD-placenta might be signs of developing placental hypertension. This study does not
652 address the impact of GDM in the development of maternal hypertension and the role of both
653 treating agents, which will be exciting to elucidate in the future work.
654 Contrary to the Gly/Met effect, the reduced upregulation of the angiogenesis-related transcripts
655 was prominently observed in the Ci-treated group. In addition, the exclusive upregulation of *Ptgs2*
656 and *Gch1*, along with the downregulation of *Ache* might reveal a potent vasodilatory effect of Ci
657 (Fig. 8B lower section). Briefly, PTGS2 in ECs and VSMCs converts the arachidonic acid to the
658 unstable endoperoxide-prostaglandin $H_2$ ($PGH_2$) which finally isomerized into prostaglandin $I_2$
659 ($PGI_2$) that relax the blood vessels [31]. Gch1 is the key enzyme of tetrahydrobiopterin (BH4)
660 generation which is essential for NO bioavailability and functionality, keeping the highest
661 efficiency of eNOS-dependent vasorelaxation and prevent its overexpression [66]. In contrast,
662 ACHE hydrolyzes acetylcholine (which is a natural vasodilator released by ECs) into choline and
663 acetate and terminate its vasorelaxant effect [67]. These transcriptional changes are consistent with
664 previous studies that Ci provokes vascular relaxation by reducing PDEs activity [96, 97], inhibiting

665 $Ca^{2+}$ influx [28, 31], improving acetylcholine induced vasodilatation [29], and inhibiting PDGF-
666 induced the intimal hyperplasia [30].

667 Transcripts encoding carboxylic acid transporters related to glucose metabolism were
668 upregulated in the diabetic placenta suggesting a high placental metabolic rate. This hypothesis
669 was confirmed through the elevated levels of OAs from glycolysis and TCA-cycle in GD-placenta.
670 Metabolically active ECs with the observed upregulation of *Mct4* might reflect extrusion of large
671 amounts of protons and lactate, generating an extracellular acidic milieu. This could induce the
672 overexpression of sugar/$H^+$-dependent *Slc45*, which could intensify the placental sugars uptake
673 creating a vicious circle (Fig. 8B2). It has recently become evident that EC metabolism relates
674 directly to the angiogenic growth factors responsivity and, thus, angiogenesis regulation [98].
675 Overexpressed *Mct2* presumably reflects lactate reuptake by ECs leading to a secondary increase
676 in *Vegf* and *Vegfr2* expression that might trigger an additional pro-angiogenic signaling and
677 exacerbates the placental hypervascularization [99, 100]. Previous human studies demonstrated
678 that GDM causes an increase in lactate concentration with a decrease in oxygen saturation in the
679 umbilical vein, indicating a high placental metabolic rate and an elevated placental oxygen
680 consumption [11, 101]. Placental ECs normally consume about 40% of the supplied oxygen in
681 glycolysis to synthesize ATP required for the ionic pumping through the fetoplacental unit, and
682 for migration, proliferation and differentiation of ECs in the new sprouting vessels in response to
683 VEGF/NO signaling [99, 102]. Thus, the observed high metabolic activity of GD-placenta may
684 reveal that ECs consumed more oxygen in the development of the hypervascularization that could
685 reduce the transvascular transfer of oxygen to the fetus generating a relative fetal hypoxic status.
686 Although the expression of the carboxylic acid transporters was significantly downregulated only
687 in response to Ci, both Ci and Gly/Met prevented the placental metabolic hyperactivity that could

688 be due to their potent hypoglycemic impact. This proposes a different mechanism for Gly/Met
689 action.

690 With respect to redox status, maternal hyperglycemia in this study seems to expose the placenta
691 to oxidative stress. Our results showed a significant increase in FRAP, SOD, CAT, GPX and Trx
692 activities in GD-placenta. These antioxidant defenses, however, were inadequate to neutralize the
693 oxidative stress as shown by the overexpressed *Cyp1a1* and *Cyp2s1*, and the increased
694 concentrations of MDA. We proposed that as placental ECs were metabolically active, the
695 mitochondrial respiration and the electron transport chain (ETC) might be enhanced resulting in
696 excessive ROS output [12, 13] (Fig. 8B3). ROS might trigger eNOS-uncoupling by oxidizing the
697 BH4 (eNOS-cofactor) generating more $O_2^{\cdot-}$ and $ONOO^-$ that exaggerated the cellular stress and
698 attenuated the placenta vascular reactivity [10]. The observed increase in the total reduced
699 glutathione content (that reflects a high level of the oxidized form glutathione; GSSG) could also
700 increase the eNOS uncoupling through the process of eNOS S-glutathionylation (eNOS-SG),
701 leading to mass $O_2^{\cdot-}$ production [103]. Notably, the induction of certain antioxidant enzymes
702 (SOD, CAT, GPX and Trx) in GD-placenta suggests that they may be the major enzymes in the
703 defense against ROS within the feto-placental unit in order to minimize the hyperglycemic-
704 oxidative damage from further insult into the fetus. Results of the current study revealed that the
705 placentae of the Ci-treated group are better protected against the oxidative damage caused by GDM
706 as compared to Gly/Met. Increasing the GSH pool through the administration of Ci, together with
707 increased expression of *Gch1*, could provide high recovery of eNOS functionality and NO
708 availability. This indicates that the total antioxidant defenses of the Ci-treated placenta were not
709 activated as a result of the absence of excessive ROS production secondary to its free radical
710 scavenging action, hypoglycemic effect and placental metabolic activity controlling [25, 85]. In

contrast, the changes in the antioxidant activity in Gly/Met-group is not effective in decreasing the placental lipid peroxidation even when the placenta metabolism was alleviated.

