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Effects of dapagliflozin on human epicardial adipose tissue: modulation of insulin resistance, inflammatory chemokine production, and differentiation ability

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Aims In patients with cardiovascular disease, epicardial adipose tissue (EAT) is characterized by insulin resistance, high pro-inflammatory chemokines, and low differentiation ability. As dapagliflozin reduces body fat and cardiovascular events in diabetic patients, we would like to know its effect on EAT and subcutaneous adipose tissue (SAT).

Methods and results Adipose samples were obtained from 52 patients undergoing heart surgery. Sodium-glucose cotransporter 2 (SGLT2) expression was determined by real-time polymerase chain reaction ($n = 20$), western blot, and immunohistochemistry. Fat explants ($n = 21$) were treated with dapagliflozin and/or insulin and glucose transporters expression measured. Glucose, free fatty acid, and adipokine levels (by array) were measured in the EAT secretomes, which were then tested on human coronary endothelial cells using wound healing assays. Glucose uptake was also measured using the fluorescent glucose analogue (6NBDG) in differentiated stromal vascular cells (SVCs) from the fat pads ($n = 11$). Finally, dapagliflozin-induced adipocyte differentiation was assessed from the levels of fat droplets (AdipoRed staining) and of perilipin. SGLT2 was expressed in EAT. Dapagliflozin increased glucose uptake (20.95 ± 4.4 mg/dL vs. 12.97 ± 4.1 mg/dL; $P < 0.001$) and glucose transporter type 4 (2.09 ± 0.3 fold change; $P < 0.01$) in EAT. Moreover, dapagliflozin reduced the secretion levels of chemokines and benefited wound healing in endothelial cells (0.21 ± 0.05 vs. 0.38 ± 0.08 open wound; $P < 0.05$). Finally, chronic treatment with dapagliflozin improved the differentiation of SVC, confirmed by AdipoRed staining [539 ± 142 arbitrary units (a.u.) vs. 473 ± 136 a.u.; $P < 0.01$] and perilipin expression levels (121 ± 10 vs. 84 ± 11 a.u.).

Conclusions Dapagliflozin increased glucose uptake, reduced the secretion of pro-inflammatory chemokines (with a beneficial effect on the healing of human coronary artery endothelial cells), and improved the differentiation of EAT cells. These results suggest a new protective pathway for this drug on EAT from patients with cardiovascular disease.

Keywords Epicardial adipose tissue • Insulin resistance • Antidiabetic drugs • Endothelium

1. Introduction

There is a clear relationship between epicardial adipose tissue (EAT) thickness, insulin resistance,[1] and coronary artery disease (CAD).[2,3] The increase in EAT thickness is thought to be caused by adipocyte hypertrophy and low pre-adipocyte differentiation ability.[4] This process combines inflammation, insulin resistance, and free fatty acid (FFA) release, which are reflected in systemic modifications.[5]

The enlargement or hypertrophy of adipocytes limits the energy storage ability of adipose tissue and its turnover, which also contributes to

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insulin resistance and cardiometabolic disease.[6] In addition, the inflammatory state of EAT can also induce insulin resistance[7] and release molecules that could interact with endothelial or myocardial cells.[8,9] These mechanisms may explain, in part, the association between EAT thickness and cardiac structure[10] and dysfunction.[11]

Dapagliflozin is a novel therapeutic agent for the treatment of patients with type 2 diabetes mellitus (T2DM) that reduces plasma blood glucose levels and increases insulin-mediated tissue glucose disposal.[12] This drug inhibits sodium-glucose cotransporter 2 (SGLT2) and reduces hyperglycaemia in an insulin- independent manner.[13]

SGLT2 belongs to a human SGLT (SLC5) family that has 12 members[14,15] and is mainly localized in the early proximal renal tubule. It is responsible for the reuptake of 80–90% of the glucose load, whereas SGLT1 is mainly expressed in the S2 and S3 segments of the proximal renal tubule and reabsorbs just the remaining 10–20% of the glucose load. Thus, SGLT2 has been proposed as the major pathway for glucose reabsorption in the kidney overall.[16] Recently, dapagliflozin has been associated with a 15% reduction of fatal and non-fatal cardiovascular (CV) disease events compared with insulin treatment.[17] There is also less risk of hypoglycaemia compared with insulin treatment. In addition, dapagliflozin reduces fat mass and improves the quality of life of patients with CV disease.[18,19]

Thus, our main objective is to investigate whether dapagliflozin improves glucose uptake, reduces pro-inflammatory factors, and improves the differentiation ability of EAT cells in patients with CV disease. In addition, we study the consequences of the EAT changes on endothelial cells.

2. Methods

2.1 Samples

After obtaining informed consent, epicardial and subcutaneous adipose tissue samples were obtained from 52 patients undergoing cardiac surgery. The exclusion criteria were previous heart surgery or severe infective diseases. The study protocol was approved by the Galician Clinical Research Ethics Committee and carried out in accordance with the Declaration of Helsinki.

2.2 SGLT2 and SGLT1 expression

RNA was extracted following the manufacturer's protocol using the RNeasy Plus Mini Kit (Qiagen GmbH, Hilden, Germany). One microgram of RNA was retrotranscribed to complementary DNA (cDNA) with a maxima first-strand cDNA synthesis kit (Thermo Fisher Scientific, Waltham, MA, USA) in a thermocycler (25 °C for 10 min, 50 °C for 15 min, and 85 °C for 5 min). Real-time polymerase chain reaction (PCR) was performed with 2 µL of cDNA, SYBR Green, and 300 nM of SGLT2 and SGLT1 primers with the following sequences and conditions: 95 °C for 10 min, followed by 40 cycles of 95 °C for 30 s and 60 °C for 30 s and then 72 °C for 30 s. SGLT2 primers: 5′-AGTGCCTGCTCTGGTTTTGT-3′ (forward), 5′-GTGAGGCTGTGGCTTATGGT-3′ (reverse) and SGLT1 primers: 5′-AGCTCCTTATACGGCCTCCT-3′ (forward) and 5′-ATTCGCAGGACAGCTCTTAC-3′ (reverse). Expression levels were normalized by housekeeping gene, actin (ACTB) as the antilogarithm of the ratio of cycle thresholds of gene and housekeeping.

2.3 Immunohistochemistry

Using Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/), we compared sequences of the following proteins from the same family: sodium/glucose cotransporter (SLC5A) for *Homo sapiens* (UniProtKB); SGLT1 (SLC5A1, P13866), SGLT2 (SLC5A2, P31639), SGLT3 (SLCA4, C7EWH7), SGLT4 (SLC5A9, Q2M3M2), and SGLT5 (SLC5A10, A0PJK1) (see Supplementary material online, *Figure S1*). Specific antibodies against SGLT2 and SGLT1 were selected and used for immunohistochemistry and western blot (see Supplementary material online, *Table S1*).

