The protective effect of adipose derived stem cells against liver injury by trophic molecules.

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Abstract:

Background

In this study we investigated whether adipose-derived stem cells (ADSCs) had any beneficial protective effects on liver injury and regeneration in vivo. Moreover, we examined whether ADSCs protect hepatocytes by trophic molecules.

Materials and Methods

We transplanted ADSCs into mice after 70% hepatectomy (Hx) and ischemia-reperfusion (I/R), and observed liver injury and regeneration after reperfusion. We co-cultured hepatocytes with ADSCs using a Transwell System for seven days and evaluated the viabilities of hepatocytes and the cytokine levels in the culture medium. Bevacizumab was used in order to confirm the effect of VEGF on hepatocytes.

Results

ADSCs improved serum liver function at six hours after reperfusion in a non-lethal model and stimulated liver regeneration at 24 hours after reperfusion in a lethal model. VEGF levels in the culture medium increased by co-culture ADSCs with hepatocytes. ADSCs improved the viabilities of hepatocytes. The inhibited production of VEGF by bevacizumab did not affect the viability of hepatocytes.

Conclusions

ADSCs were able to ameliorate liver injury and stimulate liver regeneration in

subsequent Hx and I/R injured model mice. Furthermore, hepatocytes were protected by the trophic molecules of the ADSCs. However, such protective effects might be provided by mechanisms other than VEGF signaling.

Abbreviations:

Hepatectomy: Hx

Ischemia-reperfusion: I/R

Mesenchymal stem cells: MSCs

Adipose-derived stem cells: ADSCs

Vascular endothelial growth factor: VEGF

Hepatocyte growth factor: HGF

Tumor necrosis factor-α: TNF-α

Interleukin: IL

The ratio of liver weight to body weight: Lw / Bw

Aspartate aminotransferase: AST

Alanine aminotransferase: ALT

Total bilirubin: T-Bil

Introduction:

During major hepatectomy or liver transplantation, repetitive clamping of the hepatoduodenal ligament (the Pringle maneuver) is effective to reduce blood loss. However, this procedure places the liver under severe ischemia-reperfusion (IR) stress. In addition, the volume of the liver is substantially reduced after a major hepatectomy. These procedures additively or synergistically damage postoperative hepatic function and increase the incidence of postoperative morbidity and mortality. Although various strategies against I/R [1] stress or liver volume loss [2, 3] have been reported, few studies have examined I/R and hepatectomy (Hx) specifically. Then we now have focused on “the stem cells therapy”. After transplantation these cells can support a host’s liver function and thereby can open the way to further treatment as well as liver regeneration. The preeminent candidates as a source of stem cells are mesenchymal stem cells (MSCs), which can be obtained from different sources, such as bone marrow [4], umbilical cord blood [5], amniotic fluid [6], scalp tissue [7], placenta [8], and adipose tissue [9, 10] from the human body. MSCs possess both multipotentiality and semi-infinite proliferation ability [4 - 10]. Currently, “adipose-derived stem cells” (ADSCs) have been isolated [11, 12] and shown to possess the potential to differentiate into cells and organs of mesodermal origin as well as nonmesodermal origin. As many

as 1% of adipose cells are estimated to be ADSCs, compared with 0.001% – 0.002% of those found in bone marrow [13, 14]. These cells play a role in healing tissue damage, and are being investigated in phase I clinical trials for healing recurrent fistula in Crohn's disease [15]. Therefore, ADSCs have a wide range of clinical applications in many fields of surgery [16].

Recently, the trophic effects related to MSC-secreted bioactive molecules have been elucidated [17]. Moreover, Yarmush et al. [18] proposed that the molecules produced by MSCs could directly modulate hepatocyte death and regeneration in vitro and in vivo. Weil et al. [19] reported that MSCs might increase the viability and proliferative capacity of fetal intestinal epithelial cells after hypoxic injury via the paracrine release of vascular endothelial growth factor (VEGF), interleukin-6 (IL-6) and hepatocyte growth factor (HGF), as well as the down-regulation of apoptotic signaling. It has also been reported that trophic factors, especially VEGF, secreted by human MSCs, have enhanced islet survival and function after transplantation [20]. Furthermore, several studies of gastric ulcers have suggested that MSCs might accelerate the healing of these ulcers by a VEGF-dependent mechanism [21]. VEGF is a well-known signal protein produced by cells that stimulates vasculogenesis and angiogenesis, and the most important member is VEGF-A [22, 23]. However, other functions of VEGF, such as its

anti-apoptotic effects have also been reported [24, 25].

The aim of this study was to examine the beneficial effects of ADSCs on liver injury and regeneration after subsequent Hx and I/R in vivo, and to clarify whether VEGF was an essential signaling molecule for the hepatocytes-protective effects of ADSCs in vitro.

Materials and Methods:

Isolation and culturing of ADSCs

STEMPRO® human ADSCs were purchased from Life Technologies™, Tokyo, Japan [26]. The ADSCs were isolated from human adipose tissues collected during liposuction procedures and cryopreserved from primary cultures. Before cryopreservation, the ADSCs were expanded for one passage in MesenPRO RS® medium (Life Technologies). Cells were tested for purity by flow cytometry and for their ability to differentiate into osteogenic, chondrogenic, and adipogenic lineages. These cells were positive for CD29, CD44, CD73, CD90, CD105, and CD166 (> 95%), and negative for CD14, CD31, CD45, and Lin1 (< 2%). Thawing of the cells and initiation of the culture process were performed according to the manufacturer's instructions. ADSCs were plated in tissue culture flasks and cultured with MesenPRO RS® basal medium (Life Technologies) containing 2% fetal bovine serum (FBS), MesenPRO RS® growth supplement (Life Technologies), 2 mM of L-glutamine at 37°C, 5% CO_2 and 90% humidity. When cells were attached to the growth surface, the medium was replaced with an equal volume of fresh complete MesenPRO RS® medium. Cells were utilized for experimentation between passages 2 and 5. At the time of ADSCs administration, cells were harvested with 0.05% trypsin-EDTA (invitrogen,

Tokyo) and washed twice with phosphate buffered saline (PBS). Cells were then transplanted in a state of being dissolved in PBS.