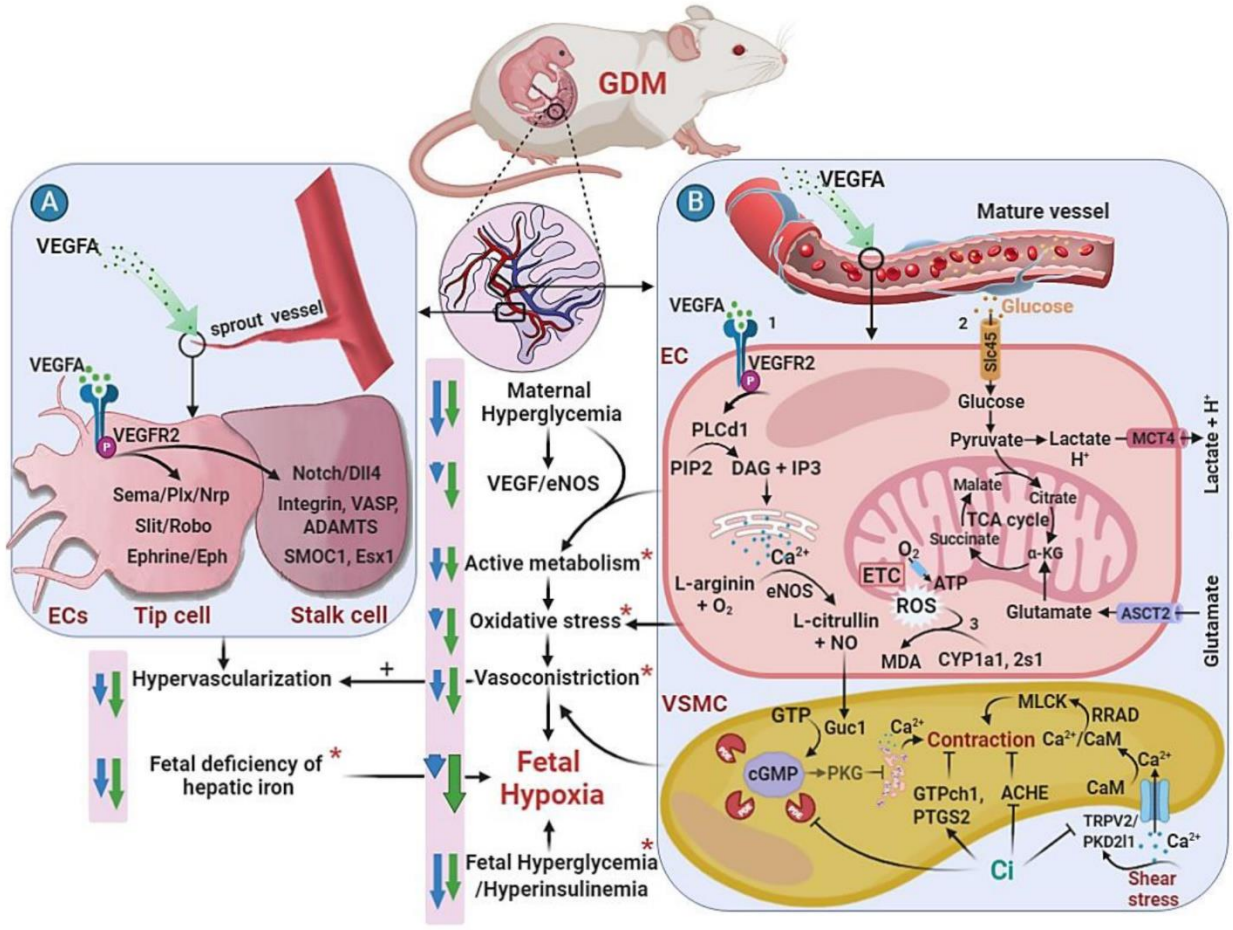



**Fig. 8.** Mechanistic model summarizes the multi-modal effect of Ci in mitigating GDM-induced placental vascular dysfunction and protection from the fetal hypoxia. **(A)** Vascular sprout formation and hypervascularization. **(B1)** Vasoconstrictive remodelling. **(B2)** Endothelial metabolic activity. **(B3)** Endothelial oxidative stress. ACHE: acetylcholinesterase, ADAMTS: ADAM-metallopeptidase, α-KG: alpha-ketoglutarate, ASCT2: glutamate transporter, CaM: calmodulin, Ci: cinnamaldehyde, cGMP: cyclic guanosine monophosphate, CYP: cytochrome P450, eNOS: endothelial nitric oxide synthase, ECs: endothelial cells, Esx1: homeobox gene, ETC: electron transport chain, GDM: gestational diabetes mellitus, GTP: guanosine triphosphate, GTPch1: GTP-cyclohydrolase 1, Guc1: soluble guanylyl cyclase, IP3: inositol 1,4,5-trisphosphate, MCT4: monocarboxylate transporter 4, MDA: malondialdehyde, MLCK: myosin light chain kinase, NO: nitric oxide, PDEs: phosphodiesterases, PLCd1: phospholipase Cd1, PKD2l1: polycystin 2 like 1 transient receptor potential cation channel, PKG: cGMP-activated protein kinase G, PTGS2: prostaglandin-endoperoxide synthase 2, ROS: reactive oxygen species, RRAD: ras-related glycolysis inhibitor and calcium channel regulator, Sema/Plx/Nrp: semaphorin/plexin/neuropilin, Slc45: solute carrier 45, SMOC1: secreted modular calcium-binding protein 1, TCA: tri-carboxylic acid cycle, TRPV2: transient receptor potential cation channel V2; VASP: vasodilator-stimulated phosphoprotein; VEGFR2: vascular endothelial growth factor receptor 2, VEGFA: vascular endothelial

