Introduction

A limitation to the long term benefits of vascular angioplasty is restenosis. Restenosis is a complex phenomenon that involves platelet aggregation and thrombus formation, smooth muscle cell proliferation and migration, excessive production of extracellular matrix and adventitial scarring (1, 2). Furthermore, acute elastic recoil and vascular remodeling can be responsible for up to 50% of late luminal loss (3-5). If intracoronary stents limit restenosis in de novo lesions (6, 7), they are associated with an increase in late luminal loss due to excessive smooth muscle cell proliferation and extracellular matrix formation (8, 9).

Summary

This study aimed to investigate the effect of percutaneous endoluminal arterial cryoenergy immediately after balloon angioplasty on vascular remodeling. Restenosis, the main complication after coronary artery angioplasty, is a complex phenomenon in which vascular remodeling plays a prominent role. Observations of reduced scarring in freeze-induced wounds suggest potential value for cryoenergy in the prevention of restenosis. Juvenile swine underwent a first oversized balloon injury in both carotid arteries (CA) (3 injury sites/artery) and a second injury at day 14. At that time, one CA was randomly assigned to endoluminal cryoenergy of the sequential segments (proximal, medial, distal) at -15°C, -30°C, and -50°C for 120 sec, and the other CA was used as control. Animals were sacrificed 28 days after the second procedure. Compared with intact reference segments, angioplasty reduced both the luminal (LA) and the external elastic lamina (EEL) areas from 6.66±0.59 to 3.13±0.54 mm² (p<0.05) and from 8.81±0.81 to 6.48±0.52 mm² (p<0.05), respectively. Cryoenergy, at the temperature with maximal benefits (-50°C), caused a temperature-related protection, as the LA was maintained (6.79±0.89 versus 7.18±0.78 mm² for reference) and the EEL area increased from 9.12±0.78 to 12.98±1.07 mm², p<0.05. Moreover, cryoenergy increased the density of collagen III (p<0.05) which correlated with the maintenance of the LA (r=0.8045, p<0.009). Cryoenergy prevents late luminal loss after double-injury angioplasty by improving vascular remodeling, and is an interesting new therapeutic approach for the prevention of restenosis after angioplasty. The increased synthesis of collagen III appears to be involved in this phenomenon and could be a potential method of stabilizing the vulnerable plaque.

Keywords

Angioplasty, remodeling, restenosis, cryoenergy, collagen

Cryoenergy has been used in the clinical management of cardiac arrhythmias (10-12). The rapid and intense freezing of tissue produces a localized and sharply demarcated wound (13) with mild scarring of the arterial wall (14) and little wound contraction due to a better preservation of the extracellular matrix (15). Moreover, a recent study has demonstrated a low incidence of thrombus formation in cryoenergy lesions, reflecting intact tissue ultrastructure and endothelial preservation (16). Considering that arterial remodeling may parallel wound healing (17), that smooth muscle cells (14, 18), but not collagen and elastic fibers (14) or endothelial cells (18), are very susceptible to cold injury, and that cold therapy results in better tissue repair...
with less fibrosis (19, 20), there is reason to believe that application of cryoenergy may prove beneficial in the prevention of restenosis after angioplasty. Indeed, cryoenergy has been associated with vascular lumen preservation in porcine coronary arteries (21). The exact mechanisms of this protection are not known, but may involve the prevention of acute elastic recoil by disturbance of autonomic regulation due to damage to vascular nerves, local release of vasodilator substances or cell necrosis within the medial and adventitial layers of the vessel wall (22).

In this study, we hypothesized that the application of cryoenergy to porcine carotid arteries might reduce restenosis after angioplasty by preventing late luminal loss and negative vascular remodeling.

**Materials and methods**

**Experimental preparation**

This study was performed after approval by the Montreal Heart Institute Animal Care and Use Committee following the guidelines of the Canadian Council on Animal Care. Seven juvenile swine weighing 20-25 kg (22.2±1.02 kg) were used (2 in the acute feasibility study and 5 in the chronic study of cryoenergy), as previously described (23-25).

