A Novel Therapy for Pancreatic Fistula using Adipose-derived Stem Cell Sheets Treated with Mannose

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Abstract

Background: Given that no studies have reported the use of adipose-derived stem cell (ADSC) sheets for the prevention of pancreatic fistulas, it is unclear whether ADSC sheets are effective at preventing this complication. The aim of this study was to evaluate the efficacy of novel therapy for the prevention of pancreatic fistulas using ADSC sheets treated with mannose.

Methods: The rat pancreatic duct (splenic duct) and surrounding pancreatic parenchyma were transected to induce a pancreatic fistula. ADSC sheets with or without mannose treatment were attached to the pancreatic transection stump. Amylase and lipase levels were measured in both the ascites and serum. The expression of 40 cytokines in human ADSCs with and without mannose treatment was investigated using a multiplex assay.

Results: The ADSC sheets remained at the initial attachment site at 48 hours after surgery. Macroscopically, more severe degeneration and adhesion in the peritoneal cavity were observed in the untreated rats than in the rats treated with ADSC sheets. The levels of ascitic amylase in the untreated, ADSC-sheet-treated and ADSC-sheet-with-mannose-treated rats were $10.7 \pm 2.9 \times 10^4$ U/L, $2.6 \pm 0.9 \times 10^4$ U/L and $1.5 \pm 0.3 \times 10^4$ U/L, respectively. The levels of ascitic lipase in the untreated, ADSC-sheet-treated and ADSC-sheet-with-mannose-treated rats were $9.5 \pm 2.9 \times 10^3$ U/L, $4.0 \pm 3.3 \times 10^3$ U/L and $0.4 \pm 0.2 \times 10^3$ U/L, respectively. Significant differences were found in both the ascitic and serum levels of amylase and lipase between the untreated rats and the rats treated with ADSC sheets with mannose ($p < 0.05$). Fibroblast growth factor 2 (FGF2) gene expression levels were significantly higher in human ADSCs treated with mannose than in human ADSCs treated without mannose.

Conclusion: ADSC sheets treated with mannose are effective for preventing pancreatic fistulas and have promising potential for clinical applications. The enhancing effect of mannose on the function of ADSCs may be partially explained by induction of FGF2.

Introduction

Although mortality after a pancreatectomy in high-volume centres has decreased to less than 5%, the rate of postoperative complications remains high, ranging from 30% to 50%. Pancreatic fistulas are a major complication that occurs after pancreatic resection,[1, 2] with an occurrence rate of 30-50% for distal pancreatectomy and 13-45% for pancreaticoduodenectomy. [3, 4, 5] Therefore, preventing pancreatic fistulas is the most important clinical issue in pancreatic surgery.[6]

Adipose-derived stem cells (ADSCs) are multipotent cells that originate from adipose tissue. Similar to mesenchymal stem cells in bone marrow,[6] ADSCs have the ability to differentiate and regenerate. With respect to the clinical application of stem cells, ADSCs are often more useful than mesenchymal stem cells because multipotent stem cells are more abundant in the adipose tissue than in the bone marrow. We previously reported that the intravenous administration of ADSCs enhanced liver regeneration after a hepatectomy.[7] In addition, several studies have reported the efficacy of ADSCs in the treatment of ischaemic colonic anastomosis,[8, 9] liver injury,[8, 9] myocardial infarction,[8, 9] and urinary incontinence.[10, 11] In our preliminary study (unpublished), ADSCs (without forming a sheet) were applied to the injured tissue. However, these cells slipped off from the initial attachment point and showed no therapeutic effect. In recent years, we have created a multilayered ADSC cell sheet using a novel magnetite tissue engineering method, and we have applied this sheet for the treatment of myocardial infarction.[12] However, the use of ADSC sheets in preventing pancreatic fistulas has not been previously reported.

Mannose is a monosaccharide and is an important component of the carbohydrate chain. Extracellular mannose is directly used for the biosynthesis of glycoproteins, particularly the biosynthesis of carbohydrate chains in several types of cells.[13] Other reports have shown that carbohydrate chains are important in promoting stem cell function.[14] Therefore, it can be hypothesized that the mannose supplementation of the ADSCs may enhance the functions of these cells. However, this hypothesis has not been investigated.

The aim of this experimental study was to evaluate 1) the efficacy of ADSC sheets in

the prevention of pancreatic fistulas and 2) the enhancing effect of mannose supplementation.

Materials and Methods

Cell Culture

Human ADSC lines (PT-5006) were obtained from Lonza Walkersville, Inc. (Lonza, Walkersville, MD). ADSCs were cultured in MesenPro (Thermo Fisher Scientific, Waltham, MA) at 37°C in a humidified atmosphere with 5% CO_2.

