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Immunohistomorphology of Pancreatic Islet Microvasculature and the Immunophenotypic Analysis of CEPC in Adult Diabetic Rats

Inmunohistomorfología de Microvasculatura de Islotes Pancreatic y el Análisis Inmunofenotípico de CEPC en Ratas Diabéticas Adultas

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SUMMARY: Hyperglycaemia is one of the main causes for the endothelial cell (EC) damage in diabetic patients. Even though circulating endothelial progenitor cells (CEPC) could be used as a prognosis for microvascular complications, there is very little information on the islet microvasculature. We analysed by immunohistochemistry and by flow cytometric immunophenotyping, the expression of CD34 on EC and the expressions of CD31, CD34, CD45 and CD133 on CEPC in Streptozotocin (STZ)-induced diabetic rats. Peripheral blood and tissue specimens were obtained from rats of different treatment regimens: STZ treatment, control saline (NS) and sodium citrate (CB) treatments. Blood cells were exposed to flow cytometric immunophenotyping for CD133, CD31, CD34, CD45 and CD133. While tissues from the pancreas, liver and kidney were routinely processed and stained immunohistochemically for CD34. There was a tendency of an increased in CD45-/CD133+/CD31+/CD34+ cells (0.04 ± 0.11 %) in diabetic rats compared to the controls (CB: 0.03 ± 0.04 %; Saline: 0.01 ± 0.03 %). But there was no significant statistical difference between them. The expression pattern of CD34 on the EC in the organs' vascular beds including arterioles, venules, capillaries and sinusoids was extremely heterogeneous across and within treatment regimens. The ECs in the sinusoids of the liver presented similar CD34 expression patterns across different treatment regimens, while the expression of CD34 on the ECs of sinusoidal capillaries in the pancreas vary with the treatment regimen. We conclude that the degree of endothelial cell damage is not uniform across organs' vascular beds in the rat, contrary to mice and humans. Furthermore, the sinusoids in the pancreas and the kidney may have the same degree of endothelial damage when exposed to the same deleterious causes.

KEY WORDS: CEPC; EC; Pancreas; Liver; Kidney.

INTRODUCTION

Circulating endothelial progenitor cells (CEPCs) are hematopoietic cells released by the bone marrow into the blood in response to vascular damage (Asahara *et al*., 1997). Circulating endothelial progenitor cells mobilise to the sites of damaged tissue to induce vascular repair, but their recruitments are impaired by hyperglycaemia (Thangarajah *et al*., 2010). Chronic hyperglycaemia, as a result of Diabetes mellitus (DM), increases reactive oxygen species (ROS) levels leading to oxidative stress (King & Loeken, 2004), which in turn causes endothelial cell (EC) activation. This inflammatory and immunological response (Hunt & Jurd, 1998) is characterised by vascular endothelial hypertrophy and thickening, leading to permanent EC damage (Zhang *et al*., 2010). A number of studies (Liao *et al*., 2010; Chen *et al*., 2013; Liu *et al*., 2013; Rigato *et al*., 2015) have been published with the intention to use the levels of CEPCs as a prognosis for chronic kidney disease, cardiovascular disease, liver disease, retinopathy and vascular endothelial function. Hence, scarce data are available on the immunohistomorphology of microvascular ECs for CD34 and the expression of CEPCs for commonly used EC markers like CD31, CD34, CD45 and CD133 in the pancreas tissue in a diabetic rat, in comparison with that of the kidneys and liver.

CD31, a member of the immunoglobulin superfamily, functions during inflammation and angiogenesis in facilitating the migration of leucocyte between ECs, respectively (Feng *et al*., 2004). It stains plasma cells, monocytes, and megakaryocytes. CD34 plays a role in hematopoietic stem cell and leukocyte recruitment by acting as a regulating blocker of cell adhesion and migration

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enhancer (Nielsen & McNagny, 2009). It is commonly used as a marker for vascular ECs and for hematopoietic stem cell purification (Nielsen & McNagny). CD45 is a receptor-linked protein tyrosine phosphatase expressed on all leucocytes. The antigen, which is also expressed on all nucleated hematopoietic cells, is important for the function and activation of leucocytes (Hermiston *et al*., 2003). CD133 however, is a member of the prominin family of pantaspan membrane proteins and is responsible for maintaining proper lipid composition within the plasma membrane (Mizrak *et al*., 2008). It is commonly used as a marker for primitive haematopoietic and neural stem cells (Mizrak *et al*.).

