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Functional survival of rat pituitary gland in hypothermic storage for pituitary transplantation

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Abstract

Purpose Deteriorated pituitary function can lead to serious complications that might need lifelong hormone replacement therapy. However, long-term hormone administration can have significant adverse effects. Thus, it would be more desirable to restore pituitary function by pituitary transplantation. In this study, we investigated functional preservation of extracted pituitary gland in special preservation solution under hypothermic condition for pituitary transplantation.

Methods We obtained nineteen pituitary glands from 250–300 g male Sprague–Dawley rats via parapharyngeal approach. These extracted glands were divided into three pieces and stored in histidine-tryptophan-ketoglutarate (HTK) solution at 4 °C and compared to their corresponding glands stored in phosphate buffer saline (PBS). Light and electron microscopic examinations were performed to identify morphological changes of pituitary gland at 0,3, and 7 days after storage. TUNEL assay to confirm cell viability, and adenosine-triphosphate (ATP) concentration were also serially examined.

Results Tissue architecture and cellular viability of specimens preserved in HTK solution for 3 days were considerably maintained and similar to those in normal pituitary gland (0 day specimen). In contrast, specimens stored in PBS were markedly destroyed after 3 days of storage. After 7 days of storage, significant degeneration occurred in tissues stored in both HTK and PBS. However, tissue architecture was preserved more in specimens stored in HTK solution than those stored in PBS. ATP concentration decreased more rapidly in specimens stored in PBS solution, but there was no statistical significance ($p=0.055$).

Conclusions Extracted rat pituitary gland supplemented with special preservation solution could be preserved for 3 days under hypothermic condition.

Keywords Histidine-tryptophan-ketoglutarate solution · Organ preservation · Pituitary gland · Simple static cold storage · Transplantation

Introduction

The pituitary gland is a key regulator of the endocrine system composed of the hypothalamic–pituitary–organ axis. The hypothalamus delivers precise signals to the pituitary

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gland, which then releases various hormones to stimulate target endocrine organs. These pituitary hormones can directly affect functions of the thyroid gland, the adrenal gland, and gonads. They can also influence growth, lactation, and water balance [1]. When the function of the pituitary gland deteriorates due to various reasons such as tumor, infection, inflammation, or surgery, serious complications including diabetes insipidus, hypothyroidism, and hypocortisolemia can develop that may need lifelong hormone replacement therapy [2]. However, the long-term steroid and thyroid hormone replacement therapy can have significant adverse effects [2, 3]. These adverse effects of long-term hormone replacement therapy may be overcome by pituitary transplantation, a curable treatment option for hypopituitarism [4, 5].

Organ transplantation remains the only effective therapy for end-stage organ failure in many cases [6]. The first and extremely important step for organ transplantation is "good organ preservation" [7]. Cooling, the first line of defense against hypoxic tissue damage, is one of the most powerful method to slow biological deterioration in organs extracted from their normal physiologic environments [6, 8]. Reducing cellular metabolism and the oxygen requirement by cooling can prevent tissue injury in extracted organs [6, 9]. Currently, simple static cold storage (SCS) is the main method for organ preservation [10]. SCS is based on cooling supplemented with special preservation solution containing macromolecular colloids, carbohydrates, antioxidants, and energy compounds. This combination of special preservation solution and a hypothermic condition can minimize the effect of ischemic injury [6, 10–12]. Hypothermia, ranging from 4 to 8 degrees in Celsius can achieved by using a refrigerator or melting ice. This can be used for short-term preservation of extracted organs for a period of several hours to a few days during which they can be transported to distant locations or storage in hospitals. SCS with special preservation solution can reliably provide good early function after organ transplantation of most solid organs such as kidney, liver, heart, and lung [10, 13]. However, to the best of our knowledge, research regarding preservation for pituitary gland transplantation has not been reported.

In this study, we investigated whether the pituitary gland could survive in special preservation solution under hypothermic condition and assessed the duration of pituitary gland preservation.

Materials and methods

Animals

Nineteen male Sprague Dawley rats weighing 250–300 g (Daehan biolink, Eumseong, Korea) were used. These animals were housed in a controlled temperature laboratory that was maintained on a 12 h light–dark cycle. Food and water were provided ad libitum. All protocols using animal were approved by Institutional Animal Care and Use Committees (IACUC) of the Catholic University of Korea (SVH IRB 17-5).

