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Assessment of the hypoglycemic effect of *Cyanthillium cinereum* (L.) H. Rob. and its dual impact on uterine contraction in gestational diabetic rats

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ABSTRACT

Objective: This study investigates the effects of *Cyanthillium cinereum* (L.) H. Rob. ethanolic extract (CCE) on gestational diabetes mellitus (GDM) in rats using biochemical, histological, and uterine contractility studies.
Methods: Diabetes was induced in pregnant rats using streptozotocin (60 mg/kg). CCE was administered orally at low (50 mg/kg BW) and high (500 mg/kg BW) doses from gestation day 7 to day 21. Maternal evaluations included body weight, gravid uterine weight, and biochemical assays for serum glucose, insulin, lipids, and liver enzymes. Fetal outcomes included fetal size. Histological analyses of maternal pancreatic and uterine tissues and uterine contractility studies using *ex vivo* muscle strip experiments were also performed.
Results: CCE and metformin (MET) significantly reduced elevated blood glucose levels and improved the Islets of Langerhans area compared to the GDM group ($P < 0.05$). Both treatments showed a trend toward increased insulin levels ($P > 0.05$) and significantly reduced lipids, AST, and ALP levels ($P < 0.05$). High-dose CCE and MET increased gravid uterine weight and fetal size ($P < 0.05$) while showing a trend toward reducing placental weight and index ($P > 0.05$). Histological analysis revealed increased fiber area and decreased interstitial space in uterine sections ($P < 0.05$). *Ex vivo*, CCE enhanced spontaneous and oxytocin-induced contractions ($P < 0.05$), while MET had no effect.
Conclusion: CCE reduces elevated glucose levels and exhibits hypolipidemic and hepatoprotective effects, improving maternal and fetal outcomes in GDM. Its uterine contractility effects suggest potential as a complementary therapy to MET for GDM management.

1. Introduction

Hyperglycemia management is an important requirement that affects the health outcomes of diabetic mothers and newborns (Wang et al., 2019). Labor dystocia (failure to progress in labor) contributes to increased cesarean birth rates in gestational diabetes mellitus (GDM), with around 40% of emergency cesarean sections arising from prolonged labor, failed induction, or obstructed labor (Olerich et al., 2022). Ineffective labor with serious complications for both mother and neonate in GDM contributes to uterine excitation-contraction dysfunction, causing a shift toward a lower amplitude and decreased frequency of contractions and a reduction in the responsiveness to clinically used agonists such as oxytocin (OT) (Al-Qahtani et al., 2012). Consistent with this, clinical data shows that women with GDM spend a longer time in active labor, both in spontaneous and induced active labor compared to women without GDM (Nevander et al., 2023). In addition, higher doses of OT are necessary to induce or augment labor in GDM (Reinl et al., 2017). Thus, both human and animal data shows that poor myometrial contractility may be an important factor found in pregnancies complicated by diabetes (Al-Qahtani et al., 2012; Nevander

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et al., 2023). In *in vitro* studies on rats, the underlying mechanism was related to a gradual loss of uterine muscle mass, reduced calcium channel expression, decreased intracellular calcium signaling, and a diminished response of OT and high potassium (KCl)-induced contraction (Prendergast, 2020). Additionally, ultrastructural studies of smooth muscle cells in diabetic pregnant rats reveal that diabetes affects the organization of the muscle layers, the contractile apparatus, and the cell proliferation profile in the pregnant myometrium (Wagih et al., 2021).

Products from indigenous plants are used in alternative medicine to aid pregnant women, and research studies are conducted to verify or refute, their efficacy and safety on uterine contractility for pregnant women (Jun et al., 2021; Bafor and Kupittayanant, 2020). *Cyanthillium cinereum* (L.) H. Rob. (CC) also named as *Vernonia cinerea* (L.), can be consumed as a herbal tea and is high in antioxidants and anti-inflammatories, and has also been popularly recommended in Thai medicine for smoking cessation (Kaji and Puttarak, 2022). Previous literature showed various therapeutic uses of *Cyanthillium cinereum* (L.) H. Rob including antibacterial, anticancer, antispasmodic, and antimalarial activity (Ramya et al., 2021). Leaves of CC extract contain polyphenols and flavonoids that inhibit alpha-amylase and alpha-glucosidase activity, suggesting their potential as an alternative or complementary treatment for diabetes (Alara et al., 2018). The whole plant CC extract modulates insulin resistance by regulating glucose and lipid homeostasis and improving insulin sensitivity in high-fat diet-induced obese mice (Naowaboot et al., 2018). A clinical trial in patients with type 2 diabetes who received herbal treatment with CC root paste at a dose of 6 g per day for 6 months showed a significant decrease in the glycemic state, including glucose, hemoglobin A1c, cholesterol, low-density lipoprotein (LDL) cholesterol, and triglyceride levels in patients with type 2 diabetes (Sayeed et al., 2013). Despite this promising result, there is however, no study conducted for treatment of GDM with CC extract, in either women or animals.

Drugs such as metformin (MET) are given to help control glucose levels, and potentially improve labour outcomes in women with GDM (Chiswick et al., 2015), but are costly, have side effects, and not always available in developing world communities. The present study was designed to determine if treatment with CC extract could be used as an alternative anti-diabetic therapeutic against GDM complications, especially ineffective labors and induction of labor.

The effect of CC extract in GDM was determined in this study by investigating blood glucose levels, lipid profiles, liver enzymes, maternal reproductive performances, and fetal outcome parameters, assessed *in vivo*. The alterations of the myometrium's ultrastructure and function in GDM in response to CC extract were investigated both *in vivo* and *ex vivo*. Some studies were also conducted using MET, to compare the CC data to a standard antidiabetic drug.

Together, these investigations aimed to evaluate the potential of CC extract as a cost-effective and accessible therapeutic option for improving glycemic control, restoring myometrial function, and mitigating adverse pregnancy outcomes associated with GDM, while providing comparative insights against the standard antidiabetic drug, MET.

2. Methods

2.1. Ethics statement

The animal care followed the guidelines set forth by the Committee of Care and Use of Laboratory Animal Resources, National Research Council of Thailand. The experimental procedures received approval from the Institutional Animal Care and Use Committee at Suranaree University of Technology, Nakhon Ratchasima, Thailand (Ethical approval numbers 13/2560).

2.2. Chemicals

Analytical-grade chemicals were obtained from Sigma-Aldrich Chemical Co., Singapore. Key chemicals included streptozotocin (Lot#WXBC2044V), citric acid (Lot#BCBL0135V), sodium citrate tribasic dihydrate (Lot#BCBP0175V), MET (1,1-Dimethylbiguanide hydrochloride, Lot#BCBR6505V), and oxytocin (Lot#SLBM4784V). All stock and working solutions were prepared and stored following the manufacturer's guidelines.

2.3. Plant preparation

Fresh CC whole plants were collected from Ayutthaya province (Pak Kran district), Thailand, during the winter season, under natural conditions. A voucher specimen was deposited and identified at the Royal Forest Department of Thailand, Bangkok, Thailand (BKF no. 193578). The CC whole plants were washed, oven-dried at a low temperature (<40 °C) for two days, ground into a powder, and weighed. Soxhlet extraction (Borosil, India) was employed to extract the plant powder with 70% ethanol (4 L) for 6 h, followed by filtration using Whatman® No.1 filter paper. Subsequently, a rotary evaporator (Buchi Rotavapor® R-210, BUCHI (Thailand) Ltd., Thailand) was utilized to evaporate the extract to dryness under reduced pressure (1000 mbar–60 mbar) at a low temperature (<40 °C), and then lyophilized (Labconco, USA). The CC extract (CCE) was stored in a sealed container at −20 °C until use.

2.4. Phytochemical analysis

CCE presented as a sticky yellow to dark brownish powder with an extraction yield of 6.95%. Preliminary qualitative screening was conducted to identify its phytochemical constituents. The chemical compositions of CCE underwent quantitative analysis using an Agilent Technologies 7890A gas chromatography, coupled with an Agilent Technologies 5975C (EI) mass spectrometer (GC-MS). All separated compounds were identified by comparing their mass spectra with those from the National Institute of Standards and Technology (NIST) and Wiley Libraries (Singh et al., 2020).

