Wellcome Film Project

Cryobiology and Transplantation

The Scientific Basis of Medicine

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<**Opening titles**>

<**Pegg to camera** >

Cryobiology is the study of the effects of cold in biology and it finds considerable applications in the fields of transplantation medicine. So in this lecture I want to deal with the applications of cryobiology in transplantation.

I've divided the lecture into 3 sections. First I want to talk about fundamental research in cryobiology to explain what we understand about the mechanisms of freezing injury and how cryoprotective agents work. Then I'll talk a little about cell preservation and tissue preservation. And finally I want to deal with organ storage.

<**Intertitle**>

1. Fundamentals of Cryobiology

<**Pegg to camera**>

Now, cooling has been used to preserve the survival of isolated cells and tissues for very many years and of course this depends upon the well-known effect of lowering temperature on chemical and physical reaction rates.

<**Pegg narrates over graph**>

It can be shown, for example, that the oxygen consumption or renal cortical slices decreases roughly exponentially with falling temperature, reaching less than 5% by 5˚C. Similar curves can be shown for the requirements of other substrates.

<**Pegg to camera**>

Clearly it would be expected that one would get longer survival of isolated cells the lower the temperature can be reduced but unfortunately as soon as freezing occurs, cell survival with most cell types drops to very low levels. So let us first look at what happens when cells are first frozen.

Now cells of course are very complicated systems so we'll look first of all at the simplest model which is sodium chloride solution.

<**Pegg narrates over graph**>

This graph shows the freezing point depression curve for sodium chloride. In this and in subsequent graphs, I've expressed the concentration of sodium chloride in mole fraction terms which is perhaps a little unfamiliar. It is however applicable to cryobiology because, as I will explain, as freezing proceeds, one reaches very high solution strengths and the conventional methods of expressing concentration are no longer strictly applicable. So to orientate yourselves, let me say that a mole fraction of 0.0027 is isotonic in the case of sodium chloride.

Now this graph as I say shows the freezing point depression of solutions of increasing strength of sodium chloride. But it's important to realise that this graph also shows what happens when a solution of a single strength, shall we say isotonic,

is cooled to its freezing point and beyond. Because what happens is, when the freezing point is reached, ice separates, which is pure water, and the solute becomes concentrated in the remaining liquid phase. And as cooling proceeds, further ice separates so that the strength of the remaining solution follows precisely that curve.

<**Pegg narrates over further graph**>

This is perhaps more clearly shown in this graph where I have represented isotonic sodium chloride by 1 on the Y axis, and have plotted the proportional increase in strength of sodium chloride as the temperature is reduced. And you can see that there is a 32-fold increase in the concentration of salt by the time the eutectic temperature -21˚C is reached. Now clearly this must have very dramatic effects on any cells that are also present in the system.

00:04:29:12

<**Pegg to camera, then turns to stand in front of a series of charts on boards which he points at with an indication stick**>

So, let us look at these very simple drawings that show diagrammatically what happens. Now here we have sodium chloride solution. The dots represent solute molecules and the circles represent the membranes of cells. There is an equal concentrate of solutes on either side of the cell membrane before freezing starts. When freezing occurs, ice separates as pure water, pure solid water, so the solute molecules become more concentrated in the remaining liquid and there is then an osmotic imbalance across the cell membrane.

The response to this is of course that the cells shrink. They lose water to equalise the osmotic pressure across the cell membrane. So you see, there is ice outside the cells, but no ice inside. Now of course the loss of water takes time which is dependent on the water permeability of the particular cell in question and it would theoretically be possible to cool so quickly that the water could not leave the cell fast

enough to freeze outside and intracellular freezing could occur. But we will come back to that question in a few minutes time.

Now these diagrams are all very well but let me just show you that this does actually happen.

< **Pegg narrates over graph**>

Here I have a graph prepared by my colleague Tony Woolgar showing the mass of cell water in red cells suspended in sodium chloride solutions of increasing strength. And you can see that the cells shrink and reach a minimum volume at around 1800 milliosmoles. Now what effect does this shrinkage have upon the cells?

< **Pegg to camera** >

Well there are many effects. One of them is that the cell membranes change their permeability characteristics.

< **Pegg narrates over series of graphs**>

Here we see what happens to cell potassium in red cells suspended in sodium chloride solutions of increasing strength and you can see at around about 1800 milliosmoles, the point where the minimum volume is reached, there is a loss of intracellular potassium.