Parapharyngeal approach for pituitary gland extraction

All mice were anesthetized with isoflurane and a midline incision was made in the anterior neck. Entry to the floor of the cranium was obtained through the omohyoid muscle at the lower edge of the sternohyoid muscle. The sternohyoid muscle, trachea, and esophagus were retracted laterally. Drilling was done under a binocular microscope with the bone burr held vertically and precisely at the midline and in front of the blue suture line. A circular piece of the last layer of bone was broken off. The hole was widened until most of the underlying pituitary gland was exposed (Online Resource 1). The gland covered with a fine membrane was obtained en-bloc by fine forceps. All extracted pituitary glands were divided into three pieces. One piece was used for immunohistochemistry, ATP measurement, or electron microscopy (EM) postoperative immediately. The other two pieces were stored in HTK solution or PBS. They were examined at postoperative 3 and 7 days.

Preservation of pituitary gland

The histidine-tryptophan-ketoglutarate (HTK) solution was used for the preservation of surgically extracted pituitary gland. HTK solution was developed in the 1970's by Bretschneider. Since then, it has been used as a preservation solution for abdominal organs such as the liver, kidney, heart, and pancreas [11]. It contains sodium chloride 876.6 mg/L, potassium chloride 671 mg/L, magnesium chloride 813.2 mg/L, histidine HCl 3.7733 g/L, histidine 27.9289 g/L, potassium hydrogen 2-ketoglutarate 184.2 mg/L, calcium chloride 2.2 mg/L, L-tryptophan 408.5 mg/L, and D-mannitol 5.4651 g/L. We placed the extracted pituitary glands in HTK solution and stored in a refrigerator to maintain a temperature at 4 °C. We also stored some of these glands in phosphate buffer solution (PBS) at 4 °C as a control.

Immunohistochemical examination

For histopathologic examination, each pituitary gland was fixed in 10% formalin and embedded in paraffin. Serial sections of the pituitary gland were cut to 4 μm thickness with a cryostat and mounted onto slides. After deparaffinization and rehydration with xylene, ethanol, and deionized H_2O,

these sections were stained with hematoxylin and eosin (H&E). Reticulin fiber staining using modified Gomori's silver impregnation method was also conducted to detect any disruption of the normal acinar architecture of anterior pituitary gland. The process of reticulin staining was carried out as described previously [14]. Deparaffinized and rehydrated sections were oxidized in 0.5% potassium permanganate solution for 5 min and rinsed with tap water. These sections were reduced in 2% potassium metabisulfite solution for 30 s. After washing with tap water, they were sensitized in 2% iron alum solution for 10 min and rinsed with distilled water. The sections were then placed in the ammoniacal silver solution for 1 min. After washing with distilled water, the sections were developed with 20% formalin solution for 90 s and incubated with 0.2% gold chloride solution for 10 s. Finally, these sections were washed with distilled water, dehydrated in an ethanol series, and mounted with coverslips.

We also performed high-mobility group box 1 (HMGB1) staining for detecting necrosis [15]. Formalin-fixed paraffin embedded 4 μm thick sections were used for the HMGB1 staining. After treatment with xylene, ethanol, and washing with PBS, these sections were incubated with 5% bovine serum albumin (BSA) (Sigma Aldrich Chemical Co., St. Louis, Missouri, USA) for 30 min at room temperature to block non-specific antibody binding. They were then incubated with monoclonal antibodies against HMGB1 (1:300 dilution, Sigma Aldrich Chemical Co., St. Louis, Missouri, USA) as the primary antibody overnight at 4 °C. After washing with PBS, tissues were incubated with the corresponding biotinylated secondary antibody at room temperature for 30 min. These sections were then stained with 3,3-diaminobenzidine (DAB) (Vector Labs, Burlingame, CA, USA). Finally, they were washed in distilled water, dehydrated in an ethanol series, and mounted with coverslips.

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay was conducted to detect apoptosis of pituitary cells. Rat pituitary slides were fixed with ice-cold 4% paraformaldehyde (Solarbio) for 15 min followed by permeabilization with 0.1% (v/v) Triton X-100 (Solarbio) for 3 min. Cells were incubated with TUNEL reaction mixture (Roche, Penzberg, Germany) for 1 h at 37 °C in a wet and dark environment. Subsequently, nuclei were stained with 6–diamidino–2–phenylindole (DAPI; Sigma). Positive TUNEL staining was visualized and photographed with a fluorescence microscope (Olympus, Tokyo, Japan). The percentage of TUNEL positive cells was calculated as the apoptosis index (number of positive cells/total number of cells × 100%).

