Effects of Procurement and Preservation Media on Cellular Apoptosis in Autologous Saphenous Vein Grafts

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KEY WORDS: Apoptosis, vein, procurement, preservation, pyruvate

ABSTRACT

Background: Procurement and temporary storage of saphenous vein grafts (SVGs) prior to grafting in coronary artery bypass surgery (CABG) may contribute to graft thrombosis and stenosis. In this study, we sought to evaluate the effects of procurement and short storage in 4 different preservation media on cellular apoptosis of SVGs procured for coronary revascularization.

Methods: Five-centimeter SVG specimens were retrieved for research purposes from 14 patients undergoing CABG. Each 5-cm specimen was then divided into five 1-cm segments giving a total of 70 segments for evaluation. For each patient, the first of the 5 segments was immediately processed and examined for apoptosis and served as control. The remaining 4 units were stored at 20°C for 1-hour in one of the following solutions: normal saline solution, Euro-Collins solution (EC), University of Wisconsin solution (UW), or a 10 mM pyruvate solution (PYR). Apoptotic cells were detected by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL assay).

Results: Across patients, the mean (SEM) number of TUNEL positive cells was similar in the control segments and those preserved with any one of the 4 solutions. However, there was a large degree of variability among the standard deviations (37-50% of mean values). When the preservation data was normalized to control for each patient and then averaged, there was a 38 ± 3.6% increase in the number of apoptotic cells in SVGs preserved with normal saline solution and EC, 25 ± 4.8% increase in SVGs preserved with UW

and only 4 ± 3.1% increase in segments preserved with PYR. When compared to the 14 segments preserved with normal saline solution for 1 hour, pyruvate preserved segments had significantly less apoptotic cells ($P<0.05$) in 10 patients (71%). EC and UW preserved segments had less apoptotic cells compared to normal saline solution in 4/13 patients (31%) and 4/14 patients (29%), respectively ($P<0.05$).

Conclusions: Procurement of SVGs is associated with a measurable degree of cellular apoptosis as seen in controls. Cellular apoptosis is amplified by short-term preservation. Pyruvate preserved SVGs had less cell damage compared to those preserved in other media. The clinical implications of these findings and their impact on the long-term SVG patency warrant further investigation.

INTRODUCTION

Coronary artery bypass grafting (CABG) with saphenous vein grafts (SVGs) has long been the standard practice for the surgical treatment of coronary artery disease.[1,2] However, the degree of SVG failure is a constant reminder that this procedure is a temporizing measure and frequently requires repeat intervention. During the first year after CABG, up to 10% of SVGs occlude, primarily due to technical issues, thrombosis and intimal hyperplasia. Between 1 and 6 years after bypass, the closure rate is 1% to 2% per year, and at 6 years it is estimated to be about 4% per year. By 10 years after surgery, only 50% of SVGs remain patent and only 20% of the patent vein grafts are free of significant stenosis.[3-5] In recent years, the use of arterial conduits has increased and that has led to improved long-term patency rates. However, the availability of arterial conduits is limited and, in view of that, continuous effort is placed on considering better methods of SVG preservation.

Failure of SVGs has been attributed to many factors including vein morphology, operative technique, ischemia-reperfusion injury, and arterialization following implantation. In recent years, ischemia-reperfusion injury has come to the forefront as the basis for vein graft failure, and as a result, a closer look at the different methods of preservation of the vein graft during the obligatory period of ischemia was undertaken in an attempt to limit the process of endothelial cell injury and to minimize the degree of graft failure.

The goals of this study are 1) to assess the degree of cellular apoptosis among endothelial and smooth muscle cells associated with manipulation and procurement of the SVG during CABG, and 2) to evaluate 4 storage media, commonly used in the clinical practice for short-term preservation of the SVG. We hypothesized that cellular apoptosis in autologous saphenous vein grafts procured for coronary revascularization occurs during the period of procurement and is amplified by short-term preservation. In addition, we hypothesized that a pyruvate-based anaerobic preservation medium may reduce the degree of cellular apoptosis compared to the aerobic media currently in use.

