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Research article

Differentiation of endogenous erythropoietin and exogenous ESAs by Western blotting

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ABSTRACT

Doping tests for the illegal use of erythropoiesis-stimulating agents (ESAs) have been developed. We developed a new Western blotting method to detect and distinguish endogenous erythropoietin (Epo, 35-38 kDa) and exogenous ESAs (epoetin α and β, 38-42 kDa; darbepoetin α, 47-50 kDa; epoetin β pegol, 93-110 kDa). Epo and ESAs are glycoproteins and deglycosylation using peptide-N-glycosidase F shifted all Epo and ESA bands except epoetin β pegol to 22 kDa. We cut the bands of Epo and ESAs from SDS-PAGE gels and analyzed them by Liquid Chromatography/Mass Spectrometry (LC/MS). LC/MS detected all endogenous Epo and exogenous ESAs as deglycosylated 22 kDa Epo, indicating that LC/MS analysis could confirm the presence of Epo or ESA, but could not distinguish between endogenous Epo and exogenous ESAs. We propose the following Epo doping tests: 1) detect Epo or ESAs by Western blotting of the glycosylated form; 2) increase the reliability by the band shift following deglycosylation; and 3) complete confirmation of Epo or ESA by LC/MS analysis using cut gels. One of the advantages of our method is that pre-purification of samples for Epo is not required in our Western blotting.

1. Introduction

Erythropoiesis stimulating agents (ESAs) have been invented to treat chronic kidney disease (CKD) patients with anemia [1]. Currently, four types of ESAs have been used in Japan: epoetin α and β, darbepoetin α and epoetin β pegol. The major clinical difference is the duration of the effect. The half-life after the injection of darbepoetin α and epoetin β pegol was increased by the addition of glucose or polyethylene glycol (PEG), respectively, causing the extension of administration interval [2, 3]. The half-life after the injection to control subjects or patients with CKD was 7, 3–5, 32–48 and 170-200 h in epoetin α and β, darbepoetin α and epoetin β pegol, respectively, based upon the manufacturer's protocol.

Illegal usage of ESAs by athletes has been checked using tests for doping [4, 5]. To detect illegally used ESAs in urine, isoelectrical focusing (IEF) and/or SAR-PAGE after enrichment and purification of Epo have been recommended by the World Anti-Doping Agency (WADA) [4, 5, 6, 7, 8, 9]. Liquid Chromatography/Mass Spectrometry (LC/MS) has also been used for the detection of ESAs [10]. Pre-purification of Epo, or loading a large amount of sample, is required to observe Epo by Western blotting or SDS-PAGE with Coomassie brilliant blue staining [11, 12, 13]. Doping tests not only reveal illegal usage of ESAs by athletes but also

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protects a player's career from incorrect results. Therefore, accurate analysis is required for a doping test.

We developed a new method for the detection of Epo in urine, plasma/serum and tissue by Western blotting [14, 15]. Pre-purification of Epo is not required in our method. We cut the 22 kDa band of deglycosylated Epo, isolated from an anemic patient's urine, and analyzed it by LC/MS. We found that the band contained human Epo, proving the correctness of our method [15]. Using this method, we tried to distinguish the difference between endogenous Epo and exogenous ESAs.

2. Materials and methods

2.1. Materials

Urine was collected from an anemic patient. Our protocols were checked and approved by the Ethics Committee at Kitasato University Medical Center (25-2, 2018032, 2019029). Informed consent was obtained from the patient. ESAs were obtained from Kyowa Kirin Co. (Tokyo, Japan) and Chugai Pharmaceutical Co. (Tokyo, Japan): Epoetin α: Espo Injection Syringe 750IU/0.5ml, Kyowa Kirin, Epoetin β: Epogin 750IU/0.5ml, Chugai, Darbepoetin α: Nesp injection plastic syrnge 5mg/0.5ml, Kyowa Kirin, Epoetin β pegol: Mircera 12.5mg/0.3ml, Chugai.