ATP concentration measurement

We measured adenosine-triphosphate (ATP) concentration using a commercial ATP assay kit (ATP assay kit, Biomax Co., Seoul, Korea) with a colorimetric method. Briefly, 10 μL of 10 mM ATP standard solution was diluted with 90 μL of distilled water to generate 1 mM ATP standard solution. Then 0, 2, 4, 6, 8, and 10 μL of the 1 mM ATP standard solution was placed into a 96 well plate. ATP assay buffer was added to each well to bring the volume to 50 μL for making standards. For sample preparation, we homogenized the tissue in 100 μL of ATP assay buffer after deproteinization using 10 kDa cut off the spin column. Then 50 μL of homogenized tissue was added into duplicate wells of a 96 well plate. Fifty μL of the reaction mix was added to each well and mixed by pipetting. The plate was incubated at room temperature for 30 min, protecting the plate from light during the incubation. Finally, the absorbance at 570 nm was measured using a microplate reader. After obtaining the ATP standard curve by measuring the ATP standards the concentration of ATP in pituitary tissue was calculated according to the ATP standard curve.

Electron microscopic examination

The ultrastructure of the extracted pituitary gland was observed by electron microscopy (EM). The tissue was placed in fixative and washed with PBS. Then it was post-fixed in phosphate-buffered 1% osmium tetroxide. After serial dehydration in increasing concentrations of alcohol, the tissue was embedded in epon resin. Ultrathin Sects. (70–80 nm) were cut using an ultramicrotome (Ultracut UCT, Leica, Austria). The sections were subsequently stained with uranyl acetate and lead citrate. Ultrastructure of these specimens including the nuclei, rough endoplasmic reticulum (RER), mitochondria, and Golgi apparatus was examined using an EM (JEM-1010, Jeol, Japan).

Statistical analysis

SPSS version 20.0 (IBM, Armonk, NY, USA) was used for the statistical analysis. We used *t* test to compare the ATP concentration and percentages of TUNEL assay positive cells between the two groups preserved with HTK solution and PBS. P values < 0.05 were considered significant.

Results

Morphological changes according to time based on immunohistochemical examination

Nine out of 19 pituitary glands were used for immunohistochemistry (3 for H&E and reticulin stain, 3 for HMGB1, and 3 for TUNEL assay). The specimens were examined at 0, 3, and 7 days after surgery. As compared to H&E and reticulin stained specimen (0 day) as control, 3 days specimens stored in PBS showed signs of necrosis, including loss of normal glandular structure with amorphous areas, reduction in cell size, decreased number of acidophils and basophils, and disruption of normal acinar architecture outlined by a reticulin stain. On the other hand, specimens stored in HTK solution showed considerably preserved glandular and acinar structures of the anterior pituitary gland. For samples stored for 7 days, extensive necrosis and destruction of normal architecture were observed for specimens preserved in PBS. In contrast, the damage was less apparent after 7 days of storage for specimens preserved in HTK solution (Fig. 1a and b).

HMGB1 staining also showed a distinct difference in tissue viability between tissues preserved with HTK solution and PBS. For specimens stored in HTK solution for 3 days, a nearly normal structure was observed. Their staining pattern was similar to that of HMGB1 stained control specimen (0 day). Specimens stored in PBS for 3 days and those stored in HTK solution for 7 days showed similar microscopic morphologies, including increased necrotic area, reduced cell size and number, and loss of normal glandular structures. The tissue preserved in PBS for 7 days showed extensive necrosis (Fig. 2).

The percentage of TUNEL-positive cells was also different between the two groups (stored in HTK solution and PBS) based on time. For samples stored for 3 days, the mean percentage of apoptotic cells was significantly higher

