Efficacy of Tokishakuyakusan on the Anemia in the Iron-Deficient Pregnant Rats

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Received January 26, 2007; accepted May 9, 2007; published online May 11, 2007

Iron-deficiency anemia not only causes insufficient oxygen delivery to the body of the mother, but creates serious conditions in the fetus, such as insufficient oxygen delivery and poor development, and its treatment has become an important issue. To elucidate the mechanism of the anemia-ameliorating action of Tokishakuyakusan, we investigated the effect of administration of Tokishakuyakusan on anemia-related parameters and the serum transferrin and erythropoietin levels, erythrocyte morphology, and bone marrow cells in pregnant rats and in pregnant rats with iron-deficiency anemia. The results showed that Tokishakuyakusan significantly improved the low erythrocyte count, hemoglobin, and hematocrit values of the pregnant rats in the iron-deficient state, increased the proportion of normal erythrocytes in terms of erythrocyte morphology, increased the proportion of erythroblasts among bone marrow cells, and also significantly increased the erythropoietin and transferrin levels in the blood. These findings suggested that Tokishakuyakusan promotes erythrocyte differentiation in iron-deficiency anemia, and that it possesses anemia-ameliorating efficacy.

Key words Tokishakuyakusan; iron-deficiency anemia; pregnancy; rat

The anemia in pregnant women is a state of physiological hemodilution, because the increase in plasma that begins around the 6th week always exceeds the increase in erythrocyte volume, and they exhibit so-called hydremia.^{1,2)} In addition, iron deficiency develops as a result of the increase in iron demand associated with fetal growth and development, *etc.*, and pregnant women develop manifestations of anemia.³⁾ Since iron deficiency and anemia are directly involved in the transport and utilization of oxygen, which is important to energy production, treatment is very important, because they not only cause insufficient oxygen delivery to the body of the mother but serious conditions in the fetus, such as insufficient oxygen delivery and poor development.^{4,5)}

Oral iron preparations are the most common means of treating anemia in pregnancy,^{6,7)} but taking them very often results in adverse effects, such as heartburn, nausea, diarrhea, and constipation,^{8,9)} and they are frequently linked to worsening of drug compliance.¹⁰⁾

The ancient medical text “Kinki Yoryaku” states that “Tokishakuyakusan is effective against intra-abdominal diseases and pain in pregnant women,” and the Chinese herbal medicine Tokishakuyakusan has been used in Japan to treat anemia of pregnancy, infertility, and dysmenorrhea. In a retrospective clinical survey the authors previously reported that Tokishakuyakusan is more useful than iron preparations in terms of efficacy against anemia, safety, and economy,¹¹⁾ that Tokishakuyakusan was not found to be teratogenic in an animal experiment conducted in rats,¹²⁾ and that Tokishakuyakusan improves iron-deficiency anemia.¹³⁾ However, many aspects of the mechanism of action of Tokishakuyakusan in improving anemia are still unknown.

In the present study we aimed to elucidate the mechanism of action of Tokishakuyakusan in improving anemia. We simulated pregnancy, a period when anemia tends to occur, and assessed the effect of Tokishakuyakusan on anemia-re-

lated parameters in blood, on transferrin and erythropoietin levels, erythrocyte morphology, and bone marrow cells in pregnant rats and iron-deficient pregnant rats.

MATERIALS AND METHODS

Animals used and animal care conditions (preparation of pregnant rats and iron-deficient pregnant rats).

Nine-week-old male and female Wistar rats were purchased from Sankyo Labo Service Corporation (Tokyo), and after preliminary care for 1 week, a pair of male and female rats was placed in each cage, and a mating environment was prepared. Pregnancy was judged to have occurred by confirming a vaginal plug in the female rats and checking for the presence of sperm under a microscope by the smear test. The day on which the presence of sperm in the vagina was confirmed was counted as pregnancy day 0, and the pregnant rats were cared for until day 20, just before delivery.

After confirming pregnancy, the “standard-diet pregnant rats” were given a diet of standard chow CE-2 (Clea Japan Inc., Tokyo; iron content: 343.5 $\mu\text{g/g}$) and the “iron-deficient-diet pregnant rats” were given an iron-deficient diet (formula AIN-93G for rats, Funabashi Farms, Chiba; maximum iron content: 3.8 $\mu\text{g/g}$). The rats were given free access to purified water and cared for in an environment maintained at a temperature of $23 \pm 1^\circ\text{C}$, a humidity of $55 \pm 5\%$, and on a 12-h lights-on cycle (08:00–20:00).