ADSCs transplantation into mice with liver injury

We used six-weeks-old female BALB/c nu-nu mice (Charles River™, Tokyo, Japan). The mice were divided into two groups: 70% hepatectomy (Hx) following ischemia reperfusion (IR) with PBS (Hx I/R group, n=15), and 70% Hx following IR with ADSCs (1.0×10^5 cells / mouse, injected from tail vein; Hx I/R ADSC group, n=22). The 70% Hx was performed using the modified technique of Higgins and Anderson [27]. The hepatoduodenal ligament was clamped for 15 minutes before Hx, and the ADSCs in a volume of 100 µl or PBS were injected just after the Hx. The mice were killed at six hours and 24 hours [28] after reperfusion (Fig. 1 A). We compared the mice's serum liver function test results between the two groups. In addition, a lethal model was used where the hepatoduodenal ligament was clamped for up to 20 minutes and the mice were killed at 24 hours [**29**] after reperfusion (Fig. 1 B). We compared the survival and liver regeneration rates of the mice. The liver regeneration rate was defined as the ratio of liver weight to body weight (Lw/Bw) [27] . The present study was conducted under the supervision of the Division for Animal Research Resources, Institute of Health Biosciences, University of Tokushima. The experiments and procedures were approved

by the University of Tokushima's Animal Care and Use Committee.

Serum liver function test

To evaluate liver injury, the levels of serum aspartate aminotransferase (AST), serum alanine aminotransferase (ALT) and total bilirubin (T-Bil) were measured using the Japan Society of Clinical Chemistry standardization matching method. All measurements were performed by Shikoku Chuken, INC. Kagawa, Japan.

Isolation of murine hepatocytes

Hepatocytes were isolated from six-weeks-old female BALB/c nu-nu mice (Charles River™, Tokyo, Japan) using a two-step collagenase perfusion method set out by Seglen [30] .and Casciano [31] . All harvests yielded hepatocytes with viability exceeding 90% as determined by trypan blue dye exclusion.

Co-culture of hepatocytes with ADSCs

A Transwell System (0.4 μm pore size membrane, Corning, Acton, MA) was used to prevent ADSCs from contacting hepatocytes. Human ADSCs (1.0×10^5) were loaded into the lower chamber of the well, and murine hepatocytes (1.0×10^5) were added to the upper chamber coated by type 1 collagen (Fig. 2). The hepatocytes were co-cultured with ADSCs for seven days (n=4), and the viabilities of hepatocytes were evaluated

over time (days 1, 3, 5 and 7). We also examined the protein levels of cytokines and growth factors, such as VEGF, HGF and tumor necrosis factor-α (TNF-α) in the medium. For the control group (n=4), hepatocytes were cultured alone without ADSCs and compared with co-culture groups.

Measurement of protein levels of cytokines and growth factors

The protein levels of human VEGF, HGF and TNF-α in the cell culture supernatants were determined by ELISA, which was performed at Shikoku Chuken, INC. Kagawa, Japan.

Cell viability

Propidium iodide (PI) and fluorescein diacetate (FDA) were used to evaluate the viability of hepatocytes. Hepatocytes were rinsed with PBS and stained with solution (10 μg/ml PI, 20 μg/ml FDA, 2% FBS, and 0.1% BSA in PBS) at room temperature for five minutes. The intracellular fluorescence was observed and documented with an inverted fluorescent microscope (Model IX70, Olympus Optical Co., Japan). The cell viability in each group was calculated by measuring the average green fluorescent area in five random low power fields.

Administration of bevacizumab

In order to confirm the effect of VEGF on hepatocytes bevacizumab was used, a

humanized anti-VEGF monoclonal antibody. Bevacizumab was purchased from Chugai Pharmaceutical™, Tokyo, Japan. Bevacizumab has been reported to have cross reactivity with mice [32] . Bevacizumab (0.25 mg/mL) was administered into the culture medium [33, 34] , and the viabilities of hepatocytes were compared with and without bevacizumab.

Cell culture grouping

Cell culturing was divided into three groups; single hepatocytes culture (Control group n=4), and co-culture ADSCs with hepatocytes (ADSC group n=4), and co-culture ADSCs with hepatocytes being administrated bevacizumab (Bev. group n=4).

Statistical Analysis

All results were presented as mean ± SD. Multiple group comparisons were performed using one-way analyses of variance followed by the Scheffe procedure for comparison of means. Comparisons between the two groups were performed using the Mann-Whitney U-test using statistical software (JMP 8.0.1., SAS Campus Drive, Cary, 27513 NC, USA). A *p*-value of less than 0.05 was considered statistically significant.

