学　位　論　文

Reticulocyte hemoglobin content changes after treatment of anemia of prematurity

(未熟児貧血治療後の網赤血球ヘモグロビン量の変化)

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Original Article

Reticulocyte hemoglobin content changes after treatment of anemia of prematurity

Running title: Chronological changes of RET-He in VLBWI

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Abstract

Background

Iron deficiency (ID) during infancy is associated with poor neurological development, but iron overload causes severe complications; thus, appropriate iron supplementation is vital. Reticulocyte hemoglobin content (RET-He) provides a real-time assessment of iron status and reveals important finding of hemoglobin synthesis in preterm infants. However, existing literature lacks detailed reports assessing changes in RET-He. The aim of this study was to assess the chronological changes in RET-He during oral iron dietary supplementation, and concomitant therapy with recombinant human erythropoietin (rHuEPO) in preterm very-low-birthweight (VLBW) infants.

Methods

VLBW infants, admitted to our neonatal intensive care unit were retrospectively analyzed. Hemoglobin (Hb), reticulocyte percentage (Ret), mean corpuscular volume (MCV), RET-He, serum iron (Fe), and serum ferritin (FER) were recorded. Data at birth (T0), the initial day of rHuEPO therapy (T1), the initial day of oral iron supplementation (T2), 1–2 weeks (T3), 3–4 weeks (T4), 5–6 weeks (T5), 7–8 weeks (T6) from the initial day of oral iron supplementation were extracted, and their changes over time were examined.

Results

RET-He was highest at birth and declined rapidly thereafter, especially after starting rHuEPO therapy. There was no upward trend in RET-He after the initiation of oral iron supplementation, with a slower increase during 5–6 weeks after the initiation of iron therapy.

Conclusions

During the treatment of anemia of prematurity, low RET-He levels may be prolonged. Anemia of prematurity should therefore be assessed and treated on a case-by-case basis, while considering the iron metabolic capacity of preterm infants.

Key words: erythropoietin, iron deficiency anemia, reticulocytes, Reticulocyte hemoglobin content (RET-He), very low birth weight infant

Introduction

Iron deficiency (ID) is the most common nutritional deficiency worldwide, and infants and young children are highest at risk (1). Most of the iron in a term infant is accreted

during the third trimester of pregnancy. Premature infants miss this rapid accretion and are frequently deficient in total body iron (2). Moreover, due to rapid growth during the first months of life and low iron intake, infants in general and preterm infants in particular are at an increased risk of iron depletion and may have iron-restricted erythropoiesis (3). Iron plays an important role in the development of the central nervous system and is essential for neural myelination and neurotransmitter function (4). Iron deficiency anemia (IDA) during infancy is associated with poor neurological development (5). Therefore, early detection and treatment of ID is extremely important for the convalescence of premature infants. On the other hand, severe complications in premature infants, such as chronic lung disease, necrotizing enterocolitis, retinopathy of prematurity, and periventricular leukomalacia, have recently been reported to be caused by increased oxidative stress associated with iron overload (6). Therefore, premature infants require appropriate supplementation of iron.

ID is usually diagnosed using biochemical parameters (e.g., serum iron (Fe), serum ferritin (FER), and transferrin saturation) (7,8). However, these parameters are unreliable for the evaluation of ID during acute inflammation. Furthermore, it is unsafe to draw high

amounts of blood from premature infants, thus making the diagnosis more challenging. The development of automated systems for hematological analysis have recently made it possible to measure reticulocyte indices. These indices are thought to reflect iron content in reticulocytes, using the same sample that is used for complete blood count tests. Reticulocytes are the youngest erythrocytes released from bone marrow into the circulating blood. Reticulocyte indices provide a real-time assessment of iron status, owing to their brief 1–2 days lifespan. There are two indices of reticulocyte hemoglobin content: "CHr" parameter of the Bayer H3 and ADVIA 120 system (Bayer Diagnostics, Tarrytown, NY), and "RET-He" parameter of the Sysmex (Kobe, Japan) (9,10). These two parameters have been reported to correlate well and are considered to be equivalent as novel parameters for iron deficiency (11).

Several reports revealed that reticulocyte indices are useful for the evaluation of ID in adults and children (12–14). Some studies have also proved reticulocyte indices useful to evaluate ID in preterm infants, as well as to reveal important findings regarding hemoglobin synthesis therein. However, to date, there are no detailed reports concerning the changes in RET-He. Thus, the aim of this study was to assess chronological changes

in RET-He during oral iron supplementation and concomitant therapy with recombinant human erythropoietin (rHuEPO) in preterm very low birth weight (VLBW) infants.