Samples were immersion fixed in 10% neutral-buffered formalin for 24 h and embedded in paraffin. Sections of 4 µm thickness were mounted on FLEX IHC microscope slides (Agilent, Carpinteria, CA, USA). After deparaffination and epitope retrieval (in EnVision FLEX target retrieval solution at high pH, for 20 min at 97 °C), immunohistochemistry was automatically performed using an AutostainerLink 48 immunostainer (Agilent). The slides were incubated at room temperature in: (i) SGLT2 (D-6) monoclonal mouse antibody (Santa Cruz Biotechnology, Dallas, TX, USA) at 1: 100 for 60 min, (ii) EnVision FLEX/HRP (dextran polymer conjugated with horseradish peroxidase and affinity-isolated goat anti-mouse immunoglobulins) for 20 min, (iii) substrate working solution (mix) (3, 3′-diaminobenzidine tetrahydrochloride chromogen solution) for 10 min, and (iv) EnVision FLEX hematoxylin for 9 min. Human kidney was used as the positive control.

2.4 Dapagliflozin treatment on epicardial and subcutaneous adipose tissue explants

Epicardial and subcutaneous fat biopsies were obtained at the time of heart surgery and immediately transferred to the laboratory. Then, the samples were split, and each 100 mg piece was cultured with 500 µL of M199 for 18 h to washout excess blood. Afterwards, the samples were treated with insulin (5 µg/mL), dapagliflozin (1–100 µM) or both for 6 h, as previously described.[20] Dapaglifozin doses (1, 10, 30, 100, and 300 µM) were tested in *ex vivo* assays according to *in vitro* experiments (0.2–20 µM). The most efficient and reproducible concentration was 100 µM.

Glucose levels in the supernatants of the EAT and subcutaneous adipose tissue (SAT) explants were determined using the Glucose Colorimetric Assay Kit (Cayman Chemical, Ann Arbor, MI, USA) following the manufacturer's protocol. The glucose uptake levels for each treatment were calculated as follows: medium glucose levels - glucose levels in fat supernatants. The medium glucose levels were 100 mg/dL. Fat explants, after treatment with insulin or dapagliflozin, with higher glucose uptake than untreated fat explants were considered insulin or dapagliflozin responders. Secretomes were stored for later study of their proteomic profile and their paracrine effect on human coronary endothelial cells (HCAECs).

2.5 Adipokine array

Arrays (R&D systems, Inc, Abindong, Oxford, UK) were used to determine adipokine profiles of proteins released from the EAT with ($n = 5$) or without dapagliflozin ($n = 5$) treatment, according to the manufacturer's protocol. Each array contained secretomes (125 µL for each array) from four independent explants with or without insulin response. The proteome profiler human adipokine array detected 36 different cytokines (see Supplementary material online, *Table S2*). Spot quantification was analysed by ImageJ software (Bethesda, MD, USA), and the intensity of spots representing dapagliflozin treatment were compared with each control treatment. Validation of chemokine (C-C motif) ligand 2 (CCL2) levels was measured by enzyme-linked immunosorbent assay (ELISA) (R&D Systems, Inc. Abingdon, NB, UK) following the manufacturer's protocol. Diluted secretomes (1:10) of EAT (100 mg/500 µL

M199) with or without previous dapagliflozin treatment from 17 patients were analysed.

2.6 FFA release quantification

FFA levels were measured in the supernatant of the EAT explants according to manufacturer's protocol with a Free Fatty Acid Fluorimetric assay kit (Cayman Chemical). The amount of FFAs released from 100 mg of fat tissue, after dapagliflozin treatment for 6 h, was measured with a fluorimeter at an excitation wavelength of 540 nm and an emission wavelength of 590 nm (FLUO Star OPTIMA, BMG Labtech, Ortenberg, Germany). To obtain FFA concentrations as μM, the following equation was used: FFA (μM) = [(corrected fluorescence) - (*y*-intercept)/slope)] × dilution.

2.7 Western blot

After treatment, adipose tissue samples were homogenized using a pestle and following the AllPrep DNA/RNA/Protein Mini Kit (Qiagen) instructions for simultaneous purification of DNA, RNA, and protein from cells and tissues. Western blot was performed on the explant protein samples, which were resolved on 8% SDS-PAGE and blotted onto polyvinylidene difluoride membranes, as previously described.[21] SGLT2, SGLT1, glucose transporter type 4 (GLUT-4), perilipin and β-actin proteins were detected using monoclonal and polyclonal antibodies. The conditions are summarised in Supplementary material online, *Table S1*. Densitometry Image J software (Bethesda, MD, USA) was used to quantify the protein levels, evaluated in duplicate and expressed with respect to β-actin levels.

2.8 Secretome effect on HCAEC

HCAECs (Sigma-Aldrich, St. Louis, MO, USA) from one batch were thawed and cultured with mesoendothelial cell growth medium (Sigma-Aldrich). They were used at passage 2 or 3. Confluent monolayers of cells were seeded in 24 wells and wounded with plastic micropipette tips. Diluted EAT secretomes from 11 patients, after being treated or not with dapagliflozin (1:1) on a mesoendothelial cell growth medium (Sigma-Aldrich) were added to the cells for 6 or 24 h. Wound width was measured microscopically (Leica DFC295, objective 10×) and quantified with ImageJ software at 0, 6, and 24 h after injury.

2.9 Viability of SVCs after dapagliflozin treatment

The label-free xCELLigence System (ACEA Biosciences, San Diego, CA, USA), which allows for real-time monitoring, was used to assess cell viability. Using this platform, the cell index (CI) was the parameter used to represent the cell status based on the measured electrical impedance.[22] SVCs obtained from adipose tissue (EAT and SAT) were seeded [6000 cells/well (E-plates, 16 wells)] and incubated for 24 h. At that point, the cells were treated with dapagliflozin (1, 10, 100, 500, and 1000 μM) and dimethyl sulphoxide (DMSO), the vehicle control. Cell behaviour was automatically measured and recorded without interruption every 15 min for aperiod of 24 h. The data obtained were presented in time-course curves.

2.10 Glucose uptake in EAT and SAT stromal cells after adipogenesis induction

The fluorescent glucose analogue, 6-NBDG [6-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-6-deoxyglucose, Thermo Fisher Scientific], was used to report glucose transport. EAT and SAT SVCs were seeded in a 24 multiwell plate (Vision plates) in medium M199 (Lonza Biologics, Basel, Switzerland) supplemented with 10% foetal bovine serum until 90% confluence. Adipogenesis was induced with the adipogenic cocktail, as previously described.[23] Then, cells were serodeprived undergoing stable isotope labelling with amino acids in cell culture (SILAC) medium (Gibco, Gaithersburg, MD, USA) 1 h before treatment with dapagliflozin (10 and 100 μM for 30 min) and later incubated with insulin (5 μg/mL) for 45 min before adding 6 NBDG (Thermo Fisher Scientific). Fluorescent glucose (75 μM) was incubated for 1 h 30 min, followed by three washes; subsequently fluorescence was visualized on a fluorescence microscope at 488 nm (Zeiss AX10) and quantified by fluorimeter at 485–520 nm (FLUO Star OPTIMA, BMG Labtech).

