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Benefit of polyvinyl alcohol (PVA) macro-encapsulated islets on islet cryopreservation

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*Abstract

Islet transplantation is a promising method for the treatment of type 1 diabetes mellitus (DM) and has been widely performed around the world. The long-term cryopreservation of islets shows many advantages in the field of islet transplantation. Previous studies have described the development of novel sheet-type polyvinyl alcohol (PVA) macro-encapsulated islets (MEIs) to treat type 1 DM without any immunotherapy. The present study examined their beneficial effects on islet cryopreservation. PVA MEIs of Wistar rats were divided into three groups of 1-day, 7-day and 30-day cryopreservation at -80℃. The 30-day group showed a lower recovery rate of the islet number and impaired insulin release in comparison to the 1-day group, whereas no significant differences of the *in vitro* results were observed between the 1-day and 7-day groups. The MEIs transplantation recipient mice in the 1-day and 7-day groups reached normoglycemia for a 4-week observation period, and the recipients in 30-day group also showed a significant decrease followed by a slightly higher non-fasting blood glucose level. These results suggest that the novel PVA MEIs are useful for islet long-term cryopreservation, and that the use of cryopreserved PVA MEIs may therefore be a promising modality for performing DM therapy.

Keywords: Polyvinyl alcohol (PVA); Macro-encapsulated islets (MEIs); Islet cryopreservation; Islet transplantation.

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1. Introduction

Islet transplantation is a promising method for the treatment of type 1 diabetes mellitus (DM) [1]. Clinical islet transplantation is still suffering from several major obstacles, including insufficient donors, the side effects of immuno-suppressive drugs and islet loss in the early stage after transplantation [2-3]. Encapsulated islets (islets enclosed in a semi-permeable membrane) emerged in transplantation studies to overcome these problems. The transplanted islets can survive in the host immune system and can be protected from inflammatory factors that induce cell loss in the early stage after transplantation because the semi-permeable membrane can prevent high-weight molecules and immune cells to contact with islets, thereby allowing low-weight molecules, such as oxygen, glucose, and insulin etc., to freely pass through it.

Recently, due to remarkable advances in both research and surgical techniques, islet transplantation is now widely performed around the world [1, 4-6]. Cryopreservation shows many advantages for research and clinical islet transplantation: 1. Isolated islets can be shipped to other institutions worldwide. 2. Islets isolated at different time can be accumulated to obtain a sufficient number for transplantation.3. Cryopreservation provides the time for quality control of islets before transplantation [7]. Previous studies described the development of novel sheet-type polyvinyl alcohol (PVA)

macro-encapsulated islets (MEIs) by 1-day freezing and succeed in confirming their positive effects on xeno-transplantation [8], allo-transplantation (manuscript in press) and prevention of diabetic nephropathy [9-10].

This study tested 2 periods (7 days and 30 days) of freezing and examined their functions *in vitro* and *in vivo* in comparison to the original method of cryopreservation (1-day freezing). The results showed that PVA MEIs in the 7-day cryopreservation group could maintain their normal functions *in vitro* and normalize non-fasting blood glucose (NFBG) of diabetic mice *in vivo*.

2. Materials and Methods

2.1. Materials

Polyvinyl alcohol (PVA, molecular weight: 387200, saponification degree: 99.8 mol %) powder was a generous gift from Prof. Suong-Hyu Hyon (Kyoto University). Dimethyl sulfoxide (DMSO) was purchased from Wako Pure Chemical (Osaka, Japan). Cell Banker was purchased from Nippon Zenyaku Kogyo (Fukushima, Japan). Type XI collagenase was purchased from Sigma-Aldrich (St. Louis, MO, USA). Dextran power was purchased from Sigma-Aldrich (Sweden). BSA was purchased from Nacalai Tesuque (Kyoto, Japan). Streptozotocin (STZ) was purchased from Sigma-Aldrich (St.

Louis, MO, USA). Rats and mice were purchased from Shimizu Laboratory Supplies Co. Ltd. (Kyoto, Japan). The approval to conduct this experiment was obtained from the Animal Care Committee of Institute for Frontier Medical Sciences, Kyoto University, and the animals were treated according to the experimental protocols under its regulations.

2.2 Islets isolation

Ten-week old male Wistar rat islets were isolated as described previously [8]. Briefly, the rat pancreas was digested by type XI collagenase and then the islets were separated by a dextran gradient. The separated islets were thereafter purified by handpicking and then were cultured in an RPMI-1640 medium with 10% heat-inactivated fetal bovine serum (FBS) and a 1% antibiotics solution overnight.

2.3. Preparation of PVA MEIs

The 3% (w/v) PVA solution contained 10% 10 times concentrated Euro-Collins (EC) electrolyte solution, 10% heat-inactivated FBS, 5% DMSO and 1% antibiotics in dd-water as described previously [8]. Rat islets were pretreated with Cell Banker and suspended in 100μl PVA solution.This mixture was sandwiched between two pieces of

1mm thick polyethylene terephthalate mesh for reinforcement. After this molding process, the MEIs were frozen in a computer-controlled program freezer (TAIYO Nippon Sanso Corporation, Tokyo, Japan). The cooling rate was set to -3ºC/min until the surface temperature of the MEIs reached -10ºC, then changed to -1ºC /min until the surface temperature of the MEIs reached -30ºC.

Thereafter, MEIs were randomly divided into 3 groups, 1-day group (original method: 1-day cryopreservation), 7-day group (7-day cryopreservation) and 30-day group (30-day cryopreservation). The MEIs were then immediately moved to an -80ºC ultra low freezer (SANYO Electric, Tokyo, Japan) and stored for 1, 7 or 30 days to form crystallized gels. These gels were thawed rapidly in 37ºC RPMI-1640 medium for a few seconds followed by immersion in 4ºC University of Wisconsin (UW) solutions for 24h. Finally, after an overnight culture in RPMI-1640 (37ºC, 5%CO_2 95% air), the MEIs were used in this experiment.

2.4. Morphological changes and islet recovery rate of MEIs after freezing-thawing

The morphological changes of MEIs were observed by microscopy and the islet numbers were counted to calculate their recovery rate. The islet recovery rate was defined as the percentage of the number of islets after thawing to the number of islets

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10 *2.5. Static incubation*
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13 Static incubation was performed in the three MEIs groups. MEIs were pre-incubated
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16 in RPMI-1640 culture medium containing 3.3mm glucose and 0.1% BSA for 1h, and
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19 then were incubated in RPMI-1640 medium containing 3.3mm glucose for another 1h.
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22 Thereafter, they were incubated in RPMI-1640 medium containing 16.7mm glucose for
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25 1 h (glucose stimulation). At last, the MEIs were incubated again in RPMI-1640
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28 medium containing 3.3mm glucose for 1 h. At the end of each incubation period, the
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31 medium were collected and frozen for an insulin assay using a rat insulin ELISA kit
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34 (Shibayagi, Gunma, Japan). The stimulation index (SI) was calculated by the ratio of
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37 the insulin content in high glucose medium to the insulin content in the first low glucose
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47 *2.6. MEIs transplantation*
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50 A type 1 DM animal model was induced in 8-week old male C57BL/6 mice by a
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53 single injection of STZ (190mg/kg body weight, i.p.) 7 days before transplantation.
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56 Mice with an NFBG level >450mg/dl were used as recipients in the experiment. Eight
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hundred encapsulated Wistar rat islets (MEIs size: 20x 15x 1mm) were transplanted into the peritoneal cavity of C57BL/6 mice (n=6). NFBG and then the body weights were observed until 4 weeks after transplantation.

2.7. Intraperitoneal glucose tolerance test (IPGTT)

IPGTT was performed 2 weeks after transplantation. Briefly, after an overnight fast, 20% glucose solution (2g/kg) was injected into the peritoneal cavity of recipient mice. The blood glucose level was then measured at 0, 15, 30, 45, 60, 80, 100 and 120 min after injection of the glucose solution.