This also coincides with an increased loss of cells, haemolysis. Here we see that haemolysis starts at around 1400 milliosmoles and reaches around 50% by 4000 milliosmoles.

< **Pegg to camera** >

So, we know that salt concentration rises during freezing but does this provide an adequate and complete explanation of freezing injury? Well it doesn't. For one thing

the amount of cell destruction occurring during freezing is much greater at a given osmolality than it is during constant temperature exposure to that osmolality. In addition one gets the same cell shrinkage and the same changing cation permeability whether the osmolality is increased with salt or with sucrose. But with freezing there is very much less damage if it is done in the presence of sucrose than if it is done in the presence of sodium chloride. We have also found that red cells become permeable to sucrose when they are frozen but not when they are exposed to high osmolality without freezing. So clearly there are additional factors at work in freezing.

Well, what are these? Well, I think it is fairly obvious that the two factors which occur in freezing but do not occur in the simple hyperosmolar model system are firstly that when cells are frozen, then thawed, they are not only exposed to high osmolality but they are also re-suspended in isotonic solution when the ice melts. Secondly of course freezing and thawing involve temperature change which the simple hyperosmolar model system does not.

Well many years ago Dr James Lovelock working at Mill Hill investigated the importance of these other two factors – re-dilution and temperature change.

<**Pegg narrates over series of graphs**>

What he did was to take a red cell system and to suspend it in sodium chloride solutions of increasing strength and then re-suspend the cells in isotonic sodium chloride and observe the haemolysis produced and you can see he obtained a sigmoid curve of this sort. The next experiment was to calculate the temperatures which produced the same sodium chloride concentrations and then to freeze the cells to these temperatures in isotonic sodium chloride and then thaw them. And when he did this, he found that he got a very similar curve – within experimental error they were the same. So that re-dilution after hypertonic exposure had in fact duplicated the effect of freezing.

Lovelock also investigated the effect of temperature change under constant hypertonic conditions and when he suspended red cells in sodium chloride solutions,

between 0.01 and 0.03 mole fraction of sodium chloride, and then cooled them from 37˚C to 0˚C, that is without freezing, he obtained greatly increased haemolysis.

00:10:23:20

< **Pegg to camera** >

So, it was shown that cells exposed to hypertonic conditions and then subjected either to dilution into their original osmolality or to temperature change, were damaged, and the addition of these two factors to the hyperosmotic effect was sufficient to explain freezing injury.

Now, all these experiments were done with relatively slow cooling rates and I mentioned initially that there is a possibility that intracellular freezing could occur when cooling is rapid. So let us see whether cooling rate does in fact have any effect on cell survival.

< **Pegg narrates over series of graphs** >

This is a graph showing the survival of human red blood cells after cooling at rates between 1˚C and 10,000˚C per minute. You can see that survival increases as the cooling rate is increased to 3000˚ per minute and then falls off. Now clearly the salt effects which we have been talking about account for the reduction in survival to the left of the peak, and the reason the peak actually occurs is that as the cooling rate increased, there is progressively less and less time for the salt effects to take place. But when a cooling rate, in this case of 3000˚ per minute has been reached, then survival falls off and we could expect that this might be accounted for by intracellular freezing.

Now Mayser has done some very interesting work on this. First of all he calculated the proportional reduction in volume of cells at different cooling rates depending on the rate at which water could leave them. This graph shows his calculations for red blood cells – the measure V/Vi is in fact the volume over the initial volume, in other

words it's a percentage of the initial volume of the cells. If the cells were able to remain in equilibrium they would follow the dotted line but obviously as they are cooled more and more rapidly, and here we have graphs for 1˚, 5˚ and 10˚ per minute, so they are less able to maintain thermodynamic equilibrium and in fact contain significant amounts of intracellular water and are therefore liable to freeze internally.

<**Pegg to camera**>

Now Mayser showed that several different cell types with known different permeabilities to water did in fact show different peak survivals at different cooling rates.

<**Pegg narrates over graph**>

The next graph shows the response to cooling rate to 4 different cell types. Yeast had a peak survival of 10˚ per minute and yeast is relatively impermeable to water, red cells on the other hand which are highly permeable to water had a peak survival at a high cooling rate and hamster tissue culture cells which are intermediate in water permeability had an intermediate peak survival rate. I would like to point out also on this graph that mouse bone marrow cells, which are a very typical mammalian nucleated cell, in fact showed no peak at any cooling rate and I will come back to that in a minute. But this work of Mayser's did supply an adequate explanation of the effect of cooling rate which has in fact since been confirmed by direct electron microscopic studies.