2.5. Animals

Female Wistar rats aged between 8 and 10 weeks and weighing between 250 and 300 g were individually housed in cages measuring 24 x 15 × 15 cm. These cages were maintained under 12-h light-dark illumination cycles at a constant temperature of 25 ± 0.5 °C and a relative humidity level of 45–50%. The rats were provided with a standard laboratory diet containing 24% crude protein, 4.5% crude fat, 5% crude fiber, 1% calcium, and 0.7% phosphorus (CP. Co. Ltd, Thailand), along with *ad libitum* access to water. Vaginal smears from adult female Wistar rats were examined daily to identify the presence of cornified epithelial cells in the pro-estrous phase and then were selected for mating with a fertile male rat (2 females: 1 male) for an overnight stay. The presence of a mucous plug of spermatozoa in vaginal smears the following morning was designated as gestational day 0.

2.6. Experimental design

Acute and chronic toxicity studies of CCE conducted previously demonstrated a no observable adverse effect level (NOAEL) at a dose of 2000 mg/kg/day in animal models (Johnson and Varghese, 2023). Based on these findings, the NOAEL limit for pregnant animals is estimated to be 1000 mg/kg/day, following Guideline 415 of the OECD (Organisation for Economic Co-operation and Development) for reproductive toxicity studies (OECD Guideline for Testing of Chemicals, 1983). In this study, CCE treatment doses of 500 mg/kg/day (1/2 NOAEL) and 50 mg/kg/day (1/20 NOAEL) were selected as the maximum and minimum testing doses for pregnant animals,

respectively. Similarly, MET treatment at a dose of 100 mg/kg/day was selected (Johnson and Varghese, 2023). Therefore, the experimental pregnant rats were divided into five groups as follows:

Group 1 (Non-GDM): Non-gestational diabetic control group received the equivalent volume of the vehicle by oral gavage once a day for 15 days, from gestation day 7–21.

Group 2 (GDM): Gestational diabetic control group received the equivalent volume of the vehicle by oral gavage once a day for 15 days, from gestation day 7–21.

Group 3 (MET): Gestational diabetic rats treated with MET at a dose level of 100 mg/kg in 1 mL by oral gavage once a day for 15 days, from gestation day 7–21.

Group 4 (LDCCE): Gestational diabetic rats treated with CCE at a dose level of 50 mg/kg in 1 mL by oral gavage once a day for 15 days, from gestation day 7–21.

Group 5 (HDCCE): Gestational diabetic rats treated with CCE at a dose level of 500 mg/kg in 1 mL by oral gavage once a day for 15 days, from gestation day 7–21.

2.6.1. Gestational diabetes model

Diabetes mellitus was induced on gestational day 5 by a single intraperitoneal injection (*i.p.*) of streptozotocin (STZ) at a dosage of 60 mg/kg body weight, dissolved in a 0.1 M cold citrate buffer solution with a pH of 6.5. This chemical-induced diabetes model selectively destroys pancreatic β-cells, leading to insulin deficiency and hyperglycemia in an experimental GDM animal model (Bueno et al., 2020). The GDM rat model, which exhibits metabolic alterations after mid-pregnancy, is a relevant tool for studying the pathophysiology of diabetes-induced complications during pregnancy. These alterations are comparable to maternal diabetes observed in humans (He et al., 2020). Afterward, a small blood droplet from a tail vein puncture was monitored using an Accu-Chek Performa glucometer with glucose strips from Roche Diagnostics on gestational day 7, and glucose levels equal to or higher than 200 mg/dL were considered indicative of gestational diabetes (Bueno et al., 2020). The percentage of diabetes induction, survival, and mortality rates were monitored. After diabetic confirmation, the rats were divided into the *in vivo* study and the *ex vivo* study.

Maternal weight was recorded each day and used to calculate the correct dose per kg body weight of rats until term.

2.6.2. Blood sampling and biochemical measurements

On gestation days 0, 7, 14, and 21, maternal blood samples were collected from the tail vein. Rats were killed using CO_2 asphyxia on gestation day 21. Blood collected from cardiac puncture was centrifuged, and serum samples were assayed for total cholesterol (TC), triglyceride (TRI), high-density lipoprotein (HDL cholesterol), low-density lipoprotein (LDL cholesterol), and liver enzymes (aspartate transaminase (AST), alanine transaminase (ALT), and alkaline phosphatase (ALP)) using a Mindray BS-120 blood chemistry analyzer. Insulin level was measured using a commercial sandwich ELISA kit (FineTest®) following the manufacturer's instructions.

2.6.3. Measurements of maternal and fetal outcomes

The gravid uterus, containing fetuses with their placentas, was removed and weighed. Uterine horns were exposed to count corpora lutea (CL), implantations, and fetus numbers for the calculation of pre-implantation (number of CL – number of implantations x 100/number of CL) and post-implantation loss (number of implantations – number of live fetuses x 100/number of implantations) rates, as well as resorptions and dead fetuses. Fetuses and placentas were removed and weighed to calculate the placental index. Fetal weight was classified as small for pregnancy age (SPA), appropriate for pregnancy age (APA), or large for pregnancy age (LPA) based on non-GDM pups' birth weight mean ± 1.0 SD. Crown-rump length (CRL) of the pups was measured to determine embryonic size.

2.6.4. Histology

Pancreas and uterine samples were taken, weighed, cut into small pieces, and immediately fixed in 10% neutral formalin. Sections at 5 μm were prepared for hematoxylin and eosin (H&E) staining and visualized at 200x (pancreas samples) and 40x (uterine samples) on a light microscope, Olympus BX51 with an Olympus DP20 camera (Olympus Optical Co., Ltd., Japan). The area of the islets' outlined in square micrometers (μm^2) was used to quantify the pancreatic islet area. Pancreatic mass index (pancreas weight/total body weight x 100) was calculated. The color threshold was determined for the relative proportion of interstitial space and muscle fiber quantification using ImageJ software, version 1.52.

2.6.5. Muscle strip experiment

The experimental pregnant rats were divided into two groups as follows: non-gestational diabetic rats (non-GDM) with glucose levels averaging 122.58 ± 30.54 mg/dL and gestational diabetic rats (GDM) with glucose levels averaging 449.25 ± 132.69 mg/dL. On gestation day 21, the pregnant rats were humanely killed under CO_2 asphyxia. Routine laboratory investigations and standard solutions were performed to examine uterine contractility (Wray and Arrowsmith, 2021). In detail, the uterine horn was removed and immediately bathed in a physiological saline solution (pH 7.4) containing 154 mM NaCl, 10 mM HEPES, 8 mM glucose, 5.4 mM KCl, 2 mM $CaCl_2$, and 1.2 mM $MgSO_4$. A high potassium solution (40 mM KCl) and oxytocin (10 nM OT) were directly prepared in the physiological solution. The uterine strips (4–6 strips preparations obtained from each animal) were mounted vertically with each end connected between metal hooks and a force transducer (ADInstruments Pty Ltd., Spain) in an organ bath apparatus containing physiological solution at 37 °C. Isometric recording under 1 g of tension and an equilibration time of 30 min were allowed. Changes in isometric force were recorded and measured using the PowerLab system software (ADInstruments Pty Ltd., Australia). The electrical signal from a force-displacement transducer was converted into a digital signal and recorded on a computer using Chart software version 5.

2.7. Statistical analysis

The results of the biochemical measurements, maternal reproductive performances, and fetal outcome parameters (except for fetal classification, which is presented as a percentage), pancreatic islet area, pancreatic mass index, and relative proportion of interstitial space and muscle fiber quantification are presented as means ± standard deviation (SD) with 'N' represents the number of animals (biological replicates). One-way analysis of variance (ANOVA) followed by Tukey's post-hoc test was utilized to compare the mean values among different treatment groups, and the chi-square test was used for fetal classification.

The results of the muscle strip experiment are presented as means ± SD with 'N' represents the number of uterine samples (each one from a different animal, biological replicates). Changes in the area under the contraction (AUC) were compared appropriately to spontaneous contractions, high-K depolarization (KCl), or oxytocin (OT) alone, which were taken as 100%. The contractility responses for each parameter were expressed as percentages of changes. A paired Student's t-test was used to test the significance of differences within the same strip (before and after the test period), and an unpaired Student's t-test was used to compare differences between groups (non-GDM and GDM strips).

All data were graphed using Microcal Origin software (©OriginLab Corporation., Massachusetts, USA) and analyzed using SPSS version 17.0 (SPSS Inc., USA) with a probability level less than 5% ($P < 0.05$) was considered statistically significant.

