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Early effects of adipose-derived stem cell sheets against detrusor underactivity in a rat cryo-injury model

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ABSTRACT

Aims: This study investigated the effects of adipose-derived stem cell sheets on a rat model of detrusor underactivity.

Main methods: Adipose-derived stem cell sheets were prepared from the subcutaneous adipose tissue of male Lewis rats. Female Lewis rats were assigned into four groups: control, sham operation, cryo-injury, and cryo-injury+sheet ($n = 8$ per group). Rats in the cryo-injury+sheet group were implanted with ASC sheets 3 days after cryo-injury induction and underwent cystometry 7 days later. Subsequently, reverse transcription-polymerase chain reaction (RT-PCR) and histopathological examinations were performed. Cell sheets expressing the green fluorescent protein were prepared and transplanted to confirm the viability and differentiation of the sheets. Fluorescence was confirmed using a fluorescence stereomicroscope on days 3, 7, 14, 21, and 28 after sheet implantation, and tissue immunostaining was performed.

Key findings: Cystometry showed that sheet implantation improved the maximum intravesical pressure ($P = 0.009$) and the residual urine volume ($P = 0.011$). Furthermore, RT-PCR indicated that the mRNA levels of the angiogenic factors vascular endothelial growth factor and hepatocyte growth factor were significantly higher in the cryo-injury+sheet group than in the cryo-injury group ($P = 0.045$, $P = 0.037$, respectively). Histologically, sheet implantation resulted in an improvement in inflammation and increased the number of blood vessels. Green fluorescent protein-positive cells fused with von Willebrand factor-positive cells and differentiated into blood vessels 7 days after sheet implantation.

Significance: Adipose-derived stem cell sheets transplanted into the bladder of cryo-injured rats differentiated into blood vessels and restored bladder contractile function 7 days after transplantation.

1. Introduction

Lower urinary tract dysfunction has been shown to greatly reduce patients' quality of life (QOL) [1] and is associated with various conditions. Among them, detrusor underactivity (DU) reduces QOL and also induces other diseases such as urinary tract infections and renal dysfunction [2]. DU is a urodynamic diagnosis defined by the International Continence Society as "a contraction of reduced strength and/or duration, resulting in prolonged bladder emptying and/or failure to achieve complete bladder emptying within a normal time span." Moreover, psychiatric disorders such as multiple sclerosis, Parkinson's disease, dementia, spinal cord disease, diabetes, and pelvic surgeries,

Abbreviations: ASC, adipose-derived stem cell; DLPP, detrusor leak point pressure; DU, detrusor underactivity; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; GFP, green fluorescent protein; MSC, mesenchymal stem cells; MIP, maximum intravesical pressure; PBS, phosphate-buffered saline; QOL, quality of life; RV, residual volume; VE, voiding efficiency; VV, voided volume.

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such as radical prostatectomy and extensive hysterectomy, may cause DU. Many risk factors for DU have also been reported, including psychotropic drugs, anesthesia, constipation, and decreased daily activities [3]. Thus, diagnosis and treatment of DU are difficult, and sufficient basic and clinical research has not been conducted. Although intermittent deurination and pharmacotherapy can be considered for DU treatment, there is no pharmacotherapeutic protocol with sufficient evidence yet. Thus, developing new treatment methods for DU is an important issue.

To address this issue, we focused on the field of regenerative medicine in the present study. With recent advancements in stem cell biotechnology and materials science, which have made the clinical application of regenerative medicine more realistic, regenerative medicine is expected to develop as a new medical field to replace organ transplantation. Mesenchymal stem cells (MSCs) have been attracting attention as stem cells for regenerative medicine because they can be harvested from various tissues. MSCs in tissues other than the bone marrow have been identified in multiple studies. In 2001, adipose-derived stem cells (ASCs) were identified by Zuk et al. [4] ASCs have the potential to serve as an innovative therapeutic option for bladder regeneration in the field of urology. However, one of the known problems in stem cell transplantation is the low survival rate of the cells after transplantation [5]. The reasons for this include cell damage caused by isolation operations, exposure to ischemic conditions, apoptosis, inflammation, and immunological rejection [6]. Among the various methods proposed for cell transplantation, the cell sheet method has the potential for higher cell viability [7].

Based on these considerations, we created a rat DU model by inducing cryo-injury to the bladder and applied ASC sheets to the bladder to investigate whether bladder contractile function could be restored. Although stem cells have been previously used to study bladder regeneration, most of these studies used bone marrow-derived stem cells, and very few reports have reported the use of ASCs. Furthermore, none of the previous reports have discussed the transplantation of ASC sheets generated in temperature-responsive culture dishes into rats and the evaluation of physiological functional recovery by cystometry, as well as confirmation of their long-term viability and differentiation. Therefore, in this study, we investigated the differentiation potential of the ASC sheets and evaluated their survival rate by marking them with the green fluorescent protein (GFP).

2. Materials and methods

2.1. Animals

For ASC sheet production, mature male syngeneic Lewis rats weighing 200–250 g were obtained from Japan SLC, Inc. (Hamamatsu, Japan). For the production of GFP-expressing ASC sheets, male Lewis rats (LEW-Tg (CAGEGFP)1Ys), which express GFP in tissue cells throughout the body under the control of the CAG promoter, were obtained from the National Bioresource Project-Rats (Kyoto, Japan). ASC sheet transplantation was performed in 10-week-old female syngeneic Lewis rats weighing 180–230 g obtained from Japan SLC, Inc. (Hamamatsu, Japan).

All rats were housed under standard experimental conditions with a 12-h light/dark cycle and *ad libitum* access to food pellets and tap water. The protocols for animal and genetic recombination experiments were approved by the Research Promotion Organization of Tottori University (32-027, 20-Y-23). All experiments were conducted in accordance with the guidelines established by Tottori University and conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1996). All procedures were performed in accordance with the Tottori University Animal Care Guidelines.

2.2. Grouping of control laboratory animals and intervention methods

The rats were assigned into four groups of eight rats each: the first group underwent induction of bladder cryo-injury followed by application of ASC sheets to the injury site (cryo-injury + ASC sheets group); the second group underwent bladder cryo-injury induction but did not receive ASC sheets (cryo-injury group); the third group underwent the surgical procedure without cryo-injury induction or application of ASC sheets (sham operation group); the fourth group did not undergo any treatment (control group).

2.3. Engineering of ASC sheets

Adipose tissues were obtained from the inguinal subcutaneous fat tissue of male Lewis rats. The obtained adipose tissues were washed with phosphate-buffered saline (PBS) and cut into small pieces. They were digested with 1% collagen-1 at 37 °C for 1 h. ASCs were obtained by centrifugation at 2500 rpm for 5 min and filtered through a 100-μm mesh filter. The cell pellets were seeded into a 10-cm dish and cultured in DMEM supplemented with 20% FBS and 1% penicillin/streptomycin. Subsequently, 35-mm temperature-responsive culture dishes (UpCell, Cell Seed, Inc. Tokyo, Japan) were used to prepare the cell sheets. After 2–3 passages, 1×10^6 ASCs were transferred to FBS-preincubated dishes and cultured for 48 h. The ASC sheets were harvested after maintaining the cells at room temperature for 1 h and then transplanted onto the site of bladder cryo-injury in the female rats. During transplantation, the cultured cells were recovered as intact sheets by leaving them in a 20 °C environment for 1 h.