**Vascular injury model**

A double angioplasty model was used (26). The angioplasty balloon 5-7 mm in diameter and 4 cm in length (Opta 5, Cordis, Roden, The Netherlands) was advanced under fluoroscopic guidance and overstretch injury was produced by three 30-sec inflations at 12 atmospheres at 3 separate sites (proximal, medial and distal) in each carotid artery, and quantitative carotid angiographic measurements were obtained (23). Fourteen days after the first angioplasty, the procedure was repeated in both carotid arteries and cryoenergy was applied to each site from one carotid artery using a cryocatheter system with a 10-mm distal tip (CryoCath Technologies Inc., Montreal). The cryocatheter consisted of a small diameter shaft containing a micro-injection tube within a long, flexible, steerable catheter. The liquid refrigerant (AZ-20) was delivered at pressures of 250-500 psi to a thermoconductive tip where boiling (liquid-to-gas phase transfer) occurred. The refrigerant evaporated in the tip to produce rapid cooling to temperatures between -10°C and -60°C (accurate to within ± 2°C) on the endoluminal surface. The temperature was recorded on-line at the distal tip via integrated thermocouples (16).

**Acute feasibility study**

In 2 swine, the cryocatheter was advanced into each carotid artery and cryoenergy was randomly applied for 120 sec at -15°C, -30°C and -50°C at the proximal, medial and distal sites of one carotid artery while the contralateral vessel served as control. Each site was separated by a minimal distance of 10 mm. One hour after cryoenergy application, the catheter was removed and the animal was euthanized. Both carotid arteries were harvested and perfusion-fixed for histological assessment.

**Chronic study of cryoenergy**

Immediately after the second angioplasty at day 14, cryoenergy was randomly applied for 120 sec at -15°C, -30°C or -50°C in the proximal, medial, and distal sites of one carotid artery (n=5). The contralateral vessel was also exposed to angioplasty and used as control. Twenty-eight days after the second angioplasty, a follow-up angiogram and quantitative measurements were performed and the carotid arteries were perfusion-fixed in-vivo (23).

**Histological and morphometric analysis**

The 3 cryotreated and the 3 corresponding angioplastied segments (including an appropriate proximal and distal reference segment that did not undergo angioplasty) were paraffin-embedded. Each segment was then divided into 3 equal sections to account for any anatomical variation, the sections were analyzed and the results pooled. Thus, the values for each segment are the mean of the 3 sections for a total of 90 sections (45 cryotreated and 45 angioplastied sections). Each section was stained with hematoxylin-eosin and Verhoeff’s elastic solutions to allow identification of the internal (IEL) and external elastic lamina (EEL), and computerized morphometric analysis was performed (23-26). The percentage of medial fracture, defined as the length of medial disruption divided by the IEL, was determined as well as the degree of vessel wall injury (23, 24). Remodeling was calculated as the ratio of the EEL at the site of treatment/EEL of the proximal reference segment. A remodeling index > 1 indicates positive remodeling, = 1: absence of remodeling and < 1: negative remodeling. The presence or absence of inflammatory cells was also determined in each segment, and graded as follows: 0 = absence of inflammatory cells, 1 = presence in less than 24%, 2 = in 25% to 49%, 3 = in 50% to 74% and 4 = in more than 75% of the segment.

**Immunohistochemistry**

In all segments, the presence of proliferating cell nuclear antigen (PCNA) identification (24), endothelial cells (26) and constitutive nitric oxide synthase (eNOS) (25) were determined and graded as described above.

**Collagen expression**

Each segment was stained with Picro sirius red to evaluate collagen I, which appeared in red, and collagen III, which appeared in green. Respective collagen subtype expression was evaluated by the relative number of pixels in the media by adjusting the threshold permitting a binary analysis. Collagen density was computed by the area of the pixels classified as collagen I or collagen III in the media divided by the total medial area.
**Data analysis**

All values are expressed as mean±SEM. The statistical significance of the difference between groups was evaluated by one-way ANOVA. A p value <0.05 was taken to indicate statistical significance. An adjusted t test with the p value corrected by the Bonferroni method was used to compare pairs of means.

**Results**

In all swine, the body weight, the hematological and hemodynamic parameters were similar before the two interventions, and they were within the normal physiological range (data not shown).

