Effect of the Cell Alive System on nerve tissue cryopreservation

Tomohisa Hashimoto[1,*], Sano Kazufumi[1], Ozeki Satoru[1], Nakadate Kazuhiko[2]

[1] Department of Orthopedic Surgery, Dokkyo Medical University Saitama Medical Center, Koshigaya City, Saitama, 343-8555 Japan

[2] Meiji Pharmaceutical University Pharmaceutical Education Research Center, Kiyose-city, Tokyo, 204-8588, Japan

[*]**Corresponding author:** Tomohisa Hashimoto

Department of Orthopedic Surgery,

Dokkyo Medical University Saitama Medical Center,

Koshigaya city, Saitama, 343-8555, Japan

Phone number: +81-48-986-111

Email: thashi@dokkyomed.ac.jp

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Abstract

Effective cellular cryopreservation while maintaining high cell viability is achieved by preventing intracellular and extracellular ice crystal formation using the Cells Alive System (CAS), a programmed freezer that applies a magnetic field. Here, the optimal temperature settings of the CAS were determined using rat sciatic nerves as a model tissue. Firstly, it was found that Schwann cell survival was increased by pre-cooling the samples in the ice crystal formation zone, increasing the freeze-thaw speed, and freezing-thawing in a magnetic field. Secondly, the setting (intensity and frequency) of the magnetic field at freezing-thawing was changed, and the optimum magnetic field strength was determined by evaluating cell viability. At the set temperature excluding previous studies, the minimum temperature was set to -50 ℃ and kept frozen for 15 minutes, and then thawed immediately. The highest cell viability (27%) was achieved at 0.67 mT (intensity 3 [29.6V] and frequency setting 10 [60 Hz]). The effects of the freeze-thaw program were assessed using transplanted sciatic nerve tissues removed after 2, 4, and 8 weeks.

Anterior tibial muscle wet weight increased at 8 weeks in the control (without freezing) and after freezing-thawing in a magnetic field, compared to that without a magnetic field. Fluorescence staining of the sciatic nerve with anti-S100 antibodies revealed that Schwann cell counts increased at the transplanted site (at 8 weeks) of nerves that were freeze-thawed in a magnetic field. Overall, the CAS prevented ice crystal formation in rat sciatic nerves and could be used to maintain cell viability during cryopreservation.

Keywords: Cell Alive System; tissue cryopreservation; magnetic field intensity; magnetic field frequency; freeze-thaw

Introduction

In clinical practice, situations involving extensive injuries to soft tissues, such as nerves, blood vessels, and tendons, are often encountered, and direct suturing of these injuries may not be possible. In such cases, transplantation is performed directly onto the injured site. However, complications, such as residual wounds, paresthesia due to nerve damage, and edema in vessels at the donor site, are often observed when transplanting soft tissues from a healthy site. Research on the development and improvement of artificial nerves and blood vessels is underway; however, no studies have reported results with efficiencies comparable to autografting from a healthy donor site, particularly for artificial nerves. Moreover, Schwann cell viability is an important factor for nerve regeneration after transplantation. Thus, viable cryopreserved nerve tissues may help overcome the problems associated with the regeneration of soft tissues, and transplantation and regeneration of cryopreserved soft tissues may lead to the development of novel treatments for such injuries.

This study aimed to establish the optimal freezing conditions for cryopreservation of neuronal tissues using the Cells Alive System (CAS; ABI Co., Ltd., Nagareyama, Chiba, Japan) to enable cryopreservation while maintaining cell viability. Cryopreservation of periodontal ligaments and rat bone tissues has been reported using the CAS in the field of dentistry (Kaku et al. 2010; Kojima et al. 2015). However, to date, no report has described the cryopreservation of neural tissues using the CAS.

