<table>
<thead>
<tr>
<th>Section</th>
<th>Text</th>
</tr>
</thead>
<tbody>
<tr>
<td>Title</td>
<td>Development of a novel rat model with pancreatic fistula and the prevention of this complication using tissue-engineered myoblast sheets.</td>
</tr>
<tr>
<td>Author(s)</td>
<td>田中 貴之</td>
</tr>
<tr>
<td>Citation</td>
<td>Nagasaki University (長崎大学) 博士 医学 (2013-08-07)</td>
</tr>
<tr>
<td>Issue Date</td>
<td>2013-08-07</td>
</tr>
<tr>
<td>URL</td>
<td><a href="http://hdl.handle.net/10069/35205">http://hdl.handle.net/10069/35205</a></td>
</tr>
</tbody>
</table>

© The Japanese Society of Gastroenterology.; The final publication is available at www.springerlink.com
Development of a novel rat model with pancreatic fistula and its prevention using tissue-engineered myoblast sheets

Takayuki Tanaka, Tamotsu Kuroki, Tomohiko Adachi, Shinichiro Ono, Amane Kitasato
Masataka Hirabaru, Mitsuhisa Takatsuki, Susumu Eguchi

Department of Surgery, Nagasaki University Graduate School of Biomedical Sciences,
1-7-1 Sakamoto, Nagasaki, 852-8501, Japan

Running title: Prevention of pancreatic fistula with myoblast sheets
Key words: pancreatic fistula, myoblast sheet, tissue engineering, rat model

Correspondence to: Tamotsu Kuroki
Department of Surgery
Nagasaki University Graduate School of Biomedical Sciences
1-7-1 Sakamoto, Nagasaki, 852-8501, Japan
Phone: +81-95-819-7316 Fax: +81-95-819-7319
Email: tkuroki-gi@umin.ac.jp
Abstract

[Objective]

Although pancreatic fistula (PF) is one of the most important complications for pancreatic surgery, there are no small experimental animal models for use in research toward the prevention of PF. The aims of this study were therefore to establish a PF model in rats and to investigate the efficacy of our new method for preventing PF by using myoblast sheets that were made by tissue engineering techniques.

[Methods]

To establish a PF model, rats underwent transection of each of four pancreatic ducts: the gastric, duodenal, common, and splenic duct. Then, the ascitic amylase and lipase levels were measured to evaluate the level of PF. To investigate the efficacy of myoblast sheets for preventing PF, a myoblast sheet was attached to the pancreatic cut surface in the PF models. Then the levels of amylase and lipase in both serum and ascites were measured. In addition, surgical specimens were investigated pathologically.

[Results]

It was supposed that a new PF model established by transecting the splenic duct in rats may be highly useful for studies on the prevention of PF. There were no significant differences in serum amylase and lipase levels between the myoblast sheet
group and control group. However, there were significant differences in ascitic amylase and lipase levels between the two groups (p<0.05). Among the pathological findings, the number of inflammatory cells in the myoblast sheet group was smaller than that in the control group. In addition, the presence of the myoblast sheets on the surface of the pancreatic stump was confirmed by immunofluorescence staining.

[Conclusion]

Our data suggested that the efficacy of the new rat model of PF presented herein, and that it might be possible to prevent PF using myoblast sheets.
Introduction

Although pancreatic resection has become much safer and the mortality from pancreatic resection has decreased considerably in the past decade, the incidence of pancreatic fistula (PF) in pancreatic surgery still remains high. Several reports have shown pancreatic fistula rates ranging from 2% to 20% after pancreaticoduodenectomy\(^1\)\(^2\) and from 20% to 30% after distal pancreatectomy\(^3\)\(^-\)\(^6\). PF induces abscess formation, vascular injuries, pseudoaneurysm rupture, and fatal hemorrhage or sepsis, all of which eventually result in a prolonged hospital stay, a potentially serious and life-threatening event, or death\(^7\)\(^8\). Although various surgical techniques and devices have been proposed to prevent fistula formation\(^9\)\(^-\)\(^13\), it remains a matter of controversy which strategy is most appropriate for managing PF. Moreover, there are no stable models for researching the prevention of PF. Consequently, it is an extremely important challenge for surgeons to develop novel methods for the prevention of PF.

