Research proposal: inflammation and oxidative stress in coronary artery bypass surgery graft: comparison between diabetic and non-diabetic patients

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Abstract

Background: Diabetes mellitus patients (DM) have more severe progression of atherosclerotic disease than non-diabetic (NDM) individuals. In situ inflammation and oxidative stress are key points in the pathophysiology of atherosclerosis, a concept largely based on animal model research. There are few studies comparing inflammation and oxidative stress parameters in medium-sized arteries between DM and NDM patients. A fragment of the internal mammary artery used in coronary artery bypass grafting (CABG) will be employed for this purpose.

Objective: To assess the expression of inflammatory markers tumor necrosis factor-α, transforming growth factor-β1, nuclear factor kappa B, the enzymes superoxide dismutase, and catalase in the vascular wall of the arterial graft used in CABG, comparing DM and NDM patients.

Results: The present study will add information to the vascular degenerative processes occurring in diabetic patients.

Keywords: Inflammation, Oxidative stress, Diabetes mellitus, Coronary artery bypass grafting

Introduction

Coronary artery disease (CAD) is the main cause of death in diabetic patients [1]. Coronary artery bypass grafting (CABG) is one of the most important strategies for CAD treatment [2]. The internal mammary artery (IMA) is the graft of choice for revascularization of the left anterior descending coronary artery [3]. During CABG, not the whole extension of IMA is used by the surgeon. A fragment of the unemployed part of the vessel provides the opportunity to access an artery with similar structure compared to the epicardial coronary arteries [4], as a model for evaluating vascular degenerative processes in these patients.

Increased inflammation, oxidative stress, and the resulting endothelial dysfunction are key factors to the severity of atherosclerosis in diabetes [5]. While research on the field is largely based on animal models [6–8], only few studies have addressed diabetes-induced molecular mechanisms in human medium-sized arteries [9, 10].

The goal of this study is to identify the contribution of diabetes to the vascular expression of a group of molecules in CAD patients. For this purpose, we will compare IMA samples obtained from diabetic (DM) versus non-diabetic (NDM) patients subjected to CABG. The selected inflammatory markers will be tumor necrosis factor-α (TNF-α), transforming growth factor β-1 (TGFβ-1), and nuclear factor kB (NF-kB). Oxidative stress pathways will be evaluated by the expression of the enzymes superoxide dismutase (SOD) and catalase (CAT).

TNF-α induces endothelial expression of cell adhesion molecules, vascular cell adhesion molecule (VCAM) and
intercellular cell adhesion molecule (ICAM), both important for the infiltration of monocytes at the intima of the vessel [11]. TGFβ-1 is a multifunctional peptide which stimulates cell proliferation, migration, and extracellular matrix deposition, contributing to the vascular remodeling of atherosclerosis [12]. NF-κB is a transcription factor activated both by hyperglycemia and reactive oxygen species (ROS) [13], regulating the expression of cytokines and adhesion proteins, modulating vascular inflammation, and the recruitment of immune cells to the vascular wall [14, 15]. Superoxide dismutase is an essential component of cell defense against ROS, converting superoxide anion to hydrogen peroxide. Catalase is an enzyme with complementary action in ROS elimination, converting hydrogen peroxide to water [16]. An excess of ROS in the vascular tissue is directly related to endothelial dysfunction [17].

**Main text**

**Methods**

**Study design and patients**

This will be a cross-sectional study with convenience sampling. Individuals will be consecutive male adult patients admitted for elective CABG. The surgical strategy will be defined by the National Institute of Cardiology heart team, with none of its members directly involved in the study. Eligibility criteria are male individuals above 18 years old and IMA use in CABG. Exclusion criteria are kidney failure with hemodialysis, known acute or chronic infectious disease, presence of autoimmune disease, or use of immunosuppressants. The study is approved by the Local Ethics Research Committee under protocol # 33705614.2.0000.5272, and informed consent will be obtained.

