

## Fetoplacental endothelial exosomes modulate high D-glucose-induced endothelial dysfunction



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### ABSTRACT

**INTRODUCTION:** Gestational diabetes mellitus (GDM) is associated with fetoplacental endothelial dysfunction, which may be induced by hyperglycemia. We hypothesized that endothelial exosomes, which are extracellular nanovesicles affecting endothelial function, play a role in the high glucose (HG)-induced endothelial dysfunction.

**METHODS:** Exosomes were isolated from HUVECs incubated with basal glucose (5.5 mmol/L; HUVEC-BG; exo-BG) and from HUVECs incubated with HG for 24 h (25 mmol/L; HUVEC-HG; exo-HG) in exosome-free medium. Exosomes were isolated and characterized by ultracentrifugation, sucrose gradient, electron microscopy, nanotracking analysis and Western blotting. HUVEC-BG and HUVEC-HG were exposed to exo-BG and exo-HG in two different concentrations: 5 µg and 1 µg exosome protein/mL. The exosomal effect on endothelial cell function was determined by wound healing assay, expression of endothelial nitric oxide synthase (eNOS), human cationic amino acid transporter type 1 (hCAT-1), vascular endothelial growth factor (VEGF) and intracellular adhesion molecule type 1 (ICAM-1) by Western blotting, qPCR or flow cytometry.

**RESULTS:** HG increased the exosomal release from HUVECs, endothelial wound healing and expression of phosphorylated (P~Ser<sup>1177</sup>)-eNOS, hCAT-1, VEGF and ICAM-1. Exo-HG also increased endothelial cell wound healing, P~Ser<sup>1177</sup>-eNOS, hCAT-1 and ICAM-1 expression in HUVEC-BG. Exo-BG reverted the effect of HG on endothelial cell wound healing and hCAT-1 mRNA expression to normal values.

**DISCUSSION:** Our results show that HG may induce endothelial dysfunction in HUVECs and that exosomes from HUVEC-HG mimicked some of the effects of HG. This study contributes to the unraveling of the mechanism by which hyperglycemia affects the fetoplacental vasculature in GDM.

### 1. Introduction

Gestational diabetes mellitus (GDM) is one of the most common pregnancy complications in the world [1], affecting 2–6% of all pregnancies in Europe [2]. It is associated with several fetal and maternal complications such as an increased risk for preeclampsia, preterm delivery and fetal macrosomia [3,4]. Moreover, GDM is associated with long-lasting health consequences for both mother and child, such as a

chance to develop type 2 diabetes in later life [5–7].

Key in the complications associated with GDM is probably vascular dysfunction in both the maternal and fetoplacental endothelium [1,8]. In human umbilical vein endothelial cells (HUVECs) from women diagnosed with GDM, an impaired endothelial function has been demonstrated. Endothelial dysfunction is characterized by impaired endothelial-dependent vasodilation caused by a loss in NO bioactivity [9,10]. Various changes associated with low NO bioactivity have been

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found in the L-Arginine/nitric oxide (NO) signaling pathway in HUVECs derived from GDM pregnancies, such as increased expression of human cationic amino acid transporter type 1 (hCAT-1), increased NO production and increased phosphorylated Ser<sup>1177</sup> endothelial nitric oxide synthase (P~Ser<sup>1177</sup>-eNOS) [9,11], as compared to HUVECs from healthy pregnancies.

However, the mechanisms behind the GDM induced fetal-placental endothelial dysfunction are largely unknown. Since GDM is characterized by maternal and fetal hyperglycemia [1,12–14], high glucose (HG) could be one of the factors inducing endothelial dysfunction in GDM [12,15]. Indeed, previous studies in HUVECs have shown that HG increased the expression of P~Ser<sup>1177</sup>-eNOS and hCAT-1, as well as enhanced the transport of L-arginine and the production of NO [16], suggesting an important role for high glucose on fetoplacental endothelial dysfunction in GDM.

Another mechanism that could be responsible for fetoplacental endothelial dysfunction may be exosomes. An enhanced release of exosomes and a change in exosomal cargo have recently been implicated in several vascular disorders [17–19]. Exosomes are extracellular nanovesicles, which are released by different cell types, including placental and endothelial cells, cells such as HUVECs [20,21]. Exosomal cargo include proteins, mRNAs and microRNAs [22]. Exosomes can transfer this cargo from one cell to another, thus acting as cell-to-cell communicators [23,24]. In this way, exosomes are for instance able to modulate endothelial cell function [25].

In the present study, we tested the hypothesis that high D-glucose induces similar endothelial dysfunction as seen in HUVECs of GDM pregnancies and, since our previous study showed a role for exosomes in inducing endothelial dysfunction in HUVECs of GDM [21], we hypothesized that this effect of high glucose may be mimicked by endothelial exosomes derived from HUVECs incubated with high glucose. To this end, we incubated HUVECs with basal and high levels of D-glucose and isolated the exosomes. These exosomes were incubated with HUVECs (both under basal or high glucose levels) after which protein and mRNA levels were studied of hCAT-1, eNOS and P~Ser<sup>1177</sup>-eNOS and VEGF. To determine endothelial cell functionality and activation, we performed a wound healing assay and evaluated ICAM-1 expression in HUVECs incubated with exosomes.

## 2. Material and methods

### 2.1. Cell culture and treatment

Human umbilical vein endothelial cells (HUVECs) from normal pregnancies were provided by the Endothelial Cell Facility of the UMCG (Groningen, Netherlands). HUVECs were isolated by collagenase digestion (0.25 mg/mL Collagenase Type II from Clostridium histolyticum; Boehringer, Mannheim, Germany), as previously described [26]. HUVECs were cultured in RPMI medium (Lonza, Basel, Switzerland) supplemented with 2 mmol/L L-glutamine, 5 U/mL heparin, 100 IE/mL penicillin, 100 µg/mL streptomycin, 50 µg/mL crude ECGF solution, and 20% FCS (37 °C, 5% CO<sub>2</sub>) on 1% gelatin-precoated tissue culture flasks (Corning® Costar®; Sigma-Aldrich, Zwijndrecht, The Netherlands).

HUVECs in passage 2 with 70% confluence in T<sub>75</sub> (12 mL) culture flask were incubated with exosome-free media for 24 h, with addition of D-glucose (HUVEC-HG; 25 mmol/L D-glucose (high glucose, HG) or without addition of D-glucose (HUVEC-BG; 5.5 mmol/L D-glucose (basal glucose, BG)). Exosome free medium was obtained after ultracentrifugation of HUVECs culture medium (100000 g at 4 °C for 70 min), supernatant was filtered (0.22 µm filter) and pellet was discarded. After 24 h, HUVECs cell number was not different when incubated in BG compared with HG medium (not shown), therefore exosomes were isolated from the same number of HUVECs in both conditions.

