Relationship between glyated haemoglobin concentration and erythrocyte survival in type 2 diabetes mellitus determined by a modified carbon monoxide breath test

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Abstract

In clinical practice, an unexplained discordance between percentage haemoglobin A1c (HbA1c) and the progression of diabetes and its complication is observed. HbA1c is determined by the blood glucose level and the red blood cell (RBC) lifespan. Whether the RBC lifespan changes in diabetic patients remains undefined because of the lack of a convenient and accurate measurement method. In the present study, we aim to observe the RBC lifespan in type 2 diabetic patients with poor blood glucose control by an endogenous carbon monoxide (CO) measurement using a rapid and simplified CO breath test machine. The RBC lifespan, age, RBC count, haemoglobin, haematocrit, fasting blood glucose (FBG) level, HbA1c, blood lipids and the liver and kidney function were compared between 38 diabetic patients and 40 healthy individuals. Compared with the control group, in the diabetic patients, the RBC lifespan was significantly decreased by 17.52 ± 4.58 (86.08 ± 18.13 d versus 103.6 ± 22.02 d, p = 0.00). Although a univariate linear correlation analysis showed that the RBC lifespan was negatively correlated with the FBG level (r = −0.386, p = 0.000), haemoglobin A1c (r = −0.346, p = 0.002) and age (r = −0.291, p = 0.010), a stepwise multiple linear regression analysis showed that the RBC lifespan was most affected by the FBG level (t = −3.554, p = 0.001), but not by HbA1c or age, while HbA1c was most affected by the FBG level (t = 13.989, p = 0.000), but not the RBC lifespan. The RBC lifespan in diabetic patients with poor glycaemic control was reduced. The decrease in the RBC lifespan caused by hyperglycaemia was not associated with HbA1c. Thus, a decrease in the RBC lifespan will lead to an underestimation of the actual level of hyperglycaemia and the progression of disease by HbA1c in type 2 diabetic patients if we do not adjust the RBC lifespan.

1. Introduction

Diabetes mellitus is a group of metabolic diseases characterized by chronic hyperglycaemia. Blood glucose and glycated haemoglobin are not only the golden standards for the diagnosis of diabetes but also important indicators of the acute and chronic complications of diabetes. Glycated haemoglobin is the product of the irreversible reaction between glucose and haemoglobin by non-enzymatic glycation, which depends on the blood glucose level, the glycation time and the red blood cell (RBC) lifespan [1]. In 2010, the American Diabetes Association (ADA) incorporated haemoglobin A1c (HbA1c), the pre-dominant component of glycated haemoglobin, as one of the criteria for the diagnosis of diabetes [2]. The significantly positive correlation between blood glucose and HbA1c has now been widely accepted. Previous studies have suggested that when the mean blood glucose (MBG) increased for 2 mmol l⁻¹, HbA1c increased by 1% [2].
However, this interpretation was built on the premise that the RBC lifespan remained unchanged. In fact, minor changes in the RBC lifespan could cause significant changes in HbA1c. Studies have found that HbA1c decreased from 7% (53 mmol mol\(^{-1}\)) to 6% (42 mmol mol\(^{-1}\)), if the RBC lifespan decreased by 15% [3]. In patients with an elevated RBC lifespan, such as patients with splenectomy or iron deficiency anaemia, HbA1c would significantly increase [4]. In contrast, in patients with haemolytic anaemia and a significantly reduced RBC lifespan, the percentage of glycated haemoglobin was abnormally low [5, 6]. These studies suggest that the RBC lifespan should be included in the adjustment of HbA1c when making a diagnosis of diabetes or assessing a related complication if the RBC lifespan changed in such patients.

However, whether the RBC lifespan changes in patients with diabetes remains undefined. The results of the previous studies were contradictory. Some studies found that hyperglycaemia could lead to a decrease in the RBC lifespan [7, 8]. Peterson further observed that the RBC lifespan increased when the high blood glucose level was brought under control in seven type 2 diabetic patients with poor glycaemic control [8]. However, other studies reported no statistically significant difference in the RBC lifespan between diabetic patients and the control group, because of the large variation of the RBC lifespan in the population [9, 10].