3. Results

3.1. Effect of CCE on blood glucose levels, body weight, and food consumption

Overall, 82% of the rats in this study exhibited high blood glucose levels after STZ injection. The survival rate of pregnant rats following STZ administration is shown in Fig. 1.

The blood glucose levels, body weight, and food consumption rates for all experimental groups throughout pregnancy are presented in Fig. 2. Blood glucose levels of non-GDM rats remained under 200 mg/dL throughout pregnancy, while significantly elevated blood glucose levels were detected in all GDM rats (compared to the non-GDM group, $P < 0.05$), persisting until term. Treatment with MET and CCE (at both doses) did not alter blood glucose levels in GDM rats as measured on gestation days 7 and 14. However, a significant reduction in blood glucose levels was observed on gestation day 21 with MET and both doses of CCE (compared to the GDM group, $P < 0.05$) as seen in Fig. 2A. Maternal body weight (BW) was comparable among groups before STZ induction. However, a significant reduction was observed in all GDM groups after STZ induction on gestation days 14 and 21 compared to the non-GDM group ($P < 0.05$). On gestation day 21, treatment with MET and CCE at both doses showed a trend toward weight restoration, although this did not reach statistical significance (Fig. 2B). Food consumption in all GDM groups did not differ from non-GDM throughout the entire pregnant period ($P > 0.05$) as seen in Fig. 2C.

3.2. Effects of CCE on insulin level, lipid profiles and liver enzymes

The effects of CCE on insulin level, lipid profiles, and liver enzymes at gestation day 21 are presented in Fig. 3. GDM rats had a significant reduction in serum insulin levels compared to the non-GDM group ($P < 0.05$). Treatment with MET and CCE at both doses showed a trend toward increased serum insulin levels compared to the GDM group (Fig. 3A). Regarding lipid profiles, gestational diabetes led to a significant increase in serum TC, TRI, and LDL levels compared to the non-GDM group ($P < 0.05$), while HDL levels showed a trend towards reduction in the GDM group. Serum TC and TRI levels were significantly reduced with MET and CCE treatment at both doses compared to the GDM group ($P < 0.05$). Although no significance was observed, at both doses of CCE treatment increased serum HDL and reduced serum LDL levels were noted. However, a significant increase in serum HDL levels

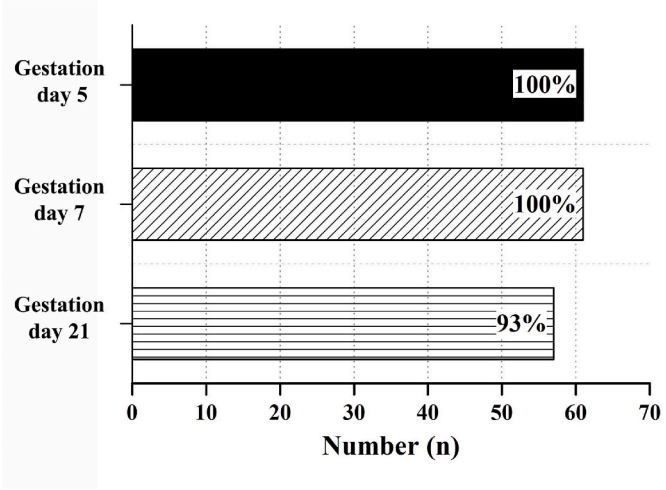

Fig. 1. Survival rate of pregnant rats following STZ administration. Survival rates of pregnant rats on gestational days 5 (after streptozotocin (STZ) administration), 7, and 21 are shown. Data are presented as percentages, representing the proportion of surviving rats throughout the experimental period.

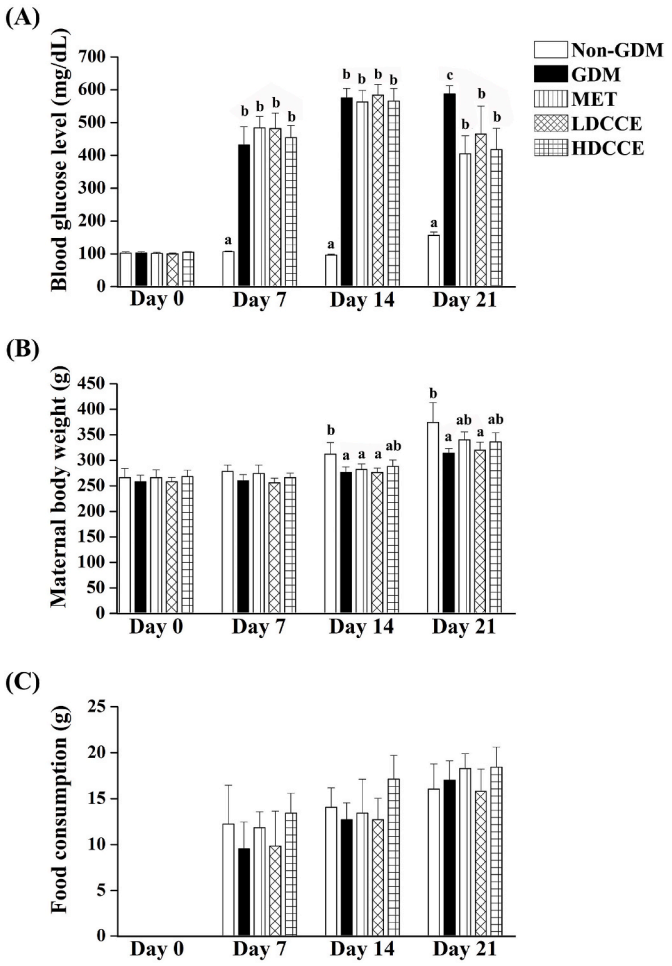

Fig. 2. Blood glucose levels, body weight, and food consumption in all experiment groups throughout pregnancy. (A–C) Effects of CCE on blood glucose levels, body weight, and food consumption on gestation day 0, 7, 14, and 21, respectively (n = 5). Data are shown as mean ± SD and analyzed by one-way ANOVA, followed by Tukey's post hoc test ($P < 0.05$). Groups bearing different superscripted letters on the bars indicate statistical significance between groups. Non-GDM = non-gestational diabetes mellitus, GDM = gestational diabetes mellitus, MET = metformin, LDCCE = low dose CCE, HDCCE = high dose CCE.

was found with MET compared to the GDM group ($P < 0.05$) as seen in Fig. 3B-E. In terms of liver enzymes, serum AST, ALT, and ALP levels were significantly increased in GDM compared to the non-GDM group ($P < 0.05$). All liver enzymes were significantly reduced with MET compared to the GDM group ($P < 0.05$). Additionally, serum AST levels were significantly reduced with CCE treatment at both doses, and serum ALT levels were significantly reduced with high-dose CCE treatment compared to the GDM group ($P < 0.05$) as seen in Fig. 3F-H.

3.3. Effects of CCE on maternal reproductive performances and fetal outcome parameters

The effects of CCE on maternal reproductive performances, fetal outcome parameters and fetal classification are presented in Table 1. For maternal reproductive performances, GDM produced a significant reduction in gravid uterus weight, number of live fetuses, and a significant increase in post-implantation loss rate compared to the non-GDM group ($P < 0.05$). Treatment with MET and high-dose CCE significantly improved gravid uterus weight, enhancing maternal reproductive performances, with no alteration in other parameters including the number of corpora lutea, number of implantations, number of live fetuses,