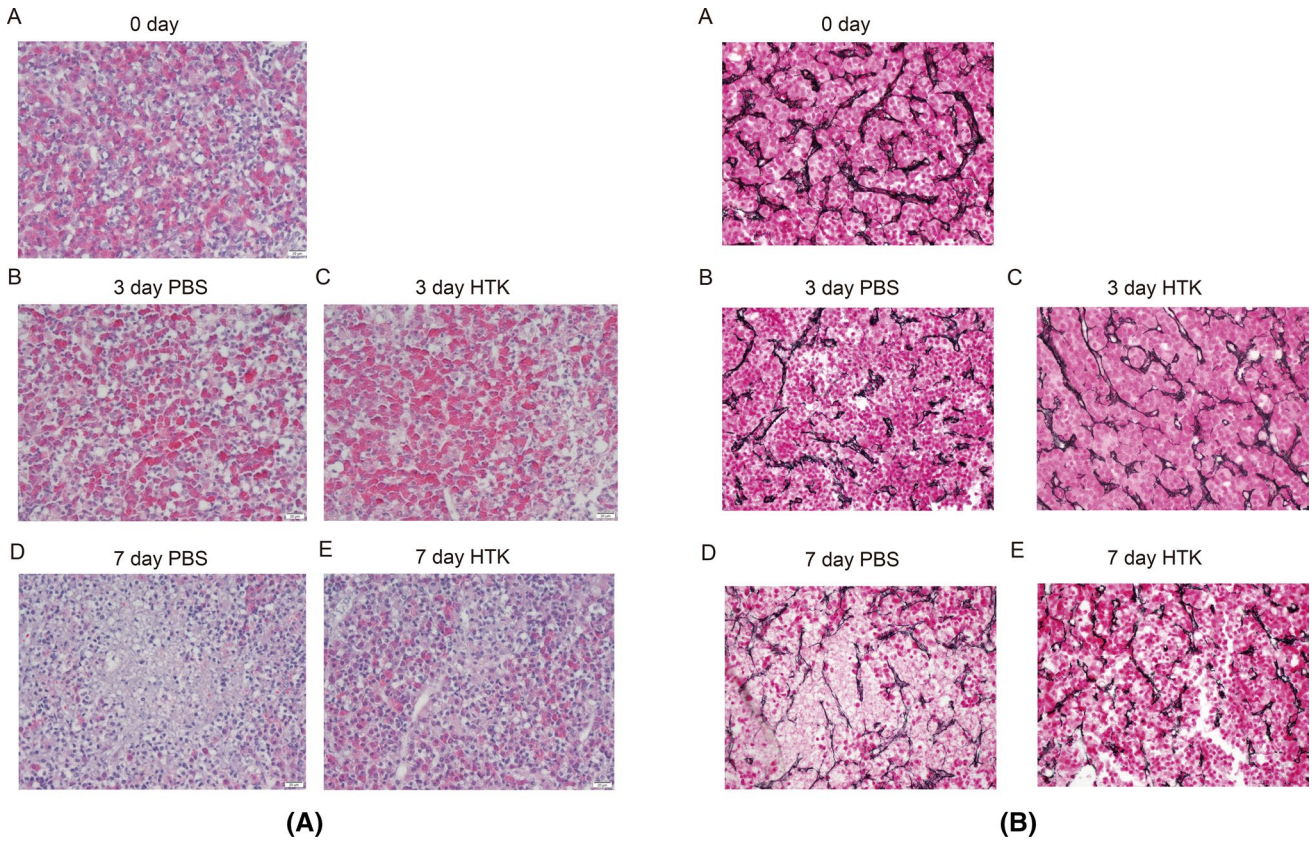


Fig. 1 **a (H&E stain) and b (reticulin stain) a** H&E and reticulin staining of normal pituitary gland tissue (day 0, original magnification × 400). Normal glandular structure and intact reticulin lobular architecture are noted. **b** Pituitary tissue stored in PBS for 3 days, showing signs of necrosis with focal loss of glandular and lobular structure and decreased cell number. **c** In contrast, Pituitary tissue stored in HTK solution for 3 days, showed considerably preserved normal glandular structures with spared cell size and number and lobular structure. **d** Pituitary tissue after 7 days of preservation in PBS solution, showed extensive necrosis with destroyed lobular structure. **e** Pituitary tissue after 7 days of preservation in HTK solution, showed less apparent destruction of the normal glandular and lobular structure. (scale bar = 20 μm)

