Title Page

Title: Effect of carbon dioxide insufflation on structural and functional viability of human saphenous vein endothelium – Role of calcium mobilisation and nitric oxide production

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Abstract

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Objective

Carbon dioxide (CO2) is used by many endoscopic vein harvesting systems to facilitate visualisation and dissection by creating a subcutaneous tunnel. We aim to investigate the potential detrimental effect of CO2 insufflation, on endothelial structural and functional viability of human saphenous vein (HSV).

Method

We performed an ex vivo experiment on HSV segments (n = 40) by applying CO2 for 40 minutes at 6 – 12 mmhg in a specially designed chamber and compared it with controls (n = 40). We have investigated the level of endothelial damage by Calcein/Propidium iodide (PI) Live/Dead assay. Functional viability of HSV was assessed by Ca^{2+} (FLUO3 AM) and nitric oxide (DAF-FM) specific indicators for calcium mobilisation and endothelial nitric oxide synthase related nitric oxide (NO) production.

Results

CO2 did not cause any immediate structural endothelial damage and there was no statistical difference between control and CO2 groups (Calcein p = 0.27 and PI p = 0.98). After CO2 exposure and bradykinin stimulation, Ca^{2+} mobilisation was significantly attenuated (p = 0.013) and NO generation was also markedly reduced in endothelial cells, without reaching statistical significance (p = 0.078).

Conclusion

We believe these results are purely due to CO2 insufflation, which can compromise the entire length of vein graft. However, more research is required to establish, whether these findings are transient in nature or they have long term implications on graft survival and clinical outcome

Introduction

Carbon dioxide (CO2) is colourless and odourless gas used routinely in laparoscopic abdominal surgery to create pneumoperitoneum. CO2 is the gas of choice for laparoscopy due to its characteristics of being easily soluble in tissues and blood, non-combustible and readily discarded through pulmonary ventilation. Moreover, CO2 insufflation used by many endoscopic vein extraction systems facilitates dissection by creating a subcutaneous tunnel. Normally, the insufflated CO2 is absorbed by the blood and must be eliminated by the lungs through increased ventilation[1]. In the literature, some cases of CO2 embolisms or hypercarbia during this procedure are described[2–4] . The mechanism of CO2 embolism during endoscopic procedures is supposed to be CO2 absorption or its direct entry, through an injured vessel, into the blood stream. The entry of CO2 in the circulation depends on the gradient between the central venous pressure and the pressure of insufflation[5]. No previous study has addressed the direct effect of carbon dioxide insufflation on quality of saphenous vein graft. We wanted to investigate the effect of CO2 in our ex vivo model to look for the endothelial preservation and functional viability of human saphenous vein graft (HSV).

Materials and Methods

The vein grafts were removed immediately after harvesting and placed in a container, prefilled with Hanks' balanced salt solution (HBSS, Sigma Aldrich®) at kept at 4°C. We collected 80 samples Samples were harvested by no touch vein harvesting technique (NTVH) with the accompanying perivascular fat and strictly not distended intraoperatively to prevent endothelial injury and immediately transported to the histology/bio imaging facility at University of Manchester for research work. The journey time from cardiac theatres to the histology lab at Manchester University was 30 – 45 minutes. After reaching the histology lab, vein grafts were cut into small 2-4 mm segments and stored in HBSS at 4°C until further use. Specimens for CO2 study were placed in a specially designed chamber with an inlet for gas supply and outlet for venting. After running 100% CO2 for a 1 minute at 6-12 mmhg, outlet and then inlet were clamped to maintain constant pressure inside the chamber. Intermittently CO2 was replaced by unclamping and running the gas for 60 seconds. Total time of CO2 exposure was 40 minutes. The structural viability of the veins was measured using the live/dead assay (Life Technologies, Carlsband, CA). Vein segments were incubated for 30 mins with a final concentration of 5 mM Calcein-AM and 5 mM propidium iodide respectively in HBSS at room temperature (37°C). After incubation, veins were washed with HBSS and mounted on slides for Confocal Microscopy.

Calcium mobilization in endothelial cells was measured using FLUO3 AM dye (Life Technologies, Carlsband, CA). Vein segments were incubated for 30 mins with a final concentration of 10 uM FLUO3 AM in HBSS at room temperature (37°C). Resting calcium levels (controls) were measured in the absence of bradykinin stimulation. The segments were stimulated with bradykinin (10 uM, final concentration) and imaged in real time over the course of 10 minutes by confocal microscopy. We used DAF-FM dye for the detection of endothelial NO in our study. (Life Technologies, Carlsband, CA). Vein segments were incubated for 30 mins with a final concentration of 10 mM DAF-FM in HBSS at room temperature (37°C). Resting NO levels (controls) were measured in the absence of bradykinin stimulation. The segments were stimulated with bradykinin (10 uM, final concentration)