The present study was undertaken to analyse immunohistochemically, and by flow cytometric immunophenotyping the expression of microvascular endothelium for CD34 and the expression of CEPCs for CD31, CD34, CD45 and CD133 in Streptozotocin (STZ)-induced diabetes rat tissue, respectively. For a comprehensive analysis, we studied two organs in comparison with the pancreas but focused mainly on the endothelial damage in microvasculature of the (pancreatic islet of Langerhans), (glomerular capsule Bowman's capsule) and the liver acinus, using the expression of CD34. We also used monoclonal CD31, CD34, CD45 and polyclonal CD133 antibodies that have been recognized as a common combination of anti-bodies in the identification of CEPCs (Hirschi *et al*., 2008).

MATERIAL AND METHOD

Animal. Adult male Wistar rats (225-245 g) were obtained and housed in a temperature-controlled room, with free access to water and food. The use of animals for this study was approved by the Stellenbosch University Animal ethics committee, in accordance with the South African National Standard (SANS 10386:2008). The rats were grouped into treatment regimens of five animals each: the saline (NS) control group, the sodium citrate buffer (CB) control group and the Streptozotocin (STZ) diabetes group.

Treatment regimens. Under light anaesthesia, rats received an IP injection of STZ (50 mg/kg; dissolved in 1 ml CB; experimental rats). Rats were monitored for ten consecutive days, the body weight (BW) was measured every two days. Only rats with blood glucose levels (BGL) ≥ 15 Mmol/mL for three consecutive days were considered diabetic. For the control groups, rats received an IP injection of saline solution (NS; 1 ml; NS control rats) or sodium citrate buffer (CB; 1 ml; CB control rats). Rats in all groups were kept for 40 days and the bodyweight and blood glucose levels (BGL) were monitored every two days.

Blood sample and tissue collection and processing. A day prior to tissue collection, blood samples were obtained and analysed for CEPC expression using flow cytometry. The anti-bodies used in this study are listed in Table I. The flow cytometer was set to acquire a maximum of 1 000 000 events during a 10 minute period. The gating strategy used in flow cytometry analysis to detect different subsets of circulating hematopoietic cells is shown in Figure 1. Viable cells which include all single and living cells were first gated from the total events and were expressed as percentage of the mean. The expression of CD45 was used to gate lymphocytes and granulocytes from viable cells. However, the expression of CD133 was gated from the resulted CD45+/- cells and grouped into CD45-/CD133+ and CD45+/CD133+ cells. Furthermore, the expression of CD31 vs. CD34 was then gated from CD45+/CD133+ and CD45-/CD133+ cells and expressed as CD45+/CD133+/CD31+/CD34+ and CD45-/CD133+/CD31+/CD34+ cells. CD45-/CD133+/CD31+/CD34+ cells were characterised as CEPCs.

Tissue sections at 3 μm each were cut from each tissue block representing each organ in the corresponding treatment groups, and were mounted on positively charged microscope slides. A BondMax™ immunoautostainer was used to stain slides using a modified F protocol for a Bond Polymer Refine detection kit (Code: DS9800) (Leica Biosystems, Wetzlar, Germany). Human tonsil was used as a positive and negative control. A Zeiss Axioskop microscope (Carl Zeiss, Oberkochen, Germany) was used to visualise and capture the stained tissue images.

Drugs. The following drugs and reagents were used in this study: Streptozotocin (Sigma-Aldrich, Missouri USA), Sodium citrate buffer (Kimix Chemicals S.A., Cape Town, RSA).

Table I. Antibodies used in the study.

Antibody	Dilution	Source	Clone	Incubation
Monoclonal CD34	1/100000	Abcam, Cambridge, UK	EP373Y	60 min
Monoclonal CD31	0.5/100000	Abcam, Cambridge, UK	TLD-3A12	60 min
Polyclonal CD133	2/100000	Abnova, Taipei city, TW	8842	60 min
Monoclonal CD45	2/100000	Biolegend, California, USA	OX-1	60 min
Monoclonal **CD34***	1/500	Abcam, Cambridge, UK	ICO-115	30 min

* Antibody used for immunohistochemistry.

