Abstract

**Objective:** To examine whether the supplementation of inosine augments ATP in vitro in human erythrocytes incubated in saline.

**Methods:** Peripheral blood was drawn from each of three subjects, i.e. one healthy male and two males with thalassemia and hemoglobinopathy. After washing the erythrocytes in saline, they were suspended in saline to which inosine was added to final concentrations of 0, 0.5 and 2.5 mM. The suspension was incubated at 37 °C for 1 or 3 hours, and 0.5 ml ice cold 8% perchloric acid was added to the 0.5 ml erythrocyte-containing solution. After removing precipitates and perchloric acid, the supernatant was submitted to HPLC for the measurement of ATP.

**Results:** Since the blood samples of the two subjects with thalassemia and hemoglobinopathy were transported from the clinics to the laboratory, ATP in the blood decreased considerably during the transportation. However, the reduction of ATP with time was observed in the erythrocytes in saline obtained from each of the three subjects during the incubation from 1 hour to 3 hours. In addition, dose-dependent suppression of the decrease of ATP with inosine was observed in all the three cases at both 1 hour and 3 hour incubation times.

**Conclusions:** Incubation of erythrocytes from a healthy subject and two thalassemia/hemoglobinopathy patients in saline at 37 °C resulted in time-dependent decreases of ATP. Supplementation of inosine to the solutions resulted in the suppression of the decreases of ATP in a dose-dependent manner.

**Background**

ATP (adenosine triphosphate) is the most important compound for storing energy in living organisms and supplying it when needed. It is considered that ATP reduction is related to the pathology of various diseases. An example is the hemolytic anemia caused by glycolytic enzyme deficiencies such as hexokinase deficiency1, pyruvate kinase deficiency2, glucose phosphate isomerase deficiency3, phosphofructokinase deficiency4 and phosphoglycerate kinase deficiency5. Since mature erythrocytes lack mitochondria, they are heavily dependent on the anaerobic generation of ATP during glycolysis for nearly all of their metabolic needs.

**Key words:** ATP, febuxostat, xanthine dehydrogenase, xanthine oxidase, inosine, gout, hyperuricemia

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energy requirements\(^6\). Therefore, glycolytic enzyme deficiencies cause ATP deficiency in erythrocytes\(^6\) and impair functions such as Na/K–ATPase that is important to maintain the ion gradients through cell membranes. As a result, intracellular fluid becomes hypotonic, the cell shrinks and cellular death occurs\(^7\).

Changes in ATP content of the blood during storage play an important role in red blood cell viability\(^8\). To suppress a decrease in ATP in the stored blood, adenine is sometimes added to enhance ATP during the storage\(^9\). These data suggest that enhancement of ATP in erythrocytes may increase cell viability in vivo when the erythrocytes are exposed to undesirable conditions such as oxygen deficiency or glucose deficiency.

In thalassemia and hemoglobinopathies, abnormalities in oxygen–carrying protein hemoglobin cause changes in erythrocyte cell membrane permeability\(^10,\ 11\). Although ATP deficiency is not the primary cause of the hemolysis in those diseases, the enhancement of ATP in the erythrocytes may increase cell viability in vivo.

We reported a method to enhance ATP in vivo in humans by clinical studies on healthy subjects\(^12\). The measurement of ATP was performed in peripheral blood. Since ATP is present at high concentrations only within cells but not in the extracellular fluid, most of ATP in blood exists in erythrocytes. Therefore, it is suggested that the method reported in the previous paper\(^12\) enhanced ATP in erythrocytes.

In the present study, we attempted to examine whether ATP is enhanced in vitro when erythrocytes from a healthy subject and patients with thalassemia and hemoglobinopathy were incubated with inosine in saline. Metabolic pathway related to the present study is shown in Fig 1. Although a xanthine dehydrogenase (XDH) / xanthine oxidase (XO) inhibitor febuxostat was added in addition to inosine in the previous clinical studies\(^12\), inosine alone was used in the present study. This is because XDH/XO activity is very low in human peripheral blood (Fig 1).

**Methods**

**Blood collection**

This study was approved by the institutional review board (IRB of Tsukuba International Clinical
Informed consent was obtained from each of the subjects. The healthy subject was a Japanese male aged 68. Case 1 was a 56-year-old male of African origin with alpha-thalassemia (homozygote), HbS (heterozygote) and liver cirrhosis. Lab data showed WBC 2500/µL (normal range 3900–9800), RBC 532 x 10^4/µL (normal range 427–570), Hb 11.3 g/dL (normal range 13.5–17.6), Ht 37.8% (normal range 39.8–51.8), reticulocyte 14% (normal range 1–4) and haptoglobin 17 mg/dL (normal range type 1–1, 43–180). Case 2 was a 32-year-old Thai male with beta-thalassemia and HbE. Lab data showed WBC 16925/µL (normal range 4000–10000), RBC 319 x 10^4/µL (normal range 450–600), Hb 7.3 g/dL (normal range 13–18) and Ht 32.8% (normal range 40–54).