**Acute feasibility study**

The acute feasibility study was designed to examine the effects of different endoluminal cryoenergy application on normal porcine carotid arteries. The 2 swine used in this study successfully underwent cryoenergy treatment. Endoluminal temperatures of -15ºC, -30ºC and -50ºC could be achieved locally in each carotid artery. No acute arterial spasm or thrombosis was observed and each vessel remained patent. Macroscopic examination revealed only localized hyperemia on the adventitial surface. Standard histology showed loss of endothelium with no or mild injury to the IEL. No difference was observed among the 3 temperatures with respect to acute vessel damage.

**Chronic study of cryoenergy**

**Quantitative carotid angiographic evaluation**

Table 1 summarizes the angiographic data obtained before and after the procedures and at sacrifice. All quantitative angiographic parameters were similar after the first injury. However, the application of cryoenergy, immediately after the second injury, resulted in a significantly greater minimal lumen diameter (Post MLD) as compared to the angioplastied segments (p<0.05). This effect was not an acute response as, at the time of sacrifice, cryoenergy resulted in the maintenance of the MLD as compared to the reference segments. Thus, the application of cryoenergy may be involved in the prevention of the negative geometric remodeling observed after double-injury angioplasty.

**Histomorphological analysis of angioplastied and cryotreated carotid arteries**

As shown in table 2, the application of cryoenergy at -30ºC and -50ºC increased the EEL area by 1.6 and 2-fold, respectively (p<0.05). When the IEL was examined, its length was significantly increased in the segments subjected to cryoenergy at -50ºC (1.6-fold increase, p<0.05) even though the percentage of IEL fracture was significantly greater (p<0.05). These observations support the hypothesis that cryoenergy at -50ºC may promote the positive remodeling of the angioplasty-injured carotid arteries, as the lumen size is larger (i.e., EEL), which is secondary to a larger overall vessel size (i.e., EEL). On the other hand, standard angioplasty was associated with negative remodeling. The injury scores were similar between segments and treated groups, although more severe injury was present in the segments treated with temperatures of -30ºC and -50ºC. Inflammatory cells were absent in the reference segments and low (grade 1) in all treated segments.

**Effects of cryoenergy on the vascular wall**

Figure 1 summarizes the changes in the morphometry of the angioplastied and cryotreated carotid arteries at the 28-day follow-up. As expected, double-injury angioplasty reduced the vascular lumen by 50% (p<0.05). In contrast, cryoenergy (-50ºC) reduced late luminal loss, as the luminal area was similar to that observed in the uninjured reference segments (6.79±0.89 versus 7.18±0.78 mm²), but increased by 117% when compared to its respective angioplasty segments (p<0.05). Partial protection was observed with treatment at -30ºC. When the medial and neointimal areas were analyzed, cryoenergy increased both areas when compared to their respective angioplasty control segments (Fig. 1, p<0.05). Collectively, these observations suggest that the overall temperature-related bene-

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<td><strong>Second Injury</strong></td>
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MLD indicates minimal lumen diameter. Results are mean±SEM. *p<0.05 versus angioplasty; † p<0.05 versus reference.
fits in lumen preservation are due to improved vascular remodeling (increase in the EEL and IEL areas), albeit neointimal hyperplasia was not prevented. Figure 2 illustrates representative sections of a control, angioplastied and cryotreated vessel.

Effect of cryoenergy on vascular remodeling

The effects of cryoenergy on the vascular wall were further evaluated by computing the remodeling index. As shown in figure 3, cryoenergy at -50°C promoted the positive remodeling of angioplastied segments (1.45±0.10 versus 0.77±0.035, p<0.05) while minimal remodeling was observed at -30°C (1.10±0.14). Representative histomorphological cross-sections depicting vascular remodeling in the angioplastied and cryotreated segments are presented in figure 2. These results stress the concept that cryoenergy, specifically at -50°C, exerts long-term beneficial effects by shifting the deleterious negative vascular remodeling associated with angioplasty into a beneficial positive remodeling. Indeed, a positive correlation was observed between the EEL area and the luminal area (r=0.7143, p<0.03). This was not due to a lesser degree of injury (Table 2) since our acute feasibility study showed no or mild injury to the IEL after cryoenergy application. These observations lend support to a unique effect of cryoenergy that is still operative even when the vascular wall is greatly damaged.