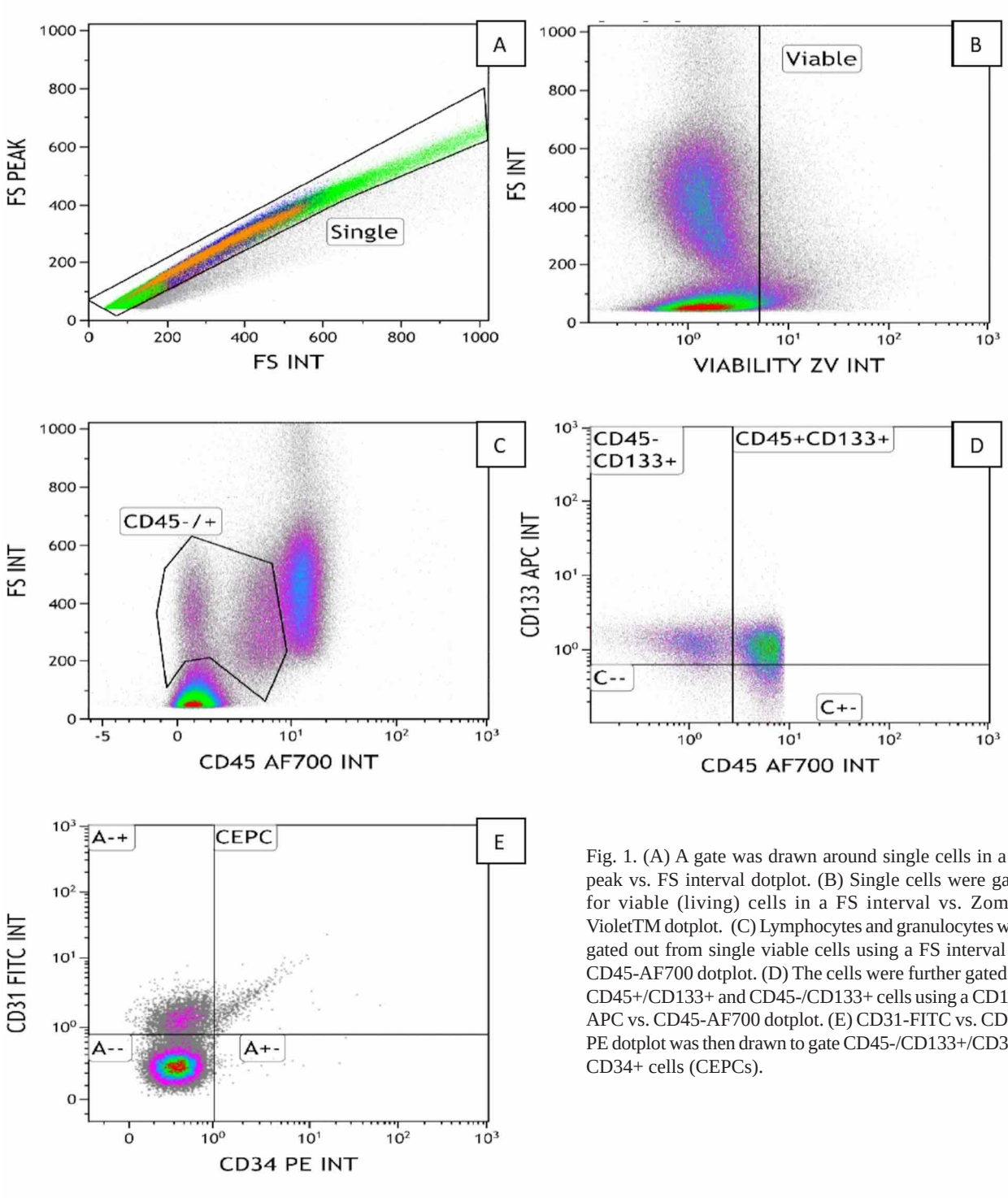

Fig. 1. (A) A gate was drawn around single cells in a FS peak vs. FS interval dotplot. (B) Single cells were gated for viable (living) cells in a FS interval vs. Zombie VioletTM dotplot. (C) Lymphocytes and granulocytes were gated out from single viable cells using a FS interval vs. CD45-AF700 dotplot. (D) The cells were further gated for CD45+/CD133+ and CD45-/CD133+ cells using a CD133-APC vs. CD45-AF700 dotplot. (E) CD31-FITC vs. CD34-PE dotplot was then drawn to gate CD45-/CD133+/CD31+/CD34+ cells (CEPCs).

Statistical analysis. The flow cytometry data was analysed using GraphPad Prism 5 software (GraphPad software Inc.). One way Kruskal-Wallis test was performed for comparison between groups followed by a Dunn's post-test. The results are presented as mean ± standard error of the mean (SEM) percentage. The significance level was set at $p < 0.05$.

