Published in final edited form as:

J Vasc Interv Radiol. 2010 June ; 21(6): 910–915. doi:10.1016/j.jvir.2010.02.016.

THE EFFECTS OF FREEZING VERSUS SUPERCOOLING ON VASCULAR CELLS: Implications for Balloon Cryoplasty

Maria Theresa G Basco, MD¹, Wai-ki Yiu, MBBS, MRCS^{1,2}, Stephen WK Cheng, MS, FRCS, FACS², and Bauer E. Sumpio, MD, PhD¹

¹ Department of Vascular Surgery, Yale University School of Medicine, New Haven, CT, USA

² Division of Vascular Surgery, Department of Surgery, University of Hong Kong Medical Centre, Queen Mary Hospital, Hong Kong

Abstract

Background—Cryoplasty as treatment for peripheral vascular disease is thought to provide greater long-term patency rates compared to conventional angioplasty although the mechanisms remain unclear. Since smooth muscle cells are intimately involved in restenosis and neointimal hyperplasia and endothelial cells modulate healing after angioplasty, we sought to investigate the effects of supercooling, a phase whereby the cells are below 0C but still in a liquid state and freezing, the phase when the cells become solid, of vascular cells in culture.

Method—Bovine aortic endothelial cells and smooth muscle cells were supercooled to −10C with or without freezing for 3, 30 or 60 seconds then rewarmed to 37C for 24 hrs. Viability was assessed via trypan blue exclusion while apoptosis was assessed by the TUNEL assay.

Results—Viability of smooth muscle cells declined 49% after freezing versus supercooling ($p < 0.05$). Endothelial cells maintained greater viability rates. A 19.5% smooth muscle cell apoptotic rate was observed after freezing while smooth muscle cell supercooling yield rates of only 11% ($p < 0.05$). A 4.17% endothelial cell apoptotic rate was observed after freezing while supercooled endothelial cells yield a 1.76% rate ($p < 0.05$).

Conclusion—Freezing results in decreased viability and increased apoptosis compared to supercooling in both cell lines. Smooth muscle cells appear more susceptible to freezing. The biologic effects of freezing on vascular cells may elucidate the mechanisms behind the enhanced patency after cryoplasty of atherosclerotic lesions.

INTRODUCTION

First described in 1964, percutaneous transluminal angioplasty was revolutionary in offering patients an alternative to open surgery and establishing the field of endovascular therapy. Four decades later, percutaneous transluminal angioplasty still remains the endovascular procedure of choice in the treatment of atherosclerosis with over 1,285,000 angioplasties done in 2004 (1). Percutaneous transluminal angioplasty has historically been associated with high technical success rates, but poor long-term patency rates remain its primary drawback and alternative endovascular therapies continue to be developed.

To whom correspondence should be addressed: Bauer E. Sumpio, Department of Surgery (Vascular Surgery Section), Yale University School of Medicine, 333 Cedar Street, New Haven, CT 06520-8062. Tel.: (203) 785-2561; Fax: (203) 785-7609. bauer.sumpio@yale.edu.

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Cryoplasty, which combines conventional angioplasty with the delivery of hypothermia to the blood vessel wall, is a relatively novel treatment which preserves the mechanical effects and technical success rates of percutaneous transluminal angioplasty, but adds the theoretical biological effects of hypothermia on the vessel (2,3). Outcomes utilizing the PolarCath balloon (Boston Scientific, Natick, MA) for atherosclerotic lesions in the femoropopliteal region have reported primary patency rates as high as 75% after 3 years(4). It has been theorized that cryoplasty, which cools and freezes the target lesion at -10°C , can have several effects on the blood vessel wall, including an increase in susceptibility to mechanical disruption, altered matrix remodeling, and induction of vascular smooth muscle cell apoptosis(5,6).

Restenosis associated with conventional angioplasty is a complex process but neointimal hyperplasia secondary to smooth muscle cell activation has been an area of intense scrutiny. Endothelial cells lining the blood vessel wall protect smooth muscle cells against circulating growth promoting factors and produce growth inhibitory factors important in smooth muscle cell proliferation(7). Therefore, the effects of cryoplasty on the endothelium, is also of interest. In addition, the proximity of vascular cells to the PolarCath balloon will determine whether some cells are frozen or simply supercooled, a phase whereby the cells are below 0°C but not frozen(3).

