Peer-reviewed version available at *Nutrients* **2020**, *12*, 533; doi:10.3390/nu12020533

*Article*

# Acai Extract Increases the Red Blood Cell Population via Erythropoietin Upregulation in Mice

**Shuichi Shibuya [1,2], Toshihiko Toda [2], Yusuke Ozawa [2] and Takahiko Shimizu [1,2,*]**

[1] Aging Stress Response Research Project Team, National Center for Geriatrics and Gerontology, 7-430 Morioka-cho, Obu, Aichi 474-8511, Japan; s-shibuya@ncgg.go.jp (S.S.)
[2] Department of Endocrinology, Hematology and gerontology, Chiba University Graduate School of Medicine, 1-8-1 Inohana, Chuo-ku, Chiba, Chiba, 260-8670, Japan; hik_toda-jac@proteome.jp (T.T.); ozawayusuke3@gmail.com (Y.O.)
* Correspondence: shimizut@ncgg.go.jp; Tel.: +81-562-44-5651; Fax.: +81-562-48-2373

**Abstract:** Acai (*Euterpe oleracea* Mart. Palmae, Arecaceae) is a palm plant native to the Brazilian Amazon. It contains many nutrients, such as polyphenols, iron, vitamin E, and unsaturated fatty acids, so in recent years, many of the antioxidant and anti-inflammatory effects of acai have been reported. However, the effects of acai on hematopoiesis have not been investigated yet. In the present study, we administered acai extract to mice and evaluated its hematopoietic effects. Acai treatment significantly increased the erythrocytes, hemoglobin, and hematocrit contents compared to controls for four days. We then examined the hematopoietic-related markers following a single injection. Acai administration significantly increased the levels of the hematopoietic-related hormone erythropoietin in blood compared to controls and also significantly upregulated the gene expression of *Epo* in the kidney. Furthermore, in the mice treated with acai extract, the kidneys were positively stained with the hypoxic probe pimonidazole in comparison to the controls. These results demonstrated that acai increases the number of blood cells through an increased erythropoietin expression via hypoxic action in the kidney. Acai can be expected to improve motility through hematopoiesis.

**Keywords:** acai; erythropoiesis; erythropoietin

## 1. Introduction

To maintain oxygen homeostasis, mammals have hematopoietic regulatory mechanisms, including erythropoiesis. Erythropoietin (EPO) is a hematological factor mainly expressed in the kidney in adults [1]. EPO is induced under conditions of reduced oxygen levels as well as blood loss [2]. The *Epo* transcription is regulated by hypoxia-inducible transcription factors (HIFs), which have two oxygen-responsive sites associated with prolyl hydroxylase and lead to degradation by ubiquitination under normoxia [3]. This evidence demonstrates that the redox states on renal proteins containing HIF are potential indicators of erythropoiesis in adult mammals.

Acai (*Euterpe oleracea* Mart. Palmae, Arecaceae) is a large palm plant found in the northern region of South America, called the "Amazon", in Brazil. Acai berries have a high polyphenol content, including anthocyanins such as cyanidine-3-glucoside (C3Glc), cyanidine-3-diglucoside, and cyanidin-3-rutinoside, which contribute to antioxidant activity [4]. In several rodent studies, the benefits of acai intervention have been reported to include improving cardiac dysfunction following myocardial infarction [5], protection from diet-induced obesity [6] and hepatic steatosis [7], prevention of brain oxidative damage [8], and modulation of age-related hippocampal inflammation [9]. However, no studies have determined the erythropoietic effect of acai on renal redox alteration.

In the present study, in order to clarify the erythropoietic action of acai, we administered acai extract to mice and examined the relationship between the erythropoietic factors and the redox change in the kidney.