745 growth factor A, VSMCs: vascular smooth muscle cells, (*): factors lead to fetal hypoxia, (+): stimulator
746 of hypervascularization, Green arrows: effects of cinnamaldehyde compared to GDM, Blue arrows: effects
747 of glyburide/metformin-HCl compared to GDM. *(Double column fitting image)*

748 It is worthwhile to mention that Gly and Met can both cross the placenta [104, 105], affecting
749 fetal physiology and causing neonatal hypoglycemia, electrolyte imbalance and other adverse
750 effects [22-24]. To our knowledge, there are no reports concerning the transfer of Ci through the
751 placenta to the fetal circulation. Using UPLC-MS, we were unable to detect Ci itself in the
752 placental tissue and the fetal blood. However, cinnamic acid (one of Ci metabolites) was detected
753 in both samples at very low concentrations (below the limit of quantification; LOQ, data not
754 shown) which might reflect that Ci (and its metabolites) do not transfer significantly through the
755 placental barriers. We working to increase the sensitivity of our technique to give more robust
756 conclusion.

757 The current study did not address the contribution of fetal sex on the impact of gestational
758 diabetes and the utility of the tested drugs, that need to be considered in the upcoming
759 investigations. Assessing placental morphology using the double labelled immunohistochemistry
760 and the stereological principals [106] would provide further mechanistic understanding for the role
761 of the treatments on GDM-induced placental pathophysiology.

762 **5. Conclusions**

763 With the rapid global rise in GDM prevalence, a deeper understanding of GDM
764 pathophysiology is required in order to develop safe and efficient therapies to protect from the
765 negative impact of GDM on pregnancy and offspring health. This study demonstrated five possible
766 factors that occurred in combination and resulted in a decrease in oxygen delivery to the fetuses
767 from GDM. Placental hypervascularization/vasoconstrictive remodelling, active metabolism and
768 oxidative stress, as well as the fetal hyperglycemia/ hyperinsulinemia and hepatic iron deficiency

may well be behind the observed fetal hypoxic status (Fig. 8). Combining the transcript profiling with biochemical and histopathological approaches provided an integrative insight into the mechanism of Ci action that indicated potent effects on multiple control points. Daily oral intake of Ci prior to and during the gestational period alleviated the materno-fetal glycemia, diminished the placental vasculopathy, restored the fetal liver iron content and protected from fetal hypoxia. The lack of effectiveness of Gly/Met on the impaired placental vascular performance draws the medical community's attention to the vital role of the placenta in developing effective medications for GDM. Ci would be a promising candidate for further clinical tests as an alternative medicine for GDM.

## Declaration of Competing Interest

The authors have declared that no competing interest exists.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## Authors' Contributions

A.A. and A.H. conceived and designed the study; G.B. and H.A. developed the research concept; A.H. performed the experimental work with the help of S.A., M.A., E.P. and H.A.; A.A., G.B., H.A. and A.H. analyzed and discussed the results; A.H. wrote the manuscript; G.B. and A.A. critically revised the manuscript and contributed to the writing. All authors revised and approved the final manuscript.


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## References

[1] L. Yuen, P. Saeedi, M. Riaz, S. Karuranga, H. Divakar, N. Levitt, X. Yang, D. Simmons, Projections of the prevalence of hyperglycaemia in pregnancy in 2019 and beyond: Results from the International Diabetes Federation Diabetes Atlas, 9th edition, Diabetes Res Clin Pract 157 (2019) 107841.

[2] J.F. Plows, J.L. Stanley, P.N. Baker, C.M. Reynolds, M.H. Vickers, The Pathophysiology of Gestational Diabetes Mellitus, Int J Mol Sci 19(11) (2018).

[3] E. Ganguly, N. Hula, F. Spaans, C.M. Cooke, S.T. Davidge, Placenta-targeted treatment strategies: An opportunity to impact fetal development and improve offspring health later in life, Pharmacol Res 157 (2020) 104836.

[4] L. Leach, A. Taylor, F. Sciota, Vascular dysfunction in the diabetic placenta: causes and consequences, J Anat 215(1) (2009) 69-76.

[5] F.R. Derubertis, P.A. Craven, Activation of protein kinase C in glomerular cells in diabetes. Mechanisms and potential links to the pathogenesis of diabetic glomerulopathy, Diabetes 43(1) (1994) 1-8.

[6] L. Xia, H. Wang, S. Munk, H. Frecker, H.J. Goldberg, I.G. Fantus, C.I. Whiteside, Reactive oxygen species, PKC-beta1, and PKC-zeta mediate high-glucose-induced vascular endothelial growth factor expression in mesangial cells, Am J Physiol Endocrinol Metab 293(5) (2007) E1280-8.