Methods

VLBW infants, admitted to the neonatal intensive care unit (NICU) of Asahikawa Medical University Hospital, between January 2014 and December 2019, were retrospectively analyzed. Infants were excluded if they were discharged within 4 weeks, died before discharge, had chromosomal abnormalities, or underwent a red blood cell transfusion. We performed a retrospective chart review of the neonates. Gestational age, birth weight, birth weight percentile, sex, number of neonates with mechanical ventilation and breastfeeding, as well as details of the management of anemia were extracted. The following data were recorded: hemoglobin (Hb), reticulocyte percentage (Ret), mean corpuscular volume (MCV), RET-He, Fe, and FER. Data at birth (T0), the initial day of rHuEPO therapy (T1), the initial day of oral iron supplementation (T2), 1–2 weeks (T3), 3–4 weeks (T4), 5–6 weeks (T5), and 7–8 weeks (T6) from the initial day of oral iron supplementation were extracted, and their changes over time were examined. Note that

Fe and FER were not measured at T0 because at birth, priority was given to minimizing volume loss due to blood sampling and stabilizing the child's general condition. Furthermore, we examined the correlation between RET-He and other indices of erythropoiesis and during each testing period. Hb, Ret, MCV and RET-He were determined using the Sysmex XN3100 (Sysmex Corporation, Kobe, Japan). Fe and FER were quantified using an automated chemical analyzer (Hitachi, Tokyo, Japan). At our NICU, we performed blood tests daily during the first 3–5 days of life. After 1 week, blood tests were routinely repeated weekly. Blood samples were obtained through a venipuncture or an arterial catheter. The criteria for IDA were Hb < 10.5 g/dL and FER < 12 μg/L. The mean corpuscular volume (MCV) was < 70 fL or Fe < 50 μg/dL (15–18) for ID without anemia. According to the guidelines for oral iron supplementation for preterm infants from the Japan Society for Premature and Newborn Medicine 2017 (19), oral iron supplementation was started at a dose of 4–6 mg/kg of soluble ferric pyrophosphate (INCREMIN® Syrup 5%, Alfresa Pharma Ltd., Osaka, Japan) when serum iron tended to decrease after starting rHuEPO therapy. All VLBW infants received rHuEPO (ESPO®, Kirin Brewery Ltd., Tokyo, Japan) subcutaneously at a dose of 200 IU/kg twice a week.

rHuEPO therapy was started when the Hb level was <12 g/dL, and continued for 8 weeks. If the patient was discharged within 8 weeks of rHuEPO therapy, the treatment was discontinued. Statistical analyses were performed using IBM SPSS Statistics for Windows, version 22.0. Differences in iron status variables between chronological time points were tested by one-way repeated measures analysis of variance and Tukey's multiple comparison tests. Pearson's correlation coefficient was used to analyze the correlations. Statistical significance was set at p <0.05. This study was approved by the Research Ethics Committee of Asahikawa Medical University.

Results

A total of 87 infants were enrolled in this study. Not assessed (n = 11), died before discharge (n = 6), chromosomal abnormalities (n = 3), and infants with transfused red blood cells (n =13) were excluded. As a result, 54 infants participated in this study (Figure 1). The clinical characteristics of the patients are summarized in Table 1. The median (minimum - maximum) for gestational age and birth weight were 29w4d (24w1d–34w5d) and 1057.5 g (400–1470 g), respectively. Twenty-nine of fifty-four infants (54%) had a

birth weight of less than 1000 g, and twenty-one infants (39%) were small for gestational age (SGA). Thirty-nine infants (72%) were breastfed. All patients received oral iron supplementation and rHuEPO therapy during hospitalization. rHuEPO therapy and oral iron supplementation were initiated at 27.5 (10–49) and 38 (11–64) days of life, respectively. The effects of birth weight and nutritional methods on postnatal iron status variables were also examined (Table 2, 3). Infants of birth weight <1000 g had significantly lower Hb and higher MCV at birth than infants of birth weight ≥ 1000 g. On the other hand, FER at initial day of rHuEPO (T1) therapy was significantly higher in infants of birth weight <1000 g. Breastfed infants had significantly lower RET-He than formula fed infants at T1. Hb levels were highest at T0 phase (median, 16.6 g/dl; range 13.6–23.7 g/dl) and declined rapidly thereafter. Hb levels gradually decreased towards the T3 phase, reaching the lowest value (median, 10.5 g/dl; range 8.6–13.6 g/dl) despite rHuEPO being administered, and then increased after the T4 phase. Hb showed significant decrease at T1 and all time points thereafter (p<0.01 vs. T0) (Figure 2A). Although Ret decreased rapidly after birth, it quickly increased after rHuEPO administration, depicting highest values (median, 74 ‰; range 31–158 ‰) at the T3 phase.