MATERIAL AND METHODS

Informed consents were obtained from 14 arbitrarily selected patients with surgically correctable coronary artery disease, who were scheduled for CABG at our center over a 6-month period between July and December of 2002. Permissions were obtained from each of the 14 patients to harvest an additional 5 cm specimen of the saphenous vein that was procured for use as a vascular conduit. The study was reviewed and approved by our institutional Internal Review Board (IRB). All SVGs were procured from the lower extremity of

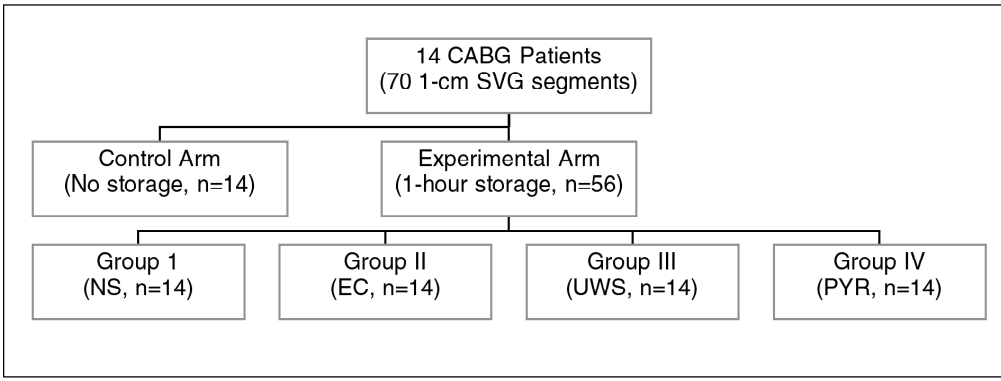

Figure 1. Study design.

each of the subjects by a single experienced surgical assistant. Great care was taken to avoid damaging the vessel during the procurement by meticulously dissecting the tissue away from the vein and by ligating the vein branches while keeping the proximal and distal ends of the vein patent to maintain blood flow through that segment until the dissection is completed and the vein segment is excised.

Five-centimeter saphenous vein specimens were taken from each of the 14 patients. Each 5-cm specimen was then divided into five 1-cm segments giving a total of 70 segments to be evaluated (Figure 1). For each patient, the first of the 5 segments was immediately processed and examined for endothelial and smooth muscle apoptosis using the TUNEL assay and served as control. This first segment that did not undergo storage was used as benchmark to which the remaining 4 segments were compared. These latter 4 segments from each of the 14 patients studied were stored for 1 hour in any one of the following four preservation solutions at 20˚C: Normal saline solution, Euro-Collins solution (EC), the University of Wisconsin solution (UW), and a 10 mM pyruvate solution (PYR, Sigma Aldrich Inc., St. Louis, Mo) that was suspended in Ringer's lactate. The segments in these four experimental groups were then assessed for endothelial and smooth muscle apoptosis using the TUNEL assay technique.[6] The assay principle is based on the cleavage of genomic DNA during apoptosis that yields double-stranded, low molecular weight DNA fragments as well as single strand breaks ("nicks") in high molecular weight DNA. Those DNA strand breaks can be identified by labeling with terminal deoxynucleotidyl transferase (TdT), which catalyzes polymerization of labeled nucleotides to free 3'-OH DNA ends in a template-independent manner (TUNEL-reaction). Incorporated fluorescein can then be detected by anti-fluorescein antibody Fab fragments from sheep, conjugated with horseradish peroxidase (POD). After L-2,4 diaminobutyric acid (DAB) substrate reaction, the stained cells are then analyzed under light microscopy.