[7] L. Lassance, H. Miedl, M. Absenger, F. Diaz-Perez, U. Lang, G. Desoye, U. Hiden, Hyperinsulinemia stimulates angiogenesis of human fetoplacental endothelial cells: a possible role of insulin in placental hypervascularization in diabetes mellitus, J Clin Endocrinol Metab 98(9) (2013) E1438-47.

[8] D.B. Chen, J. Zheng, Regulation of placental angiogenesis, Microcirculation 21(1) (2014) 15-25.

[9] N. Ghiso, R.M. Rohan, S. Amano, R. Garland, A.P. Adamis, Suppression of hypoxia-associated vascular endothelial growth factor gene expression by nitric oxide via cGMP, Invest Ophthalmol Vis Sci 40(6) (1999) 1033-9.

[10] M. Lappas, U. Hiden, G. Desoye, J. Froehlich, S. Hauguel-de Mouzon, A. Jawerbaum, The role of oxidative stress in the pathophysiology of gestational diabetes mellitus, Antioxid Redox Signal 15(12) (2011) 3061-100.

[11] C. Escudero, M. González, J. Acurio, F. Valenzuela, L. Sobrevia, The role of placenta in the fetal programming associated to gestational diabetes, in: L. Sobrevia (Ed.), Gestational Diabetes - Causes, Diagnosis and Treatment, InTechOpen, London, 2013, pp. 135-162.

[12] A. Koziel, A. Woyda-Ploszczyca, A. Kicinska, W. Jarmuszkiewicz, The influence of high glucose on the aerobic metabolism of endothelial EA.hy926 cells, Pflugers Arch 464(6) (2012) 657-69.

[13] D. Dymkowska, B. Drabarek, P. Podszywalow-Bartnicka, J. Szczepanowska, K. Zablocki, Hyperglycaemia modifies energy metabolism and reactive oxygen species formation in endothelial cells in vitro, Arch Biochem Biophys 542 (2014) 7-13.

[14] B.S. Carson, A.F. Philips, M.A. Simmons, F.C. Battaglia, G. Meschia, The effects of sustained insulin infusion upon glucose uptake and oxygenation in the ovine fetus, Pediatr Res 14 (1980) 147-152.

[15] J.A. Widness, K.A. Teramo, G.K. Clemons, P. Voutilainen, U.H. Stenman, S.M. McKinlay, R. Schwartz, Direct relationship of antepartum glucose control and fetal erythropoietin in human type 1 (insulin-dependent) diabetic pregnancy, Diabetologia 33(6) (1990) 378-83.

[16] N. Magon, V. Seshiah, Gestational diabetes mellitus: Non-insulin management, Indian J Endocrinol Metab 15(4) (2011) 284-93.

[17] H. Zhu, B. Chen, Y. Cheng, Y. Zhou, Y.-S. Yan, Q. Luo, Y. Jiang, J.-Z. Sheng, G.-L. Ding, H.-F. Huang, Insulin therapy of gestational diabetes does not fully protect offspring from diet-induced metabolic disorders, Diabetes (2019) db181151.

[18] L. Jovanovic, D.J. Pettitt, Treatment with insulin and its analogs in pregnancies complicated by diabetes, Diabetes Care 30 Suppl 2 (2007) S220-4.

[19] R.I. Holt, K.D. Lambert, The use of oral hypoglycaemic agents in pregnancy, Diabet Med 31(3) (2014) 282-291.

[20] G.M. Brawerman, V.W. Dolinsky, Therapies for gestational diabetes and their implications for maternal and offspring health: Evidence from human and animal studies, Pharmacol Res 130 (2018) 52-73.

[21] O.O. Erejuwa, S.A. Sulaiman, M.S. Wahab, S.K. Salam, M.S. Salleh, S. Gurtu, Antioxidant protective effect of glibenclamide and metformin in combination with honey in pancreas of streptozotocin-induced diabetic rats, Int J Mol Sci 11(5) (2010) 2056-66.

[22] Z. Kimber-Trojnar, B. Marciniak, J. Patro-Malysza, K. Skorzynska-Dziduszko, E. Poniedzialek-Czajkowska, R. Mierzynski, K. Galczynski, M. Trojnar, B. Leszczynska-Gorzelak, J. Oleszczuk, Is glyburide safe in pregnancy?, Curr Pharm Biotechnol 15(1) (2014) 100-12.

[23] Z. Nachum, N. Zafran, R. Salim, N. Hissin, J. Hasanein, Y. Gam Ze Letova, A. Suleiman, E. Yefet, Glyburide Versus Metformin and Their Combination for the Treatment of Gestational Diabetes Mellitus: A Randomized Controlled Study, Diabetes Care 40(3) (2017) 332-337.

[24] G. Priya, S. Kalra, Metformin in the management of diabetes during pregnancy and lactation, Drugs Context 7 (2018) 212523.

[25] R. Zhu, H. Liu, C. Liu, L. Wang, R. Ma, B. Chen, L. Li, J. Niu, M. Fu, D. Zhang, S. Gao, Cinnamaldehyde in diabetes: A review of pharmacology, pharmacokinetics and safety, Pharmacol Res 122 (2017) 78-89.