Numerous tissue engineering methods using cell sheets as biomaterials have been developed in recent years. In fact, cell sheets have been used to recreate the cornea\(^14\)\(^15\), to prevent esophageal stenosis after endoscopic submucosal dissection (ESD)\(^16\), and to repair the heart failure\(^17\)\(^18\). However, there have been no reports on the
prevention of PF with cell sheets. The aims of this study were to establish a PF model in rats and to evaluate the efficacy of myoblast sheets in preventing PF.
Materials and Methods

All rats received humane care according to the criteria outlined in the “Guide for the Care and Use of Laboratory Animals” prepared by the National Academy of Sciences and published by the National Institutes of Health (NIH publication 86-23, revised 1985). The animal protocol was approved by the Animal Experimentation Committee of Nagasaki University.

Animals

8-week-old male SD rats (Charles River Laboratories Japan Inc., Yokohama, Japan) and 10-week-old male F344 nude rats (F344/NJcl-rnu/rnu; CLEA Japan, Tokyo, Japan) were used for the experiments. The weight of the rats at the time of initiation of the experiments was 249 - 290 g. The rats were housed in plastic cages in a temperature and humidity-controlled room with a 12-hour light / dark cycle and given standard rat chow and water at the Laboratory Animal Center for Biochemical Research at Nagasaki University Graduate School of Biomedical Sciences. Rats were first placed under sedation by inhalation anesthesia using diethyl ether and then anesthetized by intraperitoneal injection of pentobarbital (50 mg/kg) as maintenance anesthesia.

Anatomical study of the rat pancreatic duct

It has been shown in previous reports that the rat pancreatic duct is composed
of four smaller ducts: the gastric duct, duodenal duct, common duct, and splenic duct\textsuperscript{19,20}. In the present study, the ducts were visualized by means of an injection of Indocyanine green (ICG; Diagnogreen Inj. Daiichi Pharmaceutical Co., Ltd., Tokyo, Japan) via the portal vein. Rats received a slow bolus injection of 5 mL ICG (2.5 mg/mL) via the portal vein for 3 min. The stream of ICG into the pancreaticobiliary ductal system was then observed macroscopically 30 min after the pigment injection.

**Development of the pancreatic fistula rat model**

Based on the anatomical study of pancreatic ducts in a rat, SD rats were divided into four groups: a gastric duct (G), duodenal duct (D), common duct (C), and splenic duct (S) transection group. The abdomen was swabbed with an iodinated solution and an 8-cm incision was made along the middle of the abdomen. The pancreas was exposed and transection of each duct in the pancreas was performed aseptically. The transectional procedures for each duct were as follows: the transection in the G group was performed in the middle of the gastric duct, the transection in the D group was performed along the common duct, the transection in the C group was performed in the middle of the common duct, and the transection in the S group was performed along the portal vein to preserve the splenic vessels. The pancreas was then returned to its original position and the abdomen was closed. The rats were sacrificed on days 1, 3, and 7 after
the operation. Five rats per group were examined at each time point. The samples of blood and ascites were obtained on the day of the operation and on days 1, 3, and 7 after the operation.

Myoblast sheets

SD rat myoblasts (American Type Culture Collection, Manassas, VA) were seeded at a density of 1x10^6 cells/dish onto 60 mm temperature-responsive culture dishes (Cellseed, Tokyo, Japan). Dulbecco's Modified Eagle's Medium (American Type Culture Collection) supplemented with Fetal Bovine Serum (American Type Culture Collection) and 1% penicillin and streptomycin (Sigma Aldrich Inc., St. Louis, MO) was used to culture the cells, and the medium was replaced with fresh medium every 48-72 hours. After 5 days of culture at 37°C in a humidified 5% CO₂ atmosphere, confluent myoblasts on the temperature-responsive dishes were transferred to another CO₂ incubator set at 20°C for about 2 hours, causing the myoblast sheets to detach spontaneously. The myoblast sheets were investigated by pathological examination.

Grafting the myoblast sheet in the PF rat model

Having established the PF rat model, we then applied it to an experiment investigating the prevention of PF with myoblast sheets. F344 nude rats were randomly divided into two experimental groups. In the myoblast group, a myoblast sheet was
attached onto the cut surface of the pancreas in the PF rat model using a Cell Shifter
(Cellseed, Tokyo, Japan). In the control group, transection of the splenic duct was
performed in the same way, but no myoblast sheet was attached. The rats were
sacrificed on days 1, 3, and 7 after the operation. Five rats per group were examined at
each time point. The samples of blood and ascites were obtained on the day of the
operation and on days 1, 3, and 7 after the operation.