Creation of the ADSC sheets

C57BL/6J8 10-week-old male mice were obtained from SLC (Nagoya, Japan). ADSCs were isolated from the inguinal fat pads (0.1-0.2 g) obtained from the mice. The ADSCs were cultured with magnetic nanoparticle-containing liposomes (MCLs) in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% foetal bovine serum (FBS) and antibiotics (100 U/mL penicillin G, and 100 μg/mL streptomycin) at 37°C and 5% CO_2. When the cells reached confluence, the attached ADSCs were resected in a similar medium as described above at a concentration of 2.0×10^3 cells/cm^2. The MCL-labelled ADSC suspension (5×10^5 cells in 50 μL) was mixed with an extracellular matrix (ECM) precursor solution comprised of 85 μL of collagen solution and 15 μL of Matrigel basement membrane matrix (BD Biosciences, San Jose, CA). Subsequently, the mixture (150 μL) was added to each well of a 24-well ultra-low attachment plate, in which cloning rings (diameter, 5 mm; height, 10 mm; Asahi Glass Co., LTD., Tokyo, Japan) were placed at the centre of each well. Thereafter, a magnet was immediately placed under the wells. The magnetised ADSCs formed multilayered cell sheets after approximately 3 hours of incubation.

Analysis of ascitic and serum samples

Amylase and lipase concentrations in both the ascites and serum were measured using standard laboratory methods (SRL, Tokyo, Japan).

Quantitative RT-PCR

Total RNA was isolated from ADSCs using a QIAcube (Qiagen, Hilden, Germany) in accordance with the manufacturer's instructions. CDNA was generated from total RNA samples using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). RT-PCR analysis was performed using the 7300 Fast Real-Time PCR System

(Applied Biosystems). Each reaction was performed in a 20 μL reaction mixture containing TaqMan Universal PCR Master Mix in accordance with the manufacturer's instructions (Applied Biosystems). The TaqMan probe and primer for FGF2 (assay identification no. Hs00201616_m1) were purchased from Applied Biosystems, and 18S rRNA (assay identification no. Hs99999901_s1; Applied Biosystems) was used as an internal control. In each experiment, the relative expression of the gene of interest was normalised to the 18S control using standard curves prepared for each gene, and the average values were used for quantification.

Histology

Rat pancreas specimens including the pancreatic transection stump were immediately fixed in neutral buffered formalin and embedded in paraffin. The samples were then dehydrated in a graded ethanol series and embedded in paraffin. Six-micrometre-thick sections were mounted on glass slides and stained with haematoxylin and eosin.

Measurement of the Levels of Multiple Cytokines in ADSCs Culture Medium

Human ADSCs were cultured in MesenPro medium (Thermo Fisher Scientific) for 48 hours. The medium was changed to DMEM without FBS and cultured for an additional 2 hours. Then, the ADSCs were cultured in DMEM with or without 0.4% mannose for 3 hours. Supernatant from the medium was collected and analysed for the expression of 40 cytokines using a multiplex assay kit (Millipore, Billerica, MA) in accordance with the manufacturer's protocol.

Animal Studies

All animal experiments were conducted in compliance with the guidelines of the Institute for Laboratory Animal Research, Nagoya University Graduate School of Medicine. Wister S/T rats (8 to 9 week-old males weighing approximately 250-314 g) were purchased from SLC (Nagoya, Japan) and housed in a temperature- and humidity-controlled environment under a 12-hour light-dark cycle. Animals were allowed ad libitum access to water and food. The rats were anesthetised with isoflurane. The pancreas was exposed via an upper abdominal midline laparotomy. The rat pancreatic duct consists of four ducts, including the gastric, duodenal, common, and splenic ducts[15]. The splenic artery and vein were

preserved, and the splenic duct and surrounding pancreatic parenchyma were transected to induce the pancreatic fistula[16]. After transection of the pancreas, an ADSC sheet or a collagen sheet was attached to the stump of the pancreas. These sheets were pre-incubated in DMEM with or without 0.4% mannose. The rats were sacrificed on day 2 after the operation. At re-laparotomy, the abdominal cavity was rinsed with 5 ml saline, and ascites was collected for the measurement of amylase and lipase concentrations. Thereafter, pancreatic tissue and blood samples were collected from each rat.

In vivo Imaging

The ADSC sheets were incubated in XenoLight DiR (Sumitomo Pharmaceuticals International Corporation, Tokyo, Japan) for 30 min. Then, these sheets were attached to the stumps of the rat pancreases. The ADSC sheet fluorescence signals were detected at the time of the operation and on day 2 after surgery using the IVIS® Imaging System (Sumitomo Pharmaceuticals International Corporation) according to the manufacturer's instructions. The excitation/emission wavelengths in the IVIS system is 710 nm /760 nm.

Statistical Analysis

All data are presented as the means ± standard error (SE). Differences were analysed using Bonferroni's method. Differences were considered statistically significant at a value of $p < 0.05$.

Results

Grafting of ADSC Sheets to the Pancreas Transection Stumps

The cultured multilayered ADSC sheet was approximately 6 mm in diameter **(Fig. 1a)**. The ADSC sheet remained attached to the pancreatic transection stump at the same site at 48 hours after surgery **(Fig. 1b)**. In vivo imaging revealed the fluorescent signal of the ADSC sheets at the pancreatic transection site on day 3 after the attachment **(Fig. 1c)**. The fluorescent signal was also confirmed in the removed pancreatic specimen **(Fig. 1d)**. These observations indicated that the ADSC sheets adhered to and did not detach from the pancreatic transection stump after surgery.