Our early studies explored the effects of supercooling on vascular smooth muscle cells and endothelial cells using a culture model simulating the temperature changes of cryoplasty and demonstrated that smooth muscle cells have significantly greater rates of apoptosis compared to endothelial cells(8,9). In this study, we directly compare the effects of supercooling versus freezing on vascular endothelial cells and smooth muscle cells exploring the hypothesis that freezing induces apoptosis to a greater degree than supercooling and that different vascular cells remain less susceptible to cold injury despite the more severe frozen state

MATERIALS and METHODS

Cell culture

Bovine thoracic aortic endothelial cells and smooth muscle cells were obtained from freshly slaughtered calves. The protocol was approved by the Yale Institutional Animal Care Use Committee. In brief, endothelial cells were harvested by gently scraping the thoracic aortic intima and seeding them onto cell culture plates containing culture medium(9). The intima was then gently scraped of the underlying media and smooth muscle cells were obtained via the explant method (9–11). Cells were cultured and maintained in Dulbecco's modified Eagle's medium F-12 (GIBCO BRL, Gaithersburg, MD) supplemented with 10% fetal calf serum (Gemini BioProducts, Woodland, CA), 5 $\mu\text{g}/\text{ml}$ deoxycytidine/thymidine (Sigma-Aldrich, St. Louis, MO), and antibiotics (penicillin 100U/ml, streptomycin sulfate 100mg/ml, amphotericin B 0.25mg/100ml) (GIBCO-BRL) at 37°C in a humidified incubator with 5% carbon dioxide. Cells of passage 9 to 13 were seeded on 6 well plates (Becton Dickinson, Franklin Lakes, NJ) prior to experimentation.

Supercooling, freezing and re-warming system

A cooling apparatus as previously described was modified slightly(8,9,11). A refrigerated circulator (VWR International, West Chester, PA) was connected to a cryostage custom-made to accommodate six well plates (Cryovascular systems, Inc, Los Gatos, CA). The design of the cryostage was optimized to deliver the same temperature to each of the six wells of the culture plate by creating symmetric flow channels. Ethylene glycol was used as coolant to bring the temperature of the cryostage to -21°C . Each well contained 1ml of medium and the temperature change of each well on the six-well plate placed atop the cryostage was recorded (Figure 1A). The temperature of the media dropped to -6.2°C by 60 seconds, -14.7°C by 120 seconds; this

part of the resulting curve follows closely the temperature curve obtained when cryoplasty is performed utilizing the PolarCath balloon(12). Beyond 120 seconds on the cryostage, medium within the wells will freeze spontaneously around 144 seconds (Figure 1A)^{9,11}. Supercooling, refers to the phase whereby the cells are below 0C but still in a liquid state in contrast to freezing, the phase when the cells become solid, This physical change is reflected in the temperature curve resulting in a brief rise in temperature. To induce freezing, ice nucleation was performed by touching the tip of a capillary tube containing frozen culture medium to the center of each well resulting in a new temperature curve (Figure 1B).

Experiments were performed when cells reached greater than 80% confluence. Freezing versus supercooling was directly compared by placing a six-well plate on the cryostage, nucleating the upper three wells after 60 seconds of cooling and leaving the lower three wells to supercool. Approximately 3 seconds passed when nucleating the top three wells. The six-well plate remained on the cryostage for periods of 63, 90 and 120 seconds before immediately being transferred to a 37°C humidified incubator for 24 hours of re-warming. This resulted in 3, 30 and 60 seconds of freezing time and approximately 3, 30 and 60 seconds of supercooling time. Trypan Blue analysis and TUNEL assay was then performed.

Trypan Blue

Cell viability was determined via trypan blue exclusion. Cells were fixed with 4% paraformaldehyde in phosphate buffered saline (PBS). To identify nonviable cells, 200ul of Trypan Blue solution (Sigma-Aldrich, UK) was added to each well and counterstained with haematoxylin. The number of nonviable cells was counted by phase-contrast microscopy (Olympus) at fields of 100x magnification radiating from the center of the well. Four fields of cells per well were examined. Due to fields which had experienced detachment, as seen in some wells which were frozen, we report percent viability as “corrected” percent viability. In our experiments, wells which were not frozen did not experience detachment. Therefore, the number of cells per field in these wells was used as the control in order to calculate the corrected percent viability. Corrected percent viability was calculated as the difference between the total number of cells in the frozen wells and the number of trypan blue positive cells in these wells divided by the total number of cells in control. Detachment rates were calculated as the difference between the total number of cells in the control and the total number of cells in the frozen wells divided by the total number of cells in the control.

Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) Assay

Apoptosis was assessed by the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay. In brief, cells were fixed and then permeabilized with 0.1% Triton X-100 containing 0.1% sodium citrate in distilled water. Non-specific endogenous peroxide was blocked with 3% hydrogen peroxide in methanol. The degree of apoptosis was detected by using an in situ cell death detection kit (POD; Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. The labeled DNA was visualized with 3,3'-Diaminobenzidine as chromogen and counterstained with haematoxylin. The number of TUNEL positive cells was counted at 100x magnification as described above. To account for detachment, a corrected percent TUNEL positive rate was calculated using the number of cell per field in the non-frozen wells as control.

Statistical Analysis

All data are represented as mean $\pm$ standard error of mean. Analysis of variance with post-hoc testing or T-test was used for statistical analysis when applicable and differences were considered significant at $p < 0.05$.

RESULTS

Vascular Cell Viability after Freezing or Supercooling

In both cell lines, greater viability was observed after supercooling versus freezing (Figure 2). Representative light microscope images indicate Trypan Blue positive endothelial cell (Figure 2A) and smooth muscle cell (Figure 2B) increased with increasing frozen time (Figure 2A, 2B). Endothelial cells remained viable after 30 seconds of freezing with a rate of 87.3% (Figure 2C) beyond which there was a statistically significant drop in viability. Supercooled endothelial cells remained viable after 60 seconds of freezing with a rate of 96.9%. Statistically significant differences exist in viability rates between supercooling and freezing at 30 and 60 seconds of frozen time ($p<0.05$). A drop in endothelial cell viability with increasing frozen time was observed with significant differences existing between 3 and 60 seconds of frozen time as well as 30 and 60 seconds of frozen time. ($p<0.05$)

There was no statistically significant difference in smooth muscle cell viability after supercooling for all three time points and viability rates were as great as 93.5% (Figure 2D). There was a statistically significant drop in smooth muscle cell viability from 72.7% after 3 seconds of freezing time to 39.5% after 60 seconds of freezing time ($p<0.05$). Statistically significant differences in viability rates exist between supercooling and freezing in all three time frames ($p<0.05$).

Vascular Cell Apoptosis after Freezing and Supercooling

In both cell lines, the highest rates of apoptosis were observed after freezing compared to supercooling (Figure 3). Representative light microscope images indicate TUNEL positive endothelial cell (Figure 3A) and smooth muscle cell (Figure 3B) increased with increasing frozen time. Endothelial cell apoptosis was greatest at 4.17% and was observed after 60 seconds of freezing (Figure 3C). Statistically significant differences in apoptotic rates exist between supercooling and freezing only at the 30 second time frame ($p<0.05$). Furthermore, statistically significant increases in TUNEL positive rates with increasing frozen time exist only at the 30 second time frame ($p<0.05$).

Smooth muscle cell freezing resulted in TUNEL positive rates as great as 19.5% while supercooled smooth muscle cells resulted in an 11% TUNEL positive rate (Figure 3D). Statistically significant differences exist between supercooling and freezing at the 30 and 60 second time frame ($p<0.05$). Rate of apoptosis was directly related to frozen time. There were statistically significant increases in TUNEL positive rates after each increase in length of frozen time from 4.8% after 3 seconds to 15.2% after 30 seconds to 19.5% after 60 seconds of frozen time ($p<0.05$). Statistically significant differences in smooth muscle cell apoptosis after supercooling exist at the 60 second time frame only ($p<0.05$).

DISCUSSION

Cryoplasty is performed in a fashion similar to angioplasty in that a balloon is inserted across the lesion over a guidewire but differs in that nitrous oxide is used to inflate and cool the balloon to -10°C . An automated process, the PolarCath balloon remains inflated at -10°C for 20 seconds before rewarming and deflating (12). Cells in the vessel wall exposed to the PolarCath balloon will experience different thermal histories depending on their proximity to the cold balloon. Cells in layers near to the lumen or in close contact with the balloon will freeze while cells in outlying layers or farther from the balloon will cool or supercool. The physical insult brought about by freezing is thought to provoke apoptosis, a form of cell death characterized by organized nuclear fragmentation, DNA degradation, cell shrinkage and blebbing (5,6). Also thought of as “active cell death”, apoptosis results in the organized removal of cell materials

by phagocytosis, thus avoiding an inflammatory response. It is the mechanical benefits of arterial dilation combined with the proposed biologic effects of freeze induced apoptosis that is thought to abrogate restenosis.

