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Cryopreservation of Skin Tissues for Skin Grafts

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1. Introduction

Preservation of living tissue and organs are very important to ensure successful transplantation. The storage of living tissue, from the time of removal of the donor tissue until transplantation, is one of the most important factors for successful tissue transplantation. The purpose of living tissue storage is to maintain the viability of the mammalian cells, and various methods have been developed to lengthen the time donor tissue can be stored without loss of cell integrity.

At present, low temperature is used as the major organ preservation method. Generally, cells are preserved in a frozen state at -196°C (1-3). The survival rate after such storage has been enhanced by controlled temperature freezing. However, cell survival after freezing can be low (namely, 20～40%), as with ES cells (embryonic stem cells),EG cells (embryonic genital cells) and induced pluripotent stem (iPS) cells. It would be of great advantage to researchers in the field of stem cell research 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. Various storage solutions for organ preservation, such as the UW solution developed at the University of Wisconsin (USA) are also in current clinical use. However, it is necessary to develop storage solutions that can maintain the viability of tissues and organs for longer periods because of the limitations of storage in UW solution.

After transplantation, many organs suffer from the generation of free radicals following reperfusion. The restoration of blood flow becomes a trigger for injury, 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 also potentially prevent the injury to the small vessel endothelium seen with freezing and to delicate tissues, such as the cornea, which do not survive freezing well. Although these tissues can be held from 4 to 24 hours at 4°C, 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 demanded 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, nerves, islet tissues, articular cartilage and myocardium, at room temperature.(24) Furthermroe, in the case of hematopoetic stem cells, the polyphenols suppress the differentiation of the cells into erythrocytes, T cells and B cells. These findings suggest the possibility of a new method for tissue banking that does not require freezing.

We have been conducting the research on the applications of green tea polyphenol (epigallocatechin-3-*0*-gallate, EGCG) to the regenerative medicine (7-14). In the field of transplantation medicine, which is a subfield of regenerative medicine, we have faced challenging problems associated with graft tissues and organs, including loss of viability and function (15-18), hyperplasia and immunological rejection of grafted tissues after transplantation (19,20). To overcome these problems, we propose the use of a preservation medium containing EGCG, which has been shown to have anti-oxidative (22-34), anti-proliferative (8)and immunosuppresive properties (19).

Polyphenols have recently attracted attention as components of functional foods, and have been shown to have various bioactivities such as anticancer activity, antimicrobal and anti-virus activity since the 1980's.(7-11) Therefore, there are many papers and patents on the use of polyphenols for various applications. However, there has been no research which applies to the use of polyphenols for the preservation of various tissues and organs. We believe that such applications are possible, and we recently found an interesting phenomena related to the effects of polyphenols on mammalian cells and living tissues. We herein describe the effects of polyphenols on living cells and tissues, and present possible applications of polyphenols for their preservation.

2. Preservation solutions for organ and tissue transplantation

Polyphenols have a hydroxyl group attached to the 2nd carbon, and have properties completely different from other phenolic chemicals such as hydroxybenzene. The chemical structure of green tea polyphenol is shown in Figure 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, and anthocyanin, which is the red pigment in red wine The antioxidative effects of green tea polyphenol and catechin, as well as proanthocyanidin, are especially potent, and these agents are known to be associated with a lower morbidity from heart disease (35-37). It has also recently been reported in ***Nature*** that the proliferation of cancer cells was suppressed by polyphenols (38-39).

The success rate of organ transplantation has increased due to the improvements in surgical techniques and the development of new immunosuppressive agents in recent ten years. For instance, in the USA where transplantation is frequently practiced with organs or tissues from brain-dead donors, there were 4 million transplants performed in 2002.

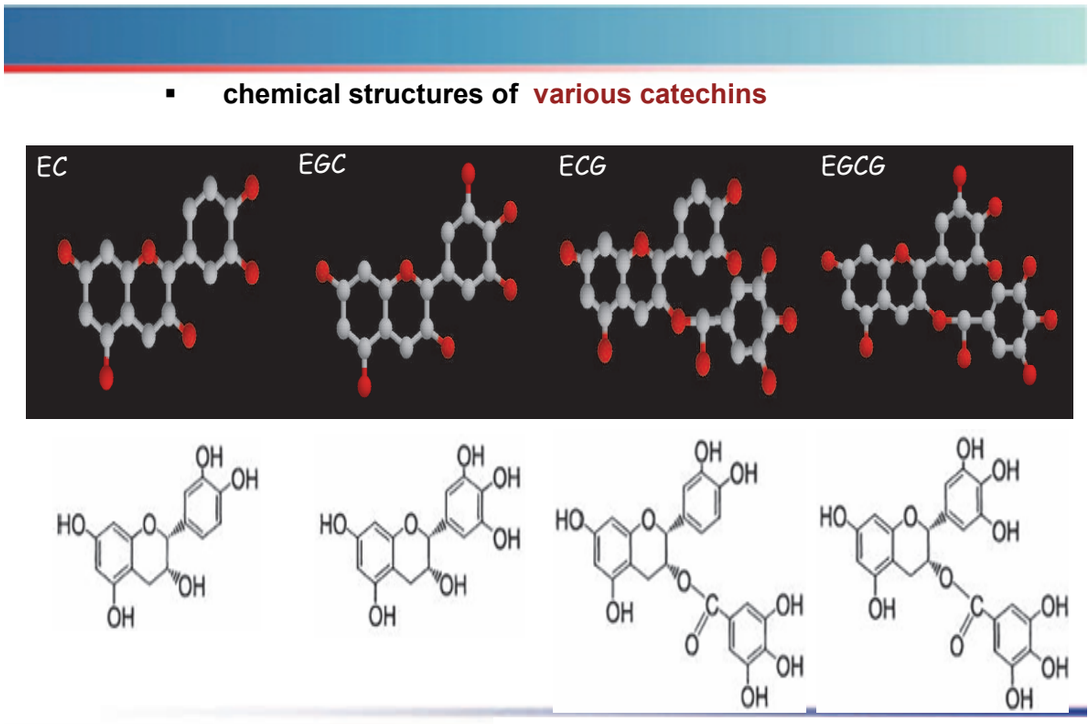

Fig. 1. The chemical structure of green tea polyphenol.