and imaged in real time over the course of 10 minutes by confocal microscopy. After loading the vein segments on TCS SP5 upright viewing plate, stored pre-set was selected from computer database as per fluorescence assay used. After processing confocal microscope images were stored in central bio-imaging server in .lif format. Lif files were opened by ImageJ (open source image processing computer programme) and background intensity was subtracted. For size filtering and intensity thresholding Imaris® Bitplane® version 7.6 was used under surpass mode. Images were transported to Imaris by using import from Fiji™ extension. The region of interest (ROI) i.e. HSV endothelium was selected by cropping the adventitial layer and background intensity. The "Surface Object" is a computer-generated representation of a specified region of interest, which is visualized as an artificial solid object. The surface object acts as a container from which statistics can be calculated with the Imaris Measurement Pro module. After cropping, endothelium loaded with fluorescence indicator was rendered with Iso-surface, imaged and quantitative data was generated in excel format (stored in the main server for analysis). The fluorescence images were exported in TIFF format for histological analysis.

Results

Endothelial viability of saphenous vein segments was studied by performing immunofluorescence (Live/Dead) assay with Calcein Am and Propidium Iodide dyes. The calcium movement & Nitric oxide production of saphenous vein segments by performing immunofluorescence assay with Calcium specific FLUO3 AM and Nitric oxide specific DAF-FM indicators.

Calcein AM (Live Cells) Fluorescence Assay

The Calcein AM fluorescence assay showed higher normalised maximum fluorescence intensity in Control group (41.15 AU ± 11.47, Figure 1) as compared to CO2 group (34.16 AU ± 15.83, Figure 1), but the difference was not statistically significant ($p = 0.27$).

Propidium Iodide (Dead Cells) Fluorescence Assay

Propidium Iodide fluorescence assay showed almost identical maximum fluorescence intensities between Control (9.46 AU ± 1.14, Figure 2) and CO2 groups (9.44 AU ± 2.8, Figure 2) with no statistical difference ($P = 0.98$).

FLUO 3 AM Fluorescence Assay (Calcium specific indicator)

Bradykinin stimulation resulted in increase in normalised maximum fluorescence intensity in control group (64.77 AU ± 14.9, Figure 3, Table 1), which was markedly higher than the CO2 group (40.62 AU ± 16.18, Figure 3, Table 1) and the difference between control and CO2 group was statistically significant ($p = 0.013$, Figure 4).

DAF-FM Fluorescence Assay (NO specific indicator)

Bradykinin stimulation resulted in increase in normalised maximum fluorescence intensity in control group (71.1 AU ± 14.0, Figure 5, Table 2), which was higher than the CO2 group (59.4 AU ± 18.4, Figure 5, Table 2) but did not reach statistical significance ($p = 0.078$, Figure 6).

Discussion

Pneumoperitoneum by CO2 insufflation in laparoscopic surgery is associated with a lesser amount of blind dissection and lesser use of materials, such as retractors. It is also associated with reduced amount of washing and drying of the peritoneum, thereby lesser contact of foreign materials and lesser tissue damage compared to conventional surgery[6,7]. By utilising the same principle of laparoscopic abdominal surgery, CO2 insufflation during endoscopically harvested saphenous vein is a standard practice in cardiac surgery in many centres around the world. The rationale behind this practice is to create a subcutaneous tunnel in the leg for better visualisation, used as an aid in dissection. CO2 is the gas of choice for laparoscopy due to its characteristics of being easily soluble in tissues and blood, non-combustible and readily discarded through pulmonary ventilation. It has, however, a side effect in its acidifying capacity by reacting with water to form carbonic acid. It is well known that routinely used pressures during robotic hysterectomy and other urogenital procedures are about 8–15 mmHg[8] and mostly higher than 10 mmHg[9,10], whereas a raised CO2 insufflation pressure is associated with blood gas acid base disturbances followed by acidemia and a linearly increased partial CO2 pressure and decreased pH lead to metabolic hypoxemia[11]. Insufflation of CO2 into the peritoneal cavity seemed to result in an immediate decrease in peritoneal pH. This peritoneal effect also seems to influence systemic acid-base balance, probably due to trans-peritoneal absorption[12]. We selected NT-OVH technique for this experiment as we wanted to minimise excessive surgical manipulation inflicted upon vein graft during harvesting and we could study the effect of CO2 on endothelium in a controlled environment. The prime objective of utilising “No Touch” samples for ex vivo study, was to alleviate physical injury at the time of harvest by preserving normal architecture of vein graft. As the names implies, harvesting vein with its surrounding perivascular tissue not only protects adventitia and network of vasavasroum but also conserve a rich source of NO, proven to be beneficial for graft performance. It also saves endothelium from crush induced injury during harvesting. For the first time in literature we have demonstrated that limited exposure (40 min) of CO2 insufflation at variable pressure of 6-12 mmhg during endoscopic extraction does not cause any immediate/accelerated endothelial damage and there was no statistical difference between control and CO2 groups.