2.8. Serum insulin and C-peptide contents

Blood samples were collected from the sacrificed mice 4 weeks after transplantation. After centrifugation, serum samples were frozen for later measurement. The serum insulin level was measured using a rat insulin ELISA kit (Shibayagi, Gunma, Japan) and the serum C-peptide level was measured using a rat C -peptide ELISA kit (Shibayagi, Gunma, Japan).

2.9. Histology of MEIs and recipients' pancreas

Transplanted MEIs and recipients' pancreas were retrieved from mice sacrificed 4 weeks after transplantation. The samples were fixed in 4% paraformaldehyde solution for 24 h, followed by immersion in 70% ethanol at 4℃ for 24~48h. Next, the samples were embedded in paraffin and 5 μm thick sequential sections were cut.

The pancreas sections were stained for H&E and MEIs were stained for insulin as described previously [11]. Briefly, glass slides were mounted with 1% rabbit normal serum in PBS for 30 min. Subsequently, primary antibody (anti-Insulin guinea pig polyclonal antibody) was applied overnight at 4℃. Thereafter, they were incubated with peroxidase conjugated rabbit anti-guinea pig immunoglobulins (second antibody) diluted to 1:100 in PBS for 30 min. After washing in PBS, coloring reaction was carried out.

2. 10. Statistical Analysis

All results were expressed as the mean ± SEM. Significant differences between the groups were tested by an analysis of variance (ANOVA). Statistical significance was defined as $p<0.05$.

3. Results

3.1 .Morphological changes and islet recovery rate

The MEIs in the three groups showed a normal morphology after freezing-thawing, without islet fragments, and no obvious differences were observed between the three groups **(Fig. 1)**. The islet recovery rate in the 1-, 7- and 30-day groups were 74.4± 1.72%, 69.6±3.97% and 62.8±3.2%, respectively (7-day vs. 1-day: $p>0.05$; 30-day vs. 1-day: $p<0.05$; **Fig. 2**).

3.2. Static incubation

Static incubation was performed in the three groups to assess the function of the PVA MEIs, after freezing-thawing. The MEIs in the three groups showed good insulin secretion abilities in response to high glucose concentration **(Fig. 3 A)**. The SI in the 1-, 7- and 30-day groups was 1.84±0.07, 1.71±0.1 and 1.66±0.07, respectively. No significant differences were found between three groups **(Fig. 3B)**. However, the insulin release in the basal (3.3 mM) and stimulation (16.7 mM) medium of the 30-day group was lower than 1-day group ($p<0.05$; **Fig. 3C**).

3.3. MEIs xeno-transplantation

Mice in the 1-, 7- and 30-day groups showed a significant decrease in the NFBG

levels in comparison with those in DM group after PVA MEIs xeno-transplantation. Moreover, mice in the 1- and 7-day groups achieved normoglycemia (NFBG<200mg/dl) within 1 week after transplantation, and maintained normoglycemia for 4 weeks. Although mice in the 30-day group did not achieve normoglycemia, the NFBG significantly decreased from 485.8±25.1mg/ml to 246.3±19.6mg/dl (at the 4th week) after transplantation **(Fig. 4A)**.The MEIs groups maintained their body weight for 4 weeks. In contrast, the DM group showed a significant decrease in body weight in a time-dependent manner **(Fig. 4B)**.

3.4. IPGTT

IPGTT was performed 2 weeks after transplantation. The blood glucose changes are shown in **Figure 5A**. The 30-day group and DM group showed significantly higher area under the curve (AUC), and the normal group showed a significantly lower AUC in comparison to the 1-day group. No significant difference was observed in the AUC between the 1-day and 7-day groups. Moreover, the AUC in 30-day group was lower than that in DM group ($p<0.05$; **Fig. 5B**).

3.5. Serum insulin and C-peptide

The 1-, 7- and 30-day groups showed higher serum insulin **(Fig. 6A)** and *C* -peptide **(Fig. 6B)** concentrations than the DM group ($p<0.05$), and no significant differences were observed among the 1-, 7- and 30-day groups. The normal mouse group was omitted in this assessment because of the species cross reaction between the rat and mouse in the insulin and C-peptide level.

3.6. Histological findings

HE staining of the pancreas of recipient mice was performed in each group to check the regeneration of islets in STZ-induced diabetic mice. No intact islets were observed in the STZ-induced diabetic mice (DM, 1-, 7- and 30-day groups). In contrast, large islets with intact morphology were found in the normal group **(Fig. 7A)**. These results indicated that the regeneration of islet did not happen in the STZ-induced diabetic mice.

Insulin staining was performed in the MEIs group (1-, 7- and 30-day groups) to confirm the surviving islets in the PVA MEIs 4 weeks after transplantation. The islets in each MEIs group were positive for insulin staining **(Fig. 7B)**. This result, together with results of the serum insulin and C-peptide, indicated that MEIs still survived with a normal insulin secretion function 4 weeks after transplantation.

4. Discussion

Encapsulated islets can be divided into two categories: namely, micro-encapsulated islets and macro-encapsulated islets. Both types have been emerged and each has their own advantages [12-14]. Micro-encapsulated islets are one or a few islets enclosed in semi-permeable membranes, which can provide a beneficial surface for diffusion, thus maintaining the functions of islets inside. However, they are irretrievable after transplantation [15]. In contrast, macro-encapsulated islets are retrievable so that the functions of islets inside can be evaluated at anytime; that is the reason why the macro-encapsulated islets were employed for transplantation.

In previous studies, islets were pretreated with Cell Banker, and 5% DMSO and 10% EC solution were used as cryoprotective agents to reduce cryo-damage. Rajotte et al. suggested that slow cooling and rapid thawing could protect islets better during cryopreservation [16]. Therefore, MEIs were frozen with a slower cooling rate (4ºC ~ -10ºC: -3ºC/min; -10ºC ~ -30ºC: -1ºC /min) by a computer-controlled program freezer to maintain the islets functions during long-term cryopreservation.

The MEIs in all three groups showed good insulin secretion ability in response to glucose concentration changes **(Fig. 3A)** without significant difference in SI **(Fig. 3B)**, indicating that MEIs in three groups maintained comparable insulin secretion function. However, **Figure 3C** showed that insulin release in the basal and stimulation medium of

the 30-day group was lower than the 1-day group. Considering the results of the islet recovery rate (Fig 2), it is assumed that the difference in the number of surviving islets after cryopreservation resulted in different insulin release *in vitro* **(Fig. 3C)** and different results *in vivo* **(Figs. 4** and **5)**.

Although MEIs in the 30-day group showed a slightly worse function *in vitro* in comparison to that seen in the 1-day group, and the recipient mice in the 30-day group did not achieve normoglycemia, there were still some therapeutic benefits with 30-day cryopreserved PVA MEIs in comparison to the DM group *in vivo* **(Figs. 4** and **5)**. In fact, the survival rate of recipients 4 weeks after transplantation was 100% in the 30-day group and 17% in the DM group. The results of the NFBG **(Fig. 4A)**, body weight **(Fig. 4B)** and IPGTT **(Fig. 5)** in the 30-day group also showed apparent improvements from the DM group. In addition, the MEIs in the 7-day group showed similar results to the 1-day group *in vitro* and *in vivo*. These results indicated that the immediate use of PVA MEIs after 1 day freezing is not mandatory, furthermore, 7 days is sufficient for islet accumulation for transplantation, islet shipping worldwide and an evaluation of islet quality before transplantation. These results lead us to conclude the use of PVA MEIs therefore appears to be an effective modality which can be used for clinical islet transplantation in the near future.

5. Conclusions

Long- term cryopreserved PVA MEIs showed similar effects to the original PVA MEIs (1-day group) both *in vitro* and *in vivo*. These results suggest that the PVA MEIs have advantages over other MEIs which may therefore make it possible to overcome the obstacles of insufficient donors and the side effects of immuno-suppressive drugs, because the encapsulation process with cryopreservation technique allows islet accumulation, as well as the shipping and quality control in the field of islet transplantation. Therefore, the use of PVA MEIs appears to be an effective modality for improving clinical DM therapy.

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Figure Captions:

Figure 1: Microscope images of PVA MEIs in the 1-day (left), 7-day (middle) and 30-day (right) groups after thawing.

Figure 2: Recovery rate of MEIs after thawing (* $p<0.05$; vs. 1-day group).