The cut vein segment sections were embedded and baked in paraffin at 60˚C for approximately 2 hours. The paraffin was then dissolved by washing in Xylene for 10 minutes and rehydrated through a graded series of ethanol (100%, 95%, 80%, 70%, and diluted H_2O). The sections were then incubated with Proteinase K (20 µg/mL in 10 mM Tris-HCl at a pH of 7.4-8.0) for 15 minutes at 37˚C. Incubating the sections with 0.3 % hydrogen peroxide in methanol for 10 minutes followed by rinsing 3 times with

Table 1. Mean (SEM) number of apoptotic cells/100 counted cells/HPF reading, expressed as % apoptotic cells, among each group of vein graft segments analyzed by TUNEL assay.*

Group	No. of SVGs	No. of HPF Readings	% Apoptotic Cells	SD (% of Mean)
Control	14	191	4.03 ± 0.40	1.50 (10%)
Normal saline solution	14	182	5.01 ± 0.44	1.64 (33%)
EC	13	161	5.40 ± 0.75	2.69 (50%)
UW	14	195	4.40 ± 0.50	1.84 (42%)
PYR	14	189	4.00 ± 0.47	1.74 (44%)

*SVGs indicates saphenous vein grafts; HPF, high power field readings; Mean ± standard error of mean (SEM); SD (% of Mean), standard deviation, also expressed between brackets as percent of mean apoptotic rate; EC, Euro-Collins solution; UW, University of Wisconsin solution; and PYR, pyruvate solution.

diluted H_2O for 2 minutes quenched the peroxidase. The TUNEL reaction mixture (TdT mediated dUTP nick end labeling solution) was next prepared using a labeling and enzyme solutions kit (Roche Diagnostics GmbH, Roche Applied Science, Mannheim, Germany). Each section was covered with 50 µL of mixture. Sections were then incubated with the TUNEL mixture for 60 minutes at 37˚C in a humid chamber, which was again followed by rinsing 3 times with diluted H_2O (2 minutes each). The sections were incubated with Converter-POD (anti-fluorescein antibody, fab fragment from sheep, conjugated with horseradish peroxidase) for 30 minutes at 37˚C in a humid chamber and rinsed again 3 times with diluted H_2O. The L-2,4 diaminobutyric acid (DAB) substrate for the antibody reaction development was prepared and incubated with the sections for 30 to 60 seconds at room temperature. The sections were finally counterstained with Mayer's hematoxylin for 30 to 60 seconds and rinsed 3 times again with diluted H_2O. The slides were then covered with glasscover slips and examined.

The microscopic slides were evaluated by light microscopy. Apoptosis was scored by an experienced investigator who was blinded to the study groups and preservation media used. Each segment had an average of 3 to 5 slides available for evaluation; 3 to 5 high power fields (HPFs) from each of these slides were randomly chosen and the number of apoptotic cells per 100 counted cells in each field was labeled as a "reading" and was expressed as a percent figure (% apoptosis). An average of the "readings" was then used as the mean percent of apoptotic cells for that particular vein segment. One 1-cm segment of SVG that was preserved with EC was damaged during processing and was, therefore, excluded from the analysis. The number of readings for each of the control, normal saline solution, EC, UW, and PYR preserved segments ranged from 161 to 195 (Table 1). Apoptosis was counted among both endothelial and smooth muscle cells together as one reading and no differentiation was made between the two cell types.

Statistical Analysis

The Student *t*-test was used to compare the percent of apoptotic cells between each of the preserved segments and controls, and also between the different preservation media. Values were expressed as mean ± standard error of mean (SEM), unless otherwise indicat-

Table 2. Apoptotic rates in SVGs preserved in any one of the four preservation media compared to controls among the 14 patients evaluated.*