Previously, the RBC lifespan was measured using \(^{51}\)Cr or biotin labelling protocols [11]. During the procedure, RBC are collected and labelled, and then, injected into the blood circulation of the subjects for the measurement of the disappearance rate of the labelled RBC. This method was not only complicated and time consuming, but also required auto-transfusion of the labelled erythrocytes, which might increase the possibility of contamination or misidentification. The other method is to determine the haemoglobin renewal rate of the metabolic precursors or products, and then, calculate the RBC lifespan [12]. The typical representative method is the measurement of endogenous carbon monoxide (CO). Endogenous CO is mainly derived from the decomposition of RBC after the destruction of haemoglobin. As long as the endogenous CO exhalation rate is obtained, we can calculate the time required for the decomposition of haemoglobin, which is the RBC lifespan [13]. After decades of development, the technique for the measurement of endogenous CO has been improved and verified [13–15].

Early endogenous CO assays required the collection of large volumes of exhaled breath, which is difficult to be carried out in clinic [16]. In the present study, a rapid and simplified CO breath test machine, the RBC Lifespan Analyser RBCS-01 (Seekya Breath Test Technology. Shenzhen, China) was used to measure the RBC lifespan of patients with type 2 diabetes mellitus and of healthy individuals. The correlations between the RBC lifespan, blood glucose level and glycated haemoglobin were further analysed.

2. Material and methods

2.1. Subjects

The subjects were type 2 diabetic patients who had been diagnosed for more than 5 years in the Nanshan Affiliated Hospital of Shenzhen University; they were examined from October 1, 2015, to August 30, 2016. The control group consisted of healthy individuals examined during the same period. The study sample included 38 patients with diabetes, 20 males and 18 females, with an average age of 48.71 ± 9.6 years, and 40 healthy controls, 20 males and 20 females, with an average age of 40.5 ± 6.5 years.

All type 2 diabetic patients met the standard of the diagnosis and classification of type 2 diabetes, which was formulated by the ADA in 1997. They were at the age between 20 and 65 years without severe acute or chronic complications of diabetes. The exclusion criteria included having diseases that could affect the RBC lifespan, in the last 6 months, such as the following: (1) haematological diseases; (2) cancer and other chronic wasting diseases such as tuberculosis and thyrotoxicosis; (3) infectious diseases; (4) liver disease or an increase in transaminases at more than 3 instances; (5) kidney disease (creatinine ≥ 1.5 mg dl\(^{-1}\)) or rheumatoid arthritis; (6) valve replacement or NYHA functional class III or above; (7) bleeding history or blood donation or blood transfusion within the previous 4 months; (8) smokers; (9) impaired pulmonary function; (10) pregnancy; and (11) use of drugs that could affect the RBC lifespan, such as ribavirin, barbitol, or phenobarbitone sodium. In women patients, we avoided the menstrual period when monitoring the RBC lifespan. The study was approved by the Nanshan Affiliated Hospital of Shenzhen University Ethical Review Board and was carried out according to the Declaration of Helsinki. Informed consent including consent to publish was obtained from all subjects.

2.2. Evaluation of clinical indexes

Clinical indexes such as the body-mass index, blood pressure, fasting blood glucose (FBG), haemoglobin, HbA1c, blood fat, liver and kidney function and urine protein were obtained on the same day as that of gas collection.

2.3. Alveolar and atmospheric gas sampling for RBC lifespan measurement

Immediately upon awakening in the morning and before 12:00 pm, the subjects were asked to keep fasting and sit quietly for 20 min. The subjects then held the alveolar air collection device in their hands with its mouth stuck close to their chest. They were asked to take a deep breath and hold the breath for 10 s, and then, exhale into a collected system through a
mouthpiece. The collection system discarded the first 500 ml (containing dead space) and then, automatically directed the subsequent 1000 ml alveolar air into a foil bag (figure 1). The bag was detached and sealed. Further, 800 ml atmospheric air samples were collected just after the breath sampling. The alveolar air and the atmospheric air samples were stored at room temperature and sent to the laboratory for analysis within 24 h of collection.

2.4. Gas analysis and determination of RBC lifespan
In the present study, the CO concentration was measured using RBC Lifespan Analyser RBCS-01 (Seekya Breath Test Technology, Shenzhen, China). The process matched the basic principles and calculation formulas of Levitt’s CO rapid breath test [16, 17]. However, because the sample collected by the human body was small, the original gas in the air chamber could not be completely replaced. An injecting-sample-into-chamber mode of small-volume, multiple-time and intermittent injecting was developed for the absorption spectroscopy. Thus, the amount of sample gas required was reduced.