Effect of ADSC Sheets with Mannose Treatment on Pancreatic Fistulas

Strong adhesion and induration in the peritoneal cavity were observed in the untreated rats (n = 9) **(Fig. 2a)**. More severe tissue degeneration was also observed in the untreated rats than in the ADSC-sheet-treated (n = 5) and ADSC-sheet-with-mannose-treated rats (n = 5). No significant macroscopic differences in adhesion and degeneration were found between the ADSC-sheet-treated and ADSC-sheet-with-mannose-treated rats. Histologically, a large number of inflammatory cells were identified in the pancreatic transection stumps of the untreated rats **(Fig. 2b)**. In contrast, fewer inflammatory cells were observed in the pancreatic transection stumps of the ADSC-sheet-treated and ADSC-sheet-with-mannose-treated rats. The histological findings demonstrated that the sheets with brownish MCL-labelled ADSCs attached to and covered the pancreatic transection stump **(Fig. 2b)**.

The severity of pancreatic fistulas were evaluated via ascitic amylase and lipase levels. The levels of ascitic amylase in the untreated, ADSC-sheet-treated and ADSC-sheet-with-mannose-treated rats were $10.7 \pm 2.9 \times 10^4$ U/L, $2.6 \pm 0.9 \times 10^4$ U/L and $1.5 \pm 0.3 \times 10^4$ U/L, respectively **(Fig. 2c)**. The levels of ascitic lipase in the untreated, ADSC-sheet-treated and ADSC-sheet-with-mannose-treated rats were $9.5 \pm 2.9 \times 10^3$ U/L, $4.0 \pm 3.3 \times 10^3$ U/L and $0.4 \pm 0.2 \times 10^3$ U/L, respectively. The levels of ascitic amylase and lipase in the ADSC-sheet-treated rats were lower than those in the untreated rats. However, the differences did not reach statistical significance (amylase, $p = 0.064$; lipase, $p = 0.567$). In contrast, the levels of ascitic amylase and lipase were significantly lower in the ADSC-sheet-with-mannose-treated rats than in the untreated rats (amylase, $p < 0.05$; lipase, $p < 0.05$). Unexpectedly, there were no significant differences in ascitic amylase and lipase between the ADSC-sheet-with-mannose-treated rats and the ADSC-sheet-treated rats (amylase, $p = 0.568$; lipase, $p = 0.342$).

The levels of serum amylase in the untreated, ADSC-sheet-treated and ADSC-sheet-with-mannose-treated rats were $6.0 \pm 1.3 \times 10^3$ U/L, $2.2 \pm 0.2 \times 10^3$ U/L and $1.7 \pm 0.1 \times 10^3$ U/L, respectively. The levels of serum lipase in the untreated, ADSC-sheet-treated and ADSC-sheet-with-mannose-treated rats were $18.5 \pm 5.9 \times 10$U/L, $2.5 \pm 0.6 \times 10$U/L and $2.6 \pm 1.2 \times 10$U/L, respectively. The serum amylase and lipase levels were also significantly

lower in the ADSC-sheet-with-mannose-treated rats than in the untreated rats (amylase, $p < 0.05$; lipase, $p < 0.05$). The levels of serum lipase in the ADSC-sheet-treated rats were significantly lower than those in the untreated rats ($p < 0.05$). However, the differences concerning serum amylase did not reach statistical significance ($p = 0.065$). There were no significant differences in serum amylase and lipase between the ADSC-sheet-with-mannose-treated rats and the ADSC-sheet-treated rats (amylase, $p = 0.384$; lipase, $p = 0.986$).

Physical Effect of Collagen Sheets without ADSCs on Pancreatic Fistula

To exclude physical effect of the "sheet", collagen sheets without ADSCs were created and attached to the pancreatic transection stump for 48 hours. The rats were assigned to the following three groups: untreated, collagen-sheet-treated, and collagen-sheet-with-mannose-treated ($n = 5$ in each group). Macroscopically, tissue adhesion and degeneration around the pancreatic transection stumps were equally observed in the three groups **(Fig. 3a)**. The histological findings of the pancreatic transection stumps were also similar among the three groups **(Fig. 3b)**. The levels of ascitic amylase in the untreated, collagen-sheet-treated and collagen-sheet-with-mannose-treated rats were $10.7 \pm 2.9 \times 10^4$ U/L, $18.4 \pm 8.7 \times 10^4$ U/L and $12.2 \pm 5.2 \times 10^4$ U/L, respectively **(Fig. 3c)**. The ascitic amylase levels at 48 hours after the attachment of the collagen sheet were high and were comparable among the three groups.

Production of Cytokines in Human ADSCs

To evaluate the effects of mannose supplementation on the cytokine-producing capacity of ADSCs, the levels of 40 cytokines in the culture medium of human ADSCs with ($n = 4$) and without mannose treatment ($n = 4$) were investigated using a multiplex assay. Four cytokines, including FGF2, IL6, VEGF, and HGF, were identified in the medium **(Fig. 4a)**. Of these, only FGF2 levels were significantly higher in the medium treated with mannose than in that without mannose treatment (FGF2, $p < 0.05$; IL6, $p = 0.063$; VEGF, $p = 0.391$; HGF, $p = 0.521$). Furthermore, the mRNA expression of FGF2 was significantly higher in the mannose-treated human ADSCs than in the ADSCs without mannose treatment ($p < 0.05$) **(Fig. 4b)**.

