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Endothelial Expression of Nitric Oxide Synthetase, Heat Shock Protein 90 and Calmodulin in Human Saphenous Vein

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Abstract

***Background*:** Endothelial preservation of the human saphenous vein (HSV) is an important determinant of long-term graft patency following coronary artery bypass grafting (CABG). Harvesting-related endothelial injury may impair nitric oxide (NO) signalling and contribute to graft failure. This study compared endothelial expression of endothelial nitric oxide synthase (eNOS), heat shock protein 90 (HSP90), and calmodulin (CaM) in HSV harvested using conventional open vein harvesting (OCVH) and two endoscopic vein harvesting (EVH) techniques.

***Methods*:** We conducted an observational comparative immunohistochemical study of 180 HSV specimens, with 60 specimens in each group: OCVH, closed-tunnel EVH (CT-EVH), and open-tunnel EVH (OT-EVH). Sections were stained using monoclonal antibodies against eNOS, HSP90, and calmodulin. Endothelial staining was assessed using a modified semi-quantitative grading system ranging from 1 (no staining) to 5 (marked staining).

***Results*:** eNOS expression did not differ significantly among OT-EVH (1.85 ± 0.81), CT-EVH (2.10 ± 0.96), and OCVH (1.85 ± 0.88). Similarly, HSP90 expression was comparable among OT-EVH (1.20 ± 0.41), CT-EVH (1.35 ± 0.48), and OCVH (1.30 ± 0.47). In contrast, calmodulin expression was significantly higher in CT-EVH (3.55 ± 0.68) than OT-EVH (2.60 ± 0.88; p=0.0001) and OCVH (2.75 ± 0.55; p=0.003). No significant difference was observed between OT-EVH and OCVH.

***Conclusions*:** eNOS and HSP90 expression were maintained across all harvesting techniques. CT-EVH was associated with greater endothelial calmodulin expression, which may reflect differences in preservation or regulation of endothelial NO signalling. Further functional and clinical studies are required to determine whether these molecular differences translate into improved endothelial function or long-term graft patency.

Keywords: Saphenous vein graft, endothelial nitric oxide synthase (eNOS), endoscopic vein harvesting, calmodulin, conventional vein harvesting, heat shock protein 90 (Hsp90), endothelial function, coronary artery bypass grafting.

1. INTRODUCTION

The saphenous vein graft (SVG) remains the most commonly used conduit in coronary artery bypass grafting (CABG) owing to its availability and adequate length for multiple distal anastomoses. SVGs can be harvested using either open vein harvesting (OVH) or endoscopic vein harvesting (EVH). Although EVH offers advantages such as reduced wound complications, better cosmetic outcomes, and shorter recovery times, concerns remain about its effects on graft integrity and long-term graft patency. Mechanical trauma and excessive manipulation during harvesting can compromise endothelial structure and function, thereby promoting thrombosis, intimal hyperplasia, and vein graft disease [1, 2].

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Endothelial integrity is essential for optimal graft function and long-term patency. The vascular endothelium maintains vascular homeostasis primarily through the production of nitric oxide (NO), a potent vasodilator with anti-inflammatory, antithrombotic, and antiproliferative properties. Reduced NO bioavailability is a key indicator of endothelial dysfunction and has been implicated in the development of vein graft failure [3, 4]. Endothelial nitric oxide synthase (eNOS) is the principal enzyme responsible for endothelial NO production. Its activity is regulated by several intracellular proteins, including heat shock protein 90 (HSP90) and calmodulin (CaM). HSP90 acts as a molecular chaperone that enhances eNOS stability and activity, while CaM facilitates eNOS activation in response to increased intracellular calcium levels [5-7]. The coordinated interaction among eNOS, HSP90, and CaM is therefore essential for maintaining endothelial function and vascular homeostasis.

Given the central role of these proteins in NO signalling, evaluating eNOS, HSP90, and CaM expression may provide important insights into endothelial preservation following different vein harvesting techniques. Comparative assessment of these molecular markers could improve understanding of the biological effects of OVH and EVH and help identify strategies that optimize graft quality and long-term clinical outcomes.

2. METHODOLOGY

The research project was undertaken at Transplant Research Laboratory, Wythenshawe Hospital & University of Manchester (Histology Laboratory, AV Hill and Bio-Imaging department, Michael Smith Building). We prospectively analysed a total of 180 samples for immunohistochemical study from 3 different treatment groups (60 samples each) over 12 month period.

