Dynamic aspects of ascorbic acid metabolism in the circulation: analysis by ascorbate oxidase with a prolonged in vivo half-life

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INTRODUCTION

The reduced form of AA (ascorbic acid) is a naturally occurring antioxidant that scavenges free radicals to form MDAA (monodehydroascorbic acid) and DAA (dehydroascorbic acid), its regeneration from the oxidized metabolites is critically important for humans and other animals that lack the ability to synthesize this antioxidant. To study the dynamic aspects of AA metabolism in the circulation, a long acting AOase (ascorbate oxidase) derivative was synthesized by covalently linking PEG (poly(ethylene glycol)) to the enzyme. Fairly low concentrations of the modified enzyme (PEG–AOase) rapidly decreased AA levels in isolated fresh plasma and blood samples with a concomitant increase in their levels of MDAA and DAA. In contrast, relatively high doses of PEG–AOase were required to decrease the circulating plasma AA levels of both normal rats and ODS (osteogenic disorder Shionogi) rats that lack the ability to synthesize AA. Administration of 50 units of PEG–AOase/kg of body weight rapidly decreased AA levels in plasma and the kidney without affecting the levels in other tissues, such as the liver, brain, lung, adrenal gland and skeletal muscles. PEG–AOase slightly, but significantly, decreased glutathione (GSH) levels in the liver without affecting those in other tissues. Suppression of hepatic synthesis of GSH by administration of BSO [L-buthionin-(-S,R)-sulfoximine] enhanced the PEG–AOase-induced decrease in plasma AA levels. These and other results suggest that the circulating AA is reductively regenerated from MDAA extremely rapidly and that hepatic GSH plays important roles in the regeneration of this antioxidant.

Key words: antioxidant, ascorbate oxidase, ascorbic acid, glutathione, oxidative stress.

In the present study we synthesized PEG [poly(ethylene glycol)]-AOase (ascorbate oxidase), an AA oxidase derivative with a prolonged in vivo half-life. Intravenously administered PEG–AOase effectively oxidizes plasma AA to MDAA in a dose-dependent manner. The present study describes the effect of PEG–AOase on plasma and tissue levels of AA, MDAA and DAA in control, STZ (streptozotocin)-treated diabetic rats and ODS (osteogenic disorder Shionogi) rats that lack the ability to synthesize AA [25,26]. The results suggest for the first time that the circulating AA is reductively regenerated extremely rapidly by using hepatic GSH.

MATERIALS AND METHODS

Chemicals

GSH and DTNB [5,5'-dithiobis-(2-nitrobenzoic acid)] were purchased from Wako Pure Chemicals. AOase (EC 1.10.3.3) from cucurbita species, AA, glutathione reductase, DTC (diethyldithiocarbamate), DTPA (diethylenetriamine pentaacetic acid) and STZ were purchased from Sigma. Activated PEG (MW = 10,000 Da) was obtained from Seikagaku Kogyo. All other reagents used were the highest grade commercially available.

Animals

Male Wistar rats (8–9 weeks old) obtained from SLC (Shizuoka, Japan) were fed laboratory chow and water ad libitum and used for experiments without prior fasting. Unless otherwise stated, they were used for the experiments as normal animals. We also

Abbreviations used: AA, ascorbic acid; AOase, ascorbate oxidase; DAA, dehydroascorbic acid; DTC, diethyldithiocarbamate; DTPA, diethylenetriamine pentaacetic acid; ESR, electron spin resonance; GLUT, glucose transporter; MDAA, monodehydroascorbic acid; ODS, osteogenic disorder Shionogi; PEG, poly(ethylene glycol); STZ, streptozotocin; SVCT, Na+-AA cotransport system; TCA, trichloroacetic acid.

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used ODS rats, which lack the ability to synthesize AA, and STZ-induced hyperglycaemic rats. To increase glucose levels in plasma, animals were intravenously administered 50 mg of STZ/kg of body weight 5 days prior to the experiments. The blood glucose levels of STZ-treated rats (435.3 ± 41.3 mg/dl) were higher than those of control animals (117.9 ± 23.0 mg/dl). Male ODS rats (6 weeks old) were obtained from Clea (Osaka, Japan) and allowed free access to normal diet and AA-containing water (1 mg/ml) for 2 weeks. The concentration of AA in the chow was 0.02%. All experiments were performed according to the Guidelines for Laboratory Animal Care Regulation of Osaka City University Medical School and were approved by the Ethical Committee of our University.