<**Pegg returns to earlier series of charts on boards which he points at with an indication stick**>

I want to show you here 2 electron micrographs of cells which have been cooled to liquid nitrogen temperatures, that is -196 at different cooling rates and have then been freeze-substituted so that we can see where the ice was present.

Here we have a cell which has been cooled rapidly. The white areas were zones previously filled by ice – you can just make out where the nucleus was <**points**> some mitochondria, but the cell is full of small ice crystals. On the other hand, when these cells were cooled at only 1˚ per minute they became shrunken and see that the cytoplasm is pulled right down upon the nucleus, this has become very dense, and there is no sign of any intracellular freezing.

So here we have a direct demonstration of the effect of cooling rate on the location of ice.

00:15:28:00

<**Pegg to camera**>

Now, cryobiology would in fact have relatively little application to transplantation if one was limited to cooling in the absence of cryoprotective agents. And in fact it was the accidental discovery by Chris Polge, Audrey Smith and Sir Alan Parks in 1949 that glycerol would protect against freezing injury that has made it possible to develop cryopreservation techniques for use in transplantation.

Now this as I say was an accidental discovery, but it is now reasonably well understood how this sort of cryoprotective agent works.

<**Pegg narrates over graph**>

I want to show you a graph of the freezing point depression of glycerol solutions. This is the same sort of graph as the one of sodium chloride which I showed earlier, the principle difference being that the minimum freezing point obtainable with a glycerol water system, the so-called eutectic point, is a much lower temperature. Another very important difference between glycerol and sodium chloride is that […]

<**Pegg to camera**>

[…] cells will withstand exposure to very high concentrations of glycerol whereas they will not stand exposure to very high concentrations of sodium chloride. Now if we construct, from that freezing point depression curve, graphs similar to the proportional increase in salt concentration graph, which I showed you earlier, […]

<**Pegg narrates over graph**>

[…] you will be able to see that glycerol has very profound effects in aqueous solutions which are cooled. On this graph I have also shown the sodium chloride, the isotonic sodium chloride as a dotted line, for comparison. Each of the other lines represents a glycerol solution, the concentrations are shown in mole fraction terms but correspond roughly speaking to 1, 2, 3 and 4 molar glycerol. As you can see, as the glycerol concentration is increased freezing occurs at a progressively lower temperature and the subsequent proportional increase in glycerol concentration is much less as the initial concentration is increased.

<**Pegg to camera**>

Of course the thing which is concentrating glycerol in this system is the separation of water as ice. And it follows that anything else which is present in the system cannot concentrate to a greater extent than the glycerol because both are being concentrated by the same event – the formation of ice. So that if we consider a 3 component system consisting of glycerol, water and sodium chloride, […]

<**Pegg narrates over graph**>

[…] the sodium chloride cannot concentrate at the same steep rate that it did in the simple system with water but must concentrate at a much slower rate.

Here we see the mole fraction of sodium chloride plotted against temperature in solutions containing no glycerol or 0.05, 1 molar or 2 molar glycerol. And you can see that as the glycerol concentration is increased so the rate of increase of sodium chloride concentration is also diminished. Now also shown on this graph, and I've

taken this again from data by Jim Lovelock, also shown on this graph are points which represent the mole fraction of sodium chloride or the temperature at which 5% haemolysis was found when red cells were also included in the system. And the important point is that the same degree of haemolysis occurred at roughly the same sodium chloride concentration but at different glycerol concentrations and at different temperatures in each case. And the higher the glycerol concentration, the lower the temperature at which the same degree of haemolysis was occurred.

00:19:38:39

<**Pegg to camera**>

I want to show you a direct demonstration of the effect of glycerol on the freezing of red blood cells. This is a film that was made by Audrey Smith at Mill Hill a number of years ago and shows red cells on a freezing microscope operation, both without and then with glycerol.

<**Pegg narrates over film showing microscopic cells in action**>

Here you see the red cells in saline on the microscope slide. The temperature is reduced and you see the ice crystals moving in from the left hand side. And you can see that the cells are still present in the preparation. Now the temperature is allowed to increase and the ice melts and you will see as it melts that all the cells disappear, they become haemolysed and there are no intact red cells left.

Now we see the same experiment repeated in the presence of glycerol. The first thing to notice is the ice structure of glycerol is rather different; you can see the ice crystals come in in a moment as the temperature falls and the liquid spaces between the ice crystals are rather larger than they were in the absence of glycerol. The red cells can still be seen clearly present in the preparation. And you can see them moving around slightly showing that there still is significant liquid phase present. During thawing the liquid phase progressively increases in volume and the cells can

be seen moving around before all the ice has gone. And finally, when all the ice does melt you can see that there are large numbers of intact red cells still present.