Group 1 – Open Tunnel - Endoscopic Saphenous Vein Harvesting (OT - EVH)

Group 2 – Closed Tunnel - Endoscopic Saphenous Vein Harvesting (CT - EVH)

Group 3 – Open Conventional Saphenous Vein Harvesting (OCVH)

2.1. Tissue Preparation

Paraffin-embedded human saphenous vein (HSV) tissue blocks were kept at room temperature until processing. Prior to sectioning, the blocks were chilled on ice to facilitate optimal tissue cutting. 5 µm thick serial sections were prepared using a fully automated rotary microtome (Leica RM2255, Leica Microsystems, Germany). Sections were floated on a 40°C distilled water bath and mounted onto polysine-coated adhesive slides (Superfrost® Plus, Thermo Fisher Scientific, USA) suitable for immunohistochemical analysis. The slides were air-dried overnight at room temperature and stored until further processing.

2.2. Immunohistochemistry

Immunohistochemical staining was performed using a standard avidin-biotin-free polymer detection method. Antigen retrieval was carried out using 10 mM citrate buffer (pH 6.0) at 90-95°C for 10 minutes. Following antigen retrieval, the slides were cooled at room temperature for 5 minutes and then rinsed with cold water for 20 minutes.

Endogenous peroxidase activity was blocked using the DAKO EnVision™ detection system (Dako, Denmark) according to the manufacturer's instructions. The following primary monoclonal antibodies were applied:

- Endothelial nitric oxide synthase (eNOS) (Cell Signaling Technology®, Danvers, MA, USA), dilution 1:200
- Heat shock protein 90 (HSP90) (Cell Signaling Technology®, Danvers, MA, USA), dilution 1:50
- Calmodulin (CaM) (Cell Signaling Technology®, Danvers, MA, USA), dilution 1:200
- Primary antibodies were diluted in phosphate-buffered saline (PBS) and incubated with tissue sections overnight at 4°C.

Subsequent to primary antibody incubation, sections were treated with horseradish peroxidase (HRP)-labelled secondary antibody using the DAKO EnVision™ detection system for 30 minutes at room temperature. Immunoreactivity was visualized using 3,3′-diaminobenzidine (DAB) chromogen. Slides were subsequently counterstained with Mayer's haematoxylin, dehydrated, mounted using Pertex mounting medium, and allowed to dry overnight at 37°C.

2.3. Immunohistochemical Evaluation

Immunohistochemical staining was assessed using a modified semi-quantitative grading system based on the extent of staining along the circumference of the intimal endothelial layer [8, 9]. The grading system was adapted from previously published methods and simplified [10] for the purposes of this study.

Staining intensity was graded on a five-point scale as follows:

- Grade 1: No staining
- Grade 2: Trace staining

- Grade 3: Minimal staining
- Grade 4: Moderate staining
- Grade 5: Marked staining

2.4. Digital Image Acquisition

The Pannoramic 250™ slide scanning system (3DHISTECH Ltd., Budapest, Hungary) was used to digitize stained slides and Pannoramic Viewer™ software version 1.15. was used to view, annotate and archive the obtained digital images.

2.5. Ethics Approval and Consent

Research project was approved and supported by the research and ethics committee of university hospital of South Manchester and storage of human tissue was covered by the HTA (Human Tissue Licence) held at the Transplant Laboratory, Wythenshawe Hospital, Manchester. All patients were given information booklets and written consent was obtained.

2.6. Statistical Analysis

Statistical analysis was performed using SPSS® version 32 (IBM Corp., Armonk, NY, USA). Continuous variables were expressed as mean ± standard deviation (SD), while categorical variables were summarized as frequencies and percentages where appropriate.

Comparisons among the three study groups (OT-EVH, CT-EVH, and OCVH) were performed using one-way analysis of variance (ANOVA), followed by Bonferroni-adjusted post-hoc pairwise comparisons. Statistical significance was defined as a two-tailed p-value ≤ 0.05.

3. RESULTS

3.1. Endothelial Nitric Oxide Synthase (eNOS)

The mean eNOS staining scores were 1.85 ± 0.81 in the OT-EVH group, 2.10 ± 0.96 in the CT-EVH group, and 1.85 ± 0.88 in the OCVH group. No statistically significant differences were observed among the groups. Pairwise comparisons showed no significant differences between OT-EVH and CT-EVH (p = 1.0), OT-EVH and OCVH (p = 1.0), or CT-EVH and OCVH (p = 1.0) (Table **1**, Fig. **1**).

Table 1: eNOS immunostaining by various extraction methods.