Figure 3: Insulin secretion test. (A) MEIs in all three groups showed good insulin secretion abilitiy in response to glucose concentration changes (* $p<0.05$). (B) No significant differences of SI were found between the three groups. (C) MEIs in 30-day group showed lower insulin release in comparison to those in the 1-day group (* $p<0.05$; vs. 1-day group).

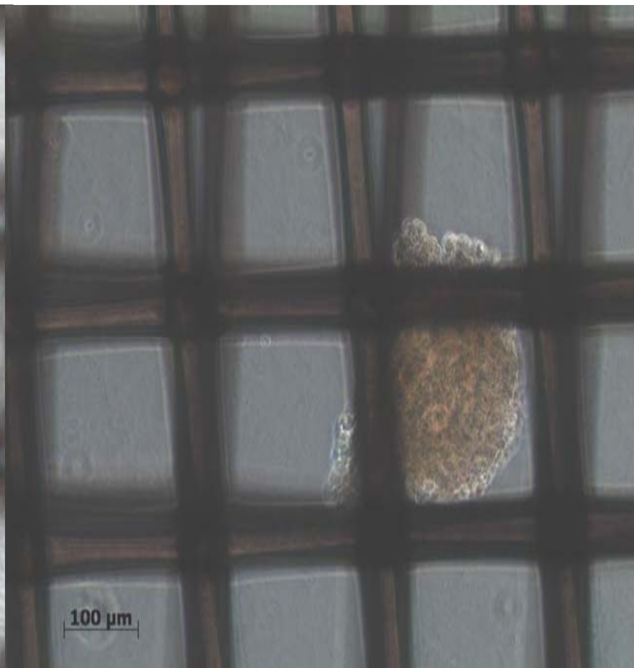
Figure 4: After MEIs transplantation, the NFBG (A) and body weight (B) changes of recipient mice (n=6).

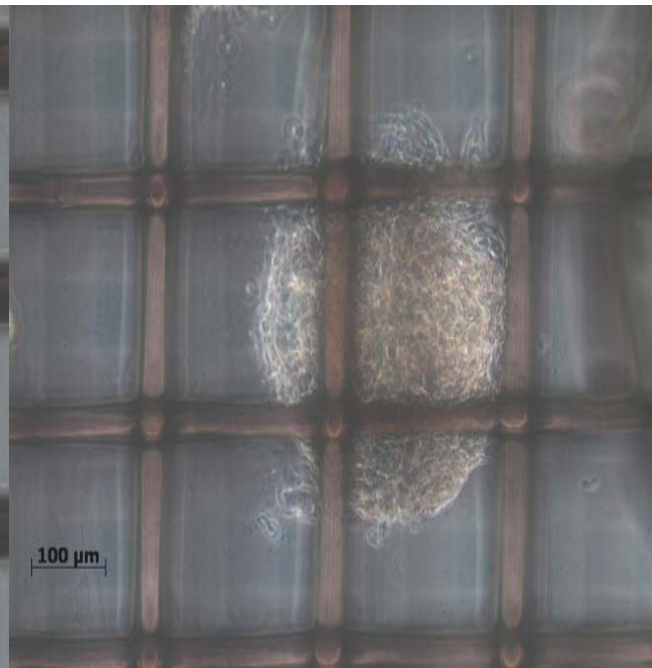
Figure 5: IPGTT was performed 2 weeks after MEIs transplantation (DM group: n=5; other groups: n=6). (A) Blood glucose changes of the recipient mice. (B) AUC (* $p<0.05$; vs. 1-day group).

Figure 6: Serum insulin (A) and C-peptide (B) levels. No significant differences were found among 1-, 7- and 30-day groups (n=3). In this evaluation experiment, at 4 weeks after transplantation, only one recipient mouse survived (n=1) in DM group, and because of the species cross reaction between rat and mouse in rat insulin and C-peptide, the normal mouse group was omitted.

Figure 7: Histological assessments 4 weeks after transplantation. (A) H&E staining of recipients' pancreas (arrow: islets). (B) Insulin staining of MEIs in 1-day, 7-day and 30-day groups (arrow: islets).