Subsequently, it decreased after the T4 phase. Ret showed significant decrease at T1, T5, and T6 (p<0.01 vs. T0), at T5, and T6 (p<0.01 vs. T2) and at T4, T5, and T6 (p<0.01 vs. T3). On the hand, Ret showed significant increases at T2, T3, and T4 (p<0.01 vs. T1) (Figure 2B). MCV was highest at T0 (median, 118.6 fl; range 105.2–141.8 fl) and gradually decreased after birth, with no upward trend by T6. MCV showed significant decrease at T1 and all time points thereafter (p<0.01 vs. T0), at T2 and all time points thereafter (p<0.01 vs. T1), at T5 and T6 (p<0.01 vs. T2), at T5 (p=0.02 vs. T3), and at T6 (p<0.01 vs. T3) (Figure 2C). RET-He was highest at T0 (median, 33.5 pg; range 25.2–39.5 pg) and declined rapidly after birth. Especially after the commencement of rHuEPO therapy (T1), RET-He decreased rapidly and reached its lowest value (median, 24.3 pg; range 19.3–33.6 pg) at the T3 phase. There was no upward trend in RET-He after the T2 phase, with a slower increase in the T4 phase. RET-He showed significant decrease at T1 and all time points thereafter (p<0.01 vs. T0), at T2, T3, T4, and T5 (p<0.01 vs. T1). RET-He showed significant increase at T6 (p=0.036 vs. T2; p=0.048 vs. T3) (Figure 2D). Fe declined rapidly after the T1 phase, but quickly increased after the T2 phase. Fe showed significant increase at T4 (p=0.027 vs. T2), at T5, and T6 (p<0.01 vs. T2) and at T6

(p<0.01 vs. T3) (Figure 2E). FER was highest at the T1 phase (median, 58.6 ng/mL; range 10.5–361.5 ng/mL) and then tended to decline. This downward trend continued even after the T2 phase, and then finally began to increase from the T6 phase. FER showed significant decrease at T2 and all time points thereafter (p<0.01 vs. T1) (Figure 2F).

We examined the correlation between RET-He and other indices of erythropoiesis during each testing phase (Table 4). Hb was only weakly positively correlated (r=0.423, p=0.016) with RET-He at the T5 phase, and Ret was only weakly negatively correlated (r=-0.539, p=0.012) with RET-He at the T6 phase. However, none of these correlations were significant at most periods. On the other hand, Fe was positively correlated with RET-He except in the T4 phase. In addition, FER did not correlate with RET-He in the early postnatal period, but was positively correlated after the T4 phase.

Discussion

RET-He is a useful index for the early detection of ID because it reflects the real-time potential availability of iron in bone marrow for the production of red blood cells. Furthermore, RET-He testing may be particularly useful for preterm infants due to its

advantage of rapid testing using a small sample volume. Recently, there have been several reports on the benefits of RET-He testing in preterm and VLBW infants for ID evaluation (20,21). However, there are no detailed reports regarding the changes in RET-He in VLBW infants admitted to the NICU during oral iron supplementation using rHuEPO therapy. The present study is considered essential for understanding iron metabolism in VLBW infants. The marked decrease in Fe and FER after the initiation of rHuEPO therapy was consistent with previous reports (22–25). This reaction may have been due to increased erythropoiesis, which may have led to an increased demand for iron and a rapid progression of iron deficiency. This can be explained by the significant decrease in RET-He after the initiation of rHuEPO therapy.