2.2. Western blot analysis with deglycosylation

Western blot analysis was performed as described previously [14, 15]. Urine samples were obtained from a 70 y.o. anemic patient. The plasma hemoglobin level was 6.8 g/dl and the urinary Epo concentration was 129 mIU/ml. The urine sample was not purified for Epo but was simply concentrated. 8.3 ml of urine was concentrated to 60 μl by Vivaspin 20 and 500 (GE Healthcare Bio-Science AB. Sweden) and used for Western blot analysis. Urine samples and ESAs were used directly, or after deglycosylation, for sodium dodecyl sulfate gel electrophoresis (SDS-PAGE) (10-20% gradient gel, Cosmo Bio No. 414893, Tokyo, Japan). Recombinant human Epo (rhEpo, 587102, BioLegend, San Diego, USA) was used as a positive control. Glycosylated and deglycosylated rhEpo were combined. After SDS-PAGE, proteins were transferred to a polyvinylidene difluoride (PVDF) membrane (Immobilon-P, Merck Millipore, IPVH00010) with 120-160 mA for 75-90 min. The membrane was blocked with 5% skim milk (Morinaga, Japan) for 60 min and incubated with the antibody against Epo (Santa Cruz, sc-5290, 1:500) for 60 min at room temperature or overnight at 4 °C. After washing, the membrane was incubated with a secondary antibody (goat anti-mouse IgG (H + L) (Jackson ImmunoResearch Laboratories, 115-035-166, 1:5,000) for 60 min. Bands were visualized by the ECL Select Western Blotting Detection System (GE Healthcare Bio-Science AB, RPN2235) and LAS 4000 (FUJIFILM).

Deglycosylation was performed as described previously [14, 15]. To 10 μl of concentrated urine or diluted ESAs, 1 μl of 10% SDS (Wako 191-07145, FUJIFILM Wako Pure Chemical Co., Osaka, Japan) was added and boiled for 3min. Then, 11 μl of 2x stabilizing buffer was added, and 2 μl of PBS or Peptide-N-glycosidase F (PNGase F, 1 mU) (Takara, 4450) was added. The samples were incubated in a water bath at 37 °C for 15-20 h. The 2x stabilizing buffer contained 62.5 mM Tris-HCl (pH 8.6), 24 mM EDTA, 2% Nonidet P-40 and 4% 2-mercaptoethanol. Urine Epo concentrations were measured by CLEIA (SRL, Tokyo, Japan, using Access Epo by Beckman Coulter, Brea, USA).

2.3. LC/MS analysis

For LC/MS analysis, SDS-PAGE was performed the same way as Western blotting. After SDS-PAGE, the gel was stained by negative staining (293-57701, FUJIFILM Wako Pure Chemical Co.) (left panel, Figure 1). The glycosylated and deglycosylated bands of rhEpo and ESAs were subjected to LC/MS. The Epo and ESAs bands were visualized by negative staining and were excised from the SDS-PAGE gel (right panel, Figure 1). Then, in-gel tryptic digestion was carried out using ProteaseMAX reagent (Promega, WI, USA) according to the manufacturer's protocol. The peptides were separated by L-column2 ODS (3 μm, 0.1 × 150 mm, CERI, Tokyo, Japan) at a flow rate of 500 nl/min using a linear gradient of acetonitrile (5%–45%). Nano-LC-MS/MS analyses were performed with an LTQ-Orbitrap XL mass spectrometer (Thermo Fisher Scientific, MA, USA) as previously described [16].

3. Results

Recombinant human Epo was detected in a broad band at 34-43 kDa (① in Figure 2) and endogenous Epo was detected at 35-38 kDa (② in Figure 2). ESAs were detected at larger sizes: epoetin α and β at 38-42 kDa; darbepoetin α at 47-50 kDa; and epoetin β pegol at 93-110 kDa (③, ④, ⑤ and ⑥ in Figure 2, respectively). The bands of endogenous Epo and exogenous ESAs, except epoetin β pegol, were shifted to 22 kDa by deglycosylation with PNGase F (Figure 2). The band of polyethylene glycol (PEG)-bound epoetin β pegol was shifted to 81-93 kDa. Although deglycosylation increased the lower limit of detection of epoetin α (left panel, Figure 3), the comparison between endogenous Epo and exogenous ESA has to be done using the glycosylated forms (right panel, Figure 3).

In contrast, LC/MS analysis detected endogenous rhEpo and exogenous ESAs both in glycosylated and deglycosylated forms as deglycosylated Epo of 22 kDa (①-⑩, Table 1). We showed that endogenous Epo is detected as a 22 kDa band in our previous report [15]. Our data clearly show that LC/MS analysis cannot differentiate endogenous Epo and exogenous ESAs since LC/MS analysis needs trypsin digestion of the samples [16]. Plasma Epo measurements also cannot differentiate endogenous Epo and ESAs. In contrast, Western blotting can easily distinguish endogenous Epo and exogenous ESAs. The shift of a band to 22 kDa confirms that the band is endogenous Epo or exogenous ESAs.