RESULTS

Body weight and blood glucose levels. The mean BW and BGL in all the groups are shown in Figure 2. The BW in the CB control group had a sharp increase above the mean value reaching 329.0 g on day 40 of the experiment, while the BGL in this group was steady and similar (5.68 mmol/L) with the NS control group. However, the mean BW in the diabetes rats dropped on day 5 post-STZ treatment (207.00 g) and rose steadily throughout the experimental period toward the NS control level (219.00 g); in the same time, the mean BGL had a sharp increase (28.4 mmol/L) following STZ injection and dropped (23.16 mmol/L) on day 5 post-STZ treatment. Thereafter, there was a fluctuation of the mean BGL levels from day 7 (24.78 mmol/L) until day 40 (23.84 mmol/L), during which a peak level of mean BGL (28.60 mmol/L) was reached on day 11 and its nadir (19.46 mmol/L) on day 15.

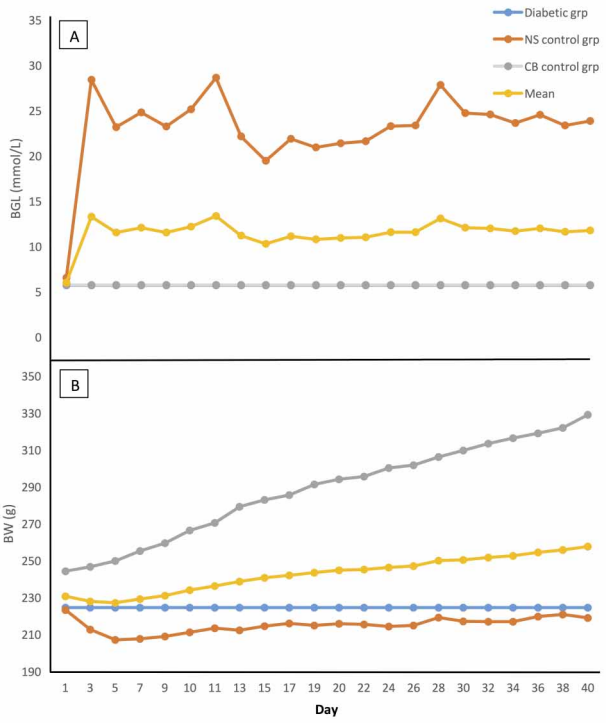

Fig. 2. Graphs (A) showing BGL in the rats of different treatment regimens and (B) their BW throughout the experimental period.

Immunophenotypic analysis. The percentage of CEPC populations and viable cells in rats of different treatment regimens is shown in Table II. These results suggest that an increase in CEPCs levels may not be associated with the number of viable cells, but with the treatment regimen used.

Immunohistomorphological study. The expression pattern for CD34 in the ECs of the vascular beds including arterioles, venules, capillaries and sinusoids of different organs in this study was extremely heterogeneous across treatment regimens, and slightly within groups; and are summarized in Table III. The immunohistomorphology of the organs represented in Figures 4, 5 and 6 presented some characteristic features specific to the treatment regimens.

Pancreatic islet : The diabetic rat pancreas had dilated acinar cells with decreased cell density in the islet, while there were some fibrotic areas indicative of beta-cell loss. The sinusoidal capillaries had a strong positivity for CD34 in the diabetic rats, but weak positivity in the interlobular capillaries (Fig. 3A). The pancreas of the CB control rats had relatively normal acini; however, the islets displayed oedema and numerous lymphocytic infiltrations. The interlobular capillaries strongly expressed CD34 in the CB control rats while the sinusoidal capillaries had low to absent positive expressions for CD34 (Fig. 3B). Conversely, the pancreas in the NS control rats had normal morphology, with healthy islets. The interlobular capillaries were strongly positive for CD34 in the NS control rats, while the sinusoidal capillaries had low positive expression (Fig. 3C).

The liver acinus: The liver in diabetic rats had more extensive hypertrophy hepatocytes in the periportal region. The portal vein was completely detached from the

Table II. Percentage of CEPC populations and viable cells in rats with different regimens.

Variable	Diabetic STZ treatment	Control CB treatment	Control NS treatment
Total events	n = 507414	n = 635529	n = 482363
Viable cells	68.80 %	76.73 %	78.85 %
CD45+ and CD45-	15.33 ± 3.87 %	12.76 ± 2.71 %	15.39 ± 1.79 %
CD45-/CD133+	4.50 ± 1.17 %	4.37 ± 1.49 %	6.03 ± 0.64 %
CD45+/CD133+	8.09 ± 3.42 %	4.02 ± 1.39 %	5.72 ± 1.60 %
CD45+/CD133+/CD31+/CD34+	0.09 ± 0.03 %	0.21 ± 0.25 %	0.05 ± 0.13 %
CD45-/CD133+/CD31+/CD34+*	0.11 ± 0.04 %	0.04 ± 0.03 %	0.03 ± 0.01 %

* CD45-/CD133+/CD31+/CD34+ cells are CEPCs.