Preserving viable tissues is important during cryopreservation. The most critical problem in

cryopreservation is intracellular and extracellular ice crystal formation, which causes cell death. The CAS, a cryopreservation system that utilizes the magnetic field, involves the application of weak energy (physical vibration) generated by the CAS apparatus to prevent crystallization of intracellular water, maintain the supercooling state, and achieve instantaneous freezing to prevent the crystallization of water. This technology has been used in primary industries, such as farming, fishery, and livestock, to conserve freshness and flavor of foods during the freezing and thawing steps. Moreover, in basic studies, this approach has been shown to yield improved viability, multipotent differentiation ability, adhesiveness, and growth in thawed cells. This approach has been evaluated for the cryopreservation of periodontal ligament cells and dental pulp tissues (tooth banking) (Kaku et al. 2010; Kojima et al. 2015). The CAS inhibits ice crystal formation during the freezing stage via vibrations generated by the magnetic field, thereby facilitating the cryopreservation of viable cells (Kaku et al. 2010; Kojima et al. 2015; Nishiyama et al. 2016).

This study was conducted to determine the applicability and utility of CAS for the cryopreservation of neuronal tissues. We optimized the temperature and magnetic field settings of the CAS and evaluated the efficacy of neuronal tissue transplantation after cryopreservation using the CAS.

Methods

Animals

The study was performed on 8–9-week-old Wistar rats (weight: 250–300 g, sex: male). Experimental protocols were approved by the Institutional Animal Care and Use Committee of Dokkyo Medical University (Permit Number: 1015), which operates in accordance with the Japanese Government for the care and use of laboratory animals.

Nerve collection, freezing, and culture methods

Under general anesthesia, induced by the inhalation of sevoflurane (Mylan Pharmaceutical Co., Ltd., Tokyo, Japan), 1–2 cm of the sciatic nerve of each rat was removed. The collected tissues were washed to remove excess blood and immediately subjected to experimental freezing, following the application of 1 mL of cryoprotectant (Cellbanker; ZENOAQ, Koriyama, Japan) in microtubes at room temperature (22 ℃–25 ℃). The freezer used in this study was the CAS Program freezer (ABI, Nagareyama, Japan) at different temperature settings. Neurons were isolated with collagenase and washed and incubated for 2 days in 35-mm dishes at 37 °C, followed by immunostaining using anti-S100 antibodies (Abcam CO., Ltd., Tokyo, Japan). Schwann cells were observed under a fluorescence microscope (10 fields of view) (Olympus IX71, Olympus CO., Ltd., Tokyo, Japan) to assess viability.

Determination of optimal conditions for freezing and thawing

Equipment and temperature settings were used as previously described by Sano et al. (2015) for the

analysis of rat tails and the CAS. The minimum tissue freezing temperature was set at −50 °C. Holding time (hereafter pre-cooling and post-cooling) has been reported to be important in conferring cryoprotection by inhibiting ice crystal formation. It has been reported that a 15-minute holding time is optimum for cryopreservation (Koseki et al., 2013). Other variables, such as pre-cooling and post-cooling temperatures, were chosen as previously described (Sano et al. 2015) (Fig. 1a), or were altered to identify the optimal conditions. These variables include the temperature settings with or without pre-cooling/post-cooling (Fig. 1b and 1c) in the ice crystal forming zone (0 °C to 5 °C) (Koseki et al. 2013), presence or absence of magnetic field (CAS+/CAS-, respectively), freezing speed (0.5 °C/min or maximum freezing speed (3.5 ℃/min) of the CAS freezer) (Hibino et al. 1995), rapid thawing in a 37 °C constant low-temperature water bath (BV100R; Yamato Scientific Co., Ltd., Tokyo, Japan), natural thawing, and thawing with magnetic field (CAS thawing). Neuron cultures of tissues frozen and thawed under various conditions were assessed using an optical microscope by hematoxylin-eosin (HE) staining and electron microscopy for viability analysis.

Analysis of optimal magnetic field settings (intensity, frequency)

Using the standardized temperature, as obtained from the results of the previous experiment, the setting (intensity and frequency) of the magnetic field during freeze-thawing was changed; then, the optimum magnetic field values were determined by cell viability analysis. Male Wistar rats were subjected to

general inhaled anesthesia, and a 1-cm segment of the sciatic nerve (n = 137) was sampled. The tissues were frozen, thawed, observed by optical（Olympus BX53, Olympus CO., Ltd., Tokyo, Japan） and electron microscopy（H-7100 electron microscope, Hitachi Co., Ltd., Tokyo, Japan), and cultured as described above to assess cell viability. Cell viability (%) was calculated as the cell count of the sciatic nerve culture obtained after freeze-thawing, using the CAS, and the percentage was calculated as a survival rate to compare with the fresh sciatic nerve.