<**Pegg to camera**>

Now glycerol of course is still very widely used as a cryoprotective agent and there are other agents of the same type in common use. Substances like dimethyl sulphoxide, dimethylacetamide and dimethylformamide. There is however another class of cryoprotective agents – the polymers like polyvinylpyrrolidone and dextran which were thought for many years to have a different mechanism of action. This thought arose because these compounds, having a high molecular weight, are present in relatively low molar concentrations when prepared in the standard sort of solutions in weight for volume terms. Now, it turns out that this is not correct.

<**Pegg narrates over graph**>

Some time ago my colleague John Farrant showed that solutions of polyvinylpyrrolidone do in fact produce profound depression of freezing point. Although there is little compression at low concentrations, the curve is markedly non-linear and at high concentrations there is a very profound effect. And of course, during freezing as ice separates very high concentrations are produced. So one can explain the cryoprotective effect of PVP with this explanation.

There is, however, an additional factor with the polymers and that is that when cells are thawed they are, as I mentioned, relatively permeable to cations and therefore the internal colloid is unopposed by external impermeable cation and the cells tend to lyse.

Now in this graph I have shown an experiment by Tony Woolgar where red cells were exposed to strong salt solution and were rediluted into solutions of differing osmolality either made up entirely of sodium chloride, and you can see that as the sodium chloride concentration is increased so the survival rate improves, but this is compared with a solution containing isotonic sodium chloride and progressively

increasing amounts of PVP – and you can see there is a marked improvement in survival. Now this is explained by the fact that the PVP is impermeant to the thawed cells and therefore balances the internal colloid and prevents colloid osmotic haemolysis.

<**Pegg to camera**>

Now this has necessarily been a rather brief review of basic cryobiology but I hope it will form an adequate background for discussion of some of the practical applications that I want now to deal with.

00:24:48:00

<**Intertitle**>

2. Cells and Tissue Preservation

<**Pegg to camera from a blood bank laboratory; Dr Blagdon in white coat to his right**>

The cryopreservation techniques for red cell storage that I have been describing find very useful application in clinical medicine and we thought it useful therefore to come to the North East Metropolitan Blood Transfusion Centre at Brentwood where Dr John Blagdon, the Deputy Director will explain the system which is used here. Dr Blagdon.

<**Pegg moves off camera. Blagdon narrates and demonstrates how blood is stored and prepared. He narrates from a variety of locations around the blood bank, close-up shots of featured equipment are shown**>

Blood donations are collected in the normal way into standard packs. They are then spun to separate the plasma from the cells. Plasma is then removed and glycerol solution is added to give a final weight volume of 20%. They are then transferred into an aluminium canister ready for freezing.

Freezing is carried out in liquid nitrogen. We make our own liquid nitrogen in the plant on the left. The storage of the units are in 4 nitrogen refrigerators, supplied from a central reservoir with automatic filling and alarm systems. The units are frozen in liquid nitrogen to bring them down to -196˚C, this takes about 9 ½ minutes. They are then transferred from this vessel into one of the nitrogen refrigerators. The storage until required. The low units are stored to about this level in liquid nitrogen at a temperature of -196˚. The upper layer is in a vapour phase with a temperature of about -180 to a maximum of about -160 at the upper level. They can be stored in this condition for about 2000 years theoretically.

Before transfusion the glycerol has to be removed. This is done by 2 methods. But first the thawing is carried out in an agitating water bath at 42˚ for 6 ½ minutes.

This is the continuous wash centrifuge method. After thawing the red cells are transferred to a plastic pack. The wash solutions are held in cradles above the blood. They then flow through the tube into the centrifuge bowl. The bowl was developed by Latham from the current fractionator in the United States. The inlet ports, the cell suspension goes into the bowl and is held by the centrifugal force while the wash solution follows, washing the glycerol from the red cells. The exit takes the supernatant out into a pack in front of the machine. After washing the red cells are siphoned off into the transfusion pack.

For the manual wash procedure the thawed red cells are transferred into a 5 tailed pack. First line is used to transfer, this is sealed and then the pipe is centrifuged, the supernatant is then separated and the first wash solution run through on the mechanical mixer. This is repeated for 2 further washes using 3 and 4. The final centrifugation and the final supernatant is then removed, the line is sealed and the pack is ready for transfusion. Using this procedure one recovers 95=97% of the cells.