[26] A.A. Hosni, A.A. Abdel-Moneim, E.S. Abdel-Reheim, S.M. Mohamed, H. Helmy, Cinnamaldehyde potentially attenuates gestational hyperglycemia in rats through modulation of PPAR gamma, proinflammatory cytokines and oxidative stress, Biomed Pharmacother 88 (2017) 52-60.

[27] E.S. Abdel-Reheim, A. Adel Abd-Elmoneim, A.A. Hosni, Fatty-Sucrosed diet/ minimal dose of streptozotocin-treated rat: a novel model of gestational diabetes mellitus, metabolic and inflammatory insight, J Diabetes Metab 5(9) (2014) 430.

[28] H.M. El-Bassossy, A. Fahmy, D. Badawy, Cinnamaldehyde protects from the hypertension associated with diabetes, Food Chem Toxicol 49(11) (2011) 3007-12.

[29] X. Guo, W. Sun, L. Huang, L. Wu, Y. Hou, L. Qin, T. Liu, Effect of Cinnamaldehyde on Glucose Metabolism and Vessel Function, Med Sci Monit 23 (2017) 3844-3853.

[30] N.E. Buglak, W. Jiang, E.S.M. Bahnson, Cinnamic aldehyde inhibits vascular smooth muscle cell proliferation and neointimal hyperplasia in Zucker Diabetic Fatty rats, Redox Biol 19 (2018) 166-178.

[31] Y.L. Xue, H.X. Shi, F. Murad, K. Bian, Vasodilatory effects of cinnamaldehyde and its mechanism of action in the rat aorta, Vasc Health Risk Manag 7 (2011) 273-80.

[32] G. Pozsgai, J.V. Bodkin, R. Graepel, S. Bevan, D.A. Andersson, S.D. Brain, Evidence for the pathophysiological relevance of TRPA1 receptors in the cardiovascular system in vivo, Cardiovasc Res 87(4) (2010) 760-8.

[33] G. Raffai, B. Kim, S. Park, G. Khang, D. Lee, P.M. Vanhoutte, Cinnamaldehyde and cinnamaldehyde-containing micelles induce relaxation of isolated porcine coronary arteries: role of nitric oxide and calcium, Int J Nanomedicine 9 (2014) 2557-66.

[34] A. Onder, D. Yilmaz-Oral, I. Jerkovic, A.O. Akdemir, S. Gur, Evaluation of relaxant responses properties of cinnamon essential oil and its major component, cinnamaldehyde on human and rat corpus cavernosum, Int Braz J Urol 45(5) (2019) 1033-1042.

[35] P. Subash Babu, S. Prabuseenivasan, S. Ignacimuthu, Cinnamaldehyde--a potential antidiabetic agent, Phytomedicine 14(1) (2007) 15-22.

[36] J. Maganha, E.d.S. Rocha, M.A.F. Brandão, V.M. Peters, M.d.O. Guerra, Embryo development alteration in rats treated with lapachol, Braz Arch Biol Technol 49 (2006) 927-934.

[37] R. Bamberg, T. Gwyn, J. Miller, M. Thompson, P. Transou, The effects of over-anticoagulated blood on hematocrit values by the microcentrifuge method, Clin Lab Sci 21(3) (2008) 146-50.

[38] R. Blust, A. Linden, E. Verheyen, W. Decleir, Evaluation of microwave heating digestion and graphite furnace atomic absorption spectrometry with continuum source background correction for the determination of iron, copper and cadmium in brine shrimp., J Anal Atom Spectrom 3 (1988) 387-393.

[39] B. Bolon, Protocols for Placental Histology, in: B. Anne Croy, A. Yamada, F. DeMayo, S. Lee Adamson (Eds.), The Guide to Investigation of Mouse Pregnancy, Academic Press2014, pp. 537-544.

[40] Q. Li, B. Yao, A. Endler, L. Chen, F. Shibasaki, H. Cheng, Int6/eIF3e Silencing Promotes Placenta Angiogenesis in a Rat Model of Pre-eclampsia, Sci Rep 8(1) (2018) 8944.

[41] M. Gamble, The hematoxylins and eosin, in: J.D. Bancroft, A. Stevens (Eds.), Theory and practice of histological techniques, Chruchill Livingstone, London, 2008, pp. 121-134.

[42] R.B. Myers, J.L. Fredenburgh, W.E. Grizzle, Carbohydrates, in: J.D. Bancroft, A. Stevens (Eds.), Theory and practice of histological techniques, Chruchill Liveingstone, London, 2008, pp. 161-186.

[43] C.A. Schneider, W.S. Rasband, K.W. Eliceiri, NIH Image to ImageJ: 25 years of image analysis, Nat Methods 9(7) (2012) 671-5.

[44] S. Ponglowhapan, D.B. Church, M. Khalid, Differences in the expression of luteinizing hormone and follicle-stimulating hormone receptors in the lower urinary tract between intact and gonadectomised male and female dogs, Domest Anim Endocrinol 34(4) (2008) 339-51.

[45] G. Neufeld, O. Kessler, The semaphorins: versatile regulators of tumour progression and tumour angiogenesis, Nat Rev Cancer 8(8) (2008) 632-45.

[46] W.X. Liao, D.A. Wing, J.G. Geng, D.B. Chen, Perspectives of SLIT/ROBO signaling in placental angiogenesis, Histol Histopathol 25(9) (2010) 1181-90.