Analysis of transplantation of nerve tissues that had been frozen and thawed

One-centimeter segments of the sciatic nerve of male Wistar rats (n = 121) were removed. The sciatic nerve segments were then transplanted without freezing or thawing (control), after freezing and thawing using the CAS (CAS+), or after freezing and thawing without using the CAS (CAS-).The minimum temperature was set to -50 ℃ and maintained for 15 minutes, after which the samples were thawed immediately. The samples were again removed 2, 4, or 8 weeks post-transplantation, and lengthwise sections of the sciatic nerve were obtained. HE staining and immunostaining with anti-S100 antibody were performed for histological assessment (Fluorescence microscope BZ-X800, Keyence CO., Ltd., Tokyo, Japan). To quantify the number of Schwann cells in the transplanted nerve, we measured the number of Schwann cells in 5 frames from each section (in regions of interest (ROI) 1.5 × 1.5 mm, 20 ROIs). The anterior tibial muscle wet weight was also measured.

Statistical analysis

The statistical significance of differences was evaluated using the analysis of variance (IBM SPSS Statistics version 26, IBM Japan, Ltd., Tokyo, Japan). Differences with p values less than 0.05 were considered significant.

Results

Analysis of the CAS

Using the temperature settings reported by Sano et al. (2015) (Fig. 1a), tissues were frozen by using the CAS and were thawed in a 37 °C water bath followed by observing under electron microscopy. Compared to the control (Fig. 2a), myelin vacuolization and axon narrowing were observed in tissues frozen with or without the CAS. However, myelin vacuolization and axon narrowing were reduced in tissues frozen with the CAS (Fig. 2~~c~~b), compared to those frozen without the CAS (Fig. 2~~b~~c). Thus, our study suggests that freezing through the CAS inhibited myelin vacuolization.

Analysis of freezing and thawing speed settings

Based on the results of the previous experiment, freezing and thawing of the tissues were carried out at the optimized temperatures, as shown in Fig. 1b using the CAS. Tissues were frozen and thawed at a slow

speed (0.5 °C/min) (Hibino et al. 1995) and at the maximum possible CAS speed (3.5 ℃/min). Tissues frozen and thawed at slow speeds showed spaces between neural fibers and myelin vacuolization in HE staining and electron microscopy (Fig. 3a, 3b arrow). A few viable cells were observed in nerve cell cultures (Fig. 3c). At the maximum speed, CAS+ freezing and thawing showed mild spacing between neural fibers and myelin vacuolization, as observed by HE staining (Fig. 3d). There was also less myelin vacuolization as compared to that observed after slow freezing and thawing, as observed by electron microscopy (Fig. 3). Nerve cell cultures showed higher cell viability (Fig. 3f), suggesting that freezing and thawing at the maximum speed (3.5ºC/min) was more effective.

Analysis of the appropriate thawing method

Because ice crystal reformation can occur during the thawing step, following crystal formation during freezing, tissues were frozen at the maximum speed at the temperature settings described in Fig. 1b. Electron microscopy imaging showed considerable myelin vacuolization and axon narrowing in tissues thawed naturally and in the tissues thawed in a 37 °C water bath (Fig. 4a, b), suggesting that cell viability was likely low. In contrast, CAS+ thawing showed reduced myelin vacuolization, by electron microscopy (Fig. 3e), and clearly higher cell viability (Fig. 3f). CAS+ thawing was therefore considered the most effective thawing method for the prevention of ice crystal reformation and for retaining cellular viability.

Analysis of pre-cooling and post-cooling settings

Nerve tissues were frozen and thawed using the CAS at the maximum speed (3.5℃/min), without pre-cooling and post-cooling, around the ice crystal formation zone. This led to the formation of intracellular and extracellular crystals (Fig. 1c). HE staining showed small spaces between neural fibers, whereas electron microscopy images showed myelin vacuolization (Fig. 5a, b). Nerve cell cultures showed very low cell viability (Fig. 5c).