2.4. Induction of cryo-injury

In this study, we used a rat bladder cryo-injury model. Cryopathy models have been used previously to induce heart and rhabdomyosinus muscle dysfunction [8–10]. These models are characterized by clear and reproducible contractile damage to the bladder and have been used to study therapies aimed at improving bladder function, such as tissue engineering [11]. Cryo-injury to the bladder has been confirmed to cause degeneration of smooth muscle cells with apoptosis due to the reduced oxygen and nutrient supply caused by reduced capillaries compared to the normal bladder wall [12]. The rat bladder in the cryo-injury model is replaced by fibrous tissue that lacks elasticity and loses its contractility. Because cryo-injury to the bladder can cause reproducible contractile dysfunction, it has long been used as a model for studying therapies aimed at improving bladder function, such as tissue engineering [11]. In this study, we created a cryo-injury model using aluminum rods cooled with dry ice, according to a previous report [12].

Three days before ASC sheet transplantation, a cryo-injury procedure was performed on the bladder. First, female Lewis rats underwent 2% isoflurane inhalation (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan), and the isoflurane concentration was subsequently maintained at 4%. A small incision was made in the midline of the lower abdomen in the supine position, and care was taken to ensure that the incision was as small as possible to avoid adhesions. A transurethral catheter (PE10) filled with saline solution was inserted into the bladder. With the bladder inflated, an aluminum rod with a diameter of 8 mm cooled with dry ice was placed in contact with the anterior wall of the bladder for 30 s to induce cryo-injury. The bladder was frozen locally when the aluminum rod was brought into contact with the bladder, but when the rod was released, the bladder thawed quickly due to body heat and became no different from normal tissue (Fig. 1). After the cryo-injury, the bladder was placed in the abdominal cavity, and the wound was closed. In the sham operation group, the bladder was similarly exposed, and only saline was injected, with no cryo-injury administered. No procedure-related deaths were recorded in the four groups.

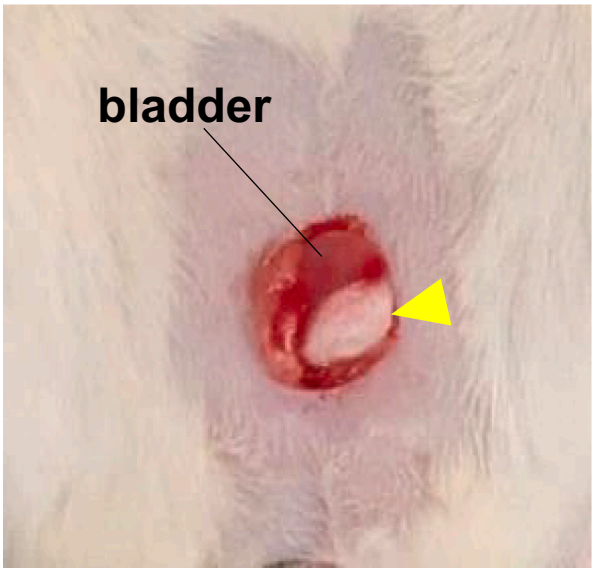

Fig. 1. Preparation of the rat DU model by cryo-injury induction. The rat was placed in the supine position under anesthesia, and an incision was made in the midline of the lower abdomen. The wound was minimized to 1 cm to prevent adhesion. An aluminum rod cooled with dry ice was placed in contact with the anterior wall of the bladder for 30 s to induce a cryo-injury (arrow). Subsequently, the wound was closed, and the rats were awakened from anesthesia.

2.5. Implantation of ASC sheets

To determine the time of implantation of the ASC sheet, we followed a previously reported study [12]. Three days after cryo-injury induction, the rats were anesthetized with isoflurane as described above, and a midline incision was made in the lower abdomen to expose the damaged part of the bladder. Then, a transurethral catheter (PE10) was inserted into the bladder, and saline was injected to fill the bladder. For the cryo-injury + ASC sheet group, the ASC sheet was applied to the cryo-injured area while the bladder was inflated. For the cryo-injury and sham operation groups, the bladder was re-exposed, and saline was injected into the bladder; however, the ASC sheet was not applied. Each operation was performed under a stereomicroscope, and the presence of a small ASC sheet was visually confirmed. Finally, the wound was closed, and the operation was completed. Seven days after these surgeries, the rats were euthanized after performing cystometry, and their bladders were removed for histological and reverse transcription-polymerase chain reaction (RT-PCR) analysis.

2.6. Cystometry

In many previous reports, intravesical pressure was measured by inserting a small-diameter catheter into the bladder transurethrally. However, with this method, the catheter stimulates the urethral afferents and triggers the voiding reflex *via* the urethral/bladder reflex. This may result in underestimation of the bladder capacity. Moreover, transurethral catheter insertion may act as an obstructing factor during voiding, causing an increase in intravesical pressure [13]. Thus, since transurethral catheter insertion reduces the accuracy of voiding muscle contraction pressure measurements and results in an inaccurate assessment of residual urine, we created a bladder fistula just before cystometry measurement.

Cystometry was performed on the 7th day after ASC sheet transplantation (10 days after cryo-injury). Anesthesia was induced with isoflurane (4%) and maintained at 2%. The rats were placed in the supine position, and a midline incision was made in the lower abdomen to expose the bladder. A small incision was made in the bladder, and a polyethylene catheter (PE60; Nippon Becton Dickinson, Tokyo, Japan) was inserted into the bladder lumen and fixed with sutures. The catheter tube inserted into the bladder was extended and connected to a three-way stopcock to monitor the intravesical pressure during cystometry. Next, a polyethylene catheter (PE10) was inserted into the internal jugular vein and fixed with sutures as the transvenous route. After surgery, isoflurane anesthesia was turned off and replaced with urethane anesthesia (0.75 g/kg subcutaneously; Sigma Chemical Co., St. Louis, MO), and additional doses of the anesthetic (0.1 g/kg/injection) were intravenously administered as needed. The level of anesthesia was confirmed by a negative response to the pinch-reflex test. Physiological saline was then continuously infused into the bladder at a rate of 0.04 mL/min at room temperature, and single cystometry findings were recorded over several sessions.