Interest in the research of freeze induced cell death has been driven primarily by its role in cryopreservation and in cryooncology. The physical mechanisms involved in cell death and survival with freezing has been widely studied and is generally accepted to be related to intracellular ice formation. Biologically, studies have shown apoptosis to be a cause of cell death induced by cold temperatures, but the cellular mechanisms are poorly understood. Results of the study by Nagle et al. (13) suggest apoptosis is induced by exposure to cold but nonfreezing temperatures. Recent studies have shown cells exposed to temperatures below -15°C died via necrosis but cells exposed the temperatures above -15°C died through apoptosis (14). Tatsutani et al. (6) found temperatures in the range of -5 to -15°C to induce apoptosis in human arterial smooth muscle cells. Our interest into the use of cryotherapy in the treatment of peripheral artery disease is driven by the lack of knowledge surrounding how apoptosis affects the complex processes leading to restenosis. Our previous studies confirmed the hypothesis that apoptosis was a mechanism governing cell death after supercooling at temperatures in the range of -6 to -14°C and that different cell lines were more susceptible to this process. Because cells within the target vessel experience different thermal histories we chose in this study to investigate how freezing compares with supercooling in affecting apoptosis. In addition to the cold thermal insult, freezing results in physical changes to the cell. With sufficiently slow cooling, ice forms in the external medium and cell contents remain supercooled. Extracellular ice formation results in a hyperosmotic environment. In response, the cell equilibrates by dehydration and cell shrinkage.

Results of our viability study suggest that more cells survived after supercooling when compared to freezing and that freezing resulted in more cell death-this was true for both endothelial cells and smooth muscle cells. In both cell lines, a statistically significant drop in viability rate was observed after 60 seconds of freezing indicating that frozen time is a factor in cell death. We also note that endothelial cells appear to have greater viability compared to smooth muscle cells. Because Trypan Blue exclusion identifies only dead cells and does not distinguish between apoptotic and necrotic cells, we investigated apoptosis via TUNEL assay. The results of this study revealed freezing induces apoptosis in both endothelial cells and smooth muscle cells and does so to a greater degree than supercooling. Statistically significant increases in rates of apoptosis with increasing frozen time indicate that frozen time is a factor in inducing cell death via apoptosis. It is also important to note that our results are presented as "corrected" therefore an unknown number of dead cells that have detached may be apoptotic. Detachment was noted with freezing and not observed in the supercooling of our cells, therefore, the true percentage of apoptotic cells in our frozen group is greater than what our TUNEL positive rates reveal. We also note that apoptosis rates for endothelial cells were much lower when compared to smooth muscle cells. Because of the low endothelial cell apoptosis rates and high endothelial cell viability rates we postulate that endothelial cells are more resistant to freeze injury.

Restenosis after angioplasty results primarily from arterial remodeling and more importantly, neointimal hyperplasia(15). Neointimal hyperplasia occurs secondary to smooth muscle cell proliferation and migration. Medial smooth muscle cells migrate across the internal elastic lamina to form a new intimal layer where they continue to proliferate and produce extracellular matrix material(16). Smooth muscle cell migration and proliferation also occurs in atherosclerotic plaques. Because of migration, smooth muscle cells are in close enough contact to the Cryoplasty balloon to be affected by freezing. Supercooling also effects smooth muscle cell apoptosis but to a lesser degree. The apoptosis observed with supercooling may affect restenosis by moderating medial smooth muscle cell proliferation and migration. The

endothelium plays an important role in blood vessel wall structure and in modulating neointimal hyperplasia⁷. Endothelium provides a nonpermeable barrier protecting smooth muscle cells against circulating growth promoting factors. Endothelial cells secrete growth inhibitory factors which govern smooth muscle cell proliferation. Endothelial cell dysfunction further contributes to restenosis when the balance between relaxing and contracting factors are disrupted(17).