Next, nerve tissues were frozen and thawed at temperature settings with pre-cooling (Fig. 1b) at the maximum CAS speed. The results showed mild spacing between neural fibers in HE staining and clearly reduced myelin vacuolization (Fig. 3). Electron microscopy also showed less myelin vacuolization (Fig. 3e) and clearly increased cell viability (Fig. 3f). Pre-cooling and post-cooling before and after the ice crystal formation zone seemed to effectively inhibit ice crystal formation.

The above results showed that freezing and thawing with the CAS at the maximum CAS speed (3.5 ℃/min) and with pre-cooling and post-cooling around the ice crystal formation zone improved viability, suggesting that this was the optimal program for freezing and thawing to use in the subsequent analyses.

Analysis of the effects of magnetic field intensity

We then analyzed the effect of magnetic field intensity on cellular viability at the temperature settings optimized through the previous experiments. The mean cell counts were 312459.7 at intensity 10 (46.9 V, 1.06 mT), 243898.4 at intensity 9 (46.1 V, 1.03 mT), 393384.6 at intensity 7 (43.4 V, 0.98 mT), 319203.5 at intensity 5 (38.6 V, 0.88 mT), 474309.4 at intensity 3 (29.6 V, 0.67 mT), 434970.9 at intensity 2 (23.3 V, 0.53 mT), and 255137.9 at intensity 1 (13.2 V, 0.26 mT). The counts were 292678.1 without CAS and 214001.2 with CAS in a 37 °C water bath. Importantly, the neuronal viability rates were 18.32%, 14.30%, 23.07%, 18.72%, 27.81%, 25.50%, 14.96%, 17.16%, and 12.55%, respectively, at the above intensities. Although the differences were not statistically significant, viability was the lowest after thawing in a 37 °C water bath (12.55%) and the highest in a magnetic field of 0.67 mT (intensity 3 [29.6 V], frequency setting 10 [60 Hz]).

Analysis of the effects of magnetic field frequency

We also analyzed the effect of magnetic field frequency on cellular viability at the temperature settings optimized through the previous experiments. As shown in Table 1b, the mean cell counts were 202312.1 at frequency setting 9 (43 Hz, 1.0 mT), 356668.7 at frequency setting 8 (9 Hz, 1.07 mT), and 349325.5 at frequency setting 7 (2.3 Hz, 1.1 mT). The viability rates were 11.86%, 20.91%, and 20.48%, respectively, at the above frequency settings; however, there were no statistically significant differences. The above

results suggested that cell viability was the highest at 0.67 mT (intensity 3 [29.6 V], frequency setting 10 [60 Hz]). Thus, these conditions were used for the cryopreservation of nerve tissues (Fig. 8a).

Analysis of HE staining and anti-S100 antibody staining of the sciatic nerve at 4 and 8 weeks after transplantation

As shown in Figures 6 and 7, there were no clear differences between the CAS+ and CAS- samples at 4- and 8-weeks post-transplantation, as observed by HE staining. However, Schwann cells increased at the transplantation site in the CAS+ samples at 4- and 8-weeks post-transplantation, as observed by fluorescence staining (Fig 6 and 7). The mean number of Schwann cells in the transplanted nerve at 4 and 8 weeks was 8.0 ± 14.09 and 22.8 ± 8.29, respectively, for the CAS- samples (5ROIs, respectively); 44.4 ± 21.13 and 42.4 ± 19.37, respectively, for the CAS+ samples (5ROIs, respectively) (Fig. 9a and 9b). Statistically, Schwann cell count was significantly different between CAS+ and CAS- samples at 4 weeks post-transplantation ($P=0.0015<0.05$). Moreover, at 8 weeks post-transplantation, a correlation test between the 2 groups showed that the correlation approached significance ($P = 0.0878$), suggesting a difference in correlation.

Anterior tibial muscle wet weight at 2, 4 and 8 weeks after transplantation

We also monitored the tibial muscle wet weight to assess the health status and growth of the sciatic nerve.