[47] N. Cheng, D.M. Brantley, J. Chen, The ephrins and Eph receptors in angiogenesis, Cytokine Growth Factor Rev 13(1) (2002) 75-85.

[48] C. Mailhos, U. Modlich, J. Lewis, A. Harris, R. Bicknell, D. Ish-Horowicz, Delta4, an endothelial specific Notch ligand expressed at sites of physiological and tumor angiogenesis, Differentiation 69 (2001) 135-144.

[49] G.H. Mahabeleshwar, W. Feng, K. Reddy, E.F. Plow, T.V. Byzova, Mechanisms of integrin-vascular endothelial growth factor receptor cross-activation in angiogenesis, Circ Res 101(6) (2007) 570-80.

[50] Z. Liu, Y. Wang, C. Dou, M. Xu, L. Sun, L. Wang, B. Yao, Q. Li, W. Yang, K. Tu, Q. Liu, Hypoxia-induced up-regulation of VASP promotes invasiveness and metastasis of hepatocellular carcinoma, Theranostics 8(17) (2018) 4649-4663.

[51] Y.Z. Jiang, K. Wang, Y. Li, C.F. Dai, P. Wang, C. Kendziorski, D.B. Chen, J. Zheng, Enhanced cellular responses and distinct gene profiles in human fetoplacental artery endothelial cells under chronic low oxygen, Biol Reprod 89(6) (2013) 133.

[52] Q. Gao, H.P. Mok, J. Zhuang, Secreted modular calcium-binding proteins in pathophysiological processes and embryonic development, Chin Med J (Engl) 132(20) (2019) 2476-2484.

[53] A.R. Duselis, P.B. Vrana, Aberrant growth and pattern formation in Peromyscus hybrid placental development, Biol Reprod 83(6) (2010) 988-96.

[54] K. Zhang, E.S. Han, T.H. Dellinger, J. Lu, S. Nam, R.A. Anderson, J.H. Yim, W. Wen, Cinnamon extract reduces VEGF expression via suppressing HIF-1alpha gene expression and inhibits tumor growth in mice, Mol Carcinog 56(2) (2017) 436-446.

[55] T.A.F. Ferro, E.B. Souza, M.A.M. Suarez, J.F.S. Rodrigues, D.M.S. Pereira, S.J.F. Mendes, L.F. Gonzaga, M. Machado, M.R.Q. Bomfim, J.B. Calixto, J.L. Arbiser, V. Monteiro-Neto, E. Andre, E.S. Fernandes, Topical Application of Cinnamaldehyde Promotes Faster Healing of Skin Wounds Infected with Pseudomonas aeruginosa, Molecules 24(8) (2019).

[56] K. Patra, S. Jana, A. Sarkar, D.P. Mandal, S. Bhattacharjee, The inhibition of hypoxia-induced angiogenesis and metastasis by cinnamaldehyde is mediated by decreasing HIF-1alpha protein synthesis via PI3K/Akt pathway, Biofactors 45(3) (2019) 401-415.

[57] H. He, V.J. Venema, X. Gu, R.C. Venema, M.B. Marrero, R.B. Caldwell, Vascular endothelial growth factor signals endothelial cell production of nitric oxide and prostacyclin through flk-1/KDR activation of c-Src, J Biol Chem 274(35) (1999) 25130-5.

[58] E. Mergia, J. Stegbauer, Role of Phosphodiesterase 5 and Cyclic GMP in Hypertension, Curr Hypertens Rep 18(5) (2016) 39.

[59] M.K. McGahon, J.A. Fernandez, D.P. Dash, J. McKee, D.A. Simpson, A.V. Zholos, J.G. McGeown, T.M. Curtis, TRPV2 Channels Contribute to Stretch-Activated Cation Currents and Myogenic Constriction in Retinal Arterioles, Invest Ophthalmol Vis Sci 57(13) (2016) 5637-5647.

[60] R. Sharif-Naeini, J.H. Folgering, D. Bichet, F. Duprat, I. Lauritzen, M. Arhatte, M. Jodar, A. Dedman, F.C. Chatelain, U. Schulte, K. Retailleau, L. Loufrani, A. Patel, F. Sachs, P. Delmas, D.J. Peters, E. Honore, Polycystin-1 and -2 dosage regulates pressure sensing, Cell 139(3) (2009) 587-96.

[61] R. Palty, A. Raveh, I. Kaminsky, R. Meller, E. Reuveny, SARAF inactivates the store operated calcium entry machinery to prevent excess calcium refilling, Cell 149(2) (2012) 425-38.

