BASIC RESEARCH STUDIES

From the New England Society for Vascular Surgery

Inhibition of vascular smooth muscle cell proliferation with red wine and red wine polyphenols

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Objective: **The potential beneficial effects of red wine consumption on the development of atherosclerotic disease have been previously suggested in the literature. Vascular smooth muscle cell (SMC) proliferation is an important component of atherogenesis. Inhibition of vascular SMC proliferation may have a beneficial effect in retarding the development of atherosclerotic disease. The goal of this study was to determine the effect of red wine, red wine polyphenol extract, and resveratrol, a polyphenol commonly found in red wine, on the proliferation of vascular SMC in culture.**
Methods: **Bovine aortic SMCs were used for all experiments. SMCs were treated with growth media supplemented with dealcoholized red wine, red wine polyphenol extract, or resveratrol at various concentrations for as long as 48 hours. SMC proliferation was assessed with ^{3}H-thymidine DNA incorporation assay. SMC viability was assessed with trypan blue exclusion studies and a colorimetric lactic dehydrogenase cytotoxicity assay.**
Results: **Our results show that red wine and red wine polyphenol extract inhibit SMC proliferation in a dose-dependent fashion. Resveratrol also inhibits vascular SMC proliferation. SMC viability studies show that this inhibition of SMC proliferation is not the result of a cytotoxic effect.**
Conclusion: **Our findings show that red wine and red wine polyphenols have an inhibitory effect on the proliferation of vascular SMCs in culture. These results suggest that the observed beneficial effects of red wine may be the result, in part, of the inhibition of vascular SMC proliferation. Furthermore, the antiproliferative properties of red wine may be caused by its component polyphenols. (J Vasc Surg 2002;35:1226-32.)**

The beneficial effects of red wine consumption on the development of atherosclerotic disease have been suggested for several years. The predominant basis for this belief is epidemiologic and has been commonly referred to as "the French Paradox." Simply stated, the typical French diet is two to three times higher in saturated fats than the usual American diet, yet the rates of cardiovascular disease in France are one third those of the United States. One hypothesis for the difference in cardiovascular disease prevalence between the two countries is based on differences in per capita red wine consumption.[1] The typical Frenchman drinks three times as much red wine as his American counterpart. The hypothesis proposes that the decreased prevalence of cardiovascular disease in France is the result of the beneficial effects of substances contained in red wine. Not surprisingly, this has created an impetus for researchers to study red wine and determine what effect, if any, it has on the development of atherosclerosis.

Atherosclerosis is a multifactorial disease process, characterized by plaque formation and progressive narrowing of the lumen diameter. The proliferation of vascular smooth muscle cells (SMCs) in the arterial intima is a key step in the formation of atherosclerotic plaques.[2,3] The purpose of this study was to elucidate some of the potential antiatherosclerotic effects of red wine. Our goal was to compare the biologic effects of red wine and resveratrol on cellular processes. Specifically, we sought to determine whether red wine, red wine polyphenol extract, or resveratrol inhibited the proliferation of vascular SMCs.

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MATERIALS AND METHODS

Materials

Tissue culture plasticware was obtained from Becton Dickinson (Franklin Lakes, NJ). Growth media (Dulbecco's modified Eagle's medium/F12) were purchased from Gibco (Carlsbad, Calif), resveratrol was obtained from Sigma (99% purity; R5010, St Louis, Mo), and tritiated (methyl ^{3}H) thymidine (2 Ci/mmol) was obtained from Amersham (Piscataway, NJ). Primary cultures of SMCs were derived from newborn calf thoracic aorta, as described previously.[4] Briefly, adventitia and intima were removed from the newly harvested aorta, and finely cut strips of media were placed in a tissue culture flask to allow for attachment. After 4 hours, growth media (Dulbecco's modified Eagle's medium/F12) containing 20% newborn calf serum from Gemini (Calabasas, Calif) were added and were changed every 3 days. After several days in culture, SMC outgrowths from the primary tissue explants became apparent. On reaching confluence, the tissue explants were discarded, and the SMCs were passaged. Passage of SMCs was accomplished with trypsinization of primary cultures and dispersion into tissue culture flasks every 6 to 9 days, followed by the addition of 10% media. All experiments were conducted on cells between passages 2 and 6.

