

1 **Title**

2 **Freeze-dried platelets promote hepatocyte proliferation in**
3 **mice**

4 *Reiko Hoshi¹, Soichiro Murata¹, MD, PhD, Ryota Matsuo¹, MD, Andriy Myronovych¹*
5 *MD, Ikuka Hashimoto¹, Hiroshi Ikeda², PhD, Nobuhiro Ohkohchi¹, MD, PhD*

6

7 1. Department of Surgery, Advanced Biomedical Applications, Graduate School of Comprehensive
8 Human Sciences, University of Tsukuba

9 2. Resarch Facility Center for Science and Technology Cryogenics Division, University of Tsukuba

10

11 **Corresponding author**

12 Nobuhiro Ohkohchi, Department of Surgery, Advanced Biomedical Applications, Graduate School
13 of Comprehensive Human Sciences, University of Tsukuba, 1-1-1 Tennodai, Tsukuba, Ibaraki,
14 305-8575 Japan

15 Tel.: +81-29853-3221

16 Fax.: +81-29853-3222

17 E-mail: nokochi3@md.tsukuba.ac.jp

18

19

20

21

22

23

1 **ABSTRACT**

2 In recent years, platelets are reported to promote liver, as well as bone
3 regeneration and dermal wound healing. Platelets are required not only for
4 thrombocytopenia treating but also for regenerative therapy. Platelets cannot be stored
5 beyond three days, therefore, shortage of fresh platelets sometimes occurs. To preserve
6 platelets for a long duration without degrading growth factors, a freeze-dried technique
7 is required. We report here that platelets can be preserved by freeze-drying, using a
8 programmed freezing method to avoid intracellular ice crystal formation. Freeze-dried
9 platelets kept their morphological countenance and response with the agonist of
10 thrombin was well maintained. Freeze-dried platelets stored adenine nucleotides, PDGF,
11 and IGF-1 the same as those of fresh platelets. Freeze dried platelets also preserved their
12 proliferative effect on hepatocytes identical to that of fresh platelets. These results of
13 our study suggest that freeze dried platelets will obviate the storage problem of fresh
14 platelets.

15

16 Words: 144

17 Keywords: freeze-dried, platelet, IGF-1, PDGF, hepatocyte, intracellular ice formation,
18 heat of crystallization, adenine nucleotides

19

20

21

22

23

24

1 **INTRODUCUCTION**

2 Blood platelets are generally stored in blood banks for 3-5 days, afterwards they
3 are discarded. This results in the problem of a chronic shortage of fresh platelets. To
4 preserve platelets for a long period without degrading growth factors, a freeze dried
5 technique is required, and several researchers have reported on the methods to make
6 freeze-dried platelets [1-6]. Bode et al. reported that platelets freeze-dried after
7 stabilization with paraformaldehyde preserved their morphology, adhesive property and
8 procoagulant property [4]. Wolkers and Crowe reported that human platelets loaded
9 with trehalose survive freeze drying [5].

10 A number of different growth factors are known to be included in the alpha
11 granules of platelets; such as platelet derived growth factor (PDGF), hepatocyte growth
12 factor (HGF), epidermal growth factor and insulin like growth factor 1 (IGF-1) [7, 8].
13 No previous studies have mentioned growth factor content in the freeze dried platelets.
14 Many studies, both in vitro and in vivo, have disclosed the effectiveness of several
15 growth factors that can enhance hepatocyte proliferation in the regenerating liver [9-12].
16 We and other researchers revealed that platelets stimulate or accelerate hepatocyte
17 proliferation [13-15]. Platelets also contribute to bone regeneration [16] and dermal
18 wound healing [17].

19 The purpose of this study is to preserve platelet shapes and to maintain contents
20 of growth factors inside platelets when making freeze dried platelets (FDPs). And we
21 also reveal that FDPs stimulate hepatocyte proliferation.

1 MATERIALS AND METHODS

2 *Animals*

3 Eight-week-old male BALB/c mice (Clea, Japan), weighing 24 to 28g, were used
4 for experiments. The animals were kept in a temperature-controlled room on a 12 hour
5 light-dark cycle. They had free access to water and standard chow throughout the
6 experiment. After an acclimation period of at least 7 days, the mice were used for
7 collecting platelets. All experiments complied with the Guidelines for Care and Use of
8 Laboratory Animals of the University of Tsukuba.