On transfusion, the post-transfusion survival of the red cells for 24 hours is about 90%.

00:30:39:02

<**Pegg, back in the studio, to camera**>

Red cell preservation is by far the most clinically important application of cryopreservation today although techniques are currently being worked out for platelets and we hope also for granulocytes.

There are 2 other applications of cryopreservation more closely allied to transplantation that I'd now like to talk about and the first of these is bone marrow transplantation.

It was found by Barnes and Loutit in the 1950s that the haemopoietic cells of the mouse could be preserved by a technique very similar to that used now for red cells.

<**Pegg narrates over series of graphs, uses indication stick**>

I have over here a graph which shows the survival of haemopoietic cells of the mouse at different cooling rates and in differing concentrations of glycerol. Now you'll remember that earlier on I showed that there was no survival of mouse bone marrow cells at any cooling rate in the absence of a cryoprotective agent. That's this line here. But as the concentration of glycerol is increased, 0.04 molar, 0.8 molar, 1.2 molar, so increasing maximum survival rates are obtained and the cooling rate giving the maximum survival rate becomes progressively slower. This of course is because glycerol is limiting the damaging effects to the left of the peak. And as more glycerol is present so more of the area to the left of the peak is revealed and the maximum survival is obtained – in this case at 1˚ per minute in a concentration of 1.25 molar of glycerol.

Here we see the bone marrow cells actually being used in irradiated mice and we have a comparison of the leukocyte count at various days post-irradiation, the transplant having been given immediately after the irradiation. And you see compared fresh marrow cells and an equal amount of frozen bone marrow cells and

you can see that there is no significant difference in survival between the 2 techniques.

<**Pegg to camera**>

So the banking of bone marrow is a perfectly feasible technique and there is no reason why this cannot be used in clinical medicine at the present time where the proper clinical indications for marrow grafting exist. These are not many but as you might know there is quite a lot of interest in this at the present time and bone marrow transplants are being done on an increasing scale.

Techniques for preservation of skin and cornea were also worked out several years ago and both of these can be used clinically. First the skin or the cornea is soaked in a solution either of glycerol or dimethyl sulphoxide – there is some evidence that it may be better to use glycerol for the epithelial surface of the cornea and dimethyl sulphoxide for the endothelial surface. In each case, a concentration of approximately 1.2 molar is used, the tissue is then cooled slowly at 1˚ per minute or thereabouts and may be stored for very long periods of time at liquid nitrogen temperatures, at -198 or even at -180 if the storage period is not required to be much longer than a year.

Both of these techniques have been used clinically and again where the clinical indications are appropriate there is no reason at all why these techniques cannot be used on a much wider scale than they are at present.

00:34:38:15

<**Intertitle**>

3. Organ Preservation

<**Pegg to camera**>

By far and away the most important organ that is clinically transplanted at the present time is, of course, the kidney. Now, the immediate requirements to make transplantation a possibility are only for relatively short-term preservation; sometimes 4 or 5 hours is sufficient but 12 or 24 is certainly enough to make the whole thing a practical proposition. However there are very good reasons, largely stemming from immunological advances, why it would be an advantage to be able to store kidneys for a much longer period of time and set up true kidney banks. However, let us first look at the immediate problem of storing kidneys for up to 24 hours to make transplantation a possibility.

When transplantation first got underway Roy Calne and I investigated this problem using dogs and we took a very simple approach which was to remove dog kidneys, cool them in crushed ice to just above zero and see how long they could be kept in that way and still support the life of a bilaterally nephrectomised dog after transplantation. We found that up to 12 hours their function was perfectly adequate although we sometimes got some acute tubular necrosis, nonetheless the dogs all survived. But after longer than 12 hours results became irreproducible and eventually one got no survivors after 24 hour storage at all.

Now two very simple modifications to this technique have made it very much successful. They were both quite empirically found. The first was to wash out the blood before commencing storage and the most probable reason why this is advantageous is that red cells in the static microcirculation become depleted of ATP […]

<**Pegg narrates over graph**>

[…] and when this happens the intracellular calcium which is normally chelated by ATP becomes bound to the cell membranes which then become rigid. This graph shows some work by Weed where he measured the pressure necessary to deform red cell membranes after the cells had been incubated for increasing lengths of time and you can see that by 18/24 hours they had become really quite rigid and when

ATP was regenerated by adding adenosine then the normal pliability of the red cell membrane returned.