Fig 1





1-day **7-day** **30-day**

Fig 2

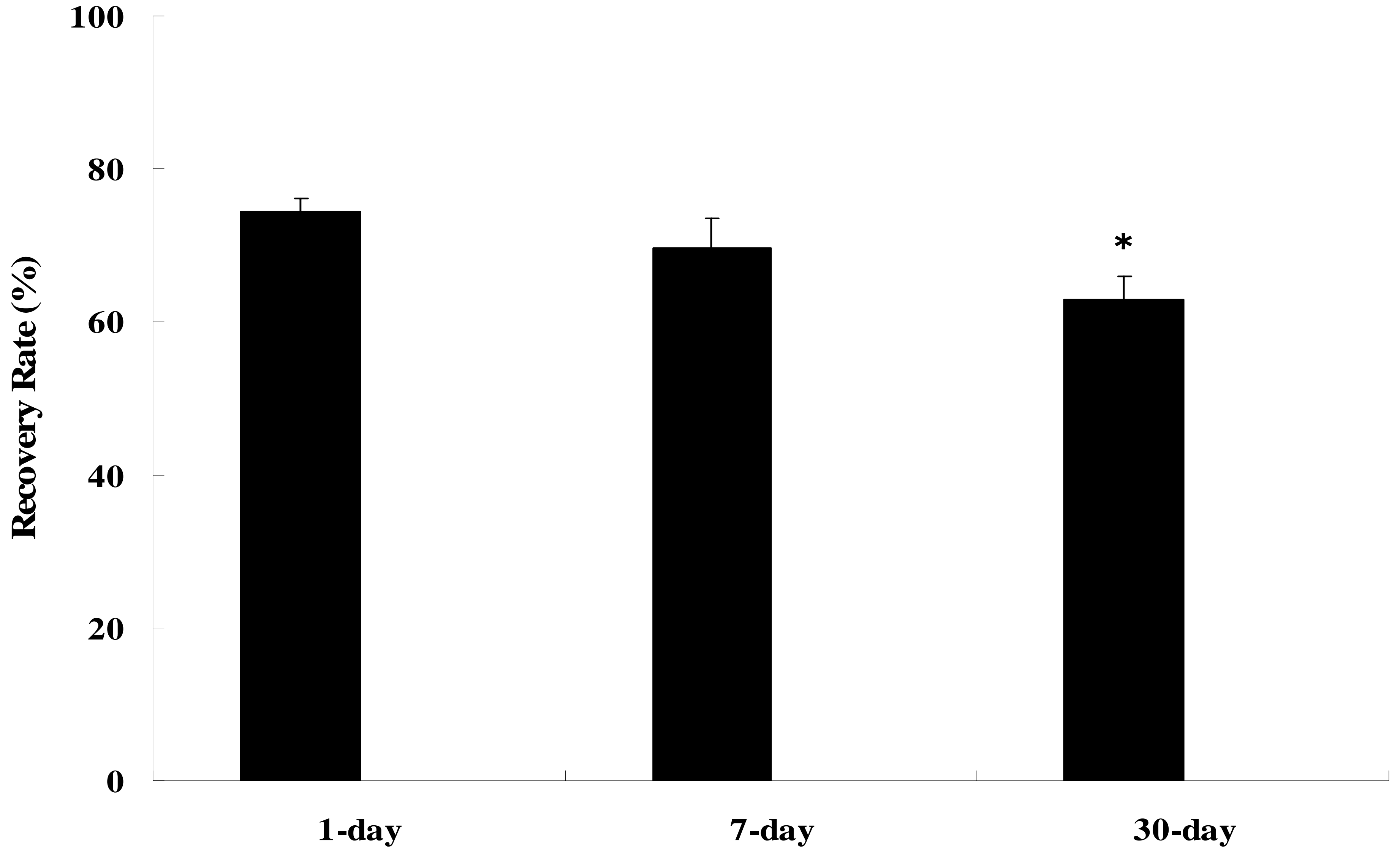


Fig 3A

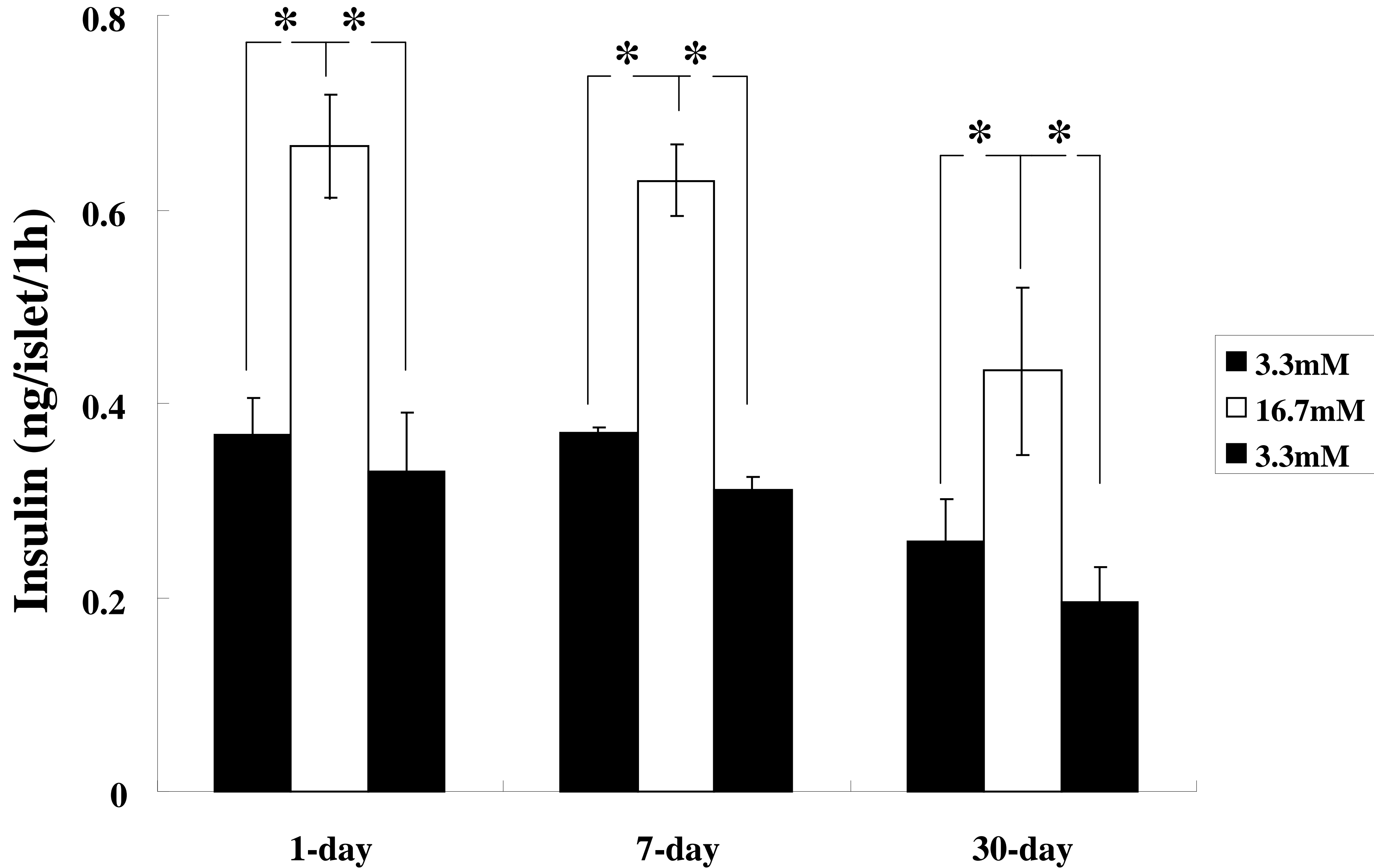


Fig 3B