9 *Platelet collection*

10 Under ether anesthesia, 1 ml of blood was taken from the heart with a 1 ml
11 syringe adding 0.1 ml heparin. After centrifugation at 200 g for 10 minutes, platelet rich
12 plasma was obtained. This platelet rich plasma was then centrifuged again at 1000 g for
13 15 minutes, and the pellet was suspended with citrate buffer which consisted of 120 mM
14 NaCl, 4.26 mM NaH₂PO₄, 5.5 mM sodium citrate, 2.35 mM citrate acid. After washing,
15 the platelets were centrifuged at 1000 g for 15 minutes at 4°C.

16 *Freezing and Freeze-Drying Procedure*

17 Washed platelets were suspended with lyophilization buffer which consisted of
18 120 mM NaCl, 4.26 mM NaH₂PO₄, 5.5 mM glucose, 4.77 mM sodium citrate, 2.35
19 mM citric acid and 1% bovine serum albumin. Typically, 0.5 ml platelet suspensions
20 were transferred in 2 ml cryogenic vials and frozen in Ice Cube 1810 (SY-LAB VmbH,
21 Purkersdorf, Austria). Vials were frozen from 20 to -50°C at a freezing rate of -1°C/min.
22 When vials reached -9°C, they were suddenly frozen to -50°C at a freezing rate of
23 -60°C/min, kept at -50°C for three minutes, warmed to -20°C at a freezing rate of
24 15°C/min, and finally returned to a freezing rate of -1°C/min by a programmed freezing

1 method for prevention of making heat of crystallization. The frozen solutions were
2 transferred to a -80°C freezer for 12 hours. Subsequently, the frozen platelet
3 suspensions were transferred in vacuum flasks that were attached to a Mechanical pump
4 1500 L/min (Japan Vacuum Equipment Co. Ltd, Tokyo, Japan) that controls shelf
5 temperature and vacuum pressure. The flasks were kept in liquid nitrogen to prevent any
6 melting and directly used for experiments. The water content of the sample was
7 calculated by weighing the sample before and after freeze-drying. The water content
8 after freeze-drying was 3.8%.

9 ***Rehydration of FDPs***

10 FDPs were washed three times with phosphate buffered saline before
11 prehydration. FDPs were prehydrated in a closed box with moisture-saturated air at
12 37°C for 2 hours prior to rehydration. Vials that originally contained 0.5 ml platelet
13 suspension were rehydrated to platelet free murine plasma.

14 ***Transmission Electron Microscopy***

15 FDPs were rehydrated with platelet free plasma and the specimens were fixed in
16 2% glutaraldehyde in 0.1 M phosphate buffer, pH7.4, and post-fixed in 1% O₃O₄ in 0.1
17 M phosphate buffer. The specimens were dehydrated through a graded series of ethanol,
18 passed through propylene oxide and embedded in EPON 812 purchased by Polysciences,
19 Inc. (Washington, PA). Ultra-thin sections mounted on copper grids were stained with
20 uranyl acetate and lead citrate and observed in a Hitachi H-7000 transmission electron
21 microscope.

22 ***Fibrinogen binding assay***

23 After rehydration, FDPs were labeled with monoclonal antibody Alexa Fluor 488
24 (Invitrogen Co, Carlsbad, CA). Excess of the antibody was removed by repeated

1 washing in PBS. Platelets were stimulated with thrombin (10U/ml) for 30 minutes.
 2 Flow cytometry analysis of the fibrinogen binded platelets was performed on a flow
 3 cytometer (FACScan, Becton Dickinson, Mountain View, CA). Particles were identified
 4 by their size and complexity using the FSC and SSC gates. Histograms of fluorescence
 5 intensity were used in comparison.