4. Discussion

We have used Western blotting and LC/MS analysis to distinguish endogenous Epo and exogenous ESAs in this study. Our Western blotting clearly differentiates endogenous Epo (35-38 kDa) and exogenous ESAs (epoetin α and β, 38-42 kDa; darbepoetin α, 47-50 kDa; epoetin β pegol, 93-110 kDa). Although the size of endogenous Epo and epoetin α/β is very close, our Western blotting could distinguish the differences in molecular size (right panel, Figure 3). In contrast, LC/MS analyses show that all endogenous Epo and exogenous ESAs are detected at 22 kDa, indicating that LC/MS analysis could confirm the presence of Epo or ESAs but could not differentiate endogenous Epo and exogenous ESAs (Table 1).

Okano, et al. reported LC/MS analysis of darbepoein α [10]. The detection limit of confirmation analysis of darbepoetin α was 5 pg/ml. Since they used 10 ml urine, the detection limit of confirmation analysis of darbepoetin α is thought to be 50 pg. This limit is very impressive. In our LC/MS analysis, 1-2 ng of recombinant Epo was required [15]. They used an EPO purification kit (MAIIA, Sweden) before LC/MS analysis to increase the Epo concentration in urine. However, we did not perform Epo purification in urine. We simply concentrated the urine and proceeded to Western blotting. The sensitivity of the detection by LC/MS analysis depends on the sensitivity of the mass spectrometer and the purity of the sample. If we added pre-purification of samples, the detection limit of Epo may have improved. In our method, Western blotting is a type of pre-purification of Epo or ESA, if the analysis proceeds to LC/MS analysis. Our Western blotting not only detects but also purifies Epo or ESAs.

One of the advantages of degycosylation is the increase of the sensitivity of detection (left panel, Figure 3). Higuchi, et al. showed deglycosylation of Epo using SDS-PAGE and Coomassie brilliant blue staining [11]. They had to load a huge amount of the samples. Epo protein

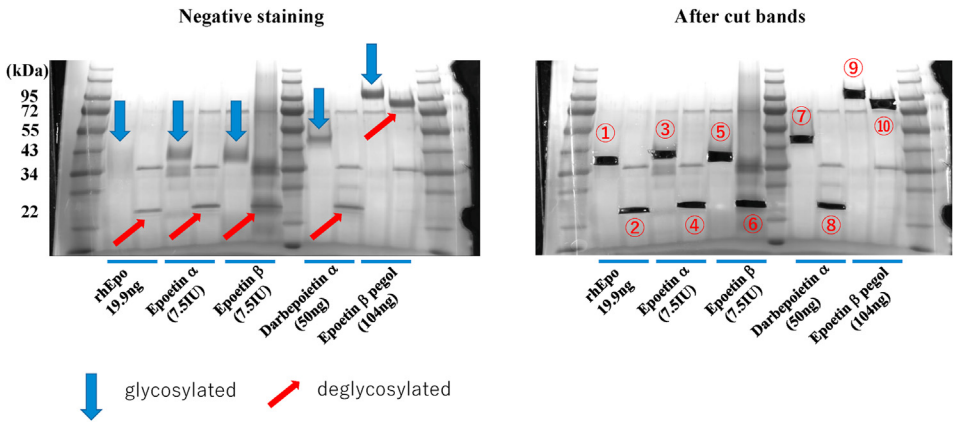
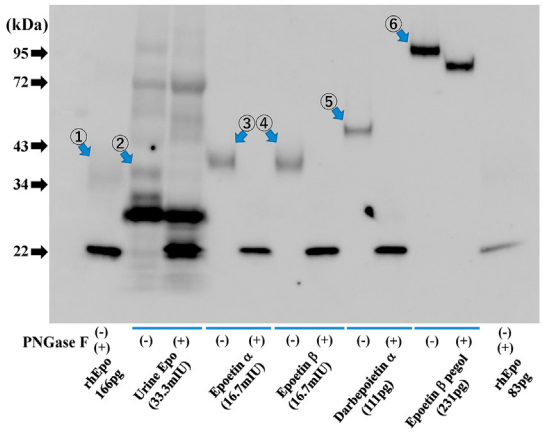