Synthesis of PEG–AOase

Lysyl amino groups of AOase were covalently linked with activated PEG as described previously [27]. Briefly, the reaction mixture contained, in a final volume of 1 ml of 50 mM bicarbonate (pH 10), 250 units of AOase, 250 mg of PEG and 50 mM AA. The mixture was incubated at 37 °C for 3 h and then for 16 h at 4 °C. The incubated mixture was dialysed at 4 °C against 3 litres of 20 mM PBS (pH 7.4). The activities of AOase and PEG–AOase were determined by measuring the rate of AA decrease. The reaction was stopped by adding 5 mM DTC, and the remaining AA was determined. One unit of the enzyme was defined as the amount of the enzyme required for the oxidation of 1 μmol of AA per min at 37 °C and pH 7.4.

Oxidation of AA by PEG–AOase

Under light ether anaesthesia, blood samples were obtained from the abdominal artery in heparinized tubes. To the fresh blood samples was added Tris/HCl (pH 7.4) to give a final concentration of 20 mM to maintain their pH. Plasma samples were obtained after centrifugation of the blood at 10000 g at 4 °C for 1 min. Blood and plasma samples were incubated with 20 m-units/ml of PEG–AOase at 37 °C in air. After incubation with the enzyme, the blood and plasma samples were collected in 1 mM DTC- and DTPA-containing tubes to stop further oxidation of AA. Blood samples were immediately centrifuged at 10000 g for 30 s. Plasma samples thus obtained were analysed for AA, MDAA and DAA.

Under urethane anaesthesia, rats were injected with various doses of PEG–AOase. At the indicated times, blood samples were collected from the left femoral vein in 1 mM DTC- and DTPA-containing tubes and analysed for AA, MDAA and DAA. Some animals were intravenously injected with 1 mmol of BSO/kg of body weight prior to the administration of PEG–AOase.

Analysis of AA and GSH levels

Fresh plasma samples were mixed with an equivolume of 10% TCA (trichloroacetic acid). The excised tissues were homogenized using a micro homogenizer (Phycosotron; Microtec) in 5 vol. of ice-cold 5% TCA containing 1 mM DTPA. After centrifugation of the samples at 10000 g at 4 °C for 10 min, the acid-soluble fractions were used for the analysis of AA, free thiols and glutathione. AA levels were determined by using HPLC equipped with a Shimazu electrochemical detection system [28,29]. DAA levels were calculated by subtraction of AA levels from total levels of AA in the samples. The total AA level was determined using HPLC analysis after reduction of the acid-soluble fractions with 3.3 mM DTT (dithiothreitol) containing 330 mM K2HPO4 for 5 min at room temperature (22 °C) as described previously [30]. Total glutathione (GSH + 2 GSSG) and low-molecular-mass thiols were determined using the method of Tietze [31] and Ellman [32] respectively.

ESR (electron spin resonance) analysis

An extracorporeal blood circulation system was established as described previously [33]. Briefly, male Wistar rats were anaesthetized with urethane, and polyethylene tubings (0.60 mm and 0.47 mm in diameter) were inserted into the left femoral artery and vein respectively. The two tubings were connected to an ESR flow cell (200 μl). The flow rate of the blood in the circuit was controlled by a Perista pump at 1 ml/min. Before and after intravenous injection of PEG–AOase, the ESR spectrum of MDAA in the circulation was recorded using a JEOL TES-TE 200 at 37 °C (8 mW of power, 0.079 mT modulation, 334.6 ± 5 mT magnetic field, 5 mT sweep width, 4 min sweep time and a 0.3 s time constant). Concentrations of MDAA were determined by using an external standard based on the signal intensity.

Statistics

Values are expressed as the mean ± S.E.M. derived from 5–15 animals. Statistical analysis was performed using ANOVA followed by a Student’s t test and the level of significance was P < 0.05.