In conclusion, freezing induces apoptosis and to a greater degree than supercooling alone. Additionally, freezing exerts differential effects on vascular cells. smooth muscle cells appear more susceptible to apoptosis after freezing than endothelial cells. Endothelial cells demonstrate some resistance to freezing and may remain viable with up to 30 seconds of freezing. Our early studies confirmed hypothermia as a mechanism behind the benefits of cryoplasty but in this study, we have demonstrated that freezing may be of greater significance. By inducing smooth muscle cell apoptosis to a greater degree while minimizing endothelial injury thus possibly preserving endothelial function, freezing may be a more important biologic mechanism behind the suppression of restenosis after cryoplasty. This may provide an explanation for the superior patency rates achieved after cryoplasty in vivo. Of course, these studies are limited by the fact that cultured cells are not exactly like the cells that are situated in vivo in a complex vessel matrix and exposed to hemodynamic forces as well as circulating chemical milieu. Nonetheless, the results are significant in that a differential response to endothelial cell and smooth muscle cell to hypothermic conditions can be clearly established.

The results of the current study also provides important information that the interventionalist could consider in determining placement of the PolarCath balloon when treating lesions. Minimizing the overlap of the balloon on normal, non-diseased arteries may be ideal. Furthermore, future designs of the catheter may take advantage of freezing and cooling regimens that would increase the freezing duration leading to optimized smooth muscle cell apoptosis. Thus further investigation into the biologic effects of freezing may provide additional insight into the putative benefits of cryoplasty.

Acknowledgments

The study was funded in part by grants from the National Institutes of Health HL R01-47345, VA Merit Review Board and the North American Center for Limb Preservation.

References

1. Rosamond W, Flegal K, Friday G, et al. Heart Disease and Stroke Statistics—2007 Update. A Report From the American Heart Association Statistics Committee and Stroke Statistics Subcommittee. *Circ* 2007;115:e69–e171.
2. Sarage, A.; Yui, W.; Blume, P.; Aruny, J.; Sumpio, BE. Aggressive revascularization options using cryoplasty in patients with lower extremity vascular disease. In: Geroulakos, G.; Reddy, D., editors. *Reinterventions in Vascular and Endovascular Surgery*. Athens: Paschalidis Medical Publications; 2008. p. 76-89.
3. Yiu WK, Basco MT, Aruny J, Sumpio BE. Cryosurgery: A Review. *Int J Angiol* 2007;16:1–6.
4. Laird J, Biamino G, McNamara T, et al. Cryoplasty for the treatment of femoropopliteal arterial disease: extended follow-up results. *J Endovasc Ther* 2006;13(Suppl 2):II52–59. [PubMed: 16511955]
5. Joye JD. The clinical application of Cryoplasty for infrainguinal peripheral arterial disease. *Tech Vasc Inter Radiol* 2005;8:160–164.
6. Tatsutani KN, Joye JD, Virmani R, Taylor MJ. Invitro evaluation of vascular endothelial and smooth muscle cell survival and apoptosis in response to hypothermia and freezing. *Cryoletters* 2005;26:55–64. [PubMed: 15772713]
7. Kipshidze N, Dandas G, Tsapenko M, et al. Role of Endothelium in Modulating Neointimal Formation. *J Am Coll Card* 2004;44:733–739.

8. Yiu WK, Aruny JE, Cheng SWK, Sumpio BE. In-vitro model for evaluation of the effects of supercooling and re-warming on vascular cells. *Int J Angiol* 2006;14:237–241.
9. Yiu WK, Aruny JE, Cheng SWK, Sumpio BE. Direct comparison of endothelial cell and smooth muscle cell response to supercooling and rewarming. *J Vasc Surg* 2007;46:557–564. [PubMed: 17826245]
10. Li W, Chen Q, Mills I, Sumpio BE. Involvement of S6 kinase and p38 mitogen activated protein kinase pathways in strain-induced alignment and proliferation of bovine aortic smooth muscle cells. *J Cell Physiol* 2003;195:202–209. [PubMed: 12652647]
11. Yiu WK, Aruny JE, Cheng SWK, Sumpio BE. Vascular Smooth Muscle Cells Apoptosis Induced by Supercooling and Re-warming. *J Vasc Interv Radiol* 2006;17:1971–1977. [PubMed: 17185696]
12. CryoPlasty® Therapy.
www.bostonscientific.com/procedure/ProcedureLanding.bsci/,/navRelId/1000.1002/id/10000821/se0.serve
13. Nagle WA, Bernard L, Soloff A, Moss AJ Jr, Henle K. Cultures Chinese hamster cells undergo apoptosis after exposure to cold but nonfreezing temperatures. *Cryobiology* 1990;27:439–451. [PubMed: 2394128]
14. Baust JG, Gage AA, Clarke D, Baust JM, Van Buskirk R. Cryosurgery-a putative approach to molecular-based optimization. *Cryobiology* 2004;48:190–204. [PubMed: 15094094]
15. Nakatani M, Takeyama Y, Shibata M, et al. Mechanisms of restenosis after coronary intervention: difference between plain old balloon angioplasty and stenting. *Cardiovasc Pathol* 2003;12:40–48. [PubMed: 12598017]
16. Clowes AW, Reidy MA, Clowes MM. Kinetics of cellular proliferation after arterial injury. I. Smooth muscle growth in the absence of endothelium. *Lab Invest* 1983;49:327–333. [PubMed: 6887785]
17. Meurice T, Vallet B, Bauters C, Dupuis B, Lablanche JM, Bertrand ME. Role of endothelial cells in restenosis after coronary angioplasty. *Fundam Clin Pharmacol* 1996;10:234–242. [PubMed: 8836697]

