Titanium Culture Vessel Capable of Controlling Culture Temperature for Evaluation of Cell Thermotolerance*1

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Surgery, radiation therapy, and chemical therapy have been proposed and established for a variety of diseases. However, the number of cancer patients continues to increase. Cancer treatments, surgery, chemotherapy, and radiation therapy have been developed, respectively; however, each strategy has problems to be solved. Surgery is highly invasive. Chemotherapy causes side effects in other healthy cells. Radiational therapy leads to inflammation in the radiation spots. As aforementioned, these problems remain to be solved. Furthermore, hyperthermia has received significant attention as a low invasive cancer therapy.1)

Hyperthermia is performed by utilizing the difference in thermal tolerance between normal cells and cancer cells, and the diseased area is exposed to thermal stimulation for cancer treatment.1) Because cancer cells have weaker thermal tolerance than normal cells, cancer cells can be selectively killed. To develop this therapy, the conditions of thermal stimulus where only cancer cells can effectively be killed should be identified. Thus, several studies to add thermal stimulus to cells for investigating a critical condition for hyperthermia have been reported. Figure 1 shows the conceptual figure, in which only cancer cells were killed with increasing temperature. The upper figure shows the cells cultured at a normal temperature of 37°C, while the lower figure shows the cells cultured at higher temperatures.

Kase et al. reported that when normal and cancer cells were exposed to a higher culture temperature than usual, the number of killed cancer cells was higher than normal cells.2) Dewey et al. demonstrated that the longer the duration of thermal stimulus, the more the cells are killed.3) Furthermore, Kitney found heat shock protein (HSP) as a key protein for developing hyperthermia.4) HSP is produced when cells are exposed to thermal stress and protects the cell nucleus from stress, such as thermal stimulus.5) Note that this phenomenon is limited to normal cells. Thus, for instance, by cyclic thermal stimulus, only normal cells can acquire thermal tolerance, which may result in effective hyperthermia.

However, previous reports regulated the culture temperature by controlling the set temperature of an incubator where plastic culture vessels were located.6) This indicates...

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that the exact temperature that the cells experienced has been unknown. Thus, it is necessary to fabricate cell culture devices that are capable of regulating culture temperature immediately and accurately to develop effective hyperthermia.

In this study, to adequately regulate the thermal stimulation, we fabricated a culture device with a metallic culture plate that was used as a culture surface and a Peltier element that regulated the temperature of the culture surface. With this device, in this study, cancer cells and normal cells were cultured and exposed to the thermal stimulation to study the thermal tolerance of each cell.

2. Culture Device Fabrication and Estimation

Figure 2 shows the fabricated culture device, which consists of an upper part for cell culture and a lower part for regulating temperature.

2.1 Fabrication of culture vessel

The cell culture chamber consists of a polydimethylsiloxane (PDMS) wall with 15 mm diameter holes (see Fig. 3) and 1 mm thick metallic plates. Two metallic plates tightly tuck the PDMS wall. One of the metallic plates was used as a culture surface where fine particle peening (FPP) was performed to improve cell adhesion to the culture surface. The other plate had 15 mm diameter holes. As for materials of the plates, pure titanium (ASTM Grade 2) and stainless steel (SUS316L) were employed, because both of them were used for biomaterials. The composition of each material is shown in Tables 1 and 2, respectively. Table 3 lists the conditions of the FPP. Note that autoclave sterilization can be performed with the fabricated culture vessels.

2.2 Evaluation of cytocompatibility of the culture vessel

To evaluate the cytocompatibility of the fabricated vessel, the proliferation rate and morphology of the cells cultured in the vessels were determined. To evaluate the proliferation rate, a mouse calvaria-derived cell line (MC3T3-E1, RIKEN BRC, Japan, Saitama) was used. The culture medium used was minimum essential medium Eagle, alpha modification (α-MEM) (Sigma-Aldrich Co. LLC, USA, MO, St. Louis) supplemented with 10% fetal bovine serum (FBS) (Funakoshi Co., Ltd., Japan, Tokyo). The number of seeded cells was $2 \times 10^4$, and the proliferation rate of the three-day culture was measured. As a control sample, cells cultured in 24 well plates (Thermo Fisher Scientific Inc., USA, MA, Waltham) were also prepared. To measure the cell proliferation rate, we performed an MTT assay with Cell Titer 96® Non-Radioactive Cell Proliferation Assay kit (Promega Corporation, USA, WI, Madison) following the protocol of the manufacturer.