The care of all animals was in accordance with NRC guidelines, and the study was conducted with the approval of the Experimental Animal Ethics Committee of Showa Pharmaceutical University.

Experimental Materials Tokishakuyakusan extract powder (iron content: 51 $\mu\text{g/g}$) was supplied by the Tsumura & Co. The bulk powder used was prepared by mixing the following ingredients in the proportions stated: Japanese Phar-

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Fig. 1. Tokishakuyakusan 3D HPLC Pattern

macopoeia (JP) Shakuyaku (peony root) 4.0 g, JP Sojutsu (*atractylodes lancea* rhizome) 4.0 g, JP Takusha (*alisma* rhizome) 4.0 g, JP Bukuryou (hoelen) 4.0 g, JP Senkyu (cnidium rhizome) 3.0 g, and JP Toki (Japanese angelica root) 3.0 g. A decoction that had been heat extracted with purified water was spray dried, and 4.0 g of it contained the daily dose of extract preparation.

A 3-dimensional high performance liquid chromatography (HPLC) chart of the Tokishakuyakusan is shown in Fig. 1. Tokishakuyakusan was extracted with methanol under ultrasonication for 30 min. The solution was filtered and then determined for HPLC with a photodiode-array detector (SPD-M10AVP; Shimadzu Co., Tokyo, Japan). The separation column was a TSK GEL ODS-80TS column (250×4.6 mm; Tosoh, Tokyo, Japan), and the column temperature was maintained at 40 °C. Binary gradient elution was performed with mobile phase A (0.05 mol/l ammonium acetate–acetic acid buffer, pH 3.6) and mobile phase B (100% acetonitrile). The separation was initiated with 90% A and 10% B, and the concentration of B was linearly increased to 100% over a period of 60 min. The flow rate was set at 1.0 ml/min. The effluents from the column were detected at absorbencies ranging from 200 to 420 nm using a photodiode-array detector. Data collection and processing for peak analysis were accomplished with CLASS-LC10 System Analysis Software (Shimadzu Co.).

Preparation of the Test Substances and Method of Administration The dose of Tokishakuyakusan extract powder used for the “standard-diet pregnant rats” and “iron-deficient-diet pregnant rats” was 0.8 g/kg, which corresponds to 10 times the usual daily oral dose in humans based on a body-weight conversion. The Tokishakuyakusan was prepared in boiled purified water before use and orally administered to the rats once daily, generally at 10:00 a.m., on days 0

to 20 of pregnancy. As control groups, purified water was orally administered in the same manner during the same period to “standard-diet pregnant rats” and “iron-deficient-diet pregnant rats” that were not given Tokishakuyakusan.

At the end of the preliminary care period the rats supplied for use in the experiment were divided into 5 groups ($n=5$) in such a manner that their hemoglobin (Hb) values and body weights would be uniform. The “standard-diet pregnant rats” given Tokishakuyakusan will be referred to as the PT group, and the control group as the PW group. The “iron-deficient-diet pregnant rats” given Tokishakuyakusan will be referred to as the PAT group, and the control group as the PAW group. A group of non-mated female rats of the same age in weeks that was cared for in the same manner for the same period was used as a normal group and will be referred to as the NW group.

Blood Examination On days 0, 7, 14, and 20 of pregnancy, under ether anesthesia, 0.5 ml of blood each was collected from the tail vein of the rats in which pregnancy had been confirmed, and the erythrocyte count, hemoglobin (Hb) concentration, hematocrit (Ht), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), and reticulocyte count were determined.

Examination of Erythrocyte and Bone Marrow Cell Morphology On day 20 after the start of the experiment blood obtained under ether anesthesia from the tail vein of all of the groups of rats was smeared on a glass slide, Giemsa stained, and prepared for microscopy. Bone marrow fluid was collected from the femur, smeared on a glass slide, stained by the May–Grünwald–Giemsa method, and prepared in the same manner. The morphology of the erythrocytes and bone marrow cells in both smear specimens was examined under a light microscope (Olympus BX50, Olympus, Tokyo). The