Preparation of dealcoholized red wine. A 2000 California Petite Sirah (Bogle Winery, Clarksburg, Calif) was chosen because of its high polyphenol content (2.5 g/L).[5,6] A 20-mL sample of wine was used to prepare the stock solutions. Ethanol was removed from the sample with rotary evaporation to 75% of the original volume with reduced pressure at 35° C. A final volume of 20 mL was achieved with the addition of ultrapure water. A 3-mL aliquot of dealcoholized wine was set aside for polyphenol extraction.

Preparation of red wine polyphenol extract. A 3-mL aliquot of dealcoholized red wine was passed through a C18-SPE cartridge (1 g Supelco, Sigma) placed in a vacuum elution apparatus that had been preconditioned with a 1:1 methanol-acetone mix and rinsed with water. The eluant was collected in a glass vial, 6 mL of water was passed through after the sample, and the eluant was rotary evaporated to a final volume of 3 mL. This water fraction contained polar organic acids, alcohols, and residual sugars.

To elute the phenolic compounds from the C18 column, an admixture of approximately 8 mL of 1:1 methanol-acetone was passed through the column. After half the solvent had passed through the C18 material, 100 μL of 2.8 N HCl was added to the remaining solvent toward the end of the flush. The eluant was rotary evaporated to a final volume of less than 2 mL and diluted with a minimal amount of water to the final volume of 2 mL.[5,6]

Methods

^{3}H-thymidine incorporation assay. SMCs were plated into 24-well culture plates and were induced to grow with the addition of 10% media. After 24 hours, the media were changed (control) or replaced with 10% media supplemented with different concentrations of dealcoholized red wine, polyphenol extract, or resveratrol. After 6 hours, 2 μCi of ^{3}H-thymidine was added to each well. The reaction was terminated at 12 hours with washing with phosphate-buffered saline solution and then incubating in 10% trichloroacetic acid for 30 minutes at 4° C. SMCs then were washed with 95% ethanol. Four hundred μL of 0.2 N NaOH was added to each sample and incubated for 1 hour at room temperature. Three hundred μL from each sample then was transferred to a scintillation vial. Rate of DNA synthesis was determined with measurement of ^{3}H-thymidine incorporation with a scintillation counter. Experiments were repeated at least in triplicate.

Morphologic analysis. SMCs were plated into 6-well plates and induced to grow with the addition of 10% media. After 24 hours, the media were changed (control) or replaced with 10% media supplemented with dealcoholized red wine, polyphenol extract, or resveratrol. At 48 hours, SMCs were photographed with low-power magnification (4×) with gross examination of cell morphology and proliferation. To confirm cell viability, SMCs were exposed to dealcoholized red wine, polyphenol extract, and resveratrol for 12 hours and then stained with 0.1% trypan blue. Cells were photographed with high-power magnification (400×). Viable cells with an intact membrane excluded trypan blue and were not stained.

Cytotoxicity assay. SMCs were plated in 6-well plates and induced to grow with the addition of 10% media. After 24 hours, the media were changed (control) or replaced with 10% media supplemented with dealcoholized red wine, polyphenol extract, or resveratrol. At 12 hours, 50 μL of media was removed from each sample population and plated in a 96-well plate. A nonradioactive colorimetric cytotoxicity assay that measures serum lactic dehydrogenase released from damaged cells was used to assess the cytotoxicity of the different substances (Promega, Madison, Wis). A 96-well plate reader was used to record the absorbance at 490 nm, which is related to the amount of LDH in the serum of the treated samples.

Statistical analysis. The results are presented as the mean ± SEM. Two-tailed analysis of variance was used to analyze the difference between control and treated groups. A P value of less than .05 was considered to be statistically significant.