Figure 4 shows a comparison of the proliferation rate with each vessel. As shown in the figure, although the proliferation rates of the cells cultured in metallic vessels seemed lower than that of the control, there was no
significant difference as a result of one-way ANOVA. This may be because of the instability of the measurement. However, the purpose of this research is not to expand culture; thus, we used the fabricated vessels for this study.

In the present study, to regulate the temperature of the culture surface, we used a Peltier element. Although there was no difference in cell proliferation between pure titanium and stainless steel, we chose pure titanium as the material of a culture vessel because of its thermophysical properties, as shown in Table 4. From the table, the thermal conductivities of pure titanium and SUS316L are similar; however, the thermal capacity of pure titanium is considerably less than that of SUS316L. Thus, the regulating temperature of pure titanium is easier than that of SUS316L. Therefore, we performed the experiment with vessels made of pure titanium.

To evaluate the cytocompatibility of the culture vessels, the morphology of a human breast cancer-derived cell line (MCF-7, RIKEN BRC) cultured with the titanium vessel was observed. Briefly, 2 × 10⁴ cells in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% FBS were seeded and cultured for 2 days, and subsequently, actin and nuclei were stained with the procedure shown in Fig. 5. Actin is a protein and is one of the cell cytoskeletons, while the nucleus is one of the cell organs regulating gene expression.¹¹,¹² Figure 6 shows the fluorescence-stained image. In this image, green and blue show actin and nucleus, respectively, and the black area corresponds to a culture surface without cells adhered. As shown, actin extends on the culture surface, which implies healthy cell adhesion to the culture surface. Furthermore, the nucleus shows a spherical morphology and is located inside the cell cytoplasm, which is a normal condition of cells cultured in a ubiquitous culture dish. As aforementioned, actin and nucleus, which are important cell organs, show their healthiness from their morphology.

The results shown in this section indicate the cytocompatibility of the culture vessels fabricated in this study. Thus, we conclude that cell thermotolerance can be investigated with the fabricated vessels.

2.3 Development and evaluation of temperature regulation part

The temperature regulation part consists of a Peltier element (TEC1-12708; Thermonamic Electronics (Jiangxi) Corp., Ltd., China, Jiangxi), NTC thermistor (NXF15XH103FA2B050, Murata Electronics Oy, Finland, Vantaa), and heatsink (see Fig. 2). The temperature of the culture surface can be regulated by feedback control with an Arduino (UNO R3, R&K Systems Co., Ltd, Tokyo, Japan) and a motor driver (Toshiba Electronic Devices & Storage Corporation, Tokyo, Japan). The developed temperature regulation system was evaluated using a thermistor attached to the center of the bottom of the culture vessel.

First, to evaluate the temperature response speed of our device, the temperature history was measured. For compar-
ison, the culture device was placed in an incubator (MCO-5AC-PJ, Panasonic Corporation, Osaka, Japan) set at 37°C, and then was changed to 41°C. Figure 7 shows the temperature history of the culture surface and the set temperature in this experiment. As shown, although a metallic culture vessel with thermal conductivity superior to that of a ubiquitous culture vessel is employed, the temperature of the culture vessels could not follow the changed temperature of the incubator. Figure 8 shows the temperature history of the culture surface when the temperature was regulated by the Peltier element. As shown, the temperature of the culture surface almost corresponded to the set temperature when using our developed system. The results shown in Figs. 7 and 8 demonstrate the effectiveness and validity of our proposed method for regulating culture temperature.

Second, to evaluate the temperature distribution on the culture surface, a thermography camera (FLIR C2, FLIR Systems, Inc., USA, OR, Wilsonville) was used, and the thermal image of the culture surface was taken from above (Fig. 9). Note that the set temperature was 41°C, and the image was captured at room temperature. Figure 9 shows that the temperature distribution on the culture surface was homogeneous, and thus cells cultured in the culture device could be exposed to a homogeneous thermal stimulus.

In most of the previous studies investigating cell thermostolerance, the temperature of the incubator where cell culture vessels were placed was regulated. Thus, the temperatures at which cells were exposed were unclear, and this uncertainty has made studying cell thermal tolerance difficult. However, because the culture device developed in this study can strictly regulate cell culture temperature, thermal stimuli on demand can be added to cells.