The donor organs or tissues are routinely transported in various preservation solutions. The UW (University of Wisconsin) solution and Euro-Collins solution are the first and the second most frequently used preservation solutions. These preservation solutions are mainly used for the transport of kidneys, liver, or pancreas. However, they cannot preserve the organs for longer than 24 hours at 4℃ (40-44). In light of the increasing demand for donor organs and tissues, preservation solutions with better preserving abilities and the research and development of such solutions are urgently needed.

When organs or tissues are isolated from a donor, their blood circulation stops, and physiological activities rapidly decrease. Varying degrees of ischemia are commonly observed for these organs, and free radicals are generated upon the restart of blood flow, which leads to lipid peroxidation of the cell membrane, causing membrane damage and various dysfunctions of the transplanted organs. The development of a preservation solution that can minimize the oxidation and cell damage can solve this problem.

One of the traditional methods used to solve this problem has been freeze preservation (45-47). Although living tissues and cells are routinely preserved at -196℃, freezing and subsequent thawing cause some structural damage. For example, frozen blood vessels have damage that often make their transplantation difficult, and corneas cannot be preserved at 4℃ for longer than one week without a significant degree of damage. Studies indicate that the cell damage is primarily caused by activated oxygen molecules emerging from the freezing and thawing processes, but also occurs even after exposing the living tissues and cells to ordinary temperatures after removal from the donor.

Recent advances in tissue engineering are now on the cusp of providing clinically useful cultured skin, cartilage and cornea specimens. However, these cultured tissues also require

preservation, and better methods for long term preservation would be beneficial for ensuring that they can be successfully applied in the clinic field. To this end, we introduce the anti-oxidant polyphenol, EGCG, as a means to prevent the cell damage associated with organ and tissue preservation. Using EGCG, we can preserve living tissues and organs allowing for longer storage and more successful transplantation (13-34).

3. Control of mammalian cell proliferation

To study the effects of EGCG on cell proliferation, the rat fibroblast cell line, L-929, was cultivated in EMEM (with kanamycin 60mg/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 effects of the polyphenol in the rat fibroblast culture are shown in Figure 2. In the polyphenol system, the cells became round, although cell proliferation was still active, and the cell population was increased to 1×10^6 cells/ml on the fourth day after cultivation, but the proliferation decreased after 1 week of treatment, and resumed when the polyphenol was removed from the culture media.

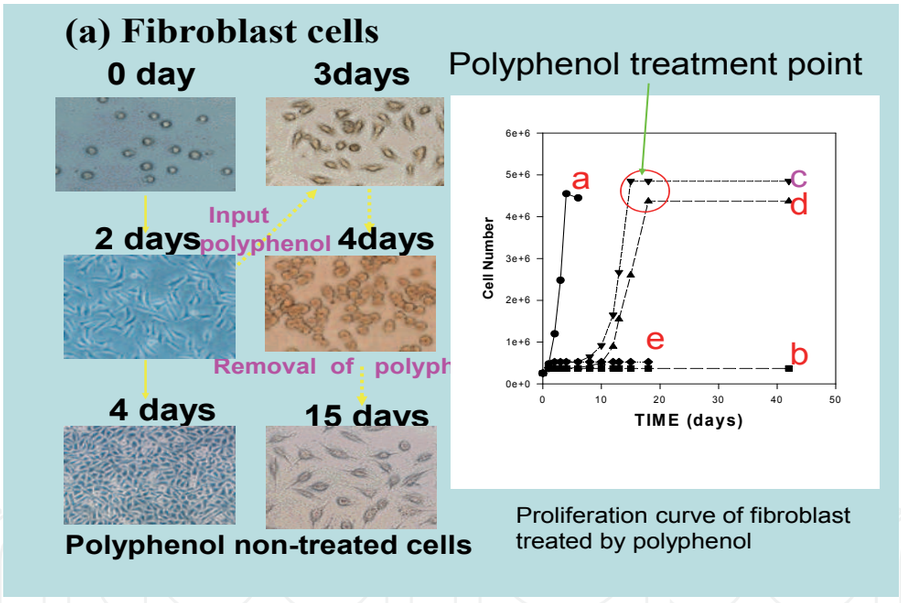

Fig. 2. The effect of polyphenols on the multiplication of the fibroblast.
(⟶) The polyphenol free. (- - →) The polyphenol addition.

We also assessed the effects of the polyphenol on the cell cycle. The results of treatment of the fibroblasts with the polyphenol as determined by flow cytometry are shown in Table 1. For the polyphenol-treated cells, the number of cells in the G0, G1 and G2/M-phases increased, although after 9 hours in culture, the number of cells in the S phase reached 0. A similar phenomenon was observed in porcine hepatocytes. In addition, the viable cell population did not decrease. Good results were also obtained for the protective effects of the green tea polyphenol against reactive oxygen species that induce oxidative stress in cultured rat calvarial osteoblasts.

Polyphenol	Cycle(%)	0	2	4	9	48*
Non-treated	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	.
Treated cells	G0G1	22.18	33.05	52.96	70.07	71.33
	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 p olyphenol from medium

Table 1. Time changes in cell cycle of untreated and polyphenol treated (250 mcg/ml) fibroblasts.

4. Green tea polyphenol affects the preservation of rats skin and improves the success rate of skin grafts

Although skin allografts are used in the treatment of serious burn injuries, skin disorders, and skin defects their use is limited because of the limited amount of normal skin that can be removed for such grafts (48). The best graft for the functional closure of wounds is the autograft. At the end of an autograft procedure, the remaining donor skin is routinely stored in a saline solution and applied to the open wound when graft loss or superficial wound breakdown occurs postoperatively. (49) However, long-time storage in saline leads to poor engraftment, and we were prompted to study other preservation media which would extend the storage time of skin grafts. Recently, it has been found that the polyphenols in green tea promote the preservation of tissues, such as the blood vessel, cornea, nerve, islet cells, articular cartilage and myocardium, at room temperature (12-34). These findings suggest the possibility of a new method for tissue banking without freezing.