RESULTS

^{3}H-thymidine incorporation assay. Our results show that red wine, red wine polyphenol extract, and resveratrol inhibit SMC DNA synthesis, as measured with ^{3}H-thymidine incorporation assay, in a dose-dependent manner. For SMCs treated with red wine, our studies showed significant inhibition of ^{3}H-thymidine incorporation into cellular DNA. Red wine, at concentrations of 0.1% and 1%, caused a 42.5% and 64.6% decrease in ^{3}H-thymidine incorporation, respectively ($P < .05$, N = 3; Fig 1). Polyphenol extract, at concentrations of 0.1% and 1%, resulted in a 57.6% and 71.4% decrease in ^{3}H-thymidine incorporation, respectively ($P < .05$, N = 3; Fig 2). Resveratrol, at

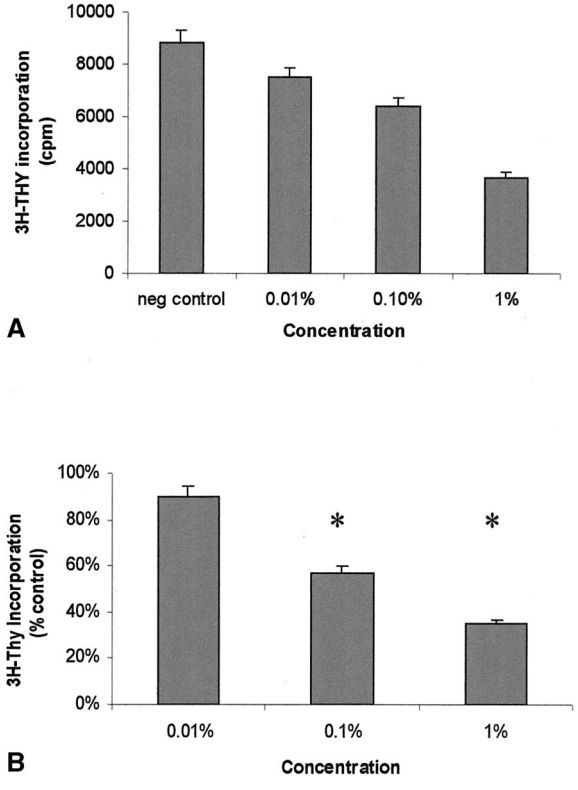

Fig 1. Twelve-hour ^{3}H-thymidine DNA synthesis assay for red wine. *x axis* represents bovine aortic SMC samples treated with different concentrations of dealcoholized red wine, shown as volume percentage. Negative control is 10% fetal bovine serum growth media. *y axis* represents ^{3}H-thymidine incorporation measured as counts per minute *(cpm)*. **A,** Results for typical experiment. **B,** Graph shows mean results for three experiments, normalized as percentage of control. *$P < .05$ compared with negative control.