Discussion

In this study, the ADSC sheets were created by a combination of magnetite tissue engineering technology and ECM-based techniques. Several previous reports showed a technique to form cell sheets that can be used in clinical settings[16,17] in which the creation of a single layered sheet required 3 to 5 days in a temperature-responsive culture dish.[17] Our procedure has some advantages compared to previously reported methods because our ADSC sheets were created in only 1 to 3 hours with a multilayered cellular structure.[12] This advantage means that our ADSC sheets can be created from autologous fat tissue and could potentially be applied during surgery. Sheet size is also an important factor for clinical applications. A large number of ADSCs, which are necessary to form a large cell sheet, can be easily and safely harvested from the patient's abundant adipose tissue. In this regard, ADSCs are superior to bone marrow-derived stem cells, which require a more complicated procedure to harvest large amount of cells from the patients.[11, 18]

Clinical trials using ADSCs are underway for myocardial infarction and urinary incontinence (unpublished study); however, ADSC sheets have never been used in humans. The tissue engineering technique enabled the creation of multilayered ADSC sheets, which were easy to attach and consistently adhered to the injured site. In this regard, ADSC sheets are considered to be more useful than ADSCs (without forming a sheet). Recently, Kuroshima et al. reported that using triple-drug therapy reduced pancreatic leakage from pancreatic fistulas in rats.[19] Combination treatment using ADSC sheets and these drugs may enhance the therapeutic effects for pancreatic fistulas. However, there are many differences in the anatomy, surgical procedure, and perioperative management between the pancreatic leak models in rats and pancreatic surgery in humans. These differences should be carefully recognized when our sheets are used in humans. Further investigations are required prior to clinical application.

Interestingly, the levels of ascitic amylase and lipase were lower in rats treated with ADSC sheets with mannose than in rats treated with ADSC sheets without mannose. This report is the first to show an enhancing effect of mannose in the preventive effect of ADSCs for pancreatic fistulas. In the in vitro experiment, we found that mannose supplementation of

the culture medium increased the production of FGF2 in human ADSCs. FGF2 belongs to the FGF family and plays significant roles in normal development and wound healing.[20] The FGF2 pathway has also been identified as a major regulatory factor of self-renewal and morphology in ADSCs.[21, 22] Based on these observations, we speculated that the enhancing effect of mannose on ADSCs sheet in preventing pancreatic fistula may partially be explained by the upregulated production of FGF2.

In summary, ADSC sheets with mannose have novel therapeutic potential for preventing pancreatic fistulas. FGF2 production was increased by mannose supplementation in the culture medium and may be partially responsible for the enhancement of ADSC function. Further investigations are needed to determine the precise preventive mechanism of ADSC sheets treated with mannose and their association with FGF2 to establish a novel preventative treatment for pancreatic fistulas.

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Conflicts of Interest

We have no conflicts of interest.

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Figure Legends

FIGURE 1. Grafting of ADSC sheets onto the pancreatic transection stump

(a) The macrograph (left) and micrograph (right) of the ADSC sheet, which was cultured and multilayered in a dish.

(b) The grafting of the ADSC sheet onto the pancreatic transection stump at 48 hours after attachment (ADSC sheet: arrowheads). The ADSC sheet was identified in the stump of the pancreas.

(c) In vivo imaging for the grafting of the ADSC sheet. The fluorescent signal was detected at 48 hours after the attachment of the ADSC sheet.

(d) Resected distal pancreas and spleen. The fluorescent signal was identified on the pancreatic transection stump.

FIGURE 2. The effect of ADSC sheets treated with/without mannose in a rat pancreatic fistula model

(a) The photographs are representative images of the peritoneal features of untreated (no treatment) and ADSC sheet treated (treated with either ADSC sheets or ADSC sheets with mannose) rat pancreatic fistulas.

(b) Histology of the pancreatic transection stump by haematoxylin and eosin staining. Brownish MCL-labelled ADSCs sheet was observed at the pancreatic transection stump (arrow heads).

(c) The levels of ascitic amylase (upper left), ascitic lipase (upper right), serum amylase (lower left) and serum lipase (lower right) in untreated (no treatment) and treated (treated with either ADSC sheets or ADSC sheets with mannose) rat pancreatic fistulas. *$p < 0.05$.

FIGURE 3. The physical effect of the collagen sheets without ADSCs in a rat pancreatic fistula model

(a) Representative images of the peritoneal features of untreated (no treatment) and treated (treated with either collagen sheets or collagen sheets with mannose) rat pancreatic fistulas.

(b) Histology of the pancreatic transection stump by haematoxylin and eosin staining.

(c) The levels of ascitic amylase.

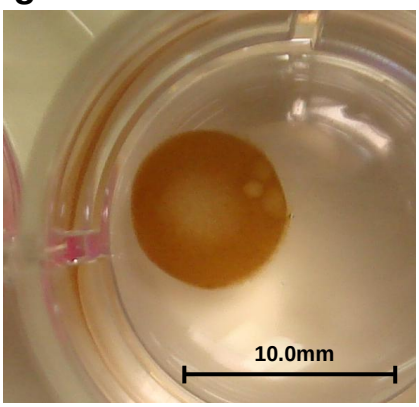
FIGURE 4. Cytokine production in human ADSCs after mannose treatment

(a) The levels of FGF2 (upper left), IL6 (upper right), VEGF (lower left), and HGF (lower right) in the supernatants of human ADSCs cultured with or without mannose as measured by a multiplex assay. *$p < 0.05$.