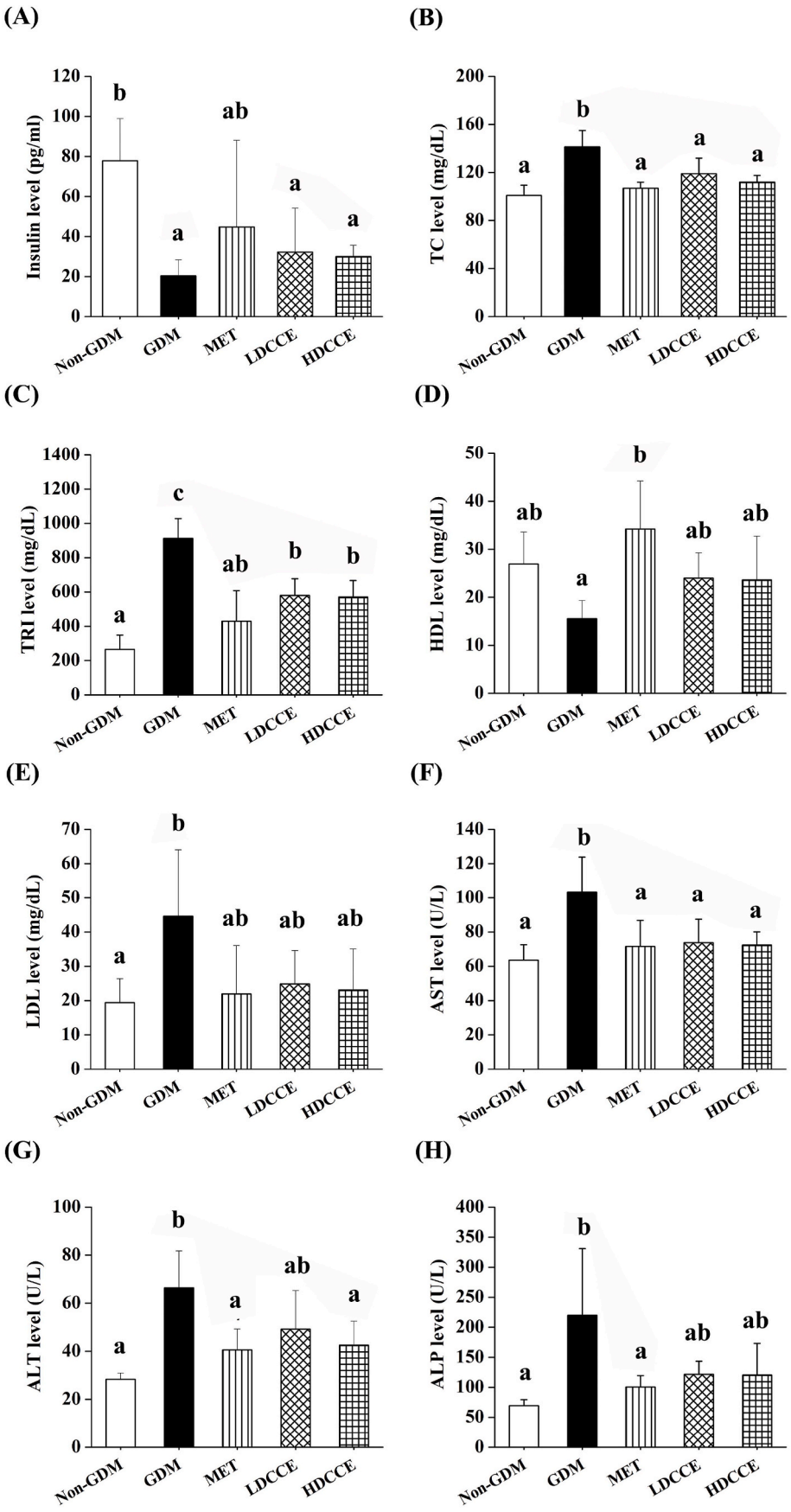

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Fig. 3. Effects of CCE on insulin level, lipid profiles and liver enzymes in GDM rats. (A–H) Effects of CCE on serum insulin, total cholesterol (TC), triglyceride (TRI), high-density lipoprotein (HDL), low-density lipoprotein (LDL), aspartate transaminase (AST), alanine transaminase (ALT), and alkaline phosphatase (ALP) levels, respectively (n = 5). Data are shown as mean ± SD and analyzed by one-way ANOVA, followed by Tukey's post hoc test ($P < 0.05$). Groups bearing different superscripted letters on the bars indicate statistical significance between groups. Non-GDM = non-gestational diabetes mellitus, GDM = gestational diabetes mellitus, MET = metformin, LDCCE = low dose CCE, HDCCE = high dose CCE.

Table 1
Effects of CCE on maternal reproductive performances, fetal outcome parameters and fetal classification.

Parameters	Treatment groups					n
	Non-GDM	GDM	MET	LDCCE	HDCCE	
Maternal reproductive performances						
Gravid uterus (g)	73.54 ± 1.21 c	43.42 ± 6.79 a	54.84 ± 2.60 b	48.86 ± 3.03 ab	54.35 ± 3.81 b	5
Number of corpora lutea	12.80 ± 1.10	13.00 ± 1.22	12.80 ± 1.30	12.60 ± 0.55	14.20 ± 0.84	5
Number of implantations	11.80 ± 1.10	10.40 ± 2.51	12.20 ± 1.10	11.00 ± 1.87	12.00 ± 1.58	5
Number of live fetuses	11.40 ± 0.89 b	8.00 ± 2.24 a	10.00 ± 0.71 ab	9.20 ± 1.30 ab	9.80 ± 0.45 ab	5
Number of dead fetuses	0.00 ± 0.00	0.20 ± 0.45	0.20 ± 0.45	0.20 ± 0.45	0.00 ± 0.00	5
Pre-implantation loss rates (%)	5.36 ± 6.84	24.39 ± 18.89	1.92 ± 3.85	8.17 ± 11.79	10.75 ± 7.22	4
Post-implantation loss rates (%)	3.85 ± 7.69 a	27.26 ± 17.33 b	15.97 ± 5.10 ab	13.41 ± 6.58 ab	14.71 ± 6.52 ab	4
Fetal outcome parameters						
Fetal weight (g)	4.73 ± 0.58 b	3.27 ± 0.56 a	3.34 ± 0.45 a	3.36 ± 0.56 a	3.45 ± 0.58 a	5
Placental weight (g)	0.56 ± 0.08 ab	0.65 ± 0.22 c	0.62 ± 0.16 bc	0.51 ± 0.10 a	0.59 ± 0.11 abc	5
Placental index (%)	12.10 ± 2.65 a	20.37 ± 7.92 c	19.03 ± 5.77 c	15.81 ± 5.26 b	17.59 ± 5.15 bc	5
Crown rump length (mm)	39.63 ± 2.45 c	35.88 ± 2.14 a	37.30 ± 2.05 b	36.85 ± 1.83 ab	37.45 ± 2.72 b	5
Fetal classification						
SPA fetuses †	7 (12.30%)	37 (92.50%)	48 (96.00%)	42 (91.30%)	42 (85.70%)	
APA fetuses †	37 (64.90%)	3 (7.50%)	2 (4.00%)	4 (8.70%)	7 (14.70%)	
LPA fetuses †	13 (22.80%)	0 (0.00%)	0 (0.00%)	0 (0.00%)	0 (0.00%)	

N = number of animals. Data shown as mean ± SD for maternal reproductive performances and fetal outcome parameters and analyzed by one-way ANOVA, followed by Tukey's post hoc test ($P < 0.05$). Groups bearing different superscripted letters on the row indicate statistical significance between groups. Data shown as proportion of total N (%) for fetal classification and analyzed by †Chi-square test statistic (X^2) = 288.33 > df = 8, 15.507 ($P < 0.05$). Non-GDM = non-gestational diabetes mellitus, GDM = gestational diabetes mellitus, MET = metformin, LDCCE = low dose CCE, HDCCE = high dose CCE. SPA = small for pregnancy age, APA = appropriate for pregnancy age, LPA = large for pregnancy age.

number of dead fetuses, and pre- and post-implantation loss rates. Regarding fetal outcome parameters, GDM showed a significant decrease in fetal weight and crown-rump length, while there was a significant increase in placental weight and index compared to the non-GDM group ($P < 0.05$). Treatment with MET and high-dose CCE significantly improved crown-rump length compared to the GDM group ($P < 0.05$). Low-dose CCE treatment significantly decreased placental weight and index compared to the GDM group ($P < 0.05$). Furthermore, for fetal classification, higher proportions of small for pregnancy age (SPA), lower proportions of appropriate for pregnancy age (APA), and absence of large for pregnancy age (LPA) were observed in all GDM cases. Both doses of CCE treatment showed a significant improvement in APA proportion, especially with high-dose CCE ($x^2 = 288.33$, $P < 0.05$).