Several cystometry parameters were measured to quantify the effects of the ASC sheet and cryo-injury on voiding function. The maximal intravesical pressure (MIP), which is the maximum intravesical pressure during urination, voided volume (VV), and residual volume (RV) were measured. The gauze was weighed beforehand and placed at the urethral opening, and VV was measured by weighing the gauze again after urination. The RV was calculated by subtracting the VV from the water injection volume (IV). Voiding efficiency (VE) was calculated as VV/IV $\times$ 100. The percentage of urine overflow was also assessed. For rats with urine overflow, the bladder pressure at which urine flow was observed was defined as detrusor leak point pressure (DLPP), not MIP. The overflow was defined as the outflow of raw food from the external urethral opening when a certain intravesical pressure was reached, even though no steep increase or decrease in intravesical pressure was observed. For data comparison, the mean of the cystometry variables obtained from the three urination cycles was compared in each group. The percentage of urine overflow seen was defined as the percentage of three urination cycles for each group of eight rats, in short, 24 urination cycles.

2.7. RT-PCR analysis

RNA was extracted from bladder tissues using an RNeasy Plus mini kit (QIAGEN) and treated with DNase I (Promega) to eliminate genomic DNA. cDNA was synthesized using SuperScriptTM II reverse transcriptase (Gibco BRL). Quantitative RT-PCR (qRT-PCR) was performed using the 7900HT Fast Real-Time PCR System, according to the manufacturer's instructions (Applied Biosystems, CA). The mRNA levels of vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), and basic fibroblast growth factor (bFGF) were expressed as ratios to the β-actin mRNA level and further normalized to the control group value (None).

The primers used were as follows:

Gene name	Probe no.	Sequence
Rattus VEGF	4#	Forward 5′-TTAAA CGA ACG TAC TTG CAG ATG-3′ Reverse 5′-TCT AGT TCC CGA AAC CCT GA-3′
Rattus HGF	49#	Forward 5′- GAT TGG ATC AGG ACC TTG TGA-3′ Reverse 5′- CCA TTC TCA TTT TGT GTT GTT CA-3′
Rattus bFGF	7#	Forward 5′-TCT TCC TGC GCA TCC ATC-3′ Reverse 5′- GCT TGG AGC TGT AGT TTG ACG-3′
Rattus β-actin	115#	Forward 5′- CTA AGG CCA ACC GTG AAA AG-3′ Reverse 5′- GCC TGG ATG GCT ACG TAC A-3′

2.8. Histopathology

The bladders were fixed in 10% formalin and embedded in paraffin. The sections were deparaffinized, rehydrated, and rinsed with PBS. Subsequently, the specimens were subjected to hematoxylin-eosin and anti-von Willebrand factor (vWF) antibody (Abcam Plc, Cambridge, UK) staining to detect capillary formation. Capillaries were counted by light microscopy in five fields of transverse sections per animal. The number of capillaries in each field was averaged and expressed as the capillary

density.

2.9. Expression analysis and immunohistological analysis of GFP

To confirm the viability and differentiation of ASC sheets, GFP-expressing ASC sheets were prepared from the inguinal adipose tissue of male LEW-Tg (CAGEGFP)1Ys rats using the same method. In accordance with the procedure described above, cryo-injury was induced in 25 female Lewis rats, and 3 days later, GFP-expressing ASC sheets were transplanted into the bladder. Five rats were euthanized 3, 7, 14, 21, and 28 days after implantation. The abdomen of the euthanized Lewis rats was cut open and observed under a fluorescent stereomicroscope (M205FA, Leica) to confirm ASC sheet viability under excitation light. The bladder was then removed and fixed in 10% formalin. For double immunofluorescence staining of GFP and vWFGFP, the deparaffinized and rehydrated specimens were blocked with serum at room temperature (20–25 °C) for 1 h and then incubated with anti-vWF (Abcam Plc, Cambridge, UK), or anti-GFP (MBL, Nagoya, Japan) primary antibodies at 4 °C overnight, after which Alexa Fluor 546 anti-rabbit or Alexa Fluor 488 anti-mouse conjugated antibodies were used as the secondary antibodies. The sections were treated with an autofluorescence quenching kit for 7 min at RT before mounting. Images were obtained using an ECLIPSE Ti fluorescence microscope (Nikon).

2.10. Statistical analysis

All data are expressed as mean ± standard error. A one-way analysis of variance (ANOVA) was used to compare items among the four groups showing equal variances and normality. Multiple comparisons using Tukey's test were conducted for items that showed significant differences after the test. For items that did not show equal variances or normality, the Kruskal–Wallis test was used as a nonparametric test for independent sample comparisons. Statistical significance was set at $P < 0.05$. All analyses were performed using SPSS software (version 27.00; IBM Corporation, Armonk, NY, USA).

3. Results

3.1. Cystometry

A cystometry test was performed to confirm the effect of ASC sheets on the bladder cryo-injury model. The typical cystometry findings in each group are shown in Fig. 2.

The results for the cystometry evaluation items are shown in Table 1 and Fig. 3. No significant differences were found in IV and VV, but significant differences were found in MIP ($P = 0.011$), RV ($P = 0.020$), VE ($P = 0.020$), and the percentage of urine overflow ($P = 0.001$).

Multiple-comparison tests were then performed on the items that showed significant differences. For MIP, significant differences were

Table 1
Results of cystometry at 1 week after adipose-derived stem cell (ASC) sheet transplantation (10 days after cryo-injury).

Mean ± SE	Control ($n = 8$)	Sham operation ($n = 8$)	Cryoinjury ($n = 8$)	Cryoinjury + ASC sheet ($n = 8$)	*P*
Maximal Intravesical pressure (cmH_2O)	30.2 ± 2.3	28.2 ± 2.3	21.2 ± 1.0	32.5 ± 3.1	0.011
Detrusor leak point pressure (cmH_2O)	NA	NA	32.6 ± 2.5	NA	NA
Injection volume (μL)	148.6 ± 17.3	151.6 ± 17.6	251.1 ± 45.6	143.1 ± 8.8	0.065
Voided volume (μL)	123.9 ± 18.4	115.0 ± 13.9	147.6 ± 26.0	118.7 ± 10.1	0.584
Residual volume (μL)	24.7 ± 5.8	36.7 ± 11.1	106.9 ± 24.9	24.5 ± 2.4	0.020
Voiding efficiency (%)	77.5 ± 7.2	76.1 ± 5.4	59.9 ± 4.4	80.9 ± 3.0	0.020
Urine overflow (%)	0.0 ± 0.0	0.0 ± 0.0	45.8 ± 15.4	0.0 ± 0.0	0.001

All data are expressed as mean ± standard error. Comparisons of parameters were performed using the Kruskal–Wallis test or ANOVA analysis. ASC, adipose-derived stem cells.