### 2.2. Exosome isolation and purification

Exosomes were isolated according to Théry and colleagues [27]. We isolated exosomes from supernatant (40 mL) of 7 HUVECs cell cultures with equal numbers of umbilical cords incubated in exosome-free medium. Exosomes were isolated and purified by differential ultracentrifugation as recently described in primary cultured HUVECs [21]. In brief, supernatant was filtered through a 0.22 µm filter and then, centrifugation was performed at 2000 g at 4 °C for 30 min, followed by 12000 g at 4 °C for 45 min. Then, the supernatant was centrifuged at 110000 g at 4 °C for 70 min (Centrikon T-1080 ultracentrifuge, Kontron Instruments, SW 41 rotor). The pellet was resuspended in phosphate buffer solution (PBS, 1 mmol/L, pH 7.4) and centrifuged at 110000 g at 4 °C for 70 min. The exosome pellet was resuspended in sucrose/HEPES (hydroxyethyl piperazineethanesulfonic acid) 2 mol/L and subsequently purified using a discontinuous sucrose gradient (2–0.25 mol/L). Thus, exosomes were mixed with 2 mL of 2 mol/L sucrose in HEPES and placed on the bottom of a SW41 centrifuge tube, overlaid in a solution with 1.5 mL of 1.35 mol/L sucrose, 0.5 mL of 1.28 mol/L sucrose, 1 mL of 1.15 mol/L sucrose, and 5 mL of 0.25 mol/L sucrose, and ultracentrifuged for 16 h at 210 000 g at 4 °C. Thus, 12 fractions were collected with 1 mL sucrose and refractive index was measured. After this, all fractions were washed and ultracentrifuged again with PBS at 110 000 g at 4 °C for 70 min. Fraction 11 showed floating density for exosomes (1.16 g/mL) and exosomal markers by western blotting and was used for the experiments described below. At the end of each isolation (n = 7), the final pellet was resuspended in 300 µL of PBS and stored at –80 °C for later incubation experiments with HUVECs. The exosomal protein concentration was determined using the BSA™ Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA, USA) (around ~ 0.35 µg/µL per isolation).

### 2.3. Preparation of exosomes for transmission electron microscopy (TEM)

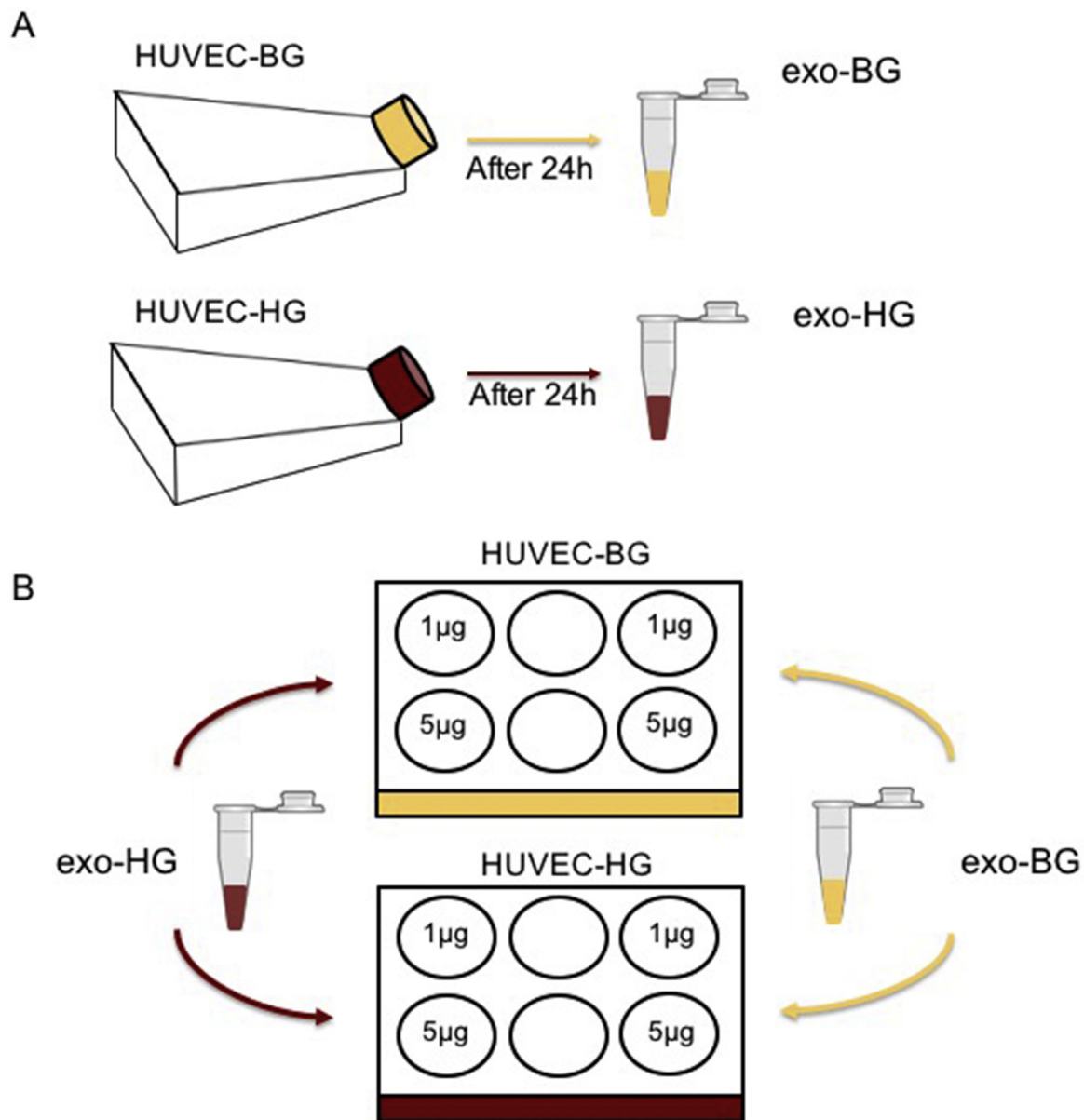
Aliquots (10 µL) of the exosomal suspension was allowed to adhere on freshly prepared Formvar coated 200 mesh electron microscopy grids for 2 min. After draining the liquid with a filter paper, samples were stained for 1 min on a drop of 2% ammonium molybdate in water. After draining the liquid and air-drying for 5 min, samples were examined in a FEI Cm100 transmission electron microscope operated at 80 KV. Pictures were taken with a Morada camera using Olympus-isis software.