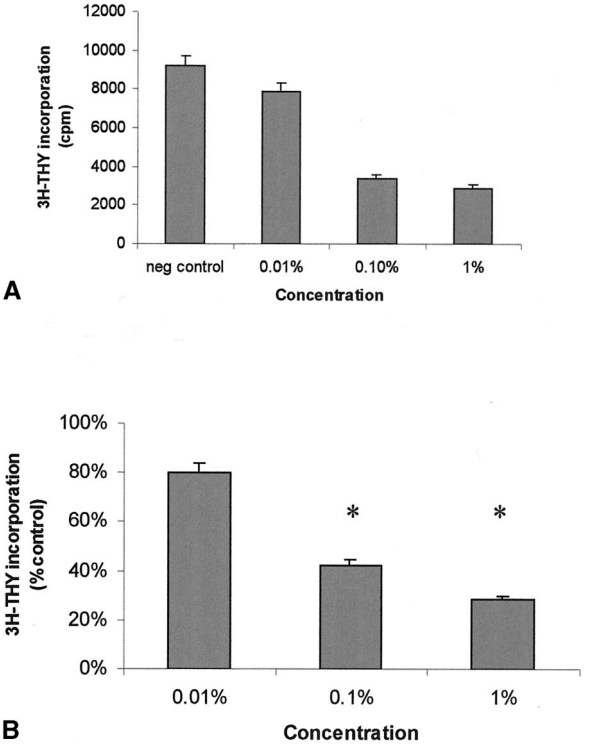

Fig 2. Twelve-hour ^{3}H-thymidine DNA synthesis assay for red wine polyphenol extract. *x axis* represents bovine aortic SMC samples treated with different concentrations of red wine polyphenol extract, shown as volume percentage. Negative control is 10% fetal bovine serum growth media. *y axis* represents ^{3}H-thymidine incorporation measured as counts per minute *(cpm)*. **A,** Results for typical experiment. **B,** Graph shows mean results for three experiments, normalized as percentage of control. *$P < .05$ compared with negative control.

concentrations of 10 μm and 100 μm, caused a 43.9% and 96.2% decrease in ^{3}H-thymidine incorporation, respectively ($P < .05$, N = 3; Fig 3).

Morphologic analysis. No gross morphologic changes were observed in SMCs exposed to red wine, polyphenol extract, or resveratrol for as long as 48 hours. However, a dose-dependent decrease was seen in the total number of SMCs in samples treated with all three substances (Fig 4). Trypan blue staining confirmed that SMCs exposed to red wine, polyphenol extract, and resveratrol excluded the trypan blue staining similar to control samples, showing their viability (Fig 5).

Cytotoxicity analysis. Analysis of serum LDH levels in treated SMC samples with a colorimetric assay showed no significant increase in the culture media of SMCs exposed to red wine, red wine polyphenol extract, or resveratrol, as compared with control samples. This is consistent with the concept that the observed antiproliferative effect of these substances is not likely the result of a cytotoxic effect.

DISCUSSION

Wine contains many polyphenolic antioxidant substances. The origin of most of these substances is the grape berry. Polyphenolic compounds are found in many foods of plant origin, such as fruit, tea, coffee, and chocolate. The level of polyphenols in a particular bottle of wine depends on two major factors. The most important consideration is that red wine is made by fermenting the grape juice in the presence of grape solids (seeds and skin) and white wine is made by pressing the juice away from the grape solids and then allowing it to ferment. The grape skin and seeds contain most of the polyphenols, and the grape flesh contains a relatively small percentage of the total polyphenols. A typical bottle of red wine contains 1.8 g/L of total polyphenols, whereas a typical bottle of white wine contains 200 to 300 mg/L total polyphenols. The second major determinant of wine polyphenol content is the winemakers themselves, who control the processing of the grapes to achieve a specific flavor.

The proliferation of vascular SMCs is a key step in the development of atherosclerosis.[2,3] It follows that a sub-