6 ***Measurement of ATP, ADP, and AMP***

7 FDPs were homogenized with 500 µl of 0.5N HClO₄ using a homogenizer at 4°C.
 8 Samples were maintained in an ice bath during homogenization. They were then
 9 centrifuged at 3000 g for 10 minutes at 4°C. The clear supernatant solution was
 10 neutralized with 5N KOH. The supernatant was centrifuged at 3000 g for 10 minutes at
 11 4°C. After filtration of the supernatant with 0.45 µl filters, FDPs were centrifuged at
 12 3000 g for 30 minutes at 4°C. ATP, ADP, and AMP of the final supernatant were then
 13 measured by high pressure liquid chromatography UV-970 (JASCO, Tokyo, Japan).
 14 Energy charge (Ec) was one of the index of the energy status of cells and was calculated
 15 by the following formula [18].

16
$$Ec = \frac{[ATP] + \frac{1}{2}[ADP]}{[ATP] + [ADP] + [AMP]}$$

17 ***Enzyme-Linked Immunosorbent Assay for HGF, PDGF, and IGF-1***

18 In the present study, to quantify the amount of PDGF and IGF-1, commercially
 19 available Quantikine ELISA kits (R&D Systems, Minneapolis, MN) and the Rat
 20 specific HGF ELISA kit (Institute of Immunology, Tokyo, Japan), which cross reacts
 21 with mouse HGF, were used. In each growth factor 4 to 6 samples were assayed.

22 ***Primary hepatocytes***

23 Eight-week-old male BALB/c mouse hepatocytes were obtained by a collagenase

1 perfusion method [19]. Hepatocytes were cultured in two groups: platelet (-) group:
2 hepatocytes cultured without platelets; and FDP group; hepatocytes cultured with FDPs.

3 ***BrdU assay***

4 Hepatocyte proliferation was evaluated using the BrdU assay kit (Roche
5 Diagnostics GmbH, Pezberg, Germany) according to the manufacture's instructions.
6 Briefly, primary hepatocytes were seeded in culture plates. Then, FDPs suspended in
7 serum-free William's E medium at various concentrations were added to each well.
8 After incubation at 37°C for 48 hours BrdU uptake was measured.

9 ***[³H]-methyl thymidine uptake assay***

10 Primary cultured hepatocytes were radiolabeled with [³H]-methyl-thymidine
11 (Amersham Bioscience, Buckinghamshire, UK). Briefly, hepatocytes were placed in
12 6-well culture plates with FDPs and grown for 24 hours. Then [³H]-methyl-thymidine
13 was added. After 48 hours of incubation, the cells were harvested and
14 [³H]-methyl-thymidine was measured using a liquid scintillation counter (LS-6500,
15 Beckman Coulter, Fullerton, CA).

16 ***Statistical Analysis***

17 All data were expressed as the mean ± standard deviation. Statistical analyses
18 were carried out with Mann-Whitney U-test and one-way ANOVA followed by
19 Bonferroni-Dunn multiple comparisons post hoc test. A *P* value <0.05 was considered
20 significant.

21

22 **RESULTS**

23 ***FDPs procedure with programmed freezing***

24 At a temperature of -9°C, ice crystal formation was induced in platelets, and as a

1 result, it released energy known as the heat of crystallization (Figure 1A). To avoid this
2 heat of crystallization, we used programmed freezing for making FDPs. Using
3 programmed freezing, the temperature around the platelets was decreased suddenly to
4 -50°C , without releasing heat of crystallization (Figure 1B). Figure 2 shows the sample
5 temperature and freeze-dryer pressure profiles after freeze-drying. The precise water
6 volume of FDPs was 3.8%.

7 ***Transmission electron microscopical findings***

8 Transmission electron microscopy showed that FDPs were swollen but cellular
9 membrane and internal granules were preserved (Figure 3A). Figure 3B shows intact
10 fresh platelet. FDPs without using the programmed freezing method are shown in
11 Figure 3C. These FDPs made heat of crystallization as shown in Figure 1A.

12 ***Coagulation ability of FDPs***

13 The coagulation response of FDPs by 10 U/ml of thrombin was almost identical
14 to that of control platelets measured by fibrinogen binding assay (Figure 4A, 4B).
15 Figure 4C shows the ratio of thrombin coagulation/natural coagulation. There was no
16 significant difference between fresh platelets and FDPs. Coagulation of FDPs and fresh
17 platelets were suppressed by citrate addition (data not shown).