Based on previous studies with vascular tissue obtained from CABG [9, 10], the total estimated number of patients necessary for this study is 50, equally divided in DM and NDM.

**Study variables**

Data from the medical records will be obtained: anthropometric data (height, body weight, abdominal circumference, and body mass index), associated diseases, social habits (cigarette and ethanol consumption), family history of cardiovascular disease, medications, presurgical transthoracic echocardiogram (ejection fraction by Teicholz method), coronary angiography (number of stenotic vessels and percent luminal stenosis), and plasma biochemistry (fasting glucose, sodium, potassium, blood urea nitrogen, creatinine, low-density lipoprotein, high-density lipoprotein, and triglycerides).

**Vessel collection and processing**

The evaluation of the selected markers will be performed by quantitative real-time polymerase chain reaction (qPCR) and immunohistochemistry. Initially, the unused fragment of IMA during CABG will be harvested by the surgeon in a glass tube containing 25 ml of cold, sterilized, phosphate buffered saline (PBS). Immediately after collection, the fragment will be delicately flushed and carefully dissected for adventitia removal. It will be divided in two parts. One will be placed in fixative solution paraformaldehyde 4% in PBS. The other portion will be weighed and frozen in liquid nitrogen, for mechanical maceration until pulverization for RNA purification.

**Real-time polymerase chain reaction**

Total RNA extraction from pulverized tissue will be performed with an extraction kit (mirVana®, Life Technologies, Carlsbad, California, USA). The protocol will be followed according to the manufacturer instructions. The resulting RNA fraction will be quantified by the use of 260 nm spectrophotometry. High Capacity kit (Life Technologies, Carlsbad, California, USA) will be employed for reverse transcriptase PCR, of an aliquot of total RNA (2 ng/μl) diluted in ultrapure water treated with diethylpyrocarbonate (DEPC). Thermocycler (Biocycler) setting will be 25 °C for 10 min, followed by 37 °C for 120 min and 85 °C for 5 s, as recommended by the kit. Complementary DNA (cDNA) will be frozen at −20 °C for posterior utilization. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) will be the house-keeping gene. The primers to be used in the study are listed in Table 1, based on literature research [18–20].

qPCR quantification will be performed at thermocycler VIIA7 (Applied Biosystems, Foster City, California, USA), with 10 μl of SYBR Green Master Mix (Life Technologies, Carlsbad, California, USA), 2 μl of cDNA, 0.8 μl of primer solution (3 μM), and 7.2 μl of DEPC water. All samples will be analyzed in triplicate. Optimal primer concentration and PCR efficiency will be determined for all markers. The results of the amplification curves will be analyzed with the software available in the thermocycler manufacturer website (http://www.thermofisher.com/en/home/cloud.html).

**Immunohistochemistry**

The fragment fixated in paraformaldehyde 4% will be stored under refrigeration until processing with alcohol dehydration, xylol clearing, and paraffin inclusion. Transversal sections 5 μm thick will be obtained for histological analysis. Primary monoclonal antibodies will be used for
Table 1 Nucleotide sequence of the primers to be used in the study

|                         | Amplicon size (bp) |
|-------------------------|--------------------|
| SOD2 F                  | 5′-CTG GAC AAA CCT CAG CCC TA-3′ |
| SOD2 R                  | 5′-TGA TGG CTG CCA GCA ACT C-3′ |
| CAT F                   | 5′-AGT GAT CGG GGG ATT CCA GA-3′ |
| CAT R                   | 5′-AAG TCT CGG CGC ATC TTC AA-3′ |
| TNF-α F                 | 5′-GTT CCT CAG CCT CTT CTC CT-3′ |
| TNF-α R                 | 5′-ACA ACA TGG GCT ACA GGC TT-3′ |
| TGF-β1 F                | 5′-TGA ACC GGC CTT TCC TGC TCT TCA TG-3′ |
| TGF-β1 R                | 5′-GGG AAA GAG AGT GGA ATG GGC AGT C-3′ |
| NF-κB F                 | 5′-CGA AGC CGA CCA CCA TGT-3′ |
| NF-κB R                 | 5′-GAA GAT GGT GAT GGG ATT TC-3′ |
| GAPDH F                 | 5′-GAA GGT GAA GGT CGG AGT C-3′ |
| GAPDH R                 | 5′-GAA GAT GGT GAT GGG ATT TC-3′ |