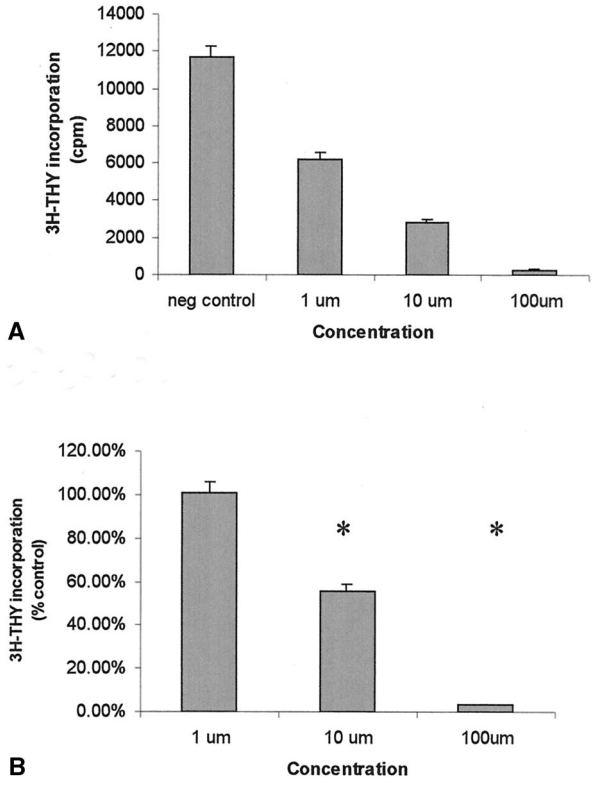

Fig 3. Twelve-hour ^{3}H-thymidine DNA synthesis assay for resveratrol. *x axis* represents bovine aortic SMC samples treated with different concentrations of resveratrol, in micromolar. Negative control is 10% fetal bovine serum growth media. *y axis* represents ^{3}H-thymidine incorporation measured as counts per minute *(cpm)*. **A,** Results for typical experiment. **B,** Graph shows mean results for three experiments, normalized as percentage of control. $*P < .05$ compared with negative control.

stance that inhibits the proliferation of SMCs may have a potential beneficial effect on the development of atherosclerotic disease. The goal of our study was to determine whether red wine, red wine polyphenol extract, and resveratrol inhibit SMC proliferation. Our results showed that resveratrol, red wine, and red wine polyphenol extract all inhibit SMC proliferation, as determined with ^{3}H-thymidine DNA synthesis assay. Our findings suggest that the beneficial health effects of red wine consumption may be the result, in part, of the inhibition of vascular SMC proliferation by red wine polyphenols.

Resveratrol is a nonflavonoid polyphenol found only in the grape skin, so it is present much more in red wine than in white. A typical resveratrol level in a bottle of red wine is approximately 7 mg/L, and white wine contains only 0.5 mg/L resveratrol.[6,7] The *trans* isomer of resveratrol is the biologically active form, and *cis* resveratrol is absent in grapes.[8] The *trans-cis* isomerization of resveratrol in wine is caused by natural light exposure.[9]

One recent study used a rabbit endothelial denudation model to investigate the effects of resveratrol consumption on vascular disease.[10] These investigators were able to show that rabbits fed a high resveratrol diet (4 mg/kg/d) had less intimal hyperplasia develop than did controls. Furthermore, they were also able to show that the number of SMCs in the thickened intima was significantly reduced in rabbits that were fed a high resveratrol diet. These results correlate with our findings with an in vitro model and suggest a potential antiatherosclerotic benefit of resveratrol.

In consideration of the potential implications of our data, some important points need to be made. Foremost among them is that our study investigates the in vitro effect of red wine and resveratrol applied directly to vascular SMCs. This methodology, although similar to that used by other investigations of the biologic effects of red wine polyphenols, does not address the potential effects of hepatic metabolism of resveratrol. In the liver, resveratrol is predominantly metabolized to its diglucuronide conjugate before it enters the systemic circulation. Whether resveratrol glucuronidation reduces its bioavailability is not fully known. In a recent study, the presence of other polyphenols commonly found in red wine was shown to decrease resveratrol glucuronidation.[11] This is believed to increase the bioavailability of free resveratrol. In this manner, the total amount of polyphenols in a bottle of red wine may be as important to its potential health effects as is the resveratrol content. In our investigations, the concentration of resveratrol we used was correlated with the amount of total polyphenols in the dealcoholized red wine solution. For example, the 0.1% wine solution contained approximately the same amount of total polyphenols as the 10-μm resveratrol solution. Diglucuronide conjugates of red wine polyphenols are, unfortunately, not available to be isolated with significant purity for study in cell culture.

Any observed antiproliferative effect in a regular red wine drinker would likely be attributable to long-term exposure (years) to these substances. Also, the pattern of exposure is different, one of repeated brief exposures to the circulating polyphenols rather than a single sustained exposure lasting several hours. Our in vitro study used a short time course. Nonetheless, it provides important observations with regard to the effects of red wine and red wine polyphenols on vascular physiology.