3.4. Effects of CCE on pancreatic islet architecture, islet area quantification and pancreatic mass index

The histological images of the pancreas at gestation day 21 are illustrated in Fig. 4, showing black tracings of islet area at 200× magnification (Fig. 4A). In non-GDM, normal distribution and well-structured islet structures with regularly shaped and uniformly distributed intercellular spaces of pancreatic cells are observed. Conversely, in GDM caused by STZ-induced islet cell death and consequent hyperglycemia, resulting in a deterioration in islet area. Additionally, MET and CCE at both doses markedly improved islet area restoration compared to the GDM group. The quantification of islet area from H&E staining slides and pancreatic mass index are presented in Fig. 4C-D. The quantification of islet area from H&E-stained slides was based on the mean values of 25 islets per group (n = 5, with 5 islets per animal). The results showed that the islet area was significantly reduced in the GDM group (5952.54 ± 2259.31 μm^2) compared to the non-GDM group (37158.85 ± 9647.31 μm^2, $P < 0.05$). Treatment with MET and CCE at both doses significantly improved the islet area (24626.68 ± 8424.96 μm^2, 20701.12 ± 9911.02 μm^2, and 22435.73 ± 7673.43 μm^2, respectively) compared to the GDM group ($P < 0.05$). The pancreatic mass index was significantly decreased in the GDM group (0.19 ± 0.00%) compared to the non-GDM group (0.30 ± 0.00%, $P < 0.05$). Conversely, treatment with MET and high-dose CCE significantly improved the pancreatic mass index compared to the GDM group ($P < 0.05$).

3.5. Effects of CCE on uterine ultrastructure, relative uterine tissues area and interstitial space proportion

The histological images of uterine tissue sections (perimetrium, myometrium, and endometrium) at gestation day 21 are illustrated in Fig. 4B, with interstitial spaces located at 40× magnification (avoiding implantation sites). Additionally, the relative uterine tissue area and interstitial space proportion from H&E staining slides are presented in Fig. 4E-F. The results from 15 uterine images per group (n = 5, with 3 uterine images per animal) revealed significant increases in interstitial space (46.07 ± 3.32%) and significant decreases in uterine tissue area (54.02 ± 6.52%) in the GDM group compared to the non-GDM group (interstitial space: 31.43 ± 4.12% and uterine tissue area: 70.69 ± 4.37%, $P < 0.05$). Treatment with MET and high-dose CCE significantly decreased uterine interstitial space and improved uterine tissue area proportion compared to the GDM group ($P < 0.05$).

3.6. Concentration dependency of CCE

After the equilibration period, the cumulative concentration-dependent effect of CCE at concentrations of 0.5, 1, 1.5, and 2 mg/mL on force measurement were investigated with myometrial strips of GDM, as illustrated in Fig. 5A. It can be seen that at the lower two concentrations there is a clear stimulation of contractility, largely due to a significant increase in frequency of contractions. As the CCE concentration was further increased the increase in frequency was such that the contraction became tonic, and was not sustained. Thus the total amount of force began to decline, as can be seen in the force integral, AUC. The

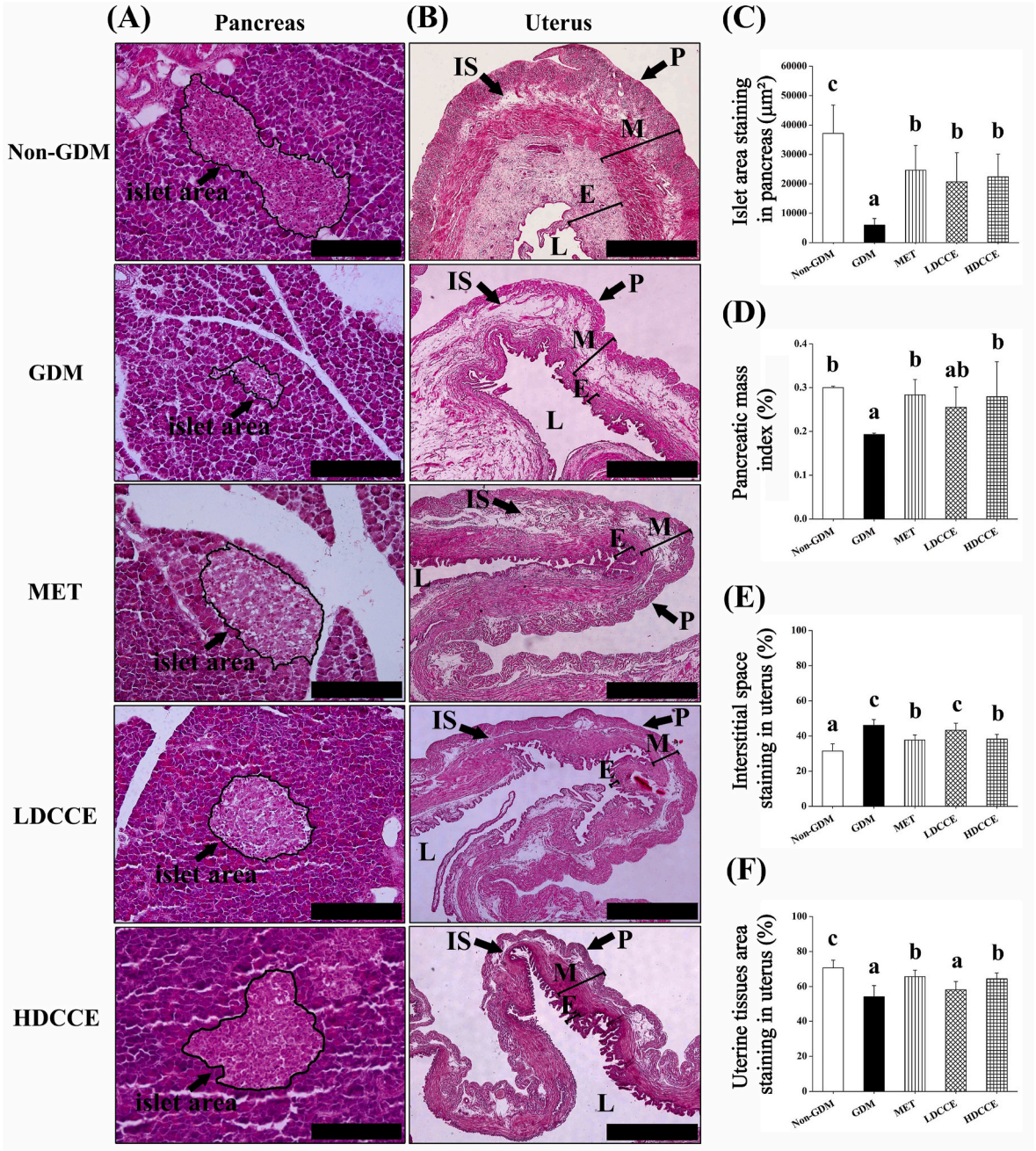

Fig. 4. Photomicrographs of H&E stained histological slides of pancreatic islet area and uterine tissue in GDM rats. (A) Black tracings of islet area in all experimental groups at 200× magnification and scale bar = 200 μm. (B) Black arrow and bracket indicating uterine tissues appearance including perimetrium (P), myometrium (M), endometrium (E) with interstitial space (IS), and lumen (L) was observed in all experimental groups at 40× magnification and scale bar = 1000 μm. (C–D) Effects of CCE on relative islet area quantification from the mean values of total 25 pancreases in each group and pancreatic mass index, respectively (n = 5). (E–F) Effects of CCE on relative interstitial space and uterine tissues cross-section area quantification from the mean values of total 15 uterine images in each group, respectively (n = 5). Data are shown as mean ± SD and analyzed by one-way ANOVA, followed by Tukey's post hoc test ($P < 0.05$). Groups bearing different superscripted letters on the bars indicate statistical significance between groups. Non-GDM = non-gestational diabetes mellitus, GDM = gestational diabetes mellitus, MET = metformin, LDCCE = low dose CCE, HDCCE = high dose CCE.

AUC during exposure to CCE at starting concentrations of 0.5 and 1 mg/mL significantly increased to 190 ± 33% and 207 ± 54%, respectively, with an increasing frequency and elevating baseline observed. Subsequently, the AUC reduced to 135 ± 38% and 63 ± 25% at concentrations of 1.5 and 2 mg/mL, compared to the equilibration period set as 100% spontaneous control ($P < 0.05$, n = 5 for each concentration). Subsequent experiments were conducted using a concentration of 1.5 mg/mL (the inhibitory concentration: IC_{50}).