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**Table 1.** Contents in 100 g of acai extract

| Contents | | Fatty acids | (%) |
|---|---|---|---|
| Energy intake | 82.0 kcal | Palmitic acid (C16:0) | 22.5 |
| Protein | 1.4 g | Palmitoleic acid (C16:1) | 3.3 |
| Total lipids | 6.9g | Stearic acid (C18:0) | 1.9 |
| Carbohydrates | 2.0g | Oleic acid (C18:1) | 61.2 |
| Dietary fiber | 3.3 g | Linoleic acid (C18:2) | 10.6 |
| Total polyphenols | 390 mg | Linolenic acid (C18:3) | 0.5 |
| Iron | 1.0 mg | | |

## 2. Materials and Methods

### *2.1. Animals*

C57BL/6NCrSlc mice were purchased from Japan SLC (Shizuoka, Japan) and inbred in our own cohorts. The animals were housed under a 12-h light/dark cycle and fed an MF diet (Oriental Yeast Co., Ltd., Tokyo, Japan) *ad libitum*. The mice were maintained and studied according to the protocols approved by the Animal Care Committee of the Chiba University (ethical code: 29-277).

### *2.2. Administration*

Acai extract (Table 1, Lot. 171115 and 180622) provided by FRUTAFRUTA, Inc. (Tokyo, Japan) was made by finely grinding whole fruit and filtrating with a #30 strainer. The extract was orally administered at 10 ml/kg/day via gavage to mice 1 time ($n = 8$) and for 4 days ($n = 4$) at 12-16 weeks of age. C3Glc (NS380102) was purchased from Nagara Science (Gifu, Japan). ASP1517 (FG-4592, #15294) was purchased from Cayman Chemical (Ann Arbor, MI, USA). The water-dissolved C3Glc (50 mg/kg, $n = 6$) and 0.5% carboxymethyl cellulose-suspended ASP1517 (80 mg/kg, $n = 7$) were administered orally once to littermate mice of the acai-treated cohort. The study was performed using the blood and kidney tissue of animals collected under anesthesia 2-3 h after the final administration.

### *2.3. Histology*

To evaluate the tissue hypoxic area, Hypoxyprobe™-1 Omni (Hypoxyprobe, Inc., Burlington, MA, USA) was used [10]. Mice were sacrificed 15 min after being intraperitoneally injected with anesthetic and 60 mg/kg of pimonidazole. The kidney was fixed in a 4% paraformaldehyde phosphate buffered saline (PBS) (Nacalai Tesque, Inc., Kyoto, Japan) and embedded in paraffin. The rehydrated sections were antigen retrieved with 10 mM citrate buffer (pH 6.0, with 0.05% Tween 20) at 95°C for 30 min, washed with PBS containing 0.1% Tween 20 three times, and intrinsic peroxidases were quenched with 3% $H_2O_2$ for 30 min. We performed blocking with 3% goat serum or Blocking One Hitsto (Nacalai Tesque) and then treated samples with 1:200 or 1:20 diluted anti-pimonidazole antibody (Hypoxyprobe, Inc.). The pimonidazole-positive area was evaluated by the QWin V3 imaging software program (Leica, Wetzlar, Germany) or a BZ-X710 analyzer (Olympus Co., Tokyo, Japan) using the ABC staining kit (Vector Labs., Inc., Burlingame, CA, USA) or the FITC-fluorescence method (goat anti-rabbit antibody #AP307F, Sigma and Fluoro-KEEPER Antifade Reagent with DAPI #12745-74; Nacalai Tesque), respectively.

### *2.4. Measurement of EPO*

The plasma EPO level was measured using the Mouse Erythropoietin Quantikine ELISA kit (#MEP00B; R&D Systems, Inc., Minneapolis, MN, USA) according to the manufacturer's instructions. In brief, thawed plasma was diluted 2-fold by calibrator diluents and then incubated on an

