5-1-1964

Experiment to determine the effect of in situ freezing on arteries in dogs

Donald E. Short
University of Nebraska Medical Center

AN EXPERIMENT TO DETERMINE THE EFFECTS OF IN SITU FREEZING ON ARTERIES IN DOGS

Donald Eugene Short

Submitted in Partial Fulfillment for the Degree of
Doctor of Medicine

College of Medicine, University of Nebraska

February 5, 1964

Omaha, Nebraska

TABLE OF CONTENTS

INTRODUCTION

This experiment describes the freezing of arterial segments in situ to determine if an artery so frozen would remain patent and functional.

In the radical attacks being made in the surgery of carcinoma, the involvement of major vessels by a tumor mass often prevents adequate tumor resection. While grafts or synthetic prosthesis would conceivably aid in the removal of tumor in certain situations, in some anatomical areas such treatment might prove difficult or impossible. In certain cases the procedure of choice would necessarily be closure of the wound without an attempt at tumor extirpation.

It is suggested that in cases of tumor involvement of vessels, the involved artery might be subjected to a freezing process which would kill the cellular components of both the tumor and the artery. Conceivably the elastic and fibrous tissue in the arterial wall would keep the artery patent and intact until tissue regeneration replaced the killed cells. Important arteries invaded by tumor cells might thus be freed of tumor while arterial flow was maintained. Such a procedure would be advantagous as compared to the la-

borious replacement of vessels by homografts or simple closure of the wound where grafting was impossible.

Few experiments in the past have been concerned with procedures to bring about the death of animal cells by freezing. Early experiments were concerned with the preservation of cellular tissue following frostbite.

Following the advent of the arterial homograft most experiments were directed primarily at preserving the cellular integrety of the homograft in the face of its preparatory freeze-drying.

The relationship between atherosclerosis and arterial injury has also prompted investigators to subject arterial walls to various forms of trauma which included freezing.[1]

These experiments provided considerable information concerning cellular death by freezing, although this information was not the primary goal.

MATERIALS AND METHODS OF PROCEDURE

The animals selected for this experiment were three large healthy mongrel dogs. The animals were given 4 mg/kg depot heparin I.M. one day prior to arterial freezing.

The coolant used was solid carbon dioxide ice; this presents a surface temperature of - 43°C.

Each artery was exposed using aseptic technic. The proximal portion of the segment to be frozen was clamped with a bulldog clamp. All blood in the segment was milked distally and excluded from the vessel by means of a second bulldog clamp. The handle of a Bard-Parker scalpel was passed beneath the ischemic segment and elevated so that the vessel was held up and apart from underlying structures. A piece of carbon dioxide ice approximately the width of the scalpel was held against the arterial segment with thumb forceps for one minute to insure complete freezing. Thus an arterial segment the width of the scalpel handle (1.5 cm) was frozen. The solidified arterial segment was allowed to thaw slowly to room temperature. The segment was marked using 6-0 silk sutures which were placed in the adventitia at the proximal and distal extremities of the

frozen area. The bulldog clamps were removed and the artery was allowed to fall back into place. The artery was palpated to insure patency and the wound was closed. The operative sites were checked until the arterial segments were removed days and weeks later. Particular attention was devoted to watching for signs of infection and to checking pulses as to whether or not thrombosis had occured.

The central one-third of the frozen segments were removed and the proximal and distal stumps ligated. The first segment was removed at the end of week one, the second was removed at the end of week two and thusly until all five segments were removed. A section of unfrozen artery was also removed from two animals for comparative studies. The arterial segments were sectioned and stained with both H&E and Virchow stains. Histological studies of the segments were made to compare the structural differences between segments.

OBSERVATIONS AND RESULTS OF EXPERIMENT

When Freezing

Blood reentering the thawed arterial segments exhibited a dark reddish-purple color which was considerably darker than the non-frozen portions of the same vessels. The pulses could be palpated equally well in the frozen and the non-frozen areas and the blood flow seemed unchanged, as viewed grossly, when compared to the flow before freezing.

Post-Freezing Inspection

Pulses could be palpated throughout the vessel lengths at all times following closure of the wounds. There was no evidence of thrombosis at any time. There was no evidence of infection at any time.

Removal of the Frozen Arterial Segments

One Week Post-Freezing

GROSS: At surgery, very few changes were evident. Blood flow was essentially normal to appearance and palpation. The frozen segment did, however, appear to be lighter in color than the rest of the artery.

MICROSCOPIC: The endothelium had apparently disappeared. The internal elastic membrane was partially flattened. Cells in both the media

and the adventitia showed loss of nuclei and karyopyknosis. Vasa vasorum were dilated. There were remarkably few inflammatory cells present, and the few present were restricted to the adventitia.

Two Weeks Post-Freezing

GROSS: The adventitia had begun to show evidence of increased vascularity. The frozen segment was now much lighter in color than was the rest of the artery.