### 2.4. Nanoparticle tracking analysis

Quantification and size distribution of exosomes was done using Nano Tracking Analysis (NTA) equipped with a 405 nm laser with LM14 module (NanoSight NTA 3.0 Nanoparticle Tracking, Version 3.0). Samples (100 µL) were diluted with PBS (1/10) prior to analysis in order to obtain particle distribution of particles per image (50 particles per image). Samples were measured using capture 60 and repeat 5. Settings were kept constant in all samples. Each video was analyzed to get the mean particle size and the concentration of particles.

### 2.5. Western blotting of exosomes

Exosomal pellets isolated from HUVEC-BG and HUVEC-HG were prepared for western blotting. Exosome pellets isolated from BG and HG HUVECs were lysed with RIPA buffer (1×) (Radio-Immunoprecipitation Assay buffer, 25 mmol/L Tris·HCl pH 7.6, 150 mmol/L NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS (Sodium dodecyl sulfate) (Pierce® RIPA Buffer, Thermo Fisher Scientific Inc, Landsmeer, Netherlands) at room temperature for 5 min and then loading buffer (5×) was added to the samples and incubated at 95 °C for 5 min. Aliquots of 20 µg of exosomal protein per well was separated by SDS-polyacrylamide gel electrophoresis (8%) (SDS-PAGE) and



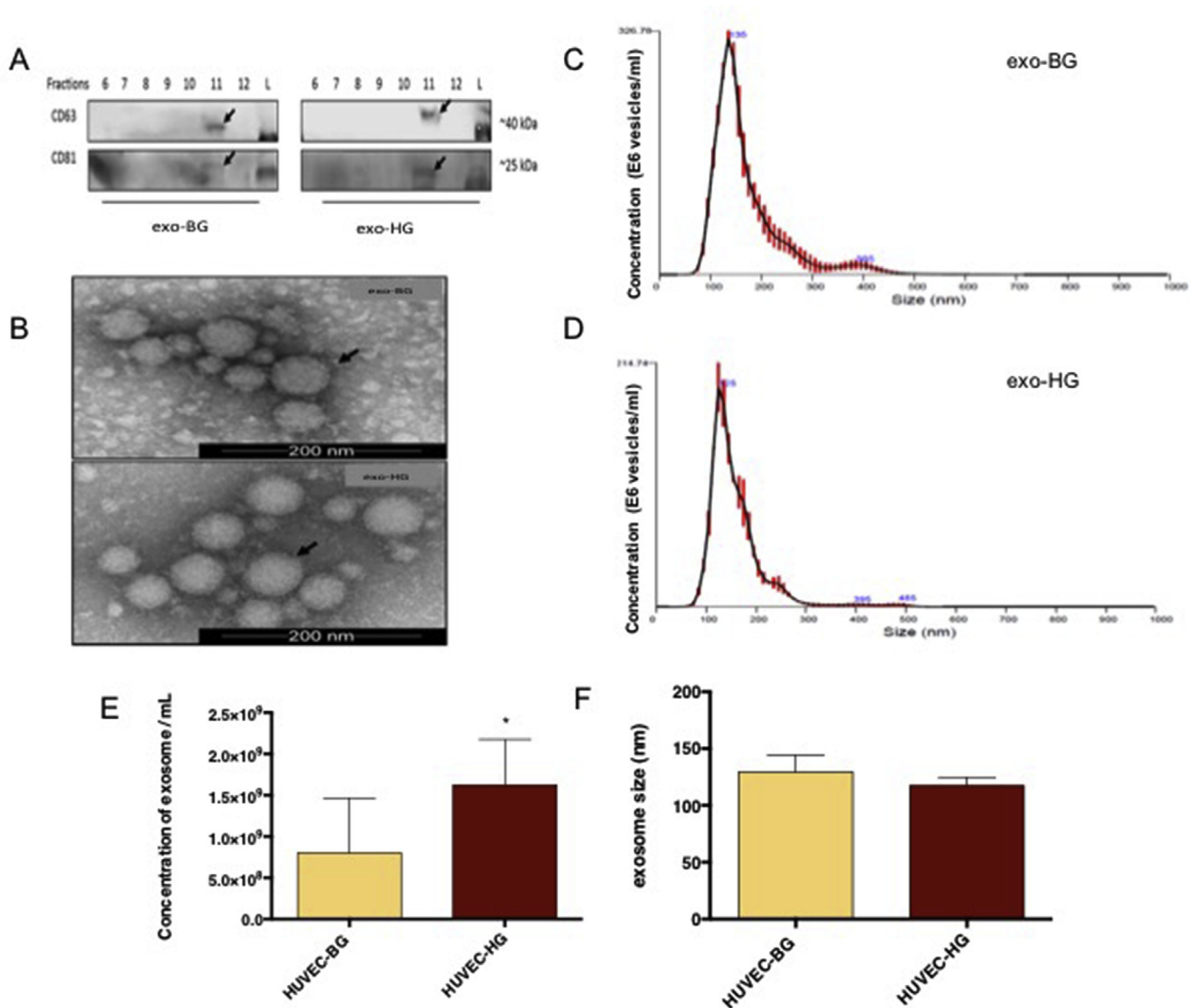
**Fig. 1. Representative scheme of exosome isolation and experimental design.** A: exosomes were isolated from the supernatant of HUVECs exposed to basal glucose (HUVEC-BG, 5 mmol/L D-glucose) or high glucose (HUVEC-HG, 25 mmol/L D-glucose) for 24 h incubation with exosome-free endothelial culture medium. Exosomes from basal glucose and high glucose (exo-BG and exo-HG, respectively) were purified and characterized. B: HUVEC-BG and HUVEC-HG were exposed for 12 h with exosomes from both conditions, using 1 or 5 µg exosome protein/mL. HUVEC-BG or HUVEC-HG without exosome treatments were used as controls.

transferred onto polyvinylidene fluoride (PVDF) membranes (Immobilon<sup>®</sup>-FL, Millipore, Amsterdam-Zuidoost, Netherlands). Membranes were probed with primary antibody, polyclonal rabbit anti-CD63 (1:200; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) and monoclonal mouse anti-CD81 (1:100; Santa Cruz Biotechnology), monoclonal anti-Grp94 (1:500, Santa Cruz Biotechnology) as negative exosome marker. After incubation at 4 °C overnight, membranes were washed in Tris buffered saline (pH 7.4) and incubated for 1 h at room temperature with secondary antibody IRDye<sup>®</sup> 800CW or 680CW Secondary Antibodies (LI-COR, Lincoln, Nebraska USA). Membranes were analyzed using LI-COR Odyssey Scanner (LI-COR, Lincoln, Nebraska USA).