<**Pegg to camera**>

Quite obviously rigid red cells of this sort would be very difficult to dislodge from the microcirculation so washout is an advantage. The next empirical advance was that if the washout solution is rendered hyperosmolar, that results are very much better.

<**Pegg narrates over series of graphs**>

Now, when kidneys were washed out with hyperosmolar solutions, and here we see lactated ringers solution rendered hyperosmolar with either glucose or sucrose, then the water content remains constant during storage. Whereas in the absence of glucose or sucrose the water content increases, in other words the kidneys become oedematous. This is probably particularly important in the case of the endothelial cells. Here we have a measurement of endothelial cell thickness in normal kidneys and in ischaemic kidneys where you can see it is, in fact, doubled, and this will have a profound effect on the vascular resistance of the kidney. In this experiment mannitol was added to the washout solution and you can see that this enabled the endothelial cell thickness to remain at its control value.

<**Pegg to camera**>

Now, many different sorts of washout solution have in fact been used and it was very difficult to be sure which was better because the experiments were all done in different laboratories and so on. So in our laboratory we did a comparison of a range of washout solutions which were modified, each from the next, in a systematic manner. And I have the results on these charts here.

<**Pegg returns to earlier series of charts on boards which he points at with an indication stick**>

We've measured the function of the kidney after preservation by measuring in an in vitro system glucose uptake and sodium uptake. This is how controlled, freshly isolated kidneys perform. This is how kidneys, removed from the animal and not washed out at all, just stored for 24 hours function – very little function. This is how kidneys function that have been washed out with a solution having the same composition as plasma, the same osmolality, no added glucose, sucrose or mannitol, again rather poor function. However, when the osmolality is increased to 400 milliosmoles per kg, by the addition of glucose, very much improved function is obtained. In this solution the same osmolality was used but the sodium concentration was reduced and the magnesium concentration was increased. This made no significant effect. In this solution the sodium concentration was reduced and the potassium concentration increased and you can see that this significantly depressed function. These two experiments were with continuous perfusion with a solution containing dextran. That had an osmolality of 300 milliosmoles per kg, and that one 400 milliosmoles per kg by adding additional glucose. And you can see that these results are by no means as good as the simple washout solution but that increasing the osmolality did improve function over the one of normal osmolality.

This chart shows the comparable experiments but using sodium uptake as a measurement of viability and you can see the pattern is much the same.

00:41:15:18

<**Pegg walks away from charts and back to camera**>

So our conclusion from these experiments is that a washout solution of high osmolality is certainly beneficial but that other modifications that have been suggested by others, such as increasing the magnesium and the potassium concentration are not in fact beneficial.

<**Pegg narrates over series of tables**>

I will now show you the actual composition of solution. You can see the ionic composition is similar to that of plasma but we have added sufficient glucose, 107 millimoles to raise the osmolality to 400 milliosmoles per kg. We also have heparin and a vasodilator, procaine, present and the solution is buffered with hepes.

Now, these experiments in the laboratory have all been done with kidneys that were removed from animals in good health, there was no period of warm ischaemic injury before preservation started. And of course although this is an ideal situation, it is not one that is commonly encountered in transplantation practice in this country at least where most kidneys have suffered some warm ischaemic injury before they can be removed. In these circumstances, washout preservation may not be the best technique.

<**Pegg narrates over graph**>

Some years ago Bob Johnson in Newcastle made direct comparison of washout with Collins Solution, which is a high osmolality washout solution, and continuous perfusion with PPF (plasma protein fraction) which is crudely fractionated albumin solution. And you can see that as the warm ischaemia time is increased so the survival decreases but at all warm ischaemia times, above very brief ones, continuous perfusion gave better preservation.

<**Pegg to camera**>

Now continuous perfusion is probably the method of choice in Scandinavia, certainly in some parts of the continent it is used a great deal and in North America it is widely used, but in this country it has not been so popular, I think largely because of the complexity and the expense of the machinery.

<**Pegg returns to earlier series of charts on boards which he points at with an indication stick**>

I have over here a diagram of the equivalent which we use in our laboratory and you can see it is a good deal more complicated than just putting a kidney in a thermos flask. The kidney is suspended, in this case, from a weight transducer so that we can continuously monitor oedema. The venous effluent falls into a reservoir where the pH is maintained by passing 5% $C0_2$ in air over the perfusate. It is then pumped through a submicron filter, a bubble trap, to an arterial cannula where the pressure is monitored and the pressure is used to feed a process control which regulates the pump speed so that we maintain a constant perfusion pressure of 40mm of mercury and both the weight and the pressure are continuously recorded. The perfusion equipment itself is enclosed in a temperature controlled cabinet at 5˚C.