Figure 1. SDS-PAGE and negative staining for LC/MS analysis. (left) rhEpo (19.9 ng) and ESAs (epoetin α, 7.5 IU; epoetin β, 7.5 IU; darbepoetin α, 50 ng; epoetin β pegol, 104 ng) were applied to SDS-PAGE and stained by negative staining. All of the glycosylated and deglycosylated bands were stained by negative staining. (right) The bands were cut and proceeded to LC/MS analysis. Blue arrows indicate glycosylated Epo and ESAs. Red arrows show deglycosylate Epo and ESAs. ①-⑩ indicate the place of cut gel.



The sizes of Epos

	glycosylated	deglycosylated	LC/MS
rhEpo	34-43	22	22
Endo hEpo	35-38		
Epoetin α	38-42		
Epoetin β	38-42		
Darbepoietin α	47-50		
Epoetin β pegol	93-110	81-93	

(kDa)

Figure 2. Detection of urinary Epo and ESAs by Western blotting with deglycosylation. Urinary Epo and ESAs were investigated by Western blotting. Endogenous urinary Epo (②) was smaller than ESAs (③-⑥). The bands for endogenous Epo and ESAs, except epoetin β pegol, were shifted to 22 kDa by deglycosylation. The band of PEG-bound epoetin β pegol was slightly reduced in size by deglycosylation. The sizes of endogenous Epo and ESAs before and after deglycosylaton by Western blotting and by LC/MS analysis (Table 1) were summarized in the Table. Glycosylated rhEpo (①), urine Epo (②) and ESAs (③-⑥) are indicated by blue arrows. The amount of samples are as follows: urine Epo (33.3 mIU); epoetin α and β (16.7 mIU); darbepoietin (111 pg); and epoetin β pegol 231 pg rhEpo, recombinant human Epo, Endo Epo, endogenous Epo. ① rhEpo, ② human urine Epo, ③ epoetin α, ④ epoetin β, ⑤ darbepoetin α, and ⑥ epoetin β pegol.

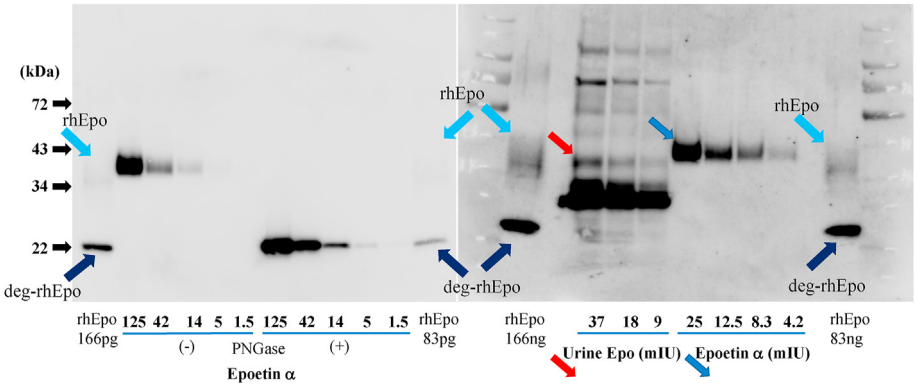


Figure 3. Detection of urine Epo and ESAs by Western blotting. (left panel) Glycosylated and deglycosylated epoetin α (38–42 and 22 kDa, respectively) were investigated by Western blotting. The detection limit was improved by deglycosylation. (right panel) Urinary Epo and epoetin α without deglycosylation were investigated by Western blotting. Urinary Epo was smaller than epoetin α. rhEpo; recombinant human Epo, deg-rhEpo; deglycosylated recombinant human Epo.

expression by Western blot has been reported but pre-purification of Epo from samples was required, which caused difficulty for quantitative analysis [12, 13]. Pre-purification of Epo from a sample is not required in our method. The most important advantage of our method is its high sensitivity and specificity. 1-2 ng of Epo is required for the detection by LC/MS analysis, while only a few pg of Epo is needed for Western blotting. The differentiation of endogenous Epo and exogenous ESAs were easily detected by the differences of band sizes in the glycosylated forms (Figures 2 and 3). The shift of a band to 22 kDa by deglycosylation is not useful for the differentiation of endogenous Epo and exogenous ESAs but is useful to confirm the presence of some kinds of Epo. To completely confirm the presence of Epo or ESAs, LC/MS analysis using cut gels is required. Combining both Western blotting with and without degycosylation and LC/MS analysis is currently the best way to perform a doping test for the illegal use of ESAs. The control band of recombinant human Epo helps the quantitative analysis of ESAs. Since our method does not need pre-purification of samples for Epo, the cost for the determination of Epo is very cheap.