2.11 Adipogenesis

Epicardial and subcutaneous SVCs were obtained from adipose tissue biopsies, as previously described.[23] Adipogenesis induction was performed with or without dapagliflozin (1 and 10 μM). Before reaching the maximum level of differentiation (after 20–28 days of induction), the cells were stained with AdipoRed assay reagent (Lonza Biologics) for the analysis of lipid droplets and quantification by fluorimeter at 485–520 nm (FLUO Star OPTIMA). After the cells were washed with phosphate-buffered saline and the cell nuclei were stained with DAPI (4',6-diamidino-2-phenylindole; NucBlue Fixed Cell Stain ReadyProbes Reagent, Molecular Probes, Waltham, MA, USA) for 1 h. Then, the lipid droplets in the cells were visualized with an AX10 microscope (Zeiss, Jena, Germany). Perilipin expression levels were detected by western blot with diluted antibody (1:500) from Sigma-Aldrich.

2.12 Statistical analysis

Categorical variables were expressed as percentages and compared using the χ^2 test or the Fisher's exact test. We used the Shapiro–Wilk test to confirm the normality of continuous variables for glucose uptake in fat explants. Non-skewed variables were summarized using the mean and standard error. Differences between treatments and controls were performed using the paired Student's *t*-test. Statistical significance was defined as $P < 0.05$, $P < 0.01$, and $P < 0.001$. In *Figure 2A*, one data point lying more than 2 SDs from the mean was considered as an outlier and removed from further analysis. All analyses were computed using SPSS 15.0 software for Windows (SPSS, Inc., Chicago, IL, USA).

3. Results

3.1 SGLT2 and SGLT1 expression

Epicardial fat biopsies were obtained from 20 patients undergoing open-heart surgery [72 ± 10 years, 27 ± 4 kg/m^2 body mass index (BMI), 55% male, 50% with CAD, 30% with T2DM, 75% with arterial hypertension (HTA), and 40% with heart failure (HF)]; the tissue was used to determine SGLT2 expression levels and SGLT2 regulation by CV risk factors. When analysing the differential expressions regarding gender, hyperlipidaemia, HTA, and obesity, higher levels of mRNA SGLT2 expression were detected in men compared with women [1.75 ± 0.04 arbitrary units (a.u.) vs. 1.71 ± 0.03 a.u.; $P = 0.017$; Supplementary material online, *Table S3*], but not with respect to the other CV risk factors in EAT. SGLT1 expression levels were very low and sometimes undetectable. The PCR amplification product for SGLT1 and SGLT2 is shown in *Figure 1A*, left panel; immunohistochemistry and western blot were used to determine protein levels in EAT (*Figure 1A* and *B*, right panel).

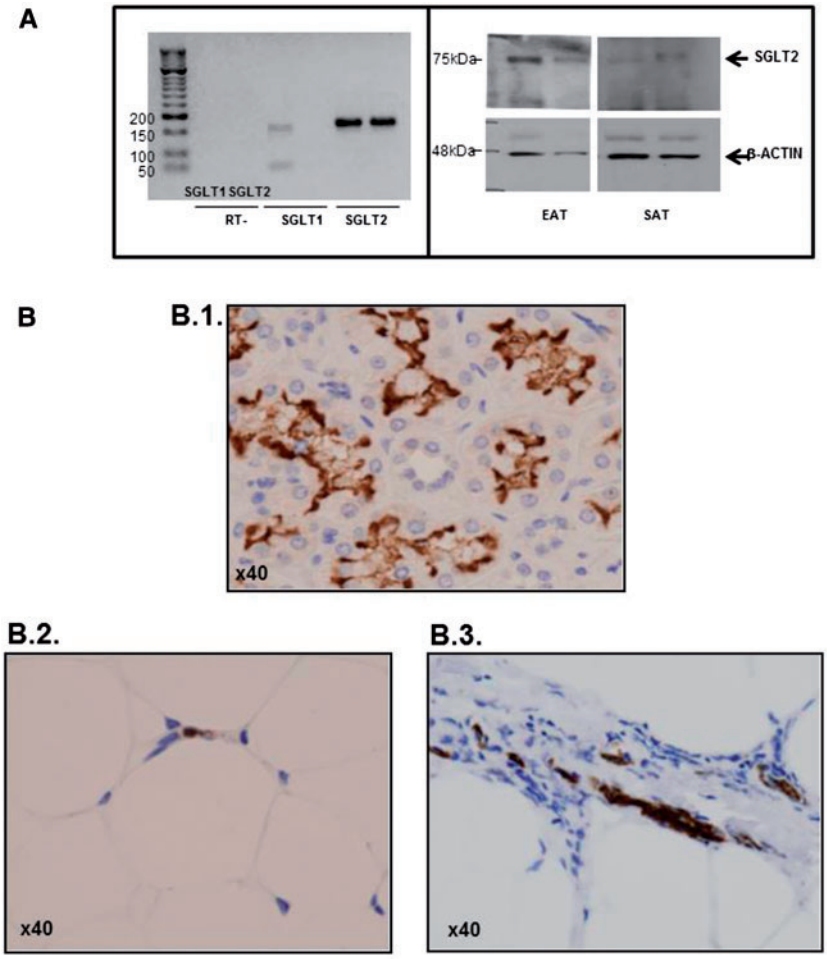

Figure 1 SGLT2 expression in fat pads. Agarose gel with SGLT2 and SGLT1 amplified products using real-time PCR or western blot with SGLT2 antibody in epicardial or subcutaneous adipose tissue (EAT or SAT) (*A*). Immunohistochemistry (*B*) with SGLT2 antibody in kidney (intense apical membrane staining of cells in proximal convoluted tubules) (*B*.1.) and in adipose tissue (adipocytes and non-adipocytes cells staining) (*B*.2, *B*.3) (objective magnification, ×40).

3.2 Dapagliflozin effect on EAT explants

3.2.1 Glucose uptake and FFA release

Dapagliflozin treatment induced an increase in glucose uptake compared with baseline control (20.95 ± 4.4 mg/dL vs. 12.97 ± 4.1 mg/dL control; $P < 0.001$) in 65% of all tested epicardial fat biopsies ($n = 21$) (*Figure 2A* and *B*). As a result of the relatively high insulin resistance of EAT (when compared with SAT, previously described by several authors[1,23]) there was a low percentage (52%) of samples that responded with an increase in glucose uptake after insulin treatment (16.5 ± 4.3 mg/dL vs. 13.0 ± 4.1 mg/dL without insulin; $P = 0.266$, *Figure 2B*). In samples with insulin resistance, dapagliflozin increased glucose uptake (29.0 ± 9.3 mg/dL vs. 14.5 ± 7.5 mg/dL with control; $P = 0.02$, *Figure 2C*). It also increased glucose uptake in the samples with insulin sensitivity (17.4 ± 5.0 mg/dL vs. 12.3 ± 5.2 mg/dL with control; $P = 0.002$, *Figure 2D*). However, the increase in glucose uptake disappeared after insulin treatment was added to dapagliflozin treatment. Since GLUT-4 is a glucose transporter translocated from the cytosol to the cell membrane by insulin in adipocytes,[24] we analysed GLUT-4 and SGLT2 protein expression levels in EAT explants after insulin or dapagliflozin treatment.

