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A Non-Frozen Living Tissue Bank for Allotransplantation Using Green Tea Polyphenols

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Generally, mammalian cells and living tissues can be cryopreserved in a frozen state at very low temperatures over a long storage term. The survival rate of cell suspensions is often acceptable however, living tissues suffer a variety of injuries. In this paper, it was demonstrated that the addition of polyphenols extracted from green tea to conventional cell culture medium and tissue compatible liquid, can control cell proliferation and also preserve tissues for several months at ordinary room temperature, including such tissues as blood vessels, cartilage, islet cells and corneas. This protocol allows a non-frozen living tissue bank to be established using the preservation fluid described.

Key Words: Polyphenol, tissue bank, proliferation control, tissue preservation, cryopreservation

INTRODUCTION

Generally, cells are preserved in a frozen state at -196℃.[1-3] The survival rate after such storage has been enhanced by controlled temperature freezing. However, cell survival after freezing can be low (20 - 40%), as with ES cells (embryo stem cells) and EG cells (embryo genital cells). It would be of great advantage to workers in the stem cell field, and potentially to future patients, to be able to preserve these cells more successfully. It would also be of benefit to be able to preserve other cells, such as platelets, over the long term without freezing. Likewise, research continues in our attempts to prolong the time for transplantation of solid organs, and in the development of optimal perfusion fluids that protect against ischemia remains an active subject of investigation.

After transplantation many organs suffer from the generation of free radicals following reperfusion. The restoration of blood flow becomes a trigger, with subsequent lipid peroxidation of the biomembrane, leading to membrane failure and as a result, the transplanted organ fails. A logical goal would then be the development of a preservation fluid that would limit cell damage by preventing peroxylipid generation. Such a preservation fluid should limit cell division and multiplication. A room temperature storage state could potentially prevent the injury to small vessel endothelium seen with freezing and to delicate tissues, such as the cornea, which do not survive freezing well. Although they can be held from 4 to 24 hours at 4℃, large organs impose a severe time limitation on the medical team.[4-6] In addition, through advances in tissue engineering, cultured skin and cultured cartilage have reached the level of clinical application and demand long term storage techniques for optimum utilization. Transplantation of xenogeneic organs from genetically prepared animal donors would likewise benefit from the possibility of longer periods of organ storage.

It has now been found that the polyphenols in green tea promote the preservation of tissues, such as blood vessels, cornea, nerve, islet cells, articular cartilage and myocardium, at room temperature. Further, in the case of hematopoetic stem cells, the polyphenols suppress the differentiation into erythrocytes, T cells and B cells. These findings suggest the possibility of a new method of tissue banking without freezing.

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THE POLYPHENOL

Polyphenol is a phenol with the hydroxyl group over the 2nd carbon, and has properties completely different from other phenolic chemicals such as hydroxybenzene. The chemical structure of green tea polyphenol is shown in Fig. 1. It is possible to classify these polyphenols into flavonoid hydrolysis type tannins and other polyphenols. Various chemical compounds are known within the polyphenol group. Representative members include catechin, which is mainly found in green tea and oolong tea, etc., and anthocyanin, which is the red pigment in red wine and tannin (the tannin is separated from the proanthocyanidin in the hydrolysis type tannin), which is a main component of the persimmon tannin of red wine, and are also companions of the polyphenol. The antioxidation power is strong for green tea as well as for catechin, which is found in red wine, and for proanthocyanidin, and these agents are known to be associated with a lower morbidity from a heart disease.[7-9] It has also recently been reported in Nature that the multiplication of cancer cells was suppressed by polyphenols.[10,11]

RESULTS AND METHODS

Multiplication control of the mammalian cell[12]

The rat fibroblast, L-929, was cultivated in EMEM (with kanamycin 60 mg/1) supplemented with 10% fetal bovine serum. A cell proliferation test was carried out at a cell density of 1.76×10^5 cells/ml. The polyphenol (250 μg/ml concentration) was added to another culture system as a control. The supplemental effect of the polyphenol in the rat fibroblast culture is shown in Fig. 2. In the polyphenol additive system, the form of the cells became round, although in the polyphenol purity system cell proliferation was active, and the cell population also increases at 1×10^6 cell/ml on the fourth day after cultivation, but the multiplication stopped for 1 week, and would resume when the polyphenol was removed.