Furthermore, in animal studies with resveratrol, decreased intimal hyperplasia was observed in rabbits fed a high resveratrol diet (4 mg/kg/d) for 5 weeks.[10] For the 75-kg man, that would be the equivalent of 300 mg/d resveratrol. Given that the average concentration of resveratrol in red wine is 7 mg/L and a single glass contains approximately 1 mg resveratrol, the doses used in the animal studies are extremely high. Although long-term exposure to low-dose resveratrol is postulated to produce a beneficial antiatherosclerotic effect, these studies have not been conducted.

Previous in vitro studies involving resveratrol have investigated its potential for use as an anticancer agent. Multiple studies have shown resveratrol to have an antiproliferative effect on several different cancer cell lines, including human breast cancer, oral squamous cell carcinoma,

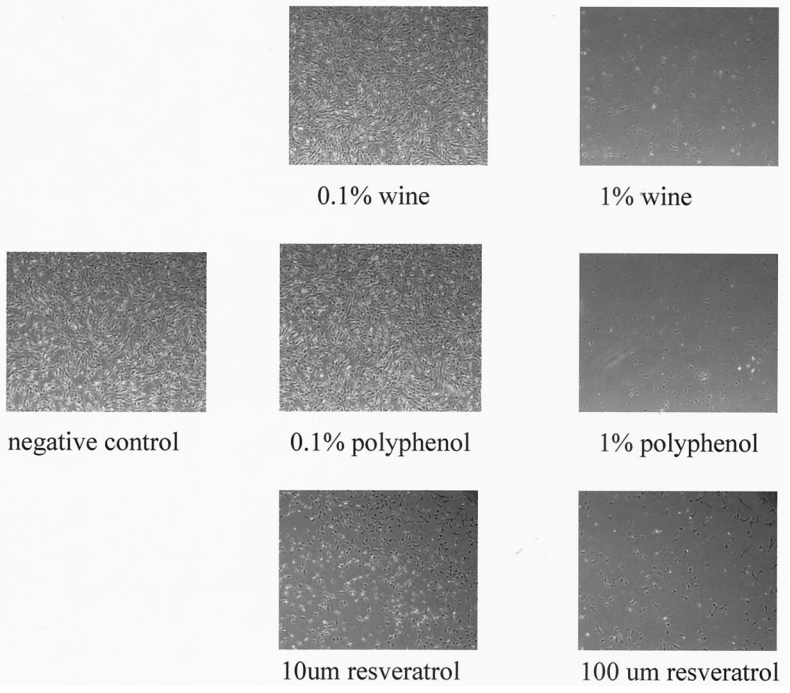

Fig 4. Gross morphologic examination of SMCs treated with dealcoholized red wine, red wine polyphenol extract, or resveratrol for 48 hours. Examination with low-power magnification (4×) shows no gross morphologic changes in treated SMC samples. However, dose-dependent decrease in cell number at higher concentrations is seen.

and different human leukemias.[12-14] The inhibitory effect of resveratrol in the previous cell culture studies was observed at concentrations similar to those used in our investigations. Furthermore, a recently published study looking solely at resveratrol showed an inhibitory effect on vascular SMC proliferation.[15] In our study, dealcoholized red wine and red wine polyphenol extract were shown to have a similar effect. Red wine contains other polyphenols in addition to resveratrol, and our findings suggest that they may also have an antiproliferative effect.

