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# Hibernation of Mammalian Cells at a Living Body Temperature

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**Abstract** The present study revealed that polyphenol induces the hibernation of mammalian cells at a living body temperature. It was found that polyphenol is a cytostatic-sleeping agent for mammalian cells, where almost all cells resume proliferation after the hibernation period and cell death seldom occurs. By changing the concentration of polyphenol, various mammalian cells can be stored under different conditions, such as temporary sleep, sound sleep, and hibernation conditions.

*Keywords*: hibernation, polyphenol, cytostatic, proliferation, cryopreservation, mammalian cell

## INTRODUCTION

Cell and organ transplantation can potentially normalize organ functions and stop the progression of clinical disease [1-3]. In general, the best chances of success are when fresh and cryopreserved cells or organs are transplanted in combination. Cryopreservation has many immunological advantages for clinical cell transplantation from multiple donors, which is required for patients with long-standing diseases [4]. However, no optimal method for the cryopreservation of mammalian cells and tissue has yet been established, plus mammalian cells show a poor survival after thawing and the recovery of function is low [4]. In addition, one of the most important requirements for success in clinical cell transplantation is the use of a large number of viable donor cells. Moreover, current methods can result in a substantial loss of function and lead to the damage and destruction of cells and tissues. Contamination is also a particularly difficult problem for immunosuppressed transplant recipients during cryopreservation [4]. Microbial contaminants introduced via donor cells can persist throughout cryopreservation. Therefore, before transplantation into human subjects the cryopreservation procedures have to be checked for cell functional loss, inadequate functional recovery, and contamination by bacteria and fungi. To resolve these drawbacks of cryopreservation, the methods currently in use need to be modified.

Accordingly, this study investigated a novel preservation method, which can control the proliferation of various types of cells and facilitate the long-term preservation of various organs or cells at a physiological temperature through the use of polyphenol [5-9] as the preservation agent.

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## MATERIALS AND METHODS

### Materials

Polyphenol extracted from green tea was purchased from PFI Inc. Kyoto Japan. It was composed mainly of (-)-epigallo-catechin-3-*O*-gallate (28%), (-)-gallocatechin -3-*O*-gallate (11.6%), (-)-epicatechin-3-*O*-gallate (4.6%), (-)-epigallocatechin (15.0%), (+)-gallocatechin (14.8%), (-)-epicatechin (7.0%), and (+)-catechin (9.5%), and its purity exceeded 90%.

### Methods

*Cell Preparation*

L-929 fibroblast cells (L-929) taken from fetal mouse skin were cultured in Eagle's MEM containing antibiotics (Kanamycin, 60 mg/L) and 10% fetal bovine serum (FBS: M.A. Bioproduct, Maryland, USA). The cell density was adjusted to $2.56 \times 10^5$ cells/10 cm dish for cell growth and a proliferation test, in both a serum-containing medium and a polyphenol mixture (250 μg/mL) for a fibroblast system. Also, porcine hepatocytes were harvested from a pig weighing approximately 20 kg according to the method reported by Sielaff *et al.* with modifications [10]. For in vitro culture, the cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 100 mL/L of fetal bovine serum (FBS), 5,000 unit/L of penicilin, 100 mg/mL of streptomycin, 0.5 ng/mL of EGF, 3.7 g/L of bicarbonate, and 0.25 mL/L insulin (Humulin R, Lilly Japan, Kobe, Japan). The cells were kept in a humidified incubator at 37°C under a 5% $CO_2$ and 95% air atmosphere. The cell density was adjusted to $3.5 \times 10^4$ cells/10 cm dish for cell growth and a proliferation test, in both a serum-containing medium and polyphenol mixture (250 μg/mL) for a hepatocyte system. At predetermined intervals, the numbers of cells grown on the cell culture dishes were counted. Also, the cells were kept for predetermined times in a humidified incubator at 37°C under

a 5% $CO_2$ and 95% air atmosphere. The numbers of cells grown on the cell culture dishes were counted as a function of the incubation time.