Pt	C	NSS	*P value*[1]	EC*	*P value*[2]	UW	*P value*[3]	PYR	*P value*[4]
1	**4.39**	**3.39**	*NS*	**2.87**	*NS*	**3.08**	*NS*	**3.78**	*NS*
2	**1.49**	**2.96**	*NS*	**3.35**	*0.00*	**2.89**	*0.04*	**3.40**	*0.04*
3	**3.85**	**3.68**	*NS*	**2.89**	*NS*	**2.83**	*NS*	**1.53**	*0.00*
4	**1.62**	**3.68**	*0.02*	**2.43**	*NS*	**4.78**	*0.02*	**1.16**	*NS*
5	**3.70**	**4.69**	*NS*	**-**	**-**	**3.31**	*NS*	**2.48**	*NS*
6	**5.89**	**5.38**	*NS*	**9.99**	*0.03*	**4.99**	*NS*	**6.87**	*NS*
7	**3.86**	**5.42**	*0.00*	**5.07**	*0.02*	**3.61**	*NS*	**3.36**	*NS*
8	**4.38**	**4.97**	*0.01*	**7.52**	*0.00*	**2.24**	*0.02*	**3.81**	*0.00*
9	**5.55**	**8.05**	*0.04*	**8.62**	*0.02*	**7.47**	*NS*	**5.15**	*NS*
10	**2.41**	**4.20**	*0.03*	**3.96**	*NS*	**2.88**	*NS*	**2.62**	*NS*
11	**6.87**	**3.58**	*0.00*	**9.36**	*NS*	**3.37**	*0.00*	**6.21**	*NS*
12	**4.51**	**7.30**	*0.00*	**2.73**	*0.02*	**6.87**	*0.00*	**5.42**	*NS*
13	**3.90**	**5.22**	*NS*	**6.01**	*0.02*	**5.66**	*NS*	**5.87**	*0.03*
14	**3.94**	**7.64**	*0.00*	**5.42**	*NS*	**7.60**	*0.00*	**4.41**	*NS*

*Pt indicates patient; C, Control; NSS, Normal Saline Solution, EC, Euro-Collins; UW, University of Wisconsin; PYR, pyruvate; NS, not statistically significant. *P* values[1-4] refer to each specific solution vs. control

ed, and $P < 0.05$ indicating statistical significance. To eliminate any bias caused by the variability in the standard deviation (SD) of the expressed mean rates of apoptosis, expressed as percent apoptotic cells for each of the preservation media, the rates were normalized to the control in each patient, then averaged for the 14 patients and compared.

RESULTS

Table 2 shows the apoptotic rates in SVGs preserved in any one of the 4 preservation media compared to controls among the 14 patients evaluated. The mean (SEM) number of TUNEL positive cells/100 cells expressed as percent apoptosis in each of the procured non-stored control segments was 4.03 ± 0.40% (Table 1). Non-normalized apoptotic rates of PYR preserved segments were closer to controls (4.00 ± 0.47% versus 4.03 ± 0.40% among controls). When the non-normalized mean rate of apoptosis among the 56 preserved segments (less one excluded EC-preserved segment that was damaged) were compared, there was no statistically signifi-