Figure 1A

Figure 1B

Figure 1.

A. Representative cooling curve of a 6 well plate containing 1 ml of culture medium containing 10% fetal bovine serum (diamond line). The re-circulating apparatus was set to -30°C allowing the conduction cryostage to cool down to a temperature of -21°C (solid line). Thermocouples connected to a digital recorder placed into the medium of each of the 6 wells of the plate atop the cryostage allowed continuous temperature recordings. Spontaneous freezing was observed after approximately 144 seconds of cooling (arrow).

B. Representative cooling curve of a 6 well plate containing 1 ml of culture medium containing 10% fetal bovine serum when freezing is induced after 60 seconds of cooling (arrow).

Figure 2A

Figure 2B

Fig. 2C

Fig. 2D

Figure 2.

A. Supercooled endothelial cells featuring Trypan Blue positive cells (arrows) at A) 3 seconds B) 30 seconds and C) 60 seconds. Frozen endothelial cells revealing Trypan Blue positive cells (arrows) at D) 3, E) 30 and F) 60 seconds. (100X)

B. Supercooled smooth muscle cells revealing Trypan Blue positive cells (arrows) at A) 3 seconds, B) 30 seconds and C) 60 seconds. Frozen smooth muscle cells revealing Trypan Blue positive cells (arrows) at D) 3, E) 30 and F) 60 seconds. (100X)

C. and D. Viability of (C) endothelial cells and (D) smooth muscle cells as assessed by Trypan Blue Exclusion after supercooling (black bar) or freezing (white bar) followed by 24 hours of re-warming. The percentage of viable cells is indicated on the y-axis and the length of supercooled or frozen time is indicated on the x-axis. Greater viability was observed after supercooling versus freezing in both cell lines. The viability of both endothelial cells and smooth muscle cells decreased with increasing frozen time. Data are represented with the means $\pm$ the standard error of mean. $^{*}(p<0.05)$

Figure 3A

Figure 3B

Fig. 3C

* $p < 0.05$
N=3

Fig. 3D

* $p < 0.05$
N=3

Figure 3.

A. Supercooled endothelial cells featuring TUNEL positive cells (arrows) at A) 3 seconds B) 30 seconds and C) 60 seconds. Frozen endothelial cells revealing TUNEL positive cells (arrows) at D) 3, E) 30 and F) 60 seconds. (100X)

B. Supercooled smooth muscle cells revealing TUNEL positive cells (arrows) at A) 3 seconds, B) 30 seconds and C) 60 seconds. Frozen smooth muscle cells revealing TUNEL positive cells (arrows) at D) 3, E) 30 and F) 60 seconds. (100X)

C and D. Apoptosis of (C) endothelial cells (ECs) and (D) smooth muscle cells (SMCs) as assessed by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay after supercooling (black bar) or freezing (white bar) followed by 24 hours of re-warming. The percentage of TUNEL positive cells is indicated on the y-axis and the length of supercooled or frozen time is indicated on the x-axis. Higher rates of apoptosis as indicated by the percentage of TUNEL positive cells were observed after freezing compared to supercooling in both cell lines. Apoptosis of both endothelial cells and smooth muscle cells increased with increasing frozen time. Data are represented as mean $\pm$ standard error of mean. *($p < 0.05$)