Immunohistochemical analysis

As expected, the percentage of positive cells expressing PCNA was less than 1% in all segments. On the other hand, immunostaining for the Dolichos biflorus agglutinin (DBA) lectin indicated that complete neointimal reendothelialization was

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<td><strong>Angioplasty</strong></td>
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<td>Fracture/EEL length (%)</td>
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IEL indicates internal elastic lamina; EEL, external elastic lamina; N/A, not available. Results are mean±SEM. † p<0.05 versus reference. * p<0.05 versus respective angioplasty segments.

Figure 1: Morphometric analysis of carotid arteries after angioplasty and cryoenergy at the 28-day follow up. Results are mean±SEM, * p<0.05 versus reference, † p<0.05 versus respective angioplasty segments.
Figure 2: Representative light micrographs (× 40) from carotid segments after angioplasty and cryoenergy. The left panel represents an angioplastied segment (upper) with its distal reference segment (lower). The right panel represents the effect of cryoenergy at -50°C after angioplasty (upper) and in the noninjured distal reference segment.

Figure 3: Remodeling index. The remodeling index was defined as the ratio of the EEL area at the angioplastied segment to the EEL area at the proximal reference segment. A remodeling index > 1 indicates positive remodeling; = 1: absence of remodeling; and < 1: negative remodeling. * p<0.05 versus angioplasty segments.

Figure 4: Collagen density in the medial area of the angioplastied and cryotreated carotid arteries. Results are mean±SEM, * p<0.05 versus angioplasty, p<0.05 versus collagen I.
achieved in the angioplastied (grade 4) and the cryotreated (grade 3.75) segments at the 28-day follow-up. As well, the presence of ecNOS on the neointima was similar between the angioplastied (grade 3.38) and cryotreated (grade 3) segments.

Collagen expression

The distribution of collagen I and III in the medial layer of the reference, angioplastied and cryotreated segments is presented in figures 4 and 5. The expression of both collagen I (6.87±0.21 %) and III (6.90±0.26 %) was similar in the reference uninjured segments. Angioplasty induced a slight increase in their expression (p<0.05), while cryoenergy at -30°C and -50°C resulted in a 93% and 310% increase in collagen I and III, respectively (p<0.05). Our results also revealed that the density of collagen III in the media was always significantly greater than that of collagen I after angioplasty and cryoenergy. Specifically, collagen III was found to be a predictor of some of the morphometric changes observed after cryoenergy. A highly significant and positive correlation was observed between collagen III and the luminal area after the application of cryoenergy at -50°C (r=0.8045, p<0.009), but not after angioplasty (r=0.0502, p<0.91). The increase in the density of collagen III was not due to a greater vascular injury as no relationship was observed between the two parameters. It appears that the synthesis of collagen III, but not collagen I, is involved in the positive remodeling and reduction of late luminal loss observed after cryoenergy.

Discussion

Application of percutaneous endoluminal cryoenergy, immediately after angioplasty of porcine carotid arteries, resulted in vascular lumen preservation and increase in the overall vessel size. The density of both collagen I and III was also increased, but only collagen III was correlated with the enlargement of the luminal area. This study is the first to provide evidence that cryoenergy prevents the development of restenosis by favoring positive arterial remodeling.

Vascular effects of endoluminal arterial cryoenergy

At the 28-day follow-up, application of cryoenergy at -50°C resulted in a net 117% enlargement of the vascular lumen of the angioplastied segments, which was similar to that of the reference segments (Fig. 1, 2). This protection against late luminal loss was correlated with an overall 1.6 and 2-fold increase in both the IEL and EEL (Table 2). Partial protection was observed with the intermediate temperature of -30°C. Complete reendothelialization and ecNOS expression was achieved in all treated segments while the inflammatory response was minimal.