As previously stated the dysfunction of transplants occurs as a result of free radicals due to ischemia, which triggers lipid peroxidation of the cell membrane when blood flow is restarted. It is reported that EGCG prevents peroxylipid generation. (50)

To determine whether the addition of EGCG to conventional cell culture medium could enhance the viability of stored skin grafts and also extend storage time, we performed a study using the skin from rats. The storage solution chosen for this study was Dulbecco's Modified Eagle Medium (Sigma, St. Louis, MO, USA) supplemented with 10% Fetal Calf Serum (FCS; Sigma), add and 1% antibiotic solution (including 10000U penicillin and 10 mg streptomycin, Sigma). EGCG was dissolved in the storage solution at a final concentration of

1 mg/ml. Transgenic Sprague-Dawley rats (8 weeks old, male) expressing the green fluorescent protein (GFP) (green 2 Kim et al., produced with the constructs used for the green mouse (51)), were a kind gift from Dr. Masaru Okabe (Genomic Information Research Center, Osaka, Japan).

For anesthesia, pentobarbital (50 mg/Kg) was administered intraperitoneally to the GFP transgenic rats. After shaving and depilating the backs of the rats, the back skins were biopsied. The muscular layers were immediately stripped from the skin biopsies. Skin samples measuring 1×1 cm were kept in sterile containers with 50ml preservation solution at 4℃ and 37℃ for up to 8 weeks. Periodically, some of preserved skin (30 min, 1, 2, 4, 6, 7, 8 weeks) were used for grafts in nude mice or were directly examined histologically.

From histological examinations of the 4℃ preservation skin, it was noted that there was a decrease in the GFP value in the non-EGCG skin noted during the second week (Figure 3), as was a slight degeneration of the epidermal layer. The degeneration of the epidermal and dermal layers were noted beginning at 5 weeks in all 4℃ groups (Figure 4).

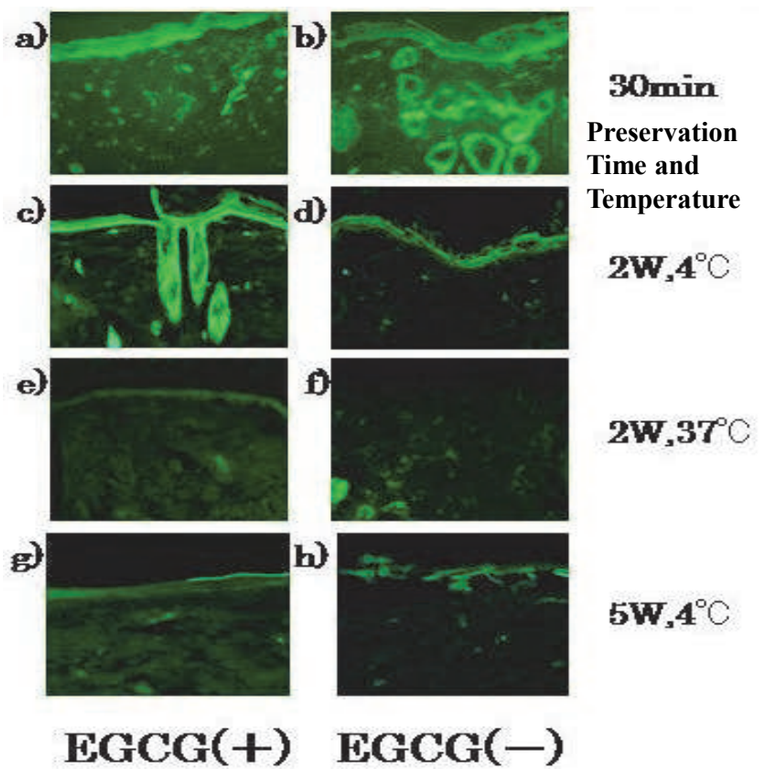

Fig. 3. Photographs showing cryosection of the preserved GFP rat skins. GFP value was slightly decreased according to preservation time.

In the 37℃ preserved groups, degeneration and flakiness of the epidermal layer were noted beginning after one week of preservation, both with or without EGCG, and a decrease in the GFP value was observed, regardless of the presence of EGCG, at 2 weeks. Moreover, the epidermis was damaged in the tissue samples preserved without EGCG, although not in the EGCG group.

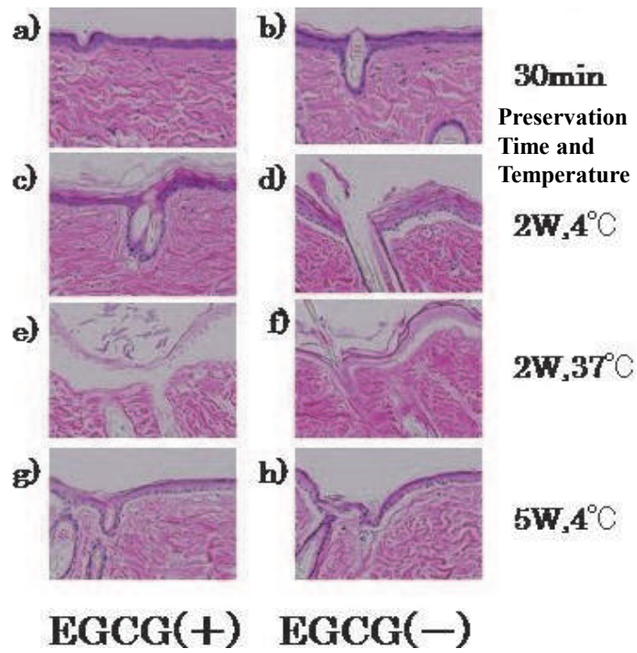

Fig. 4. Photographs showing H.E. stain of preservation GFP rat's skins. Degeneration of the epidermis and dermis was observed, according to preservation time. The degeneration is improved in EGCG groups compared to EGCG(-) groups.