[62] C. Reynet, C.R. Kahn, Rad: a member of the Ras family overexpressed in muscle of type II diabetic humans, Science 262(5138) (1993) 1441-4.
[63] J.S. Moyers, P.J. Bilan, J. Zhu, C.R. Kahn, Rad and Rad-related GTPases interact with calmodulin and calmodulin-dependent protein kinase II, J Biol Chem 272(18) (1997) 11832-9.
[64] I. Kim, H.D. Je, C. Gallant, Q. Zhan, D.V. Riper, J.A. Badwey, H.A. Singer, K.G. Morgan, Ca2+-calmodulin-dependent protein kinase II-dependent activation of contractility in ferret aorta, J Physiol 526 Pt 2 (2000) 367-74.
[65] A. Rokolya, H.A. Singer, Inhibition of CaM kinase II activation and force maintenance by KN-93 in arterial smooth muscle, Am J Physiol Cell Physiol 278(3) (2000) C537-45.
[66] S. Wang, J. Xu, P. Song, Y. Wu, J. Zhang, H. Chul Choi, M.H. Zou, Acute inhibition of guanosine triphosphate cyclohydrolase 1 uncouples endothelial nitric oxide synthase and elevates blood pressure, Hypertension 52(3) (2008) 484-90.
[67] C. Wilson, M.D. Lee, J.G. McCarron, Acetylcholine released by endothelial cells facilitates flow-mediated dilatation, J Physiol 594(24) (2016) 7267-7307.
[68] R. Bartolke, J.J. Heinisch, H. Wieczorek, O. Vitavska, Proton-associated sucrose transport of mammalian solute carrier family 45: an analysis in Saccharomyces cerevisiae, Biochem J 464(2) (2014) 193-201.
[69] A.M. Pajor, Sodium-coupled dicarboxylate and citrate transporters from the SLC13 family, Pflugers Arch 466(1) (2014) 119-30.
[70] Y.D. Bhutia, V. Ganapathy, Glutamine transporters in mammalian cells and their functions in physiology and cancer, Biochim Biophys Acta 1863(10) (2016) 2531-9.
[71] F. Grimolizzi, L. Arranz, Multiple faces of succinate beyond metabolism in blood, Haematologica 103(10) (2018) 1586-1592.
[72] A.P. Halestrap, The monocarboxylate transporter family--Structure and functional characterization, IUBMB Life 64(1) (2012) 1-9.
[73] R.B. Ceddia, G. Sweeney, Creatine supplementation increases glucose oxidation and AMPK phosphorylation and reduces lactate production in L6 rat skeletal muscle cells, J Physiol 555(Pt 2) (2004) 409-21.
[74] L.J. van Loon, R. Murphy, A.M. Oosterlaar, D. Cameron-Smith, M. Hargreaves, A.J. Wagenmakers, R. Snow, Creatine supplementation increases glycogen storage but not GLUT-4 expression in human skeletal muscle, Clin Sci (Lond) 106(1) (2004) 99-106.
[75] L. Gehre, O. Gorgette, S. Perrinet, M.C. Prevost, M. Ducatez, A.M. Giebel, D.E. Nelson, S.G. Ball, A. Subtil, Sequestration of host metabolism by an intracellular pathogen, Elife 5 (2016) e12552.

[76] S. Furukawa, S. Hayashi, K. Usuda, M. Abe, S. Hagio, I. Ogawa, Toxicological pathology in the rat placenta, J Toxicol Pathol 24(2) (2011) 95-111.

[77] J.C. Kovacic, S. Dimmeler, R.P. Harvey, T. Finkel, E. Aikawa, G. Krenning, A.H. Baker, Endothelial to Mesenchymal Transition in Cardiovascular Disease: JACC State-of-the-Art Review, J Am Coll Cardiol 73(2) (2019) 190-209.

[78] Y. Wen, J. Gu, S.L. Li, M.A. Reddy, R. Natarajan, J.L. Nadler, Elevated glucose and diabetes promote interleukin-12 cytokine gene expression in mouse macrophages, Endocrinology 147(5) (2006) 2518-25.

[79] J.R. Doherty, J.L. Cleveland, Targeting lactate metabolism for cancer therapeutics, J Clin Invest 123(9) (2013) 3685-92.

[80] M. Gul, N. Bayat, A. Cetin, R.A. Kepekci, Y. Simsek, B. Kayhan, U. Turhan, A. Otlu, Histopathological, Ultrastructural and Apoptotic Changes in Diabetic Rat Placenta, Balkan Med J 32(3) (2015) 296-302.

[81] D. Bhattacharjee, S.K. Mondal, P. Garain, P. Mandal, R.N. Ray, G. Dey, Histopathological study with immunohistochemical expression of vascular endothelial growth factor in placentas of hyperglycemic and diabetic women, J Lab Physicians 9(4) (2017) 227-233.

[82] P.H. Bui, E.L. Hsu, O. Hankinson, Fatty acid hydroperoxides support cytochrome P450 2S1-mediated bioactivation of benzo[a]pyrene-7,8-dihydrodiol, Mol Pharmacol 76(5) (2009) 1044-52.

[83] B. Huang, J. Bao, Y.R. Cao, H.F. Gao, Y. Jin, Cytochrome P450 1A1 (CYP1A1) Catalyzes Lipid Peroxidation of Oleic Acid-Induced HepG2 Cells, Biochemistry (Mosc) 83(5) (2018) 595-602.

[84] O.M. Cecilia, C.G. Jose Alberto, N.P. Jose, C.M. Ernesto German, L.C. Ana Karen, R.P. Luis Miguel, R.R. Ricardo Raul, R.C. Adolfo Daniel, Oxidative Stress as the Main Target in Diabetic Retinopathy Pathophysiology, J Diabetes Res 2019 (2019) 8562408.

[85] P. Subash-Babu, A.A. Alshatwi, S. Ignacimuthu, Beneficial Antioxidative and Antiperoxidative Effect of Cinnamaldehyde Protect Streptozotocin-Induced Pancreatic beta-Cells Damage in Wistar Rats, Biomol Ther (Seoul) 22(1) (2014) 47-54.