The result of the cell cycle measurement of the fibroblast by flow cytometry is shown at Table 1. For in polyphenol additive system, the G0, G1 and G2 M-phases increased, although after 9 hours, the S phase reached 0. It was then recognized that the S phase increased with the absorption of the polyphenol. This suggested that the polyphenol

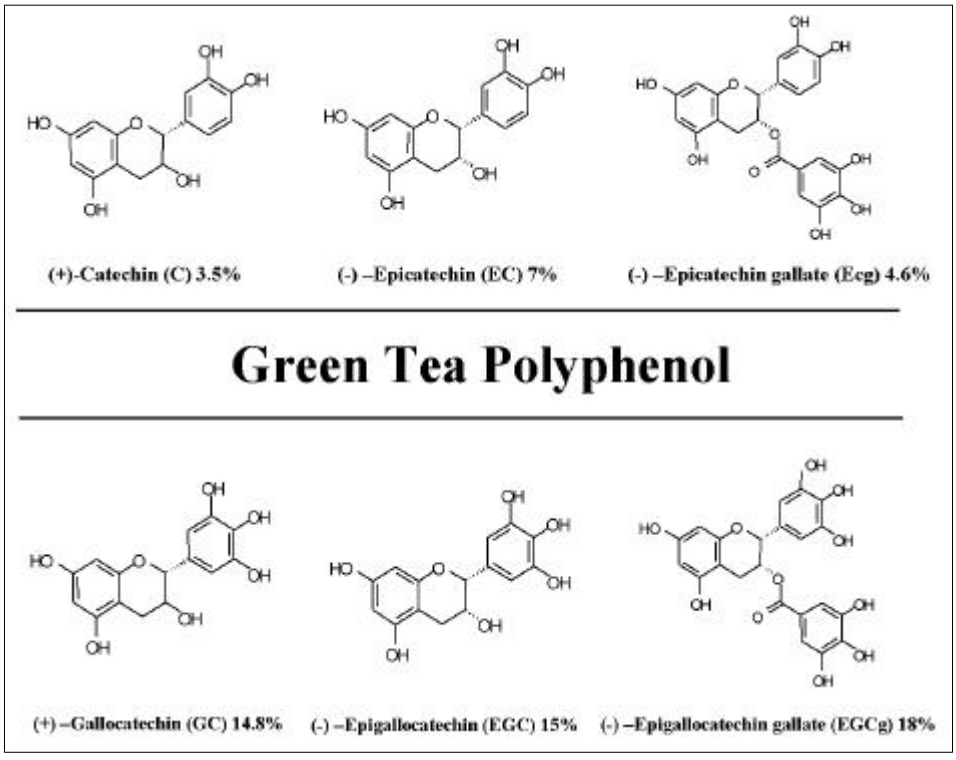

Fig. 1. The chemical structure of phenol and green tea polyphenol.

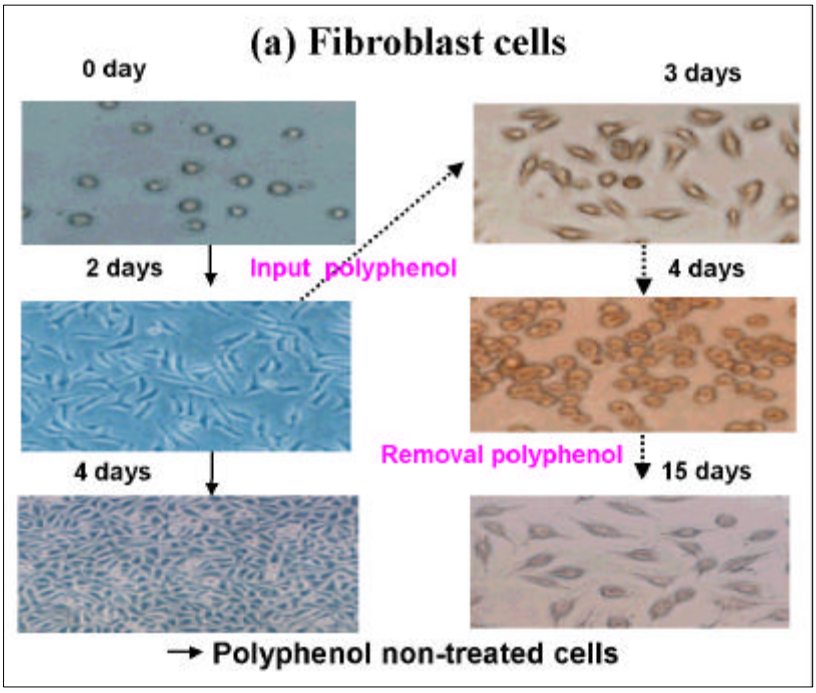

Fig. 2. The effect of polyphenols on the multiplication of the fibroblast. (→) Polyphenol free. (→) Polyphenol addition.