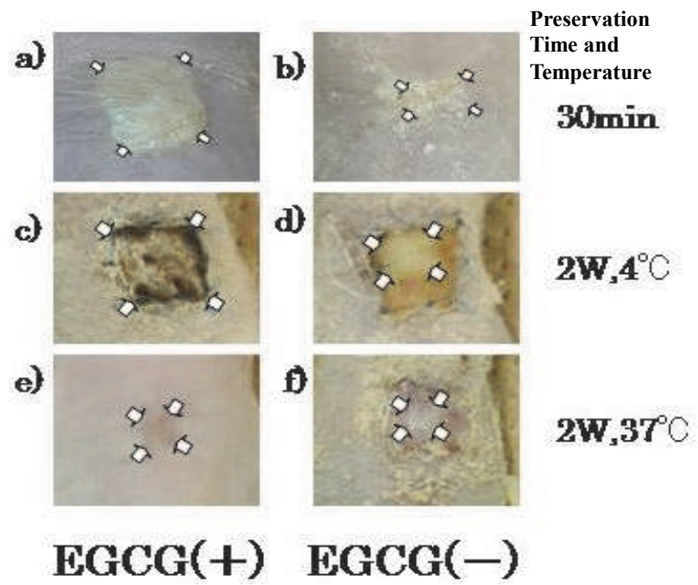

Fig. 5. Appearances 4 weeks after grafting the preserved skins to the nude mice. Thirty minutes preserved skin with EGCG, holds hair (a). On the other hand, skin without EGCG (b) has no hair and the graft size is smaller than that with EGCG groups (a). After 2 weeks preservation with EGCG at 4℃, grafts color were dark like necrosis, but it only remain EGCG. Grafts size were smaller or only scar were observed at all groups, except preservation under 4℃ with EGCG, after 2weeks preservation.

The rat skin grafted onto the nude mice after 30 minute preservation with EGCG showed a better condition than those implanted without EGCG, which showed loss of hair and shrinkage. In the EGCG-preserved groups, the skin color was dark and looked like there was necrosis superficially, however, the color was found to be due to the presence of the EGCG (Figure 5). After 2 to 7 weeks of preservation in EGCG at 4℃, there was good graft survival. In the other groups, the grafts shrunk or were only scar-like because of contracture or rejection.

In the histological analysis, grafts preserved at 4℃ with EGCG were found to have been completely accepted, with both epidermal and dermal layers remaining. On the other hand, grafts preserved at 4℃ without EGCG had no GFP positive keratinocytes or fibroblasts observed, and only phagocytes were observed in the dermal layer (likely targeting the GFP). In the 37℃ preserved groups, no GFP positive rat cells were observed, with or without EGCG (Figure 6). No grafts were successful from the 37℃ preserved groups (with or without EGCG). However, the 4℃ preserved grafts were accepted, and the success rate for the EGCG-treated grafts was 100% even after 4 weeks of preservation (Figure 7).

Wide skin defects caused by burns or trauma should be covered with skin grafts, but sometimes skin grafts fail due to various causes such as hematoma. When skin grafts are partially unsuccessful, small skin defects can be repaired, using the skin leftover from the primary grafting, if it is preserved. Moreover, injuries leading to complicated tears or small flap-like. In such cases, if the skin can be temporarily preserved, it can later be used as part of the graft after the wound condition is improved. Freeze-drying porcine skin, xenografts,

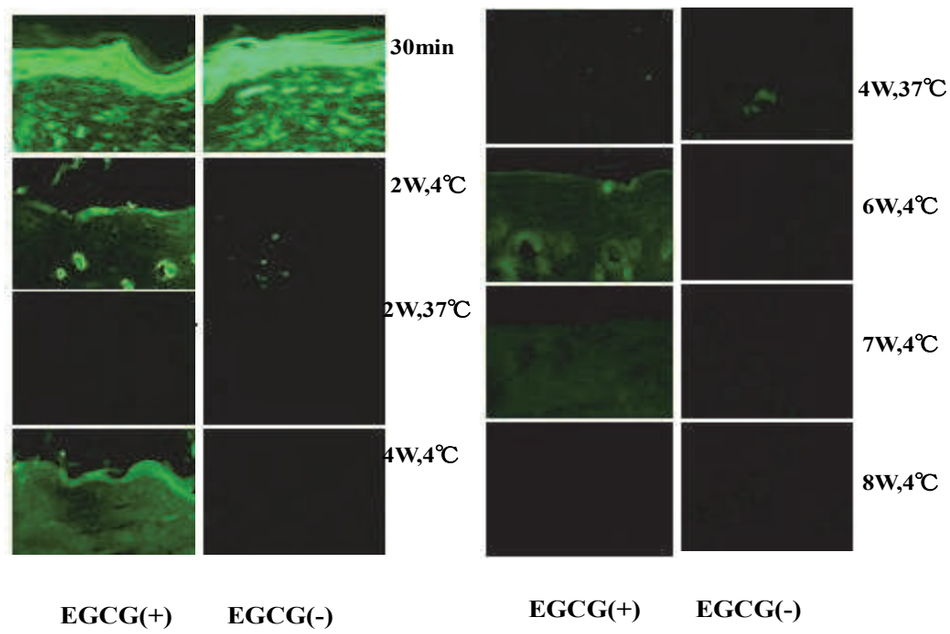

Fig. 6. Photographs showing cryosection of the GFP rat skins 28 days after grafting to nude mice (n=4-6). EGCG positive keratinocytes were observed only in the grafts preserved in 30 minutes preservation groups and 4℃ preservation groups with EGCG, 2weeks to 6 weeks (a, c, g, k). EGCG positive fibroblasts were seen only in grafts preserved at 4℃ with EGCG, 30 minutes to 7 weeks (a, c, g, k, m). In other groups, only phagocytes which ate the GFP were observed.

and frozen allografts are also used for wound coverage, but they are not usually permanently incorporated. Therefore, it is very useful to develop better technologies for the preservation of autografts.