Results:

Transplantation of ADSCs into mice with liver injury

In the Hx I/R ADSC group (n=11), serum levels of AST, ALT, and T-Bil at six hours after reperfusion were significantly lower than those in the Hx I/R group (n=11). (AST: 3,793±306 vs. 5,748±710 U/l, p=0.024, ALT: 2,443±163 vs. 3,672±353 U/l, p=0.017, T-Bil: 0.17±0.02 vs. 0.48±0.08 mg/dl, p<0.01) (Fig. 3). Additionally, at 24hours after reperfusion, serum levels of AST, ALT, and T-Bil in the Hx I/R ADSC group (n=7) tended to be lower than those in the Hx I/R group (n=4). (AST: 3,886±364 vs. 6,920±1978 U/l, p=0.07, ALT: 3,213±328 vs. 4,668±1679 U/l, p=0.29, T-Bil: 0.18±0.04 vs. 0.90±0.47 mg/dl, p=0.07) (Fig. 3). In the lethal model, the liver regeneration rate (Lw/Bw) at 24 hours after reperfusion in the Hx I/R ADSC group (n=7) tended to be stimulated compared to the Hx I/R ADSC group (n=4) (2.68±0.49 vs. 2.16±0.19, *p*=0.07) (Fig. 4). The survival rate at 24 hours after reperfusion in the Hx I/R ADSC group was 41.2% (7/17), while in the Hx I/R group it was 23.5% (4/17).

Identification of ADSCs-secreted molecules

In the ADSC group, VEGF concentration levels in the culture medium were significantly higher than in the control group. Furthermore, VEGF levels in the ADSC group increased in a time-dependent manner until day 7. HGF levels were significantly

higher than those in the control group at day 5. On the other hand, there were no significant differences in TNF-α levels between the two groups (Fig. 5).

Quality assessment of hepatocytes co-cultured with and without ADSCs

The cell viabilities of the ADSC group were significantly better than those of the control group at days 3, 5 and 7. (89.6±9.5 vs. 54.9±7.4% at day 3 $p<0.05$, 51.0±6.0 vs. 26.0±7.3% at day 5 $p<0.05$, 25.0±7.9 vs. 7.3±5. % at day 7 $p<0.05$) (Fig. 6).

The effect of VEGF signal on the quality of hepatocytes

After administration of bevacizumab into the culture medium, VEGF concentration (1,703±289 pg/ml at day7) substantially decreased (12±3 pg/ml at day7) in the ADSC group. However, the inhibited production of VEGF caused by the bevacizumab did not affect the cell viability of hepatocytes (Fig. 6). Therefore, the protective effects of ADSCs on hepatocytes might be provided by molecular mechanisms other than VEGF signaling.

Discussion

Recent studies have suggested that the therapeutic capacity possessed by ADSCs for liver disorders might stem from trophic effects resulting from factors such as the various types of cytokines and chemokines they produce [35,36]. Herein, we demonstrated the following findings: (1) In vivo, ADSCs had beneficial effects for liver injury after subsequent Hx and I/R. (2) In vitro, ADSCs' trophic molecules, including VEGF and HGF, protected hepatocytes. However, suppression of VEGF by the administration of bevacizumab did not affect the protective effects of ADSCs.

In recent years, MSCs transplantation has been widely used in animal models of cerebral infarction, myocardial infarction, and renal I/R injury to regenerate the damaged tissues. Some of these reports have demonstrated the effectiveness of MSCs transplantation against drug-induced liver injury [35, 37]. However, the effects of MSCs on hepatic I/R injury remain uncertain. In a model of drug-induced chronic liver disease, it was shown that the transplanted MSCs provided antifibrotic effects. They also showed that these MSCs scattered mostly in the hepatic connective tissue and survived in the liver for four weeks after the transplantation, but did not differentiate into hepatocytes expressing albumin or alpha-fetoprotein [37]. This suggests that a variety of bioactive cytokines secreted by the transplanted MSCs, such as VEGF, HGF and FGF, might be

involved in restoring liver function and promoting regeneration.

In models of acute liver disease due to I/R injury, free radicals generated during the acute phase of I/R injury initiate the inflammatory cascade, giving rise to the second attack, which is characterized by the infiltration of activated neutrophils in the liver promptly after reperfusion. Activation of Kupffer cells and T lymphocytes promotes neutrophil recruitment, assisted by increased endothelial expression of adhesion molecules [38] . Therefore, it seems that the transplanted MSCs should work efficiently just after the reperfusion. In the present study, the administration of ADSCs ameliorated the increase in serum transaminase and T-Bil levels at six hours after reperfusion, which serves as the most sensitive marker for clinical and experimental hepatic I/R injury evaluation. These findings suggest that the ADSCs were viable and able to function shortly after the transplantation. In our lethal model, our preliminary data suggests that the ADSCs tended to stimulate the remnant liver regeneration at 24 hours after reperfusion. Kanazawa H. et al. [38] reported that MSCs stimulated liver regeneration after I/R injury by several cytokines secreted by the transplanted MSCs. Indeed, ADSCs have been reported to secrete anti-inflammatory [39]and anti-oxidative [40]cytokines, and these effects attenuate I/R liver injury and stimulate liver regeneration.

ADSCs have been reported to secrete various cytokines, including VEGF, HGF,

NGF, and FGF, being active in hepatocyte proliferation. However, these cells are also rich in the production of anti-inflammatory cytokines, such as IL-1Ra and IL-10, thus indicating a relationship between mechanisms for suppressing concentrations of inflamed cells and recovery from liver damage [35] . In our study, the culture medium in the ADSC group had a higher content of VEGF and HGF. Furthermore, the VEGF levels in the ADSC group elevated in a time-dependent manner. In our previous study, ADSCs secreted sufficient amounts of VEGF, IL-6 and IL-8 by co-culturing with injured porcine islets, especially in the VEGF (data not shown). Therefore, we speculated that the VEGF signal should have a key role in hepatocytes protection. In fact, Park et al. [20] reported that the VEGF signal plays a key role in the cell protective effects of MSCs. They co-cultured the MSCs with mice's islets and VEGF was detected at higher concentrations and demonstrated a critical correlation with islet quality. Furthermore, co-cultured islets demonstrated increased levels of VEGF-receptor 2, being a VEGF-A-induced signaling [41] , and the phosphorylation of Akt. VEGF is a well-known signal protein produced by cells that stimulates vasculogenesis and angiogenesis, and the most important member is VEGF-A [22, 23] . On the other hand, it was reported that VEGF might suppress the apoptosis of K562 cells through its influence on the bcl-X(L)/Bax expression ratio in K562 cells [24] . Kosaka N et al.