Table 1. Time Changes in the Cell Cycle of Untreated and Polyphenol Treated (250 μg/ml) Fibroblasts

Polyphenol	Cycle (%)	Time (hr)				
		0	2	4	9	48*
	G0G1	22.18	15.69	22.86	18.99	
	G2M	2.58	1.12	11.93	20.85	
	S	75.24	83.19	65.22	60.16	
	G0G1	22.18	33.05	52.96	70.07	71.33
Treated cells	G2M	2.58	11.00	13.83	29.93	20.54
	S	75.24	56.95	33.21	0	8.13

*Cell cycle fibroblasts cultured in medium only during 48hr after removal of polyphenol from medium.

normally controls the multiplication of the cell. A similar phenomenon was confirmed in the hepatocyte of the pig. In addition, the viable cell population did not decrease.[13] Good results were abtained for the protective effects of green tea polyphenol against reactive oxygen species that induce oxidative stress in cultured rat calvarial osteoblast.[14]

Storage of rat pancreas islets of Langerhans cells[15]

In the pancreas, insula Langerhans (islets) are the endocrine cells that secrete insulin. This islet consists of several thousand β cells, which together number about 100,000 per pancreas. The development of artificial islets and research into medical transplantation of porcine islets are actively advancing in research all over the world, as the number of global diabetics is increasing year by year. Although immune rejection remains a major problem, islet preservation is also an important problem. Now that islet cells are able to be frozen, the survival rate is still only 10 - 30%. Thus, our attempt at their long term preservation with polyphenols has been encouraged.

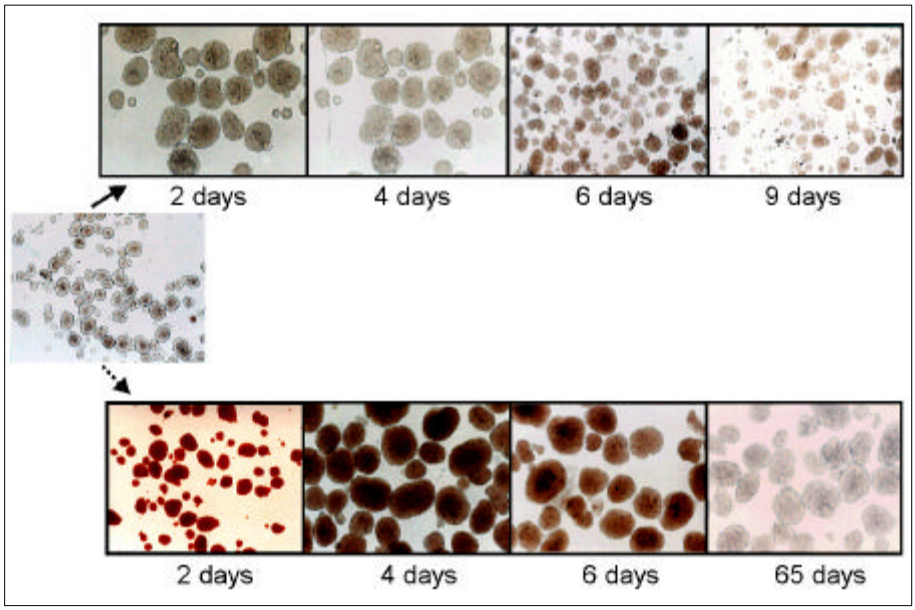

Fig. 3. The effect of the polyphenol on the preservation of the rat islet at 37℃. (→) Polyphenol free. (⇢) Polyphenol addition.