Data sampling

Blood samples were drawn from the right femoral vein on the operative day
and from the inferior vena cava on days 1, 3, and 7 after the operation, and centrifuged
to obtain the serum. Ascitic samples were obtained from the abdominal cavity after
irrigation with 5 ml of normal saline and centrifuged to obtain the supernatant on the
operative day and on days 1, 3, and 7 after the operation. Subsequently, the blood
samples and ascitic samples were stored at -80°C until analysis. The levels of serum
amylase, serum lipase, ascitic amylase, and ascitic lipase were measured on the day of
the operation and on days 1, 3, and 7 after the operation. After the animals were
sacrificed, a total pancreatectomy was performed and the pancreas was subjected to
pathological examination.

Pathological examination
The specimens were fixed in 10% formalin and sectioned, and serial sections were carefully cut from the paraffin-embedded blocks and stained with hematoxylin and eosin. To prove that the myoblast sheets had engrafted onto the pancreas surface, immunofluorescence examinations were performed as follows. Sections were deparaffinized in various ethanol concentrations and washed in phosphate-buffered saline (PBS). The sections were then treated with a microwave oven (500 W) for antigen retrieval (Target Retrieval Solutions, pH 9.0; Dako, Tokyo, Japan) for 10 min. After being washed in PBS, the samples were treated with 0.03% hydrogen peroxide in methyl alcohol. Thereafter, they were treated with 0.25% casein in PBS containing stabilizing protein and 0.015 mol/L sodium azide (Real Peroxidase-Blocking Solution; Dako) at room temperature for 5 min and covered with a mouse monoclonal antibody to Myo-D (M-318): sc-760 (Santa Cruz Biotechnology Inc., Santa Cruz, CA) at a dilution of 1:50 in PBS at room temperature for 60 min. After the samples were rinsed with PBS, the sections were treated with goat anti-rabbit IgG FITC: sc-2012 (Santa Cruz Biotechnology) at room temperature for 45 min and washed with PBS.

**Statistical analysis**

Continuous data are expressed as the mean ± SD and were compared using a Mann Whitney-U test. We assigned statistical significance at <0.05.
Results

**Development of the PF model in rats**

As shown in Figure 1, the rat pancreatic ducts are comprised of four ducts: the gastric duct, duodenal duct, common duct, and splenic duct. In the G group, ascitic amylase levels were $33.2 \pm 10.8$, $94.2 \pm 4.8$, $25.8 \pm 4.8$, and $25.0 \pm 3.2$ (IU/l), respectively. Ascitic lipase levels were $2.6 \pm 0.4$, $24.0 \pm 4.4$, $11.8 \pm 1.8$, and $2.0 \pm 0.9$ (U/l), respectively (Figure 2a,b). In D group, ascitic amylase levels were $40.4 \pm 6.8$, $588.6 \pm 99.7$, $51.0 \pm 12.9$, and $38.0 \pm 1.4$ (IU/l), respectively. Ascitic lipase levels were $2.8 \pm 0.7$, $179.2 \pm 17.0$, $14.0 \pm 5.3$, $3.0 \pm 1.1$ (U/l), respectively (Figure 2c,d). All rats in the G and D groups lived for a long time. In the C group, scitic amylase levels were $34.0 \pm 4.0$, $56586.4 \pm 17118.6$, and $49849.2 \pm 15420.1$ (IU/l), respectively. Ascitic lipase levels were $2.6 \pm 0.5$, $2958.2 \pm 681.6$, and $2858.2 \pm 593.6$ (U/l), respectively (Figure 2e,f). Ascitic total bilirubin levels were $0.0 \pm 0.0$, $3.7 \pm 0.8$, and $4.0 \pm 0.7$ (mg/dl) due to the leakage of bile, respectively. All rats in the C group died by day 3 after operation. In the S group, ascitic amylase levels were $40.0 \pm 8.6$, $8867.3 \pm 4525.1$, $2537.4 \pm 431.1$, and $43.2 \pm 6.7$ (IU/l), respectively. Ascitic lipase levels were $9.4 \pm 1.5$, $854.2 \pm 69.1$, $372.3 \pm 75.9$, and $33.4 \pm 8.9$ (U/l), respectively (Figure 2g,h). Three of five rats in the S group died within four weeks after operation. In terms of the macroscopical findings,
the intraabdominal change was poor, and adhesion was uncommon in the G group and slight in the D group (Figure 3a, b). In the C group, the intraabdominal change was serious, and circumference organs were melted by pancreatic and bile leakage (Figure 3c). In the S group, the intraabdominal change was moderate, and the degree of adhesion was high (Figure 3d). Thus, it was possible to establish a PF model by transection of the splenic duct, and this model was used for the experiment on the efficacy of myoblast sheets for preventing PF.