More recent studies involving resveratrol have sought to understand the basis of its antiproliferative effects. A number of reports suggest that resveratrol arrests cell cycle progression at the S (DNA synthesis) phase of mitosis.[16,17] These findings have been correlated with other studies that have shown that resveratrol, in the presence of copper ions, is capable of causing DNA strand breakage.[18] Resveratrol bound to cellular DNA has been postulated to form a complex with Cu(II), reducing it to Cu(I). This causes redox cycling of copper, which leads to the generation of various reactive oxygen species, which serve as DNA cleaving agents.[19] On the basis of the previous mechanism, wine drinkers may theoretically more fully enjoy the beneficial effects of resveratrol than those who get their resveratrol from other food sources because red wine is an excellent source of dietary copper. Preliminary evidence in our laboratory with cell cycle analysis indicates that a similar arrest in S/G2 phase transition may also occur in SMCs exposed to resveratrol. In addition to the cell cycle arrest mechanism, researchers have proposed that resveratrol may inhibit cell proliferation by causing apoptosis. Recent investigations have shown that resveratrol exposure causes increased apoptotic cell death and a decreased expression of the anti-apoptotic protein Bcl-2 in human leukemia cells.[20] Diminished expression of Bcl-2 has been observed in certain cell lines undergoing apoptosis. Thus, resveratrol appears to inhibit cell proliferation by more than one pathway.

Although our research has focused on smooth muscle proliferation, red wine polyphenols are postulated to limit the development of atherosclerosis by other mechanisms as well. One study published in *Nature* (during the review of this manuscript) has shown that endothelin-1 synthesis with cultured bovine aortic endothelial cells could be inhibited by red wine polyphenol extract. With comparison of polyphenol extracts from several different red wines, white wine, and grape juice, inhibition of endothelin-1 synthesis was directly related to the polyphenol content of the tested wines. Furthermore, an MTT assay was performed, and as in our study, no cytotoxic effect was observed in cell samples exposed to red wine polyphenols.[21]

Another potential atheroprotective effect of red wine polyphenols is by inhibition of LDL oxidation. With an in vitro model, investigators were able to show that macrophages treated with red wine showed a significant inhibition of LDL oxidation.[22] Evidence also shows that dealcoholized red wine and red wine polyphenols inhibit platelet aggregation and thromboxane synthesis.[23,24] These findings suggest that red wine may have multiple beneficial

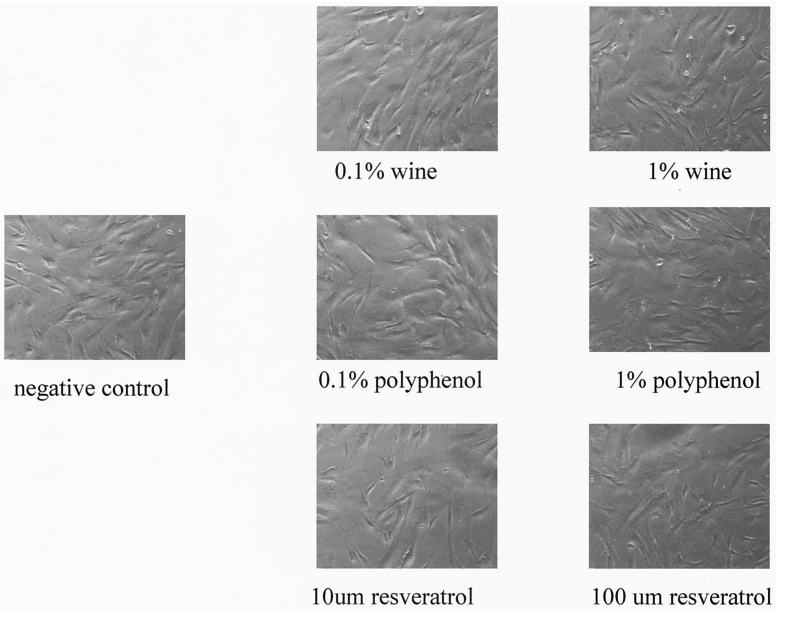

Fig 5. Trypan blue exclusion staining of SMCs treated with red wine, red wine polyphenol extract, and resveratrol for 12 hours. Trypan blue staining was performed to assess cytotoxicity of dealcoholized red wine, polyphenol extract, and resveratrol. Cytotoxic substances cause cell membrane damage, which allows cells to absorb trypan blue stain and thus appear blue. Examination with high-power magnification (400×) shows occasional blue-stained cells in all samples with no significant difference between treated and control groups.

effects in limiting the development of atherosclerotic disease.