Table 1. Variation in the Blood Tests after Drug Administration for Pregnancy

Laboratory parameter	Group 9W ^{I)}	(Baseline)	7 d ^{II)}	14 d	20 d
RBC (10 ⁴ /μl)	NW	707±74	746±15	753±20	773±19
	PW	740±41	722±27	651±17^{a)}	581±21^{a)}
	PT	722±43	710±30	644±22^{a)}	586±33^{a)}
	PAW	736±19	673±22^{a,b)}	599±32^{a,b,c)}	466±16^{a,b,c)}
	PAT	697±27	675±25 ^{a)}	608±34^{a,b,c)}	524±3^{a,b,c,d)}
Hb (g/dl)	NW	16±1	16±0	16±1	16±0
	PW	16±1	15±1^{a)}	14±0^{a)}	12±0^{a)}
	PT	16±1	15±1^{a)}	13±0^{a)}	12±1^{a)}
	PAW	16±0	14±0^{a)}	12±0^{a,b,c)}	9±0^{a,b,c)}
	PAT	15±0	14±1^{a)}	12±0^{a,b,c)}	11±0^{a,b,c,d)}
Ht (%)	NW	46±4	47±1	47±2	49±2
	PW	48±3	45±2	40±1^{a)}	37±1^{a)}
	PT	46±3	45±2	40±3^{a)}	40±2^{a)}
	PAW	49±2	41±2^{a,b,c)}	35±2^{a,b,c)}	27±1^{a,b,c)}
	PAT	47±2	42±2^{a)}	36±2^{a,b,c)}	30±1^{a,b,c,d)}
MCV (fl)	NW	63±1	63±1	63±1	64±1
	PW	65±1	62±1	61±1	63±2
	PT	63±1	63±2	63±3	65±3
	PAW	67±1	61±1	58±1^{a,c)}	57±1^{a,b,c)}
	PAT	67±2	62±1	59±1^{a,c)}	57±1^{a,b,c)}
MCH (pg)	NW	21±1	22±1	21±1	21±1
	PW	22±1	21±1	21±1	21±0
	PT	22±1	21±1	21±1	21±0
	PAW	21±1	21±1	20±1	19±1^{a,b,c)}
	PAT	22±1	21±1	20±1	19±1^{a,b,c)}
MCHC (%)	NW	34±1	35±1	34±1	33±1
	PW	33±1	34±1	34±1	34±1
	PT	34±1	33±1	33±2	33±1
	PAW	32±1	35±1	35±1	34±2
	PAT	33±1	34±1	34±1	33±1
Reticulocyte (0/00)	NW	26±5	22±1	30±7	34±2
	PW	26±5	27±3 ^{a)}	50±5^{a)}	43±4^{a)}
	PT	23±2	27±3 ^{a)}	49±5^{a)}	37±3^{a)}
	PAW	25±5	37±7^{a,b,c)}	43±4^{a)}	20±8 ^{a,b,c)}
	PAT	25±4	36±2^{a,b,c)}	40±5^{a,b,c)}	6±1^{a,b,c,d)}

I) Rat age in weeks. II) The gestational period of Rat. Each value represents mean±S.D., *n*=5. Significantly different among the following at *p*<0.05 by Bonferroni test: Nw, Pw, PT, PAW and PAT. Bonferroni test result is shown; a) NW vs. PW or PT or PAW or PAT, b) PW vs. PT or PAW or PAT, c) PT vs. PAW or PAT, d) PAW vs. PAT. Bold-faced type and underline; significant difference before and after the drug administration at *p*<0.05 by Wilcoxon signed-ranks test.

proportions of cells in three microscope fields of the erythrocyte and the bone marrow smear specimens were calculated respectively.

Measurement of Serum Erythropoietin and Transfer-rin On day 20 after the start of the experiment blood obtained under ether anesthesia from the tail vein of every group of rats was allowed to stand for 30 min at room temperature, and serum was obtained by centrifuging for 15 min at 654×*g*. The erythropoietin concentration by RIA (double-antibody method) and the serum transferrin concentration was measured by EIA.

Statistical Analysis The Bonferroni multiple comparison test was used to compare each of the parameters between groups. The Wilcoxon signed rank test was used to analyze changes before and after each of the parameters within groups only in the Table 1. A significant difference was judged to exist when the *p* value was less than 0.05.