The mean anterior tibial muscle wet weight at 2, 4, and 8 weeks was 0.26 ± 0.055, 0.25 ± 0.056, and 0.23 ± 0.029 g, respectively, for the CAS- samples (n = 18, n = 14, and n = 14, respectively); 0.30 ± 0.060, 0.24 ± 0.054, and 0.29 ± 0.104 g, respectively, for the CAS+ samples (n = 14, n = 15, and n = 14, respectively); and 0.28 ± 0.046, 0.25 ± 0.074, and 0.25 ± 0.111 g, respectively, for the controls (n = 10, n = 10, and n = 12, respectively). Although the differences were not statistically significant, the muscle wet weight at 8 weeks in the CAS+ samples had increased to a level similar to that of the controls (Fig. 8b). Furthermore, testing for a common correlation in the CAS+- groups at 8 weeks approached significance ($P = 0.0981$), suggesting a difference in correlation.

Discussion

Intracellular and extracellular ice crystal formation represents a major problem in cryopreservation and can cause cell death, regardless of the speed at which the tissue passes through the ice crystal forming zone (between 0 °C and -5 °C) (Mazur, 1977; Bank and Mazur, 1972). The CAS has been reported to inhibit ice crystal formation through vibrations induced by the magnetic field, and encouraging results have been reported for cryopreservation of periodontal ligament cells, dental pulp tissues, and neural stem cells (Kaku et al. 2010; Kojima et al. 2015; Nishiyama et al. 2016). Cell death, induced by ice crystal

formation, increases due to an increase in ice crystal volume. This mainly happens when the temperature changes while passing through the ice crystal formation zone is slower (Kawata 2016). For stem cells, good outcomes have been reported by applying a magnetic field during freezing and thawing to inhibit ice crystal formation and reformation (Shikata et al. 2016). In this study, tissues were observed to identify the optimal temperature settings, that is, pre- and post-ice crystal formation zone cooling to accelerate passage through the ice crystal formation zone. Also, the effects of a magnetic field in controlling the intracellular and extracellular ice crystal formation and reformation during the freeze-thaw process were studied. The results showed that increasing the freezing speed promoted intracellular ice crystal formation, but the CAS controlled this process to maintain cellular viability. Moreover, the findings showed that cellular viability was the highest in the presence of a magnetic field of 0.67 mT (intensity 3 [29.6 V], frequency setting 10 [60 Hz]). The viability of Schwann cells in transplanted nerves has been reported to be important for nerve regeneration (Kornfel et al. 2019). The viable cryopreserved nerve grafts are expected to support axonal regeneration more efficiently than a dead or acellular graft (Fansa et al., 2000). Based on this, CAS was suggested for nerve preservation. In addition, there is concern about rejection after nerve transplantation; however, it has been reported that changes in MHC and rejection decline due to freezing, opens up a possibility of future allotransplantations (Evans et al. 1998). However, regardless of the presence of a magnetic field, the overall survival rate of cells was lower than that reported for cells from other body tissues. This could be attributed to the sensitivity of nerve cells to

collagenase, which was used in the culturing process. Neurons are histologically more fragile and show greater sensitivity to collagenase than hepatic cells and other cells in culture. Cryopreservation agents themselves are also toxic. These factors were all thought to have affected the experimental results. In addition, the lack of a statistically significant difference in the wet weight of the anterior tibial muscle was thought to be due to the smaller number of sciatic nerves and to the fact that rats were agitated after nerve transplantation, leading to suture failure.

Studies on the development of optimal freezing technologies have suggested that the establishment of cryopreservation media, better adapted for tissue freezing, is necessary. Moreover, the optimal magnetic field intensity of the CAS used in this study for nerve tissue freezing was 29.6 V, but 60 Hz, 3.5 ℃/min was the maximum frequency and speed settings of this freezer, suggesting the need to develop machines with even higher maximum frequency and speed settings. In addition, as the currently available allogeneic nerve tissue is decellularized by chemicals, allogeneic nerve tissue with living cells need to prove its value before used in clinical settings. Immunogenicity must be evaluated, and comparative studies would be needed.