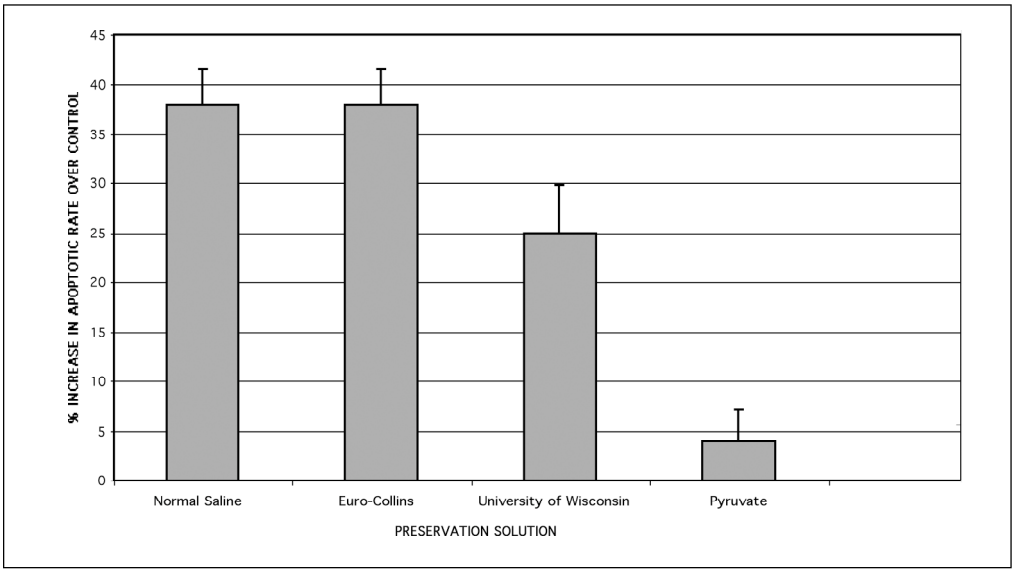

Figure 2. Percent increase in the rate of apoptosis compared to controls, expressed as mean percent increase over control ± SEM. The preservation data was normalized to controls for each patient, then averaged for the 14 patients, whose saphenous vein grafts were preserved in normal saline solution, University of Wisconsin solution, or pyruvate solution, and for the 13 patients in the Euro-Collins group.

cant difference in the mean apoptotic rate between the four preservation groups and the 14 control segments. However, there was a significant degree of variability between the patients in the standard deviation of the mean number of apoptotic cells per 100 cells counted (% apoptosis). As shown in Table 1, the standard deviation varied from 10% of the mean value for controls to 33% and 50% of the mean values for normal saline solution and EC, and to 42% and 44% of the mean for UW and PYR, respectively. Figure 2 shows the percent increase in the number of apoptotic cells after normalization of the values among each of the four preservation media compared to controls. There was a 38 ± 3.6% increase in the number of apoptotic cells in SVGs preserved with normal saline solution and EC compared to controls (P<0.05). Likewise, there was a 25 ± 4.8% increase in apoptosis in UW preserved segments compared to controls (P<0.05). However, there was only 4 ± 3.1% increase in the rate of apoptosis among PYR preserved segments as compared to controls (not statistically significant).

For the groups preserved by normal saline solution, EC, UW and PYR, the number of patients in whom the preserved segments had similar rates of apoptosis compared to controls (P>0.05) was identified. PYR preserved segments had similar rates of apoptosis to controls in 10/14 patients (71%), whereas UW preserved segments had similar rate of apoptosis to controls in 8/14 patients (57%) compared to controls. The remaining two groups, namely EC and normal saline solution, had similar rates of apoptosis as controls in 6/13 patients (46%) and in 6/14 (43%), respectively (Figure 3).

Figure 4 shows the number of patients in whom SVGs preserved with PYR, EC or UW had significantly lower apoptotic rates than those preserved with normal saline solution. Ten of the 14 patients (71%) whose SVGs were preserved in PYR had significantly less apoptotic rates than those stored with normal saline solution (P<0.05), whereas