<**Pegg to camera**>

Now this, as I say, is a piece of equipment that was built for experimental use but comparable apparatus is in use clinically.

00:44:47:20

<**Pegg narrates from in front of the Gambro Perfusion Machine, Mr O'Donaghue to his right**>

To show you some organ perfusion equipment in actual clinical use we have come to St Bartholomew's Hospital where the Institute of Urology Gambro Perfusion Machine will be demonstrated by Mr O'Donaghue. You can probably hear the machine operating in the background but I'm afraid we don't have a kidney today. But Mr O'Donaghue will now show you how it operates. Thank you.

< **O'Donaghue to camera, demonstrates the Gambro Perfusion Machine**>

This is the Gambro Kidney preservation machine which we have been using at the Institute of Urology and at St Bartholomew's Hospital for some time now. In this particular view, the front cabinet of the machine has been opened so that you can see the perfusion circuit. Here we have two separate organ containers so that two

kidneys can be perfused at the same time. Here one sees the pump by which the perfusate is pumped around here and up through a filter, then to a bubble trap and then around to the organ container and so through the kidney. The perfusate is continually recirculated through each kidney at a low temperature. The temperature range being 5-9˚C. During perfusion, perfusate pressure may be monitored here and perfusate flow from the control panel at the side.

The original machine has proved effective in use but is rather bulky and so is difficult to transport between transplant centres. This is the latest Gambro machine which as you can see is very much smaller and more compact and so is much more readily transportable and I think will be of much greater use in clinical transplantation.

<**Pegg to camera**>

In continuous perfusion techniques it is very important to get the right perfusate. I showed earlier on some experiments in which a dextran solution was used and as you saw the results were not as good as our best washout method. Now, the machines in current use employ either cryoprecipitated plasma which is complex and is expensive to produce, or more commonly highly purified albumin solution and this is the one which is usually used in the Gambro machine.

I think that the reason continuous perfusion gives better results with ischaemic kidneys than with washout is again to do with the maintenance of a patent vascular system. Clearly, if the vascular system is not open at the time of transplantation then it will be impossible for the recipient blood to flow through the kidney and even if the kidney tissue itself is well preserved it will rapidly die if it doesn't have a blood supply. So continuous perfusion can be used for preservation under optimal conditions for 2, perhaps 3 days.

Now, the extension of the preservation time from 1 day to 3 days is occasionally useful, but to take advantage of immunological techniques that are coming along it will be necessary to have very much longer preservation than that, and I cannot see how continuous perfusion methods could ever provide an adequate solution to this

problem. They would tie up too many machines, 1 machine to each kidney and it would never be possible to have a large bank using this sort of technology. Therefore we are very actively investigating the possibility of using true cryopreservation techniques for kidney storage.

The first problem one comes up against in trying to apply these methods to renal preservation is the simple one of trying to get the cryoprotective agent actually into all the cells of the organ and then to remove it subsequently without damage. That is even without going to sub-zero temperatures.

We've studied 3 cryoprotective agents so far – glycerol, dimethyl sulphoxide and ethanediol and from our studies it appears that glycerol is the least toxic.

<**Pegg returns to earlier series of charts on boards which he points at with an indication stick**>

I have here 2 charts which show the effect on vascular resistance and on weight gain, that is oedema, of perfusion with 2 molar glycerol at 2 temperatures, at 37˚ and at 5˚. You can see that when the cryoprotective agent is introduced into the perfusion circuit, the vascular resistance decreases. This is due to osmotic shrinkage of the endothelial cells widening the capillary channels. And thereafter the vascular resistance stays constant.

Another problem that one is worried about in organ perfusion is the development of oedema and you can see that when kidneys are perfused with 2 molar glycerol at 37˚ there is a large increase in weight, gross oedema develops. Whereas when they are perfused at 5˚ the weight remains at just below the starting weight and there is no progressive oedema.

<**Pegg returns to camera**>

So we have studied 2 molar glycerol perfusion of isolated kidneys and are able to get it in all right. The next problem, however, is to get it out again and here one comes up against much greater difficulty.