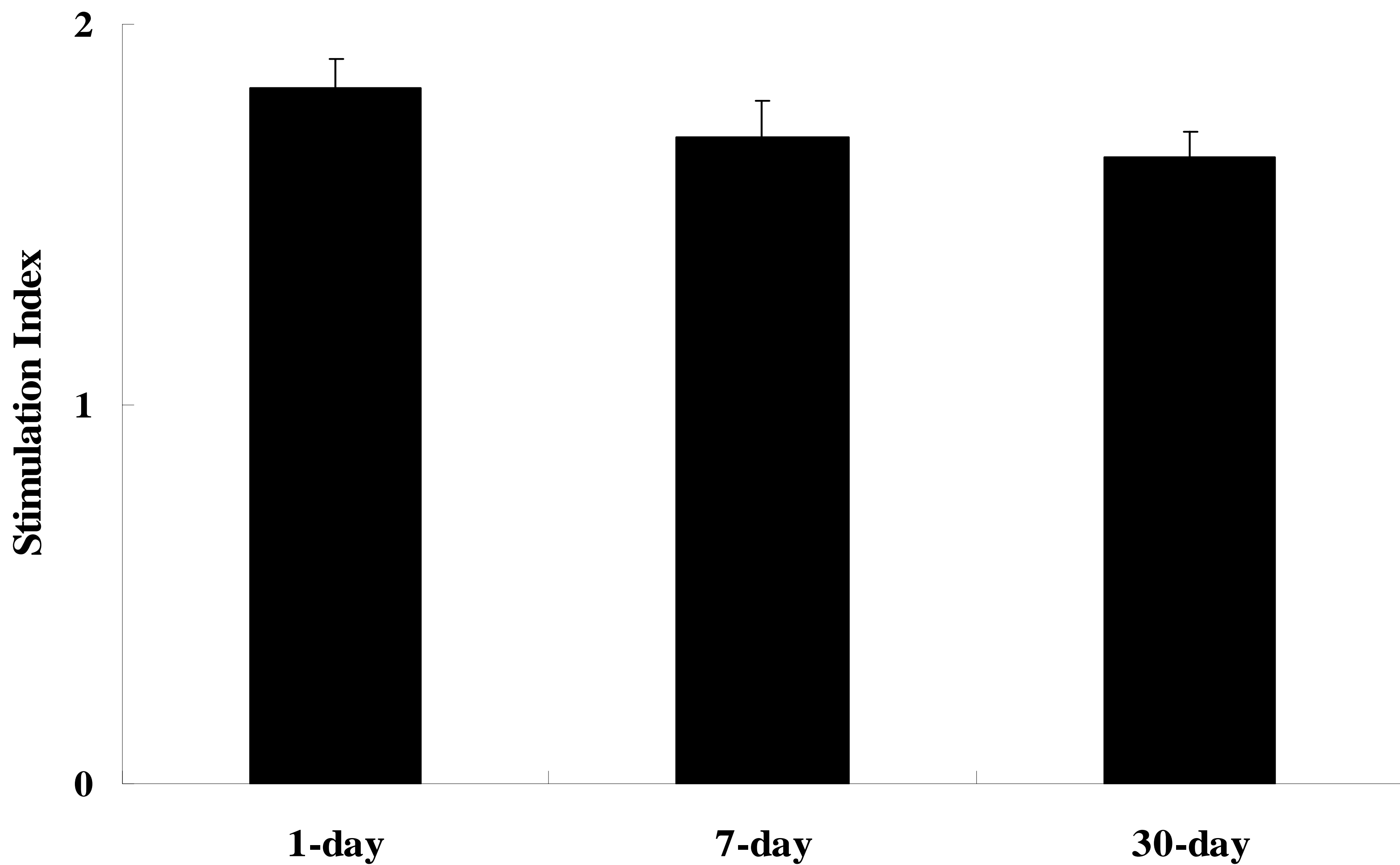


Fig 3C

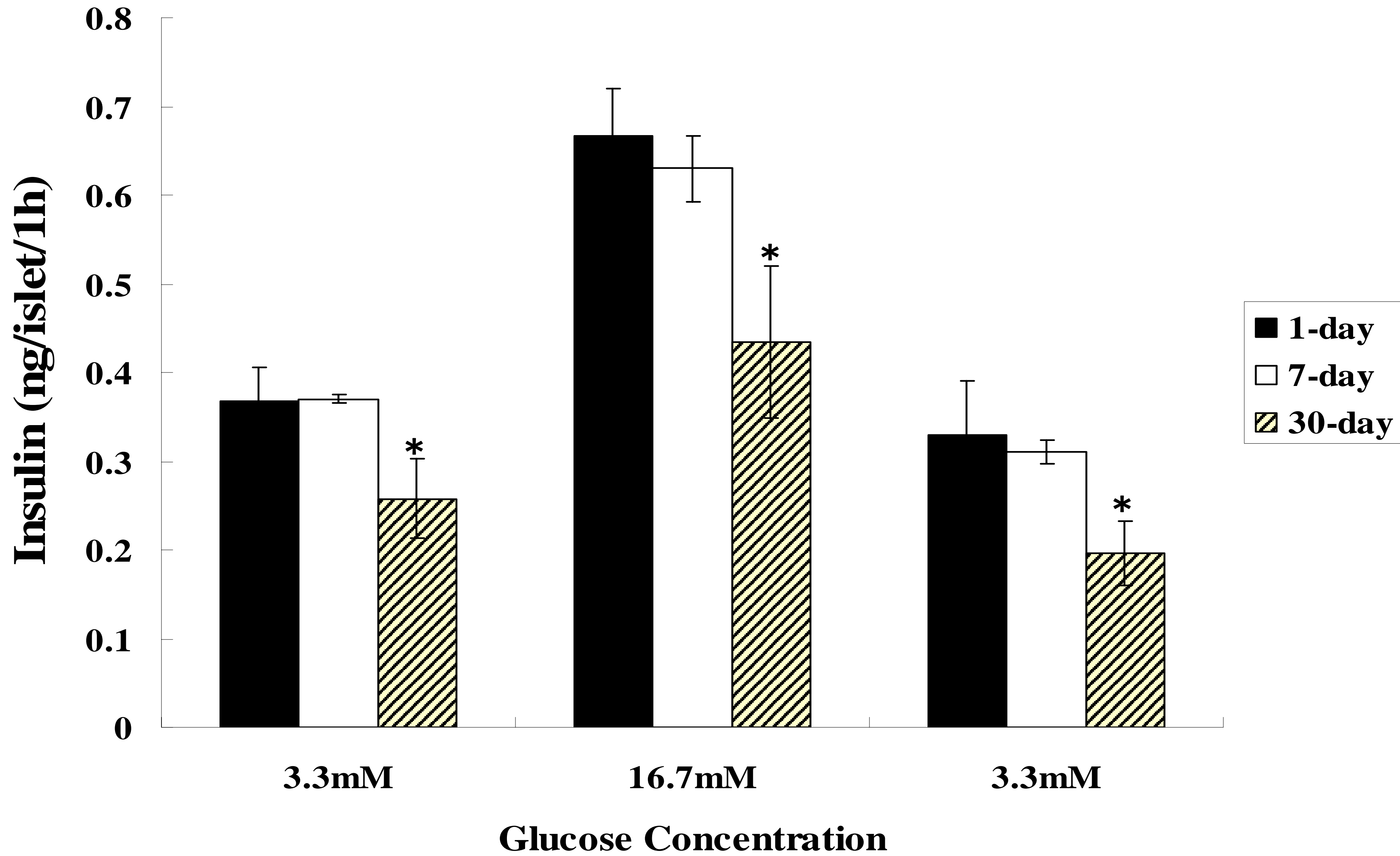


Fig 4A

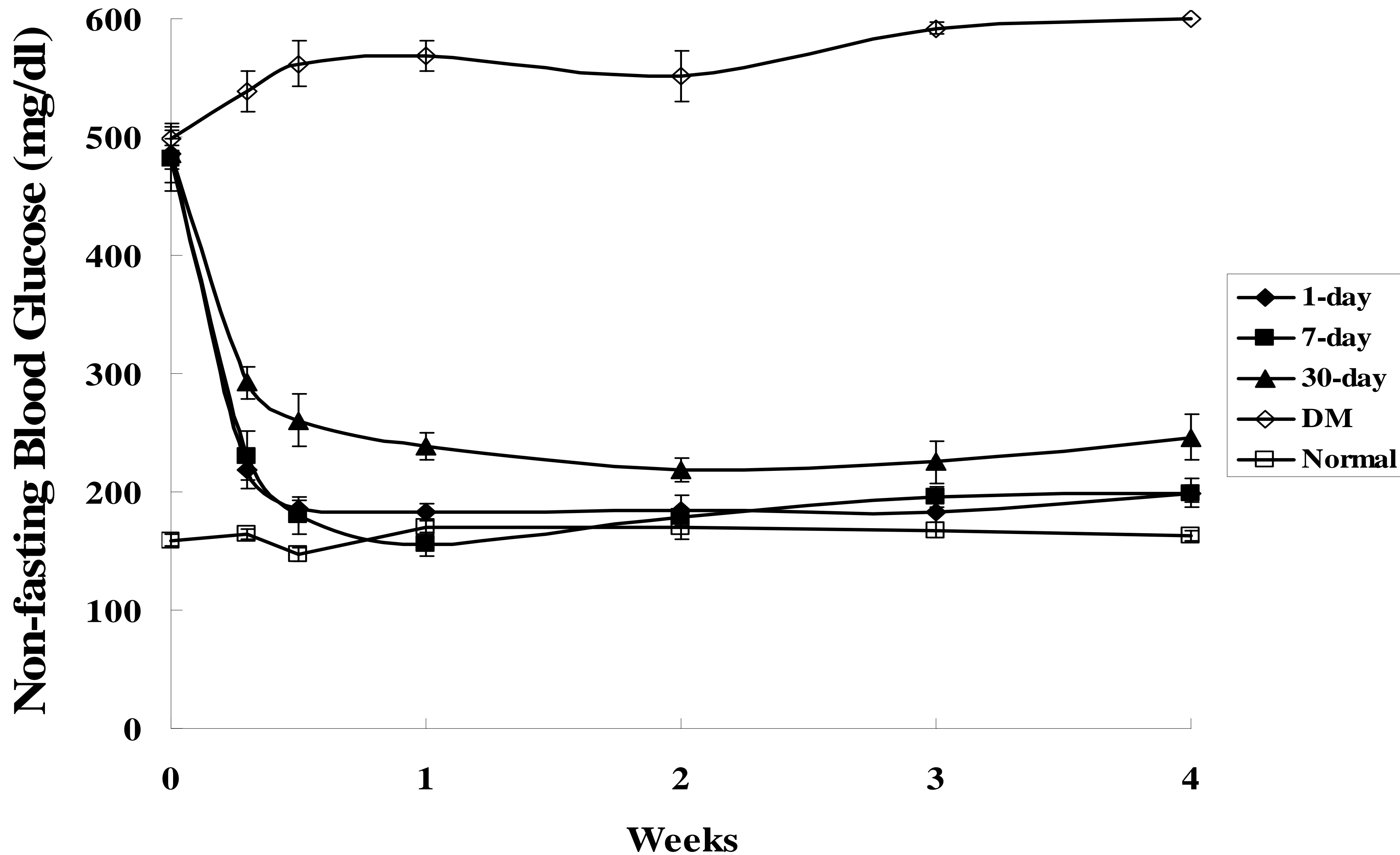


Fig 4B