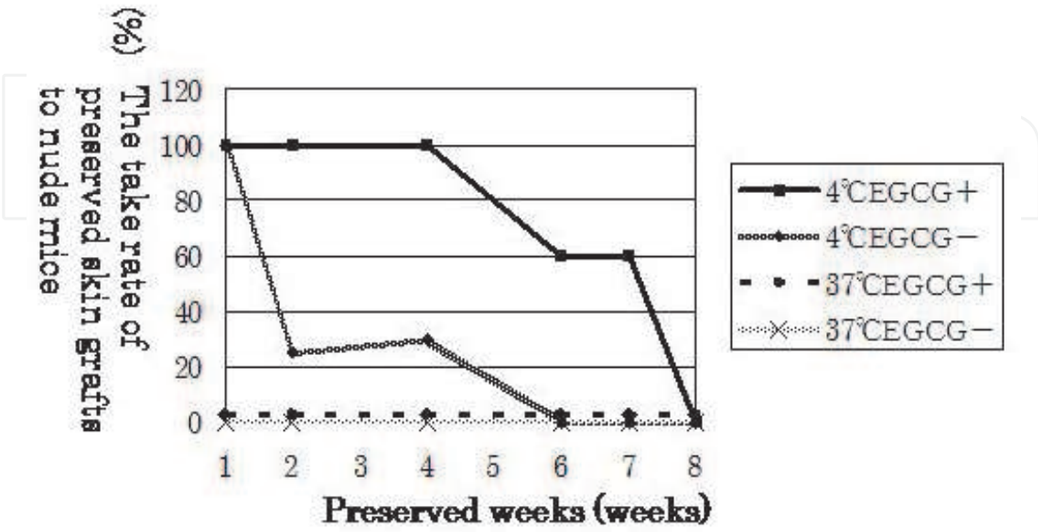

Fig. 7. The take rate of preserved skin grafts to nude mice, judged 28 days after surgery. No grafts took in 37℃ preserved groups with or without EGCG. Take rates were improved by addition of EGCG, at 4℃ preservation.

There have been a few reports on the preservation of the cornea, blood vessels, nerves, pancreatic islet cells, saphenous veins and peripheral nerves using EGCG. In the first reported experiment, histological examination revealed that EGCG improved the length of preservation time a skin sample. With time, skin samples start to degenerate from the epidermal layer to the dermal layer. Skins samples preserved at 4℃ seem to undergo less degeneration than the ones at 37℃. However, it was difficult to judge the cell viabilities based on only histological examinations, because GFP was still present in all of the skin samples. Therefore, we investigated the viability of the skin specimens by examining whether the samples could lead to successful grafts. It has been reported that the dead tissue or skin is rejected by around two weeks after implantation. (52, 53)

We used immunodeficient mice as recipient animals and judged the success of skin grafts from GFP-Tg rats. The results indicated that the preservation of the skin with 37℃ is not possible even if EGCG is added. Similarly, AE CRAM et al. reported that only 1/3 of their grafts were successful at ten days after transplanting skin samples preserved at 4℃ for two weeks. In our study, at 4 weeks after transplantation of skins preserved for 4 weeks at 4℃ with EGCG were successful, and one-third were still successful even after preservation for 7 weeks at 4℃ with EGCG. We therefore concluded that EGCG was beneficial for the preservation of skin samples by decreasing the degeneration of the epidermal layer and suppressing the postoperative graft contraction.

There might be several reasons why EGCG improved the preservation of the skin. EGCG has strong anti-oxidative activities. The strong anti-oxidative activity of EGCG might inhibit the lipid peroxidation of cell membrane of the preserved skin. M. Kapoor et al. reported that

EGCG had anti-inflammatory and free radical scavenging effects *in vitro*. (54) EGCG was also reported to control cell division. We believe that a combination of these effects improved the condition of the preserved skin. In addition, the actions of EGCG, such as was strengthening the scaffold structure, and its antibacterial activity also seem to have contributed to its preserving effects. EGCG also improved the quality of scars after full thickness incisions in rats, likely because EGCG increases iNOS (inducible nitric oxide synthase), COX-2 (cyclooxygenase-2), and VEGF (vascular endothelial growth factor) expression, leading to the formation of new blood vessels. EGCG also has been shown to decrease arginase-I activity and protein levels. (54)

Our results suggest the possibility of the future clinical use of EGCG for skin preservation without freezing, although the mechanism underlying how EGCG exerts its beneficial effects on skin preservation still remains unclear.

5. Long term preservation of rat skin tissue by EGCG

EGCG enhances the viability of stored skin grafts and also extends the storage time for up to 7 week at 4°C. The addition of EGCG to conventional freezing medium, "Cell Banker" (CB), could enhance the viability of skin grafts stored at −196°C and also extend their storage time. Metabolic assays have been used as a surrogate measure of overall viability in the grafts. Skin tissue was transplanted from GFP transgenic rats into immunodeficient mice, and cell migration in graft tolerance of GFP positive cells was investigated after transplantation (55).

For anesthesia, pentobarbital (50 mg/kg) was administered intraperitoneally. After shaving and depilating the back of the rats, the back skin was elevated. After procurement, the muscular layer was immediately stripped from the skin biopsies. Skin samples from GFP rats measuring 1 × 1 cm were kept under sterile containers and refrigerated with PBS solution with or without EGCG for 1 night. Skin samples were then transferred to CB solution with or without EGCG and were stored in liquid nitrogen (-196°C) for up to about 24 weeks. Periodically, the preserved skin grafts of GFT rats were transplanted into nude mice (2, 8, and 24 weeks). Circular skin biopsies (8 mm in diameter) were also made using a sterile dermal biopsy punch (Kai Industries Co., Ltd. Gifu, Japan) and preserved in the same way for in vitro analysis.

A full-thickness excisional square wound (1 × 1 cm) was created on the dorsum of each nude mouse, and the GFP rat skin specimens were sutured to the adjacent normal skin with 6-0 ProleneTM thread. After surgery, the mice were housed in separate cages. Skin grafts were excised with 2–3 mm of the surrounding tissue, bisected, and processed for histology 14 days after transplantation (n =4–6).