2.5. Measurement of CO₂ concentration
Because the alveolar gas may be diluted in the process of sampling, and the human alveolar gas CO₂ is stable at 5%. The measured value of the CO₂ concentration is used to calculate and compensate for the alveolar gas CO concentration that may be diluted by air during the sampling process, making the measurement accurate. The formula used was \( D = \frac{A}{B} \). Here, \( A \) represents the measured value of the CO₂ concentration of alveolar air, \( B \) denotes the measured value of the CO concentration of alveolar air by CO₂. Then, 200 ml of the alveolar sample gas was injected into the first measurement chamber, and the CO₂ concentration was measured at the infrared wavelength of 4.26 \( \mu m \).

2.6. Measurement of CO concentration
Next, 800 ml of the alveolar sample gas and 800 ml of the environmental background gas were slowly injected into the second measurement chamber, and the endogenous CO concentration was measured at the infrared wavelength of 4.65 \( \mu m \). The endogenous CO concentration was corrected according to the CO₂ concentration of the alveolar sample. The sensitivity of the RBC Lifespan Analyser RBCS-01 for the measurement of the CO concentration was 0.02 PPM. The accuracy was 0.08 PPM, and the repeatability was 0.08 PPM.

2.7. Determination of RBC lifespan
Breath endogenous CO mainly comes from the destruction of RBC and is produced during the process of haemoglobin degradation and heme oxidation. By measuring the difference in the CO concentration between the breath and the environment, we could obtain the endogenous CO concentration. According to the amount of alveolar ventilation, the amount of CO exhaled and the amount of haemoglobin decomposition in unit time were calculated. Then, the overall time needed for the entire blood haemoglobin decomposition could be calculated, which was the estimated RBC lifespan in vivo [16].

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\text{RBC lifespan (days)} = \frac{4 \times \text{Hemoglobin concentration} \times 22400 \times \text{Blood volume}}{0.7 \times \text{Breath endogenous CO concentrations} \times 64400 \times 1440 \times \text{Alveolar ventilation}}
\]

\[
= \frac{\text{Hemoglobin concentration} \times 1380}{\text{Breath endogenous CO concentrations}}
\]

2.8. Statistical analysis
A one-sample \( t \) test was used for the comparison between the control group and the diabetic group. Regression analysis was used for the analysis of the correlations between variables. Differences were considered statistically significant at a \( p \) value of less than 0.05. All data were expressed as means ± standard deviation (SD). The statistical analyses were performed automatically in SPSS13.
3. Results

3.1. Clinical feature and RBC lifespan between diabetic patients and healthy controls

No statistically significant difference in the serum RBC count, haemoglobin, haematocrit, alanine aminotransferase (ALT), aspartate aminotransferase (AST), creatinine, total cholesterol (TC) and low-density lipoprotein cholesterol (LDL-C) was observed between the diabetic patients and the healthy individuals ($p > 0.05$). In contrast, the age, FBG, HbA1c and triglyceride (TG) of the diabetic patients were significantly higher than those of the control group ($p < 0.05$), while the RBC lifespan and high-density lipoprotein cholesterol (HDL-C) were significantly lower than those of the healthy controls ($p < 0.05$) (table 1). Compared with that of the control group, the RBC lifespan of the diabetic patients decreased by 17.52 ± 4.58.

3.2. Correlation analysis of RBC lifespan

The RBC lifespan was significantly and negatively correlated with age (figure 2), FBG (figure 3) and HbA1c (figure 4) ($p < 0.05$) by a univariate linear correlation analysis (table 2). The relatively wide spread of data may be attributed to the large variation of the RBC lifespan in the population [9, 10] and the relatively small sample.

To determine which variables had the greatest effect on the RBC lifespan, a stepwise multiple linear regression analysis was conducted. The RBC lifespan was used as the dependent variable, and age, FPG, HbA1c, haemoglobin, haematocrit, ALT, AST, creatinine, TG, TC, HDL-C, LDL-C and RBC count were used as the independent variables. A stepwise multiple linear regression analysis suggested that the RBC lifespan was mostly and only affected by FPG (table 3).