Calcium is the key second messenger in mammalian cells, which regulates a variety of cellular functions. Selecting a Ca^{2+} indicator appropriately for a particular experimental study depends upon several considerations including Ca^{2+} binding affinity, dynamic range and ease of loading. A number of studies have used calcium orange dye to measure calcium mobilization in the endothelial cells of HSV[13–16]. For our study, we have used FLUO3 AM, which has been one of the most popular and widely used Ca^{2+} indicators[17]. Because FLUO3 is a single wavelength dye with fluorescein-like spectral characteristics, it can easily be excited with an argon laser (488 nm) for confocal microscopy[18]. A comparative study Thomas and colleagues, described the in-situ calibration of a number of commonly used fluorescent indicators, and Fluo3 proved to be the indicator of choice due to its large dynamic range, which provides images with the best contrast[19]. In literature, FLUO3 has not been utilised as a calcium indicator in any previous study on human saphenous vein. The most successful indicator for NO has been 4,5-diaminofluorescein diacetate (DAF-2 diacetate)[20]. It has been successfully used in previous studies on HSV[13–15,21]. We have used DAF-FM reagent for our study as it has some important advantages over DAF-2. NO adduct of DAF-FM is significantly more photostable than that of DAF-2[22], which means additional time for image capture, making it more sensitive for NO detection.

In our ex vivo model, we exposed the human saphenous vein segments to 100% CO2, harvested by NT-OVH to study the local effect of gas insufflation on vasomotor function. We opted for NT-OVH technique instead of OCVH for our study, as our previous work on histological and immunohistochemical analysis of HSV [23] demonstrated more endothelial damage in OCVH method. The samples were strictly not distended during harvesting to avoid any pressure related endothelial injury and periadventitial fat was preserved around the vein. A number of studies[24–26] investigating mechanisms underlying the improved performance of NT-OVH have identified various features contributing to its success from the advantages of reducing vascular damage and preservation of the vasa vasorum to preserving the perivascular fat and various tissue factors beneficial to the maintenance of a healthy graft.

We primarily, looked at the normalised maximum fluorescence intensity (Figure 3 and 4, table 1) and found bradykinin stimulated calcium mobilisation was significantly decreased in the CO2 group ($p = 0.013$) as compared to the control group. Similarly, eNOS mediated NO generation (Figure 5 and 6, table 2) was markedly attenuated in CO2 group, without reaching statistical significance ($p = 0.078$). For the first time in literature we have demonstrated local effect of CO2 on HSV endothelium, which not only directly depresses eNOS related NO release from endothelium but also impairs intracellular calcium activated calmodulin (CaM) coupling, resulting in overall decreased NO synthesis[27–29].

Calcium mobilisation is known to be an important step in the pathway for NO production. Rousou and colleagues[14] reported decreased calcium mobilization in response to bradykinin stimulation in the EVH group when compared with the OCVH group. Accordingly, it was also confirmed that NO production was markedly decreased in the EVH group compared with the OCVH group. A similar study was performed by Hussaini and colleagues[13] in 2011. The response of calcium mobilization and NO production to bradykinin stimulation, they discovered significant differences between EVH and OCVH groups. The endoscopic vein grafts showed markedly decreased calcium mobilization and NO production in response to bradykinin stimulation as compared to open vein grafts.

In summary, these studies showed endoscopic vein grafts do not maintain their functionality, especially with respect to the NO signaling cascade and NO production, unlike OCVH. They fail to establish the link between CO2 insufflation and impaired vasomotor function due to lack of Ca2+ and NO as a direct effect of CO2 gas on HSV endothelium. Low level of NO induces vasoconstriction, platelet and neutrophil adhesion and atherosclerosis. The inability of the endoscopically harvested SV endothelium to produce NO by an external stimulus like CO2 insufflation, likely has significant implications on graft patency and the loss of functional integrity.

We believe, these results are purely due to CO2 insufflation, as other potential mechanisms of injury were minimised with NT-OVH technique and vein segments were not distended during harvesting or procurement. This is an important finding as disruption of acute physiological processes can

compromise the entire length of vein graft, whereas mechanical injuries would rather localize to a specific area. However more research is required to establish, whether these findings are transient in nature or they have mid to long term implications on graft survival and clinical outcome.

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Conflict of interest statement

None declared

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Figure legends

Figure 1: Calcein AM (Live Cells) Assay – **(A)** Control Group **(B)** CO2 Group showing viable HSV endothelial cells by green fluorescence staining (L – lumen). The graph showing normalised maximum fluorescence intensity between groups in arbitray units (AU).

Figure 2: Propidium Iodide (Dead Cells) Assay - **(A)** Control Group **(B)** CO2 Group showing dead HSV endothelial cells by red fluorescence staining (L – lumen). The graph showing normalised maximum fluorescence intensity between groups in arbitray units (AU).

Figure 3: FLUO3 AM fluorescence assay – Control and CO2 Group

(A) Pre & **(B)** Post Bradykinin stimulation of HSV endothelial cells (L – lumen)

Figure 4: Normalised maximum fluorescence intensity by FLUO3 AM assay

Figure 5: DAF-FM fluorescence assay – Control and CO2 Group

(A) Pre & **(B)** Post Bradykinin stimulation of HSV endothelial cells (L – lumen)

Figure 6: Normalised maximum fluorescence intensity by DAF-FM assay