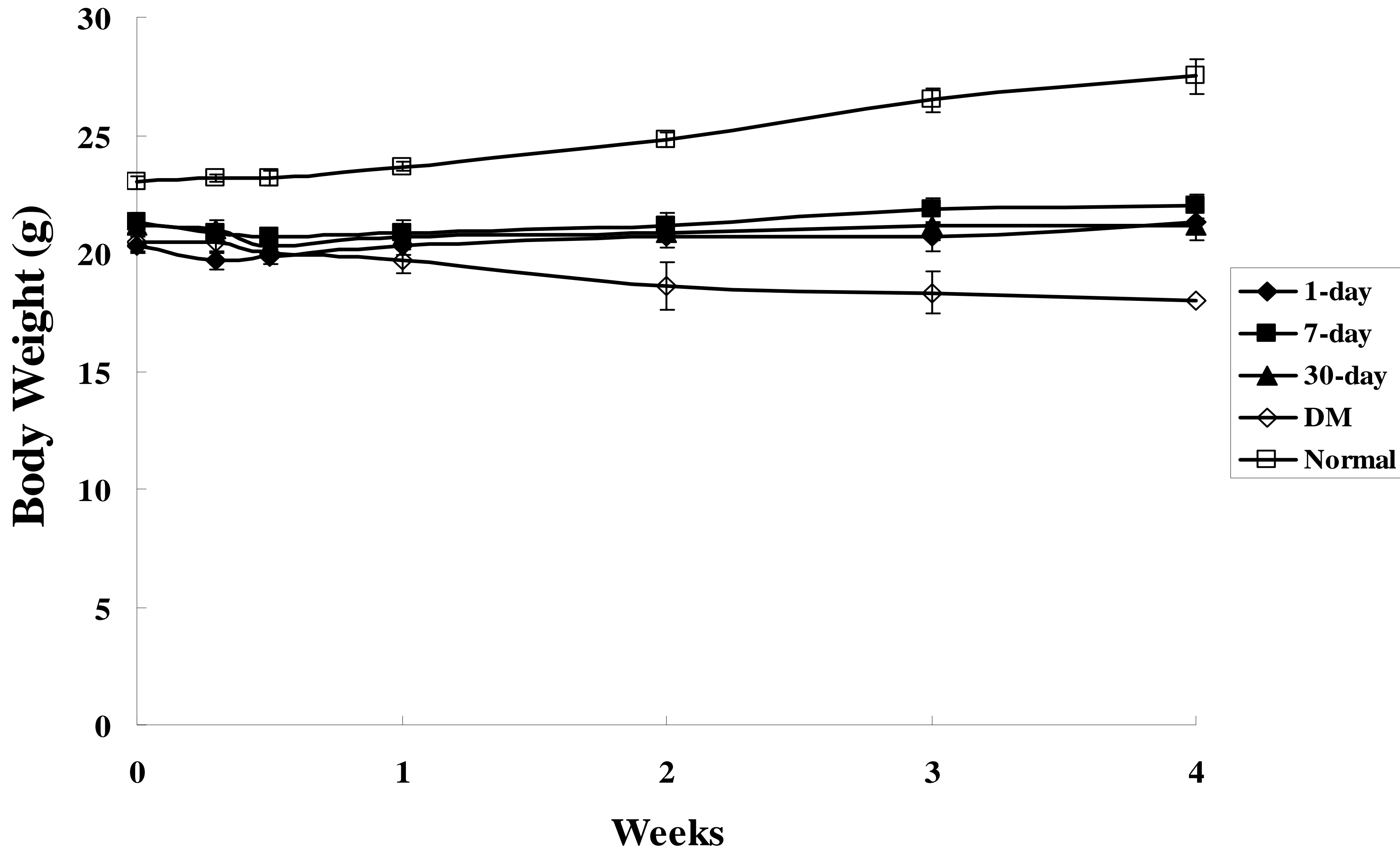


Fig 5A

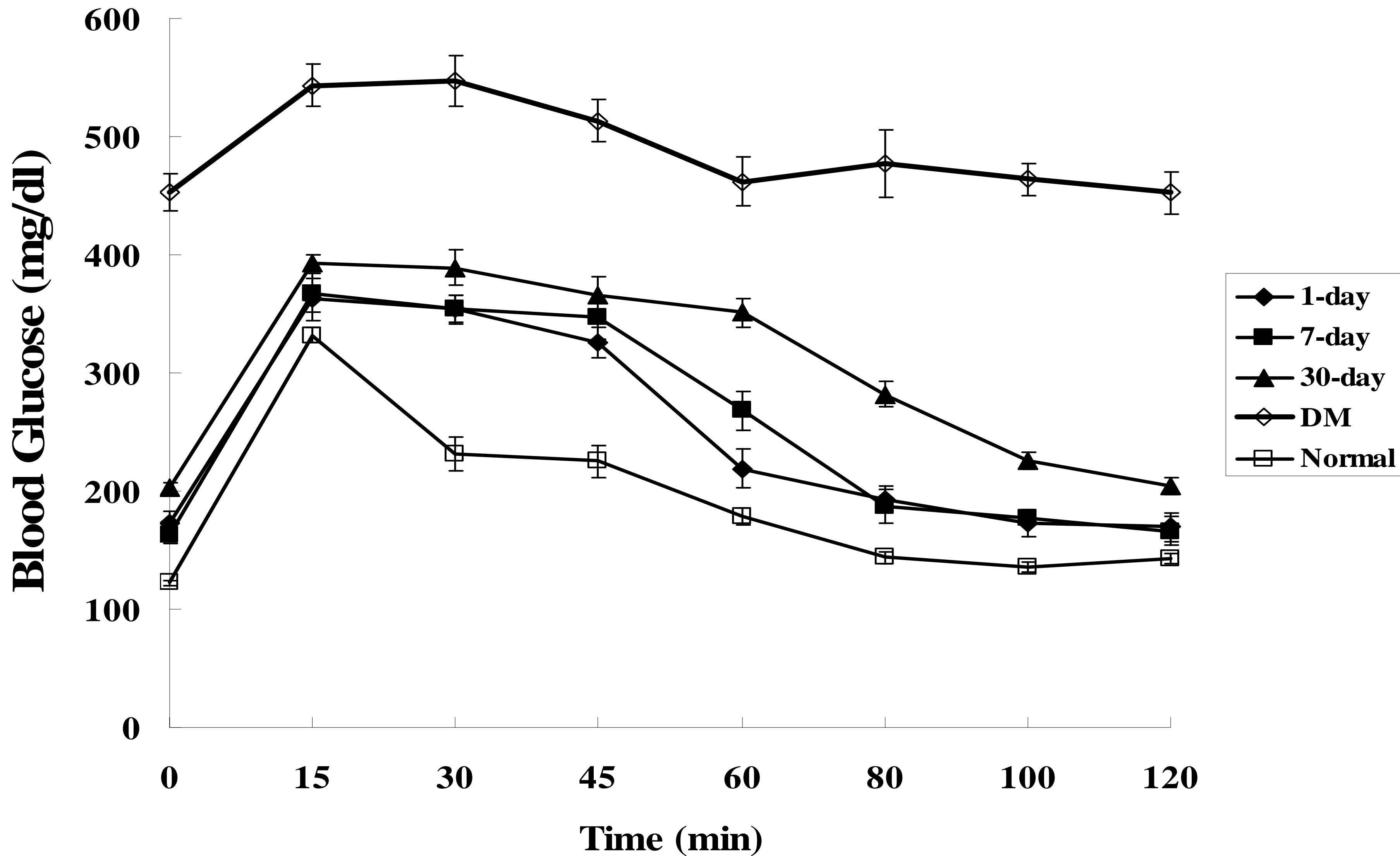


Fig 5B

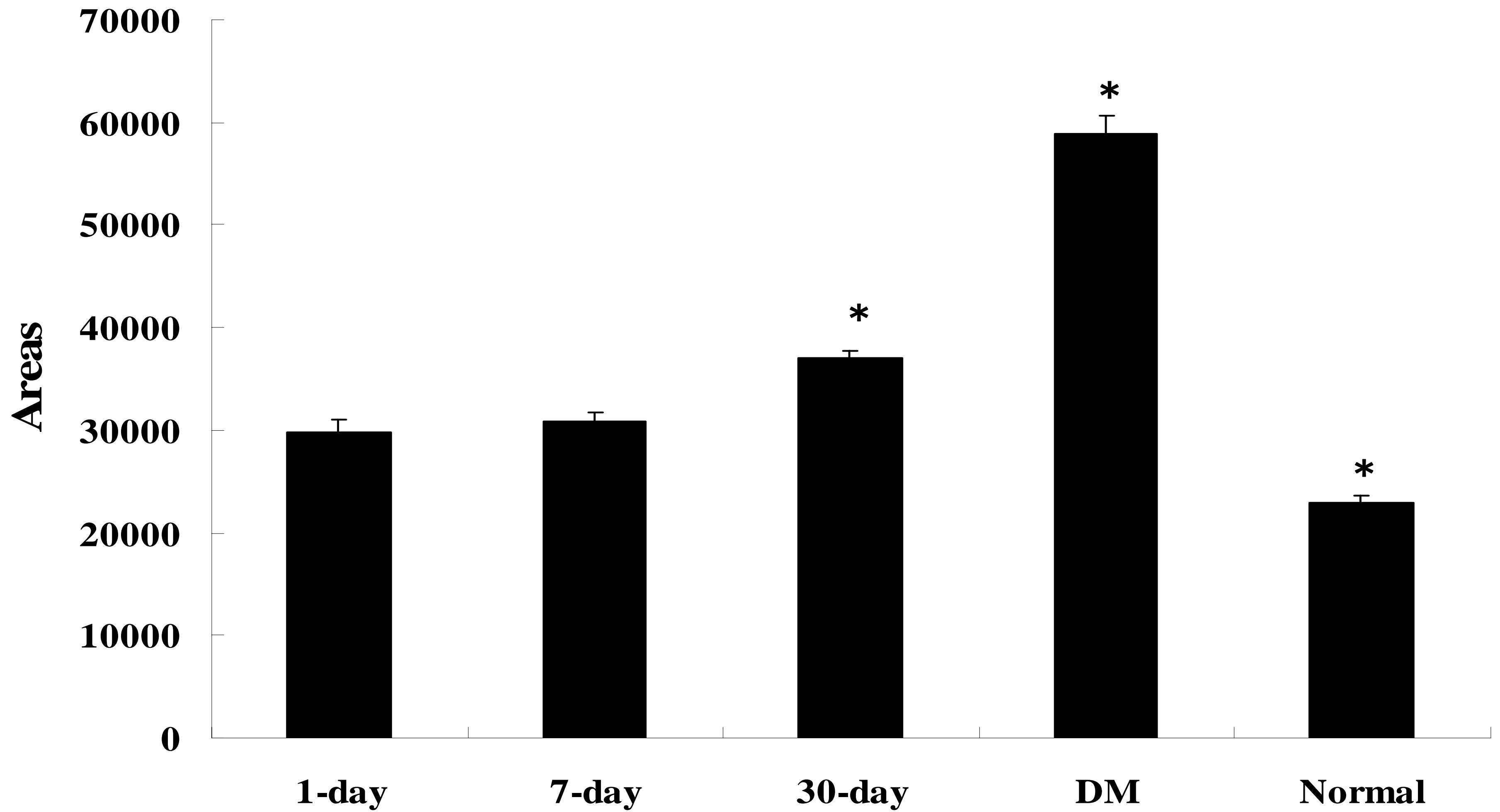


Fig 6A