Table 1. LC/MS analysis of rhEpo and ESAs. The results of LC/MS analyses are shown. The numbers ①-⑩ corresponds to the numbers in the right panel of Figure 1. The matched peptide with Epo is shown by bold. LC/MS analysis shows that all of the bands contained 22 kDa Epo. Mr: molecular mass.

①. **Glycosylated rhEpo (Mr: 21578) Protein sequence coverage: 27%**
001 MGVHECPAWL WLLLSLLSLP LGLPVLGAPP RLICDSRVLE RYLLEAKEAE
051 NITTGCAEHC SLNENITVPD TK**VNFYAWKR MEVGQQAVEV WQGLALLSEA**
101 VLRGQALLVN SSQPWEPLQL HVDKAVSGLR **SLTTLLR**ALG AQKEAISPPD
151 AASAAPLR**TI TADTFRK**LFR **VYSNFLR**GKL KLYTGEACRT GDR
②. **Deglycosylated rhEpo (Mr: 21578) Protein sequence coverage: 16%**
001 MGVHECPAWL WLLLSLLSLP LGLPVLGAPP RLICDSRVLE RYLLEAKEAE
051 NITTGCAEHC SLNENITVPD TK**VNFYAWKR** MEVGQQAVEV WQGLALLSEA
101 VLRGQALLVN SSQPWEPLQL HVDKAVSGLR **SLTTLLR**ALG AQKEAISPPD
151 AASAAPLR**TI TADTFRK**LFR **VYSNFLR**GKL KLYTGEACRT GDR
③. **Glycosylated epoetin α (Mr: 21578) Protein sequence coverage: 24%**
001 MGVHECPAWL WLLLSLLSLP LGLPVLGAPP RLICDSRVLE RYLLEAKEAE
051 NITTGCAEHC SLNENITVPD TKVNFYAWK**R MEVGQQAVEV WQGLALLSEA**
101 **VLR**GQALLVN SSQPWEPLQL HVDKAVSGLR **SLTTLLR**ALG AQKEAISPPD
151 AASAAPLR**TI TADTFRK**LFR **VYSNFLR**GKL KLYTGEACRT GDR
④. **Deglycosylated epoetin α (Mr: 21578) Protein sequence coverage: 27%**
001 MGVHECPAWL WLLLSLLSLP LGLPVLGAPP RLICDSRVLE RYLLEAKEAE
51 NITTGCAEHC SLNENITVPD TK**VNFYAWKR MEVGQQAVEV WQGLALLSEA**
101 VLRGQALLVN SSQPWEPLQL HVDKAVSGLR **SLTTLLR**ALG AQKEAISPPD
151 AASAAPLR**TI TADTFRK**LFR **VYSNFLR**GKL KLYTGEACRT GDR
⑤. **Glycosylated epoetin β (Mr: 21578) Protein sequence coverage: 35%**
001 MGVHECPAWL WLLLSLLSLP LGLPVLGAPP RLICDSRVLE RYLLEAKEAE
051 NITTGCAEHC SLNENITVPD TK**VNFYAWKR MEVGQQAVEV WQGLALLSEA**
101 VLRGQALLVN SSQPWEPLQL HVDKAVSGLR **SLTTLLR**ALG AQK**EAISPPD**
151 AASAAPLRTI TADTFRKLFR **VYSNFLR**GKL KLYTGEACRT GDR
⑥. **Deglycosylated epoetin β (Mr: 21578) Protein sequence coverage: 27%**
001 MGVHECPAWL WLLLSLLSLP LGLPVLGAPP RLICDSRVLE RYLLEAKEAE
051 NITTGCAEHC SLNENITVPD TK**VNFYAWKR MEVGQQAVEV WQGLALLSEA**
101 VLRGQALLVN SSQPWEPLQL HVDKAVSGLR **SLTTLLR**ALG AQKEAISPPD
151 AASAAPLR**TI TADTFRK**LFR **VYSNFLR**GKL KLYTGEACRT GDR
⑦. **Glycosylated darbepoietin α (Mr: 21578) Protein sequence coverage: 35%**
001 MGVHECPAWL WLLLSLLSLP LGLPVLGAPP RLICDSRVLE RYLLEAKEAE
051 NITTGCAEHC SLNENITVPD TK**VNFYAWKR MEVGQQAVEV WQGLALLSEA**
101 VLRGQALLVN SSQPWEPLQL HVDKAVSGLR **SLTTLLR**ALG AQK**EAISPPD**
151 AASAAPLRTI TADTFRKLFR **VYSNFLR**GKL KLYTGEACRT GDR
⑧. **Deglycosylated darbepoietin α (Mr: 21578) Protein sequence coverage: 35%**
001 MGVHECPAWL WLLLSLLSLP LGLPVLGAPP RLICDSRVLE RYLLEAKEAE
051 NITTGCAEHC SLNENITVPD TK**VNFYAWKR MEVGQQAVEV WQGLALLSEA**
101 VLRGQALLVN SSQPWEPLQL HVDKAVSGLR **SLTTLLR**ALG AQK**EAISPPD**
151 AASAAPLRTI TADTFRKLFR **VYSNFLR**GKL KLYTGEACRT GDR
⑨. **Glycosylated epoetin β pegol (Mr: 21578) Protein sequence coverage: 39%**
001 MGVHECPAWL WLLLSLLSLP LGLPVLGAPP RLICDSRVLE RYLLEAKEAE
051 NITTGCAEHC SLNENITVPD TK**VNFYAWKR MEVGQQAVEV WQGLALLSEA**
101 VLRGQALLVN SSQPWEPLQL HVDKAVSGLR **SLTTLLR**ALG AQK**EAISPPD**
151 AASAAPLRTI TADTFRKLFR **VYSNFLR**GKL **KLYTGEACR**T GDR
⑩. **Deglycosylated epoetin β pegol (Mr: 21578) Protein sequence coverage: 50%**
001 MGVHECPAWL WLLLSLLSLP LGLPVLGAPP RLICDSRVLE RYLLEAKEAE
051 NITTGCAEHC SLNENITVPD TK**VNFYAWKR MEVGQQAVEV WQGLALLSEA**
101 VLRGQALLVN SSQPWEPLQL HVDKAVSGLR **SLTTLLR**ALG AQK**EAISPPD**
151 AASAAPLRTI TADTFRKLFR **VYSNFLR**GKL **KLYTGEACR**T GDR