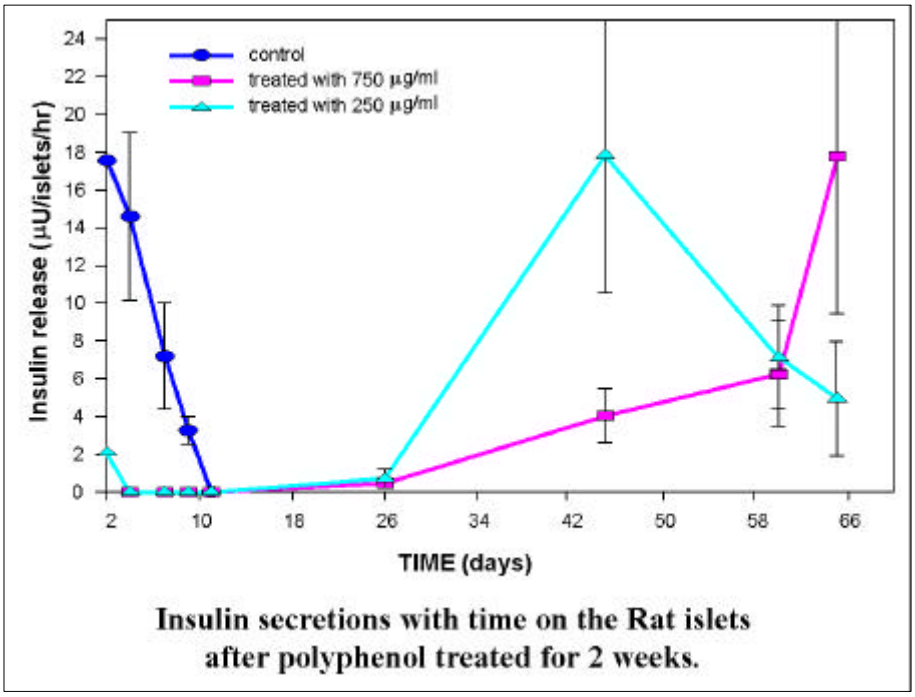

Fig. 4. Insulin release behavior from the rat islet during long term storage at 37℃. ●: The control (polyphenol free). ▲: Polyphenol addition (250 μg/ml). ■: Polyphenol addition (750 μg/ml).

2000 Islets were collected from the pancreata of Wistar rats (380g body weight), and the cells of 200 were cultivated using RPMI-1640 at 37℃. The morphological change of the islets with culturing is shown in Fig. 3. In the polyphenol free system 40 and 100% of the islets had been destroyed by the fourth and ninth days, respectively. In the polyphenol additive system, the form of initial grape-like aggregate persisted for 1 month. Ultimately, the initial form was maintained for 65 days of culture, with no abnormality.

Next, the insulin secretion capacity was examined in order to evaluate the function of the preserved islets and the results are shown in Fig. 4: Insulin release of the polyphenol free system and the islets of the additive system. The poly-

Table 2. The Recovery Rate and Viability of Human Islets after Treatment of Different Concentration of Polypohenol

Concentration of Polyphenol (μg/ml)	Survival rate (%)		Viability (%)	
	After 24 h	After 48 h	After 24 h	After 48 h
	55 ± 9.6	18 ± 2.6	96 ± 1.5	93 ± 1.3
30	93 ± 5.7[a]	45 ± 9.5	95 ± 1.3	94 ± 1.4
60	92 ± 8.3[b]	57 ± 14	95 ± 0.9	95 ± 1.1
125	97 ± 13[b]	77 ± 17[b]	95 ± 1.0	94 ± 1.8
250	95 ± 9.9[b]	63 ± 11[b]	96 ± 1.0	95 ± 1.4
500	85 ± 15	52 ± 15	95 ± 9.4	93 ± 2.0

[a]$p<0.05$, [b]$p<0.01$.

phenol free system islets showed an initially high insulin release, which was reduced to zero after the 10th day. In the polyphenol additive system, no insulin was secreted during the initial 18 days, but thereafter the secretion was significant, and showed a maximum at a polyphenol concentration of 250 μg/ml after 6 weeks. In the case of a polyphenol concentration of 750 μg/ml, the maximum insulin secretion value was shown after 9 weeks, and continued for about 1 - 2 months thereafter.

Increases Culture Recovery Rats of Isolated Islets from Human pancreata[16]

Based on above research, the effect of the addition of polyphenol to islets isolated from human and non-human primate pancreata was examined. The islets were isolated from human pancreata not meeting the criteria for clinical transplantation (n=6) and from nonhuman primate pancreata (n=5). The recovery rates of human islets cultured with and without polyphenol are show in Table 2. After 24-h of culturing, the islet yields were significantly higher in cultures with polyphenol at concentrations of 30 ($p<0.05$), 60 ($p<0.01$), 125 ($p<0.01$) and 250 μg/ml ($p<0.01$). However, no significant difference in islet recoveries was noted between cultures with 500 μg/ml of polyphenol and the control cultures. After 48h of culturing, significant differences (Fig. 5) with the addition of polyphenol were only observed at concentrations of 125 ($p<0.01$) and 250 μg/ml ($p<0.01$).

In summary, both the human and nonhuman primate islet recovery rates after short-term cultures were substantially improved with the addition of polyphenol, with no deterioration in the viability and morphology. Furthermore, it was possible to demonstrate that the addition of polyphenol apparently improved the culture recovery rates by precluding apoptotic events in isolated islets. The effect of polyphenol has important implications for improving islet survival rates prior to transplantation. This strategy will be further evaluated *in vivo* to determine whether it might be beneficial for maintaining islets prior to clinical transplantation.