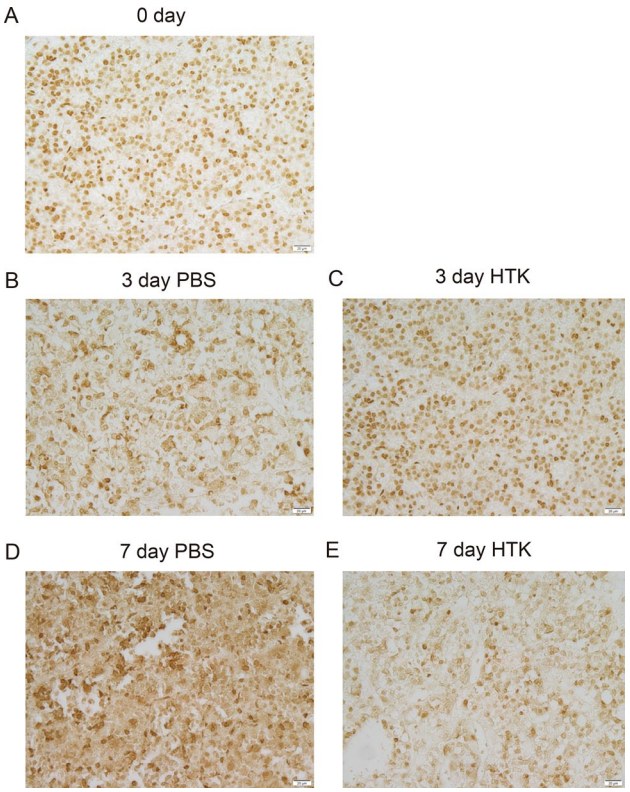


Fig. 2 **a** HMGB1 staining of normal pituitary gland (day 0, original magnification x 400). **b** Pituitary tissue stored in PBS for 3 days, showing increased necrotic area, reduction in cell size and number, and loss of normal glandular structure. **c** Pituitary tissue stored in HTK solution for 3 days, showing nearly preserved normal architecture. **d** Extensive necrosis after 7 days of preservation in PBS. **e** Signs of necrosis after 7 days of preservation in HTK solution. (scale bar = 20 μm)

in specimens stored in PBS (79.3 ± 3.2%) than those stored in HTK solution (30.1 ± 3.0%, $p < 0.001$). However, there was no significant difference in the number of apoptotic cells between the two groups preserved with HTK solution (51.3 ± 2.5%) and PBS (66.1 ± 3.4%, $p = 0.307$) after storage for 7 days (Fig. 3).

Comparison of ATP concentration

A total of six pituitary glands (3 with HTK solution and 3 with PBS) were used to compare ATP concentrations between the two groups. The ATP concentration was measured at 0, 3, and 7 days after surgery. After 3 days, mean ATP concentration levels were decreased to 72.4% and 60.1% of their initial levels in the tissue preserved with HTK solution and PBS, respectively, and had no significant difference between the two groups ($p = 0.183$). At 7 days after surgery, the mean ATP concentration in the tissue stored in PBS was markedly decreased to 25.6% of its initial level, while that in the corresponding tissue stored in HTK solution was

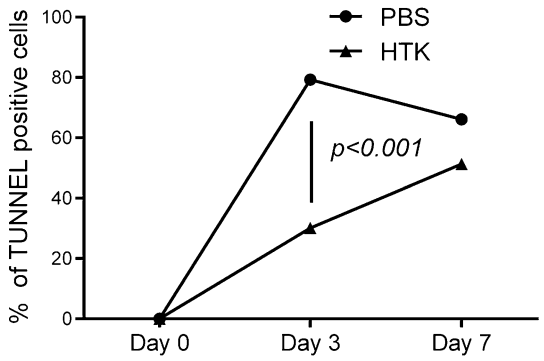


Fig. 3 TUNEL assay of the normal pituitary gland (day 0), showing no apoptotic cells. Pituitary tissue preserved with PBS for 3 days, showing a significantly higher proportion of apoptotic cells compared to corresponding tissue preserved with the HTK solution. The occurrence of apoptosis is decreased in the PBS group while that in the HTK solution group is increased after 7 days of preservation

decreased to 60.6% of its initial level. However, there was no significant difference between the two groups ($p = 0.055$) (Fig. 4).

Ultrastructural changes of pituitary gland on electron microscopic examination

We examined various ultrastructural parameters including mitochondrial damage, change in the nucleus, rough endoplasmic reticulum (RER), secretory granules (SGs), and area of necrosis. After 3 days of preservation, almost all mitochondria and RER was destroyed without having normal architectures, and numbers of SGs were markedly decreased in tissues preserved with PBS. In addition, there were also some necrotic areas in these specimens. In contrast, tissues preserved with HTK solution for 3 days maintained relatively normal structures compared to corresponding tissues preserved with PBS, although pyknotic nuclei and vacuolar