The effects of cryoenergy on neointimal growth are less clear. In a landmark study by Gage and colleagues (14), they found that smooth muscle cells were very susceptible to cold injury. Similar results were also observed in the epigastric arteries of rats (27) and in human coronary artery smooth muscle cells (18). In contrast, Cheema et al. (22) found that cryoenergy stimulated neointimal hyperplasia in a rabbit model of iliac artery angioplasty. Our results are in accordance with their study in that cryoenergy at -50°C promoted neointimal growth by approximately 5-fold (Fig. 1) although the vascular lumen was maintained to its original size. These observations lend credence to the contention that neointimal hyperplasia may not be as important as previously speculated in the development of restenosis (28, 29) and that cryoenergy may have a direct impact on the vessel wall by favoring the positive remodeling of the angioplastied arteries.

Cryoenergy for the prevention of negative arterial remodeling

Based upon the EEL area (Table 2) or expressed as a remodeling index (Fig. 3), application of cryoenergy at -50°C, and to a lesser extent at -30°C, improved vascular remodeling through the expansion of the injured arteries. Although Terashima et al. (21) did not evaluate remodeling per se, one can assume that cryoenergy also resulted in the positive remodeling of the coronary arteries since no loss in lumen diameter or increment in the percentage of stenosis were observed. In contrast, our results are in disagreement with those of Cheema et al. (22) who suggested that the cryotherapy-induced increase in the collagen content was responsible for the late luminal loss and overall negative

Figure 5: Representative histological sections (× 100) from carotid segments after angioplasty and cryoenergy. Presence of collagen I and collagen III in the medial area appears in red and green, respectively. A: reference segment, B: angioplastied segment and C: cryotreated segment (-50°C).
remodeling. We have also found that the density of both collagen I and III was increased after cryoenergy (Fig. 4) despite positive remodeling. In fact, the density of collagen III was correlated with the increase in the EEL area. Our study corroborates that of Coats and colleagues (30) who demonstrated that the increased collagen density in nonrestenotic vessels was correlated with preserved lumen area and positive remodeling. On the other hand, some studies have correlated the increase in collagen density with constrictive remodeling after angioplasty (22, 31, 32). A possible explanation for this discrepancy may relate to the fact that these latter studies did not discriminate between the specific collagen subtype(s).

Collectively, the results of the present study support the premise suggesting that arterial remodeling may be more important than neo-intimal hyperplasia in the development of restenosis. However, it is not known how cryoenergy can shift the balance from negative to positive remodeling, but the synthesis of collagen III appears to be involved in this process. This suggests that cryoenergy may modulate the extracellular matrix turnover. Indeed, inhibition of matrix metalloproteinases (33) or activation of apoptosis (34) prevent constrictive arterial remodeling without inhibiting neo-intimal hyperplasia.

**Clinical implications**

Unstable atherosclerotic lesions associated with plaque vulnerability are more commonly associated with positive than negative remodeling (35). The inflammatory state that accompanies the active atherosclerotic lesion favors the decreased synthesis of collagen by increasing its degradation through the activation of matrix metalloproteinases, which leads to a weaken fibrous cap prone to rupture (36). Since application of cryoenergy, immediately after angioplasty, promotes the synthesis of collagen, it may very well strengthen the fibrous cap, thus stabilizing the lesion. However, it will have to be determined if a similar vascular response can be obtained in atherosclerotic arteries, since non-atherosclerotic vessels can remodel sufficiently to accommodate extensive neo-intimal growth (37).

**Study limitations**

The carotid artery was chosen because of catheter limitations, but we suspect that the coronary artery would respond to cryoenergy application in a similar manner. A double cycle of injury, 14 days apart, can simulate mild stenosis, but the lesion are usually not angiographically significant. The effect of cryoenergy in an atherosclerotic vessel with a severe stenosis can not be extrapolated from this experimental work. However, the temperature-related effect with positive vessel remodeling remains significant and very promising. These findings need to be placed in perspective with the current use of stents and the success in reducing restenosis with newer drug eluting stents. No data is currently available on the potential synergy between cryoenergy and stenting but favoring positive remodeling could be of clinical interest in small vessel and bifurcation lesions.

**Conclusions**

To the best of our knowledge, this is the first report to demonstrate that endovascular cryoenergy at -50°C for 120 sec prevents restenosis by favoring positive arterial remodeling. The increased synthesis of collagen III appears to be involved in this phenomenon and may be a key mediator to the beneficial effects of cryoenergy and plaque stabilization.

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**References**