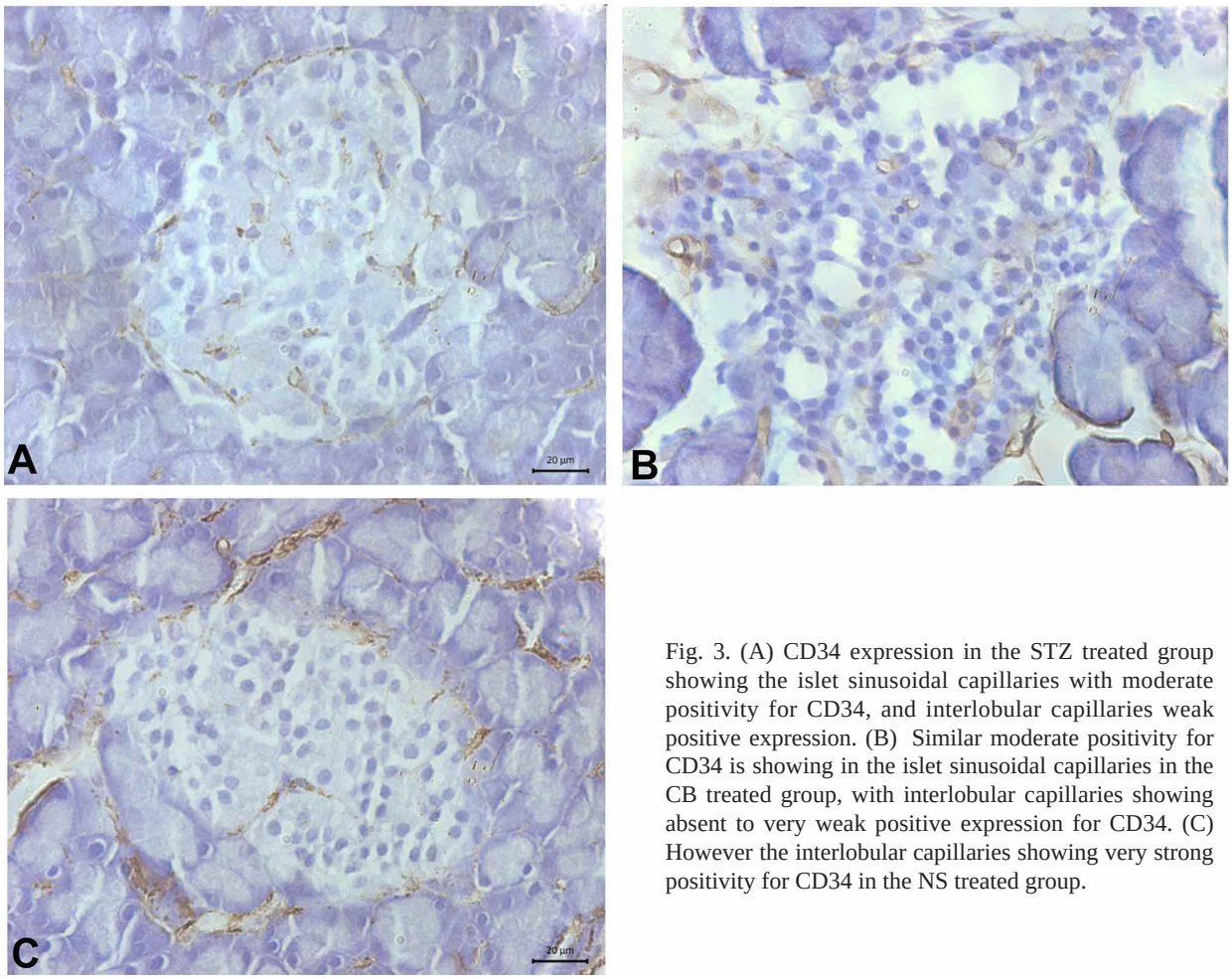

Fig. 3. (A) CD34 expression in the STZ treated group showing the islet sinusoidal capillaries with moderate positivity for CD34, and interlobular capillaries weak positive expression. (B) Similar moderate positivity for CD34 is showing in the islet sinusoidal capillaries in the CB treated group, with interlobular capillaries showing absent to very weak positive expression for CD34. (C) However the interlobular capillaries showing very strong positivity for CD34 in the NS treated group.

underlining connective tissues. Weak positivity for CD34 was observed in the ECs of the portal vein and hepatic artery in this group (Fig. 4A), while moderate positivity for CD34 was expressed in the CB control rats. However, the swelling of periportal hepatocyte was observed, having numerous lymphocytic infiltrations (Fig. 4B). The ECs in the portal vein and hepatic artery were highly positive for CD34 in NS control rats; the morphology of the periportal hepatocytes and that of the portal triad was normal (Fig. 4C). The sinusoids in all the groups were negative for CD34.