8bp base pairs, CAT catalase, F forward, GAPDH glyceraldehyde 3-phosphate dehydrogenase, NF-κB nuclear transcription factor kappa B, R reverse, SOD2 superoxide dismutase-2, TGF-β1 transforming growth factor β-1, TNF-α tumor necrosis factor-α

Discussion
This study will analyze clinical, laboratory, and histological data from CABG patients, divided in two groups, diabetic versus non-diabetic. We will first identify whether there are other clinical differences between the groups, besides the diagnosis of diabetes mellitus and the use of diabetes medications. Previous analysis of the clinical profile of CABG patients at our Institution verified the same level of exposure to all other risk factors for CAD in the two groups, except for diabetes (data not shown).

A previous study has demonstrated an increase of TGF-β1 in vascular tissue from rats with diabetes-induced nephropathy [21]. Prior animal studies have demonstrated by qPCR the increase of CAT and SOD expression in vascular tissues [22, 23] in diabetes. This could represent a tentative compensatory response to increased oxidative stress in the vascular tissue of diabetic patients. In a single study with human tissue, there was no difference in the expression and activity of SOD, when comparing the saphenous vein with IMA from CAD patients [9]. However, this analysis did not address the contribution of diabetes.

In the study of Wildhirt et al. using morphology and histopathology analysis of the radial artery (RA) in CABG, no association between intima thickness and diabetes, hypertension, or smoking was found. However, only four diabetic patients were included [24]. Another study, with a geographically distinct population, found the association between diabetes and media calcification in RA [25].

Preil et al. studied human IMA from CABG, comparing DM and NDM. The authors found an increased deposition of the basement membrane components alpha-1 and alpha-2 type IV collagen, gamma-1 and beta-2 laminin in diabetic patients [10].

Recent studies have identified plasma markers with prognostic properties in CABG patients. Examples are the measurements of endothelial-leukocyte adhesion molecule 1 (ELAM-1), interleukin 6 and 8, and TNF-α [26]. In the future, follow-up of the patients in the present study could eventually lead to the identification of additional prognostic markers, to be obtained from a sample of IMA used in CABG.

Limitations and strengths of the study
The present study will be performed on a convenience sample from a single healthcare center, and therefore may not be representative of the entire population of CAD patients.

There is no healthy control in the study, as it would not be ethical to obtain IMA samples from individuals not subjected to open-chest CABG. All samples will be
obtained from advanced CAD patients, and the relative contribution of diabetes could be more relevant in the early stages of the disease. However, recent work has demonstrated differences in proteomic analysis between diabetic and non-diabetic patients with established CAD [10]. The exclusion of female subjects was chosen, in order to avoid estradiol-induced attenuation of vascular inflammation [27]. While this aims to eliminate a possible source of variability of the results, it implies the study can be translated to phenomena occurring only in human male vascular tissue.

Finally, vascular degeneration and arteriosclerosis affect vessels in heterogeneous fashion across their length. The fragment available for evaluation in this study may not necessarily represent the condition of the entire grafted segment during CABG.

The main strength of the present study is the access to IMA fragments, a distinctive opportunity to obtain human vascular tissue from living individuals, in order to evaluate in fresh material for protein transcription and histological analysis.