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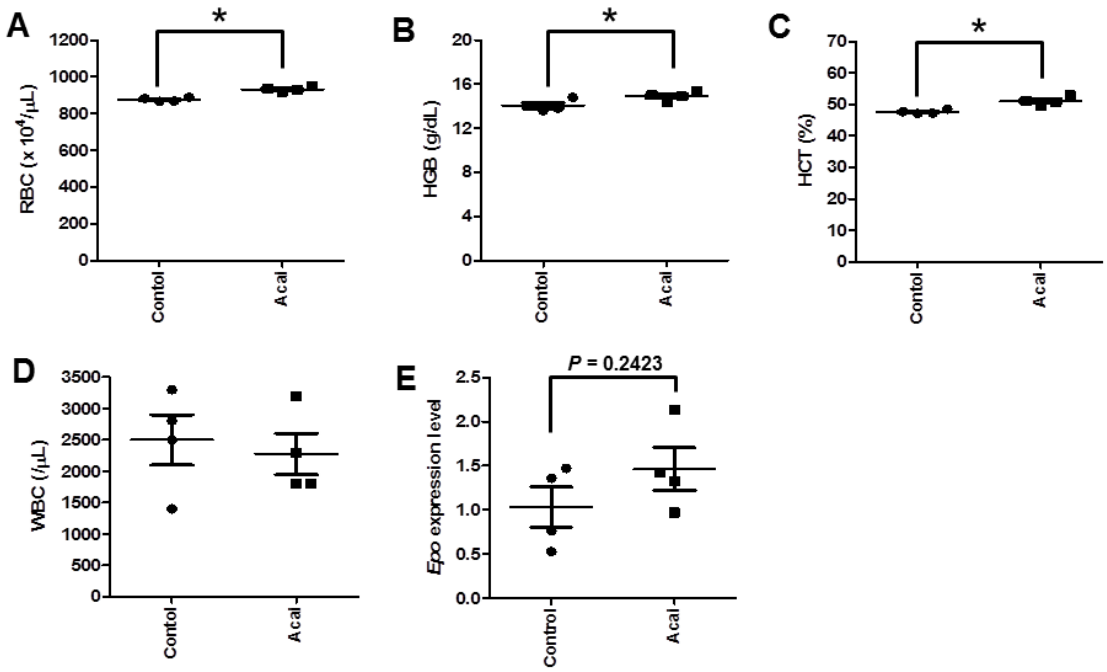


**Figure 1.** Acai extract alters hematological parameters. (A-D) The RBC (A), HGB (B), HCT (C) and WBC (D) in blood treated with acai extract (10 g/kg) daily for 4 days orally, as well as indicated in Table 2. (E) The relative *Epo* transcript level in kidney after the oral administration of acai extract (10 g/kg) daily for 4 days. Error bars indicate the standard deviation. *P < 0.05 by t-test.

antibody-coated microplate with each volume of assay diluents for 2 h using an orbital shaker. Washed wells were treated with antiserum conjugate for 2 h and with substrate mixture for 30 min. The optical density measured at 450 and 540 nm was analyzed with the standards curve using the 4-parameter logistic curve-fit.

*2.5. Quantitative polymerase chain reaction (PCR)*

Total RNA was extracted from kidney using the RNAlater (ThermoFisher Scientific) and the Sepasol-RNA I Super G reagent (Nacalai Tesque) according to the manufacturer's instructions. The cDNA was synthesized from 1 mg total RNA using ReverTra Ace qPCR RT Master Mix (Toyobo, Osaka, Japan). Real-time PCR was performed with the SsoAdvanced SYBR Green Supermix (Bio-Rad) on a Mini-Opticon (Bio-Rad) according to the manufacturer's protocols. All data were normalized to the level of the housekeeping gene β-Actin/*Actb*. The following primers were used for the analysis: *Actb*, forward, 5'-GCC CTA GGC ACC AGG GTG TGA-3', and reverse, 5'-TCC TCA GGG GCC ACA CGC A-3'; *Epo*, forward, 5'-TCA TCT GCG ACA GTC GAG TTC TG-3', and reverse, 5'-GGT ATC TGG AGG CGA CAT CAA TTC-3'.

*2.6. Hematological cytometry*

The number of red blood cells and leukocytes, hemoglobin levels, and hematocrit levels were measured by the Oriental Yeast hematology analyzing service (Tokyo, Japan).

*2.7. Statistical analyses*

The statistical analyses were performed using Student's *t*-test for comparisons between two groups and a one-way analysis of variance/Tukey's test for comparisons of three or more groups.

Differences between the data were considered significant when the *P* values were less than 0.05. All data are expressed as the mean ± standard deviation (SD).