3.7. Effects of CCE on spontaneous contraction

In Fig. 5B, the trace represents the effects of CCE (1.5 mg/mL) on spontaneous contractions in non-GDM and GDM myometrial strips. Unlike in Fig. 5A, where the concentration was applied cumulatively, here a single concentration was applied directly, resulting in a significant increase in spontaneous force (AUC) to 128.54 ± 17.65% compared to the 100% spontaneous control in non-GDM strips and 152.84 ± 33.31% in GDM strips ($P < 0.05$, n = 5). These results indicate a stronger

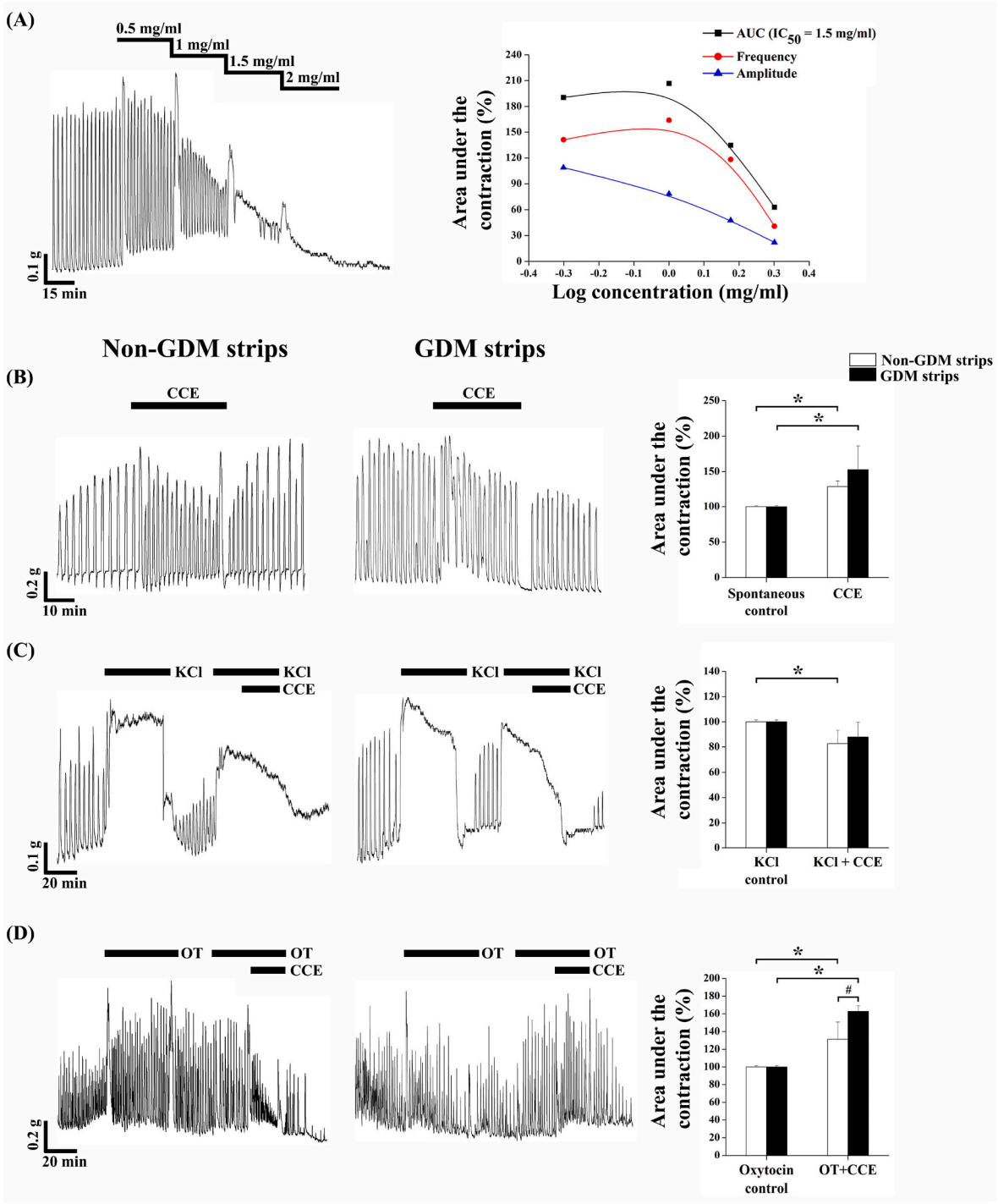

Fig. 5. Effects of CCE on uterine contraction in non-GDM and GDM rats. (A) Typical traces showing the concentration dependency of uterine response to CCE (frequency, amplitude, and AUC), with the lower concentrations showing stimulation and higher concentrations showing inhibition (n = 5). (B–D) Effects of CCE on spontaneous contraction, high K depolarization, and oxytocin-induced contraction in non-GDM and GDM strips, respectively (n = 4). Data shown as mean ± SD. *Significant difference compared within each control (paired Student's t-test, $P < 0.05$) and #Significant difference compared between non-GDM and GDM strips (unpaired Student's t-test, $P < 0.05$). AUC = area under the contraction, IC_{50} = a half-maximum inhibitory concentration, KCl = high K solution, OT = oxytocin.

stimulatory effect in GDM.

3.8. *Effects of CCE in the continued presence of high K depolarization and oxytocin-induced contraction*

Further experiments were performed to investigate whether CCE could enhance the highly coordinated contractile response during the labor process by using agonist-induced uterine contractility such as high K depolarization (Fig. 5C) and oxytocin-induced contraction (Fig. 5D). During the application of the KCl solution, tonic force was produced. The sustained force gradually decreased during control (KCl alone) and KCl with CCE in both non-GDM and GDM strips. The decrease was 82.65 ± 10.58% in non-GDM strips ($P < 0.05$) and 87.93 ± 11.75% in GDM strips compared to the 100% KCl control. No statistically significant difference was observed between groups (n = 4). In the presence of oxytocin (OT), CCE facilitated the uterotonic effect. The AUC increased

significantly to 131.31 ± 19.55% in non-GDM strips and 162.81 ± 6.31% in GDM strips compared to the 100% OT control. Statistical significance was observed between groups ($P < 0.05$, n = 4).

3.9. Concentration dependency of MET and effect of MET on spontaneous contraction

The cumulative concentrations of MET (100, 200, 300, 400, 500, and 600 μM) were applied to the myometrial strips of GDM, as illustrated in Fig. 6A. The contractile response in AUC showed a slight decrease at the initial concentration of 100 μM (97 ± 10%), followed by a slight increase at 200 μM (104 ± 17%), and then a continued decrease with increasing concentrations (89 ± 23% at the highest concentration).

In contrast, amplitude and frequency were reduced from the starting concentration of 100 μM to the highest concentration of MET administration (decreasing from 92 ± 15% to 55 ± 22% for amplitude and from 88 ± 3% to 57 ± 16% for frequency). The responses in AUC, amplitude, and frequency were not significantly different from the 100% spontaneous control ($P > 0.05$, n = 5 for each concentration). Thus, a concentration of 417.5 μM (IC_{50}) was used for further experiments.

The spontaneous contractile response in non-GDM and GDM myometrial strips under exposure to a single concentration of MET (417.5 μM) is shown in Fig. 6B. The AUC in both non-GDM and GDM strips (98.68 ± 15.11% and 104.20 ± 4.29%, respectively) was not significantly different compared to the 100% spontaneous control. Similarly, no significant differences were observed between groups ($P > 0.05$, n = 3).

4. Discussion

GDM poses significant risks to maternal and fetal health, necessitating effective therapeutic interventions to manage hyperglycemia and associated complications. While MET is a widely used treatment, there is growing interest in natural alternatives with multifunctional benefits and minimal side effects. This study investigates the potential of CCE as a natural therapy for GDM, evaluating its reduced elevated glucose levels, hypolipidemic, and hepatoprotective properties, as well as its effects on uterine contractility and fetal outcomes.

Key findings indicate that CCE improves glucose and lipid profiles, supports pancreatic function, and enhances maternal and fetal growth parameters. Notably, its biphasic effects on uterine contractions highlight a dual role in regulating uterine activity, suggesting potential applications in managing labor. These findings underscore CCE's promise as a complementary or alternative therapy to conventional treatments like MET, offering a foundation for developing herbal remedies for GDM management.