## 2.6. Treatment of HUVECs with exosomes for western blotting and qPCR

To study the effect of exosomes from endothelial cells in BG or HG, exosomes from HUVEC-HG or HUVEC-BG (exo-HG and exo-BG,

respectively) were incubated with either HUVEC-BG or HUVEC-HG (Fig. 1). HUVECs in passage 2 were plated in 6 wells plates (Corning<sup>®</sup> Costar<sup>®</sup>; Sigma-Aldrich, Zwijndrecht, The Netherlands). When 100% confluency was reached ( $\sim 3 \times 10^5$  cells per well) HUVECs were pre-incubated for 24 h with medium containing 25 mmol/L (high D-glucose) or 5.5 mmol/L (basal D-glucose). Cells were then washed and exposed to exo-HG or exo-BG in exosome-free medium for further 12 h containing two different exosomal concentrations: 5 µg protein/mL (exo-BG 5 µg exosomes from HUVEC-BG or exo-HG 5 µg exosomes from HUVEC-HG, equivalent to  $\sim 2 \times 10^5$  vesicles per  $\text{cm}^2$ ) or 1 µg protein/mL (exo-BG 1 µg for exosomes derived from BG or exo-HG 1 µg for exosomes derived from HG, equivalent to  $\sim 4 \times 10^4$  vesicles per  $\text{cm}^2$ ). The protein concentration of exosomes used in each experiment was based on previous reports [21,28]. After the incubation period, cells were washed with PBS and prepared for Western blotting or qPCR as [21,29] (see supplemental material). Briefly, the following primary antibodies were used for Western blotting, monoclonal mouse anti-total eNOS (eNOS



**Fig. 2.** Exosomes from HUVECs exposed to basal or high-glucose. A: exosomes were isolated by the ultracentrifugation protocol from HUVECs cultured for 24 h in basal glucose (HUVEC-BG, 5 mmol/L D-glucose) or high glucose (HUVEC-HG, 25 mmol/L D-glucose). After sucrose gradient, western blotting for CD63 and CD81 in each fraction was performed. CD63 and CD81 were detected only in fraction 11 (arrows). B: Photographs from electronic microscopy showing exosomes in fraction 11 from cells as in A. C and D: Representative graphs for exosomes from fractions 11 using Nanosight (median = 120 nm for exosomes from high glucose (exo-HG), median = 135 nm for exosomes from basal-glucose (exo-BG)). E: Concentration of exosomes from HUVEC-BG or HUVEC-HG. Data is shown as median with inter-quartile range for NTA analysis for all experiments (n = 7). F: particle size as measured with NTA for all experiments (n = 7).  $P < 0.05$  significant (\*), Wilcoxon signed paired test.

1:2000; BD Transduction Laboratories™, CA, USA), monoclonal mouse anti-phosphorylated eNOS at serine 1177 (P ~ Ser<sup>1177</sup>-eNOS 1:1000; BD Transduction Laboratories™, CA, USA), polyclonal rabbit anti-human cationic transporter type 1 (hCAT-1 1:200; abcam®, Cambridge, UK), polyclonal rabbit anti-vascular endothelial growth factor (VEGF 1:200; abcam®, Cambridge, UK), monoclonal mouse anti-β-actin (1:2500; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). Intensity of protein bands were quantified by densitometry using IMAGE J software (<https://imagej.nih.gov/ij/>). For qPCR experiments the following primers were used: hCAT-1 (hCAT-1 (F): 5'-GAGTTAGATCCA GCAGACCA-3'; hCAT-1 (R): 5'-TGTTACAATTAGCCCAGAG-3'), eNOS (eNOS (F): 5'-CCAGCTAGCCAAAGTCACCAT-3'; eNOS (R): 5'-GTCTCG GAGCCATACAGGATT-3') and VEGF (VEGF-A (F): 5'-TGCGGATCAAA CCTCACCAA-3'; VEGF-A (R): 5'-TGTCACATACGC TCCAGGACTT-3') [30]; 28S (28S (F): 5'-TTGAAAATCCGGGGGAGAG-3'; and 28S anti-sense (R): 5'-ACATTGTTCCAACATGCCAG-3') was used as a reference

gene. For both experiments, HUVEC-BG and HUVEC-HG without exosomes were used as controls.

### 2.7. Wound healing assay

HUVECs were plated in 6 wells plates (Corning® Costar®; Sigma-Aldrich, Zwijndrecht, The Netherlands) and the assay was performed as described [21]. When 100% confluence was reached, about  $3 \times 10^5$  cells per well, HUVECs were exposed for 24 h to high D-glucose (25 mmol/L) or basal D-glucose (5.5 mmol/L). Cells were washed with PBS and incubated with exo-HG or exo-BG using 5 or 1 μg of exosome protein as above. After 12 h incubation with exosomes a scratch was made on a uniform confluent layer of HUVECs using a sterile micropipette tip and cells were washed with PBS to remove debris. Photographs (Leica MC 120 HD camera, ©Leica Microsystems, Amsterdam, The Netherlands) of the same area of the wound were taken

immediately after the scratch (0 h), after 4 h and 8 h, to measure the area of the wound. The area under the curve, equivalent to wound healing from time 0 to time 8, was measured using ImageJ (<https://imagej.nih.gov/ij/index.html>), and expressed in arbitrary units for wounded area at 4 and 8 h compared with time 0 h.

## 2.8. Flow cytometry analysis

To measure activation of HUVECs, ICAM-1 expression was measured using flow cytometry. HUVECs were exposed for 24 h to culture medium with HG or BG, followed by treatment with exo-HG or exo-BGHG as described above (Fig. 1). After exosomal exposure, HUVECs were prepared for flow cytometry as described (see supplementary material).

## 2.9. Statistical analysis

The normality of data distributions was evaluated using the Shapiro-Wilk test. For comparison between HG and BG for exosome concentration and size the Wilcoxon signed test was used. For all other data, two-way ANOVA test was used for testing the effect of glucose concentration and exosome incubation. For post-hoc test, a multiple comparison test was conducted on the main effects using the Sidak adjustment. Data were analyzed using GraphPad Prism software (version 7, San Diego). Values are expressed as median  $\pm$  interquartile range (exosomal concentration and size) or mean  $\pm$  SD (other data).  $P < 0.05$  was considered to be significant.

## 3. Results

### 3.1. High glucose enhanced the release but did not change the size of the exosomes

Exosomes were characterized by anti-CD81 and anti-CD63 positive staining detected only in fraction 11 (Fig. 2A). This fraction was used for the subsequent experiments. Fraction 11 was studied by electron microscopy and exosomes were found in all fractions 11 analyzed (Fig. 2B). Exosomes were negative for Grp94 protein (Supplementary Fig. 2). NTA showed that exosomes from HUVEC-BG had a median size of  $\sim 135$  nm (Fig. 2C), which is the expected size for exosomes [31]. HG significantly increased the concentration of exosomes in the supernatant (Fig. 2E). The median size of the exo-HG was similar to the median size of exo-BG (Fig. 2F).