**Table 2.** Effect of acai superfine extract on the hematological parameters in mice ($n = 4$).

| | Control | 10 g/kg of acai for 4days |
|---|---|---|
| WBC (/μL) | 2500 ± 804 | 2275 ± 660 |
| RBC (× $10^4$/μL) | 876 ± 11 | 931 ± 12* |
| HGB (g/dL) | 14.1 ± 0.5 | 14.9 ± 0.4* |
| HCT (%) | 47.6 ± 0.6 | 51.1 ±1.4* |
| MCV (fL) | 54.4 ± 0.7 | 54.9 ± 0.8 |
| MCH (pg) | 16.0 ± 0.5 | 16.0 ± 0.4 |
| MCHC (%) | 29.5 ± 0.8 | 29.3 ± 0.9 |
| PLT (× $10^4$/μL) | 95 ± 5 | 97 ± 13 |
| RET (‰) | 27.8 ± 4.8 | 23.0 ± 1.2 |

WBC, leukocyte content; RBC, erythrocyte content; HGB, hemoglobin; HCT, hematocrit; MCV, mean cell volume; MCH, mean cell hemoglobin; MCHC, mean cell hemoglobin concentration; PLT, platelet content; RET; reticulocytes. *indicates a significant difference

## 3. Results

### *3.1. Acai extract alters hematological parameters*

First, to investigate the hematological effects, we administered acai extract and measured the hematological parameters in mice. Treatment with acai extract significantly increased the erythrocyte content (RBC), hemoglobin level (HGB) and hematocrit level (HCT) but not the leukocyte content (WBC) in blood for 4 days (Figure 1A-D and Table 2). Acai extract failed to increase the reticulocyte (RET) for 4 days, but an increased trend in the *Epo* expression was observed in the kidney in the same period (Table 2 and Figure 1E). Acai extract also induced no significant change in the erythrocyte mean cell volume (MCV), mean cell hemoglobin (MCH), mean cell hemoglobin concentration (MCHC) or platelet content (PLT) (Table 2). These results suggest that acai induced a hemopoietic effect via the enhancement of maturation and intact erythrocytes.

### *3.2. Acai extract acutely upregulates the EPO contents in blood*

In general, the *Epo* expression is transiently stimulated by a hypoxic condition [2]. In this context, we performed a transient experiment, administering acai extract to mice and measuring the EPO contents in plasma 2-3 h after treatment. The acai treatment caused a significant increase in the plasma EPO level compared with vehicle control (Figure 2A). Furthermore, acai upregulated the *Epo* transcript level in the kidney compared with the control (Figure 2B). The erythropoiesis inducer ASP1517 also upregulated both the EPO contents in plasma and the *Epo* transcript level in kidney (Figure 2A and 2B). In contrast to acai, the administration of C3Glc caused no significant change in either the EPO contents or the *Epo* level (Figure 2A and 2B). Furthermore, the relationship between the plasma EPO concentration and the kidney *Epo* transcript level was positive (Figure 2C). These results suggest that acai extract transcriptionally induced EPO production in the kidney.

### *3.3. Acai extract induces a renal hypoxic condition*

Finally, to investigate the relationship between EPO induction and renal hypoxia under acai administration, we administered the hypoxic probe pimonidazole to mice and histologically detected renal hypoxia. By immunostaining with pimonidazole, which accumulates in hypoxic areas, acai extract was shown to induce renal hypoxia (Figure 3A). A quantitative analysis with the fluorescence intensity of pimonidazole staining showed that acai created a significantly larger hypoxic area than was seen in the controls (Figure 3B and 3C). Furthermore, acai induced renal hypoxia mainly at the corticomedullary junction where erythropoietin is produced (Figure 3A and

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3B). These results suggest that renal erythropoietin production caused by acai depends on the hypoxic reaction in the kidney.