3. Comparison of the Tolerance between Cancer and Normal Cells

To investigate the difference in thermal tolerance between cancer cells and normal cells, thermal stimuli of several temperatures were applied to the cells with the developed culture device. Figure 10 shows the plan of an experiment by applying thermal stimulus to cells. MCF-7 and normal human...
dermal fibroblasts (NHDF) (Cosmo Bio Co., Ltd., Tokyo, Japan) were used as representatives of cancer cells and normal cells, respectively. The number of seeded cells was $8 \times 10^4$ in both cases, and DMEM supplemented with 10% FBS was used as the culture medium. As shown in Fig. 10, the cells were cultured for 24 h and subsequently exposed to 2 h of thermal stimulation. Thereafter, the culture medium was replaced because the protein in the medium could be denatured owing to the thermal stimulus. To consider the effect of thermal stimulation on cell proliferation, the number of cells after further culture was measured by the MTT assay. Figure 11 shows the viabilities of cells with a 2 h temperature stimulus at each temperature. From this figure, with 2 h thermal stimulation at 47°C, both types of cells died, while the viabilities of both cell species were intact up to 43°C thermal stimulation. Conversely, with 45°C thermal stimulation, the viability of each cell species showed a different trend, where normal cells showed a stronger thermal tolerance than cancer cells. This result is consistent with a previous study, which argued that the thermal tolerance of cancer cells is weaker than that of normal cells. However, some cells died, while some remained alive. This indicates that the thermal tolerance of each cell was different. A previous study reported that the density of cells on culture surface affects thermal tolerance. The local densities of cells differ depending on the position because it is difficult to seed cells homogeneously. Thus, the thermal tolerance of the seeded cells varied.

The relationship between the duration of thermal stimulation at 47°C and the cell viability of cancer cells was studied, as shown in Fig. 12. From this figure, it is demonstrated that the relation between the duration of thermal stimulus and cell viability should be a monotone decreasing function.

4. Discussion

The aim of this study was to develop a methodology for hyperthermia, known as a cancer therapy, that is capable of being combined with other therapies. Lee et al. reported a combination of hyperthermia and chemotherapy. In this study, MCF-7 cells were exposed to a culture temperature of 47°C for 45 min; thus, 15% of the cells died. This result was quantitatively different from ours, as shown in Fig. 12. This difference is believed to be caused by the developed temperature regulation system. In previous research, the temperature of the incubator, not the culture surface, was regulated. It was confirmed that approximately 20 min was required for the culture temperature to reach the set temperature. Hence, in that study, the actual duration for the cells to be exposed to the culture temperature of 47°C was approximately 25 min. Furthermore, in the middle of increasing temperature, cells were exposed to moderate temperature stimulation, which might have provided the cells with thermal tolerance. Lee et al. mentioned the possibility that cells exposed to the thermal stimulus express HSP and can achieve thermal tolerance. Therefore, it is difficult to evaluate the thermal tolerance of cells using the previous temperature regulation method.

Immediately after cells receive thermal tolerance, the reaction of cells to thermal stimulation is changed. The temperature regulation method of the previous reported studies required a longer duration before the thermal stimulation of a certain temperature, which may allow the cells to experience thermal tolerance. However, with our developed device, the required culture temperature can be realized immediately. Therefore, it was possible to evaluate the original thermal tolerance of the cells using our device.
In future work, the proposed device can be used for adding several patterns of thermal stimulation to cells to investigate the optimum conditions for hyperthermia. Furthermore, it has been reported that thermal stimulation regulates cell activities.\textsuperscript{16–18} Recently, culture method for three-dimensional tissue has been developed, so the present device may be used for such tissues in future works.\textsuperscript{19,20} Thus, our device can be utilized for the investigation of the optimum culture temperature for each bioengineering application.

5. Conclusion

Recently, among cancer therapies, hyperthermia with relatively lower invasiveness and smaller side effects has attracted attention. The present study conducted fundamental research to develop hyperthermia conditions.

(1) To evaluate the response of cells to thermal stimulation, a device where cell culture temperature can be regulated is required. Thus, we developed a culture device with a metallic culture surface, and subsequently evaluated the cytocompatibility and temperature control ability. Consequently, it was demonstrated that cells can be healthily cultured in the developed device, and immediate temperature regulation is possible.

(2) With the developed culture device, the viabilities of cancer and normal cells exposed to several conditions of thermal stimulus were evaluated. Note that in this study, as a model cells of cancer and normal cells, MCF-7 and NHDF were utilized, respectively. Thus, it was confirmed that cancer cells have weaker thermal tolerance than normal cells, and that the viability of cancer cells depends on the temperature and duration of thermal stimulus. These findings are consistent with the idea previously reported, which demonstrates the validity of the experiment performed with the developed device.

Therefore, we believe that our developed device can be used to optimize conditions for hyperthermia and further for fundamental research in every bioengineering field.

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