Animal models of diabetic pregnancy were established through intraperitoneal injection of STZ at a dose of 60 mg/kg BW on day 5 of gestation in this study. Hyperglycemia with glucose level >200 mg/dL was seen on day 7 of gestation and developed throughout pregnancy. Traditional herbal products, with ingredients containing CCE, have long been utilized in rural Thai medical systems for essential pharmacology (Urumarudappa et al., 2022). Several pharmacological activities associated with higher phenolic compounds and flavonoids in CCE have been reported (Alara et al., 2018). Phytochemical analysis in this study revealed the presence of alkaloids, flavonoids, tannins, phenolic compounds, terpenoids, sterols, and reducing sugars in CCE. Oral administration of CCE at both doses in rats did not induce maternal toxicity. High survival rate (93%) was observed. The changes in maternal body weight and food intake after CCE treatment did not significantly differ from non-GDM at term, consistent with previous safety evaluations of CCE in experimental animals (Johnson and Varghese, 2023).

Administration of CCE at both doses exhibited a significant reduction in elevated glucose levels in GDM rats in late gestation (as measured on

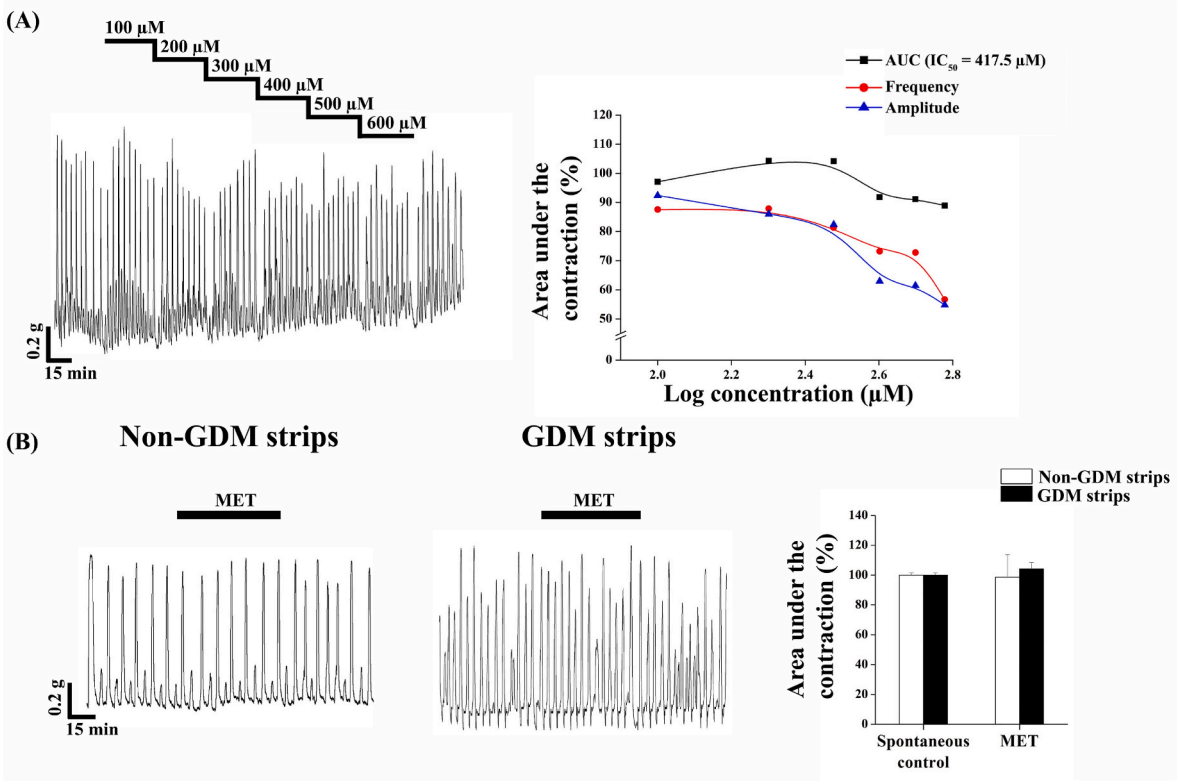

Fig. 6. Effects of MET on uterine contraction in non-GDM and GDM rats. (A) Typical traces showing the concentration dependency of uterine response to MET (frequency, amplitude, and AUC), with no significant difference compared to the 100% spontaneous control (n = 5). (B) Effects of MET on spontaneous contraction in non-GDM and GDM strips, respectively (n = 3). Data shown as mean ± SD. AUC = area under the contraction, IC_{50} = a half-maximum inhibitory concentration, MET = metformin.

day 21 of gestation), especially with high-dose CCE treatment (500 mg/kg). Additionally, increased staining of the islet area in CCE treatment at both doses was observed, suggesting a protective effect against STZ-induced histological changes in the pancreatic islet area. A trend towards an increased pancreatic mass index was observed in high-dose CCE treatment, suggesting a potential role in the regulation of glucose homeostasis and insulin level accumulation. Clinically, hyperglycemia or GDM results from insufficient insulin reserve in the second and third trimesters of normal pregnancy (Taschetto et al., 2021). Insulin levels significantly decreased in GDM in late gestation (as measured on day 21 of gestation). The gradual increase of insulin levels was found in the CCE treatment groups, suggesting potential effective restoration of pancreatic β-cell activity and pancreatic enzyme for hyperglycemic management.

The metabolic dysfunction caused by STZ-induced diabetes was investigated. CCE treatment exerted hypolipidemic effects by significantly decreasing TC and TRI and showing trends toward elevated HDL and reduced LDL levels. Previous studies found that a long-chain fatty acid isolated from CCE possesses anti-diabetic effects (Mohamud Maitheen and Prabha, 2022). Glucose and lipid metabolism are regulated by increased glucose transport activity and inhibited glucose production to enhance insulin sensitivity (Saha and Saha, 2022). The highest bioactive constituents of CCE identified by GC-MS in this study, including lupeol, lupenone, and β-sitosterol, mediate oxidative stress, lipid profile, and glycogen concentration, influencing insulin resistance and glucose transporter (GLUT-4) expression in target muscles (Kottireddy et al., 2019; Xu et al., 2022; Krishnan et al., 2021).

Abnormal liver function tests with elevated liver enzymes during pregnancy in the third trimester are the leading causes of various maternal and perinatal morbidities. Additionally, ALP levels progressively increase and reach their highest values, correlating with markedly increased cesarean delivery rates, premature deliveries, and macrosomia neonates (Titaux et al., 2023). In this study, all three liver enzymes (AST, ALT, and ALP) significantly increased in the GDM group due to STZ-induced diabetic liver injury. Treatment with CCE at both doses significantly reduced AST levels, while ALT levels were significantly reduced only at the high CCE dose, with no alterations in ALP levels. Thus, our data support the hepatoprotective effect of CCE against STZ-induced diabetic liver injury, which may be attributed to its antioxidant compounds, including terpenoids (lupeol and β-amyrin) and phytosterols (β-sitosterol). These bioactive compounds exert protective effects through mechanisms such as mitigating oxidative stress, inhibiting xanthine oxidase activity, and regulating apoptosis via the PI3K/Akt pathway (Chen et al., 2020; Coremen et al., 2022; Viet et al., 2021).

High maternal glucose concentration is a common cause of adverse neonatal effects (Picón-César et al., 2021). Consistent with these findings, GDM rats exhibited intrauterine fetal growth restriction, as evidenced by decreased gravid uterus weight and number of live fetuses, increased post-implantation loss rates, lower fetal weight, and reduced crown-rump length, alongside higher placental weight and placental index. Conversely, treatment with CCE at both doses significantly improved fetal growth, as indicated by increased gravid uterus weight, reduced placental weight and placental index, and increased crown-rump length. Moreover, the proportion of fetuses classified as appropriate for pregnancy age (APA) increased in a dose-dependent manner following CCE treatment, suggesting its potential to ameliorate maternal-fetal complications in diabetic pregnancy, likely through antioxidative and anti-inflammatory mechanisms that reduce oxidative stress, improve placental efficiency, and support fetal nutrient supply.

Treatment with MET in this study showed hypoglycemic, hypolipidemic, and hepatoprotective effects, resulting from the stimulation of pancreatic islet repair mechanisms with increased insulin levels. A prospective clinical study showed that MET could be safely taken by women with GDM to achieve glycemic control with fewer hypoglycemic events, and fewer maternal and neonatal complications (Picón-César et al., 2021). In addition, an increase in gravid uterine weight, crown rump length, and the proportion of uterine tissues area were found in this study, demonstrating a beneficial effect of MET on maternal and fetal outcomes in GDM.