### 3.2. High D-glucose and HUVEC-HG-exosomes increased endothelial cell wound healing

HUVEC-HG showed increased endothelial cell wound-healing compared with HUVEC-BG (Fig. 3A, left panels), as also evidenced by a lower area under the curve (Fig. 3B). To evaluate whether this effect of HG was due to the presence of exosomes, HUVEC-BG were exposed to exo-HG and HUVEC-HG to exo-BG in two concentrations (1 and 5  $\mu$ g protein/mL; the effects for both concentrations were mainly similar, although the effects of 1  $\mu$ g protein/mL were often less pronounced, so data for 1  $\mu$ g protein/mL exosomes are shown in the supplemental files). Exo-HG increased endothelial cell wound healing in HUVEC-BG as compared with HUVEC-BG without exosomes. Exo-BG did not alter the wound healing in HUVEC-BG. In HUVEC-HG incubation with exo-BG inhibited wound healing while exo-HG had no effect on the wound healing in HUVEC-HG.

### 3.3. High D-glucose and HUVEC-HG-exosomes activate eNOS

HG increased the protein level of P  $\sim$  Ser<sup>1177</sup>-eNOS, hCAT-1, and VEGF but did not change total eNOS protein level (Fig. 4). Exo-HG significantly increased the protein levels of P  $\sim$  Ser<sup>1177</sup>-eNOS in

HUVEC-BG (Fig. 4C). Although the P  $\sim$  Ser<sup>1177</sup>-eNOS protein level was lower in HUVEC-HG in the presence of exo-BG, this result was not statistically significant. Total eNOS, hCAT-1, and VEGF protein levels were unaltered by exosomes.

### 3.4. High D-glucose and HUVEC-exosomes modulate eNOS, hCAT-1 and VEGF mRNA expression

The mRNA levels of eNOS and hCAT-1 were higher in HUVEC-HG as compared with HUVEC-BG (Fig. 5). The eNOS and hCAT-1 mRNA relative abundance was unaltered by exo-BG or exo-HG in HUVEC-BG. However, exo-HG increased VEGF mRNA relative expression in HUVEC-BG (Fig. 5C). Exo-BG reversed the HG-increased hCAT-1 but not eNOS or VEGF mRNA relative expression. Exo-HG did not alter the HG-increased eNOS or hCAT-1 but increased VEGF mRNA expression.

### 3.5. High D-glucose and HUVECs-exosomes modulate ICAM-1 expression

HG increased the expression of ICAM-1 in HUVECs (Fig. 6A and B). Exo-HG and exo-BG increased ICAM-1 expression in HUVEC-BG as compared to HUVEC-BG (Fig. 6A and B); however, HG-increased ICAM-1 expression was unaltered by exo-BG or exo-HG.

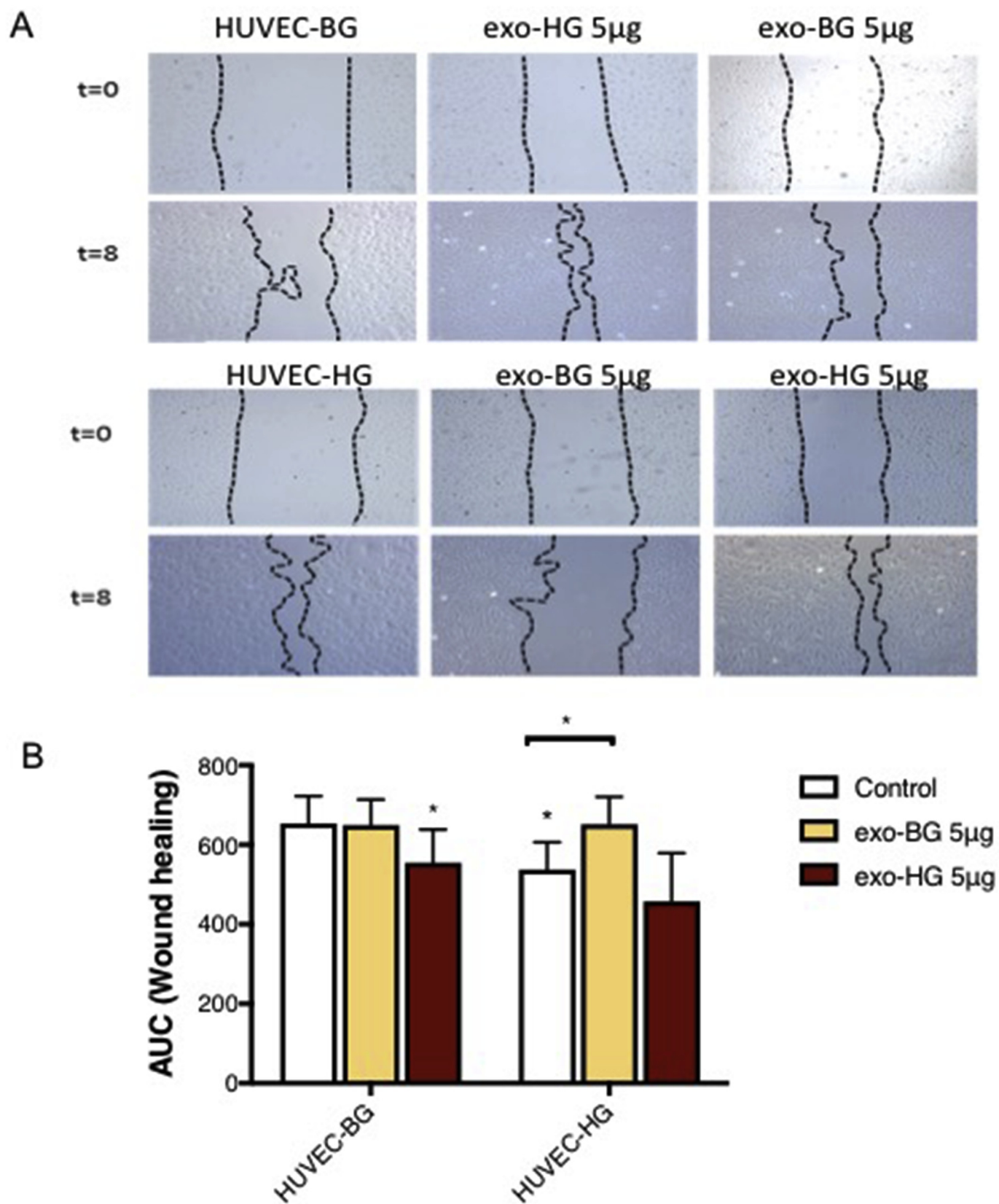
## 4. Discussion

Our data confirm our hypothesis that HG can induce endothelial cell dysfunction in HUVECs by affecting exosomes. Our results show enhanced endothelial cell wound healing, a stronger activation of the L-arginine/NO signaling pathway and increased endothelial cell activation after incubation with HG. This effect could be partly mimicked by exposing HUVEC-BG to exosomes from HUVEC-HG. The resulting endothelial phenotype has similarities with the phenotype of HUVECs from GDM [21,23].