Table III. Staining of ECs for CD34 in various rats with different treatment regimens.

Tissue	Diabetic	Control	Control
Pancreas			
Sinusoidal capillaries	+++	++	+
Interlobular capillaries	+	0/+	+++
Liver			
Hepatic arteries	++	++	+++
Portal veins	++	++	+++
Sinusoids	0	0	0
Kidney			
Glomeruli	+++	++	+
Capillaries	+++	++	0/+

Intensity of immunohistochemical staining: 0, absent; +, low; ++, medium; +++, high.

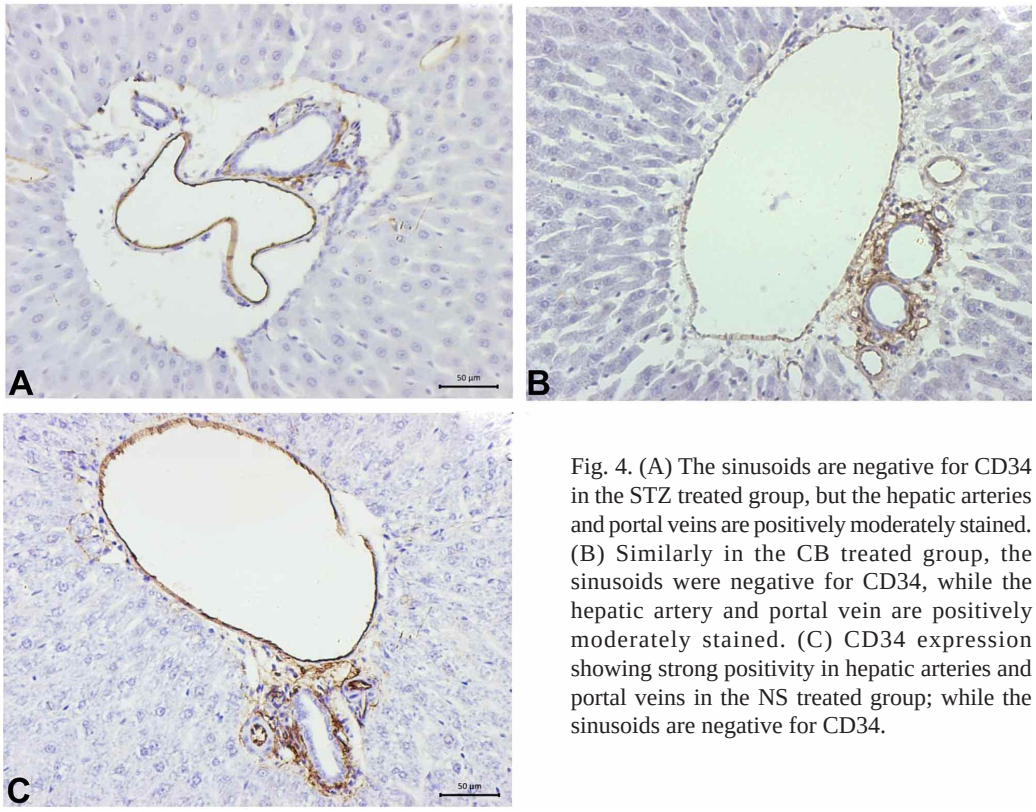

Fig. 4. (A) The sinusoids are negative for CD34 in the STZ treated group, but the hepatic arteries and portal veins are positively moderately stained. (B) Similarly in the CB treated group, the sinusoids were negative for CD34, while the hepatic artery and portal vein are positively moderately stained. (C) CD34 expression showing strong positivity in hepatic arteries and portal veins in the NS treated group; while the sinusoids are negative for CD34.