RESULTS

Oxidation of AA by PEG–AOase in isolated plasma and blood

To elucidate the dynamic aspects of AA metabolism in isolated plasma and blood, the effect of PEG–AOase on the plasma levels of AA and its metabolites were analysed in vitro. Incubation with PEG–AOase (20 m-units/ml) rapidly decreased AA levels in plasma with a concomitant increase in MDAA and DAA levels (Figure 1A). Then, the increased MDAA and DAA rapidly decreased at similar rates. When incubated with fresh blood, PEG–AOase also decreased plasma levels of AA and increased MDAA and DAA (Figure 1B). The rate of MDAA increase in blood samples was slightly lower than that observed in the experiment with plasma samples. Under identical conditions, the levels of AA in the plasma (59.9 ± 0.88 μM) and blood (57.1 ± 1.50 μM) remained unchanged (57.9 ± 0.64 μM and 60.5 ± 1.65 μM respectively) at least for 10 min in the absence of PEG–AOase.
Mechanism of regeneration of the circulating ascorbic acid in vivo

Figure 2  Effect of PEG–AOase on the concentration of AA and its metabolites in the circulation

At 1 min after intravenous administration of various doses of PEG–AOase to normal rats, the levels of the circulating MDAA were determined using the ESR method as described in the Material and methods section. Time-dependent changes in AA (C), MDAA (D) and DAA (E) levels were also measured after administration of 1 unit of PEG–AOase/kg of body weight (B). At the indicated time (arrow), the catalytic activity of PEG–AOase was blocked by intravenous administration of 0.1 mmol of DTC/kg of body weight. After 10 min of administration of 1 unit of the enzyme/kg of body weight, blood samples were collected and incubated for 1, 3 and 5 min at 37°C in air (C). Then, plasma levels of AA and DAA were also determined. Values are the means ± S.E.M. (n = 8–15).

Oxidation of AA by PEG–AOase in the circulation

To elucidate the dynamic aspects of the metabolism and transport of AA in the circulation, the effect of PEG–AOase on the circulating plasma levels of AA and its metabolites were analysed in vivo using the ESR blood circulation system as described in the Material and methods section. Intravenous administration of PEG–AOase increased plasma levels of MDAA in a dose-dependent manner (Figure 2A). Figure 2(B) shows the time-dependent changes in AA and its oxidized metabolites in plasma after administration of PEG–AOase. Although PEG–AOase (1 unit/kg of body weight) rapidly increased plasma levels of MDAA in the circulation, no significant decrease was found to occur with circulating AA levels (Figure 2B). The increased MDAA in the circulation rapidly decreased after administration of DTC, a chelating agent for Cu²⁺ to inactivate PEG–AOase, suggesting that PEG–AOase continuously oxidized AA while the generated MDAA rapidly disappeared from the circulation.

Although a fairly high dose (1 unit/kg of body weight) of the enzyme failed to decrease AA levels in the circulation, its plasma levels rapidly decreased after isolation of the blood with a concomitant increase in DAA levels as observed with in vitro experiments (Figure 2C). Thus, we tested the effect of high doses of the enzyme on AA levels in circulation (Figure 3). PEG–AOase started to decrease the plasma AA levels in normal rats at doses higher than 2 units/kg of body weight (Figure 3A). At PEG–AOase doses higher than 10 units/kg of body weight, AA levels in the circulation rapidly decreased.

Effects of AA synthesis and GLUT on the circulating AA

To test the possible involvement of de novo synthesis of AA in the mechanism for the maintenance of the circulating AA, the effect of the enzyme was also investigated with ODS rats that lack the ability to synthesize AA (Figure 3B). At doses higher than 2 units/kg of body weight, PEG–AOase also decreased plasma AA levels in ODS rats in a similar manner as in normal rats. We also studied the possible involvement of glucose transporters in the regeneration of circulating AA using STZ-induced diabetic rats (Figure 3C). Although steady-state levels of plasma AA in STZ-treated rats (30.74 ± 5.53 μM) were lower than those of control rats (57.41 ± 2.57 μM), the dose-dependency of the PEG–AOase-induced decrease in the circulating AA was similar with the two animal groups.

Effect of a high dose of PEG–AOase on plasma and tissue AA levels

Administration of a high dose of PEG–AOase (50 units/kg of body weight) rapidly diminished circulating AA levels (Figure 4). Although the plasma levels of DAA were elevated by PEG–AOase, the extent of their increase was similar to that of animals that were injected with 1 unit of the enzyme/kg of body weight.
Figure 4  Effect of a high dose of PEG–AOase on the circulating AA and DAA

After intravenous administration of 50 units of PEG–AOase/kg of body weight to normal rats, plasma levels of AA (○, △) and DAA (●) were determined in control (△) and PEG–AOase-treated rats (○, ●). The control animals were treated with the same volume of saline (1 ml/kg of body weight). Values are means ± S.E.M. (n = 5 ~ 6).