Incubation of erythrocytes with inosine

0.5 ml solution containing erythrocytes in saline with or without inosine from each subject was incubated for 1 or 3 hours in test tubes in a shaking bath at 37 °C.

Measurement method of ATP

Measurement of ATP was performed according to the literature. Briefly, 500 µL solution containing ATP was mixed with 500 µL ice cold 8% perchloric acid (PCA), and the mixture was immediately vortexed. Thereafter, the mixture was centrifuged at 12,000 x g for 10 minutes at 4 °C. 40 µL of 2 M K₂CO₃ in 6 M KOH was added to 650 µL of the supernatant to simultaneously precipitate PCA and neutralize the solution. This was centrifuged at 12,000 x g for 10 minutes at 4 °C, and then 160 µL of mobile phase was added to 40 µL of the supernatant and subjected to HPLC. The conditions of HPLC are the same as described previously. The amount of ATP was expressed by the molar amount contained in the solution after removing PCA.

Results and Discussion

Fig 2 shows the results from experiments using blood samples from the three subjects. The amounts of ATP differed greatly between the samples from the three subjects. One of the reasons for the difference is anemia in Case 1 and Case 2. Thus, hemoglobin concentrations were 11.3 g/dL in Case 1 and 7.3 g/dL for Case 2, respectively (In the healthy subject, it was not measured). However, a larger factor that influenced the ATP contents was the time between the blood sampling and the experiment. Thus, for the healthy subject, the experiment was performed immediately after the blood sampling while it took 13 hours for Case 1 and 2 days for Case 2. The blood from Case 1 was transported without cooling while it was kept at 4 °C during the transportation for Case 2. During the transportation, ATP levels apparently decreased considerably for Case 1 and Case 2. Although the conditions before the experiments differed between the three subjects' samples, changes of ATP in the blood can be examined by comparing the data for the same sample for different incubation times and different inosine concentrations. The reduction of ATP in the erythrocytes for 1 hour versus 3 hours incubation time in saline was observed in each of the three subjects' samples except for the sample from the healthy subject incubated at 2.5 mM inosine (Fig 2 A). In addition, dose-dependent suppression of the decrease of ATP with addition of inosine was observed in all three cases at both 1 hour and 3 hour incubation times (Fig 2).

Suppression of a decrease in ATP in blood from a healthy subject with inosine was reported by previous studies. In the study by Ogasawara et al., the whole blood was kept at 4 °C for 20–58 days to examine the decrease of ATP. In the present manuscript, we incubated erythrocytes in saline at 37 °C for 1 to 3 hours and were able to observe clear decreases of ATP. This is probably because we incubated the erythrocytes in saline that does not
contain glucose. Since erythrocytes generate ATP by the glycolysis pathway, ATP is expected to decrease faster in glucose-deficient conditions. Despite such differences in the procedure, our results are in accord with previous reports that inosine enhances ATP in erythrocytes. In addition, our data indicated that the augmentation of ATP with inosine occurs in the erythrocytes from thalassemia and hemoglobinopathy patients as well.

Our results suggest that a therapy equivalent to the present study may be useful for the treatment of hemolytic anemia caused by genetic glycolytic enzyme deficiencies since ATP depletion is considered to be the cause of these disorders. The same treatment may also be useful for other hemolytic anemias such as thalassemia and hemoglobinopathy since although the primary cause of these diseases is not the ATP depletion, enhancement of ATP in the erythrocytes may increase the viability of erythrocytes exposed to undesirable conditions. Although we examined the effect of inosine to augment ATP in vitro, we should add a XDH/XO inhibitor such as febuxostat in addition to inosine when applied to humans in vivo. This is because, unlike human blood, XDH/XO is abundant in such organs as liver and kidney. When inosine alone was orally administered to humans, hypoxanthine did not increase because it was degraded by XDH/XO. In the present study, hypoxanthine accumulated but neither xanthine nor uric acid increased during the incubation thereby indicating a negligible activity of XDH/XO (data not shown).

Limitations of our study are as follows. (a) Since

Figure 2: Changes of ATP when erythrocytes were incubated in saline
Erythrocytes from a healthy subject (A), Case 1 with alpha-thalassemia and HbS (B) or Case 2 with beta-thalassemia and HbE (C) were incubated in saline with or without inosine for 1 hour (open column) or 3 hours (closed column), and the amounts of ATP in the solution was measured.
the transportation of the samples from the two patients should have damaged the erythrocytes, the present experiments had better performed using fresh samples.

(b) Since the number of the samples used in this study is too small, further studies are necessary to confirm the results obtained. (c) Although the addition of inosine enhanced ATP in vitro, it is not clear whether it inhibits hemolysis. Since the hemolysis does not necessarily occur in blood vessels, this effect should be examined in clinical studies.

Thus, our present study suggests that the combined use of inosine and a XDH/XO inhibitor may be useful for the patients with hemolytic anemia caused by glycolytic enzyme deficiencies, and other abnormalities including thalassemia, and hemoglobinopathy.

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Conflicts of interest
Naoyuki Kamatani is paid from StaGen Co. Ltd and hold stocks of the same company that is the sponsor of this study.

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