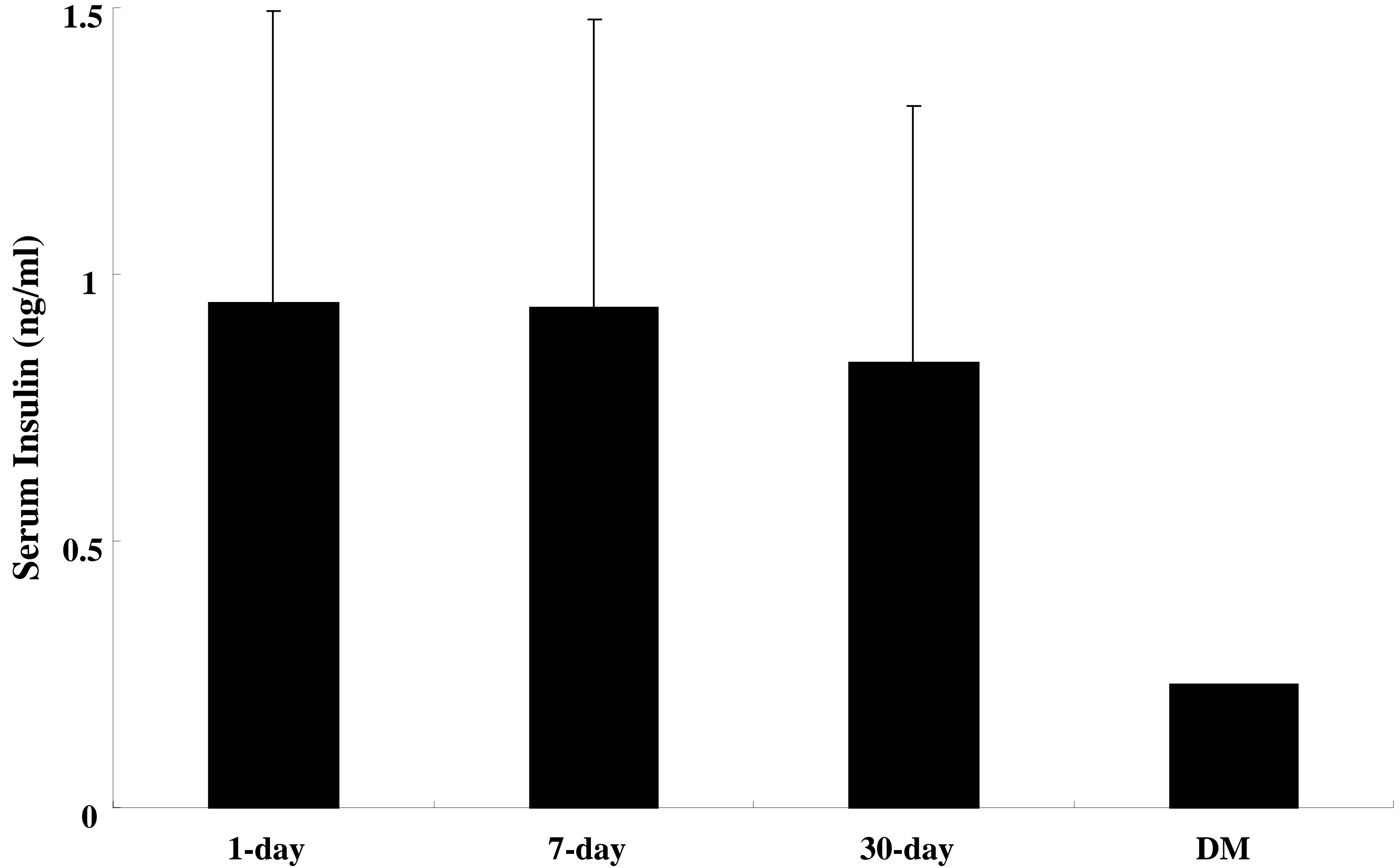


Fig 6B

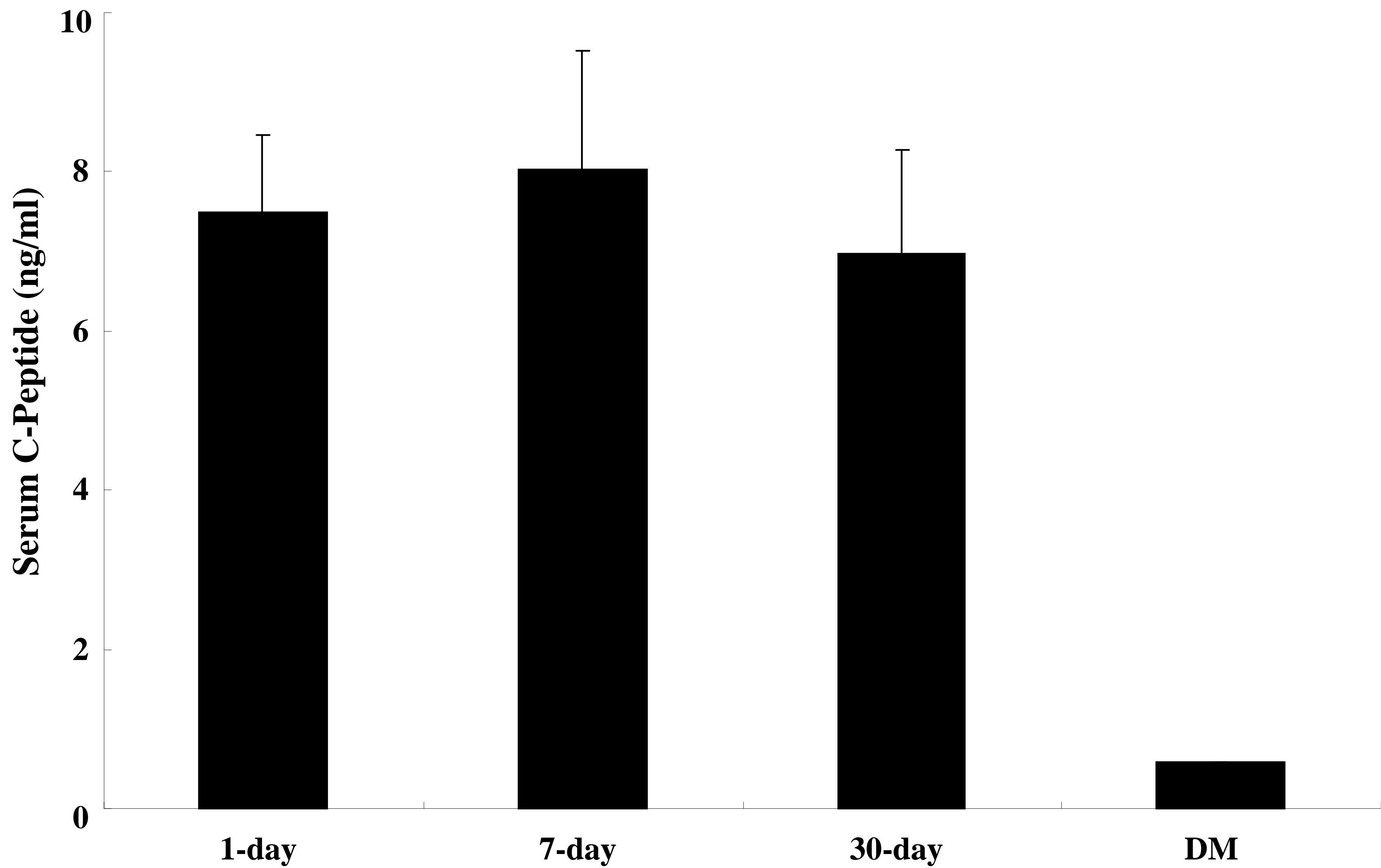
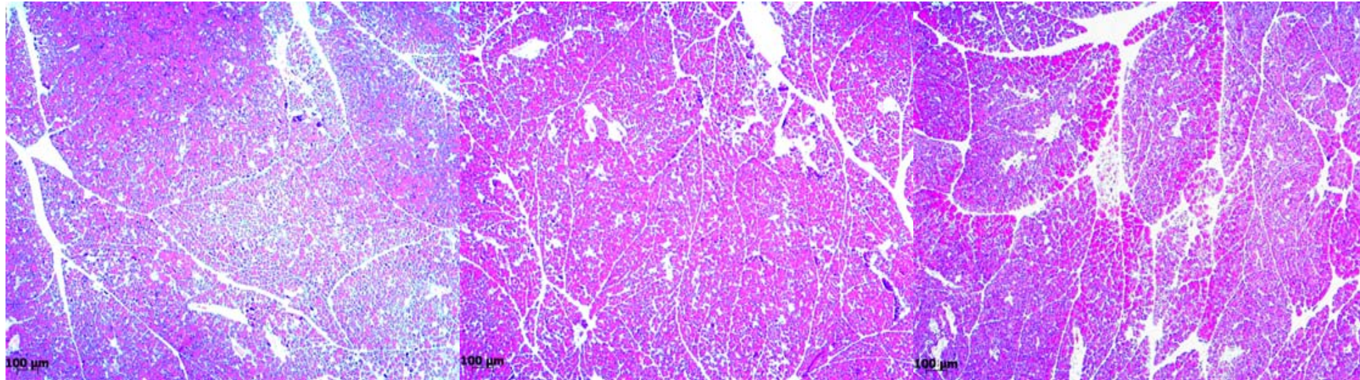