reported VEGF suppressed granulosa cell apoptosis by inhibiting the release of caspase-activated DNase (CAD) [25] . That is to say, VEGF might not only correlate to vasculogenesis and angiogenesis but also have anti-apoptotic effects. In the current results, however, the inhibition of VEGF production by bevacizumab did not affect the viability of hepatocytes (Fig. 6). Therefore, the protective effects of ADSCs on hepatocytes might be provided by mechanisms other than VEGF signaling. In fact, HGF concentration of the culture medium in the ADSC group was significantly higher at day5. Since several cytokines, such as HGF, FGF, IGF, NGF, FGF, IL-1Ra, and IL-10 were reported to have cytoprotective or anti-inflammatory effects on hepatocytes [35, 36] , it is possible that such cytokines might be key molecules in the protection of hepatocytes, although further studies are required.

In conclusion, the ADSCs ameliorated the liver injury and stimulated remnant liver regeneration after Hx and I/R injury in mice. Furthermore, the administration of ADSCs protected hepatocytes due to their trophic molecules, although such protective effects might be provided by mechanisms other than VEGF signaling. Therefore, an ADSCs-based strategy might have therapeutic potential in the prevention of liver failure.

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Figure legends:

Figure 1

(A) Experimental protocol. Non-lethal model. BALB/c nu-nu mice were divided into two groups: 70% hepatectomy (Hx) following ischemia reperfusion (Hx I/R n=15), 70% Hx following IR with ADSC (1.0×10^5 cells / mouse, injected from tail vein; Hx I/R ADSC, n=22). ADSC was injected just after Hx. The hepatoduodenal ligament was clamped for 15 minutes before Hx. The mice were killed at six and 24 hours after Hx.

(B) Lethal model. The hepatoduodenal ligament was clamped for up to 20 minutes, and the mice were killed at 24 hours after reperfusion.

Figure 2

A Transwell System. Human ADSCs (1.0×10^5) were loaded into the lower chamber of the well, and murine hepatocytes (1.0×10^5) were added to the upper chamber coated by type 1 collagen.

Figure 3

Serum transaminase and T-Bil levels at six and 24 hours after reperfusion. ADSCs significantly improved serum liver function at six hours after reperfusion in Hx and I/R injured mice.

Figure 4

Lw / Bw at 24 hours after reperfusion. ADSCs tended to stimulate liver regeneration in severe Hx and I/R injured mice.

Figure 5

Identification of ADSCs-secreted molecules. In the ADSC group, VEGF and HGF concentration in the culture medium increased more than in the control group.

Figure 6

Quality assessment of hepatocytes. ADSCs improved the viabilities of hepatocytes on days 3, 5 and 7. The inhibited production of VEGF by bevacizumab did not affect the cell viability of hepatocytes.