Domellöf et al. (26) reported a cut-off value of 40 μg/L for FER in patients with ID at less than 2 months of age. In the present study, FER at T2 was 46.2 (10.5–157.8) μg/L, and 18 of the 41 infants (43.9%) had FER below 40 μg/L. On the other hand, several cut-off values of reticulocyte hemoglobin content for ID have been reported in the past. Brugnara et al. (27) reported a cut-off value of 26 pg for CHr in children with ID, Mateos et al. (28) reported 25 pg, and Ullrich et al. (29) reported 27.5 pg. The difference in the cut-off values

may be due to a difference in the target population and the definition of iron deficiency. In the present study, RET-He in T2 was 24.6 (16.1–31.7) pg, and 30 of 54 (55.6%) cases showed RET-He below 25 pg. In this study, we found that RET-He testing can determine ID at an earlier stage, as the results were similar to those reported in the past. The most important finding of the present study were the changes seen in RET-He after the initiation of oral iron supplementation. After the initiation of oral iron supplementation (after T3), only three patients met the diagnostic criteria for ID. The iron dosage in the present study was considered reasonable compared to those in previous reports. However, there was no upward trend in RET-He after initiation of oral iron supplementation, with a slower increase from 3–4 weeks after the initiation of iron therapy. Ennis et al. (30) reported that reticulocyte hemoglobin was the only red cell marker affected prior to the onset of brain iron deficiency, and the use of anemia as a preferred biomarker for the diagnosis of iron deficiency may need to be reconsidered in clinical practice. These RET-He trends obtained in the present study suggest that ID in the central nervous system may be present for a longer period in VLBW infants. Previous report suggest that preterm infants absorb ingested iron at a high rate, although a very small proportion of the

absorbed iron is used promptly for Hb synthesis (31,32). The relatively large amount of iron that is not used immediately likely enters storage sites. Despite having sufficient stored iron, such poor utilization of iron in preterm infants may explain the prolonged low RET-He in this study. The increased erythropoiesis and iron demand due to the administration of rHuEPO may have also contributed to the prolonged low RET-He. We believe that rHuEPO therapy increases hematopoiesis and iron demand, however, iron transport to the bone marrow is insufficient to meet the demand, resulting in a supposed functional deficiency at the hematopoietic level. Functional iron deficiency with increased doses of erythropoiesis-stimulating agents has been previously reported in adults (33), and a similar phenomenon is expected to occur in preterm infants. In the present study, RET-He increased slowly from 3–4 weeks after iron supplementation, while Ret decreased slowly. We hypothesize that this trend may represent a decrease in responsiveness to rHuEPO therapy over time, as erythropoietic activity subsides and iron supply meets demand. In the present study FER did not correlate with the RET-He in the early postnatal period, but was positively correlated after the T4 phase. Although FER usually correlate with the RET-He, there was no correlation between FER and the RET-

He in preterm infants from the early postnatal period to the T3 phase. This suggests that preterm infants may not be able to utilize iron well in the early postnatal period and that this situation may persist for some time even after rHuEPO therapy administration enhances hematopoiesis.

The rHuEPO therapy was originally intended to avoid red blood cell transfusions in preterm VLBW infants. However, rHuEPO therapy may increase hematopoiesis beyond the ability to metabolize iron in preterm VLBW infants. Therefore, the dosage and timing of rHuEPO therapy should be considered on a case-by-case basis. Specifically, functional iron deficiency states may require a reduced dosage or other measures. A problem could also arise with the uptake of iron into the red blood cells. In addition, although RET-He is a very sensitive indicator of iron deficiency, its low value indicates that the dosage of iron should be carefully determined, since an increase in the dosage of iron supplements may lead to iron overload. In preterm infants, iron absorption from the gastrointestinal tract is high, but the rate at which iron is incorporated into red blood cell is low. In addition, the absorbed iron is used more in other body tissues (eg, brain) (34). Iron metabolism in preterm infants differs from that in adults, and much remains unknown. It is hoped that

the mechanism of this metabolism will be clarified in the future.

The limitation of the present study was that it was not designed to identify the mechanism responsible for the changes observed in RET-He, since it was a retrospective study performed at a single NICU. Furthermore, since we excluded cases in which red blood cell transfusions were performed, we were not able to assess VLBW infants with severe anemia. Another limitation was that we did not measure the biomarkers of iron toxicity. Although non-transferrin bound iron (NTBI) is biomarker for assessing iron toxicity, it is not easily measured in clinical laboratories (35). If this biomarker can be easily measured, it is expected that iron dosing in preterm infants with functional iron deficiency can be assessed in more detail. Furthermore, the relationship between RET-He and neurological development needs to be investigated.

Conclusion

In this retrospective study, we assessed the changes in RET-He during oral iron supplementation with rHuEPO therapy. We report the prolongation of low RET-He during the treatment of anemia of prematurity. It is possible that the changes in RET-He may be

related to iron metabolism and rHuEPO therapy in preterm infants. We believe that this information will prove useful in determining the dosage for treatment of ID in VLBW infants. Nevertheless, further studies are required for understanding these mechanisms.