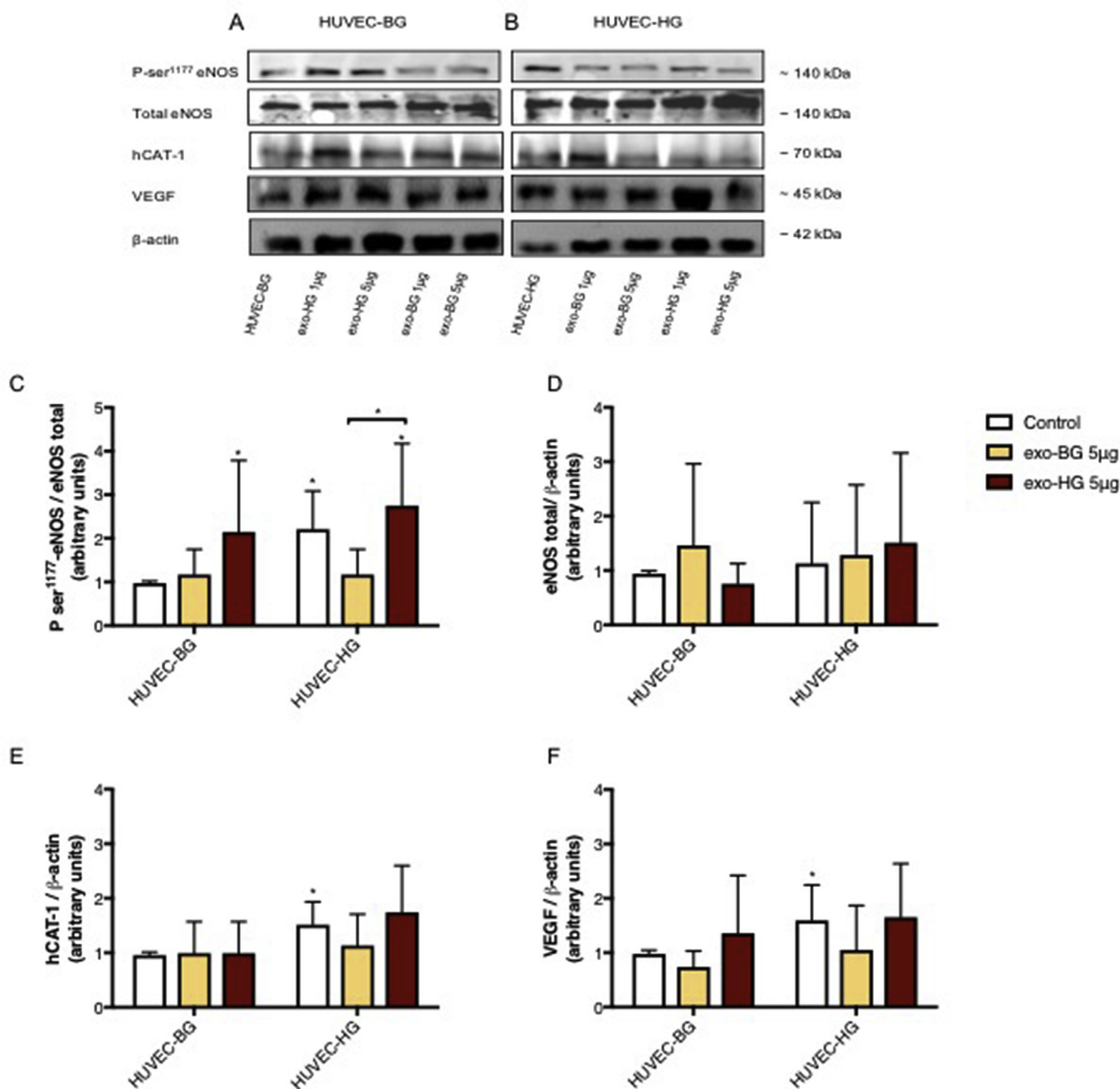
Incubation of HUVECs with HG increased the release of exosomes without affecting the size of these exosomes. This finding does not agree with the findings reported by de Jong and colleagues [20] who was not able to demonstrate an effect of hyperglycemia on exosomal release from human microvascular endothelial cells [20]. This might be explained by differences in the experimental setup such as using an adult endothelial microvascular cell type instead of an immature neonatal macrovascular endothelium and by the use of a lower D-glucose concentration (18 mmol/L) compared with our study (25 mmol/L). However, our results are in line with the findings reported by Rice and colleagues showing that hyperglycemia increased the release of exosomes from trophoblasts [25]. Interestingly, also higher concentration of exosomes are found in maternal blood from women diagnosed with GDM [32], also suggesting that high glucose may induce the release of exosomes.

HUVEC-HG showed increased endothelial cell wound healing compared with HUVEC-BG. This is in line with other studies in which exposure to HG induced an expedited retinal endothelial cell wound healing capacity [33,34]. The increased wound healing capacity in HG may result from increased endothelial cell proliferation or endothelial cell migration. However, we did not discriminate between these possibilities in the present study. A possible mechanism for the increased wound healing capacity is the enhanced level of VEGF released by HUVECs when exposed to HG [35]. In our study, although VEGF protein levels were increased in HUVEC-HG, this was not statistically significant. Interestingly, HUVECs isolated from GDM pregnancies grew slower than HUVECs from healthy pregnancy, despite increased levels of VEGF [8].

Our results show that exo-HG increased endothelial cell wound healing, suggesting that the effect of HG on endothelial cell wound healing may be mediated by exosomes. The exo-HG effect on wound healing is associated with upregulation of VEGF production in HUVECs. Our results are similar to those from Li and colleagues where exosomes



**Fig. 3. Effect of high glucose and exosomes on endothelial cell wound healing.** HUVECs cultured for 24 h in medium containing basal glucose (HUVEC-BG, 5 mmol/L D-glucose) or high glucose (HUVEC-HG, 25 mmol/L D-glucose) were exposed to exosomes (5 µg protein/mL, 12 h) isolated from HUVEC-BG (exo-BG) or HUVEC-HG (exo-HG). A: Wound healing assay in HUVECs. Area indicated with dotted lines was the wound area at time of scratch (t = 0) and after 8 h (t = 8) of incubation with exoBG or exo-HG. B: the mean ± SD of the area under curve (AUC) of the wound healing from 0 to 8 h (n = 8). Two-way ANOVA and Sidak post-hoc analysis was performed. *P* < 0.05 significant (\*) vs HUVEC-BG control and between corresponding groups.