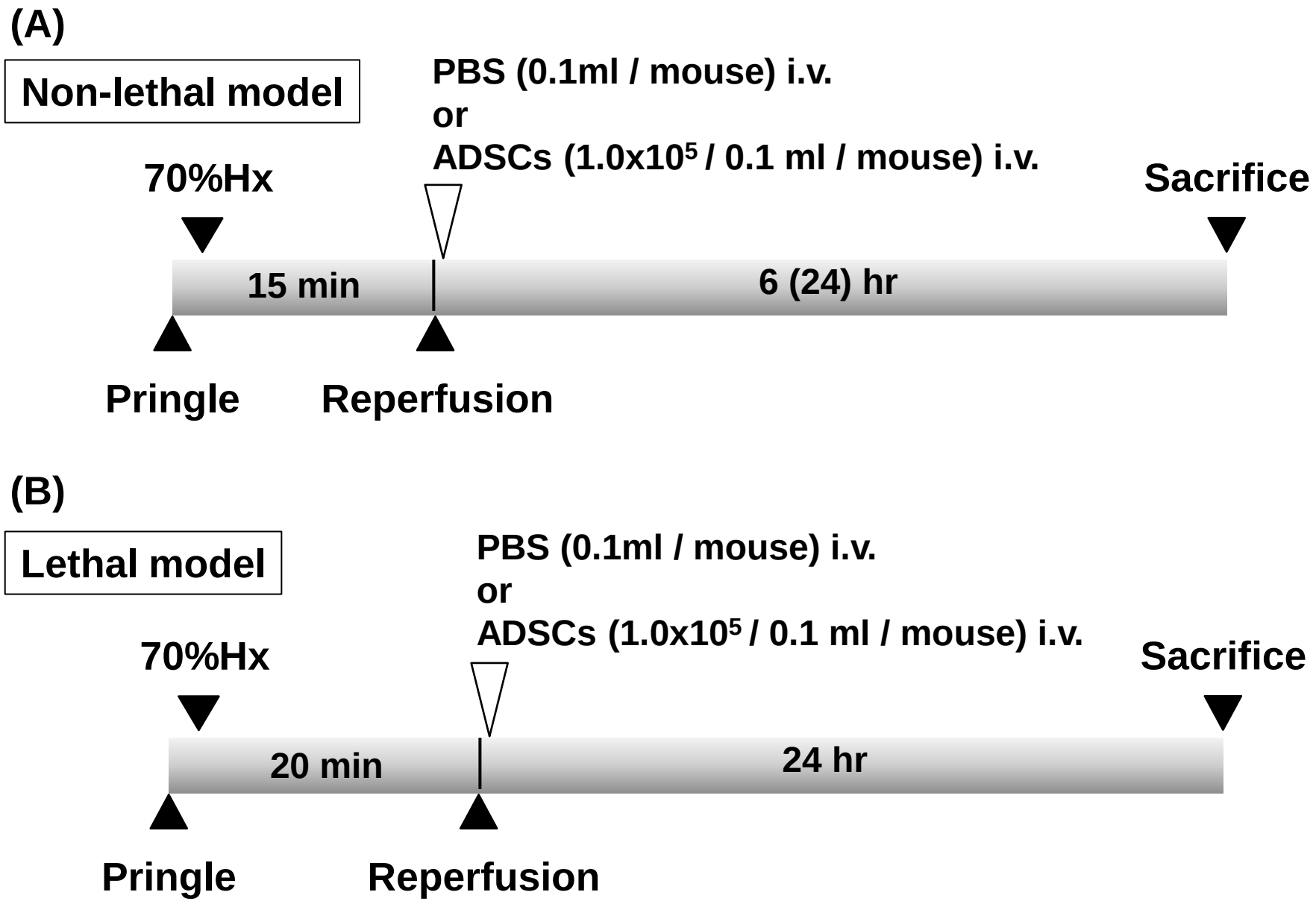

(A)
Non-lethal model
PBS (0.1ml / mouse) i.v.
or
ADSCs (1.0x10^5 / 0.1 ml / mouse) i.v.
70%Hx
Sacrifice
15 min
6 (24) hr
Pringle
Reperfusion
(B)
Lethal model