**Formation of the myoblast sheets**

The myoblasts were cultured on temperature-responsive dishes. When the cultured myoblasts reached confluence, the dish was transferred to another incubator, so that the cultured myoblasts were detached from the dish (Figure 4a). Pathological examination showed that the harvested myoblast sheet was composed of myoblasts that had retained their cell-to-cell interactive connections (Figure 4b). Moreover, the myoblast cells were confirmed by Myo-D immunofluorescence examination (Figure 4c).

**Prevention of PF with myoblast sheets**

The operative procedure for the control and myoblast groups was as follows. 1) Transection of the pancreas was performed to preserve the splenic vessels. 2) A
myoblast sheet was transplanted to cover the surface of the pancreatic stump. 3) The myoblast sheet was attached to the pancreatic surface and stump macroscopically (Figure 5a-c). In the control group, serum amylase levels were 700.2 ± 111.5, 3975.8 ± 1878.8, 1315.2 ± 270.5, and 493.6 ± 98.4 (IU/l), respectively. Serum lipase levels were, 9.6 ± 1.6, 155.1 ± 73.3, 17.4 ± 1.4, and 5.4 ± 2.8 (U/l), respectively. Ascitic amylase levels were 39.6 ± 11.0, 8267.4 ± 5525.1, 2237.4 ± 531.1, and 41.0 ± 4.7 (IU/l), respectively. Ascitic lipase levels were 9.6 ± 1.6, 848.2 ± 67.1, 382.2 ± 77.6, and 31.4 ± 9.9 (U/l), respectively. In the myoblast group, serum amylase levels were 661.8 ± 73.1, 3290.2 ± 2293.5, 669.4 ± 369.7, and 531.0 ± 131.2 (IU/l), respectively. Serum lipase levels were 9.4 ± 1.5, 356.8 ± 419.5, 85.0 ± 161.0, and 6.0 ± 3.0 (U/l), respectively. Ascitic amylase levels were 39.4 ± 10.3, 4871.2 ± 4835.5, 406.0 ± 719.7, and 36.8 ± 15.3 (IU/l), respectively. Ascitic lipase levels were 9.4 ± 1.5, 356.8 ± 419.5, 85.0 ± 161.0, and 6.0 ± 3.0 (U/l), respectively. Although there were no significant differences in serum amylase and lipase levels, there were significant differences in ascitic amylase and lipase levels on days 1 and 3 after the operation (p<0.05) (Figure 6a-d).

When the intraabdominal conditions were macroscopically compared between the control group and the myoblast group, there were no significant differences in the intraabdominal conditions on days 1 and 3 after the operation. However, although
strong adhesion was observed between the pancreas and the circumference organs such as the stomach, liver, and intestinal tract and mass formation was observed at the pancreas stump on day 7 after the operation in all rats of the control group, the degree of adhesion was milder in the myoblast group than in the control group and it was possible to exfoliate the pancreas and the circumference organs easily (Figure 7a,b). In the myoblast group, there was no induration in the pancreas stump. Pathological examinations revealed that the pancreas stump in the control group showed colonization by a large number of inflammatory cells (Figure 8a). On the other hand, fewer inflammatory cells and different cells from the cells of the pancreas parenchyma were found on the surface of the pancreas stump in the myoblast group (Figure 8b). In addition, Myo-D-positive cells were detected on the surface of the pancreas stump by Myo-D immunofluorescence examination (Figure 8c).
Discussion

This study demonstrated the establishment of a new PF rat model and the prevention of PF using tissue-engineered myoblast sheets. A grading system for PF has been developed by the International Study Group on Pancreatic Fistula Definition (ISGPF), and PF in ISGPF is defined by ascitic amylase level and serum amylase level on day 3 after the operation. Clinical research on PF has advanced since the initial report of the ISGPF. However, the available experimental animal models for the study of PF have been limited to large animals such as dogs or pigs and there have been no reports in which small animals such as rats have been used for the PF experiments. In this study, transection of the gastric and duodenal duct led to a slight degree of PF, and transection of the common duct led to all rats dying within 3 days after the operation. When considering the degree of PF in transection of the splenic duct and the definition of ISGPF, although ascitic and serum amylase levels were almost improved to the normal level by 7 day after the operation, we considered that it would be possible to establish a new PF model with rats by transection of the splenic duct as an ideal short-term observation model.