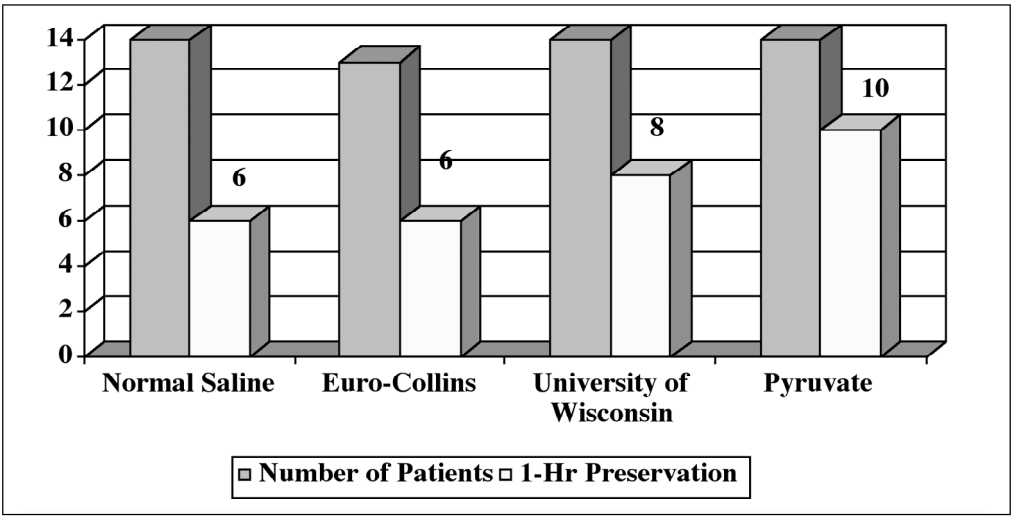

Figure 3. Number of patients (Y-axis) who had similar rates of apoptosis as controls in each of the four storage groups (normal saline solution, Euro-Collins solution, University of Wisconsin solution, and pyruvate solution).

only 4/13 patients (31%) whose SVGs were preserved with EC and 4/14 patients (29%) whose SVGs were preserved with UW had less apoptosis compared to normal saline solution ($P<0.05$).

DISCUSSION

The transient ischemia that veins undergo by necessity during coronary artery bypass, followed by reperfusion, has been shown indirectly to be one mechanism by which intimal hyperplasia occurs.[7] The process of vessel damage via intimal denudation, platelet adherence, inflammatory cell activation, and the multiple chemotactic factors that increase the rate and extent of intimal hyperplasia have been previously described by our group.[8] The ischemia-reperfusion injury with subsequent oxygen free radical formation has been found to cause chemical changes within the endothelial and smooth muscle cells that promote chemotaxis, proliferation, and deposition of extracellular matrix by activated smooth muscle cells, thus leading to a progressive increase in intimal fibrosis and a reduction in cellularity.[9] In addition, superoxide radical formation has been found to directly promote smooth muscle proliferation.[10] The result is a luminal surface that is prone to extensive hyperplastic response and an increased incidence of vein graft failure.

When ischemic tissue is reperfused with oxygenated blood, neutrophils accumulate and rapidly adhere to the hypoxically injured endothelium.[11] Leukocyte-mediated injury follows as a result of the release of a variety of substances such as oxygen-derived free radicals, thromboxanes, leukotrienes, and proteases.[12] Therefore, endothelial ischemia leads to endothelial damage, and thus, activation of the coagulation cascade that may lead to acute thrombosis of the saphenous vein graft.[7,12] Nearly all veins implanted into the arterial circulation develop some intimal wall thickening within 4 to 6 weeks from the time of implantation, thereby reducing the vessel lumen by up to 25%. However, this is not usually flow limiting. It has been strongly suggested that the hyperplasic zones associated with the extensive endothelial denudation and destruction may become atheroscle-