18 ***ATP, ADP, and AMP in FDPs***

19 The intracellular energy of FDPs and fresh platelets is shown in Table 1. There
20 was no significant difference in ADP and AMP between FDPs and fresh platelets. ATP
21 of FDPs was slightly decreased but still contained 75% of fresh platelets. Energy charge
22 is one of the indexes of the energy status of cells. There was no significant difference of
23 energy charge between fresh platelets and FDPs.

24 ***Growth factors in FDPs***

1 The intracellular growth factors of FDPs and fresh platelets are shown in Table 2.
2 IGF-1 was decreased in FDPs compared to control with significant difference, but 62%
3 of IGF-1 was still present in FDPs. PDGF was significantly increased in FDPs
4 compared to fresh platelets. HGF was not detected in either control or FDPs in 10^8 of
5 platelets.

6 *Proliferation effect of primary cultured hepatocytes*

7 We examined whether exogenous FDPs might stimulate the DNA maturation of
8 the primary cultured hepatocytes. Effectively, FDPs induced a stimulatory effect on the
9 DNA maturation of the primary cultured hepatocytes cultured in serum free conditions
10 examined by BrdU assay and [^3H]-thymidine uptake assay. This effect was recognized
11 in proportion to the dose of FDPs (Figure 5A and Figure 5B).

12

13 **DISCUSSION**

14 In this study we established a new method of making FDPs maintaining the
15 countenance and viability. We used a new program to freeze the chamber suddenly to
16 -50°C for 3 minutes then freezing at a slow rate at about -20°C to avoid making heat of
17 crystallization. This program maintained the structure of FDPs better than normal
18 cooling method. In our study, fibrinogen binding assay revealed that FDPs could react
19 to the agonist thrombin like fresh platelets. In our FDPs, adenine nucleotides, energy
20 charge, and growth factors; i.e. PDGF, and IGF-1; were almost identical to those of
21 fresh platelets. Further, we indicated that FDPs promoted proliferation of hepatocytes
22 when seeded together.

23 Human blood platelets are stored in blood banks at 22°C for only 3 days. After
24 this safety period, risk of bacterial infection with platelet transfusion increases. Blood

1 transfusion centers have the task of producing platelet concentrates for transfusion
2 under strict conditions. Freeze-drying method as in this study with proper storage
3 conditions would enable to store platelet contents, i.e., growth factors, ATPs and
4 coagulation ability. Further study is needed to investigate whether the function of our
5 FDPs is identical to fresh platelets, including CD62p expression, extent of shape change,
6 hypotonic shock response, morphology, in vivo circulation, etc.

7 No previous studies have mentioned adenine nucleotides and growth factor
8 content in the freeze dried platelets. In this study, we assayed ATP, ADP, AMP, energy
9 charge, IGF-1, PDGF, and HGF to check the viability of FDPs. Values of ADP, AMP
10 and energy charge of FDPs were almost identical to fresh platelets. ADP is released
11 from platelet-dense granules and contributes to a positive feedback mechanism of
12 ADP-induced platelet aggregation by acting through ADP receptors on the platelet
13 surface [20]. For investigation of the function of FDPs we examined aggregation
14 activity by fibrinogen binding assay. Because our FDPs might contain sufficient
15 amounts of ADP in their dense granules, aggregation activity was well preserved.

16 The levels of ATP and IGF-1 in FDPs were lower than those of fresh platelets.
17 On the other hand, PDGF in FDPs is higher than control. In our previous study,
18 intra-platelet IGF-1 was important in the proliferation of hepatocytes [15]. ATP is also
19 reported to promote hepatocyte proliferation [21]. In the preliminary study the
20 hepatocyte DNA maturation activity measured by [³H]-methyl thymidine uptake
21 revealed that FDPs are less effective than fresh platelets (data not shown). One of the
22 reasons is that the contents of IGF-1 and ATP were less than those of fresh platelets.
23 Therefore, establishing a well-developed method is required for the maintenance of
24 IGF-1 and ATP. For bone regeneration therapy in the dental field, PDGF is the most

1 important growth factor [22-26]. Actually, dentists use platelet rich plasma and bone
2 mixture for implanting in mandibular bone for quick bone regeneration [27]. Our FDPs
3 would be applicable for substitution of PRP in implantation treatment.