(b) The expression of FGF2 mRNA by RT-PCR in human ADSCs cultured with or without mannose. *$p < 0.05$.

Fig.1

Fig.1a



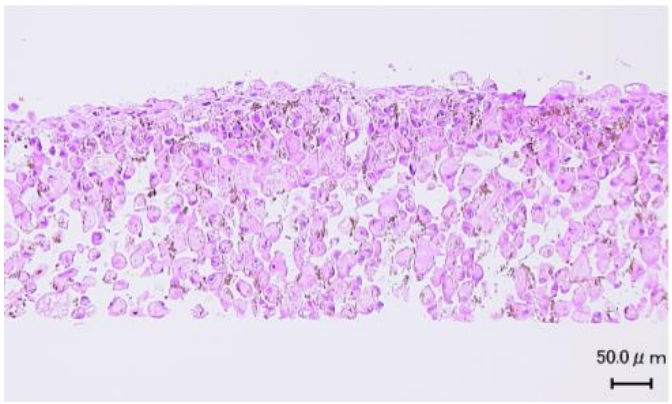


Fig.1b

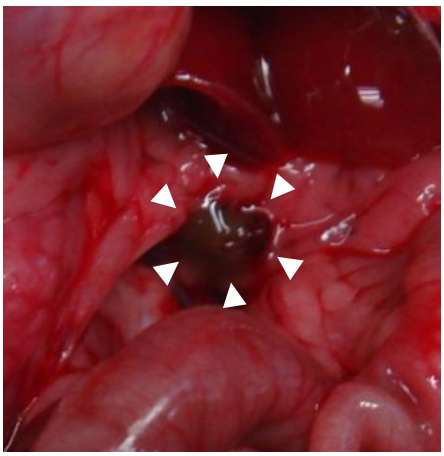
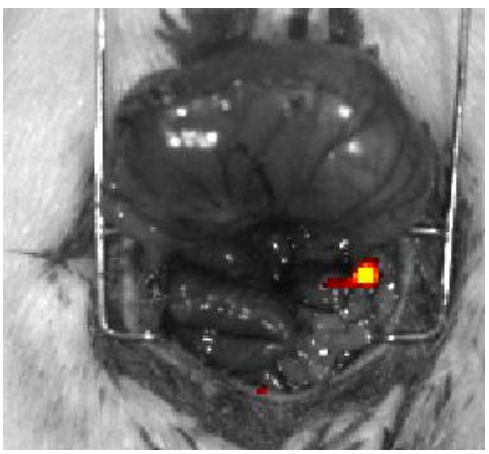
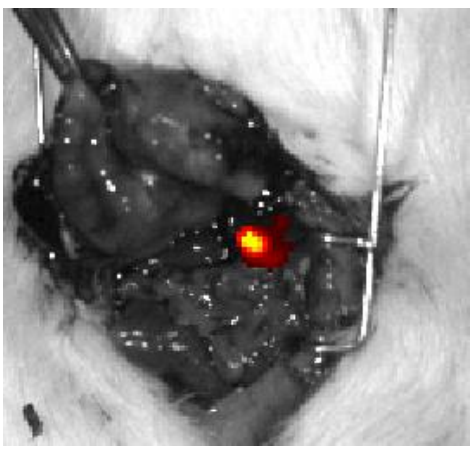


Fig.1c





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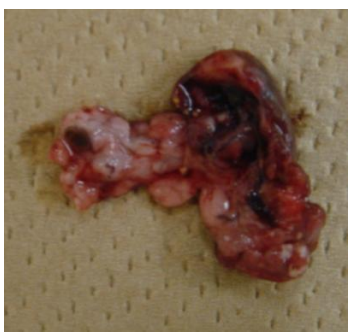
48hr

Fig.1d



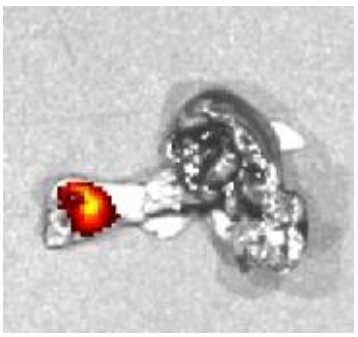
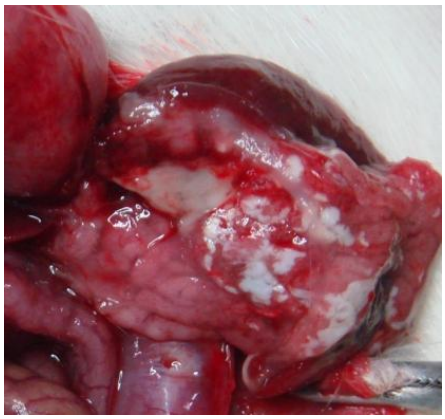
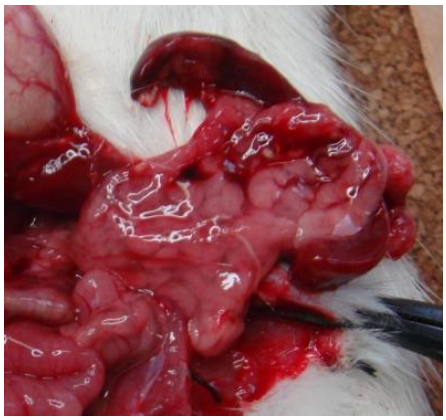