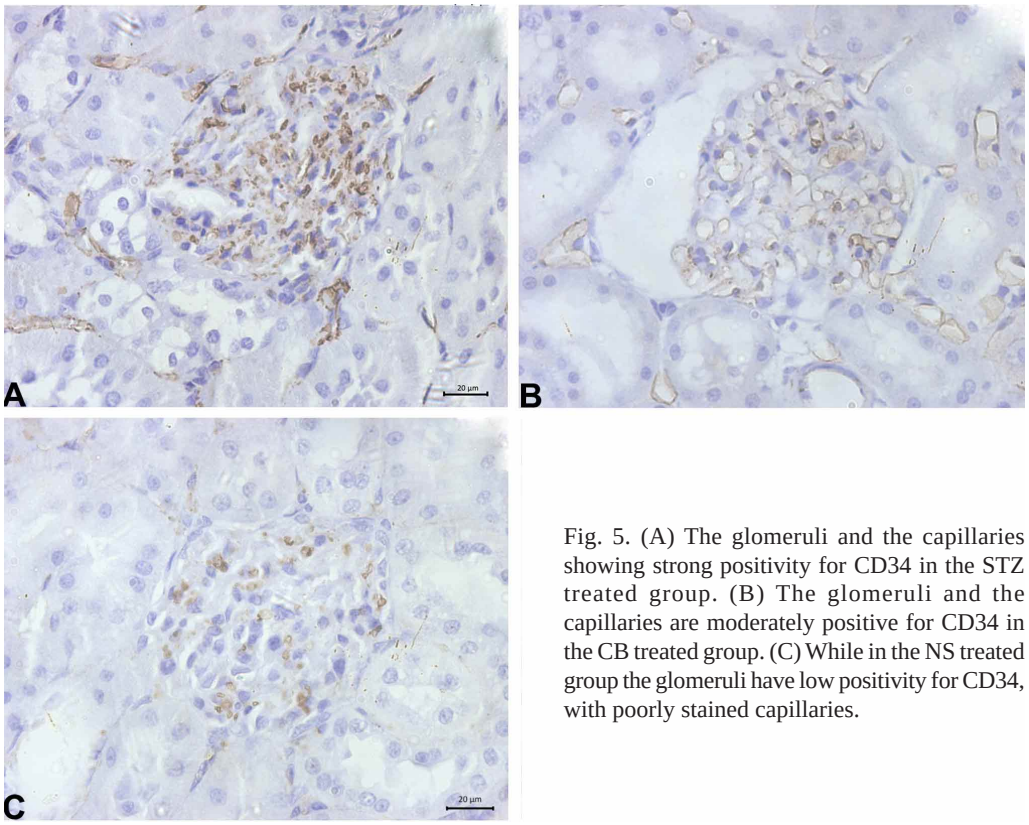

Fig. 5. (A) The glomeruli and the capillaries showing strong positivity for CD34 in the STZ treated group. (B) The glomeruli and the capillaries are moderately positive for CD34 in the CB treated group. (C) While in the NS treated group the glomeruli have low positivity for CD34, with poorly stained capillaries.

The glomerular capsule of the kidney: The kidney tissue section in the diabetic rats had an atrophy of the glomerular capsule characterised by a decreased glomerular vessel density. The ECs of the glomeruli and the capillaries had a strong positive expression for CD34 (Fig. 5A). In the CB control rats, the urinary space and the mesangial cells within the glomerulus was swollen due to oedema; while enlarged proximal convoluted tubule and distal convoluted tubules was observed. The glomeruli and the capillaries were weakly or moderately positive for CD34 (Fig. 5B). However, the NS control rats presented with diffuse positivity for CD34, having a normal kidney morphology and healthy renal corpuscles and tubules (Fig. 5C).

The irrational intensity of the immunohistochemical staining observed between various vascular beds of different organs within individual groups of treatment regimen strongly advocate that, the endothelial cell damage in vascular beds is limited in its distribution across organs. Interestingly, there were similarities in the staining intensity between the sinusoidal capillaries in the pancreas and in the glomeruli of the kidney under the same treatment regimen. This is an indication that sinusoids in the pancreas and the kidney may have the same degree of endothelial damage when exposed to the same deleterious causes.

DISCUSSION

In this study, we analysed the expression of microvascular endothelium for CD34 and the expression of CEPCs for CD31, CD34, CD45 and CD133 in the pancreas in comparison with the liver and the kidney in the diabetic rat. Our study confirms that the EC vascular damage is restricted in its distribution in Wistar rats (Testa *et al*., 2009). This was elucidated by the staining intensity of ECs for CD34 that was not identical in various vascular beds under the same treatment regimen (Table III). There were remarkable similarities in the staining intensity between the sinusoidal capillaries in the pancreas and the glomeruli of the kidney under the same treatment regimen.