Figure 5  Effect of PEG–AOase on tissue AA levels

PEG–AOase (50 units/kg of body weight) was administered intravenously to normal rats and tissue AA levels were determined after 2 h (A) as described in the Materials and methods section. The dose-dependency of the PEG–AOase-induced decrease in renal AA was determined (B). Time-dependent changes in renal AA levels were also analysed in animals treated with 50 units of PEG–AOase/kg of body weight (C). Values are means ± S.E.M. (n = 5). *P < 0.05 compared with controls.

Figure 6  Effect of PEG–AOase on tissue GSH levels

At 2 h after intravenous administration of 50 units of PEG–AOase/kg of body weight to normal rats (hatched columns), total glutathione levels in tissues were determined as described in the Materials and methods section. Control groups (open columns) were administered with 1 ml of saline/kg of body weight. Values are means ± S.E.M. (n = 5). *P < 0.05 compared with controls.

Figure 7  Effect of PEG–AOase on the circulating AA and DAA in BSO-treated animals

After intravenous administration of 1 mmol of BSO/kg of body weight to normal rats, changes in glutathione levels in the liver (○, ●) and kidney (△, ▼) were determined (A). At 3 h after administration of either saline (○, ●) or BSO (△, ▼), 1 unit of PEG–AOase/kg of body weight was administered to rats. Then, the plasma levels of AA (○, △, □) and DAA (●, ▼, ■) were determined (B) as described in the Materials and methods section. Control rats were administered with BSO and saline (○, ●). Most of the glutathione was found to be in the reduced form (GSH). Values are means ± S.E.M. (n = 5). *P < 0.05 compared with the control.

Role of GSH in the maintenance of the circulating AA

Since GSH is an important cofactor for the reductive regeneration of AA [24], the effects of PEG–AOase on tissue GSH status were studied (Figure 6). Administration of a high dose of PEG–AOase (50 units/kg of body weight) slightly, but significantly, decreased GSH levels in the liver but not in plasma and other tissues, such as the kidney, brain, adrenal gland, lung and gastrocnemius muscle. To get further insights into the relationship between GSH and AA regeneration, effects of PEG–AOase on plasma AA levels were also observed with BSO-treated rats. Administration of BSO markedly decreased GSH levels in the liver and kidney (Figure 7A). Although administration of 1 unit of PEG–AOase/kg of body weight did not appreciably affect plasma AA levels in control rats, the same dose of the enzyme markedly decreased plasma AA levels in BSO-pretreated rats (Figure 7B). The increased levels of plasma DAA in PEG–AOase-treated animals were similar in the control and BSO-treated groups.
DISCUSSION

The present study demonstrates that AA oxidized in the circulation is reductively regenerated extremely rapidly to maintain its steady-state levels in plasma and that hepatic GSH plays an important role in the regeneration mechanism.

It has been well documented that MDAA rapidly undergoes dismutation to form AA and DAA and the resulting DAA rapidly decomposes to di-keto-L-gulonic acid under physiological conditions [2,4]. Although PEG–AOase rapidly oxidized AA to MDAA both in isolated plasma and blood samples, the amounts of MDAA and DAA formed were fairly small as compared with that of the oxidized AA. Furthermore, both MDAA and DAA disappeared at similar rates (a half-life of 4 min) irrespective of the presence of blood cells. Biochemical analysis revealed that DAA spontaneously decomposed with half-lives of 5 and 4 min in PBS and fresh plasma respectively; most DAA as well as AA oxidized by the enzyme in vitro was recovered as oxidative metabolites including di-keto-L-gulonic acid (results not shown). Although di-keto-L-gulonic acid is one of the major oxidation products of AA, MDAA and DAA, it undergoes non-enzymic decomposition to form other metabolites including 3,4-dihydroxy-2-oxobutanal [4]. It should be noted that erythrocytes uptake DAA via GLUT and the transported DAA is subsequently reduced intracellularly to AA [34–36]. Previous studies have also revealed that erythrocytes directly reduce extracellular ascorbate radicals [34,37–39]. Because the transient increase of MDAA in PEG–AOase-treated blood was smaller than that in plasma and the rate of DAA disappearance in plasma and blood were similar (see Figure 1), the two mechanisms for AA regeneration by erythrocytes seem to operate minimally, particularly when plasma AA is oxidized rapidly.