PBS (0.1ml / mouse) i.v.
or
ADSCs (1.0x10^5 / 0.1 ml / mouse) i.v.
70%Hx
Sacrifice
20 min
24 hr
Pringle
Reperfusion

Fig. 1

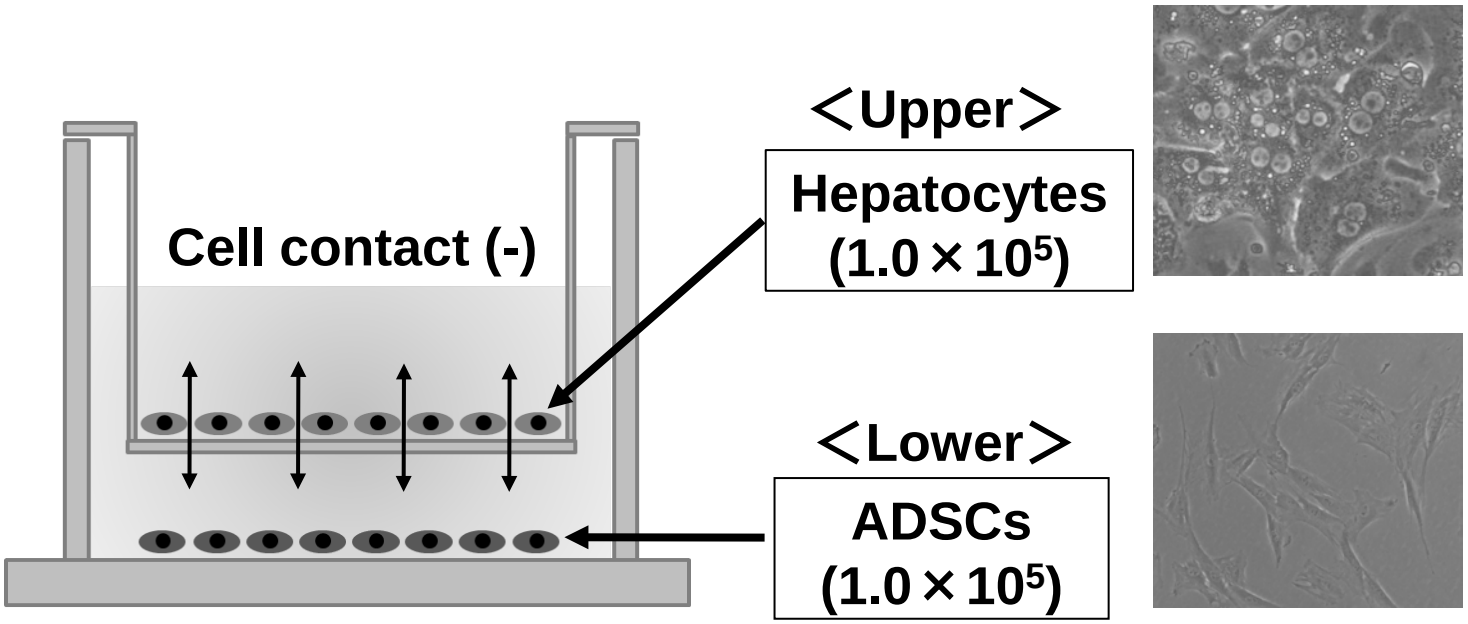
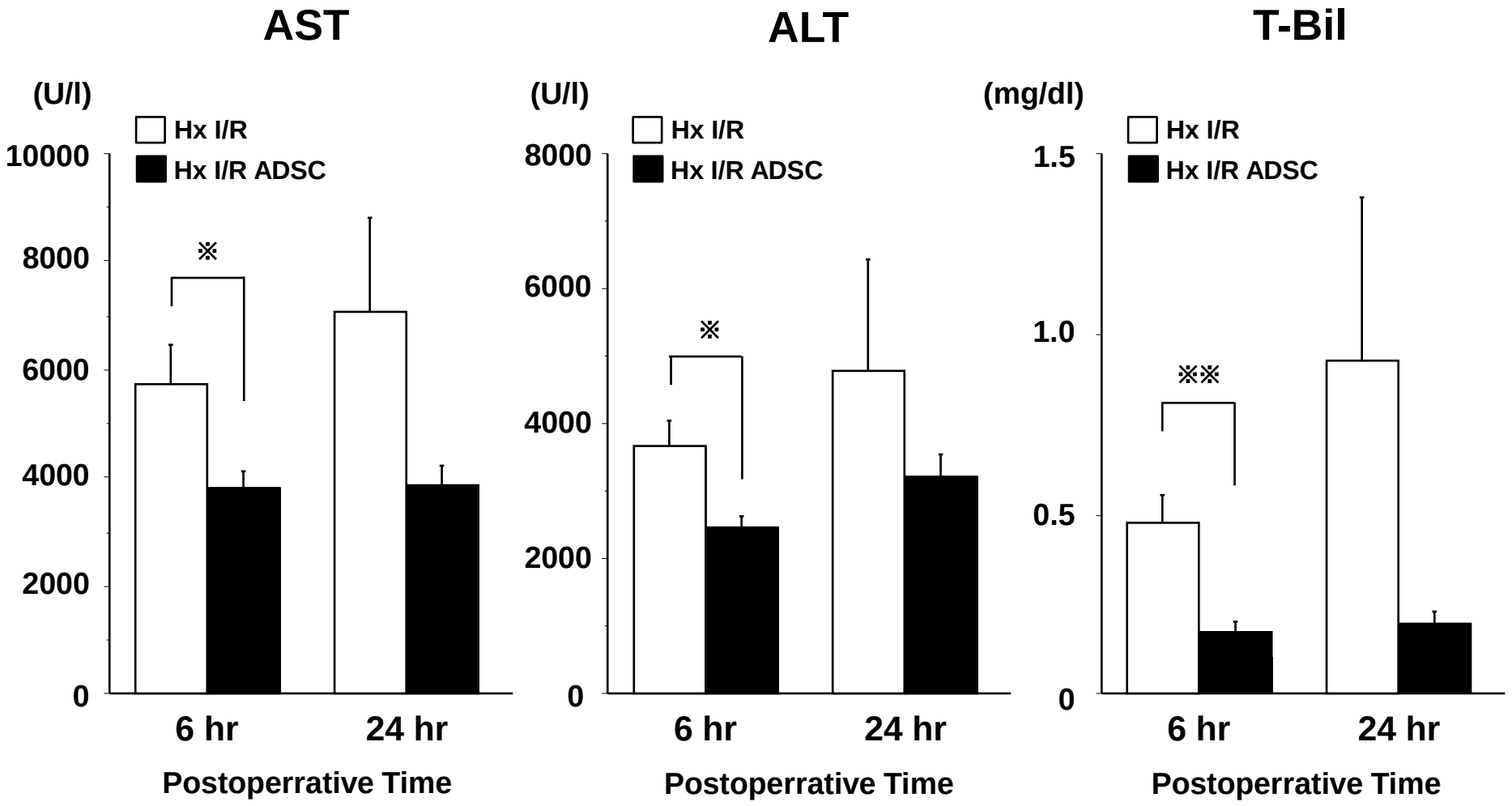