MICROSCOPIC: The endothelium was still absent. Although occasional intimal cells were present which were thought to be regenerating endothelium, their origin could not be determined by using the present stains. The cellular components of the media showed evidence of necrosis. The adventitia was quite vascular.

Three Weeks Post-Freezing

GROSS: The adventitia showed evidence of being still more vascularized. The frozen segment appeared to remain lighter in color.

MICROSCOPIC: The endothelium had reappeared as a unicellular layer. Areas of necrosis in both the media and adventitia persisted. Connective tissue cells were appearing in the media. Again, however, it could not be stated whether

these were fibroblastic or collagen cells.

Four Weeks Post-Freezing

GROSS: The frozen segment demonstrated about the same properties as noted in the third segment. However, the adventitia appeared greatly thickened and much more vascular than previously noted.

MICROSCOPIC: The endothelium was still a single layer of cells. Fibrous proliferation was much more in evidence than previously noted. Elastic fibers showed minimal fragmentation. The adventitia was more vascularized.

Five Weeks Post-Freezing

GROSS: The adventitia exhibited about the same quality as noted in the description of the fourth week segment. The color of the segment was more translucent than had been previously noted.

MICROSCOPIC: The intima was two to three cells thick and covered the entire intimal surface. The elastic membrane appeared to have fewer breaks than previously and appeared not to be quite as flat. There was essentially no change in the adventitia.

To sum up, the endothelium was destroyed originally but began to show regeneration by the

second week. The cellular components of the arterial walls became necrotic. The adventitia ultimately became highly vascularized. There was minimal inflammatory reaction in evidence throughout the experiment.

DISCUSSION

Cell Death by Freezing

"The physiological trauma suffered by cells subjected to cooling a tissue from body temperature to -70°C, followed by thawing, involves the following danger sequence:

1. Thermal shock - between 37°C and varying points below 0°C.
2. Osmotic shock - from increased electrolyte concentration in the medium as extracellular ice forms.
3. Harmful effects due to salt increase but distinct from osmotic shock; e.g.; protein and lipids being denatured, dissolved or precipitated.
4. Mechanical damage - produced by crystalization of water around and within the cell.
5. A second exposure to excessive electrolyte concentration during thawing."[2]

In order to insure complete cell death by a freezing procedure; it is helpful to understand the mechanism of cellular injury as incurred in the freeze-thaw cycle. An attempt can then be made logically so to produce maximum trauma to cells.

The rate of cellular cooling becomes quite important. Very rapid freezing, short of vitrification, presumably forms intracellular ice crystals and appears to be almost uniformly lethal to mammalian cells, with a few exceptions.[3] The extent of cellular contact with the coolant affects the rate of cooling. Air exposures are unreliable, while contact with a cold liquid provides excellant dissipation of heat and lowers the temperature rapidly and efficiently.[4] Rapid cooling rates can be obtained by employing an alcohol-carbon dioxide ice bath (-78°C) which gives a temperature drop of 1.7°C/second or approximately 100°C/minute.[5] Direct contact of a large blood vessel of man or dog with carbon dioxide ice for one minute is considered ample time in which to complete freezing.[6] Carbon dioxide ice was therefore selected for the coolant in this experiment because it adequately freezes cells, is cheap, handles easily, is readily available and will not stick to tissues.

The speed of thawing is also important in determining the degree of damage produced by freezing. In Luyet's experiments it was found that rapid thawing tended to avoid crystalization within the cells and allowed most of the cells to live, whereas fro-

zen cells thawed slowly in the air were destroyed. In general it is stated that show thawing is more lethal to cells than rapid thawing because of the increased formation of ice crystals as the temperature rises slowly.[7]

The results of early studies evaluating freezing temperatures and consequent cell death were vague. For example, Lake, in 1917, stated that a sudden drop in temperature below -6°C for as brief a period of time as 35 seconds to as long as 18 minutes produced cell death.[8] Meryman and other investigators have since felt that freezing of tissues could be considered complete when the temperature reached -15°C. However, it is accepted practice to continue cooling to -25°C to compensate for a lag in specimen temperature.

Thrombus Formation

Pate and Sawyer, by measuring the electric potential differences across the walls of vessels, have shown that dying, injured or degenerating cells have a positive injury potential on their intimal surfaces as compared to the adventitia.[9] These positive intimal potentials attract the negatively charged blood cells and a thrombus is formed on the vessel where the potential at the

intima is negative, and to the freeze-dried graft which (being dead) is incapable of maintaining any potential.[10]

In this experiment the method of freezing was such that the above conditions were met. Cellular death of the frozen arteries was virtually certain in the opinion of this investigator.