![Figure 2. Relationship between RBC lifespan and age. A significant negative correlation was observed between the RBC lifespan and age in 38 patients with diabetes and 40 healthy controls, in a univariate linear correlation analysis ($r = -0.291, p = 0.010$).](image-url)

### Table 1. Comparison of clinical data between the control group and the diabetic group. One-sample t test was used for the comparison between the control group and the diabetic group. The data are expressed as mean ± SD according to the distribution of variables. Further, $p$ values of $<0.05$ are considered significant.

|                         | Control group ($n = 40$) | Diabetic group ($n = 38$) | $t$ | $p$  |
|-------------------------|--------------------------|---------------------------|-----|------|
| RBC lifespan (d)        | 103.6 ± 22.03            | 86.08 ± 18.13             | 3.84 | 0.000|
| Age (y)                 | 40.50 ± 6.493            | 48.71 ± 9.603             | -4.44 | 0.000|
| Hemoglobin (g l\(^{-1}\)) | 137.78 ± 20.394 | 139.53 ± 16.815             | -0.415 | 0.680          |
| RBC ($\times 10^{12}$ l\(^{-1}\)) | 4.774 ± 0.5533 | 4.727 ± 0.5334 | 0.380 | 0.705      |
| Hematocrit (%)          | 42.64 ± 5.308            | 42.49 ± 4.513             | 0.126 | 0.900|
| FBG (mmol l\(^{-1}\))   | 4.74 ± 0.43              | 12.34 ± 3.15              | -14.93 | 0.000|
| HbA1c (%)               | 5.56 ± 0.39              | 10.72 ± 2.33              | -13.83 | 0.000|
| HbA1c (mmol mol\(^{-1}\)) | 37.5 ± 4.5             | 93.5 ± 25.5              |       |      |
| ALT (U l\(^{-1}\))     | 18.80 ± 14.96            | 23.30 ± 13.89             | -1.368 | 0.175|
| AST (U l\(^{-1}\))     | 17.50 ± 4.92             | 19.49 ± 9.645             | -1.125 | 0.266|
| Creatinine (umol l\(^{-1}\)) | 62.71 ± 13.65 | 62.90 ± 17.48 | -0.053 | 0.958 |
| TC (mmol l\(^{-1}\))   | 4.92 ± 0.74              | 4.92 ± 1.37               | -0.004 | 0.997|
| TG (mmol l\(^{-1}\))   | 1.21 ± 0.70              | 2.29 ± 1.96               | -3.213 | 0.002|
| HDL-C (mmol l\(^{-1}\)) | 1.48 ± 0.30             | 0.99 ± 0.24               | 7.805  | 0.000      |
| LDL-C (mmol l\(^{-1}\)) | 3.09 ± 0.76             | 2.84 ± 1.04               | 1.168  | 0.247      |
3.3. Correlation analysis of glycated haemoglobin (HbA1c)
To determine which variables had the greatest effect on HbA1c, a stepwise multiple linear regression analysis was conducted. HbA1c was used as the dependent variable, and age, FBG, RBC lifespan, haemoglobin, haematocrit, ALT, AST, creatinine, TG, TC, HDL-C, LDL-C and RBC count were used as the independent variables. A stepwise multiple linear regression analysis suggested that FPG was the only influencing factor for HbA1c (table 4).

4. Discussion
Percentage HbA1c has been used as a routine golden index of glycaemic control for more than 30 years [18]. HbA1c has been thought to be positively correlated to the MBG and to closely reflect diabetic complications [18]. However, in clinical practice, HbA1c and blood glucose do not match perfectly. Although the specificity of the diagnosis by HbA1c relative to OGTT is considerably high, exceeding 95%, the sensitivity is very low, varying between 40% and 60% in most populations.
Table 2. Univariate linear correlation analysis of the RBC lifespan and the related variables. This analysis was conducted to evaluate the relationships between the RBC lifespan and the related variables. The correlations between the RBC lifespan and the related variables were determined on the basis of the corresponding correlation coefficients (r). Further, p values of <0.05 were considered significant.