Although a fairly high dose of PEG–AOase (1 unit/kg of body weight) oxidized the circulating AA to form MDAA as effectively as in isolated blood and plasma, it failed to decrease the steady-state levels of plasma AA in vivo (compare Figures 1 and 2). Assuming 10 ml for the plasma volume of 200 g rats, the initial concentration of the administered enzyme would be approx. 20 m-units/ml of the circulating plasma; this dose of the enzyme would have been sufficient for rapidly oxidizing the AA in isolated plasma (approx. 60 μM). However, the administration of this dose of the enzyme failed to decrease AA levels in the circulation. Under identical conditions, plasma AA levels decreased rapidly only after isolation of the blood samples from the PEG–AOase-induced animals as observed in in vitro experiments with fresh plasma (see Figure 2B), suggesting that the administered enzyme continuously oxidized the circulating AA and the oxidized AA metabolite(s) was reductively regenerated to AA extremely rapidly by some unknown mechanism to maintain its steady-state level in plasma.

To investigate the ability of animals to maintain steady-state levels of the circulating AA, we tested the effect of various doses of a long-acting PEG–AOase. The enzyme started to decrease the circulating AA at doses higher than 2 units/kg of body weight in control, ODS and STZ-treated rats. This observation suggests that the activity of animals to regenerate the circulating AA from its oxidized metabolite(s) for the maintenance of its steady-state levels in plasma is as high as 2 μmol/min per kg of body weight.

Because PEG–AOase dose-dependently decreased the circulating AA in control, ODS and STZ-treated rats in a similar fashion, de novo synthesis of AA and uptake of DAA by a GLUT system followed by its intracellular reduction may not account for the rapid regeneration of the circulating AA from its oxidized metabolite(s) particularly when the rate of AA oxidation is high. It should be noted that, although the dose-dependency of the enzyme to decrease the circulating AA in STZ-treated rats was similar to that of control animals, the steady-state levels of plasma AA in the former (30.7 ± 5.5 μM) were significantly lower than those of the latter (57.4 ± 2.6 μM). This observation is consistent with the findings that plasma AA levels in patients with diabetes mellitus are lower than those of healthy subjects [40]. Thus the activity to scavenge toxic free radicals in and around the circulation would be decreased in diabetic subjects. Alternatively, the generation of reactive oxygen species would be increased in diabetic subjects, thereby resulting in low steady-state levels of the circulating AA.

The steady-state levels of AA in the circulation remained unchanged even in the presence of PEG–AOase (1 unit/kg of body weight) sufficient for decreasing AA levels in fresh plasma. Biochemical analysis revealed that PEG–AOase selectively decreased renal AA levels in a dose-dependent manner with a concomitant decrease of the circulating AA (see Figure 5). When the circulating AA was depleted by a high dose of PEG–AOase (50 units/kg of body weight), renal AA levels rapidly decreased by approx. 50% and remained at low levels for 180 min. Assuming 1 g for the renal weight of 200 g rats, approx. 17 nmol of renal AA would have been consumed within 1 min in animals administered with 50 units of the enzyme/kg of body weight. This amount of AA is identical with that of plasma AA that could be filtered by the glomerulus [41]. Since bilateral nephrectomy did not affect plasma levels of AA and administration of PEG–AOase dose-dependently decreased plasma AA levels similarly in control and nephrectomized rats (results not shown), the kidney may not play a major role in the reductive regeneration of AA, although the renal AA level depends on its plasma levels. It is not clear at present why renal AA did not decrease to lower levels than 50% even if extremely high doses of PEG–AOase were administered. It has been well documented that AA and DAA are transferred across plasma membranes of various cells and tissues via active SVCT [42] and bidirectional GLUT systems [43–46] respectively. To our surprise, the levels of AA in liver, brain, lung, muscle and adrenal gland remained unchanged even after depletion of plasma AA (see Figure 5). This observation suggests the presence of a special mechanism that maintains the steady-state levels of cellular AA in these tissues. In contrast, the renal level of AA was affected significantly by PEG–AOase. It should be noted that most low-molecular-mass nutrients filtered by the glomerulus, such as glucose and amino acids, are reabsorbed across renal tubule cells by some transepithelial transport systems in the kidney [41]. Because renal proximal tubules are highly enriched with both SVCT [42] and GLUT [43–46], the filtered AA and DAA would be reabsorbed and transferred to the circulation to maintain their steady-state levels in the kidney and plasma. Thus the renal AA level seems to reflect the balance between the two systems, the one utilized AA within the kidney and the other utilized AA derived from plasma via glomerular filtration (and/or peritubular) mechanism and transepithelial transport to the circulation (salvage system). Depression of plasma AA might decrease not only glomerular filtration of AA (and DAA) but also renal accumulation of the two metabolites. The decreased levels of AA in PEG–AOase-treated animals suggests the importance of the filtered AA and DAA for the maintenance of their steady-state levels in the kidney. It should be noted that the renal levels of AA remained unchanged after 30 min of PEG–AOase administration. Since most of the nutrients including AA are reabsorbed effectively by renal brush border membranes of proximal tubules, the filtered AA and DAA may not affect their steady-state levels in the lower portions of nephrons than proximal tubules, such as Henle’s loop, distal tubules and collecting ducts.
Thus the dynamic aspects of metabolism and transport of AA and DAA at proximal tubules and the lower portion(s) of nephron structures seem to differ; they occur rapidly in the former and slowly in the latter. The possible presence of such zonation of metabolism and transport of AA and DAA within nephron structure should be studied further.