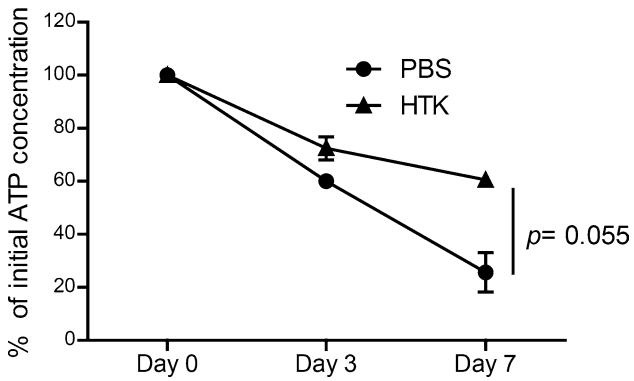


Fig. 4 ATP concentration measured at baseline, 3 and 7 days after surgery in both preservation solutions were analyzed. Levels of ATP concentration were higher in the HTK solution group compared to those in the PBS group throughout the preservation time

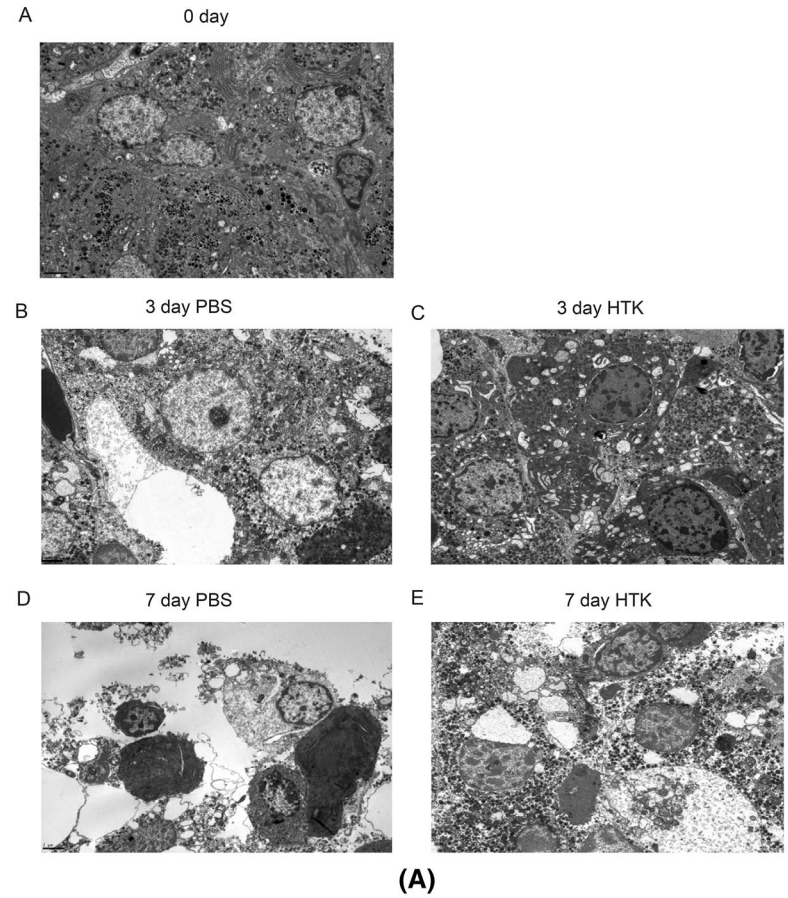
RER were observed. After 7 days of preservation, there were few normal structures with extensive necrosis for tissues with PBS. On the other hand, a moderate amount of unaffected areas were still observed for tissues after 7 days of preservation in the HTK solution (Fig. 5).

Discussion

Preserving the quality of donor organs is one of the major determinants for the good outcome of organ transplantation. Organ preservation has been described as "the supply line for organ transplantation". In a logistic sense, "preservation" buys "time" to transport organs from the donor to the recipient by minimizing organ damage [7]. Cooling and specific preservation solutions are essential for the good outcome of solid organ transplantation. SCS and hypothermic machine perfusion (HMP) are currently the two main preservation methods used for most transplantable organs [10, 16, 17]. SCS depends on cooling combined with special preservation solution. It aims to modify and delay inevitable cellular and molecular changes [10]. In contrast, HMP relies on activating residual metabolism by generating energy through oxygen supply by vascular perfusion [17, 18]. So far, only SCS is approved for most solid organs (liver, lung, pancreas, and heart) except kidney (both SCS and HMP are approved) due to technical difficulties, complex equipment, and lack of evidence for the success of HMP [16]. Therefore, we used the SCS method for our experiments.