There is evidence that STZ-induced diabetes disrupts uterine smooth muscle cells, leading to inflammatory infiltration related to impaired uterine contractility (Wagih et al., 2021). Treatment with CCE potentially improved uterine structure by decreasing the proportion of relative interstitial space and increasing the proportion of uterine tissues area, particularly at higher doses.

Impaired and dysfunctional myometrial contractility plays an important role linked to an increased cesarean section rate in gestational diabetes caused by the failure in active labor, whether spontaneous or induced (Nevander et al., 2023). The outcome of this study demonstrated that CCE had a biphasic effect on uterine contraction in gestational diabetic rats. Uterine stimulation and relaxation were mediated in a concentration-dependent manner, with maximal stimulant effect of CCE displayed at lower concentrations (1 mg/ml), whereas a relaxant effect was displayed at higher concentrations (1.5 and 2 mg/ml) in rat myometrium. This effect can be explained by the higher concentrations producing such an increase in frequency that phasic contractions shift to tonic activity, that can be maintained. The increase in frequency suggests that CCE may lead to a decrease in membrane potential, resulting in increased excitability and firing of action potentials (Wray and Arrowsmith, 2021). Although we have not yet studied its effect on membrane potential, it would be interesting to conduct whole-cell patch clamping for further validation. Stimulation of force with aqueous CCE (0.25–25 mg/ml) has been shown in isolated rat duodenal smooth muscles, with spasmodic activity seen (Pandey et al., 2012). The stimulant effect of CCE further enhanced oxygen stimulation of uterine contractility. This may be due to mobilization of calcium from intracellular stores, particularly in cases of gestational diabetes. Active ingredients in CCE, such as β-sitosterol showing a spasmodic response on smooth muscle contraction, whereas β-amyrin exhibiting antispasmodic response (Akinmurele et al., 2023; Jonathan and Okieimen, 2020). These relevant pharmacological effects could be due to the different presence of bioactive compounds in the plant extract.

In contrast, MET did not alter spontaneous contraction, as observed in our work and others, and did not augment oxytocin-induced contractility (Hehir et al., 2009). This suggests that MET may have a limited role in directly modulating uterine contractility, emphasizing the need for alternative approaches in managing uterine dysfunction during gestational diabetes.

The findings of this study demonstrated the potential usefulness of CCE in GDM rats. Unlike MET, CCE is a natural extract with multifunctional properties, including hypoglycemic, hypolipidemic, and hepatoprotective effects in late gestation, especially at higher doses. These findings suggest that CCE could alleviate pathological symptoms in GDM while also improving maternal gravid uterus weight and placental-fetal development, thereby addressing diabetic complications. Furthermore, CCE exhibited biphasic activity on uterine smooth muscle in both GDM and non-GDM conditions, showing a uterotonic effect at lower concentrations and a tocolytic effect at higher concentrations. This dual activity, particularly in response to oxytocin-induced contractility during labor, highlights CCE's potential role in augmenting labor induction and postpartum care as a complementary therapy to conventional treatments like MET. Importantly, additional experiments using non-GDM muscle strips revealed no adverse effects of CCE treatment in the absence of GDM, supporting its safety profile. These observations strengthen the evidence that CCE may serve as a safe and effective candidate for herbal remedies during pregnancy, labor, and postpartum care.

However, there are some limitations to the present study. For instance, the tension recordings in the organ bath apparatus, conducted *ex vivo* to assess concentration-response relationships in uterine smooth muscle contraction in 21-day pregnant rats, may not entirely reflect the

effect of CCE on uterine smooth muscle throughout pregnancy. There is significant potential for future research to uncover the dual effects of CCE, as well as its combined effects with other medications, in evaluating the efficacy and safety of CCE in managing clinical gestational diabetes.

5. Conclusion

The comparison between MET and CCE demonstrates that both treatments exert beneficial effects in managing GDM in rats, although CCE offers unique advantages that may complement those of MET, as summarized in Table 2. Both treatments effectively reduce blood glucose levels and improve lipid profiles, including reductions in total cholesterol and triglycerides. However, high-dose CCE (HDCCE) exhibited a tendency to further enhance maternal body weight and showed a more notable reduction in placental weight and index compared to MET.

In terms of insulin and pancreatic function, both treatments positively influenced islet area and pancreatic mass index, with MET exhibiting a slightly greater impact on insulin levels, suggesting its stronger role in enhancing insulin production. Nevertheless, CCE demonstrated additional benefits, particularly in supporting pancreatic function.

Regarding uterine effects, both MET and CCE contributed to decreasing uterine interstitial space and increasing fiber area, indicating structural improvements. Notably, CCE displayed biphasic effects on uterine contractions, promoting contraction at lower doses and inducing relaxation at higher doses. This dual activity implies that while CCE at higher doses may modulate uterine contractility, lower doses could offer a safer approach to managing uterine contractions during pregnancy.

Maternal and fetal outcomes were similarly improved by both treatments, as evidenced by increases in gravid uterus weight and crown-rump length, reflecting positive fetal growth. CCE at lower doses (LDCCE) demonstrated a more pronounced effect in reducing placental weight and index compared to MET, which may help address complications associated with GDM.

These findings suggest that CCE has the potential to serve as a natural alternative or adjunct therapy to MET for GDM management, offering additional multifunctional benefits such as biphasic uterine modulation and hepatoprotection. Its minimal side effects and multifunctional properties highlight the potential for CCE to be developed as a novel therapeutic approach in GDM treatment, especially in pregnancy, labor, and postpartum care.

CRediT authorship contribution statement

Sasitorn Kerdsuknirund: conducted the experiments and analyzed the data. **Arreeya Kosinan:** performed ELISA. **Panida Khunkaewla:** provided suggestions and expertise input. **Pakanit Kupittayanant:** provided suggestions and expertise input. **Pattama Tongdee:** provided suggestions and expertise input. **Porntip Nimkuntod:** provided suggestions and expertise input. **Susan Wray:** offered insights into the data and critically revised the manuscript. **Sajeera Kupittayanant:** supervised the experiments and wrote the full manuscript, All authors approved the final version of the manuscript and its submission for this article.

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Declaration of competing interest

The authors declare that they have no conflicts of interest.

Table 2
Comparison of the effects of metformin (MET) and CCE on various parameters in gestational diabetes mellitus (GDM) rats.

Parameters		Non-GDM	GDM	MET	LDCCE	HDCCE
Blood glucose level		↓	↑	↓ *	↓ *	↓ *
Maternal body weight		↑	↓	-	-	-
Food consumption		-	-	-	-	-
Insulin level		↑	↓	-	-	-
Lipid profiles	Total cholesterol level	↓	↑	↓ *#	↓ *#	↓ *#
	Triglyceride level	↓	↑	↓ *#	↓ *	↓ *
	HDL level	↑	↓	↑ *#	-	-
	LDL level	↓	↑	-	-	-
Liver enzymes	AST level	↓	↑	↓ *#	↓ *#	↓ *#
	ALT level	↓	↑	↓ *#	-	↓ *#
	ALP level	↓	↑	↓ *#	-	-
Islet area staining in pancreas		↑	↓	↑ *#	↑ *#	↑ *#
Pancreatic mass index		↑	↓	↑ *#	-	↑ *#
Interstitial space staining in uterus		↓	↑	↓ *	-	↓ *
Uterine tissue area staining in uterus		↑	↓	↑ *	-	↑ *
Maternal reproductive performances	Gravid uterus (g)	↑	↓	↑ *	-	↑ *
	Number of corpora lutea	-	-	-	-	-
	Number of implantations	-	-	-	-	-
	Number of live fetuses	↑	↓	-	-	-
	Number of dead fetuses	-	-	-	-	-
	Pre-implantation loss rates	-	-	-	-	-
	Post-implantation loss rates	↓	↑	-	-	-
Fetal outcome parameters	Fetal weight	↑	↓	-	-	-
	Placental weight	↓	↑	-	↓ *#	-
	Placental index	↓	↑	-	↓ *	-
	Crown rump length	↑	↓	↑ *	-	↑ *

↑ = increase, ↓ = decrease, - = no change, effects were different from GDM (*) and similar to Non-GDM (#).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.crphys.2025.100139.

Data availability

Data will be made available on request.

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