Such studies substantiate that there is a direct relationship between thrombus formation and the presence of injured cells in the wall of the artery. To state the converse, the absence of living cells in the wall of an artery correlates with the absence of any electrical potential and therefore a reduction in the possibility of thrombosis. One might assume in the present experiment that the cells in the center of the frozen segment were dead, and they were without an injury potential. However, at the juncture between the normal arterial cells and the frozen arterial cells there were certainly cells which were only injured and therefore possessed of an injury potential. These could easily have caused thrombosis. To avoid thrombosis due to such injury potentials, Davis suggested that the animals be heparinized adequately before the freezing process, and that a dosage of

4 mg/kg depot heparin be given I.M. at least 1 day prior to surgery.[11]

Late thrombosis very often occurs in frost bitten extremities, at times being delayed for as much as seventy-two hours. Lange and Boyd feel that this is due to massive infection which almost always occurs in severe frostbite, unless proper precautions are taken.[12] It was felt that this would not be a great problem in this experiment since tissue damage was minimal as compared to the freezing of a complete extremity and because aseptic technic was carefully observed. However, animals were watched closely for signs of infection; none appeared.

Post-Freezing Rupture

The cellular components of grafts used in early arterial work were dead as for example those tissues preserved in alcohol. Since essentially this effect was desired in the present experiment, it was hoped that the end result - uneventful healing - would be the same. The early changes that Hufnagel described for frozen homologus grafts would be anticipated in segments of arteries frozen in situ. Hufnagel's description recorded the histologic changes seen in each graft:

1. The intimal surface is destroyed and is rapidly replaced by a new endothelium.
2. The subendothelial layer becomes thickened.
3. The elastic lamina is well preserved and remains in good condition.
4. The adventitia becomes slightly thickened, and new blood vessels grow into the graft soon after transplantation.[14]

In the present experiment it was felt that the arterial wall would be weakest and in greatest danger of rupture within five to seven days after freezing. At this time necrosis would be maximal and regeneration would have progressed

only a limited degree. Since homologus grafts have withstood arterial blood pressure arteries frozen in situ could be expected to do the same.

CONCLUSION

It has been shown that arteries frozen in situ suffer complete cell death. Such vessels do not thrombose but remain patent. The vessel walls remain functionally intact and the circulation continues unimpeded. The procedure might prove useful in eradication of tumor from tumor involved major vessels.

The procedure warrants further investigation.

SUMMARY

An experiment to determine the effects of in situ arterial freezing is described. Five femoral arteries of dogs were frozen in situ by a dry ice freezing technic. The arteries remained patent. No post-freezing thrombosis or other complications arose. Frozen segments were removed at weekly intervals and histologic studies were made to reveal chronological changes. Complete cell death was produced. Elastic and fibrous connective tissue maintained a skeletal frame work and provided a functional arterial wall. The endothelium quickly regenerated and became two to three cells thick by the end of the fifth week. Evidence of collagen formation was noted by the end of the third week and increased greatly by the end of the fifth week.

The problem of surgical treatment of major vessels involved by tumor was discussed.

A method of eradication of such tumor from arterial walls by in situ freezing is proposed.

ACKNOWLEDGEMENTS

I would like to thank Dr. John Latenser for his advice and assistance in the laboratory, and for spending so many hours correcting this thesis. I would like, also, to thank my wife, Judy Short, for spending many laborious hours typing this thesis.

Don Short

BIBLIOGRAPHY

1. Taylor, C.B. in Symposium on Atherosclerosis, Publication 338 National Research Council, 74.

2. Lovelock, J.E., Biophysical Aspects of the Freezing of Living Cells in Preservation and Transplantation of Normal Tissue, (Ciba Found, Symposia), Boston, Little Brown and Co., 131-33, 1954

3. Meryman, H.T., Preservation of Living Cells, Fed. Proc. 22: 81-9 Jan. - Feb. 63

4. , Tissue Freezing and Local Cold Injury, Phys. Rev., 37: 233, 1957

5. Hauschka, T.S. and others, A Reliable Frozen Tissue Bank, Canc. Res. 19: 643, 1959

6. Elbin, J., The Carbon Dioxide Snow-Electrocautery Technic for occlusion of Large Veins: Suggested application to venous angioma of the Brain, Surg., Gynec. and Obst., 76: 43-50, 1943

7. Luyet, B.J. and Hodapp, M., Revival of Frog Spermatazoa in Liquid Air, Proc. Soc. Exper. Biol. and Med., 39: 433-34 1938

8. Lake, N.C., Report upon an Investigation into the Effects of Cold Upon the Body, Lancet 2: 557, 1917

9. Pate, J.W. and Sawyer, P.N., Freeze-dried Aortic Grafts. A Preliminary Report of Experimental Evaluation, A.J. Surg., 86: 3, 1953

10. , Armed Forces Med. J., 1: 23, 1953

11. Davis, H.L., Personal communication to the author

12. Lange, K. and Boyd, L.J., The Functional Pathology of Experimental Frostbite and the Prevention of Subsequent Gangrene, Surg, Gynec. Obst., 80: 346 1945

13. Hufnagel, C.A. and Eastcott, H.G., Histological Changes in Homologus Grafts, Bull. Georgetown Med. Center, 4: 119, 1951