It is well known that postoperative PF is an important complication after pancreatic resection and PF might lead to secondary complications such as abscess
formation, vascular injuries, pseudoaneurysm rupture, and fatal hemorrhage or sepsis \(^7\)\(^8\). Many surgical methods, new devices and medications have been used in an attempt to prevent PF. Surgical techniques such as omental roll-up, gastric wall-covering method, and vertical mattress suture have been tried and proposed to prevent PF \(^24\)-\(^26\). Many devices, including fibrin glue, sealants, stapler closure, electrocautery and suture have been tested in numerous studies \(^27\)-\(^32\). In addition, as medications, octreotide and its analogues have been used to prevent postoperative fistula since the 1990s \(^33\)-\(^34\). Although the mortality of PF has decreased to \(< 3-5\%\) due to these recent technical and medical advances \(^35\), the morbidity of PF still remains high \(^1\)-\(^6\). Therefore, we considered that a novel method was needed for the prevention of PF, and in the present study we focused on the use of cell sheets to this end. Tissue engineering with cell sheets has advanced since Langer et al. \(^36\) first reported the technique. In addition, cell sheets have attracted attention in conjunction with the many new therapies for injury and deficiency of tissues and organs. The advantageous characteristics of cell sheets are that they can be easily harvested from dishes, then transferred and attached to other surfaces by using temperature-responsive cultures, and that they are composed of cells that retain their cell-to-cell interactive connection \(^37\). In addition, in clinical trials, oral epithelial cell sheets have been proven effective for reconstruction of the cornea \(^14\)-\(^15\).
and myoblast sheets have been proven effective for tissue repair after ischemic heart failure and dilated cardiomyopathy\textsuperscript{17,18}). In the present study, we attempted to prevent PF using cell sheets in a new rat model based on these previous reports. In a previous study using cell sheets for the prevention of oesophageal constriction after ESD, Ohki et al.\textsuperscript{16} showed that the sheets promoted the proliferation and migration of host epithelial cells to the wound site, as well as the reduction of normal inflammatory reactions that might lead to pain and oesophageal scarring. These findings supported the present results that there were fewer inflammatory cells and a milder degree of adhesion between the pancreas and the circumference in the myoblast sheet group than the control group. Moreover, serum amylase and lipase levels in the myoblast sheet group tended to be lower than those in the control group, and there were significant differences in ascitic levels between the myoblast sheet group and the control group. These results indicated that the myoblast sheet method developed herein was the ideal method for preventing PF effectively without inducing inflammation of the pancreatic stump.

In summary, we established a new PF model in rats that has good potential for use in a range of future experiments on pancreatic fistula. In addition, it was found that myoblast sheets might be a novel method to prevent PF effectively. Further studies in
humans will be needed to confirm the potential therapeutic efficacy in the future.

Acknowledgements

This work was supported by Takeda Science Foundation Grants.
References


20. Githens S. Differentiation and development of the pancreas in animals. In: Lang V,


33. Buchler M, Friess H, Klempe I, et al. Role of octreotide in the prevention of


Figure Legends

Figure 1: Anatomy of a rat pancreatic duct.

Pancreatic ducts were comprised of four ducts: the gastric duct, duodenal duct, common duct, and splenic duct.