*Cell Cycle Analysis using Flow Cytometry*

A cell cycle analysis of the polyphenol-treated and non-treated (L-929) fibroblast cells was performed as a function of the incubation time and polyphenol treatment time at predetermined intervals. The cells were washed with a phosphate buffer and fixed with an ice-cold 70% ethanol solution for 20 min at 4°C. After filtration through a 50 μm nylon mesh, the cells were fixed and stained with 0.5 mL RNase (250 U/mL) and 0.5 mL of propidium iodide (100 μg/mL) for 20 min. The DNA contents of about 10,000 stained cells in each group were measured using flow cytometry.

*Biochemical Function of Cells*

The cells were cultured as a monolayer in collagen coated dishes containing Dulbecco's modified Eagle's medium (DMEM) and 10% fetal bovine serum. The cell density was adjusted to $2.1 \times 10^6$ cells/10 cm dish for a cell function test. The cells were kept for predetermined times in a humidified incubator at 37°C under a 5% $CO_2$ and 95% air atmosphere. At predetermined intervals, the lidocaine clearance on the hepatocytes was measured as a function of time.

## RESULTS AND DISCUSSION

Figs. 2 and 3 show the effects of polyphenol on the growth and proliferation of cells, such as fibroblasts (L-929) and hepatocytes. The polyphenol treatment exhibited dose- and treatment time-dependent effects on cell proliferation and growth. The cells treated with polyphenol (concentration: 250 μg/mL) did not increase in number at the beginning of the treatment, however, the number increased slowly relative to the polyphenol treatment time after the removal of polyphenol from the medium. In contrast, the cells treated with a higher polyphenol concentration (100 mg/mL) did not increase in number, and there was no proliferation of cells after removal of polyphenol from the medium. However, the proliferation of hepatocytes showed a little different expression to that of fibroblasts. The proliferation recovery of hepatocytes after the treatment is faster than that of fibroblasts at the same treatment concentration and duration (Fig. 3). Interestingly, the cell shape at the beginning of the experiment was maintained for 2 months or more, regardless of the treatment time and concentration (Fig. 1). Polyphenol had no effect on cell viability, and almost all cells survived. One of the most peculiar observations was the difference in morphology between the fibroblasts and the hepatocytes (Fig. 1). The fibroblasts started to show a loss of cytoplasm in the proliferated cells except for the nucleus, whereas the hepatocytes exhibited no significant change in cell morphology. These results suggest that the polyphenol-treated cells may have partially lost their mechanism of feedback control for proliferation during treatment. However, the proliferation control was slowly enhanced by the contact of cells with each other and returned to a normal level after the removal of polyphenol from the medium. These results suggest that polyphenol acts as a cytostatic-sleeping agent for mammalian cells. These phenomena may also have been due to the biological activities of polyphenol, which exhibits a strong anti-