Moreover, the analysis of the BW and the BGL in this study showed a considerable weight gain in the CB control group, while the BGL in the control groups were similar. These findings confirm the oedematous effect of CB alone (Cunha *et al*., 2009) in the rats, and may suggest that the CB used as a solute to dissolve STZ drug does not have any remarkable impact on the histomorphology of STZ treated organs. The severity of STZ-induced diabetes and its effects were apparent on the morphology of the pancreas (Fig. 3A), the liver (Fig. 4 A) and the kidney (Fig. 5A). Additionally, the BGL in the diabetic rats remained high in the study and there was a tendency of increased CEPCs in the diabetic rat compared to the controls. These findings suggest that CEPC recruitments to the sites of damaged tissue are not impaired by hyperglycaemia, contradicting a previous observation (Thangarajah *et al*.). Consequently, the mobilization of CEPCs could be due to the deleterious effects of the treatment regimen and that hyperglycemia participates very little in its recruitments.

Previous studies (Jin *et al*., 2010; Ling *et al*., 2012; Zhang & Yan, 2013) have shown that there is a significant reduction in CEPC levels in STZ-induced diabetic rats. The discrepancies between our findings and these reports in the literature can be partly explained by the selection of the STZ dosage (55 mg/kg; 30 mg/kg; 50 mg/kg), the timing of its application (not indicated) and other unknown environmental factors. Furthermore, a relation between severity of diabetic peripheral arterial disease and reduced CEPC levels was reported (Fadini *et al*., 2006), while suggesting CEPCs as a novel biomarker for peripheral atherosclerosis in DM. In fact, the latter postulate was from a study using human participants; it would be difficult to compare such results with studies done on rats, while mimicking diabetes using STZ does not represent all the physiological conditions present in a human.

CONCLUSION

This study confirms that the degree of endothelial cell damage is not uniform across organs' vascular beds in the rat, contrary to mice and humans. However, sinusoids in the pancreas and the kidney may have the same degree of endothelial damage when exposed to the same deleterious causes. We therefore, recommend that a choice of animal, dosage and timing of the administration of the drug become a necessary pillar in any study on circulating endothelial progenitor cells when used as a novel biomarker for peripheral atherosclerosis using animal model.

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RESUMEN: La hiperglucemia es una de las principales causas del daño de las células endoteliales (EC) en pacientes diabéticos. A pesar de que las células progenitoras endoteliales circulantes (CEPC) podrían utilizarse como pronóstico de las complicaciones microvasculares, hay muy poca información sobre la microvasculatura de los islotes. Se analizaron por inmunohistoquímica y por inmunofenotipificación citométrica de flujo, la expresión de CD34 en EC y las expresiones de CD31, CD34, CD45 y CD133 en CEPC en ratas diabéticas inducidas por estreptozotocina (STZ). Se obtuvieron muestras de sangre y tejidos periféricos a partir de ratas de diferentes regímenes de tratamiento: tratamiento con STZ, solución salina control (NS) y citrato de sodio (CB). Las células sanguíneas fueron expuestas a inmunofenotipado por citometría de flujo para CD133, CD31, CD34, CD45 y CD133. Mientras que los tejidos del páncreas, el hígado y el riñón fueron rutinariamente procesados y teñidos inmunohistoquímicamente para CD34. Se observó una tendencia a un aumento en las células CD45- / CD133 + / CD31 + / CD34 + (0,04 ± 0,11 %) en ratas diabéticas en comparación con los controles (CB: 0,03 ± 0,04 %; Salino: 0,01 ± 0,03 %). Pero no hubo diferencias estadísticamente significativas entre ellos. El patrón de expresión de CD34 en la EC en los lechos vasculares de los órganos incluyendo arteriolas, vénulas, capilares y sinusoides fue extremadamente heterogéneo a través de y dentro de los regímenes de tratamiento. Las EC en los sinusoides del hígado presentaron patrones de expresión de CD34 similares a través de diferentes regímenes de tratamiento, mientras que la expresión de CD34 en las CE de capilares sinusoidales en el páncreas varía con el régimen de tratamiento. Concluimos que el grado de daño de las células endoteliales no es uniforme en los lechos vasculares de los órganos en la rata, en comparación de los ratones y los seres humanos. Además, los sinusoides en el páncreas y el riñón pueden tener el mismo grado de daño endotelial cuando se exponen a las mismas causas deletéreas.

PALABRAS CLAVE: CEPC; CE; Páncreas; Hígado; Riñón.

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