	P value
OT-EVH vs. CT- EVH	1.0
CT-EVH vs. OCVH	1.0
OT-EVH vs. OCVH	1.0

P = ≤ 0.05 is considered statistically significant

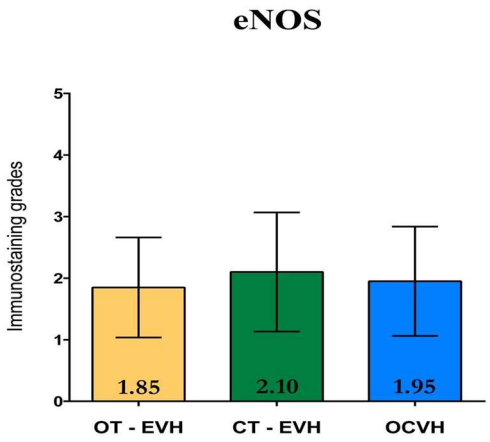


Figure 1: eNOS immunostaining by various extraction methods.

3.2. Heat Shock Protein 90 (HSP90)

The mean HSP90 staining scores were 1.20 ± 0.41, 1.35 ± 0.48, and 1.30 ± 0.47 in the OT-EVH, CT-EVH, and OCVH groups, respectively (Figures 5–8). No statistically significant differences were detected among the groups. Pairwise comparisons demonstrated no significant differences between OT-EVH and CT-EVH (p = 0.91), OT-EVH and OCVH (p = 1.0), or CT-EVH and OCVH (p = 0.91) (Table **2**, Fig. **2**).

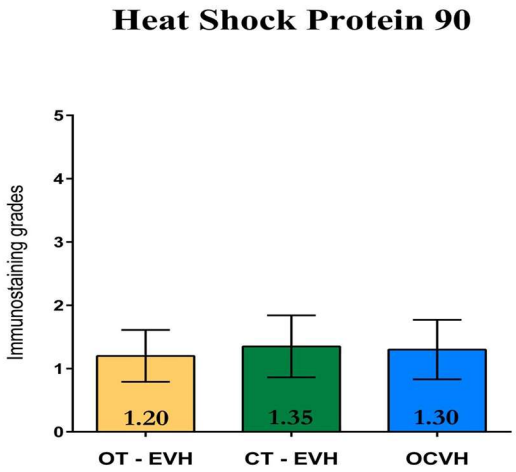


Figure 2: HSP90 immunostaining by various extraction methods.

Table 2: ANOVA post hoc analysis between groups (HSP90).

	P value
OT-EVH vs. CT- EVH	0.914
CT-EVH vs. OCVH	1.0
OT-EVH vs. OCVH	1.0

P = ≤ 0.05 is considered statistically significant

3.3. Calmodulin (CaM)

The mean CaM staining scores were 2.60 ± 0.88 in the OT-EVH group, 3.55 ± 0.68 in the CT-EVH group, and 2.75 ± 0.55 in the OCVH group. Pairwise comparisons demonstrated significantly higher CaM expression in the CT-EVH group compared with both the OT-EVH group (p = 0.0001) and the OCVH group (p = 0.003). No significant difference was observed between the OT-EVH and OCVH groups (p = 0.51) (Table **3**, Fig. **3**).

Table 3: ANOVA post hoc analysis between groups (CaM).

	P value
OT-EVH vs. CT- EVH	0.0001*
CT-EVH vs. OCVH	0.003*
OT-EVH vs. OCVH	0.51

** P = <0.05 considered statistically significant*

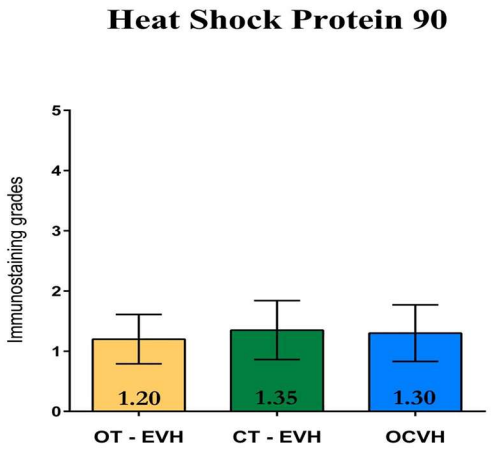


Figure 3: CaM immunostaining by various extraction methods.

4. DISCUSSION

The harvesting technique is an important determinant of saphenous vein graft (SVG) quality, as endothelial injury sustained during conduit procurement may adversely affect graft adaptation and long-term patency [11-12]. Mechanical manipulation, excessive traction, distension, and thermal injury can disrupt endothelial integrity and impair nitric oxide (NO)-mediated vascular homeostasis. Endothelial dysfunction is recognized as an early event in vein graft disease and contributes to thrombosis, inflammation, intimal hyperplasia, and accelerated atherosclerosis [4, 1].