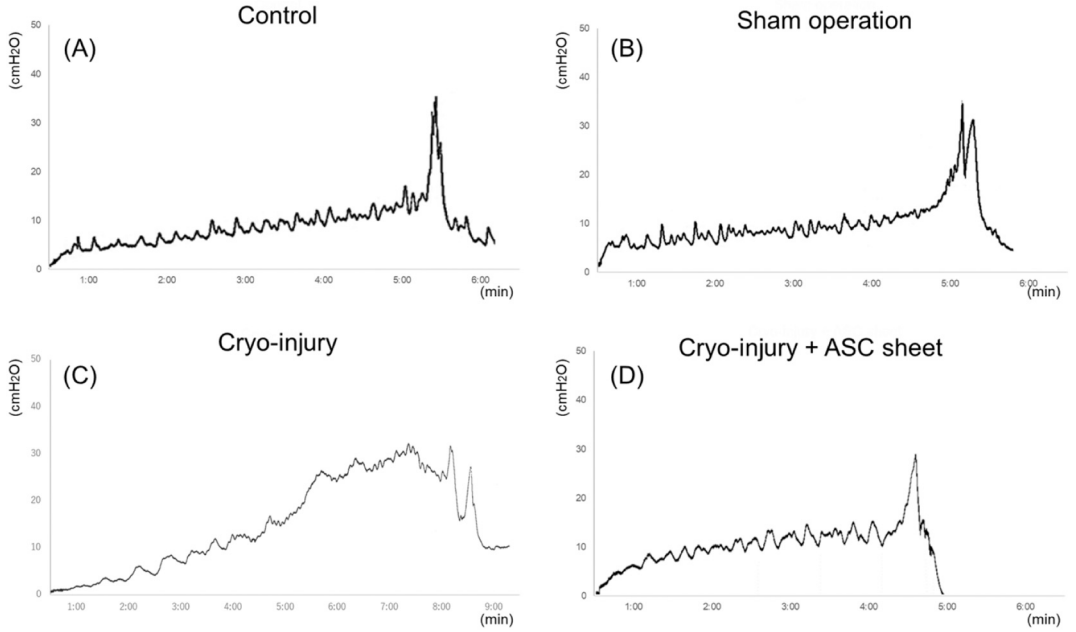

Fig. 2. Typical cystometry findings in each group. (A) Control group; (B) sham operation group; (C) cryo-injury group; (D) cryo-injury + ASC sheet implantation group.

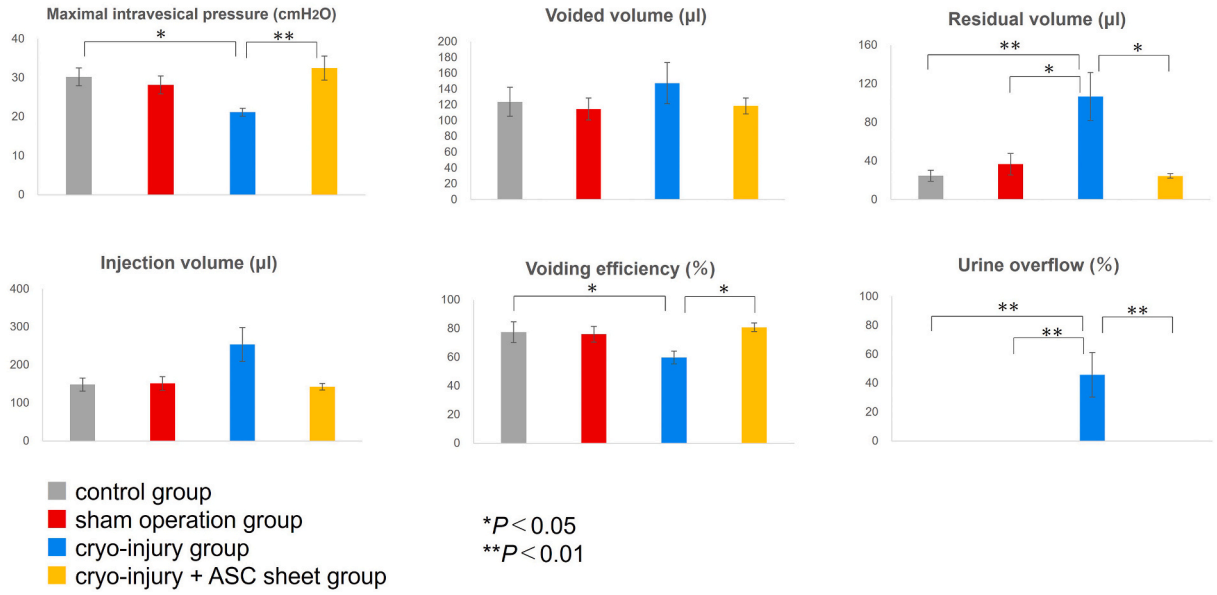

Fig. 3. Cystometry. Cystometry was performed 7 days after adipose-derived stem cell (ASC) sheet transplantation (10 days after cryo-injury). Then, for evaluation of bladder function, maximal intravesical pressure (MIP), injection volume (IV), voided volume (VV), residual volume (RV), voiding efficiency (VE), and the percentage of urine overflow were compared among a normal control group with no treatment (black bars), a sham operation group that underwent laparotomy without cryo-injury or ASC sheet transplantation (red bars), a group that only underwent cryo-injury (blue bars), and a group that underwent bladder cryo-injury followed by ASC sheet transplantation (orange bars) ($n = 8$ each; $^{*}P < 0.05$, $^{**}P < 0.01$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