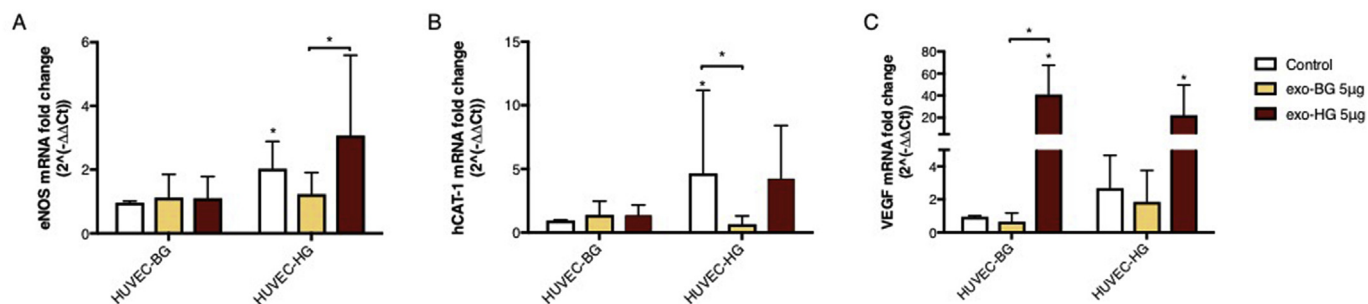


**Fig. 4.** Effect of high glucose and exosomes on eNOS, hCAT-1, and VEGF protein expression. HUVECs cultured for 24 h in medium containing basal glucose (HUVEC-BG, 5 mmol/L D-glucose) or high glucose (HUVEC-HG, 25 mmol/L D-glucose) were exposed to exosomes (1 or 5 µg protein/mL, 12 h) isolated from HUVEC-BG (exo-BG) or HUVEC-HG (exo-HG). A: Representative Western blots for phosphorylated endothelial nitric oxide synthase (eNOS) at serine<sup>1177</sup> (P ~ Ser<sup>1177</sup>-eNOS), total eNOS, human cationic amino acid transporter 1 (hCAT-1), and vascular endothelial cell growth factor (VEGF). β-Actin was a loading control. B–E: Analysis of Western blots for phosphorylated eNOS (P ~ Ser<sup>1177</sup>-eNOS)/total eNOS (B), total eNOS/β-actin (D), hCAT-1/β-actin (C) and VEGF/β-actin (E) ratios (n = 8). Analysis of the results using exosomes at 1 µg protein/mL are shown in [Supplementary Fig. 3](#). Data are expressed as mean ± SD and two-way ANOVA and Sidak post-hoc analysis was performed. *P* < 0.05 significant (\*) vs HUVEC-BG control and between corresponding groups.

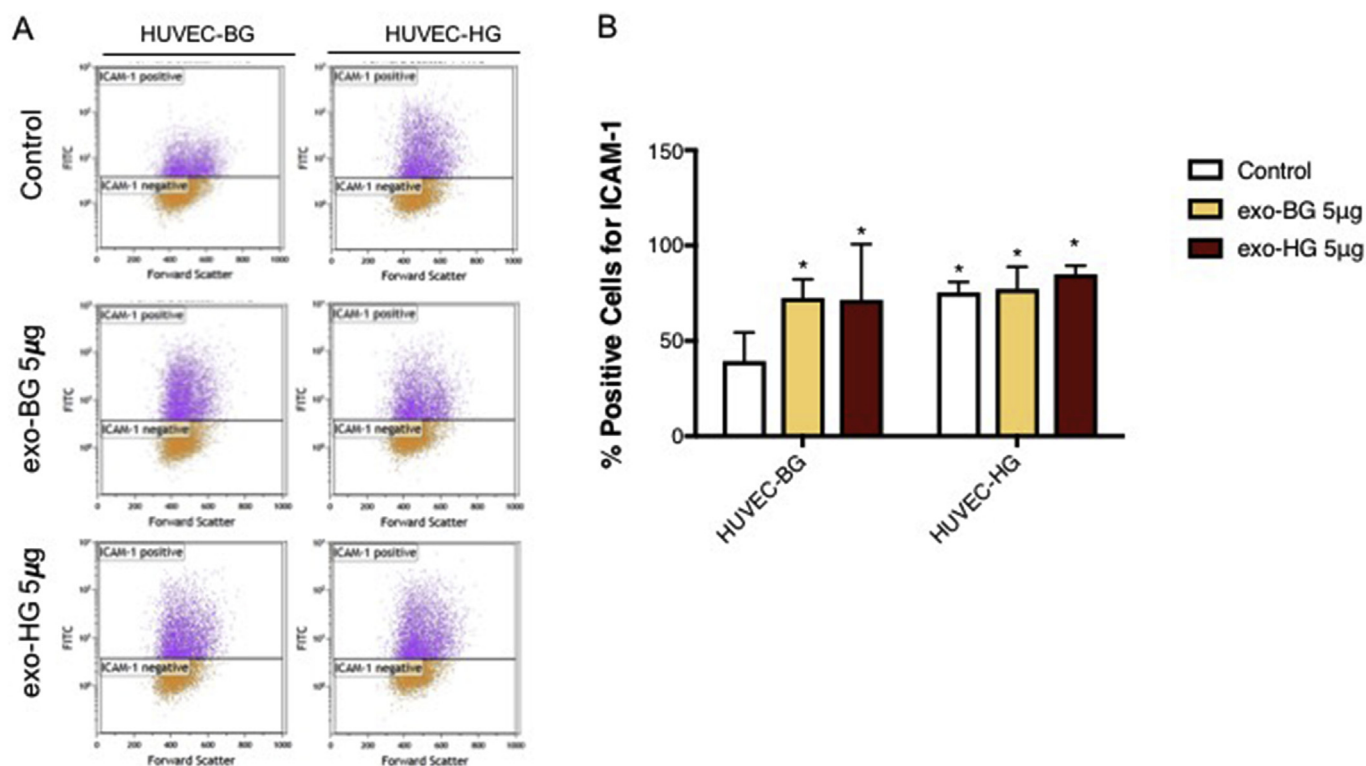
from endothelial progenitor cells increased migration of a human microvascular endothelial cell line in a wound healing assay [36]. Similar to our results, in the latter study upregulation of proangiogenic molecules such as VEGF was induced by exosomes [36]. Exo-BG incubated with HUVEC-HG seemed to be able to restore HUVEC-HG wound healing capacity to that seen in HUVEC-BG.