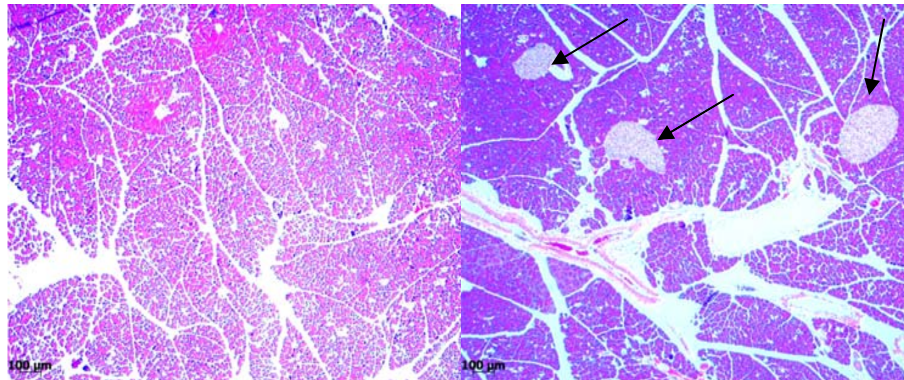


Fig 7A

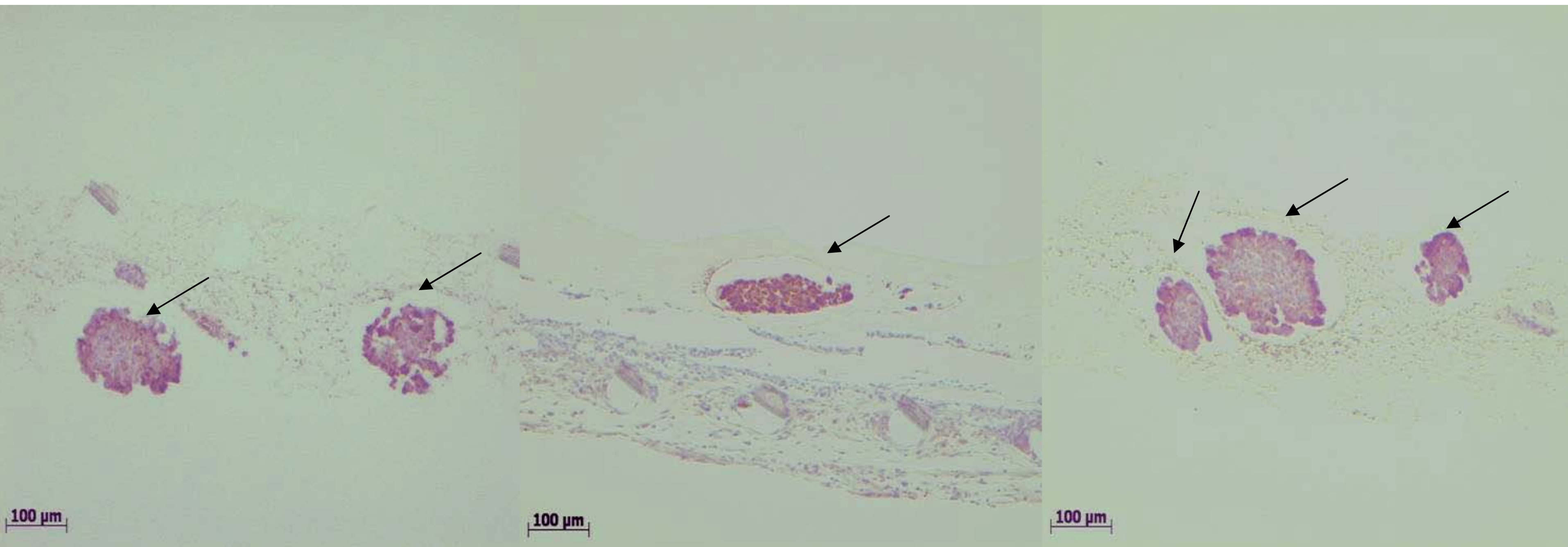


1-day **7-day** **30-day**



DM **Normal**

Fig 7B



1-day 7-day 30-day