Conclusions

In this study, the optimal program for CAS freezing was determined to promote the preservation and maintain the viability of neural tissues using the CAS. Pre-cooling and post-cooling increased the speed

of passage through the ice crystal formation zone. Additionally, applying a magnetic field during the freeze-thaw process, prevented intracellular and extracellular ice crystal formation and reformation. Furthermore, assessment of the optimal intensity and frequency settings for the magnetic field showed that a magnetic field of 0.67 mT (intensity 3 [29.6 V], frequency 10 [60 Hz]) was associated with the highest viability. However, a limitation of this study was the decrease in the survival rate of cells while evaluating the viability of nerves, which can be improved further by optimizing other processes involved in the study. Moreover, the optimal magnetic field strength of CAS used for freezing neural tissue in this study was 29.6 V, but the maximum frequency setting and speed setting of this freezer were 60 Hz and 3.5°C/min, respectively, which is even higher. The necessity of developing a machine with maximum frequency and speed setting was suggested.

Compliance with ethical standards

Funding: None.

Conflicts of interest: The authors declare that they have no conflict of interest.

Research involving animals: Experimental protocols were approved by the Institutional Animal Care and Use Committee of Dokkyo Medical University (Permit Number: 1015), which operates in accordance with the Japanese Government for the care and use of laboratory animals.

References

Bank H, Mazur P (1972) Relation between ultrastructure and viability of frozen-thawed Chinese hamster tissue culture cells. Exp Cell Res 71:441-454. https://doi.org/10.1016/0014-4827(72)90314-x.

Evans PJ, Mackinnon SE, Levi AD, Wade JA, Hunter DA, Nakao Y, Midha R (1998) Cold preserved nerve allografts: changes in basement membrane, viability, immunogenicity, and regeneration. Muscle Nerve 21:1507-1522. doi:10.1002/(sici)1097-4598(199811)21:11<1507::aid-mus21>3.0.co;2-w

Fansa H, Lassner F, Kook P, Keilhoff G, Schneider W (2000) Cryopreservation of peripheral nerve grafts. Muscle Nerve 23:1227-1233. doi:10.1002/1097-4598(200008)23:8<1227::aid-mus11>3.0.co;2-6

Hibino H, Hatake K, Sugimura Y (1995) Experimental study on cryopreservation methods of cultured mucosal epithelium. Jpn J Oral Maxillofac Surg 41:32-38. [Article in Japanese] doi:10.5794/jjoms.41.616

Kaku M, Kamada H, Kawata T, et al. (2010) Cryopreservation of periodontal ligament cells with magnetic field for tooth banking. Cryobiology 61:73-78. https://doi.org/10.1016/j.cryobiol.2010.05.003.

Kawata T (2016) Freezing of premolar and wisdom teeth extracted for orthodontic treatment. Kanagawa Shigaku 51:9-19. [Article in Japanese]

Kojima SH, Kaku M, Kawata T, et al. (2015) Cranial suture-like gap and bone regeneration after

transplantation of cryopreserved MSCs by use of a programmed freezer with magnetic field in rats. Cryobiology 70:262-268. https://doi.org/10.1016/j.cryobiol.2015.04.001.

Kornfeld T, Vogt PM, Radtke C (2019) Nerve grafting for peripheral nerve injuries with extended defect sizes. Wien Med Wochenschr 169:240-251. https://doi.org/10.1007/s10354-018-0675-6

Koseki H, Kaku M, Kawata T (2013) Cryopreservation of osteoblasts by use of a programmed freezer with a magnetic field. Cryo Letters 31:10-19.

Mazur P (1977) The role of intracellular freezing in the death of cells cooled at super optimal rates. Cryobiology 14:251-272. https://doi.org/10.1016/0011-2240(77)90175-4

Nishiyama Y, Iwanami A, Kohyama J (2016) Safe and efficient method for cryopreservation of human induced pluripotent stem cell-derived neural stem and progenitor cells by a programmed freezer with a magnetic field. Neurosci Res 107:20-29. https://doi.org/10.1016/j.neures.2015.11.011.

Sano K, Hashimoto T, Ozeki S, et al. (2015) Histological alteration of amputated tail of a rat after CAS freezing and its possibility of replantation. J Jpn Soc Surg Hand 32:95-97. [Article in Japanese]

Shikata H, Kaku M, Kojima S (2016) The effect of magnetic field during freezing and thawing of rat bone marrow-derived mesenchymal stem cells. Cryobiology 73:15-19. https://doi.org/10.1016/j.cryobiol.2016.06.006.