Nitric oxide is a key regulator of vascular function and exerts vasodilatory, antithrombotic, anti-inflammatory, and antiproliferative effects within the vessel wall [10, 12]. Endothelial NO production is mediated by eNOS, whose activity is regulated through a complex network of protein interactions and post-translational modifications rather than protein expression alone [12, 13]. Among the most important regulators of eNOS are heat shock protein 90 (HSP90) and calmodulin (CaM). HSP90 functions as a molecular chaperone that facilitates eNOS activation and stabilizes the enzyme within signalling complexes, while CaM acts as a calcium-dependent regulator that is essential for eNOS catalytic activity and NO generation [1, 4, 5].

In the present study, no significant differences were observed in the expression of eNOS or HSP90 among the CT-EVH, OT-EVH, and OCVH groups, perhaps due to our small sample size. These findings suggest that the harvesting techniques did not substantially affect the abundance of these proteins within the endothelial layer. However, preservation of eNOS expression does not necessarily indicate preservation of endothelial function, as eNOS activity is highly dependent on intracellular signalling pathways and interactions with regulatory proteins [6, 15]. Likewise, HSP90 participates in multiple cellular stress-response mechanisms and may remain relatively stable despite endothelial injury [16].

In contrast, calmodulin expression was significantly greater in the CT-EVH group compared to both OT-EVH and OCVH. The closed tunnel technique of harvesting provides better visualisation and less tissue handling, hence preserving calmodulin production. This observation may have important functional implications because CaM is a critical mediator of calcium-dependent eNOS activation. Under basal conditions, eNOS is associated with caveolin-1, which suppresses its enzymatic activity. Following an increase in intracellular calcium concentration, CaM binds to eNOS and displaces caveolin-1, resulting in enzyme activation and enhanced

NO production [5, 13, 14]. Experimental studies have demonstrated that increasing calmodulin availability can reverse caveolin-mediated inhibition of eNOS and restore NO synthesis, highlighting the pivotal role of CaM in endothelial signalling [14].

Beyond its direct effects on eNOS activation, calmodulin is also involved in endothelial mechanotransduction pathways that allow endothelial cells to respond to haemodynamic forces such as shear stress and pressure [17]. Preservation of calmodulin expression may therefore reflect maintenance of endothelial signalling competence rather than simple structural preservation of the endothelial monolayer. The significantly increased expression of calmodulin in the CT-EVH group suggests that this harvesting technique may better preserve calcium signalling, mechanotransduction, and NO production, hence provides strong mechanistic support of higher calmodulin expression [18, 19].

Although NO bioavailability was not directly measured in the present study, the selective preservation of calmodulin expression supports the hypothesis that CT-EVH may provide superior protection of endothelial function compared with OT-EVH and conventional open vein harvesting. Preservation of CaM-dependent signalling pathways could potentially enhance endothelial responsiveness, maintain vasodilatory capacity, and limit thrombotic and proliferative processes that contribute to vein graft failure. Further studies incorporating functional assessments of NO production and long-term clinical outcomes are required to determine whether the observed molecular differences translate into improved graft patency and clinical benefit.

5. CONCLUSION

eNOS and HSP90 expression were maintained irrespective of harvesting technique, suggesting preservation of key endothelial nitric oxide signalling components. However, CT-EVH demonstrated significantly greater endothelial calmodulin expression due to less tissue manipulation, suggesting improved preservation of a regulatory component of eNOS activation but a larger study is required to confirm our findings. Our study suggest, CT-EVH technique may better maintain endothelial functional integrity and nitric oxide signalling pathways within harvested saphenous vein grafts.

6. LIMITATION OF THE STUDY

This study has several limitations. Immunohistochemical assessment of eNOS, HSP90, and calmodulin reflects protein expression but not functional activity or nitric oxide bioavailability. Endothelial markers were evaluated only immediately after harvesting, precluding assessment of post-implantation vascular remodelling and long-term endothelial function. Furthermore, although calmodulin expression was significantly higher in the CT-EVH group, its relationship with nitric oxide production, graft function, and long-term clinical outcomes requires further investigation.

ETHICAL APPROVAL AND CONSENT TO PARTICIPATE

Research project was approved and supported by the research and ethics committee of university hospital of South Manchester and storage of human tissue was covered by the HTA (Human Tissue Licence) held at the Transplant Laboratory, Wythenshawe Hospital, Manchester. All patients were given information booklets and written consent was obtained.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interests.

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None.

AUTHOR CONTRIBUTIONS

SH: Conceptualization, data curation, formal analysis, methodology, visualization, writing original draft.

VM: Conceptualization, data curation, formal analysis, methodology, writing original draft.

RT: Conceptualization, data curation and writing original draft.

ID: Conceptualization, formal analysis and writing original draft.

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