4 In conclusion, we reported a novel method of making freeze-dried platelets by
5 using a programmed freezing method of preventing heat of crystallization. The FDPs in
6 our study contained ATPs and growth factors and promoted hepatocyte proliferation.
7 Freeze-dried platelet would be an effective novel agent not only for thrombocytopenia
8 but also for bone and liver regeneration. Further study in animal models will be
9 necessary for application of FDPs to liver disease in clinical use.

1 **REFERENCES**

- 2 [1] W.F. Wolkers, N.J. Walker, F. Tablin, J.H. Crowe, Human platelets loaded with
3 trehalose survive freeze-drying, *Cryobiology* 42 (2001) 79-87
- 4 [2] J.H. Crowe, F. Tablin, W.F. Wolkers, K. Gousset, N.M. Tsvetkova, J. Ricker,
5 Stabilization of membranes in human platelets freeze-dried with trehalose, *Chem.*
6 *Phys. Lipids* 122 (2003) 41-52
- 7 [3] E.P. Landi, E.G. Roveri, M.C. Ozelo, J.M. Annichino-Bizzacchi, A.F. Origa, A R.
8 de Carvalho Reis, C.A. de Souza, J.F. jr. Marques, Effects of high platelet
9 concentration in collecting and freezing dry platelets concentrates, *Transfus. Apher.*
10 *Sci.* 30 (2004) 205-212
- 11 [4] A.P. Bode, M.S. Read, Lyophilized platelets: continued development, *Transfuse Sci.*
12 22 (2000) 99-105
- 13 [5] J.H. Crowe, F. Tablin, W.F. Wolkers, K. Gousset, N.M. Tsvetkova, J. Ricker,
14 Stabilization of membranes in human platelets freeze-dried with trehalose, *Chem.*
15 *Phys. Lipids* 122 (2003) 41-52
- 16 [6] M.S. Read, R.L. Reddick, A.P. Bode, D.A. Bellinger, T.C. Nichols, K. Taylor, S.V.
17 Smith, D.K. McMahon, T.R. Griggs, K.M. Brinkhous, Preservation of hemostatic
18 and structural properties of rehydrated lyophilized platelets: potential for long-term
19 storage of dried platelets for transfusion, *Proc. Natl. Acad. Sci. U.S.A.* 92 (1995)
20 397-401
- 21 [7] T. Nakamura, H. Teramoto, A. Ichihara, Purification and characterization of a
22 growth factor from rat platelets for mature parenchymal hepatocytes in primary
23 cultures, *Proc. Natl. Acad. Sci. U.S.A.* 83(1986) 6489-6493
- 24 [8] Y. Oka, D.N. Orth, Human plasma epidermal growth factor/ β -urogastrone is