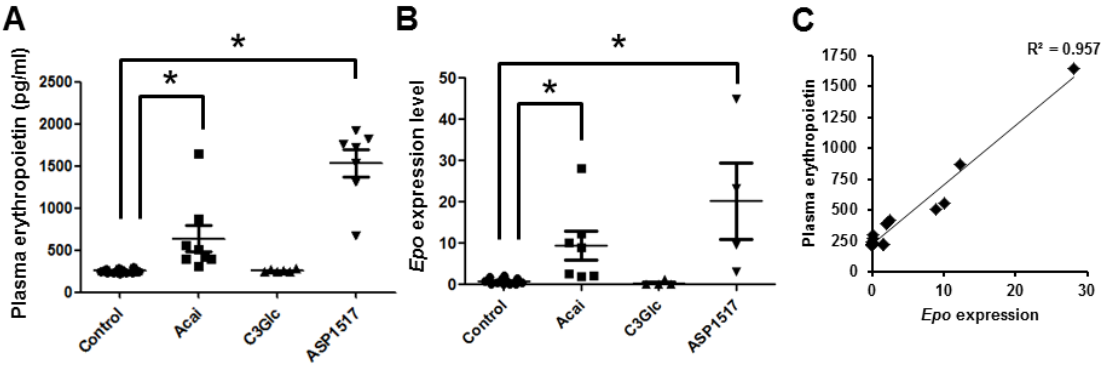


**Figure 2.** Acai extract upregulates both the plasma EPO concentration and kidney *Epo* expression. (A) The plasma EPO concentration in mice 2-3 h after the oral administration of acai extract (10 g/kg), C3Glc (50 mg/kg), and ASP1517 (80 mg/kg). (B) Relative *Epo* transcript levels in kidney 2-3 h after the oral administration of acai extract (10 g/kg), C3Glc (50 mg/kg) and ASP1517 (80 mg/kg). (C) Relationship between the plasma EPO concentration and *Epo* transcription in kidney. Error bars indicate the standard deviation. *P < 0.05 by an ANOVA/Tukey's test.

*4. Discussion*

Since EPO, a hematopoietic hormone that controls erythropoiesis, is produced in response to tissue hypoxia, athletes often incorporate high-altitude training. The induction of EPO expression mainly involves the transcription factor HIF induced by hypoxia [3]. Under hypoxic conditions, degradation of HIF is inhibited by ubiquitination due to the suppression of proline hydroxylase, thereby promoting hematopoiesis [3]. ASP1517 induces the production of erythrocytes via the prevention of HIF degradation by inhibiting the proline hydroxylase [11]. Acai significantly induced hypoxia in the kidney (Figure 3), suggesting that this hypoxic condition enhances the HIF degradation inhibitory switch and increases the EPO levels. Acai seed extract induced an anti-hypertensive effect in experimental hypertension [12], suggesting that hypoxia induction by acai may be due to a blood flow reduction in the kidney.

Acai contains large amounts of iron and long-chain fatty acids, such as oleate, palmitate and linoleate (Table 1). The monounsaturated oleic acid depresses the cytokine expression *in vitro* [13]. Furthermore, acai treatment attenuated renal ischemia/reperfusion injury via protection against renal oxidative stress in diabetic and spontaneously hypertensive rats [14, 15]. In a rat study, acai treatment significantly decreased the 8-isoprostane immunostaining level and thiobarbituric acid reactive substances level in the kidney [15], suggesting the transition from an oxidative to a reductive condition in the kidney. These findings show that acai induces erythropoietic action via an altered redox status in the kidney.

Polyphenols protect against reactive oxygen species-induced hemolysis via increased red blood cell integrity associated with the inhibition of lipid peroxidation [16-19]. Similarly, red cabbage extracts rich in anthocyanins rescue oxidative hemolysis in streptozotocin-induced diabetes [20]. Anthocyanins also exert antisickling activity by stabilizing red blood cells and their membranes and inhibiting polymerization on hemoglobin S [21, 22]. These results suggest that acai enriched in polyphenols protects against anemia of various causes. Conversely, Mn abundant in acai, suppresses Fe absorption, suggesting a risk for anemia [23]. Further research on the anti-anemic effects of acai is needed.