As shown in *Figure 3*, SGLT2 expression levels do not differ compared with control, but GLUT-4 expression levels were higher after insulin (1.7 ± 0.3-fold change over control; $P = 0.001$) or dapagliflozin treatment (2.1 ± 1.0-fold change over control; $P = 0.025$). GLUT-4 expression levels were dapagliflozin dependent and correlated with glucose uptake, as has been recently described in fat from insulin-resistant and hyperglycaemic rats.[25] Because EAT has higher insulin resistance than SAT,[1,23] we analysed the dapagliflozin effect on glucose uptake in SAT biopsies. While insulin treatment increased glucose uptake in 82% of samples, dapagliflozin treatment increased glucose uptake in 59% of SAT samples (see Supplementary material online, *Figure S2B*), (15.6 ± 3.9 mg/dL vs. 4.7 ± 3.5 mg/dL; $P = 0.001$). Insulin treatment also produced an increase in glucose uptake (15.6 ± 3.5 vs. 4.7 ± 3.5 mg/dL, $P = 0.009$) (see Supplementary material online, *Figure S2B*). Finally, FFAs were measured in EAT supernatants after treatment with or without dapagliflozin, but we did not observe any differences (see Supplementary material online, *Figure S3*).

3.2.2 Adipokine secretion profile

To measure the secretory products of EAT after dapagliflozin treatment, protein arrays (5 control and 5 dapagliflozin treatment) were performed in duplicate grouping of the secretomes with or without an insulin response. In samples with an insulin response, dapagliflozin treatment for 6

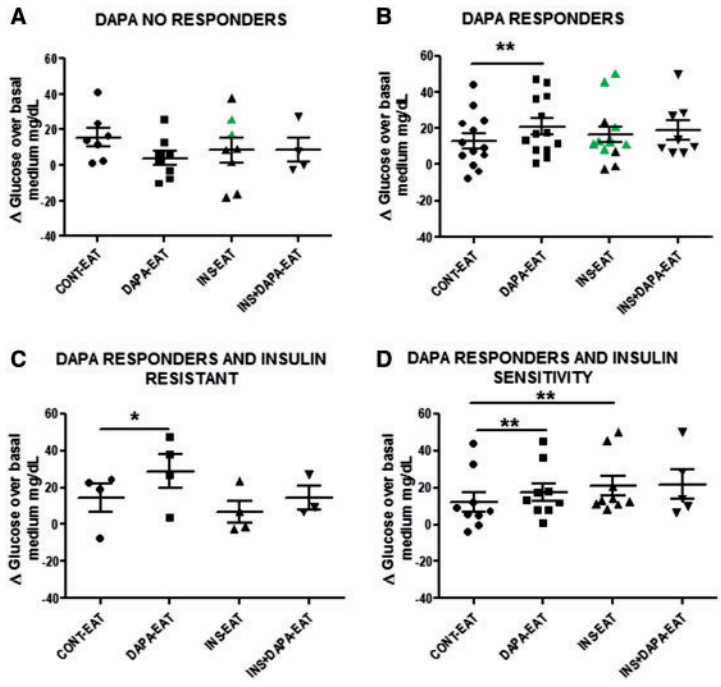

Figure 2 Glucose uptake in EAT tissue induced by dapagliflozin. Scatter/dot plots present mean ± SEM of glucose levels in EAT supernatants after insulin and/or dapagliflozin for 6 h, measured by colorimetric assay. Explants were obtained from 21 patients. Samples were grouped based on dapagliflozin response without (*A*) or with (*B*) increase in glucose uptake. Green symbols represent insulin-sensitive samples. Those explants with an increase in glucose uptake after dapagliflozin treatment (65%) were grouped as insulin resistant (*C*) or insulin sensitive (*D*). Paired *t*-test represents statistical significance. $^{*}P < 0.05$, $^{**}P < 0.01$.

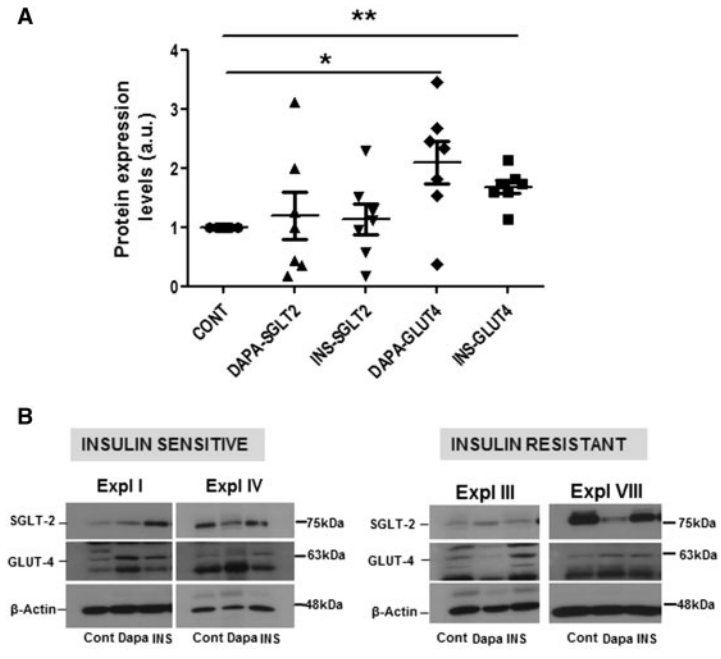

Figure 3 Regulation of SGLT2 and GLUT-4 protein expression, in EAT tissue, by dapagliflozin. (*A*) Scatter/dot plot presents mean ± SEM of SGLT2 and GLUT-4 expression levels in EAT with or without insulin or dapagliflozin treatment for 6 h. Values are expressed in fold changes over control ($n = 7$ with glucose uptake after dapagliflozin treatment). (*B*) Representative western blots for SGLT2 (75 kDa) and GLUT-4 (55-60 kDa) protein analysis, after dapagliflozin or insulin treatment for 6 h, measured using SDS-PAGE gel (12%). Ten explants were analysed in duplicates. Paired *t*-test for statistical significance. $^{*}P < 0.05$, $^{**}P < 0.01$.