A potential limitation of our study refers to the fact that HUVECs were exposed for 24 h to HG and then further incubated with exo-BG for 12 h in the presence of basal glucose levels. Thus, the possibility exists that reversal of the wound healing capacity may be due to restoration of

HUVECs functionality due to withdrawal of HG. However, HUVEC-HG cultured in BG in the absence of exo-BG did not show restoration of the wound healing capacity following removal of high extracellular concentrations of D-glucose. Moreover, there is increasing evidence showing that normalizing maternal glycaemia in gestational diabetes mellitus results in normoglycaemia in the growing fetus and the offspring at delivery [10,29,37]. However, even when reaching a normal glycaemia in the mother, fetus and offspring following diet, insulin therapy or a change in life-style, alterations in the fetoplacental endothelial function are still seen. Together these data show that removal



**Fig. 5. Effect of high glucose and exosomes on eNOS, hCAT-1 and VEGF mRNA expression.** HUVECs cultured for 24 h in medium containing basal glucose (HUVEC-BG, 5 mmol/L D-glucose) or high glucose (HUVEC-HG, 25 mmol/L D-glucose) were exposed to exosomes (5 µg protein/mL, 12 h) isolated from HUVEC-BG (exo-BG) or HUVEC-HG (exo-HG). Fold change of relative mRNA expression for (A) endothelial nitric oxide synthase (eNOS), (B) human cationic amino acid transporter 1 (hCAT-1), and (C) vascular endothelial cell growth factor (VEGF) are shown. Values were normalized to a 28S mRNA. Data are express as mean ± SD and two-way ANOVA and Sidak post-hoc analysis was performed. *P* < 0.05 significant (\*) vs HUVEC-BG control and between corresponding groups.



**Fig. 6. Effect of high glucose and exosomes on ICAM-1 expression.** HUVECs cultured for 24 h in medium containing basal glucose (HUVEC-BG, 5 mmol/L D-glucose) or high glucose (HUVEC-HG, 25 mmol/L D-glucose) were exposed to exosomes (5 µg protein/mL, 12 h) isolated from HUVEC-BG (exo-BG) or HUVEC-HG (exo-HG). A: Representative dot plots for intracellular adhesion molecule type 1 (ICAM-1) in HUVECs in the absence (Control) or presence of exosomes. B: Data are presented as percentage of ICAM-1 positive cells for the various conditions (n = 5). Data are express as mean ± SD and two-way ANOVA and Sidak post-hoc analysis was performed. *P* < 0.05 significant (\*) vs HUVEC-BG control.

of D-glucose from the medium may not be a plausible explanation, but that HUVEC-derived exosomes may induce this restoration.

The present study shows that HG increased the activator Ser<sup>1177</sup>-phosphorylation of eNOS, hCAT-1 and VEGF protein expression in HUVECs. Similar results data have been shown by Vasquez and colleagues [16], where exposure of HUVECs to 25 mmol/L for 24 h increased the phosphorylation of Ser<sup>1177</sup> at eNOS and NO synthesis, and hCAT-1 expression in addition to increased L-arginine transport. We show that some of the effects of HG on HUVECs can also be induced by exo-HG in HUVECs incubated under BG conditions. We found that exo-HG increased the level of P~Ser<sup>1177</sup>-eNOS, and hCAT1 and VEGF protein expression, although this was only significant for P~Ser<sup>1177</sup>-eNOS expression. Although similar data were found for mRNA expression, the protein data are not always consistent with mRNA data in our study. This may be due for instance to the well-known fact that

there are many post-transcriptional modifications during translation of mRNA into protein [38]. These results show that exo-HG can partly induce a HG phenotype in HUVEC-BG and are therefore in line with the hypothesis that HG induces the production of exosomes in fetal endothelium and that these exosomes negatively impact fetal endothelial function. Interestingly, exo-BG restored the P~Ser<sup>1177</sup>-eNOS level in HUVEC-HG to values in HUVEC-BG. Exo-BG also seemed to be able to restore the endothelial expression of hCAT-1 and VEGF, although this was not statistical significant. As indicated above, these results for exo-BG incubation may also be due to restoration of endothelial function following removal of HG. Future studies are needed to evaluate whether differences in exosomal cargo between exo-HG and exo-BG are responsible for the differences in response of HUVECs to these exosomes. Proteomic analysis seems to be necessary to explain this difference.

HUVECs incubated under HG showed increased endothelial cell



activation, as quantified by the expression of ICAM-1. Previous studies have also shown that HG can activate endothelial cells by increasing expression of this adhesion molecule [39,40]. Since HUVECs from GDM pregnancies also express high levels of ICAM-1 [41], HG may be one of the responsible factors for fetoplacental endothelial cell activation in GDM. Moreover, the expression of ICAM-1 is lower in HUVECs from women with GDM with HbA1c levels lower than 6% as compared with women with normal pregnancies, suggesting these differences can be due to differences in D-glucose management [42]. Although we found that this upregulation of ICAM-1 could also be induced by exo-HG, which is in line with our hypothesis, in contrast to our hypothesis, endothelial cell activation was also induced by exo-BG. The reason for this is unknown, but since ICAM-1 has been described as constitutive protein of exosomal cargo in other cell types, such as immune cells [27], it is possible that endothelial exosomes may also contain ICAM-1, which could be transferred to endothelial cells even under basal D-glucose condition.

Interestingly, exosomes derived from HUVEC-HG induced changes in wound healing capacity and eNOS activity in HUVEC-BG supporting previous results from our lab seen in HUVECs from GDM [21]. Exosomes derived from HUVECs from GDM increased the wound healing capacity and eNOS activity in HUVECs from normal pregnancies. Therefore, a paracrine action of exosomes in fetal endothelial cells in GDM pregnancies is likely, a phenomenon that may result in spreading the GDM-associated pathological phenotype to the fetoplacental endothelium inducing endothelial dysfunction of this vascular bed [23].

In summary, HG induced endothelial cell dysfunction and activation in HUVECs that is partly similar to the endothelial cell dysfunction and activation seen in this cell type from GDM pregnancies [21]. The results showed that HUVECs-derived exosomes have the capacity to modulate HUVECs function, such as their wound healing capacity and eNOS activation, under normal or high extracellular D-glucose concentration. Future studies are needed to evaluate whether exosomes are internalized by HUVECs and whether high extracellular D-glucose affects the internalization of these nanovesicles. Although incubation of HUVECs with HG mimicked several features of a state of endothelial cell dysfunction as seen in HUVECs from GDM pregnancies, exposure of HUVECs to HG cannot totally explain the phenotype of this cell type in GDM. Thus, it is suggested that other factors, such as fetal hyperinsulinemia and elevated levels of proinflammatory factors, may also play a role. Our findings suggest that fetoplacental endothelial exosomes are one of the mechanisms behind endothelial dysfunction induced by an intrauterine environment high in D-glucose concentration as seen at some stages in GDM.

### Conflicts of interest

There is no conflict of interest.

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### Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.placenta.2018.04.010>.

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