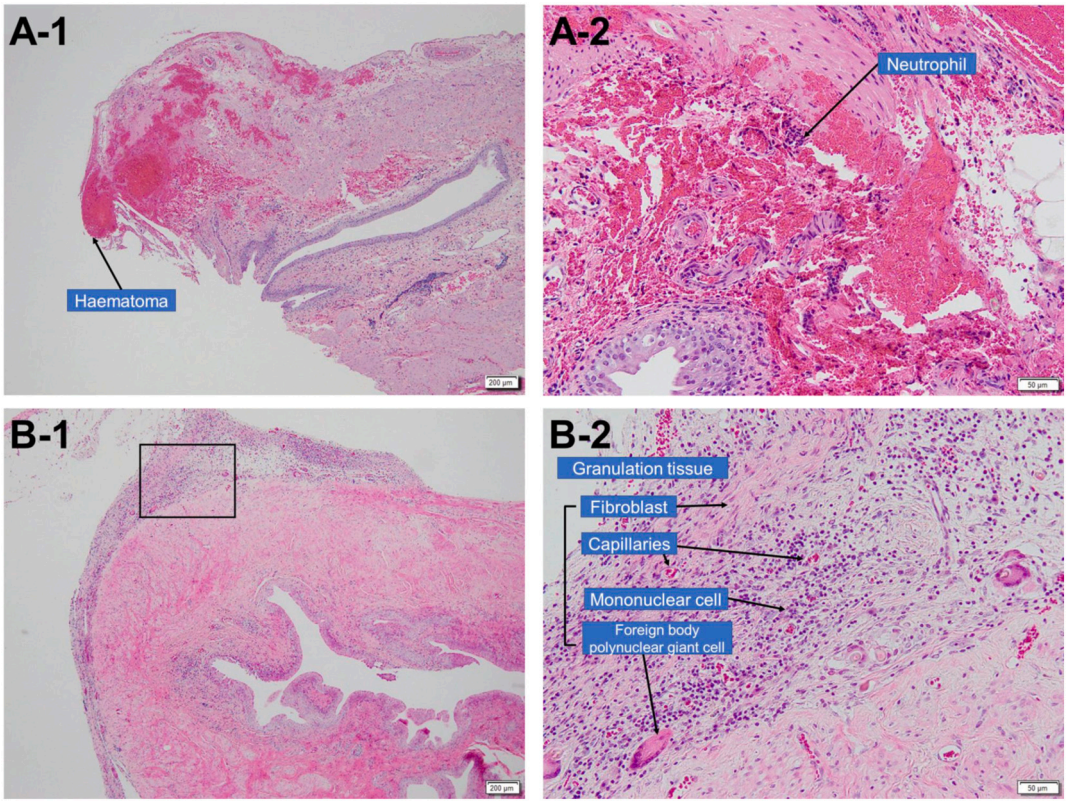

Fig. 4. Findings of hematoxylin-eosin staining. (A) Cryo-injury group. (A-1) Hematoma and necrotic tissue were observed in the area consistent with cryo-injury, and inflammatory cells such as neutrophils were seen migrating around the area. (A-2) Infiltration of migrating inflammatory cells was observed around the blood vessels, and the inflammatory stage was confirmed even 10 days after cryo-injury. (B) Cryo-injury + ASC sheet group. (B-1,2) Despite the presence of chronic inflammatory findings, granulation was confirmed to have begun from microvessels and fibroblasts generated during the wound-healing process.

found between the cryo-injury group and the control group and the cryo-injury group and the cryo-injury + ASC sheet group between the freeze-damaged and sheet groups ($P = 0.046$ for the former and $P = 0.009$ for the latter). For RV, significant differences were found between the cryo-injury group and the control group ($P = 0.004$), sham operation group ($P = 0.039$), and the cryo-injury + ASC sheet group ($P = 0.011$). For VE, significant differences were observed between the cryo-injury and control groups ($P = 0.046$) and the cryo-injury + ASC sheet group ($P = 0.043$). A comparison of the percentage of urine overflow also showed a significant difference between the cryo-injury group and the other groups ($P = 0.004$ for each comparison).

3.2. Histopathology

The findings of hematoxylin-eosin staining in the cryo-injury and cryo-injury + ASC sheet groups 7 days after cell sheet transplantation (10 days after cryo-injury) are shown in Fig. 4.

In the cryo-injury group, hematoma and necrotic tissue were observed in the area consistent with the cryo-injury. Moreover, inflammatory cells such as neutrophils, macrophages, and lymphocytes were found to migrate around the wound, and strong migration of inflammatory cells such as neutrophils, macrophages, and lymphocytes was observed around the wound, indicating acute inflammatory findings. In contrast, in the cryo-injury + ASC sheet group, chronic inflammation by inflammatory cells was observed, but the degree of tissue necrosis was low, and the formation of granulation, which occurs during the wound-healing process, was also confirmed. Fibrosis was also assessed using Masson's trichrome staining, but the results of this experiment were not significant because they were observed early after cryo-injury when injury-induced fibrosis had not yet occurred.

In addition, bladder tissue from each group was stained with the anti-vWF antibody, and the mean number of capillaries in the 4× field of view was compared (Fig. 5). The number of capillaries was significantly higher in the cryo-injury + ASC sheet group than in the other groups ($P = 0.018$ in comparison with the control group, $P = 0.015$ in comparison with the sham operation group, and $P = 0.002$ in comparison with the cryo-injury group).

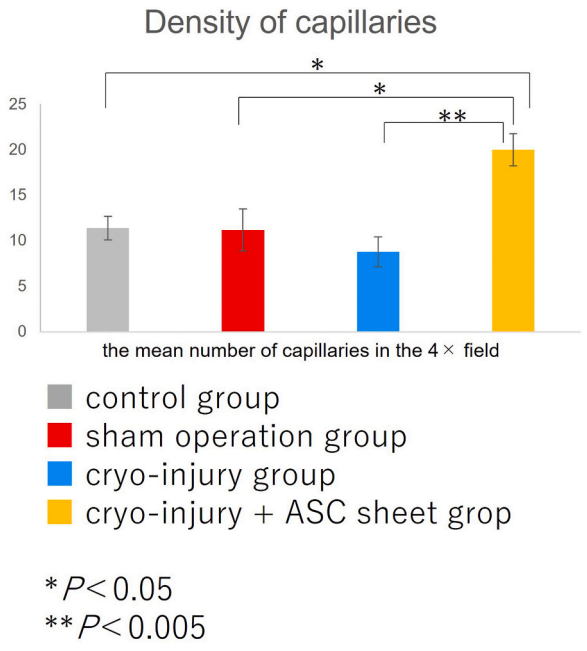

Fig. 5. Comparisons of capillary density. The bladder in each group was stained with an anti-von Willebrand factor (vWF) antibody, and the number of capillaries was measured. Five fields of transverse sections were counted under an optical microscope. The mean values were compared among a normal control group with no treatment (black bars), a sham operation group that underwent laparotomy without cryo-injury or ASC sheet transplantation (red bars), a group that only underwent cryo-injury (blue bars), and a group that underwent bladder cryo-injury followed by ASC sheet transplantation (orange bars; $*P < 0.05$, $**P < 0.005$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.3. RT-PCR analysis

After cystometry, rats were euthanized, their bladders were removed, and the mRNA levels of angiogenic factors were quantified for comparison among the four groups (Fig. 6). The mRNA levels of VEGF and HGF were significantly higher in the cryo-injury + ASC sheet group than in the cryo-injury group ($P = 0.045$ for the former and $P = 0.037$ for the latter). The four groups showed no significant difference in bFGF levels, although the mRNA expression levels tended to be higher in the cryo-injury + ASC sheet group.

3.4. Expression analysis and immunohistological analysis of GFP

3.4.1. Stereomicroscopic findings

Immediately after the application of GFP-expressing ASC sheets, the green fluorescence of the sheets was observed under excitation light. Green fluorescence in the anterior bladder wall was observed in all rats up to day 14 (100%), and fluorescence was observed in four of five rats on day 21 (80%). At 28 days post-implantation, fluorescence was observed in one out of five rats (20%). At all time points, green fluorescence was confined to the bladder implantation site and was not visible in other organs (Fig. 7).

3.5. Immunohistological analysis

Immunostaining of the removed bladder showed many GFP-positive cells at the ASC sheet application site. The GFP-expressing cells in the recipient tissue were thought to be transplanted adipose-derived cells. On day 3, GFP-positive cells did not merge with vWF-positive cells. However, after 7 days of sheet transplantation, the GFP-positive cells merged with vWF-positive cells, indicating that the cells had differentiated into blood vessels (Fig. 8).