- 1 associated with blood platelets, *J. Clin. Invest.* 72 (1983) 249-259
- 2 [9] G.K. Michalopoulos, M.C. DeFrances, Liver regeneration, *Science* 276 (1997)
- 3 60-66
- 4 [10] M. Borowiak, A. N. Garratt, T. Wüstefeld, M. Strehle, C. Trautwein, C. Birchmeier,
- 5 Met provides essential signals for liver regeneration, *Proc. Natl. Acad. Sci. U.S.A.*
- 6 101 (2004) 10608-10613
- 7 [11] J. Okano, G. Shiota, H. Kawasaki, Protective action of hepatocyte growth factor for
- 8 acute liver injury caused by D-galactosamine in transgenic mice, *Hepatology* 26
- 9 (1997) 1241-1249
- 10 [12] N. Fausto, A.D. Laird, E.M. Webber, Role of growth factors and cytokines in
- 11 hepatic regeneration, *FASEB J.* 9 (1995) 1527-1536
- 12 [13] M. Lesurtel, R. Graf, B. Aleil, D.J. Walther, Y. Tian, W. Jochum, C. Gachet, M.
- 13 Bader, P.A. Clavien, Platelet-derived serotonin mediates liver regeneration, *Science*
- 14 312 (2006) 104-107
- 15 [14] S. Murata, N. Ohkohchi, R. Matsuo, O. Ikeda, A. Myronovych, R. Hoshi, Platelets
- 16 promote liver regeneration in early period after hepatectomy in mice, *World J. Surg.*
- 17 31 (2007) 808-816
- 18 [15] R. Matsuo, N. Ohkohchi, S. Murata, O. Ikeda, Y. Nakano, M. Watanabe, K.
- 19 Hisakura, A. Myronovych, T. Kubota, H. Narimatsu, M. Ozaki. Platelets strongly
- 20 induce hepatocyte proliferation with IGF-1 and HGF in vitro, *J. Surg. Res.* (2007)
- 21 in press
- 22 [16] R. Gruber, F. Varga, M.B. Fischer, G. Platelets stimulate proliferation of bone cells:
- 23 involvement of platelet-derived growth factor, microparticles and membranes. *Clin*
- 24 *Oral Implants Res.* 13 (2002) 529-535

- 1 [17] C.A. Carter, D.G. Jolly, Sr. C.E. Worden, D.G. Hendren, C.J. Kane. Platelet-rich
2 plasma gel promote differentiation and regeneration during equine wound healing.
3 *Exp. Mol. Pathol.* 74 (2003) 244-255
- 4 [18] D. E. Atkinson, G. M. Walton, Adenosine triphosphate conservation in metabolic
5 regulation. *J. Biol. Chem.* 242 (1967) 3239-3241
- 6 [19] Y. Enomoto, N. Ohkohchi, H. Doi, S. Satomi, Platelets promote hepatocyte
7 proliferation in early period of liver regeneration, *Acta Hepatologica Japonica* 44
8 (2003) 383-394
- 9 [20] B.N. Kahner, H. Shanker, S. Murugappan, G.L. Prasad, S.P. Kunapuli. Nucleotide
10 receptor signaling in platelets. *J Thromb Haemost.* 11 (2006) 2317-2326
- 11 [21] S. Thevananther, H. Sun, D. Li, V. Arjunan, S.S. Awad, S. Wyllie, T.L.
12 Zimmerman, J.A. Goss, S.J. Karpen, Extracellular ATP activates c-jun N-terminal
13 kinase signaling and cell cycle progression in hepatocytes, *Hepatology* 39 (2004)
14 393-402
- 15 [22] Y. Ogino, Y. Ayukawa, T. Kukita, K. Koyano, The contribution of platelet-derived
16 growth factor, transforming growth factor- β 1, and insulin-like growth factor-I in
17 platelet-rich plasma to the proliferation of osteoblast-like cells, *Oral Surg, Oral*
18 *Med. Oral Pathol. Oral Radiol. Endod.* 101 (2006) 724-729
- 19 [23] R.E. Marx, E.R. Carlson, R.M. Eichstaedt, S.R. Schimmele, J.E. Strauss, K.R.
20 Georgeff, Platelet-rich plasma: growth factor enhancement for bone grafts, *Oral*
21 *Surg. Oral Med. Oral Pathol. Oral Radiol. Endod.* 85 (1998) 638-646
- 22 [24] E. Anitua, Plasma rich in growth factors: preliminary results of use in the
23 preparation of future sites for implants, *Int. J. Oral Maxillofac. Implants* 14 (1999)
24 529-535

- 1 [25] N.E. Carlson, R.B. Roach, Platelet-rich plasma-clinical applications in dentistry, J.
2 Am. Dent. Assoc. 133 (2002) 1383-1386
- 3 [26] J.D. Kassolis, P.S. Rosen, M.A. Reynolds, Alveolar ridge and sinus augmentation
4 utilizing platelet-rich plasma in combination with freeze-dried bone allograft: case
5 series, J. Periodontol. 71 (2000) 1654-1661
- 6 [27] X.Y. Ouyang, J. Qiao. Effect of platelet-rich plasma in the treatment of periodontal
7 intrabony defects in humans, Chin Med J. 119 (2006) 1511-1521

1 **ACKNOWLEDGEMENT**

2 The authors thank Yuko Jinzenji and Akiko Sato, University of Tsukuba, for
3 technical assistance. The authors also thank Chikako Sato, Tohoku University, for
4 measuring adenine nucleotides.