Fig.2

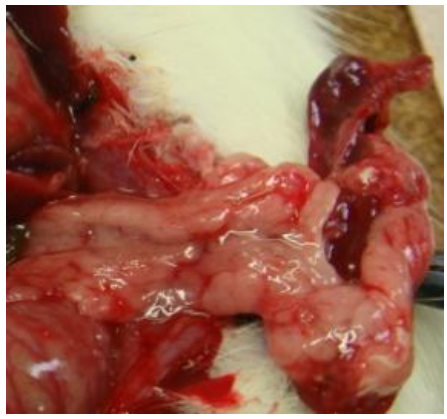
Fig.2a



Untreated

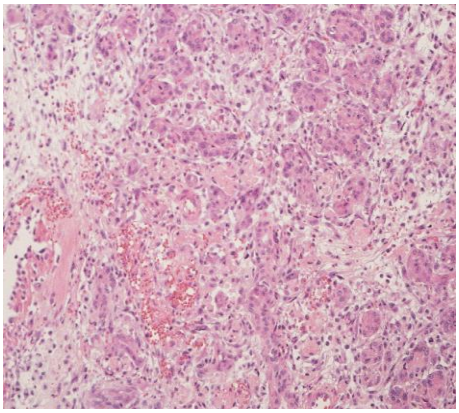


ADSC sheets

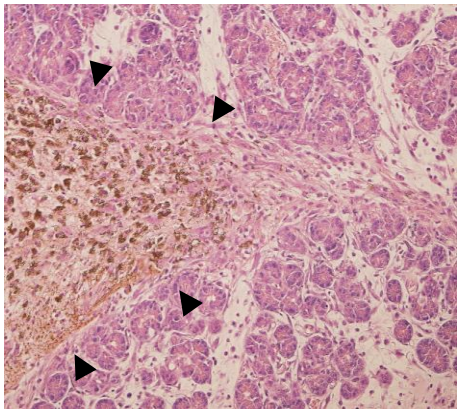


ADSC sheets
with mannose

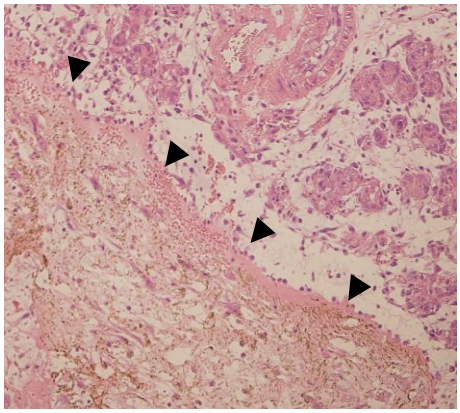
Fig.2b



Untreated



ADSC sheets



ADSC sheets
with mannose

Fig.2

Fig.2c

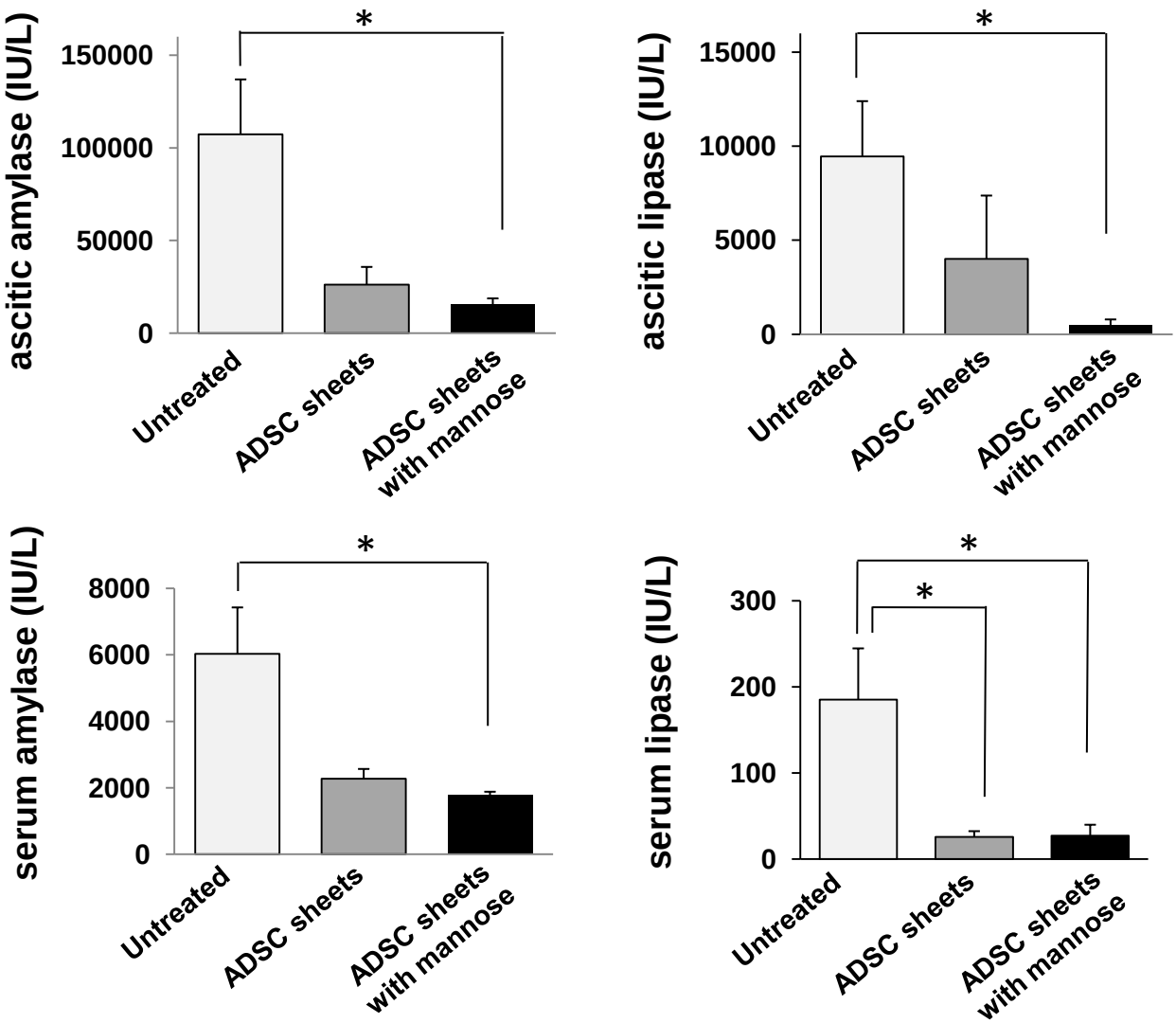
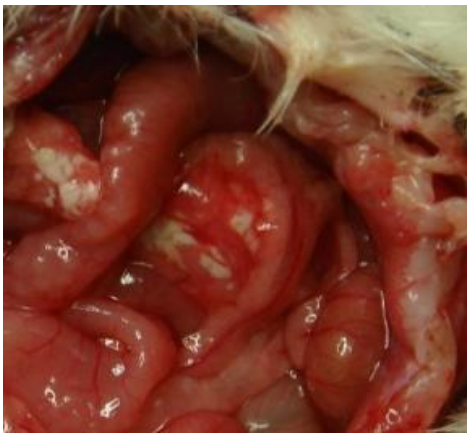