Saving in the long term of the rat peritoneal cavity aorta[17]

The authors tested the long term storage of blood vessels in polyphenol solution. The aorta (3-5 cm) of the rat (11 weeks, Lewis 355g) was preserved in Dulbecco's Modified Eagle's Medium (DMEM) at 37℃ when supplemented with polyphenol solution (1 mg/ml). The cylinder of the aorta collapsed after 1 week of storage without polyphenol, but the cylindrical architecture was maintained in the polyphenol even after 2 months. The photograph in Fig. 6 shows the preserved aorta after polyphenol treatment for 2 months under tension at 37℃.

The tensile strength (Fig. 7) of the aorta preserved in the DMEM polyphenol free system at 37℃ for 2 weeks was about one-fifth that at the time of harvest. However, the polyphenol treated aorta maintained its tensile strength and extensibility after two months of storage, which was equivalent to that at the time of harvesting.

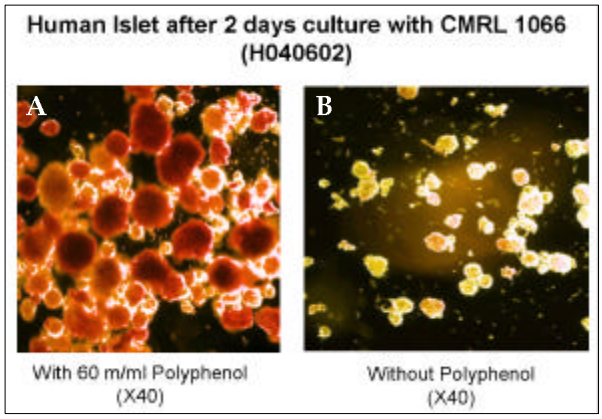

Fig. 5. Human islet after 48 hours of culturing (A) in CMRL 1066 medium supplemented with 60 μg/ml Polyphenol, (B) in CMRL 1066 medium only.

Fig. 6. Photograph of the dog peritoneal cavity aorta preserved for 2 months at 37℃ dealt with in polyphenol (1 mg/ml).

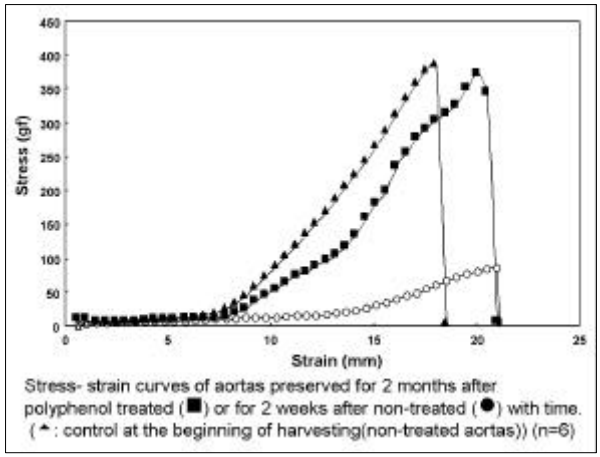

Fig. 7. Stress-warped curve of the tensile test of the rat peritoneal cavity aorta. ▲ : The blood vessel immediately after the extraction. ○ : The blood vessel preserved with the addition of polyphenol to DMEM at 37℃ for 2 weeks. ■ : The blood vessel preserved for 2 months with polyphenol (1 mg/ml) treatment, in DMEM, at 37℃.

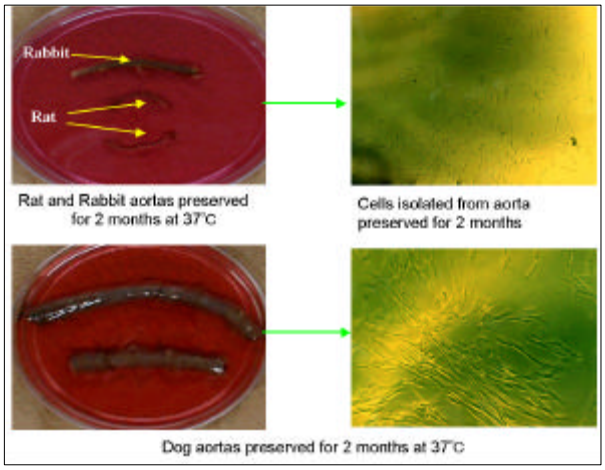

Fig. 8. The appearance of the cells in the aorta preserved after the polyphenol (1 mg/ml) treatment for 2 months at 37℃.