**Abbreviations**
- Bp: base pairs
- CABG: coronary artery bypass grafting
- CAD: coronary artery disease
- CAT: catalase
- cDNA: complementary DNA
- DAB: diaminobenzidine
- DM: diabetes mellitus
- DEPC: diethylpyrocarbonate
- ELAM-1: endothelial-leukocyte adhesion molecule 1
- F: forward
- GAPDH: glyceraldehyde 3-phosphate dehydrogenase
- ICAM: intercellular cell adhesion molecule
- IMA: internal mammary artery
- IMA fragments: a distinctive opportunity to obtain the entire grafted segment during CABG.
- LPA: lipoprotein (a)
- LDL: low-density lipoprotein
- MAPK: mitogen-activated protein kinase
- MDA: malondialdehyde
- MIRNA: micro-RNA
- MPO: myeloperoxidase
- NADPH: nicotinamide adenine dinucleotide phosphate
- NF-kB: nuclear transcription factor kB
- NO: nitric oxide
- PBS: phosphate buffered saline
- pPCR: real-time polymerase chain reaction
- qPCR: real time-polymerase chain reaction
- RA: radial artery
- RNA: ribonucleic acid
- ROS: reactive oxygen species
- SD: standard deviation
- SOD: superoxide dismutase
- TGFβ-1: transforming growth factor β-1
- TNF-α: tumor necrosis factor-α
- VCAM: vascular cell adhesion molecule
- VSP: visna virus protein
- WTR: wall thickness ratio
- W/p: wall–lumen ratio

**Authors’ contributions**
- DK, AL, AC, JC and GD contributed to the conception and design of the study and to the analysis and interpretation of data; DK, AL, AC and GD were involved in the drafting of the manuscript, and literature review. All authors have given final approval of the version to be published and are publicly responsible for its content. All authors read and approved the final manuscript.

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Not applicable.

**Competing interests**
The authors declare that they have no competing interests.

**Availability of data and materials**
Data sharing is not applicable to this article as no datasets were generated or analysed during the current study.

**Consent for publication**
Not applicable.

**Ethics approval and consent to participate**
The present study will be conducted in accordance with the Declaration of Helsinki, and this study is approved by the Institutional Review Board of the National Institute of Cardiology in Rio de Janeiro, Brazil, under protocol # 33705614.2.0000.5272. Written informed consent to participate in the study will be obtained from all participants.