Effects of cooling include the following; 1) suppressing global metabolic rate, 2) maintaining metabolic pathways capable of sustaining and/or delivering minimal essential energy supply, and 3) enhancing defense mechanisms to allow a cohesive return to normal metabolism during arousal. Tissue damage can be minimized by reducing cellular metabolism and oxygen requirements even in the absence of oxygen supply by these cooling effects [6, 10]. However, cooling has limitations to achieve successful survival of organ because some metabolic rates still remain at 4 °C. Inhibition of the Na^+/K^+ ATPase leads to cell edema and rapid depletion of ATP reserves. ATP depletion causes degradation of adenosine following accumulation of hypoxanthine and xanthine oxidase, leading to cell membrane depolarization and breakdown of ion homeostasis. Combined with other intracellular and membrane-associated cascades, this finally culminates in cell death by either apoptosis or necrosis [19]. In addition, inhibition of endoplasmic reticulum Ca^{2+} ATPase by ATP deficiency, inhibition of Na^+/Ca^{2+} antiporter by increased cytosolic Na^+ concentrations, and reverse function of Na^+/Ca^{2+} antiporter by transporting Ca^{2+} into the cytoplasm can lead to increased cytosolic Ca^{2+} concentration. This can trigger mitochondrial dysfunction by disrupting its membrane permeability. It can also cause Ca deposit, sodium and water within the cell [20]. Based on this theoretical background, we conducted investigations to measure ATP concentration. In our study, the level of ATP concentration was higher in the HTK solution group compared to that in the PBS group at all preservation times (3 and 7 days after preservation) and the ATP concentration decreased more rapidly over time in specimens preserved in PBS. However, there was no statistical significance between the two groups ($p = 0.055$). We thought that this might be due to a small number of specimens. We also examined the ultrastructure, focusing on structural changes of mitochondria and endoplasmic reticulum by electron microscopy. In addition, TUNEL assay was used to confirm apoptosis in specimens stored in HTK solution and PBS over time. Our results demonstrated decreased ATP concentration and structural changes in specimens stored in both PBS and HTK solution over time. However, specimens stored in HTK solution maintained nearly normal structures with spared cell size and number at 3 days. Thus, the extracted pituitary gland could be preserved for 3 days under hypothermic condition supplemented by HTK solution. Results of TUNEL assay in this study showed that apoptosis occurred earlier and more extensively in specimens stored in PBS than in specimens stored in HTK solution. In addition, the percentage of apoptotic cells decreased gradually in the PBS group, suggesting that apoptosis decreased and switched to necrosis over time. On the other hand, the percentage of apoptotic cells increased gradually in the HTK solution group suggesting that residual ATP level was relatively maintained steady and apoptosis was still occurring. Cold-induced cell dysfunction will manifest as apoptosis or necrosis depending on the ATP concentration which is associated with the duration of ischemia. Therefore, residual ATP levels play a key role in converting these two types of cell death. Prolonged ischemia causes the transition of mitochondrial membrane permeability along with ATP depletion, blocked apoptosis, and switches to necrosis [6, 20].

Based on previous studies, the University of Wisconsin (UW) solution has been used as the standard preservation solution for storing extracted solid organs [21–23]. HTK solution has shown similar results compared to organs stored in UW solution regarding kidney and liver function when organs are preserved within the safe preservation periods in previous studies [24–26]. In addition, HTK solution has the advantage of having lower preservation costs. Thus, it has been increasingly popular over the last 20 years [24–26]. Therefore, we used the HTK solution for preserving the extracted pituitary gland in this study. HTK solution is mainly composed of histidine as a strong buffer, mannitol as an osmotic barrier, and tryptophan and alpha-ketoglutaric acid as low-permeable amino acids as substrates for anaerobic metabolism to keep cell membranes stable. Relatively low concentration of electrolyte components such as Na^+,