RESULTS

Time Course of Changes in Blood Components First, we investigated the blood data of the “standard-diet pregnant rats” and “iron-deficient-diet pregnant rats” in regard to the

anemia indices, and then we investigated the effect of Tokishakuyakusan on them. The results for each of the parameters measured in the blood examinations are shown in Table 1. As the days of pregnancy passed in the control group for the “standard-diet pregnant rats” (PW group) significant decreases in erythrocyte count and Ht were observed from day 14 onward, and in Hb from day 7 onward, but the reticulocyte count rose, and after peaking on day 14 of pregnancy, tended to gradually decline. From pregnancy day 7 onward there were minor differences in the values for MCV, MCH, and MCHC, which allow differential diagnosis of anemia, but a significant decrease in MCV was observed. The data in relation to the changes in these parameters in the group given Tokishakuyakusan (PT group) showed a significant increase in Ht alone, and no ameliorating effect was seen on any of the other blood parameters. Although MCV had increased to the level in the normal (NW) group, it was not a significant effect. In the control group for the “iron-deficient-diet pregnant rats” (PAW group) the erythrocyte count, Hb concentration, and Ht were all significantly decreased from day 7 onward, and the reticulocyte count had significantly increased from day 7 onward, peaked on day 14, and declined thereafter. Moreover, examination of the MCV, MCH, and MCHC

values revealed a significant decrease in MCV from day 7 onward and a significant decrease in MCH from day 14 onward, but no effect was seen on MCHC. By contrast, in the group given Tokishakuyakusan (PAT group) a significant improvement in the erythrocyte count, Ht, Hb concentration, and reticulocyte count was seen on day 20, but no ameliorating effect was observed on MCV, MCH, or MCHC.

Observations of Erythrocyte and Bone Marrow Cell

Fig. 2. Microscopic Observation of Typical RBC of Rats in NW, PW, PT, PAW and PAT Groups after 20 d ($\times 1000$)

RBC of: NW; water administered normal rats, PW; water administered pregnant rats, PT; Tokishakuyakusan administered pregnant rats, PAW; water administered pregnant anemia rats, PAT; Tokishakuyakusan administered pregnant anemia rats.

Fig. 3. Comparison of Ratio of Erythrocyte Cells Each Group after 20 d
Each value represents mean $\pm$ S.D. $n=5$. The Bonferroni test was used to compare each of the parameters between groups. The result of the significant difference was shown in the article.

Morphology Photomicrographs of erythrocytes in the blood smear specimens are shown in Fig. 2 and the proportions of each of the cells are shown in Fig. 3. Photomicrographs of erythrocytes in the bone marrow specimens are shown in Fig. 4, and the proportions of each of the cells are shown in Fig. 5.

Counts of the hypochromic erythrocytes having a white, punched-out looking center, which is characteristic of iron-deficiency anemia, revealed that they accounted for

Fig. 4. Microscopic Observation of Bone Marrow of Rats in NW, PW, PT, PAW and PAT Groups after 20 d ($\times 300$)

NW; water administered normal rats, PW; water administered pregnant rats, PT; Tokishakuyakusan administered pregnant rats, PAW; water administered pregnant anemia rats, PAT; Tokishakuyakusan administered pregnant anemia rats.

Fig. 5. Comparison of Ratio of Bone Marrow Cells Each Group after 20 d
Each value represents mean $\pm$ S.D. $n=5$. The Bonferroni test was used to compare each of the parameters between groups. The result of the significant difference was shown in the article.

4.3±0.4% of the erythrocytes in the PW group ($n=5$, $p<0.05$ vs. PT or NW), and hemolyzed erythrocytes were also seen. By contrast, an increase in normal erythrocytes was observed in the PT group ($n=5$, $p<0.05$ vs. PW), and hypochromic erythrocytes accounted for 2.3±0.3%. Hypochromic erythrocytes accounted for 38±0.4% of the erythrocytes in the PAW group ($n=5$, $p<0.05$ vs. PAT or NW), as opposed to 19±0.5% in the PAT group ($n=5$, $p<0.05$ vs. PAW), and normal erythrocytes had increased (Figs. 2, 3).