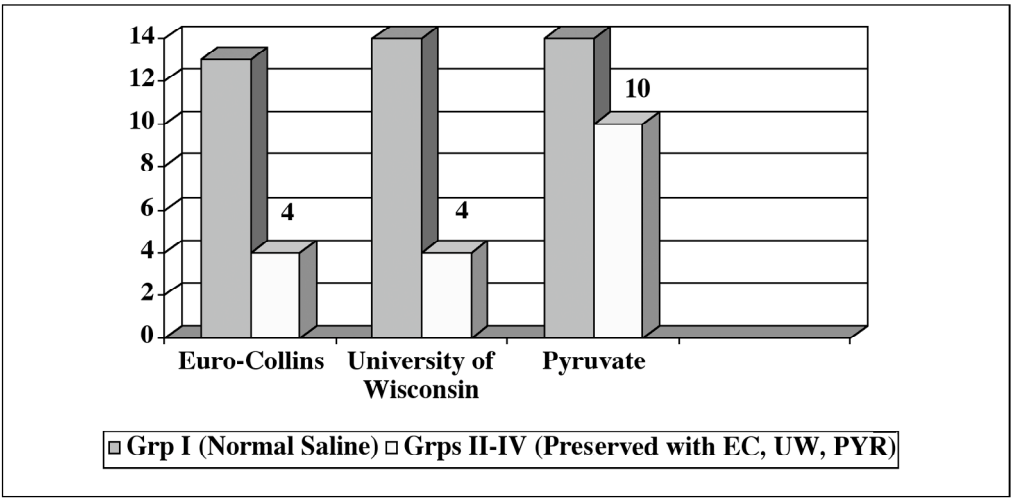

Figure 4. Number of patients (Y-axis) who had significantly less apoptosis ($P<0.05$) in the Euro-Collins solution (EC), University of Wisconsin solution (UW), and pyruvate solution (PYR) groups compared to normal saline solution.

rosis-prone regions and may lead to subsequent re-stenosis.[8,13,14]

Normal saline solution and other crystalloid solutions have repeatedly been proven to cause significant irreparable damage to the endothelium of the SVG.[14] To date, no solution stands most prominent in the prevention of endothelial damage. Studies using the UW solution for preservation have shown contrasting results of benefit and impairment.[15-17] Cavallari and associates found that veins stored in UW had more preserved smooth muscle cell function compared to those stored in normal saline solution or autologous whole blood.[15] In contrast, investigations performed by Anostasiou et al showed attenuation of endothelial dependent relaxations to acetylcholine and enhancement of the response to 5-HT.[16] Mankad et al, however, revealed a temperature dependent endothelial dysfunction in the isolated rat heart. He hypothesized that the effect might have been partly in response to the high potassium concentration.[18]

Pyruvate solutions have been found to protect against ischemic insult through the degeneration of oxygen free radicals that are produced by the degradation of the ischemic cell and are theorized to be an alternative source of energy during nutrient loss.[19-25] Ramakrishnan et al found that pyruvate prevented hydrogen peroxide-induced apoptosis in mouse thymocytes.[19] Pyruvate had also been demonstrated to have metabolic inotropic properties that increase cardiac energy reserves and bolster the endogenous glutathione antioxidant system.[20] Another study suggested that pyruvate neutralizes peroxides directly by decarboxylation-reaction and, indirectly, by augmentation of the glutathione antioxidant system through the generation of NADPH reduction power.[21] Yet, still another study by Lee et al showed that pyruvate had remarkable protective effects against zinc neurotoxicity through free radical degradation in laboratory rats.[22] Lee also demonstrated that the anti-apoptotic effect of pyruvate could be a result of modulation of key regulatory signal pathways in the cytosol and mitochondrial matrix as shown in Figure 5, thereby inactivating the endothelial cell death pathways signaling.[26]

Appropriate preservation solutions

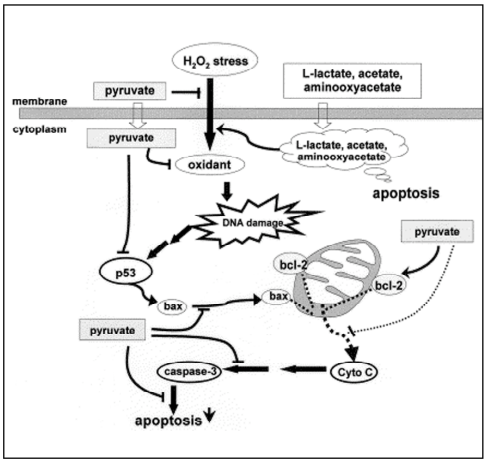

Figure 5. Schematic diagram showing the involvement of p53 and its apoptotic downstream bcl-2 and bax in the oxidant-induced endothelial apoptosis and the effect of pyruvate on modulation of these genes. As depicted, H_2O_2-derived oxidants induce expression of p53, and its downstream bax in which expression ratio of bcl-2 and bax is decreased. Pyruvate inhibits this direct apoptotic signaling and prevents against apoptosis. (Lee et al, *Microvasc Res.* 2003;66:91,[26] reprinted with permission from Elsevier Publishers).