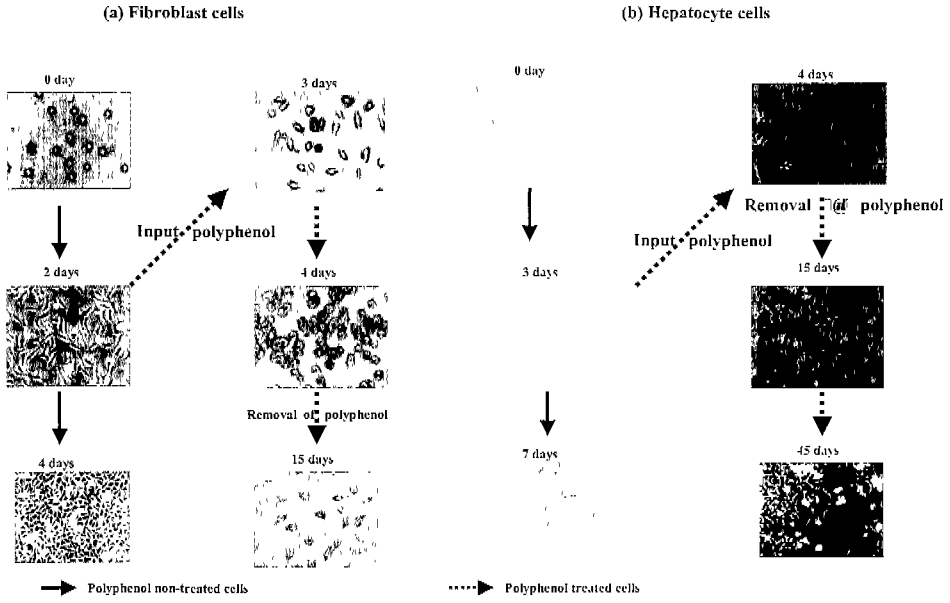


**Fig. 1.** Photographic comparison of proliferation and growth in fibroblasts and hepatocytes treated with (⟶) or without (⋯▶) polyphenol (250 μg/mL).

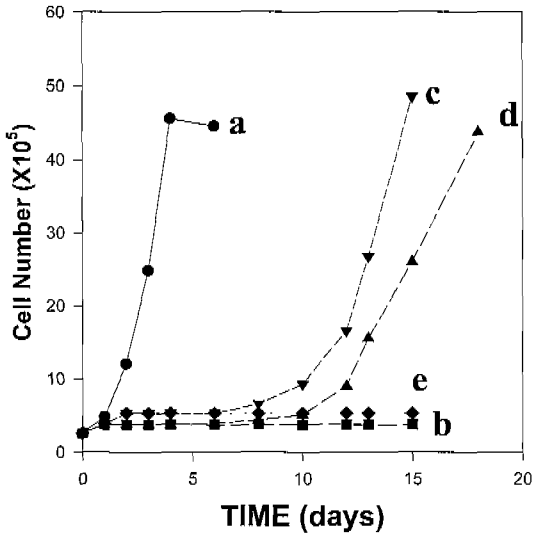


**Fig. 2.** Cell numbers and proliferation of cells with (250 μg/mL) or without polyphenol in the fibroblasts; (a): control (only MEM Eagle's medium containing 10% fetal bovine serum), (b): polyphenol treatment whenever medium was changed, (c) and (d): polyphenol treatment for 1 day, after 24 and 48 h incubation from the beginning of culture, (e): polyphenol treatment with higher concentration (100 mg/mL) for 1 day.

**Table 1.** Analysis of cell cycle in polyphenol non-treated and treated cells (250 μg/mL) relative to time

| Polyphenol | Time(h) / Cycle(%) | 0 | 2 | 4 | 9 | 48* |
|---|---|---|---|---|---|---|
| Non-treated cells | 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 of fibroblasts cultured in medium only during 48 h after removal of polyphenol from medium.

oxidant activity [3]. Polyphenol may play an important role in the prevention of carcinogenesis due to DNA damage by reactive oxygen radicals, as polyphenol would appear to bind to specific sites and thus interrupt the exogenous signals required for the proliferation and growth of cells.

Table 1 shows the results of the cell cycle analysis using flow cytometry. The percentage of non-treated cells in the G1, S and G2-M phases were 22.18%, 2.58%, and 75.24% in the first stage, respectively. The proportions of non-treated cells in the G2-M and S phases did change with time, whereas the percentage of polyphenol- treated cells in the G0-G1 phases increased from 22.18% to 70.07% and thereafter did not change significantly during the experimental period. That is, cells did

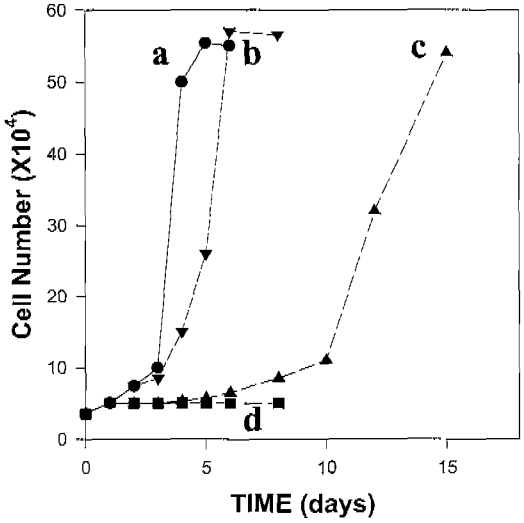


**Fig. 3.** Cell numbers and proliferation of cells with (250 μg/mL) or without polyphenol in the (a): control (only DMEM medium containing 10% fetal bovine serum), (b) and (c): polyphenol treatment for 1day, after 24 and 48 h incubation, (d): polyphenol treatment whenever medium was changed.