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**References**
1. Aronson D, Edelman ER. Coronary artery disease and diabetes mellitus. Cardiol Clin. 2014;32(3):439–55.
2. Patel MR, et al. ACC/AATS/AHA/ASE/ASNC/SCAI/SCCT/STS 2017 appropriate use criteria for coronary revascularization in patients with stable ischemic heart disease: a report of the American College of Cardiology appropriate use criteria task force, American Association for Thoracic Surgery, American Heart Association, American Society of Ecchocardiography, American Society of Nuclear Cardiology, Society for Cardiovascular Angiography and Interventions, Society of Cardiovascular Computed Tomography, and Society of Thoracic Surgeons. J Am Coll Cardiol. 2017;69(17):2212–41.
3. Windecker S, et al. 2014 ESC/EACTS guidelines on myocardial revascularization: the task force on myocardial revascularization of the European Society of Cardiology (ESC) and the European Association for Cardio-Thoracic Surgery (EACTS) Developed with the special contribution of the European Association of Percutaneous Cardiovascular Interventions (EAPCI). Eur Heart J. 2014;35(37):2541–619.
4. de Vries MR, et al. Vein graft failure: from pathophysiology to clinical outcomes. Nat Rev Cardiol. 2016;13(8):451–70.
5. Raja SG. Bilateral internal mammary artery grafting in diabetics: outcomes, concerns and controversies. Int J Surg. 2015;16(Pt B):153–7.
6. Belmadi S, et al. Elevated epidermal growth factor receptor phosphorylation induces resistance artery dysfunction in diabetic db/db mice. Diabetes. 2008;57(6):1629–37.
7. Adachi T, et al. S-glutathiolation of Ras mediates redox-sensitive signaling by angiotensin II in vascular smooth muscle cells. J Biol Chem. 2004;279(28):29857–62.
8. Kong L, et al. PKCbeta promotes vascular inflammation and acceleration of atherosclerosis in diabetic ApoE null mice. Arterioscler Thromb Vasc Biol. 2013;33(8):1779–87.
9. Guzik TJ, et al. Superoxide dismutase activity and expression in human venous and arterial bypass graft vessels. J Physiol Pharmacol. 2005;56(2):313–23.
10. Preil SA, et al. Quantitative proteome analysis reveals increased content of basement membrane proteins in arteries from patients with type 2 diabetes mellitus and lower levels among metformin users. Circ Cardiovasc Genet. 2015;8(5):727–35.
11. Lin CC, et al. Tumor necrosis factor-alpha induces VCAM-1-mediated inflammation via c-Src-dependent transactivation of EGF receptors in human cardiac fibroblasts. J Biomed Sci. 2015;22:53.
12. Toma I, McCaffrey TA. Transforming growth factor-beta and atherosclerosis: interwoven atherogenic and atheroprotective aspects. Cell Tissue Res. 2012;347(1):155–75.
13. Sena CM, Pereira AM, Seica R. Endothelial dysfunction—a major mediator of diabetic vascular disease. Biochim Biophys Acta. 2013;1832(12):2216–31.
14. Brasier AR. The nuclear factor-kappaB-interleukin-6 signalling pathway mediating vascular inflammation. Cardiovasc Res. 2010;86(2):211–8.
15. Wang Y, et al. NF-κB activity-dependent P-selectin involved in ox-LDL-induced foam cell formation in U937 cell. Biochem Biophys Res Commun. 2011;411(3):543–8.
16. Weydert CJ, Cullen JJ. Measurement of superoxide dismutase, catalase and glutathione peroxidase in cultured cells and tissue. Nat Protoc. 2010;5(1):51–66.
17. Mikhed Y, Daiber A, Steven S. Mitochondrial oxidative stress, mitochondrial DNA damage and their role in age-related vascular dysfunction. Int J Mol Sci. 2015;16(7):15918–53.
18. Strzalka B, et al. Quantitative analysis of transforming growth factor beta isoforms mRNA in the human corneal epithelium. Folia Biol (Praha). 2008;54(2):46–52.
19. Cavalcante LO, et al. Quantititation of glucocorticoid receptor alpha and NF-kappaB pathway mRNA and its correlation with disease activity in rheumatoid arthritis patients. Genet Mol Res. 2010;9(4):2300–10.
20. Andrews M, Soto N, Arredondo M. Effect of metformin on the expression of tumor necrosis factor-alpha, Toll like receptors 2/4 and C reactive protein in obese type-2 diabetic patients. Rev Med Chile. 2012;140(11):1377–82.
21. Wang T, et al. Reduced beta 2 glycoprotein I improve diabetic nephropathy via inhibiting TGF-beta1-p38 MAPK pathway. Int J Clin Exp Med. 2015;8(5):6852–65.

22. Perlman AS, et al. Serum inflammatory and immune mediators are elevated in early stage diabetic nephropathy. Ann Clin Lab Sci. 2015;45(3):256–63.
23. Rossoni Junior M, et al. Annatto extract and beta-carotene enhances antioxidant status and regulate gene expression in neutrophils of diabetic rats. Free Radic Res. 2012;46(3):329–38.
24. Wildhirt SM, et al. Graft function, histopathology and morphometry of radial arteries used as conduits for myocardial revascularization in patients beyond age 70. Eur J Cardiothorac Surg. 2006;30(2):333–40.
25. Chowdhury UK, et al. Histopathology and morphometry of radial artery conduits: basic study and clinical application. Ann Thorac Surg. 2004;78(5):1614–21.
26. Preeshagul I, et al. Potential biomarkers for predicting outcomes in CABG cardiothoracic surgeries. J Cardiothorac Surg. 2013;8:176.
27. Bowling MR, et al. Estrogen effects on vascular inflammation are age dependent: role of estrogen receptors. Arterioscler Thromb Vasc Biol. 2014;34(7):1477–85.