Next, examination of bone marrow cells to detect changes (Figs. 4, 5) that were not manifested in the peripheral blood revealed decreases in erythroblasts (5±1%) and erythrocytes (25±2%) in the PW group ($n=5$, $p<0.05$ vs. PT or NW), compared to the marrow picture in the NW group (Figs. 4, 5: NW: Erythroblast 10±2%, RBC 55±3%, Myeloblast 25±2%, Other cell 10±1%), and an increase in bone marrow cells (Myeloblast 54±1%, Other cell 16±1%) was observed ($n=5$, $p<0.05$ vs. NW). By contrast, an increase in both erythroblasts (10±2%) and erythrocytes (32±1%) was observed in the PT group ($n=5$, $p<0.05$ vs. PW) in comparison with the PW group, and a marrow picture closer to that in the NW group (Fig. 4, NW, PT) was seen. There was an increase in the neutrophil series of cells (45±6%) in the PAW group ($n=5$, $p<0.05$ vs. PAW or NW), and the erythroblasts (2±1%) had decreased ($n=5$, $p<0.05$ vs. NW). In the PAT group, on the other hand, increases were observed in erythrocytes (57±2%) ($n=5$, $p<0.05$ vs. PAW) and erythroblasts (6±3%), particularly in normochromic erythroblasts, which are the final stage of differentiation (Fig. 4, PAT).

Changes in Serum Erythropoietin and Transferrin

The changes in erythropoietin concentration in the serum on pregnancy day 20 are shown in Fig. 6, and the changes in transferrin concentration are shown in Fig. 7.

Assessment of the serum erythropoietin concentration showed increases in both the PW group and the PAW group as a result of pregnancy, in comparison with the NW group. In contrast to these changes in the control groups, even greater significant increases were seen in the PT group and PAT group, which were given Tokishakuyakusan. The serum transferrin concentrations showed a significant decrease in the PW group as a result of pregnancy, in comparison with the NW group, and there was a significant increase in the PAW group. The serum transferrin concentrations were significantly increased by Tokishakuyakusan treatment (Fig. 7).

DISCUSSION

As stated above, in experiments conducted on male rats the authors had previously demonstrated that Tokishakuyakusan has an ameliorating action on the anemia that develops as a result of an iron-deficient diet and hemodilution.¹³ We then performed regular blood examinations, examined erythrocyte and bone marrow cell morphology, and measured serum concentrations of transferrin and erythropoietin, which are used to make the differential diagnosis of anemia. After 1 week of preliminary animal care, pairs of male and female rats were housed together, and pregnancy was almost always confirmed the same day. The erythrocyte counts, Hb concentrations, and Ht values of the pregnant rats significantly decreased as the

Fig. 6. Rat Serum Erythropoietin after 20 d in NW, PW, PT, PAW, and PAT

Each value represents mean±S.D. (NW: 0.1±0.1), $n=5$. * Significantly different among the following at $p<0.01$ by Bonferroni test: NW, PW, PT, PAW and PAT.

Fig. 7. Rat Serum Transferrin Concentration after 20 d in NW, PW, PT, PAW, and PAT

Each value represents mean±S.D., $n=5$. * Significantly different among the following at $p<0.05$ by Bonferroni test: NW, PW, PT, PAW and PAT.

days of pregnancy passed, and the reticulocyte count significantly increased and peaked on day 14, and thus the manifestations of anemia had developed. Even more severe manifestations of anemia were observed in the rats that consumed the iron-deficient diet. Although the pregnancy periods in humans and rats are different, these findings suggest that circulating plasma volume also increases in rats in response to the hormones secreted by the fetal-placental system as a result of becoming pregnant, and thus they exhibit hydremia, in which the erythrocyte counts, Hb concentrations, and Ht values decrease. Hematopoiesis becomes more active to compensate for it, and the reticulocytes increase, suggesting that rats may undergo a series of changes in physiological function the same as in humans. The reticulocyte count appeared to have tended to decrease from day 14 onward as a reflection of the fact that the iron deficiency had progressed. MCV is calculated from the erythrocyte count and the volume ratio of erythrocytes and allows differential diagnosis of anemia. MCV is an indicator of iron-deficiency anemia and is known to be decreased when iron-deficiency anemia is present.¹⁴ MCV decreased significantly in the PW group, which was given iron in the diet, but there was hardly any difference in numerical value from the NW group, and the microcytic anemia did not seem to be very severe. MCV and MCH both decreased in the PAW group, in which iron intake was limited to about 1% of the amount of iron consumed by the groups fed the standard iron-containing diet, suggesting that the iron-deficiency anemia, which is considered to be a microcytic hypochromic anemia, had progressed. When Tokishakuyakusan was given to the rats in this anemic state, it