can help mitigate the extent of the oxygen free radical damage and thereby decrease the resulting hyperplastic response. Non-published pilot studies from our laboratory have found a significant reduction in the upregulation of ICAM 1, an adhesion molecule that is upregulated during inflammatory states, in order to promote lymphocyte adherence, on the endothelial cell of vein segments after incubation in a pyruvate solution and subsequent stimulation by TNF-□.

In this study, the rate of apoptosis was more than controls in 50 preserved SVG segments (89%) and less than controls in 5 (11%). It is difficult to explain why the rate of apoptosis in these 5 segments preserved for one hour was less than the controls. It is hard to believe that the preservation media might have revived some of the dying endothelial and smooth muscle cells before they became apoptotic. Possible explanations for the lower rate of apoptosis seen in 11% of the preserved SVGs may relate to the learning curve associated with the quantitation of the number of apoptotic cells per 100 cells in a given HPF, particularly that the investigator who interpreted these slides was blinded to the SVG groups. Besides, the 5 cm long SVG specimen from each of the 14 patients may not be homogeneous throughout its course and several factors such as stretch injury, variations in intimal and smooth muscle layer thickness, segmental vein sclerosis and the number of ligated tributaries might have affected the apoptotic rate within any or all of the five 1-cm segments in a given SVG specimen.

One limitation of this study is that it did not account for the damage to the endothelial and smooth muscle cells created by the 1-hour storage at 20˚C compared to storage at 37˚C. The reason why the SVG segments that were placed in preservation media were stored at 20˚C is to simulate the operating room temperature at which these veins are usually kept (68˚F or 20˚C). It is conceivable that the observed apoptosis could have been due, at least in part, to the operating room temperature storage (20˚C) in comparison to body temperature (37˚C). However, cold storage alone cannot account for the variations seen in the degree of apoptosis seen within each of the preserved SVG segments. A second limitation of this study is that the degree of apoptosis related to pyruvate preservation might be dose dependent and might need to be assessed at different pyruvate concentrations. The concentration of pyruvate used in this study (10 mM) was chosen because similar concentrations have been used in previous studies that demonstrated its anti-apoptotic effects.[26] It is conceivable that the degree of apoptosis seen with 10 mM pyruvate may be more amplified with higher concentrations.

There is, admittedly, a large gap between the demonstrated reduction in the rate of apoptosis with the use of pyruvate or other preservation media and demonstrating that this reduction will lead to a decrease in intimal hyperplasia formation and higher graft patency rates. However, the possibility of a link cannot be denied. The mechanisms that cause the hyperplastic response of the intima are multi-factorial and, therefore, much work is still required. There are still questions that need to be answered. One such question is how exactly does pyruvate execute its properties of free radical reduction. Also, studies need to be performed to show that there is a significant relationship between pyruvate's reduction of free radical formation, and its effects on intimal hyperplasia. Pyruvate needs to be seriously investigated as a preservation medium for vein grafts.

CONCLUSIONS

The present study demonstrates that manipulation and procurement of SVGs is associated with a measurable degree of cellular apoptosis as seen in controls. Although the mean rate of apoptosis was similar among the 4 preservation media, the large degree of variability among the standard deviations seems to have masked the salutary effect of pyruvate on endothelial and smooth muscle cell preservation and on the rate of apoptosis. Cellular apoptosis is amplified by short-term preservation (1-hour) regardless of the type of preservation medium used. Apoptosis among pyruvate preserved grafts was less than that among the other tested preservation media, and in 71% of the patients the apoptotic rates of pyruvate preserved segments were similar to control.

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