1 **TABLE 1**

	Contents of ATP, ADP,AMP	
	Fresh	FDPs
ATP pmol/ 10⁸ platelets	85.57 ± 11.56	64.10 ± 8.00*
ADP pmol/ 10⁸ platelets	27.79 ± 3.52	28.43 ± 8.88
AMP pmol/ 10⁸ platelets	3.85 ± 0.43	8.98 ± 7.48
Energy charge	0.82 ± 0.04	0.78 ± 0.11

Note. The contents of FDPs and controls (n=4-6) with a standard deviation. FDPs; freeze dried platelets, Fresh; fresh platelets. ATP, ADP, and AMP in the final supernatant were then measured by high pressure liquid chromatography. Energy charge was calculated by the formula by materials and methods. * $P=0.041$ vs control, Mann-Whitney U test.

2

1 **TABLE 2**

	Contents of PDGF and IGF-1	
	Fresh	FDPs
PDGF pmol/ 10⁸ platelets	89.04 ± 7.77	134.39 ± 9.34*
IGF-1 pmol/ 10⁸ platelets	1292.00 ± 53.30	800.14 ± 22.35**

Note. The contents of FDPs and controls (n=4-6) with a standard deviation. FDPs and control platelets were dissolved in PBS. FDPs; freeze dried platelets, Fresh; fresh platelets. * $P = 0.0253$ vs fresh platelets, ** $P=0.0068$ vs fresh platelets, Mann-Whitney U test.

2

1 **FIGURE LEGEND**

2 **Figure 1**

3 Temperature of platelets (solid line) and freezing course of vials (dotted line) are
4 indicated. (A) Vials were frozen from 20 to -50°C at a freezing rate of -1°C/min without
5 programmed freezing. Platelets made heat of crystallization when platelets were frozen
6 to -9°C (arrow). (B) Vials were frozen from 20 to -50°C at a freezing rate of -1°C/min.
7 When vials reached -9°C, they were then suddenly frozen to -50°C at a freezing rate of
8 -60°C/min, kept at -50°C for three minutes, warmed to -20°C at a freezing rate of
9 15°C/min, and finally returned to a freezing rate of -1°C/min by a programmed freezing
10 method for prevention of making heat of crystallization.

11

12 **Figure 2**

13 The sample temperature (dotted line) and freeze drying pressure (solid line) are
14 indicated. Samples were kept for more 11 hours at 4 mTorr and 26.8°C.

15

16 **Figure 3**

17 Transmission electron microscopical findings of FDP (A), fresh platelet (B), and
18 FDP without using the programmed freezing method (C). Magnification x 30, 000.
19 FDPs were slightly swollen compare to fresh platelets, but cellular membrane and
20 intracellular granules were preserved. Destruction of the native structure; i.e.,
21 destruction of cellular membrane and organelle; was observed in FDPs when the
22 programmed freezing method was not used.

23

24 **Figure 4**

1 Fibrinogen binding assay of fresh platelets (A) and FDPs (B). Gray lines indicate
2 natural coagulation. Black lines indicate thrombin treatment (10 U/mL) for 30 minutes.
3 (C) Thrombin coagulation/natural coagulation ratio of fresh platelets and FDPs, number
4 of each group was 6.

5

6 **Figure 5**

7 (A) Brd U uptake of primary cultured hepatocytes with FDPs. FDPs were seeded
8 10-, 100-, and 500-fold of hepatocytes. n=6. * $P<0.05$, ** $P<0.01$ vs. without platelets,
9 one-way ANOVA. (B) [^3H]-methyl-thymidine uptake assay. FDPs were seeded 500-fold
10 of hepatocytes, number of each group was 6. * $P=0.0068$ vs. without platelets, Mann
11 Whitney-U test.