In summary, ours is the first study to document that red wine, red wine polyphenol extract, and resveratrol have a dose-dependent inhibitory effect on the proliferation of vascular SMCs in culture. Because SMC proliferation is an important step in the development of atherosclerotic disease, our results suggest that the beneficial health effects of red wine may be the result, in part, of the inhibition of vascular SMC proliferation by its component polyphenols. The focus of future studies should be to further elucidate the atheroprotective properties of red wine polyphenols and to compare the effects of individual polyphenols on different cellular functions.

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DISCUSSION

Unidentified speaker A. This is a really interesting paper. However, your doses that you used for your polyphenols do not look physiologic as compared with the doses that you used in the concentrations for the wine. You used 0.1% for the wine concentrations, and for the polyphenols, it looks like you should have used 1 order of magnitude less. You should have had a result with 1 order of magnitude less, 0.001. The doses that you used were not physiologic for the polyphenols.

Dr Omar Araim. Regarding the doses for the polyphenols, the concentration of polyphenols in a bottle of wine is 2 g/L, so that corresponds to a concentration at the 0.1% wine solution of 10 μm for the polyphenols.

Unidentified speaker A. Are you going to be following mechanistic data? Your preliminary data look quite interesting also. This would suggest some problem with a molecular mediator that would usually take the cells from S phase into G2. Which molecular mediators are you focusing on?

Dr Araim. Our next step is to look at regulation of cell-cycle proteins, and that is what we are planning on doing to confirm these studies and to provide supportive data for this.

Unidentified speaker B. Have you considered this effect on other cells? These experiments I think were confined to smooth muscle cells. Does this effect require a single exposure to the polyphenols, or do they continuously have to be present? This gets to the implications of this under physiologic conditions because I think, and correct me if I am wrong, to get a 1% level of wine requires drinking about a bottle of wine an hour. If that has to be sustained to inhibit coronary artery disease that could affect the applicability of this.

Dr Araim. As far as the first question, there has been some evidence in some leukemia cell lines and some lymphoid cell lines that resveratrol has some sort of antiproliferative effect, and it has been thought to be a potential anticancer agent in that regard.

As far as the second question, you are right. What we are dealing with is 12-hour and 48-hour exposures, which even for the excessive drinker would be a long time to be exposed to those polyphenols. Those polyphenols are metabolized by the liver; therefore, the physiologic doses that we talk about are not related to the amount of time that a normal person would be exposed to those polyphenols.

Dr Joseph Raffetto (Boston, Mass). I enjoyed your paper very much. My question was, there are good epidemiologic data that show that red wine, white wine, and beer are somewhat effective in reducing overall cardiovascular risk effects. I was just wondering if you looked at the ethanol component, if it also has some of these effects on the smooth muscle proliferation, some of the other findings.

Dr Araim. As far as what we did, we have not looked at the specific effects of ethanol. The other effects of alcohol itself on inhibiting atherosclerosis point to an antiplatelet effect and prostaglandin pathways effect, which we did not really explore because what we were specifically looking at was just red wine and the basis of our data was pretty much from that perspective. Our impetus for doing this work was our first statement, namely that in France their per capita red wine consumption is three times as much. Even in other parts of Europe, where they drink the same amounts of alcohol but not the same amounts of red wine, they do not have the lesser cardiovascular mortality and morbidity that they do in France.

Dr Colleen Brophy (Phoenix, Ariz). One final question. You looked at one step in the process of intimal hyperplasia, namely, proliferation, and I know your laboratory is capable of looking at some of the more subsequent steps. In other words, the smooth muscle cells need to migrate and produce matrix for the hyperplastic response to progress. Have you examined these events?

Dr Araim. Not yet, but those are part of our future plans as well, to explore those specific effects that you had mentioned. As well, we were planning on looking at perhaps different types of wine and wines with different polyphenol contents and comparing them in that specific way.