ascitic amylase (IU/L)
150000
100000
50000
0
*
Untreated
ADSC sheets
ADSC sheets with mannose
ascitic lipase (IU/L)
15000
10000
5000
0
*
Untreated
ADSC sheets
ADSC sheets with mannose
serum amylase (IU/L)
8000
6000
4000
2000
0
*
Untreated
ADSC sheets
ADSC sheets with mannose
serum lipase (IU/L)
300
200
100
0
*
*
Untreated
ADSC sheets
ADSC sheets with mannose

Fig.3

Fig.3a



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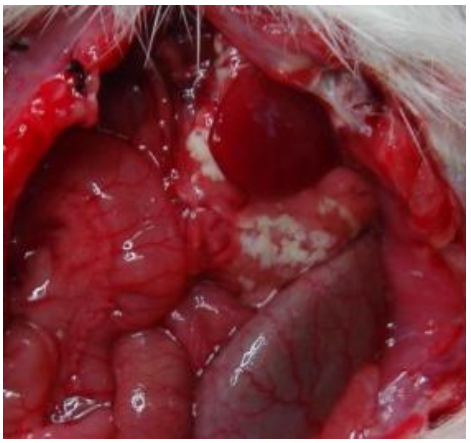
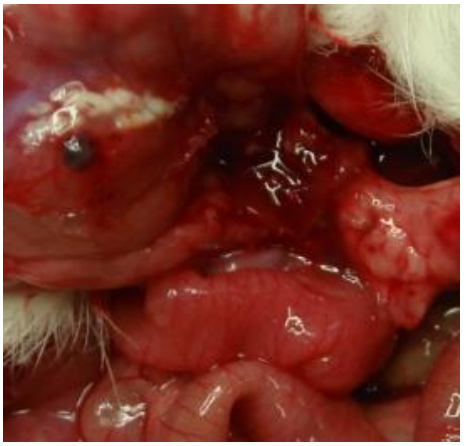
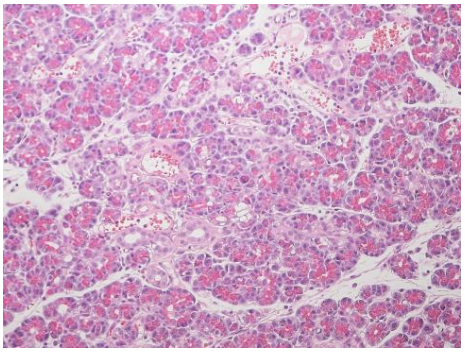