The *in vitro* results showed that 1 mg/ml EGCG had the highest protective effect on skin samples when it was used at −196℃ for 2 weeks. At concentrations higher than 1.5 mg/ml, EGCG delayed the recovery of the metabolic activity of skin samples after thawing (Figure 8). Figure 9 shows that glucose consumption was gradually increased with time and the skin samples preserved with EGCG showed higher glucose consumption in all groups after preservation. The in vitro data showed decreased glucose consumption in the 8-week-preserved group in comparison to the 2-week-preserved group. By comparing Figures 8 and 9, We concluded that the presence of DMSO and EGCG in the freezing medium led to more effective maintenance of glucose consumption than freezing medium alone. Glucose

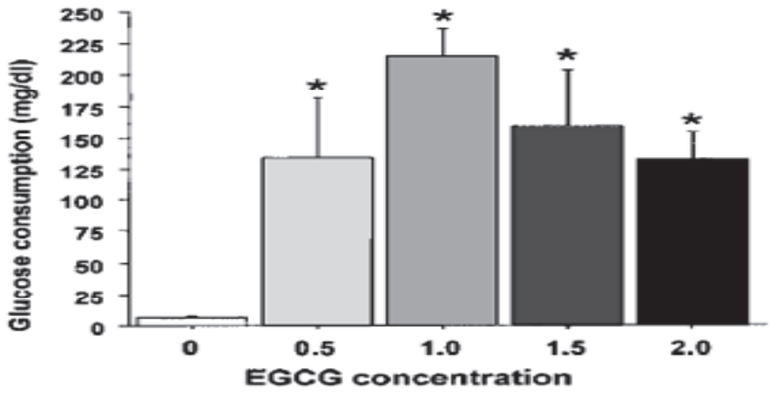

Fig. 8. The optimum concentration of EGCG for rat skin preservation was determined by measuring the metabolic activity. Skin biopsy samples were preserved in cell culture medium with various concentrations of EGCG and preserved at −196°C for 2 weeks. Glucose consumption was measured after 6 days of 37°C incubation after thawin.

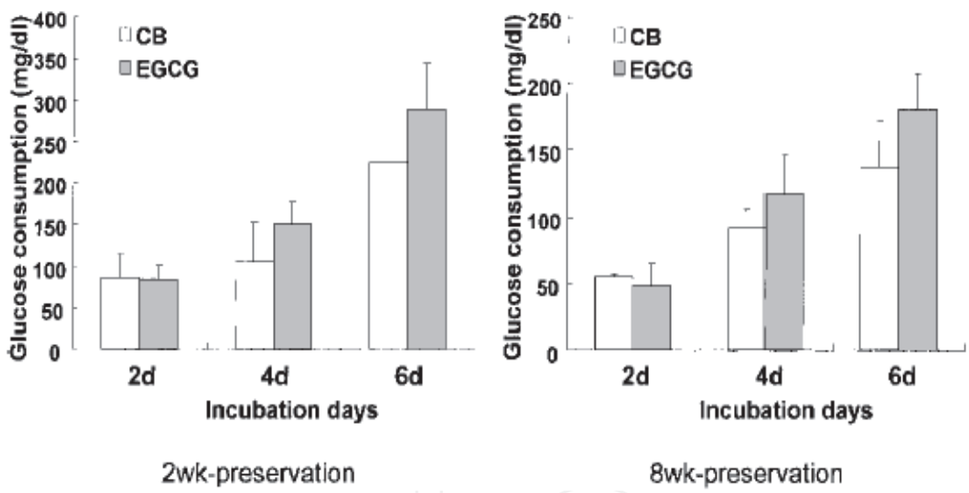

Fig. 9. Glucose consumption of the preserved skin biopsies after thawing. Skin biopsy samples were preserved in freezing medium (CB) with or without EGCG at −196°C for 4 and 8 weeks. There was no significant difference; however, the amount of glucose consumption in the EGCG groups showed faster metabolic recovery in comparison to the CB groups.

consumption by the 2-week-preserved skin samples treated with only EGCG after 6 days was about 220 mg/dl while the 4-week skin samples preserved with EGCG and DMSO was almost 300 mg/dl. After day 7 the possibility of bacterial contamination increased and false-positive data were obtained. All skin samples were washed with PBS containing antibiotics every 2 days. The GFP fluorescence appeared to be similar between the 2- and 8-week-preserved groups (Figure 10). The degradation of the epidermal layer to the dermal layer was observed in all groups, regardless of preservation. However, while the Skin grafts of GFP rats preserved in CB showed only green florescence around the dermis of the transplanted area in the 24-week-preserved group, the fluorescence of the epidermal layer

and some viable cells were confirmed in the EGCG- preserved skin grafts. The degradation was therefore improved by EGCG in comparison to the CB group. An evaluation of the viability of the transplanted skin grafts was difficult with GFP confirmation only. The histology of the transplanted skin grafts of the GFP rats and nude mice was also therefore evaluated using H&E-stained sections (Figure 11). The grafts preserved with EGCG showed a dense dermal matrix and an intact epidermal layer, as shown in the GFP pictures. Micrographs of CB preserved skin grafts were not fully rejected; however, separation between the transplanted skin of the GFP rat and immunodeficient mouse was observed near the location of the original epidermal layer. The histological score was marked with micrographs of H&E-stained specimens based on the attached area of the transplanted skin graft and the condition of the surviving skin grafts (Figure 12). Dead and completely rejected skin grafts scored 0. The surviving skin grafts with a widely attached area, intact epidermis, and dermis with hair follicles and a dense matrix scored 5. Neither the survival rate of the skin grafts nor the histological scores indicated a clear difference between the 2-, 8-,and 24-week-preserved groups. However, both the survival rate of the skin grafts and the histological scores were increased by EGCG in all groups.

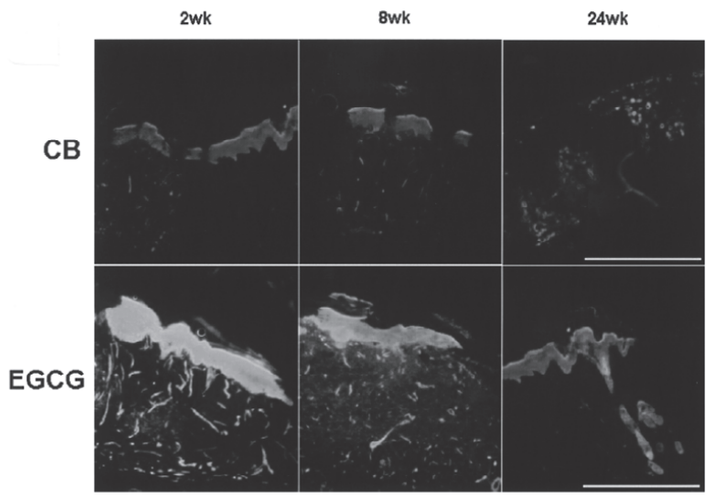

Fig. 10. Cryosections of GFP rat skin graft to a nude mouse (left: 2-week-preserved group, middle: 8-week-preserved group, fight: 24-week-preserved group). These micrographs are representative of four to six independent experiments, and showed similar results.