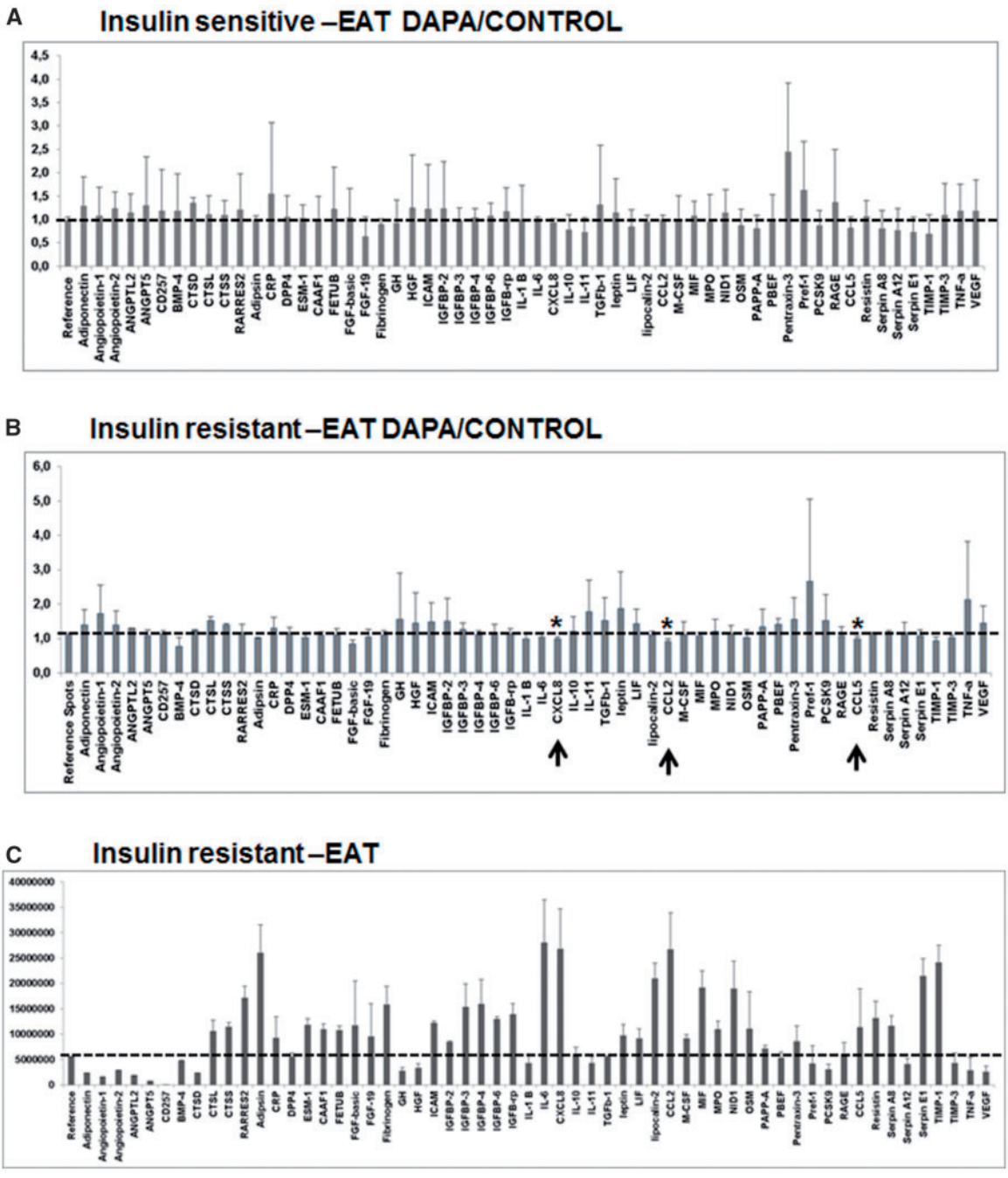

Figure 4 Adipokine profile array on EAT secretomes after being treated or not with dapagliflozin. EAT secretomes were grouped as with (*A*) or without insulin response (*B*, *C*). Bar graphs present the fold change of proteins after dapagliflozin treatment over control (*A*, *B*) or total levels in EAT secretomes from samples without insulin response (*C*). Values are from spots densitometry (mean ± SEM arbitrary units) of three or two independent arrays in duplicates (each array was performed with EAT secretomes from four patients). Paired *t*-test for statistical significance. *$P < 0.05$.

h did not modify the secreted adipokine profile with respect to the control (*Figure 4A*). However, a statistically significant reduction in the levels of CXCL8, CCL2, and CCL5 was observed in samples with insulin resistance after dapagliflozin treatment when compared with the control (*Figure 4B*). After these results, we analysed the levels of proteins released by EAT in patients without insulin response and without dapagliflozin treatment. The secreted adipokine profile in these samples revealed a higher secretion of IL-6, CXCL8, CCL2, adipsin, and tissue inhibitor of metalloproteinases-1 (TIMP-1) (*Figure 4C*). After analysing CCL2 by ELISA in secretomes of EAT with or without dapagliflozin treatment from 17 patients, our results showed that samples without treatment released 4796 ± 3711 pg/mL of CCL2. After dapagliflozin treatment, these levels tended to decrease (3818 pg/mL ± 2999 pg/mL), although not statistically significant ($P = 0.06$). For the subgroup of EAT samples with increased glucose uptake after

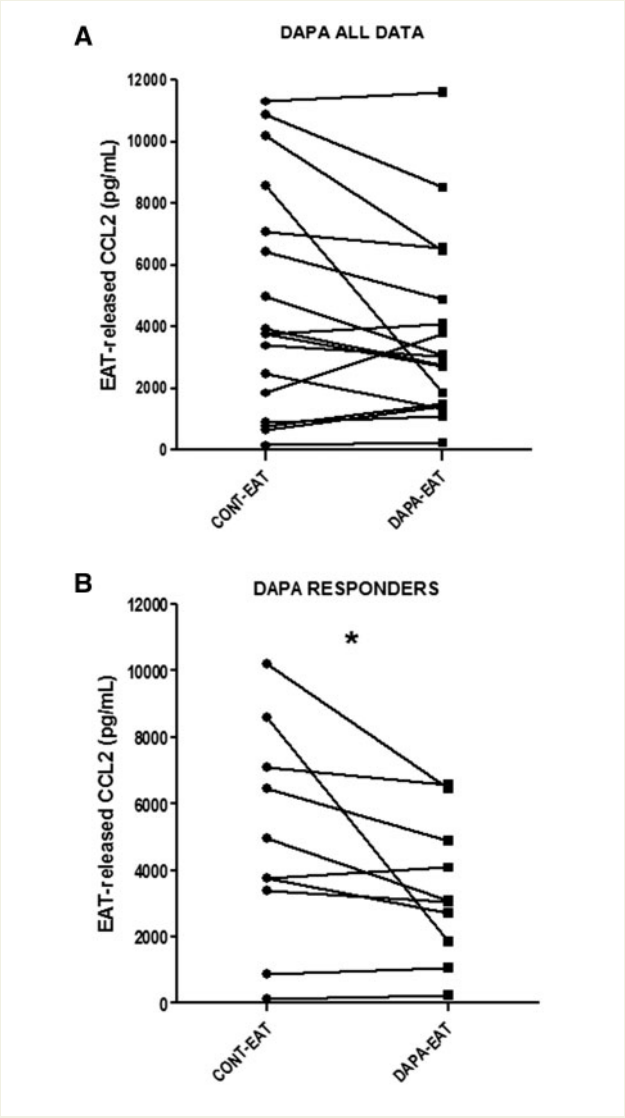

Figure 5 CCL2 levels in EAT secretomes after being treated or not with dapagliflozin. Scatter/dot plots present mean ± SEM of CCL2 values in EAT secretome with or without dapagliflozin treatment ($n = 17$) (*A*) All samples ($n = 17$). (*B*) Only samples with an increase of glucose uptake after dapagliflozin treatment. Paired *t*-test for statistical significance. *$P < 0.05$.