A doping test is useful to exclude illegal athletes from a fair competition. However, a doping test is also useful for the protection of athletes' career. Incorrect and inaccurate results of a doping test may terminate a fair player's career. We hope that our method will ensure accurate and reliable doping tests for the illegal use of ESAs to ensure a fair competition by the athletes.

5. Conclusion

In conclusion, the highly specific and sensitive detection of endogenous Epo and exogenous ESAs became possible with our Western blotting method. Western blotting of glycosylated forms could differentiate the molecular sizes of endogenous Epo and exogenous ESAs. The shift of bands by deglycosylation is useful to confirm that the band is Epo or an ESAs. Although LC/MS analysis is useful to detect urine Epo, LC/MS cannot differentiate endogenous Epo and exogenous ESAs. Combining Western blotting with and without deglycosylation and LC/MS analysis using cut gels would be the best method for a doping test for illegal use of ESAs. We propose the following Epo doping tests: 1) detect Epo or ESA by Western blotting of the glycosylated form; 2) increase the reliability by the band shift following deglycosylation; and 3) complete confirmation of Epo or ESA by LC/MS analysis using cut gels. Pre-purification of samples for Epo is not required by our method.

Declarations

Author contribution statement

Y. Yasuoka, T. Fukuyama and H. Nonoguchi: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Y. Izumi: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

T. Yamashita: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Y. Nakayama, H. Inoue, K. Yanagita, Y. Shimada and Y. Nagaba: Performed the experiments.

T. Yamazaki, T. Uematsu and N. Kobayashi: Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

M. Mukoyama, Y. Sato and K. Kawahara: Analyzed and interpreted the data.

J. Sands: Analyzed and interpreted the data; Wrote the paper.

T. Oshima: Contributed reagents, materials, analysis tools or data.

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Declaration of interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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