EGCG therefore enhances the viability of stored skin grafts and extends the storage time up to 7 weeks at 4℃. In this study, the storage time of the skin grafts was extended to 24 weeks by cryopreservation with both DMSO and EGCG. The survival rate of the transplanted skin grafts reached almost 100% in the 24-week-preserved group, indicating that long term storage is possible.(62)

Cryopreserved cells and tissues are increasingly being used for stem cell transplantation and tissue engineering. However, they are highly sensitive to freezing, storage, and thawing, which suggests the need for improved cryopreservation methods. When storing any living tissue, the destructive effects of hypoxic metabolism must be controlled. A tissue removed from its blood supply will die unless cellular metabolic activity is decreased or nutrients are provided. By reducing metabolism and providing nutrients, viability can be improved (56). EGCG was reported to control cell division, which causes the energy metabolism to decrease

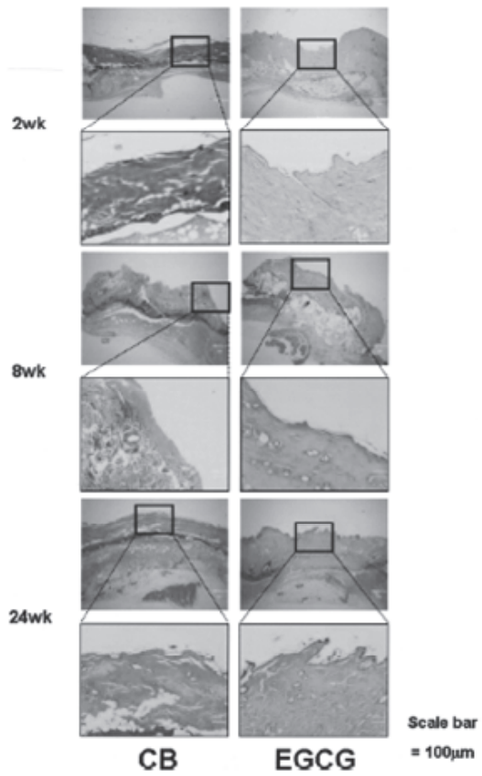

Fig. 11. Histological observation in skin grafts with H&E-stained micrographs (top: original magnification 5·, bottom: original magnification 20·). Degradation of the epidermal layer to the dermal layer was observed in the CB groups. The degradation was improved by EGCG in comparison to the CB groups. These photographs are representative of four to six independent experiments, and showed similar results.

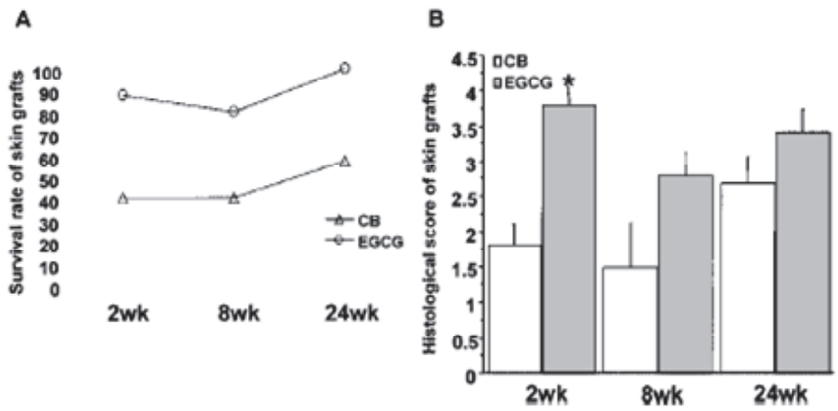

Fig. 12. (A) The survival rate of grafted GFP rat skin after 2 weeks. (B) Considering the epidermal morphology and dermal integrity of the transplanted grafts, a score was given from 0 to 5. A higher score indicates better condition of the skin grafts. The results are reported as the mean ± SE (n = 4–6) and analyzed by Fisher's PLSD test. The value marked with an asterisk is significantly ($p < 0.05$) different from the nontreatment groups.

by inhibiting the cell cycle. Therefore, the combination of such effects improves the condition of the preserved skin. In addition, EGCG reduces ischemia/reperfusion injury by attenuating nitric oxide synthase expression and activity, and improving hypoxic metabolism in preserved skin (57). Furthermore, other activities of EGCG, such as strengthening of the scaffold structure, immunomodulatory effects, and antibacterial activity, may also contribute to its preservative effects.

In the present study, the protection afforded by EGCG was similar for all three groups. EGCG and DMSO in the freezing medium were more effective for maintaining glucose consumption. These results suggest that oxygen radicals scavenged by EGCG may be responsible for damage at the membrane level, and thus, exogenous EGCG can protect cell membranes. These phenomena may be related to the intrinsic characteristics of polyphenol compounds, which readily penetrate cell membranes due to their amphipathic properties. These compounds are easily absorbed by lipid bilayers, extracellular matrices (e.g., collagen, fibronectin), and various cell membrane receptors. The absorption of polyphenolic compounds by such proteins is rapid, while the adsorption rates are slow. Consequently, the skin graft could be protected from freeze–thaw injuries due to absorption of EGCG by various membrane proteins and lipids (28). The glucose consumption of thawed tissues was assessed after 2, 4, and 6 days of incubation under tissue culture conditions. This post-thaw incubation period was selected to allow for recovery of the metabolic activity in damaged cells. These delayed measurements may be more characteristic of the actual viability of the skin allografts than measurements taken immediately after thawing and dilution. The cells may sustain lethal damage during the thawing and dilution process but have not yet completely disintegrated; thus, there may be residual metabolic activity in the lethally damaged cells immediately after thawing.