dapagliflozin treatment, the change was significant (3400 ± 2123 pg/mL vs. 4920 ± 3204 pg/mL in control, $n = 10$; $P < 0.05$; *Figure 5*). Moreover, as is known, CCL2 secretion by EAT was higher in patients with, than those without, CAD (7502 ± 3416 pg/mL; $n = 6$ vs. 3278 ± 3046 pg/mL; $n = 11$; $P < 0.05$). However, this difference disappeared in EAT samples after dapagliflozin treatment (5719 ± 3863 pg/mL vs. 2781 ± 1891 pg/mL).

3.2.3 Paracrine effect of EAT secretomes on HCAEC

Secretomes from EAT with or without dapagliflozin treatment were tested in HCAECs. A wound on these endothelial cells was induced and incubated with secretomes from EAT for 6 or 24 h. The healing was greater in endothelial cells that were incubated with secretomes from EAT that had undergone dapagliflozin treatment compared with those that had not undergone treatment for 6 h (0.8 ± 0.035 vs. 0.7 ± 0.05; $P = 0.01$) or 24 h (0.4 ± 0.086 vs. 0.2 ± 0.06; $P = 0.02$). Improved wound healing was observed with EAT secretomes that responded to dapagliflozin treatment with increased glucose uptake. This effect was not observed with EAT secretomes that had not responded to treatment with dapagliflozin (*Figure 6*).

3.3 Dapagliflozin effect on SVCs

3.3.1 Viability of SVCs after dapagliflozin treatment

A real-time cell analyser was used to monitor any possible cytotoxic effects or changes in the properties of human stromal cells under different concentrations of dapagliflozin (1, 10, 100, and 1000 μM) with DMSO as the vehicle control. The CI values after 24 h of treatment were compared for the different concentrations. Dapagliflozin did not reduce the proliferation rate of SVCs even at the highest concentrations (see Supplementary material online, *Figure S4*).

3.3.2 Glucose uptake after adipogenesis induction

The effect of dapagliflozin on glucose uptake was also measured in SVCs after adipogenesis was induced in the epicardial and subcutaneous fat biopsies and *t*-tested in primary cultures from 11 patients (74 ± 8 years, 30 ± 5 BMI, 80% men, and 20% T2DM). Dapagliflozin did not enhance glucose uptake in adipogenesis-induced EAT SVCs, at any tested dose, with insulin resistant (*Figure 7A*) or sensitivity. Even, in these cells, insulin did not increase with statistical significance the glucose uptake *Figure 7B*). However, in adipogenesis-induced SAT SVCs, treatment with dapagliflozin alone increased glucose uptake (with statistical significance) at different concentrations (1.9 ± 1.2-fold change at 100 μM; 1.5 ± 0.4-fold change at 10 μM). In these cells, insulin treatment also increased glucose uptake compared with the control (*Figure 7C*).

3.3.3 Differentiation potential

SVCs from five patients (77 ± 5 years, 29 ± 3 kg/m^2 of BMI, 3 men, 2 with CAD, 1 with T2DM, 4 with HTA, and 1 with HF) were used for adipogenesis induction with dapagliflozin addition at 1 μM and 10 μM. Our results showed that the addition of 1 μM of dapagliflozin to the adipogenic cocktail improved the differentiation process of EAT stromal cells, as shown in the perilipin expression analysis (*Figure 8A*) and AdipoRed quantification (121± 23 a.u. vs. 85 ± 15 a.u., $P = 0.007$). The differentiation also did not decrease at 10 μM (*Figure 8C*) (85 ± 15 a.u. vs. 84 ± 33 a.u.) (*Figure 8B* and *C*). Because, SAT SVCs had greater differentiation ability, dapagliflozin was tested in these cells. The results showed less variability among patients. However, the addition of dapagliflozin did not enhance the differentiation of SAT SVCs induced by the adipogenic cocktail (325 ± 167 a.u. with control, 384 ± 226 a.u. with dapagliflozin 1 μM, and 325 ± 166 a.u. with dapagliflozin 10 μM) (*Figure 8C*).

4. Discussion

For the first time, we describe the effect of dapagliflozin treatment on EAT from patients with CV disease. This novel oral and selective antidiabetic drug inhibits SGLT2 (glucose transporter) and, in consequence, glucose reabsorption by the proximal tubule of the kidney.[26] Its activity helps to avoid the deleterious effects of hyperglycaemia and facilitates weight loss in T2DM patients.[13,27] Dapagliflozin also achieves its effects with a low risk of hypoglycaemia.[13,28] In clinical trials, a reduction of 2 kg in weight in patients being treated with dapagliflozin reduced the glycated haemoglobin and the systolic blood pressure.[29] Previous studies have demonstrated that the reduction of body weight is mostly due to fat