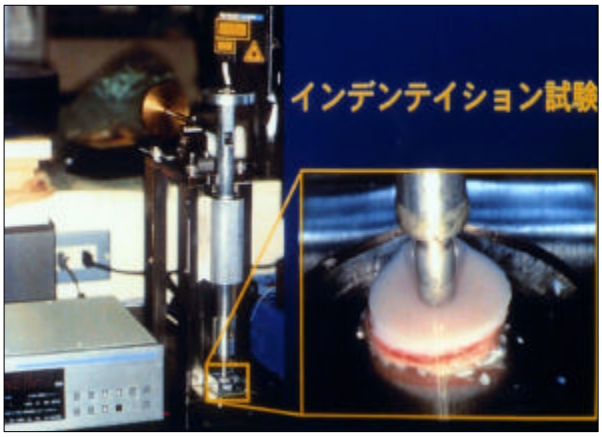

Fig. 9. Indentation test of the articular cartilage collected from the pig patella.

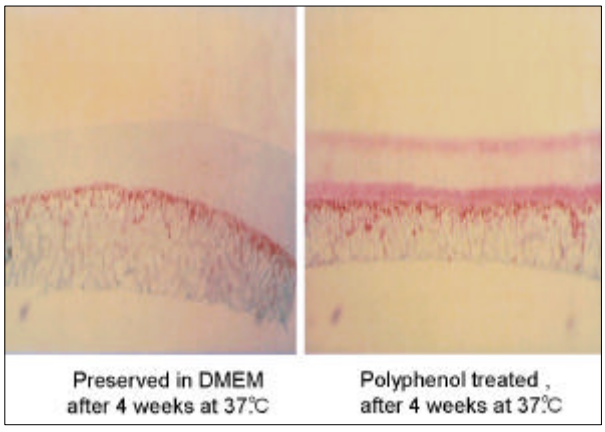

Fig. 10. Photograph of the safranin O dyed tissue section of the articular cartilage preserved for 4 weeks. (Left) Polyphenol free. (Right) Polyphenol (1

The histological appearance of the aorta preserved for 2 months with the polyphenol treatment at 37℃ is shown in Fig. 8. In addition, the homotransplantation of the aorta preserved at 3 7℃ for 2 months with polyphenol treatment was carried out through an abdominal median line incision, with end-to-end interposition. Angiography after 2 months showed the blood flow to be good. When the transplanted aorta was recovered and examined after hematoxylin and eosin staining, its endothelial lining had a normal appearance. The good results obtained were similar, even in the human vein.[18]

Articular cartilage preservation[19]

At present, the cryopreservation of cartilage beyond one week is unsatisfactory due to the deterioration of the mechanical properties when transplanted. To test our new method, a pig patella was placed in a new preservation liquid, which included the addition of polyphenol to the marketing culture solution, and was preserved at 4 and 37℃. The histological evaluation and indentation test shown in Fig. 9 were carried out. In the polyphenol treatment group, the resistance to deformation was similar to the fresh cartilage of the control. Cartilage tissue dyed with safranine 0 is shown in Fig. 10. The dye studies showed a degeneration of the cartilage stored for one month at 37℃. The staining characteristics of the polyphenol preserved cartilage were almost normal, with minimal signs of degeneration.

The long term storage of the rat cornea[20]

The cornea is a complex tissue that consists of cells in discrete layers and mucopolysaccharide that permits the transmission of light with minimal distortion. Freezing is especially damaging to the cornea, and for these reasons they are currently stored cold in a preservation liquid at 4℃. Therefore, various cornea saving liquids have been developed.[21] At present, a representative cornea preserving liquid used by ophthalmology clinics is shown in Table 3. OPTISOL appears to be the best of these preservation solutions, but the period of storage is still only 7-10 days at 4℃. However, the cornea should be used within two weeks, which is very limiting. Therefore, Kinosita et al. tried to extend the length of storage by the addition of green tea polyphenol in OPTISOL. The

Table 3. Type of Cornea Preservation Liquid Currently Marketed and Components

	Constituents of K-sol, Dexsol, and Optisol		
Constituent	K-sol	Dexsol	Optisol
Base medium	Tissue culture medium 199 and Earle's balanced salt solution	Minimal essential medium	Tissue culture medium 199, Earle's balanced salt solution, and minimal essential medium
Buffer	HEPES	HEPES	HEPES
Antibiotic	Gentamicin	Gentamicin	Gentamicin
Chondroitin sulfate	2.5%	1.35%	2.5%
Dextran			
Adenosine	No	1%	1%
Triphosphate precursors	No	No	Adenosine, inosine, and adenine
Iron	No	No	Yes
Cholesterol	No	No	Yes
L-hydroxyproline	No	No	Yes
Vitamins	No	No	Cobalamin, ascorbic acid, α-ocopherol, D-biotin, calciferol, niacin, pyridoxine, and p-aminobenzoic acid

scanning electron microscope photograph of the rat corneal endothelium in Fig. 11 documents the preservation after four weeks in OPTISOL at 4℃. The morphological features of the corneal cells of mammalia include a hexagonal organization. Preservation in the polyphenol containing solution preserves this architectural feature after four weeks of storage.