Fig. 2



※ $p<0.05$ v.s. Hx I/R group

※※ $p<0.01$

Fig. 3

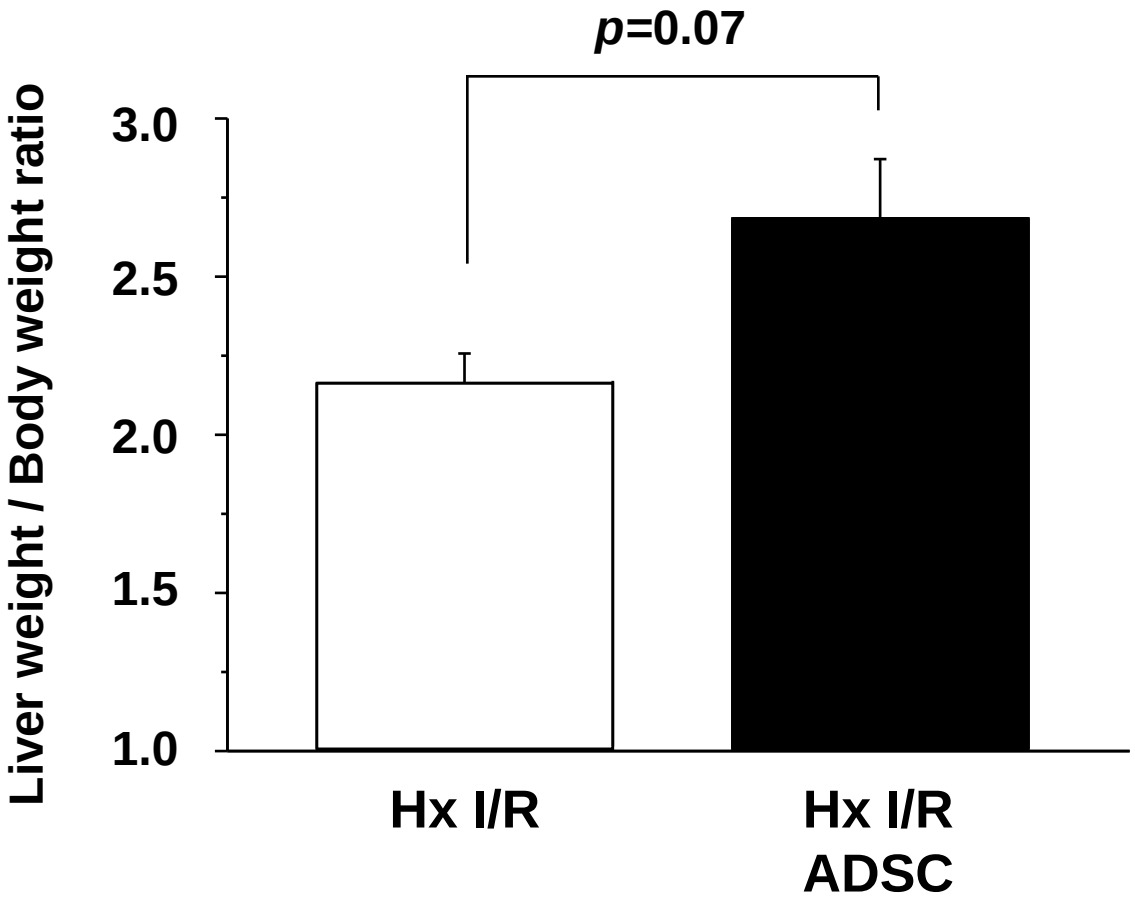
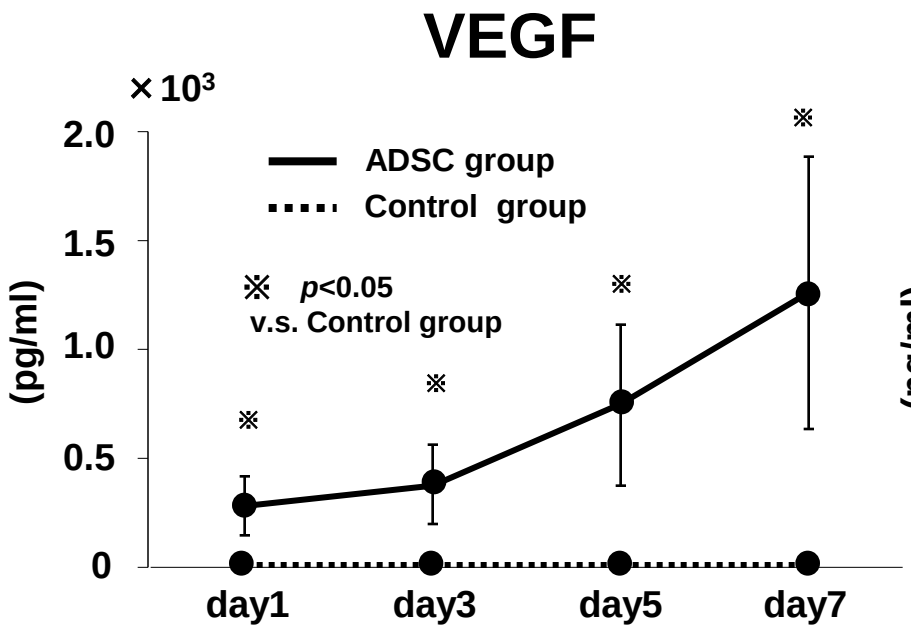
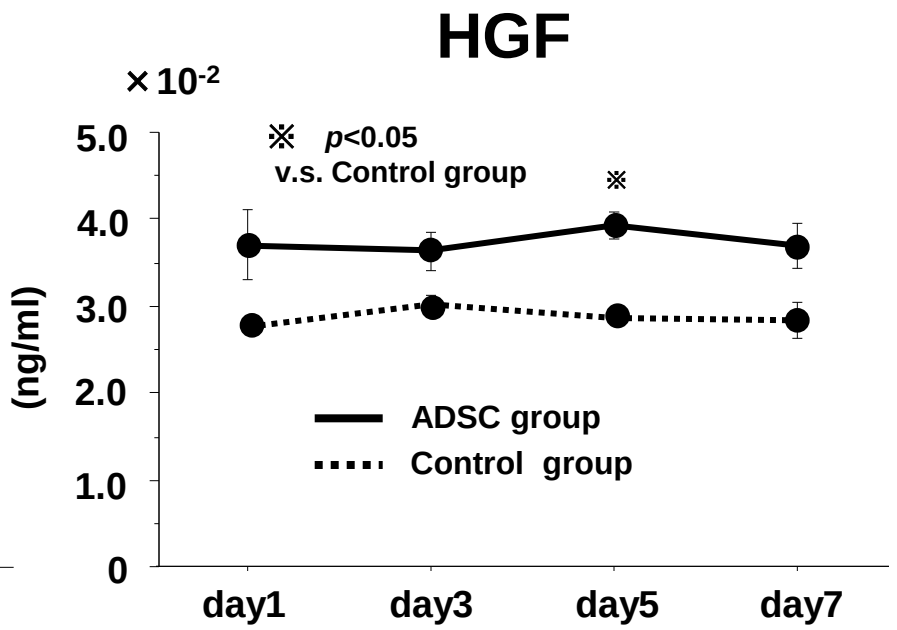

p=0.07
Liver weight / Body weight ratio
3.0
2.5
2.0
1.5
1.0
Hx I/R
Hx I/R
ADSC

Fig. 4


VEGF
×10³
2.0
1.5
1.0
0.5
0
(pg/ml)
ADSC group
Control group
※ p<0.05
v.s. Control group
※
※
※
※
day1
day3
day5
day7


HGF
×10⁻²
5.0
4.0
3.0
2.0
1.0
0
(ng/ml)
※ p<0.05
v.s. Control group
※
ADSC group
Control group
day1
day3
day5
day7