00:50:55:00

<**Pegg narrates over series of graphs**>

This graph shows the vascular resistance of a kidney in an experiment where 2 mole of glycerol was introduced where it is shown and perfused for just under 100 minutes and then a change was made instantly to a perfusate without glycerol – and you can see there is a vast increase in vascular resistance, in fact virtually no perfusate was passing through the kidney, indicating gross vascular damage.

Now we thought we might be able to improve the situation by including in the perfusate a compound which would not penetrate the cells and would therefore provide an osmotic balance to the intracellular glycerol while it was being removed.

The next graph shows what happened when we added 100 millimole of mannitol and repeated the experiment. And you can see the increase in vascular resistance was very much less but still at the end of the experiment we had roughly 5 times the vascular resistance and there must have been considerable damage to the microcirculation.

It seemed logical that changing the glycerol concentration more slowly might produce less damage. We therefore repeated the experiment and changed the glycerol concentration at 1% per minute, as shown here, still keeping 100 millimole of mannitol in the perfusate and you can see this did result in a considerable improvement. And when the rate of glycerolisation and deglycerolisation was slowed still further, we got an even better result.

<**Pegg to camera**>

We are currently investigating the viability of kidneys, glycerolised and deglycerolised both by an in vitro technique and by transplantation, and when this work is complete we shall go on to cool as the glycerol concentration is increased. This is work following on an original suggestion by John Farrant who showed that smooth muscle tissue would survive cooling to very low temperatures if the glycerol concentration, or in his case the dimethyl sulphoxide concentration was increased during cooling and increased during warming in order to prevent the occurrence of freezing at all.

Now, to apply this sort of idea to a whole organ is some engineering undertaking and I want to conclude this lecture by visiting my lab at Northwick Park where you will see such an experiment in progress.

00:53:49:10

<**Pegg narrates from within lab at Northwick Park**>

Now this is the equipment which we are using for the experiments in which rabbit kidneys are being infused with increasing concentrations of glycerol and are being cooled at the same time. The perfusate is being prepared in this thermostatically controlled mixing chamber and is continuously circulated by the pump, here, through a refractometer, which measures the glycerol concentration continuously, and around through the piping back into the reservoir. A mixture of 5% $C0_2$ and oxygen is also passed through the reservoir continuously to maintain a constant pH of 7.4. Some of the perfusate is aspirated at this point here by the perfusion pump at the back of the equipment which passes it through a submicron filter and down through a heat exchanger to the arterial cannula which is tied to the renal artery of a rabbit kidney. The venous effluent passes into the small collecting chamber below and then is taken by this pump and returned back to the mixing chamber. Now, the kidney is suspended from a weight transducer, which is actually mounted just below, so that we can monitor the kidney weight continuously. And the perfusate pressure is monitored by a connection to the cannula which leads to a pressure transducer here. The perfusate temperature is monitored by a thermocouple in the perfusate tubing just at that point, here.

Now these 3 variables are continuously recorded on a 3-channel chart recorder and in addition we make intermittent measurements of perfusate flow rate so that we can calculate the vascular resistance of the kidney.

The glycerol concentration and the temperature of the perfusate are continuously controlled by the equipment in this rack. And this unit contains the programmers and the temperature control is continuously displayed on this meter and the glycerol concentration programme on this meter. The programmes are actually generated by a linear ramp generator and by a card reader which can produce curved programmes.

In this unit we have displayed the actual measured temperature and the actual measured glycerol concentration in the perfusate, so that we have available the desired temperature <**sound cuts out for 3 seconds**> of glycerol concentration and the measured glycerol concentration. These data are fed into 2 process controllers here and here and the outputs of these controllers operate the perfusion equipment to produce the desired temperature and glycerol concentration throughout the experiment. The concentration controller operates this pump which takes fluid from this reservoir and injects it into the mixing chamber in the required amounts to produce the necessary concentration at that time. During glycerolisation the reservoir contains 40% glycerol in perfusate and during deglycerolisation it contains plain perfusate with no glycerol.

The temperature controller controls a refrigeration unit which alters the temperature of coolant flowing through the heat exchanger, here, and thereby controls the temperature of perfusate flowing through the kidney.

Now the experiment that is going on at the present time is one of a series which are designed to determine the optimum rate of addition and rate of removal of glycerol from rabbit kidneys at a constant temperature of 5˚C.

When these experiments are complete we shall then go on to add cooling to the programme; glycerol concentration will be increased during cooling, the kidneys will then be stored at a low, sub-zero temperature, and during rewarming the glycerol concentration will be reduced again on this equipment. The ultimate object being of course to develop a method of long-term storage of kidneys for transplantation.

<**End credits**>