The reductive regeneration of AA from its oxidized forms requires reducing cofactors, such as NAD(P)H and GSH [19,21]. Because both GSH and NAD(P)H are localized ubiquitously with high concentrations, most cells and tissues may have sufficient capacity to catalyse the reductive regeneration of AA. It should be noted, however, that administration of a high dose of PEG–AOase (50 units/kg of body weight) slightly, but significantly, decreased GSH levels in the liver (Figure 6). Under identical conditions, PEG–AOase had no appreciable effect on AA levels in tissues except for the kidney. Thus hepatic GSH might play important roles in the maintenance of the circulating AA. To test this possibility, we investigated the effect of BSO, a specific inhibitor of GSH synthesis, on the steady-state levels of the circulating AA in animals treated with PEG–AOase. When hepatic GSH levels had been decreased by BSO, PEG–AOase (1 unit/kg of body weight) significantly decreased AA levels in the circulation (see Figure 7B). Thus hepatic GSH seems to be responsible, at least in part, for the reductive regeneration of AA from its oxidized metabolite(s) in the circulation. In this context, the presence of a transmembranous enzyme that catalyses the reduction of ascorbate free radicals has been described in hepatocytes, neuronal cells and red blood cells [34,47–49]. It has also been reported that treatment of erythrocytes with N-ethylmaleimide at concentrations sufficient for alkylating most intracellular GSH increased the concentration of extracellular ascorbate radicals [38]. Although AA levels in fresh plasma and blood were similarly decreased by a low dose of PEG–AOase, the transient increase of MDAA, but not DAA, was slightly lower in the latter than in the former (see Figure 1). Thus the transmembranous enzyme in the liver and erythrocytes to reduce MDAA to AA may also operate, at least in part, to regenerate the circulating AA. Since the rates of AA decrease by PEG–AOase were similar with isolated blood and plasma samples, hepatic GSH seems to play predominant roles in the regeneration of the circulating AA. A transmembrane mechanism to catalyse the GSH-dependent reduction of MDAA to form AA, similar to that of the NADH-dependent reductase, might operate in the liver. This possibility should be studied further.

Because DAA is unstable and irreversibly decomposed to form di-keto-L-gulonic acid, reductive regeneration of AA from MDAA might be of critical importance for the maintenance of AA levels in the circulation irrespective of the ability of animals to synthesize AA, a potent antioxidant that protects aerobic life from oxidative stress.

**AUTHOR CONTRIBUTION**

Emiko Kasahara, Misato Kashiba, Mika Jikumaru, Daisuke Karatsune, Kumi Orita, Yurika Yamate and Kenjiro Hara performed the study. Atsuo Sekiyama and Eisuke Sato contributed to this study with their critical discussion and suggestion. Masayasu Inoue provided most of the ideas of the experiments and his grant supported the study.

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