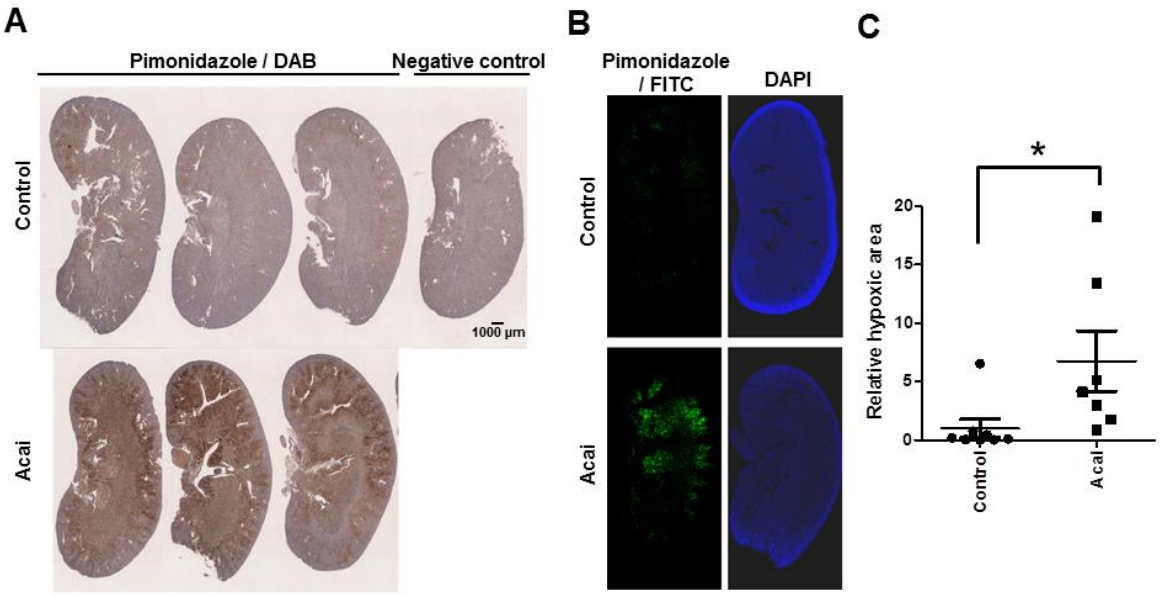


**Figure 3.** Acai extract induces a hypoxic reaction in kidney. (A-C) The pimonidazole-positive hypoxic area in kidney sections 2 h after the oral administration of acai extract (10 g/kg) was detected by 3,3′-diaminobenzidine (A) and fluorescein isothiocyanate (B and C). Scale bar denotes 1 mm. Error bar indicates the standard deviation. *P < 0.05 by *t*-test.

Recently, a number of useful effects of acai treatment have been reported. In a rodent study, acai reduced the incidence of tumor, tumor cell proliferation, and multiplicity and size of tumors [24-28], suggesting anticarcinogenic activities. Stoner et al. reported that acai inhibited the progression of esophageal tumorigenesis by reducing the serum IL-5 and IL-8 levels and increasing the serum antioxidant capacity and IFNγ levels [24]. Fragoso et al. also reported that acai prevented urinary bladder carcinogenesis by reducing the DNA damage and expression of p16 and PCNA [25]. Furthermore, polyphenolics containing acai reduced the proliferation of HL-60 leukemia cells through apoptosis induced by caspase-3 activation [29]. Velutin, a flavonoid contained in acai, also suppressed the LPS-induced inflammation in macrophage-derived cells [30]. Acai showed no toxicity in experimental models [25, 26, 28, 31-33] nor any significant differences in the body weight or food consumption [24-26, 33]. These results suggest that acai therapy is a novel and safe strategy for treating various pathologies, including cancer.

In summary, acai induces an erythropoietic effect associated with renal hypoxia. These findings provide valuable insight into the potential utility of acai for future research on hematopoiesis in humans.

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**Author Contributions:** SS, TT, and TS designed the study. SS and TS wrote the manuscript. SS, YO, and TT performed the experiments. SS analyzed the data, and TS edited the article. TS coordinated and directed the project.

**Funding:** This research was funded by FRUTA FURUTA, Inc.

**Conflicts of Interest:** The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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