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Disclosure statement

The authors declare no conflict of interest.

Author contribution

M.N. drafted the initial manuscript. T.S. and A.A. collected and analyzed data. T.O. and K.N. critically reviewed the manuscript and supervised the entire study process. All authors have read and approved the final version of the manuscript.

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Tables

Table 1. Clinical characteristics of 54 preterm infants in this study

Characteristic	**Infants**
Gestational age	29w4d (24w1d–34w5d)
Birth weight (g)	1057.5 (400–1470)
Sex	
Male, n (%)	20 (37)
Female, n (%)	34 (63)
<1000 g, n (%)	29 (54)
1000~1499 g, n (%)	25 (46)
SGA, n (%)	21 (39)
Mechanical ventilation, n (%)	45 (83)
Breast feeding, n (%)	39(72)
Oral iron therapy, n (%)	54 (100)
rHuEPO therapy, n (%)	54 (100)
Initial day of rHuEPO therapy	27.5 (10–49)

Initial day of oral iron therapy	38 (11–64)

SGA: small for gestational age; rHuEPO: recombinant human erythropoietin. All data are presented as median (minimum-maximum), n (%).

Table 2. Differences in iron status variables between infants of birth weight <1000 g and ≥1000g at birth and the initial day of rHuEPO therapy

	at birth			**at the initial day of rHuEPO therapy**		
	<1000g	≥1000g	*p*	<1000g	≥1000g	*p*
Hb, g/dL	16.2±1.7	17.5±3.0	0.039*	11.2±1.2	10.9±1.6	0.26
Ret, ‰	69±32	76±28	0.47	47±24	39±19	0.13
MCV, fL	124±8.6	116±17	<0.01*	106±7.4	96.7±15	<0.01*
RET-He, pg	33.2±2.1	33.5±6.0	0.77	28.5±3.7	29.0±4.6	0.55
Fe, μg/dL	N/A	N/A	N/A	69.3±19	69.0±21	0.97
FER, ng/mL	N/A	N/A	N/A	155±123	79.8±94	0.03*

Hb: hemoglobin; Ret: reticulocyte percentage; MCV: maen corpuscular volume; RET-He: reticulocyte hemoglobin content; Fe: serum iron; FER: serum ferritin; rHuEPO:

recombinant human erythropoietin; N/A: not available. All data are presented mean ± SD. Statistical significance is based on the Mann-Whitney *U* test. * $p<0.05$.

Table 3. Differences in iron status variables between breastfed infants and formula fed infants at the initial day of rHuEPO therapy

	at the initial day of rHuEPO therapy		
	breastmilk	formula	*p*
Hb, g/dL	11.1±0.93	11.1±1.1	0.94
Ret, ‰	45±20	37±18	0.21
MCV fL	101±8.0	102±8.0	0.49
RET-He, pg	28.1±3.2	30.5±2.7	0.01*
Fe, µg/dL	67.2±19	74.4±20	0.35
FER, mg/mL	107±109	84.6±42	0.55

Hb: hemoglobin; Ret: reticulocyte percentage; MCV: maen corpuscular volume; RET-He: reticulocyte hemoglobin content; Fe: serum iron; FER: serum ferritin; rHuEPO: recombinant human erythropoietin. All data are presented mean ± SD. Statistical significance is based on the Mann-Whitney *U* test. * $p<0.05$.

Table 4. Correlation coefficients of RET-He levels with laboratory variables

	Correlation coefficient (*p*-value)						
RET-He	T0	T1	T2	T3	T4	T5	T6
Hb	-0.041 (0.814)	0.088 (0.615)	0.271 (0.086)	0.248 (0.096)	0.310 (0.061)	0.423* (0.016)	0.228 (0.319)
Ret	0.341 (0.042)	-0.274 (0.111)	-0.224 (0.158)	-0.078 (0.607)	-0.255 (0.128)	0.124 (0.498)	-0.539* (0.012)
MCV	0.203 (0.236)	0.053 (0.704)	-0.080 (0.567)	0.308* (0.027)	0.220 (0.162)	0.322 (0.060)	-0.325 (0.130)
Fe	N/A	0.531* (<0.01)	0.565* (<0.01)	0.330* (0.025)	0.285 (0.087)	0.413* (0.019)	0.557* (<0.01)
Fer	N/A	0.292 (0.089)	0.128 (0.424)	0.205 (0.172)	0.330* (0.046)	0.376* (0.034)	0.507* (0.019)

Hb: hemoglobin; Ret: reticulocyte percentage; MCV: maen corpuscular volume; RET-He: reticulocyte hemoglobin content; Fe: serum iron; FER: serum ferritin; rHuEPO: recombinant human erythropoietin; N/A: not available. Pearson's correlation coefficient was performed. * $p<0.05$.