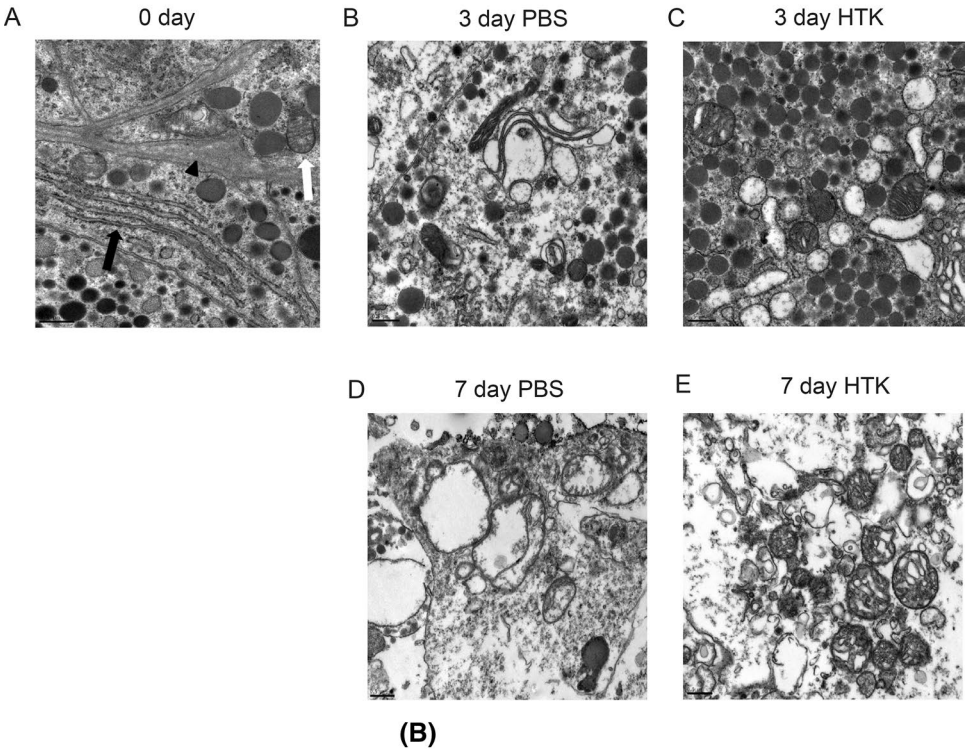


Fig. 5 a (original magnification x1500) and b (original magnification x6000) a Normal ultrastructure of pituitary gland on EM, showing viable cells containing normal mitochondria (white arrow), RER (black arrow), and SG (arrowhead). **b** After 3 days of preservation in PBS, EM showed destroyed mitochondria, vacuolar RER, and markedly decreased number of SGs as well as necrotic area. **c** After 3 days of preservation in HTK, normal architecture is relatively maintained although pyknotic nucleus and vacuolar RER are shown **d** Pituitary tissue stored in PBS for 7 days shows near absence of normal architectures with extensive necrosis. **e** EM showing a moderate amount of unaffected area after 7 days of preservation in HTK solution

K^+, and Mg^{2+} can allow a safe infusion into the recipient's circulation system. Additionally, the low viscosity of HTK solution enables effective flushing and rapid cooling of organs [6, 11, 23].

Harvey Cushing was the first man who attempted pituitary transplantation in 1908. Since then, several investigators have tried to restore pituitary functions by hypophyseal implantation [4, 5, 28–30]. After Cushing's pioneering study, Harris and Jacobshon used pituitary grafting to reveal the hypothalamic control of the pituitary gland in 1952. Halasz et al. coined the term "hypophysiotrophic area" for the anterior region of the hypothalamus that could support pituitary transplantation in 1962 [28, 31]. Thereafter, in 1980, Knigge et al. performed pituitary grafting into the third ventricle of hypophysectomized rats [30]. They found that these grafted rats showed weight gain. Thus, they proposed that cerebrospinal fluid was a possible route of hypothalamic influence from the grafted hypophysis. Tulipan et al. primarily demonstrated restitution of pituitary hormone in plasma in 1985. Their result was then reinforced by Maxwell et al. in 1998 [4, 32]. However, further investigations regarding pituitary transplantation have not existed since 1998.

In this study, we investigated the duration of preservation for extracted pituitary gland stored in HTK solution at 4 °C as a precedent research for pituitary transplantation. Additional experiments including comparing results according to different preservation solutions (HTK solution versus UW solution or two layers oxygenated solution) and various methods (hypothermic storage versus cryopreservation) might be needed to identify the most suitable method for pituitary preservation. However, this is the first research to clarify the period of pituitary storage. It could be used as a cornerstone of interest in the research of pituitary gland storage and transplantation.

Conclusion

This study demonstrates that the extracted rat pituitary gland can be preserved in commercially available media (HTK solution) under hypothermic condition (4 °C) for 3 days.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All procedures performed in studies involving animals were in accordance with the ethical standard of Institutional Animal Care and Use Committees (IACUC) of the Catholic University of Korea (SVH IRB 17-5).

Informed consent For this type of study, formal consent is not required.

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