not enter the S-phase during the experimental period plus polyphenol induced a significant G0-G1 phase arrest. This suggests that increases in the number of cells and DNA synthesis during the culture were inhibited by polyphenol. However, the proportion of cells in the S-phase increased from 0% to 8.13% after the removal of polyphenol from the medium. In vascular endothelial cells and smooth muscle cells, Matsuda et al., recognized the similar phenomena [11]. The cell cycle arrest may have been due to adsorption or blocking at specific sites on the membrane or DNA that effected the proliferation of cells and DNA replication.

Fig. 3 shows the biochemical function of the polyphenol non-treated and treated cells (hepatocytes) preserved for 2 months. The results indicate that the sensitivities of the lidocaine cycle were higher in the freshly isolated cells than in those treated with polyphenol for 2 months. However, the functions in freshly isolated cells were examined with no increase in the cell number during the culture. If two cell types have similar growth curves, when plated measurement of the lidocaine cycle parameters will yield the same values. Therefore, the cells retaining their original function would appear to have hibernated for the period of the polyphenol treatment.

## CONCLUSION

The results of the present study confirmed that polyphenol can control the proliferation of common cell types, such as fibroblasts and hepatocytes. This is an important observation as to date only freezing methods have been available for the preservation of mammalian

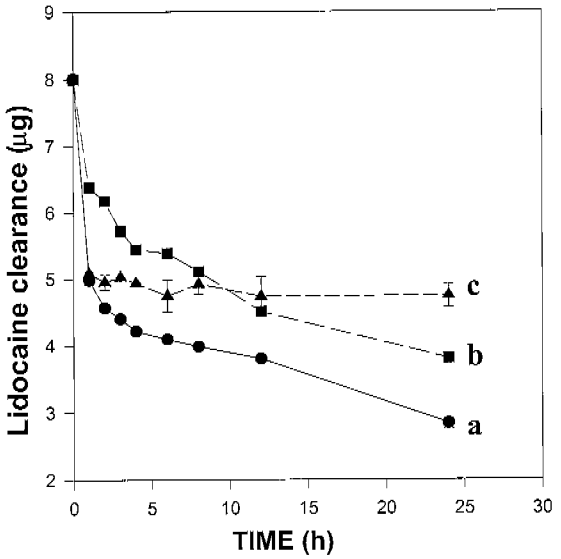


**Fig. 4.** Measurement of biochemical function (lidocaine clearance) in polyphenol non-treated (a) and treated cells (b,c) (250 μg/mL) for 1 and 45 days relative to time.

cells. The extension of this observation to the preservation of organs for transplantation will make it possible to store organs for long periods by adjusting the con-centration of polyphenol. These phenomena may be related to intrinsic characteristics of polyphenol; this compound passes easily through the extracellular matrix and cell membrane due to its amphipathic properties, and is readily adsorbed on protein. Therfore, polyphenol combines easily with receptor on the cell surface. The absoption of polyphenol to the protein is early generated but the desorption is very slow. Resulting, various mammalian cells could be physiologically hibernated by absorption of polyphenol to receptor on the cell membrane due to block proliferation signals and prevent lipid oxidation of cell membrane. Polyphenol could reversibly block cellular enzyme activities, and these activities could be restored by removal of the polyphenols [12]. Therefore, mammalian cells can be physiologically hibernated by the adsorption of polyphenol into receptors on the cell membrane that block proliferation signals. It would seem likely that along with its has not only antioxidant activity, polyphenol also has a direct effect on DNA. Polyphenol can reversibly block cellular enzyme activities, and these activities can then be restored by the removal of the polyphenols [7-9]. The adsorption and desorption of polyphenol on the cell membrane were confirmed by light microscopy, because the cells were colored by polyphenol. The results of the present study provide a basis for the development of simple and economical methods for tissue and organ preservation.

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