The viability of the preserved skin biopsy samples was investigated by examining whether skin specimens could be successfully grafted after preservation. It is reported that dead tissue or skin is rejected in around 2 weeks (58,59). Therefore, immunodeficient mice were used as recipient animals to judge the success of skin grafts from GFP rats (61). GFP fluorescent protein does not need any chemical substrate for visualization. By using a GFP transgenic animal, transplanted cells or tissues having GFP can be detected by direct visualization. Therefore, the GFP transgenic rat is thought to be a suitable skin donor for skin storage and transplantation studies.

A histological analysis demonstrated that the preserved skin began to degenerate from the epidermis to the dermis. The epidermis is easily degenerated during the preservation processes. Considering the histological data presented in previous studies, the results obtained after 2 weeks of preservation seem to be better for samples stored at 4℃ than those with cryopreservation. However, the success of the 4℃ preserved skin grafts was limited to a 7-week or shorter period, as the samples at 8 weeks no longer led to successful grafts.

EGCG therefore enhances the viability of stored skin grafts and extends the storage time up to 7 weeks at 4℃ (61). In this study, the storage time of skin grafts was extended to 24 weeks by cryopreservation using EGCG, and the survival rate was almost 100% in the samples preserved for 24 weeks. These findings suggest that there may be future clinical applications for EGCG as an agent for skin preservation without freezing, although the mechanism by which EGCG promotes skin preservation remains to be elucidated (62).

6. Induction of hibernation of the cells and long term storage of living tissues

The control of cellular proliferation may be important for the prolonged storage of tissues in polyphenol solutions without freezing. Polyphenol is a kind of antioxidant that has similar antioxidant potential as vitamins C and E and superoxide dismutase (SOD). Because of its amphiphilic properties, it also dissolves well in both water and oil. It also has a very high affinity for protein, which allows it to combine with the protein, and yet dissociate 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 between the polyphenol and proteins, the polyphenol can adsorb to a receptor on the cell surface that is integral to the process of cell division when cells are cultured, as shown in Figure 13. The signaling between cells is then blocked when the polyphenol bonds to this receptor, and the cell cycle is inhibited, resulting in no cells entering the S phase, inducing a type of hybernation. As the polyphenol reversibly leaves the cell membrane with the progression of time, the S phase of the cell cycle, and cell proliferation, 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 proteins have demonstrated interesting reversible absorption phenomena.(63)

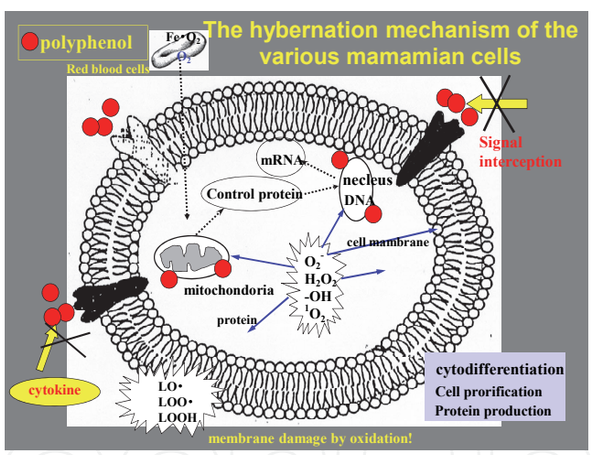

Fig. 13. The cell hibernation mechanism induced by the polyphenol.

7. Potential mechanisms for non-frozen preservation of mammalian tissues and organs by polyphenols

All of these studies were started based on the author's observation that polyphenols appeared to be useful for the physiological preservation of tissues or organs, particularly rat pancreatic islet cells [13]. Since then, evidence has been accumulating showing the beneficial effects of polyphenols on cell preservation. The extension of that observation to the preservation of tissues or even organs for transplantation will make it possible to store them for longer periods by regulating the concentration of polyphenols present in the storage solutions. Recently, it was reported that polyphenols can reduce the ischemia/reperfusion injury in rat lungs and preserve various types of tissues or organs including canine lungs,

rat aorta, rat peripheral nerves and mammalian pancreatic islet cells (12-34), supporting the author's hypothesis.

As shown in Figure 13, this non-frozen preservation of mammalian cells, tissues or organs might be mediated by hibernation, a reversible regulation of cell proliferation and survival through modulation of cell cycle-related genes by the polyphenols at the cellular level. With regard to this hypothesis, it has already been reported that the hibernation phenomenon triggered by polyphenols might be related to their intrinsic characteristics, including their bind with and penetration into the cell membrane or tissue matrix due to their amphipathic properties. The absorption of polyphenolic compounds to the proteins is generated early, but the desorption rate is very slow. Consequently, mammalian cells, tissues or organs could be physiologically preserved through adsorption of the compounds to membranous proteins and extracellular matrices, leading to both the reduction in structural deterioration and the prevention of oxidative damage (12-34).

8. Conclusion

As described above, the polyphenol was able to control the proliferation of various cell types, and was demonstrated to be very useful for the long term storage of various tissues, including islet cells, blood vessels, cartilage, corneas, nerves, and skin. 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. Cryopreservation methods are being used for the long term storage of blood vessel, cartilage and skin. However, current research is still being directed at improving the effectiveness of cryopreservation and of preservation fluids, such as the University of Wisconsin solution, for tissue transplantation. We have herein 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 of the tissue. The authors have also succeeded in preserving the rat sciatic nerve, guinea pig periodontal ligament and rat myocardium in a non-frozen condition for long term storage prior to preservation. This has been made possible by the development of a new preservation fluid with polyphenol as an additive antioxidant. Therefore, the cryopreservation of many tissues may eventually be superseded by their storage in the newly developed polyphenol tissue banking fluid. This method of cell and tissue preservation would be of great benefit, not only in Japan, but throughout the world, especially in developing countries where cryopreservation is often not possible.

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The procedure of skin grafting has been performed since 3000BC and with the aid of modern technology has evolved through the years. While the development of new techniques and devices has significantly improved the functional as well as the aesthetic results from skin grafting, the fundamentals of skin grafting have remained the same, a healthy vascular granulating wound bed free of infection. Adherence to the recipient bed is the most important factor in skin graft survival and research continues introducing new techniques that promote this process. Biological and synthetic skin substitutes have also provided better treatment options as well as HLA tissue typing and the use of growth factors. Even today, skin grafts remain the most common and least invasive procedure for the closure of soft tissue defects but the quest for perfection continues.

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