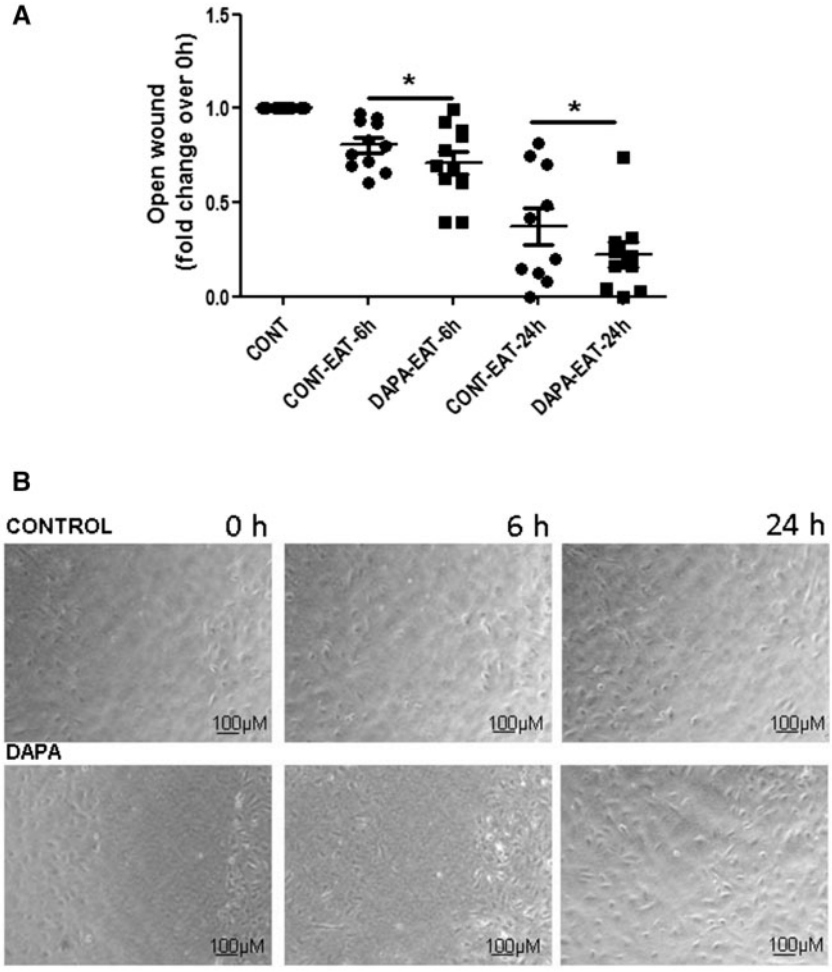

Figure 6 Wound healing of HCAECs cells with secretomes from EAT with or without dapagliflozin treatment. (*A*) Scatter/dot plot presents the fold change mean ± SEM of open wounds at 6 h and 24 h with respect to time 0 h in HCAECs after treatment with EAT secretomes from 11 patients. Paired *t*-test for statistical significance. *$P < 0.05$. (*B*) Representative photographs of EAT secretomes with or without dapagliflozin treatment at different hours on coronary endothelial cells.

mass reduction, visceral and subcutaneous.[19] Thus, dapagliflozin treatment in patients with T2DM promotes weight loss and improves quality of life.[18] The reduction of body fat may be explained, in part, by a decrease in circulating glucose levels. Other unknown mechanisms may be involved in this effect, because dapagliflozin reduces weight and blood pressure in patients with Stage 3 chronic kidney disease (CKD). In these patients with CKD, the pharmacodynamic activity of dapagliflozin is decreased and hyperglycaemia is not controlled.[30] The binding specificity of dapagliflozin to SGLT2 in the kidney has been demonstrated in a mouse model,[31] where dapagliflozin was not bound to the heart,[32] which expresses SGLT1.[33] Moreover, rodents have no EAT that can express SGLT2, as our results revealed. Therefore, this suggests that the effect of dapaglaflozin on EAT may be through SGLT2. The thickness of EAT is associated with insulin resistance in obese patients,[1] CAD asymptomatic T2DM patients,[34] and in cardiac dysfunction.[35] Under normal conditions, this tissue has protective properties acting as a buffer against lipotoxicity and glucotoxicity in the coronary arteries and myocardium.[36] Thus, the insulin–glucose uptake and lipolysis is diminished in the fat pads from patients with HF.[37] Our results show that dapagliflozin increases glucose uptake in 65% of all tested EAT samples. The increases in glucose uptake were observed in EAT samples whether insulin resistant or insulin sensitive. The absence of a response to dapagliflozin treatment in some biopsies may be caused by their dysfunction or other genetic or exogenous factors. The differences in behaviour between EAT and SAT, with regard to insulin and dapagliflozin responses, may suggest poor quality of sample or other cardiometabolic differences between both tissues. Nevertheless, this antidiabetic drug increases glucose uptake in EAT and SAT explants with or without insulin resistance. The absence of effect on SVCs from EAT, with low adipogenic ability, and presence on SAT cells, with high adipogenic ability,[23] suggests a mature adipocyte-dependent mechanism for the action of dapagliflozin. Thus, the higher expression of GLUT4 after dapagliflozin treatment and its role in mature adipocytes[38] may explain a part of the glucose uptake mechanism in adipose tissue.

Increased epicardial fat is associated not only with ventricular dysfunction[39] but also with coronary artery calcification.[40] In these patients, EAT has a pro-inflammatory profile with a higher production of inflammatory cytokines[41] and a lower production of anti-inflammatory

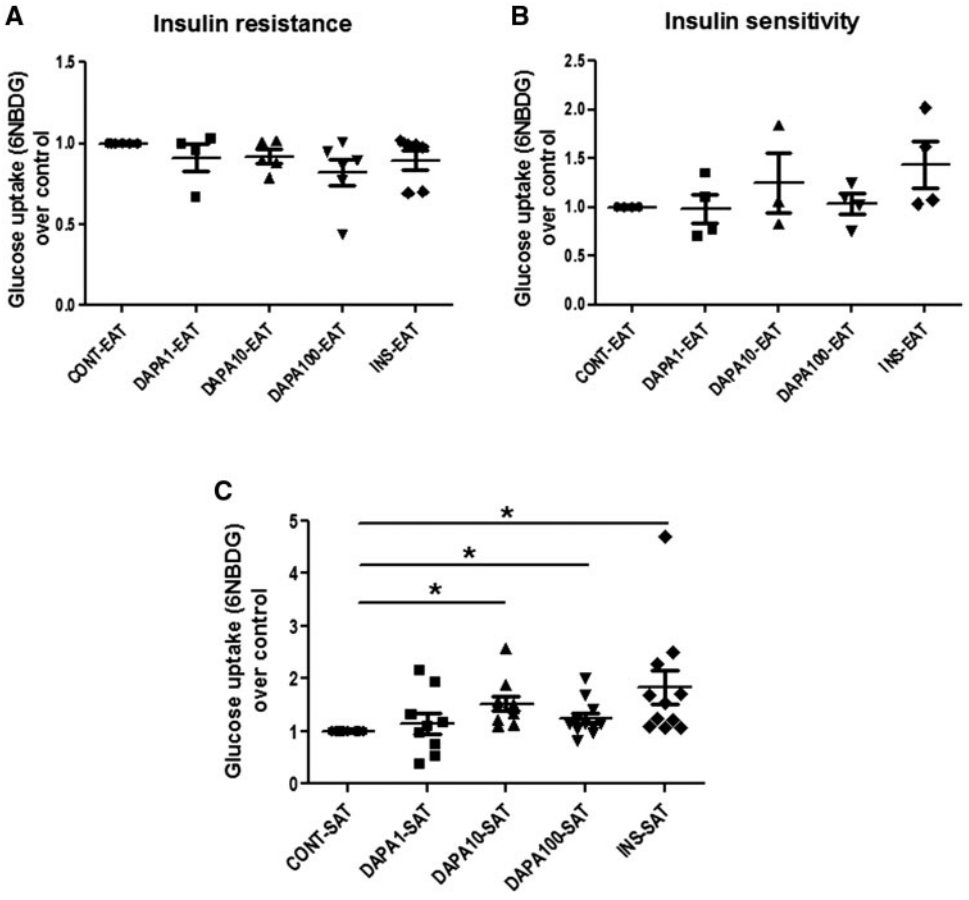

Figure 7 Dapagliflozin effect on glucose uptake of stromal vascular cells SVCs, from EAT and SAT, after adipogenesis induction. Scatter/dot plots present mean ± SEM of fluorescent glucose (6-NBDG) incorporation into EAT (*A* and *B*) or SAT (*C*) adipogenic primary culture from 11 patients after being treated with dapagliflozin for 20 min. Paired *t*-test for statistical significance. *$P < 0.05$.