Figure 2: Development of the PF model in rats.

a) In the G group, ascitic-amylase levels were 33.2 ± 10.8, 94.2 ± 4.8, 25.8 ± 4.8, and 25.0 ± 3.2 (IU/l), respectively.

b) In the G group, ascitic-lipase levels were 2.6 ± 0.4, 24.0 ± 4.4, 11.8 ± 1.8, and 2.0 ± 0.9 (U/l), respectively.

c) In the D group, ascitic-amylase levels were 40.4 ± 6.8, 588.6 ± 99.7, 51.0 ± 12.9, and 38.0 ± 1.4 (IU/l), respectively.

d) In the D group, ascitic-lipase levels were 2.8 ± 0.7, 179.2 ± 17.0, 14.0 ± 5.3, and 3.0 ± 1.1 (U/l), respectively.

e) In the C group, ascitic-amylase levels were 34.0 ± 4.0, 56586.4 ± 17118.6, and 49849.2 ± 15420.1 (IU/l), respectively.

f) In the C group, ascitic-lipase levels were 2.6 ± 0.5, 2958.2 ± 681.6, 2858.2 ± 593.6 (U/l), respectively. All rats in C group died by day 3 after operation.

g) In the S group, ascitic-amylase levels were 40.0 ± 8.6, 8867.3 ± 4525.1, 2537.4 ±
431.1, and 43.2 ± 6.7 (IU/l), respectively.

h) In the S group, ascitic-lipase levels were 9.4 ± 1.5, 854.2 ± 69.1, 372.3 ± 75.9, and 33.4 ± 8.9 (U/l), respectively.

Figure 3: The intraabdominal change after the transection of each duct.

a) The intraabdominal change was poor, and adhesion was rare in the G group.

b) The intraabdominal change was poor, and the adhesion was also slight in the D group.

c) The intraabdominal change was serious, and circumference organs were melted by pancreatic juice and bile leakage in the C group.

d) The intraabdominal change was moderate, and the degree of adhesion was high in the S group.

Figure 4: Making of myoblast sheets and pathological examination of myoblast sheets.

a) The cultured myoblasts were detached from the temperature-responsive culture dish.

b) Pathological examination showed that the harvested myoblast sheet was composed of myoblasts that had retained their cell-to-cell interactive connections.

c) The fluorescence by MyoD-immunofluorescence examination was confirmed on the surface of the sheet.

(b: HE x 200; c: Myo-D x 200)
Figure 5: Procedure with grafting of the myoblast sheets.

a) Transection of the pancreas was performed to preserve the splenic vessels.

b) The myoblast sheet (indicated by an arrow) was transplanted to cover the pancreatic surface and stump.

c) The myoblast sheet (indicated by an arrow) was attached to the pancreatic surface and stump macroscopically.

Figure 6: Data on the prevention of PF with myoblast sheet.

a) There were no significant differences in serum amylase levels between the groups.

b) There were no significant differences in serum lipase levels between the groups.

c) There were significant differences in ascitic amylase levels on day 1 and 3 after operation between the groups (p<0.05).

d) There were significant differences in ascitic lipase levels on day 1 and 3 after operation between the groups (p<0.05).

Figure 7: Intraabdominal change in the control group and myoblast group.

a) The intraabdominal change was moderate, and the degree of adhesion was strong in the control group.

b) The degree of adhesion was milder in the myoblast group than in the control group and it was possible to exfoliate the pancreas and the circumference organs easily in

the myoblast sheet group.

Figure 8: Pathological findings in the control group and myoblast group.

a) Pathological examinations revealed that the pancreas stump in the control group exhibited colonization by a large number of inflammatory cells.

b) In the myoblast group, there were fewer inflammatory cells on the pancreatic stump and the cells found on the surface of the pancreas stump were different from those found on the pancreas parenchyma.

c) The different cells from the pancreas parenchyma showed positivity for MyoD immunofluorescence.

(a, b: HE x 200, c: Myo-D x 200)
Figure 1

- duodenum
- common duct
- gastric duct
- splenic duct
- duodenal duct
- pancreas
Figure 2

**ascitic amylase in G group**

**ascitic lipase in G group**

**ascitic amylase in D group**

**ascitic lipase in D group**
Figure 2

(e) Ascitic amylase in C group

(g) Ascitic amylase in S group

(f) Ascitic lipase in C group

(h) Ascitic lipase in S group
Figure 4
Figure 6

(a) (IU/l) vs. POD0, POD1, POD3, POD7

(b) (U/l) vs. POD0, POD1, POD3, POD7

(c) (IU/l) vs. POD0, POD1, POD3, POD7

(d) (U/l) vs. POD0, POD1, POD3, POD7

※ p<0.05

Legend:
- Sheet(-)
- Sheet(+)

Note: The graphs compare the levels of certain substances over time, with different conditions indicated by sheet(-) and sheet(+).