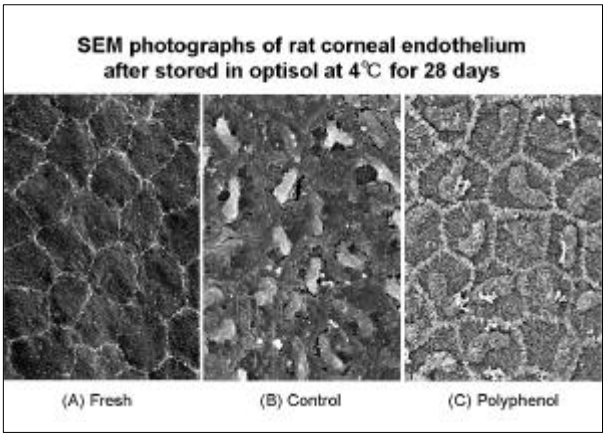

Fig. 11. Scanning electron microscope photograph of the rat corneal endothelium disorder cell. (A) The rat conea disorder immediately after extraction. (B) Rat cornea disorder in OPTISOL preserved at 4℃ for the 4 weeks. (C) Rat cornea disorder preserved after the polyphenol (500 μ g/ml) treatment in OPTISOL at 4℃ for 4 weeks.

The storage of peripheral nerve prior to transplantation using green tea polyphenol[22]

Whether peripheral nerve segments could be kept viable in a polyphenol solution for one month was inestigated. Sciatic nerve segments, 20

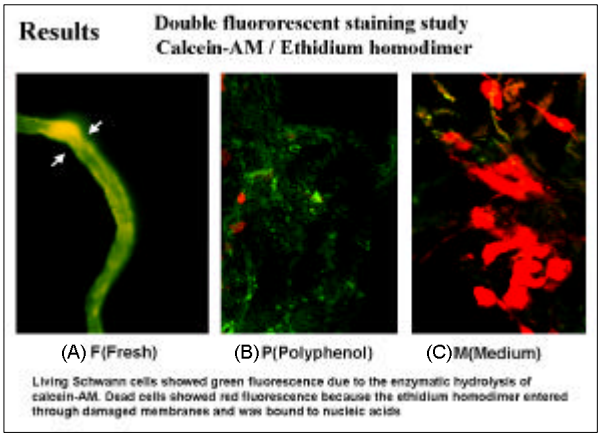

Fig. 12. Electron microscopy of cross-sections of F-, P- and M-nerves after four weeks of preservation. (A): F-nerve group. Intact nuclei and cytoplasmic membranes of Schwann cells are seen. (B): P-nerve graft. Demyelinization was seen, but the cytoplasmic membranes (small arrows) and nuclei (large arrows) of Schwann cells are preserved. (C): M-nerve graft. This graft stored for four weeks in DMEM alone demonstrated the breakdown of myelin, but the Schwann cells were absent (arrow). Scale bars: 5 μm.