Figure captions

Fig. 1 Determination of optimal conditions for freezing and thawing

(a) Temperature was set according to a previous report by Sano et al. (2015). Maximum freezing speed (3.5 ℃/min); magnetic field intensity, 29.6 V; frequency setting, 60 Hz. (b) Pre-cooling and post-cooling ice crystal formation zone. (c) No pre- or post-cooling. Enzymes become inactive at –30 °C; therefore, the minimum freezing temperature was set at –50 °C

Fig. 2 Analysis of the CAS

(a) Control, (b) Cell Alive System (CAS)+, (c) CAS-. Electron microscopy images show myelin vacuolization (*) and axonal compression (**), whereas the control does not. Myelin sheath (#); axon (##); mitochondria (###). The myelin vacuolization and axon narrowing were reduced in tissues frozen with the CAS.

Fig. 3 Analysis of freezing and thawing speed settings

Slow speed (0.5 ℃/min): (a) Hematoxylin and eosin (HE) staining. Spaces were observed between neural fibers (arrows). (b) Electron microscopy. Myelin sheaths are indicated by arrows. (c) Nerve cell cultures.

Viable cells were observed. Maximum speed (3.5 ℃/min). (d) HE staining. Spaces were observed between neuron fibers. (e) Electron microscopy. Myelin sheaths are indicated by arrows. (f) Anti-S100 antibody immunostaining of nerve cells. In histological findings, the rate of 3.5 °C/min was better than 0.5 °C/min.

Fig. 4 Analysis of the thawing method

(a) Thawing in a 37 °C constant temperature water bath. (b) Natural thawing. Myelin vacuolization (#) and axonal compression (##). Electron microscopy imaging showed considerable myelin vacuolization and axon narrowing in tissues thawed naturally and in the tissues thawed in a 37 °C water bath.

Fig. 5 Analysis of pre-cooling and post-cooling settings

(a) Hematoxylin and eosin (HE) staining. Narrow spaces were observed between nerve fibers. (b) Electron microscopy of myelin vacuolization. (c) Anti-S100 antibody immunostaining of nerve cells. HE staining showed small spaces between neural fibers, whereas electron microscopy images showed myelin vacuolization. Nerve cell cultures showed very low cell viability.

Fig. 6 Transplanted nerve tissues from Cell Alive System (CAS) (at 4 weeks). (a) Hematoxylin and eosin (HE) staining, (b) anti-S100 antibody immunostaining. ↑, sutured site. Transplanted nerve tissue from

CAS+ rats at 4 weeks. (c) HE staining, (d) anti-S100 antibody immunostaining. ↑, sutured site. Schwann cells increased at the transplant site in the CAS+ samples at 4 weeks post-transplantation, as observed by fluorescence staining.

Fig. 7 Transplanted nerve tissues from Cells Alive System (CAS) (at 8 weeks). (a) Hematoxylin and eosin (HE) staining, (b) anti-S100 antibody immunostaining. ↑, sutured site. CAS+ transplanted nerves at 8 weeks. (c) HE staining, (d) anti-S100 antibody immunostaining. ↑, sutured site. Similar to 4 weeks after transplantation, Schwann cells increased at the site of the CAS+ sample at 8 weeks post-transplantation.

Fig. 8 Analysis of the effects of magnetic field frequency

(a) Effects of magnetic field intensity with a fixed frequency setting (10, 60 Hz). Cell viability was the highest at 0.67 mT (intensity 3 [29.6 V] and frequency setting 10 [60 Hz], (b) Effects of the CAS on anterior tibial muscle wet weight. Muscle wet weight at 8 weeks in the CAS+ samples increased, similar to the level of the controls.

Fig 9 Comparison of the number of Schwann cells in transplanted nerves

(a) ROI (1.5 × 1.5 mm white square, 20 ROIs) was set, and Schwann cells were counted at 5 sections in the transplanted nerve. (b) At 4 weeks and 8 weeks after transplantation, the number of Schwann cells

increased significantly when compared to the CAS+ or CAS- samples.