Figure legends

Figure 1.

Outline of our study

Figure 2.

Changes over time in RET-He, erythrocyte indices and iron-related indices. Blood test results at birth (T0), the initial day of EPO therapy (T1), the initial day of oral iron supplementation (T2), 1−2 weeks (T3), 3−4 weeks (T4), 5−6 weeks (T5), and 7−8 weeks (T6) from the initial day of oral iron supplementation. (A) Hb showed significant decrease at T1 and all time points thereafter ($p<0.01$ vs. T0). (B) Ret showed significant decreases at T1, T5, and T6 ($p<0.01$ vs. T0), at T5 and T6 ($p<0.01$ vs. T2) and at T4, T5, and T6 ($p<0.01$ vs. T3); Ret showed significant increases at T2, T3, and T4 ($p<0.01$

vs. T1). (C) MCV showed significant decrease at T1 and all time points thereafter (p<0.01 vs. T0), and at T2 and all time points thereafter (p<0.01 vs. T1) and at T5 and T6 (p<0.01 vs. T2), at T5 (p=0.02 vs. T3) and at T6 (p<0.01 vs. T3). (D) RET-He showed significant decrease at T1 and all time points thereafter (p<0.01 vs. T0), and at T2, T3, T4, and T5 (p<0.01 vs. T1); RET-He showed significant increase at T6 (p=0.036 vs. T2 and p=0.048 vs. T3). (E) Fe showed a significant decrease at T2 (p<0.01 vs. T1); Fe showed significant increase at T4 (p=0.027 vs. T2), at T5 and T6 (p<0.01 vs. T2) and at T6 (p<0.01 vs. T3). (F) FER showed significant decrease at T2 and all time points thereafter (p<0.01 vs. T1). Box plots representing the median, upper and lower quartiles and the 1.5 interquartile range. * p<0.05, ** p<0.01.

Figure 1.

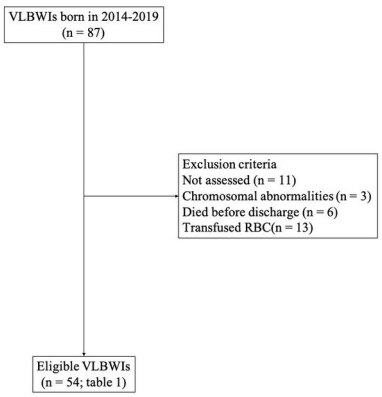

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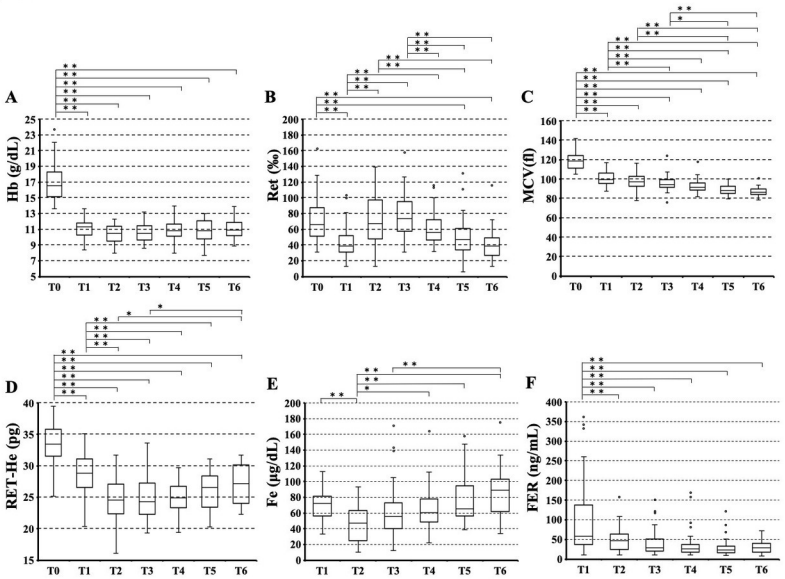
Figure 2.



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