significantly increased the Ht in the PT group, and it significantly increased the erythrocyte count, Hb concentration, and Ht values in the PAT group. When iron-deficiency anemia develops, erythrocytes fail to take up sufficient iron during erythropoiesis, and hypochromic erythrocytes having a doughnut-like appearance with white centers that appear to have been punched out are formed. These erythrocytes have low oxygen carrying capacity, and when many of them are produced as a result of iron-deficiency anemia, tissues throughout the body are at risk of becoming oxygen-deficient.¹⁵⁾ Occasional hypochromic erythrocytes were seen in the examinations of erythrocyte morphology in the blood smear specimens, but about 9 times as many were seen in the PAW group as in the PW group, and clear progression of the iron-deficiency anemia was observed as a result of the iron-deficient diet. The effect of Tokishakuyakusan in relation to these changes was to increase normal erythrocytes and to significantly reduce the proportion of hypochromic erythrocytes.

Based on these findings in peripheral blood, Tokishakuyakusan apparently did not have much impact on the hematological blood values in the mild dilutional anemic state, but it was found to have an ameliorating effect on the blood morphologically and to have a clear ameliorating effect both on the hematological values in iron-deficiency anemia and morphologically. Moreover, sometimes changes in anemia that are not manifested in peripheral blood are displayed in bone marrow cells. Examination of the morphology of the cells in the bone marrow fluid smear specimens revealed decreased numbers of erythroblasts and erythrocytes and an increased number of bone marrow cells in the PW group compared to the NW group. By contrast, an increase in number of erythroblasts and erythrocytes was seen in the groups given Tokishakuyakusan, and a picture close to that in the NW group was observed. In the PAW group, on the other hand, cells of the neutrophil series increased, and erythroblasts had decreased, but in the group given Tokishakuyakusan there was no increase in neutrophil series cells, and among the erythroblasts, increases in normochromic erythroblasts, which are close to the final stage of differentiation, and erythrocytes were observed. This suggests that Tokishakuyakusan promotes the differentiation of erythrocyte precursor cells to erythrocytes by the hematopoietic process in bone marrow, and as a result causes an increase in normal erythrocytes in the peripheral blood and relieves the manifestations of anemia. Erythropoietin, which is involved in erythrocyte production, and transferrin, which is involved in Hb synthesis, are known to promote erythrocyte differentiation.^{16,17)} An increase in the blood concentration of erythropoietin as a result of pregnancy was seen on pregnancy day 20 in both the PW group and the PAW group, compared to the NW group, and the increase was approximately 5 times greater in the PAW group, in which the iron deficiency was severe. Tokishakuyakusan significantly increased these changes in the control groups even more, suggesting that Tokishakuyakusan particularly promotes differentiation and proliferation of late-stage erythrocyte precursor cells among erythrocyte precursor cells in anemia due to pregnancy, and that its erythropoietic action is promoted. The serum transferrin concentrations measured at the same time showed a significant reduction in

the PW group compared to the NW group, and a significant increase in the PAW group. In contrast to these changes in the control groups, significant increases were observed in the PT group and the PAT group. Part of the decrease in the serum transferrin concentration in the PW group appeared to be due to dilutional anemia, and the increase in the PAW group appeared to reflect the fact that the iron-deficiency anemia was advanced. The increasing effect on the serum transferrin concentration in the PT group and PAT group suggested that Tokishakuyakusan is involved in promoting Hb synthesis.

Tokishakuyakusan significantly improved the low erythrocyte count, Hb, and Ht values of the iron-deficient pregnant rats, increased the proportion of normal erythrocytes in terms of erythrocyte morphology, increased the proportion of erythroblasts among bone marrow cells, and also significantly increased the erythropoietin and transferrin levels in the blood.

These findings suggested that Tokishakuyakusan promotes erythrocyte differentiation in iron-deficiency pregnant anemia, and that it possesses anemia-ameliorating efficacy.

Acknowledgments This work was partly supported by "High-Tech Research Center" Project for Private University: matching fund subsidy from MEXT (Ministry of Education Culture, Sports, Science and Technology) 2004—2008 of Japan. Upon completion of this work, we would like to express our sincere gratitude to Dr. Sumiko Murakami for her generous guidance and cooperation. We also wish to express our deep appreciation to Tsumura & Co. for supplying the Tokishakuyakusan powder.

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