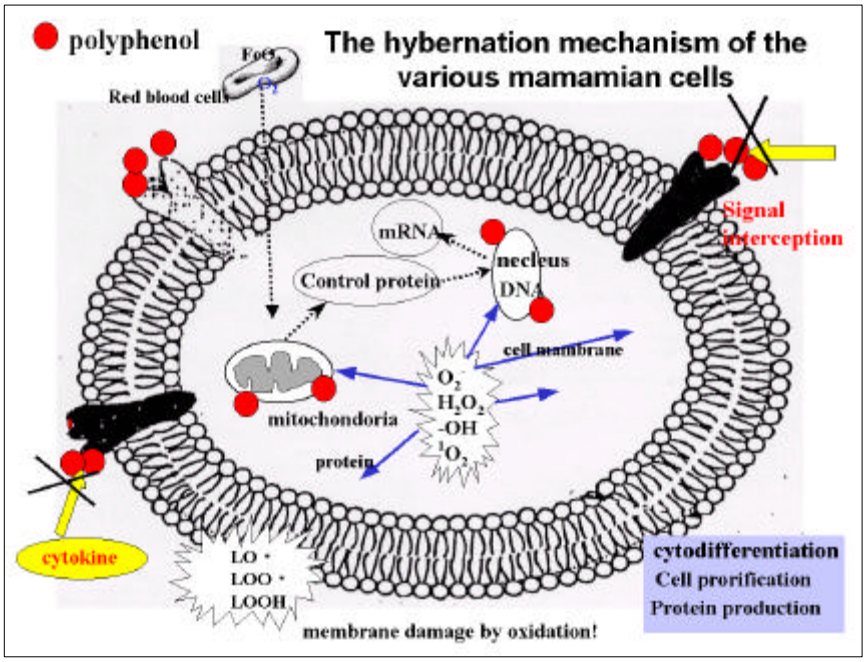

Fig. 13. The cell hibernation mechanism induced by the polyphenol.

mm long, were harvested from Lewis rats and treated in three different ways prior to transplantation to recipient Lewis rats to bridge sciatic nerve gaps created by the removal of 15 mm long nerve segments and the result shown in Fig. 12. Group F: Nerve segments were transplanted immediately after harvesting. Group P: Nerve segments were transplanted after storage in Dulbecco's Modified Eagle's Medium (DMEM) containing polyphenol for seven days at 4℃ and then in DMEM for 21 days at 4℃. Group M: Nerve segments were stored in DMEM solution alone for 28 days at 4℃. The viability of the nerve segments was assessed by vital staining (calcein-AM/ethidium homodimer), electron microscopy and genomic studies prior to transplantation. The nerve regeneration was evaluated using electrophysiological and morphological studies 12 and 24 weeks after transplantation. The neural cell viability of the preserved nerve segments was confirmed in group P, where the nerve regeneration was similar to that in group F, and superior to that in group M. Peripheral nerve segments can be successfully preserved for one month using green tea polyphenol.

MECHANISM OF HIBERNATION OF THE CELL AND LONG TERM STORAGE OF THE LIVING TISSUE

The control of cellular proliferation may be important for the prolonged storage of tissues in polyphenol solutions without freezing. The authors considered the following mechanism as a likely explanation. Polyphenol is a kind of antioxidant that resembles the antioxidant potential of vitamins C and E and superoxide dismutase (SOD), and although unlike, that of glutathione. One of its features is an amphiphilic property, and it also dissolves well in both water and oil. It also has a very high affinity property with protein that allows it to combine with the protein, and yet it dissociates with the progression of time, which is characteristic of a reversible adsorption. For the control of cell proliferation and tissue preservation, the latter characteristic is very important. Due to the high affinity characteristic between polyphenol and protein, the polyphenol adsorbs to a receptor of the cell surface that is integral to the process of cell division when cells are cultured, as shown in Fig. 13. The signal between cells is blocked when the polyphenol combines with this receptor, the S phase in the cell cycle becomes 0 and the multiplication stops under hibernation conditions. As the polyphenol reversibly leaves the cell membrane with the progression of time, the S phase in the cell the cycle and multiplication can resume. The successful long-term storage in the unfrozen state is probably also due to the adsorption of the polyphenol to the collagen and proteoglycan of the extracellular matrix, where it may easily generate temporary cross-linking reactions. Basic research on the interaction between polyphenols and protein has demonstrated interesting reversible absorption phenomenon.[23]

CONCLUSION

As seen above, the polyphenol was able to control the multiplication of various cells, and was demonstrated to be very useful for the long term storage of various tissues, for example, islet cells, blood vessel, cartilage and the cornea. Currently in the U.S.A. approximately 850,000 tissue allografts are transplanted into patients annually. Much of this tissue is stored frozen. The method of cryopreservation at -196℃ was adopted at the University of Tokyo Hospital and the Osaka National Cardiovascular Center in April 1999 here in Japan. Cryopreservation methods are being used for the long term storage of blood vessel, cartilage and skin. Current research is still being directed at improving the effectiveness of cryopreservation and of fluids, such as the University of Wisconsin solution, for tissue transplantation. Here, it has been demonstrated that the preservation of various tissues for up to three months without freezing is now possible, with excellent maintenance of the histological and biomechanical characteristics. The authors have also succeeded in preserving for study long term transplantation rat sciatic nerve, guinea pig periodontal ligament and rat myocardium in a non-frozen condition. This has been made possible by the development of a new preservation fluid with polyphenol as an

additive antioxidant. Therefore, the day is foreseen when the cryopreservation of many tissues through freezing will be superseded by their storage in the newly developed polyphenol tissue banking fluid. This method of cell and tissue preservation will be of great benefit, not only in Japan, but in the world at large, especially in developing countries where cryopreservation is often not possible.

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