4. Discussion

The present study revealed that transplantation of ASC sheets after cryo-injury resulted in bladder contraction recovery at a relatively early stage. Thus, in addition to showing paracrine activity for the secretion of angiogenic factors such as VEGF and HGF, ASC sheets may also show anti-inflammatory activity. Furthermore, this study showed that ASCs marked with GFP differentiated into cells that provided structural elements of the capillary system and survived up to 28 days after transplantation. Thus, angiogenic factors secreted from ASC sheets and ASCs differentiate into vascular endothelial cells, maintaining stable blood perfusion and contributing to tissue repair.

We also add some additional remarks on interpreting the present cystometry results. Various methods of creating rat cryo-injury models have been reported, but to create the DU model by cryo-injury in this study, we used a procedure similar to that reported in some previous articles to create the model rats [11,14]. In the present study, urine overflow was observed in about half of the cystometry in the cryoinjury group, and a decrease in MIP was also observed in those that developed voiding reflex. We believe that both conditions are due to the reduction of bladder contractility induced by cryoinjury. In addition, when urinary overflow occurred, it was clearly different from the normal voiding reflex. In other words, there was a lack of a steep increase in bladder pressure, and when a certain bladder pressure was reached, urine flowed out of the external urethral opening, and the bladder pressure became steady. The present results, which showed no decrease in urinary

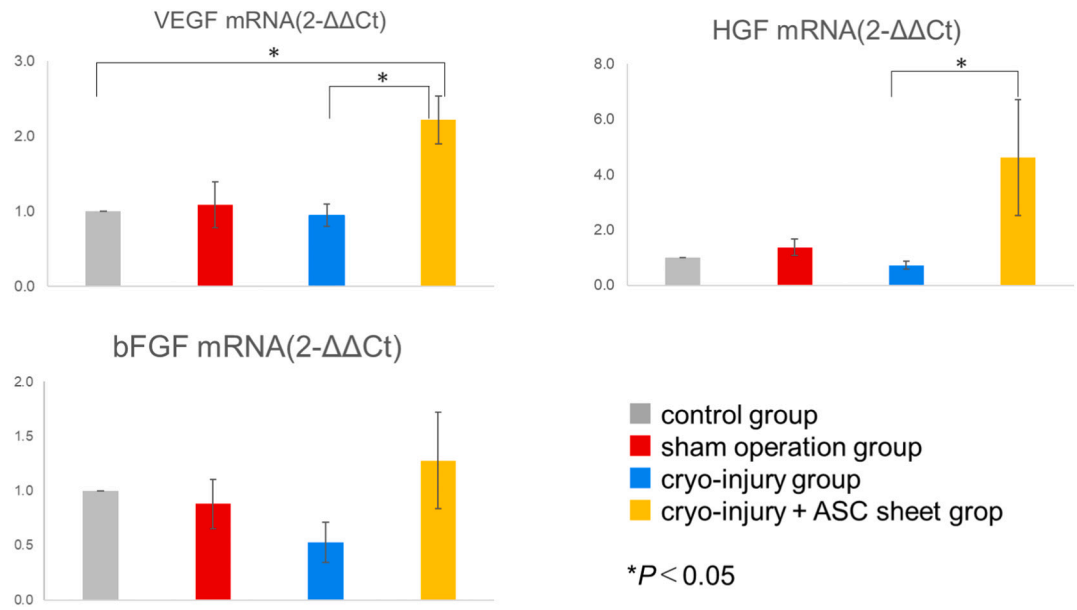

Fig. 6. Results of RT-PCR. Seven days after implantation of adipose-derived stem cell (ASC) sheets (10 days after cryo-injury induction), the rats were euthanized, and their bladders were removed. The mRNA expression levels of angiogenic factors, namely, vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), and basic fibroblast growth factor (bFGF), were quantified and compared among the four groups, namely, a normal control group with no treatment (black bars), a sham operation group with laparotomy but no cryo-injury or ASC sheet transplantation (red bars), a group with only cryo-injury (blue bars), and a group with bladder cryo-injury followed by ASC sheet transplantation (orange bars) ($n = 8$ each). *$P < 0.05$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

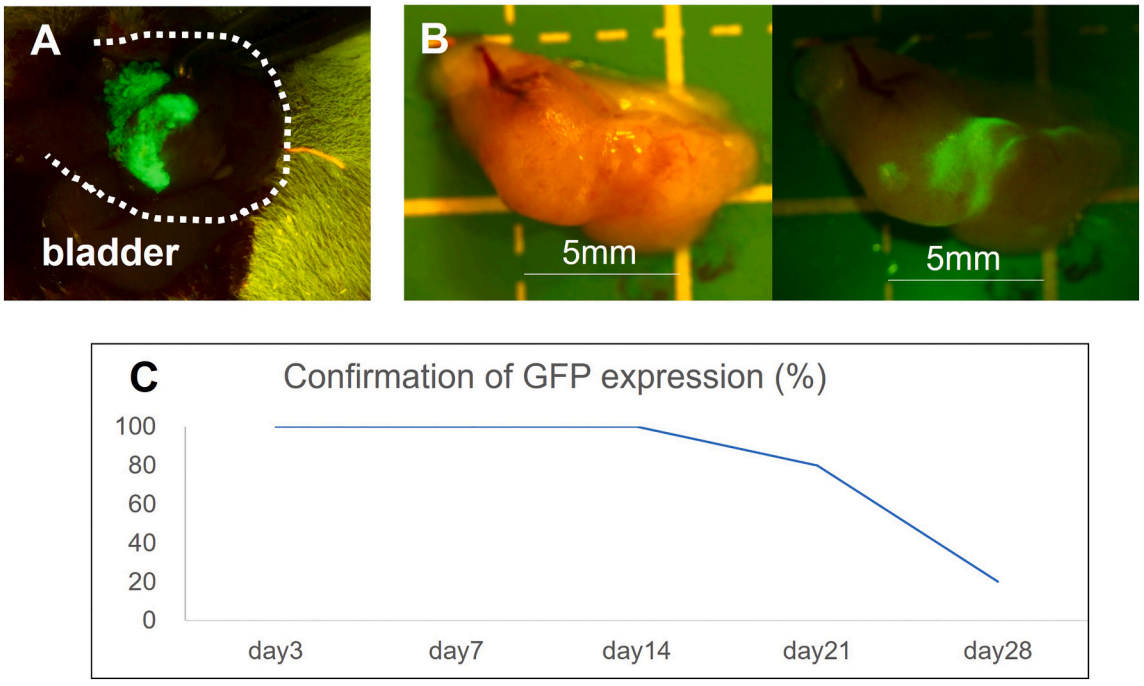

Fig. 7. Stereomicroscopic findings in the transplantation of GFP-ASC sheets. (A) Macroscopic findings obtained immediately after implantation. GFP-ASC sheets were applied to the anterior wall of the bladder at the site of the cryo-injury. Green fluorescence was observed by excitation light. (B) Bladder immediately after removal. (C) Macroscopic findings in the bladder immediately after removal. Green fluorescence by excitation light was observed mainly on the anterior wall of the bladder to which the ASC sheet was attached. No fluorescence in organs other than the bladder was observed. The scale bar corresponds to 5 mm. (D) Five rats were euthanized each day, and the green fluorescence of the sheet-grafted area was checked. Green fluorescence in the fluorescence stereomicroscope diminished as the days progressed. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

overflow or MIP in the cryoinjury + ASC sheet group, support the restoration of bladder function by the ASC sheets.