adipokines.[42] This tissue also produces proteins with paracrine effects over monocytes,[9] endothelial,[9] and myocardial cells.[8,43] Dapagliflozin reduces the secretion levels of chemokines in EAT samples without insulin response. In these samples, the adipokine profile shows increased secretion of IL-6, CCL2, CXCL8, adipsin, and TIMP-1. However, the release of CCL2 by EAT were decreased by treatment with dapaglifozin in almost all patients. This reduction might decrease atherosclerosis, as the CCL2 protein has known roles in this pathological process.[44] The increased secretion of adipsin in samples with insulin resistance was also shown in postmenopausal women with insulin resistance.[45] However, treatment with dapagliflozin does not just modify the secretion of adipsin or TIMP-1. The fact that adipsin is associated with atherosclerosis[46] and chemokines may suggest an inhibitory effect of dapagliflozin on cells producing chemokines, but not TIMP-1. Our results showed a slight beneficial effect in the healing of HCAECs exposed to EAT secretomes after being treated with dapagliflozin. The DECLARE-TIMI 58 trial will reveal the incidence of CV events in patients with CV disease or CV risk factors after dapagliflozin treatment.[47] Researchers have already demonstrated that dapagliflozin reduces the incidence of myocardial infarction (MI) in diabetic patients.[48] The improvement on glucose uptake, the decrease in release of chemokines, and the beneficial paracrine effects over endothelial cells may explain the benefits of dapagliflozin in preventing MIs. More clinical trials are necessary, because the EMPA-REG OUTCOME trial[49] did not show statistically significant reductions of non-fatal MI or hospitalization for unstable angina in diabetic patients at high risk for CV disease treated with empagliflozin (an SGLT2 inhibitor).

Epicardial fat contains several types of cells (fibroblasts, adipocytes, pre-adipocytes, mesenchymal cells, macrophages, mast cells, and others). The correct function and crosstalk among these cells support the protective role of epicardial fat on the myocardium and coronary arteries. We describe a positive effect of dapagliflozin on the glucose uptake of mature adipocytes, as well as, reductions in the secretion of chemokines in these same mature adipocytes after treatment with dapagliflozin. Our group also described the low differentiation ability of EAT SVCs and the contribution of glucose and inflammatory cells[23] to this impairment.[50] Our results show the improvement in the differentiation process after treatment with dapagliflozin. Thus, treatment of EAT with dapagliflozin might improve the renewal of mature and functional adipocytes with glucose tolerance and preserved insulin response protecting adjacent tissues.

4.1 Limitations

Small adipose tissue biopsies did not allow for the analysis of several dapagliflozin concentrations and their comparisons with several treatments.

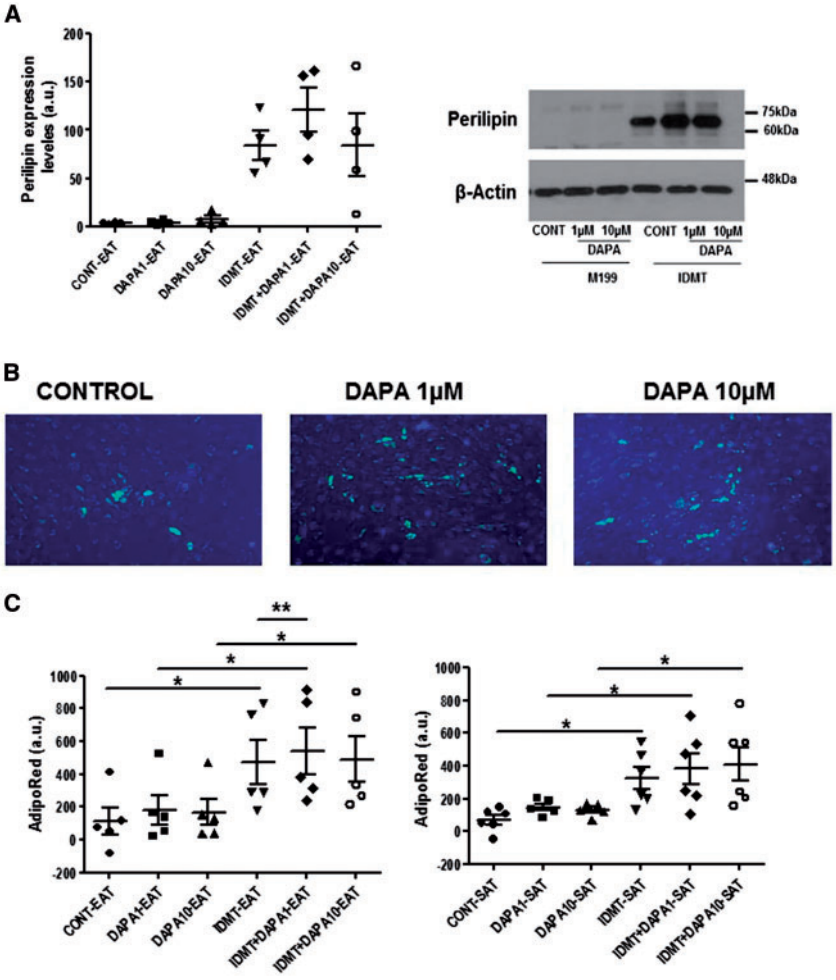

Figure 8 Dapagliflozin effect on adipogenesis induction in EAT and SAT SVCs. Adipogenesis was induced with IDMT (adipogenic cocktail) and addition or not of dapagliflozin at 1 and 10 μM. This adipogenesis was determined following perilipin expression by western blot (*A*) and lipid droplets staining by AdipoRed (*B*). Scatter/dot plots represent the mean ± SEM of fluorescent levels on adipogenesis-induced stromal cells from EAT and SAT samples ($n = 5$) (*C*). Paired *t*-test for statistical significance. $^*P < 0.05$.

5. Conclusions

Dapagliflozin improves glucose uptake, increases the differentiation ability of stromal cells, and reduces the secretion of chemokines in EAT from patients with CV disease. EAT secretomes (after treatment with dapagliflozin) improve the healing of wounds in HCAECs, suggesting additional secondary beneficial effects.

Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

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