[86] F. Zhang, C. Ye, G. Li, W. Ding, W. Zhou, H. Zhu, G. Chen, T. Luo, M. Guang, Y. Liu, D. Zhang, S. Zheng, J. Yang, Y. Gu, X. Xie, M. Luo, The rat model of type 2 diabetic mellitus and its glycometabolism characters, Exp Anim 52(5) (2003) 401-7.

[87] J. Pedersen, The pregnant diabetic and her newborn. Problems and management, Munksgaard, Copenhagen,, 1967.

[88] M. Abdul-Hamid, A.A. Hosni, A. Abdel Moneim, N. Moustafa, E.S. Abdel-Reheim, Maternal and fetal hepatic injury in gestational diabetic rats: protective role of cinnamaldehyde and ellagic acid, Asian J Pharm Clin Res 11(7) (2018) 105-112.

[89] B. Dallaqua, F.H. Saito, T. Rodrigues, I.M. Calderon, M.V. Rudge, E. Herrera, D.C. Damasceno, Treatment with Azadirachta indica in diabetic pregnant rats: negative effects on maternal outcome, J Ethnopharmacol 143(3) (2012) 805-11.

[90] M. Balsells, A. Garcia-Patterson, I. Sola, M. Roque, I. Gich, R. Corcoy, Glibenclamide, metformin, and insulin for the treatment of gestational diabetes: a systematic review and meta-analysis, BMJ 350 (2015) h102.

[91] K.A. Teramo, J.A. Widness, Increased fetal plasma and amniotic fluid erythropoietin concentrations: markers of intrauterine hypoxia, Neonatology 95(2) (2009) 105-16.

[92] C.D. Petry, M.A. Eaton, J.D. Wobken, M.M. Mills, D.E. Johnson, M.K. Georgieff, Iron deficiency of liver, heart, and brain in newborn infants of diabetic mothers, J Pediatr 121(1) (1992) 109-14.

[93] J. Zaugg, H. Melhem, X. Huang, M. Wegner, M. Baumann, D. Surbek, M. Korner, C. Albrecht, Gestational diabetes mellitus affects placental iron homeostasis: Mechanism and clinical implications, FASEB J 34(6) (2020) 7311-7329.

[94] E.S. Abdel-Reheim, A.A. Hosni, Ameliorative effects of cinnamaldehyde and ellagic acid on hematological alterations associated with pathophysiology of gestational diabetes mellitus in albino rats, Egypt. J. Zool. 60 (2013) 375-396.

[95] D.K. Kakuda, M.J. Sweet, C.L. Mac Leod, D.A. Hume, D. Markovich, CAT2-mediated L-arginine transport and nitric oxide production in activated macrophages, Biochem J 340 ( Pt 2) (1999) 549-53.

[96] A. Yanaga, H. Goto, T. Nakagawa, H. Hikiami, N. Shibahara, Y. Shimada, Cinnamaldehyde induces endothelium-dependent and -independent vasorelaxant action on isolated rat aorta, Biol Pharm Bull 29(12) (2006) 2415-8.

[97] M. Dell'Agli, G.V. Galli, E. Dal Cero, F. Belluti, R. Matera, E. Zironi, G. Pagliuca, E. Bosisio, Potent inhibition of human phosphodiesterase-5 by icariin derivatives, J Nat Prod 71(9) (2008) 1513-7.

[98] G. Eelen, P. de Zeeuw, M. Simons, P. Carmeliet, Endothelial cell metabolism in normal and diseased vasculature, Circ Res 116(7) (2015) 1231-44.

[99] N. Sawada, Z. Arany, Metabolic Regulation of Angiogenesis in Diabetes and Aging, Physiology (Bethesda) 32(4) (2017) 290-307.

[100] V.L. Payen, E. Mina, V.F. Van Hee, P.E. Porporato, P. Sonveaux, Monocarboxylate transporters in cancer, Mol Metab 33 (2020) 48-66.

[101] E. Taricco, T. Radaelli, G. Rossi, M.S. Nobile de Santis, G.P. Bulfamante, L. Avagliano, I. Cetin, Effects of gestational diabetes on fetal oxygen and glucose levels in vivo, BJOG 116(13) (2009) 1729-35.

[102] A.M. Carter, Placental oxygen consumption. Part I: in vivo studies--a review, Placenta 21 Suppl A (2000) S31-7.

[103] J.L. Zweier, C.A. Chen, L.J. Druhan, S-glutathionylation reshapes our understanding of endothelial nitric oxide synthase uncoupling and nitric oxide/reactive oxygen species-mediated signaling, Antioxid Redox Signal 14(10) (2011) 1769-75.

[104] E. Vanky, K. Zahlsen, O. Spigset, S.M. Carlsen, Placental passage of metformin in women with polycystic ovary syndrome, Fertil Steril 83(5) (2005) 1575-8.

[105] E. Sivan, B. Feldman, M. Dolitzki, N. Nevo, N. Dekel, A. Karasik, Glyburide crosses the placenta in vivo in pregnant rats, Diabetologia 38(7) (1995) 753-6.

[106] K. De Clercq, J. Lopez-Tello, J. Vriens, A.N. Sferruzzi-Perri, Double-label immunohistochemistry to assess labyrinth structure of the mouse placenta with stereology, Placenta 94 (2020) 44-47.