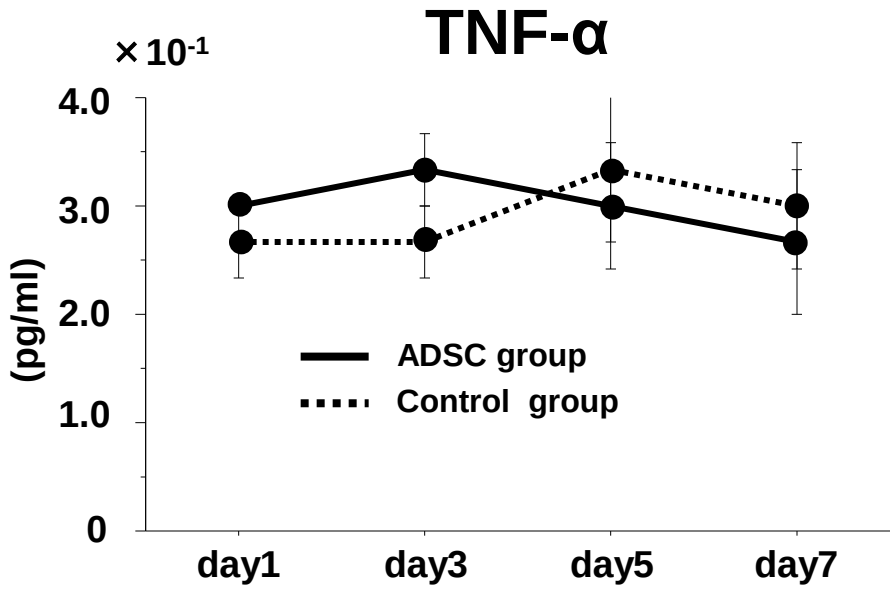

TNF-α
×10⁻¹
4.0
3.0
2.0
1.0
0
(pg/ml)
ADSC group
Control group
day1
day3
day5
day7

Fig. 5

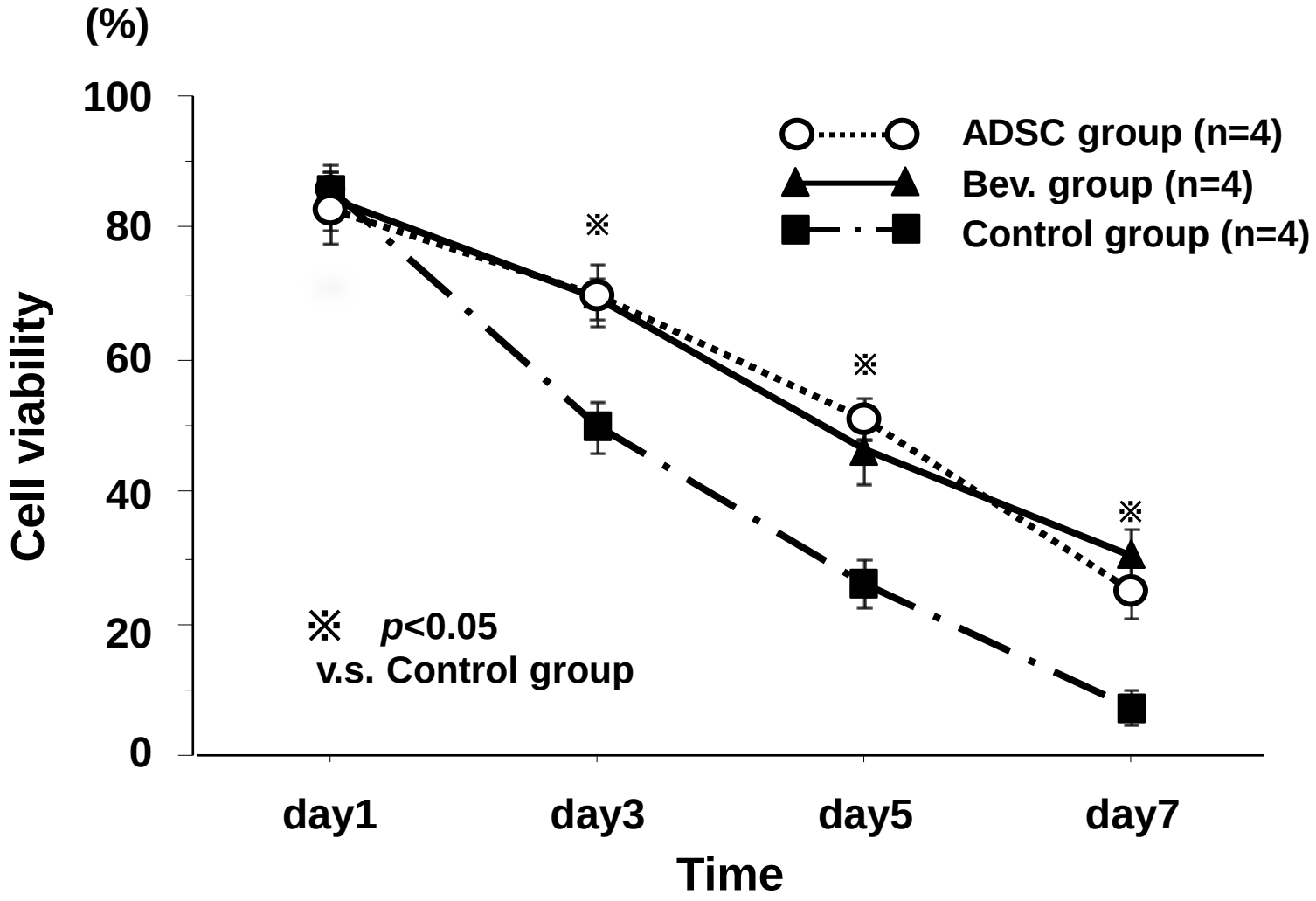

(%)
100
80
60
40
20
0
Cell viability
ADSC group (n=4)
Bev. group (n=4)
Control group (n=4)
※
※
※
※ p<0.05
v.s. Control group
day1
day3
day5
day7
Time

Fig. 6