Induced pluripotent stem cells, embryonic stem cells, and MSCs are stem cells used in regenerative medicine [15–17]. MSCs have several advantages over other types of stem cells, including fewer concerns related to tumorigenicity compared with pluripotent stem cells and greater proliferation and differentiation potential than conventional adult stem cells. They also show higher proliferative and differentiation

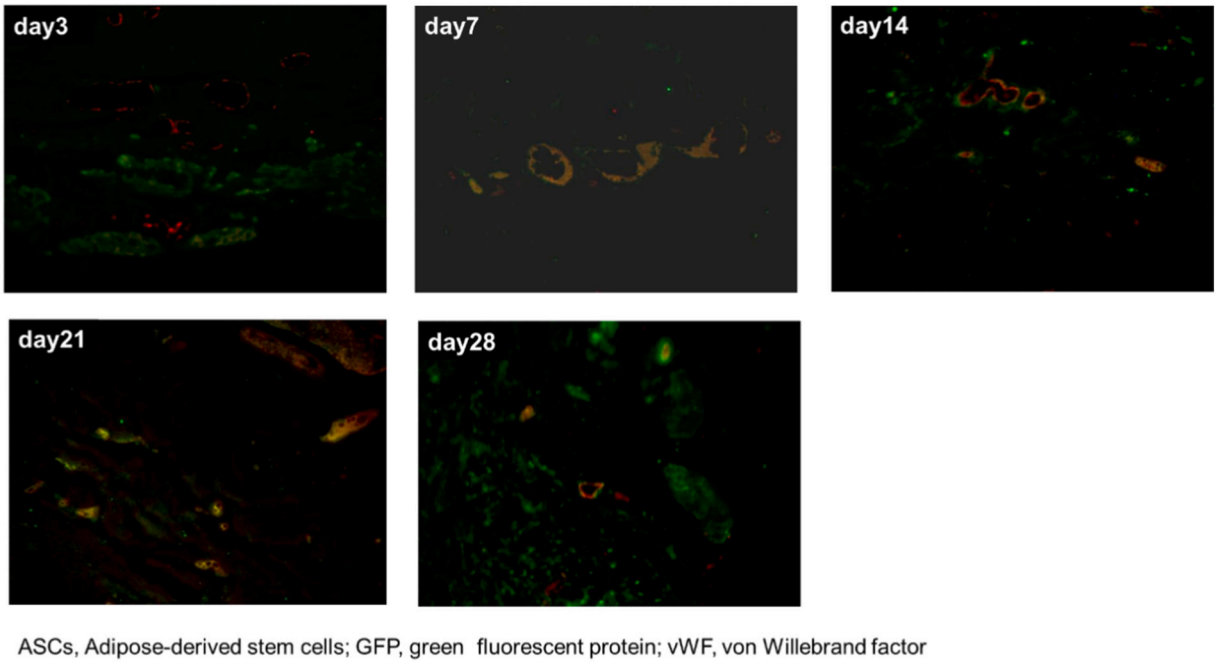

Fig. 8. Double immunofluorescence staining of GFP and vWF. On day 3, the match between GFP-positive cells and vWF-positive cells could not be confirmed, but after day 7, the match between the two pairs of positive cells was confirmed. The match rate was particularly strong on days 7–21. The observation magnification was 20 × .

potential than traditional adult stem cells. Among harvested tissues, the bone marrow has long been used as a cell source for clinical applications. However, harvesting bone marrow-derived stem cells is problematic because of the high invasiveness of the harvesting procedure in actual clinical practice. In this regard, MSCs have been recently found in various tissues other than the bone marrow, including adipose tissue, fetal appendages (umbilical cord, umbilical cord blood, placenta, and amnion), endometrium, dental pulp, synovium, and dermis [18].

ASCs have been reported to be adherent cells with strong proliferative potential obtained by treating aspirated adipose tissue harvested from the abdominal cavity with collagenase and seeding the resulting cell population into cell dishes [19]. The use of ASCs has several advantages, one of which is the high MSC content. The number of MSCs in the bone marrow is very low, constituting 0.001–0.1% of the total number of nucleated cells. One milliliter of bone marrow contains approximately 1 million cells; however, the number of MSCs therein is only about 10–100 [20]. On the other hand, approximately 5×10^3 MSCs can be collected from 1 g of adipose tissue, which is approximately 500 times the recovery rate from the bone marrow [21]. Furthermore, ASCs have low immunogenicity among the same species and are expected to have clinical applications [22]. Thus, ASCs are pluripotent and can be harvested in large quantities per individual, making them an ideal cell source for regenerative medicine.

The low survival rate of stem cells after transplantation in regenerative medicine using stem cells is a major problem [5]. Various methods have been used to improve the survival rate of stem cells [24]. One such method is stem cell administration. Studies on bladder regeneration have shown other routes of administration of stem cells other than cell sheets, including direct injection into the bladder submucosa, transurethral injection, and administration *via* the tail vein. Although transurethral injection is the least invasive method of administration, rat urine has a pH of about 6.0–7.0 and contains waste products that expose MSCs to an inappropriate environment for their survival, which may reduce cell survival and activity. The cytotoxicity of urine on MSCs has been confirmed *in vitro* [25,26]. MSCs can also migrate to damaged tissues in response to microenvironmental factors associated with inflammation and ischemic gradients, leading to damaged target tissues [27,28]. In such models, transvenous administration can be considered a relatively less invasive and effective method of administration. However, because stem cells migrate through the body, they are not only easily trapped by the lungs but also eliminated in the spleen, which reduces the number of administered stem cells that can be established in the injured area [29]. In a study comparing three methods of administration: direct injection into the submucosa, systemic urethral injection, and intravesical administration, direct injection into the submucosa of the bladder predominantly enhanced bladder regeneration and had a strong anti-inflammatory effect [30]. This may also be largely due to the survival and viability of the stem cells.