Collagen sheets

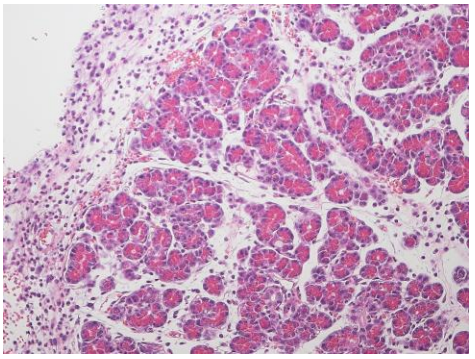


Collagen sheets with mannose

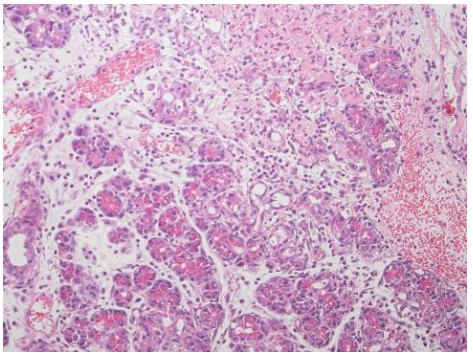
Fig.3b



Untreated



Collagen sheets



Collagen sheets with mannose

Fig.3c

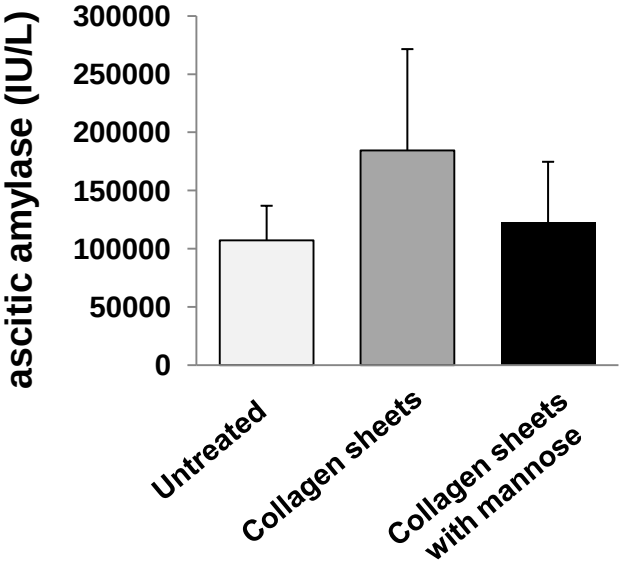


Fig.4

Fig.4a

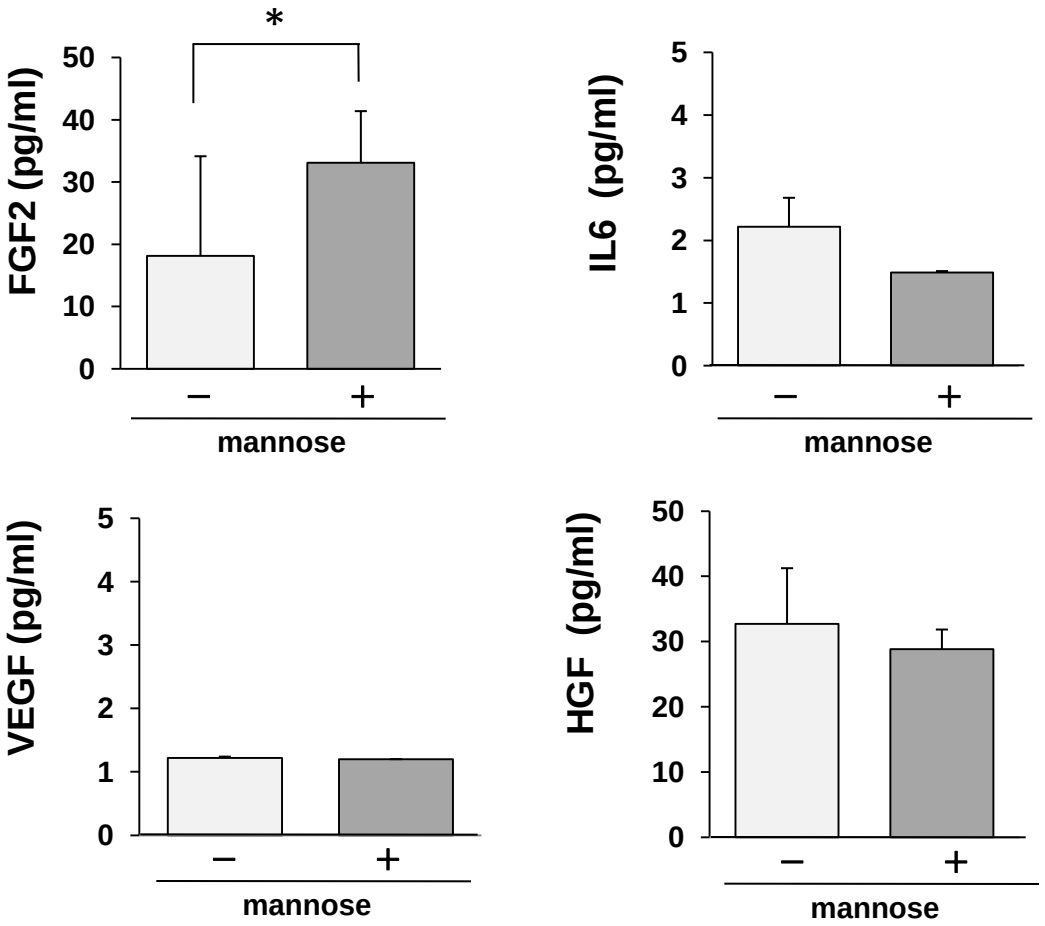


Fig.4b