For cell sheets, synthetic polymers can be used as scaffolds [31], but temperature-responsive culture dishes can be used to create cell sheets without the need for scaffolds. Cells grow confluently on the temperature-responsive culture dish and can be collected as sheets by reducing the temperature, thereby avoiding the use of enzymes. Cell sheets are cell-dense tissues that resemble actual living tissues and are believed to maintain their structure and function [32]. Therefore, cell sheet engineering can improve the efficacy of stem cell transplantation [33]. The cell sheets themselves are thin, flexible, and changeable, allowing them to adhere to organs such as a beating heart [34]. Therefore, they may be suitable for organs that expand and contract, such as the bladder. Cell sheet technology is widely used for tissue reconstruction and has therapeutic effects [35,36]. In a myocardial infarction model, ASC sheets were shown to survive for two weeks after transplantation and create vWF-positive capillaries [37].

In addition, the ASC sheet implantation method in this study involved simply having the sheet affixed to the surface of the bladder. The technique for ASC sheet implantation was similar to that in a previous paper [38]. In this study, ASC sheets were implanted on rat heart surfaces to confirm their retention, although the implantation site was different. The fact that the ASC sheet was confirmed to be viable even in a highly motile organ such as the heart suggests high adhesion. In this study, ASC sheets were applied patently to the site of frozen injury, *i.e.*, the serous membrane of the anterior wall of the bladder. The bladder was then carefully placed back into the abdominal cavity, and the wound was closed. Unlike humans, the bladder of rats exists

intraperitoneally. In other words, the bladder is close to the intestinal tract and other organs. In a study of GFP fluorescence expression on ASC sheets, the early implantation rate at the application site was 100%, and no fluorescence was observed outside of the implantation site. The ASC sheet, which can be fixed at the application site without sutures or adhesives, is considered to have significant advantages in actual clinical practice.

Several mechanisms have been proposed for stem cell wound healing, including differentiation, homing, and immunoregulation. Nonetheless, these mechanisms remain to be elucidated [39]. Recently, the release of secretory factors by paracrine action has been proposed as the main mechanism of action [15,40]. Furthermore, both *in vitro* and *in vivo* studies have reported that stem cells interact with a wide range of immune cells, suppressing excessive responses of T cells, B cells, dendritic cells, macrophages, and natural killer cells and inducing regulatory T cells [41].

A large body of evidence suggests that neovascularization induction is strongly involved in tissue repair and wound healing in ASCs. Recently, it has been reported that ASCs secrete factors such as VEGF, PDGF, IGF, HGF, b-FGF, SDF-1, TGF-β, and GDF11, which promote the differentiation of ASCs and fibroblasts into endothelial cells [42–46]. In addition, VEGF expression was increased in the ASC sheet group in this experiment, and the importance of VEGF in angiogenesis by ASCs has been described in other papers. ASCs interact with endothelial cells and macrophages and have been reported to increase MCP-1 and VEGF secretion to regulate angiogenesis [47]. Zhou X et al. also reported a strong association between angiogenesis and increased VEGF expression in a study in which autologous ASCs were administered to burn wounds in an animal model [42]. The above reports also corroborate our experimental results. In addition, HGF is thought to play an important role in ASC-mediated antifibrotic effects [13]. In the histological evaluation in our study, the acute inflammatory response was reduced in the group to which ASC sheets were applied. As with other stem cells, there are many reports on the anti-inflammatory effects of ASCs; ASCs have been reported to contribute to tissue regeneration by controlling inflammatory responses during wound healing through their immune effects [22,48–51]. *In vitro*, conditioned media of ADSCs were reported to stimulate macrophages, increase secretion of TNF and IL-10 anti-inflammatory cytokines, and promote wound healing [22]. Furthermore, vascular endothelial cells have been reported to interact with ADSCs to increase the secretion of inflammatory growth factors such as IL-6, IL-8, and MCP-1 [47]. Based on previous reports, it is thought that undifferentiated ASC sheets, which were undifferentiated before transplantation, differentiated into vascular endothelial cells at an early stage and that interaction by angiogenic factors secreted from the ASC sheets occurred. As a result, stable blood perfusion to the injured bladder wall is maintained, and acute inflammation is suppressed, suggesting that these combined effects may lead to a relatively early recovery of injured bladder function.

Immunological and oncological safety are discussed as issues for the clinical application of ASC sheets. Immunological safety, or rejection, is an issue in allogeneic xenografts. Previous studies on allogeneic xenografts of ASCs have shown that CD4/CD8, cytokine levels, and nonspecific IgM and IgG did not show an activated immune response to the transplanted cells [59,60]. Reports of testing for donor-specific antibodies found that 19–34% of recipient patients developed antibodies, but the physical impact of these reactions is unknown at this time [48,61]. In addition, several characteristics of pluripotent stem cells have been associated with carcinogenic risk in cell transplantation therapy using pluripotent stem cells. These include the risk of tumor growth and tumor metastasis *via* the production of a wide range of growth factors, cytokines, immunosuppressive factors, and other bioactive molecules. It has also been postulated that stem cells can stimulate epithelial-mesenchymal transition, which is involved in the development and progression of tumor disease [62]. However, in the field of urology, several articles have described the clinical use of ASCs in the treatment of patients with urinary incontinence who have a history of prostate cancer [63–65]. In these reports, there was no evidence of prostate cancer recurrence or other side effects during follow-up. However, we believe a longer follow-up period is needed to determine long-term oncologic safety.

There are limitations to this study. Although this study demonstrated short-term recovery of bladder function, long-term studies are needed to determine whether this recovery is sustainable. In addition, although the clinical application of ASCs is gaining ground in many medical fields, the implementation of cell therapy must be based on standard principles of evidence-based medicine. In addition, although clinical evidence for ASCs is scarce, cell therapy with ASCs in the research phase has shown great potential to date. Our data in this study also support the beneficial effects of ASC sheets.

5. Conclusions

We have confirmed that ASC sheets transplanted into the bladder of cryo-injured rats differentiated into blood vessels and restored bladder contractile function 7 days after transplantation. It promoted bladder tissue repair in the acute stage, suggesting the possibility of adaptation to acute bladder injury caused by accidents or delivery. It is necessary to understand the comprehensive regeneration mechanism further and investigate the long-term effects of transplantation for clinical applications.

CRediT authorship contribution statement

Shogo Teraoka, Masashi Honda; Conceptualization.
Shogo Teraoka, Hideto Iwamoto, Panagiota Tsounapi, Ryutaro Shimizu; Data curation.
Masashi Honda, Shogo Teraoka, Tetsuya Yumioka; Formal analysis.
Shogo Teraoka, Peili Li, Shuichi Morizane; Investigation.
Masashi Honda, Ichiro Hisatome, Katsuya Hikita; Methodology.
Atsushi Takenaka, Ichiro Hisatome; Project administration.
Atsushi Takenaka, Ichiro Hisatome; Resources.
Ichido Hisatome, Masashi Honda; Supervision.
Masashi Honda, Peili Li; Validation.
Shogo Teraoka, Peili Li, Karen Makishima; Visualization.
Shogo Teraoka, Peili Li, Masashi Honda; Writing – original draft.
Masashi Honda, Atsushi Takenaka; Writing – review & editing.

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Declaration of competing interest

The authors declare that there are no conflicts of interest.

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