| RBC lifespan | Age (y) | FBG (mmol l⁻¹) | HbA1c (%) | ALT (U l⁻¹) | AST (U l⁻¹) | Creatinine (umol l⁻¹) | RBC (×10¹² l⁻¹) | Hemoglobin (g l⁻¹) | Hematocrit (%) | TC (mmol l⁻¹) | TG (mmol l⁻¹) | HDL-C (mmol l⁻¹) |
|--------------|---------|----------------|-----------|-------------|-------------|---------------------|----------------|----------------|--------------|-------------|-------------|---------------|
| r            | -0.291  | -0.386         | -0.346    | 0.002       | 0.067       | -0.092              | -0.023         | -0.017         | -0.053       | -0.009      | 0.093       | 0.222         |
| p            | 0.010   | 0.000          | 0.002     | 0.985       | 0.560       | 0.435               | 0.844          | 0.885          | 0.648        | 0.938       | 0.427       | 0.059         |

At HbA1c = 6.0% (42 mmol mol⁻¹), MBG can vary from 100 to 152 mg dl⁻¹. At MBG = 150 mg dl⁻¹, HbA1c ranges between 5.9 (41 mmol mol⁻¹) and 7.5% (58 mmol mol⁻¹) [19]. In other words, if we rely on HbA1c in the diagnosis of diabetes, we can sometimes lose persons with diabetes or falsely diagnose those without the disease [18]. Many studies used the value of blood glucose and fructosamine to predict HbA1c, and then, the predicted HbA1c values and the actual values were compared. These studies reported that the difference between the measured HbA1c and the predicted value can be quantified as the glycation gap (G-gap). If the difference were due to measurement errors, they would not be repeated between individuals. However, Nayak showed that the values of the G-gap were stable and repeatable in 2263 individuals with diabetes, which suggested that the discordance between HbA1c and blood glucose was not caused by a single measurement error, but by a systematic error between individuals [20]. It is not clear why HbA1c could not truly reflect the level of blood glucose. However, we have known that glycated haemoglobin depends on the blood glucose level and the RBC lifespan. Presumably, a variation in the RBC lifespan can sufficiently alter the measured HbA1c and finally, contributes to the discordance between HbA1c and blood glucose.

The measurement of the RBC lifespan through an endogenous CO assay was first proposed in 1966. The biggest advantage of such a measurement of the RBC lifespan is that the assay is a non-invasive and reliable method. The downside of the method is that it cannot be used for the assay of the RBC lifespan of patients who smoke or have lost blood or use drugs such as ribavirin that affect the concentration of endogenous CO. After decades of validation and improvement, a reliable technique for measuring endogenous CO has been developed, making it possible to be widely used in clinics. In the current study, endogenous CO was measured with RBC Lifespan Analyser RBCS-01, whose accuracy has been verified [21]. The RBC lifespan in 40 cases of healthy controls was 103.6 ± 22.02 d, which was lower than the RBC lifespan measured using the ⁵¹Cr labelling method for approximately 120 d. This finding can be attributed to the fact that the endogenous CO measured by a breath test simultaneously reflects the destruction of RBC in the bone marrow and in the circulating blood, while the ⁵¹Cr labelling assay only reflects the RBC destruction in the circulation. The RBC lifespan data obtained with RBCLifespan Analyser RBCS-01 were similar to those of other endogenous CO measurement studies with the RBC lifespan measured to be around 101 ± 19 d [16], suggesting that this method has good accuracy and repeatability.

In the current study, the RBC lifespan of the diabetic patients was 86.08 ± 18.13 d, which was significantly lower than that of the healthy individuals. This result was consistent with that of Strocchin’s and Ma’s studies [7, 16], but was contrary to the results of Cohen and Sim [9, 22]. Their results suggested that the RBC lifespan did not statistically significantly differ between the diabetic patients and the healthy controls. In the present study, we ruled out the known factors affecting the RBC lifespan, including all types of anemia, haemorrhage, severe renal and liver insufficiencies, heart valve replacement and the use of drugs such as ribavirin. The univariate linear correlation analysis showed that except for age and glucose metabolism, other parameters were not significantly correlated with the RBC lifespan. However, the stepwise multiple regression analysis excluded age or HbA1c factors, suggesting that blood glucose was the only factor that affected the RBC lifespan. Further, the reduced RBC lifespan caused by hyperglycaemia may be due to high glucose toxicity. Hyperglycaemia can cause oxidative stress and inflammatory response, and leads to the consumption of a large number of antioxidants such as glutathione, which can reduce the antioxidant capacity of the RBC membranes, leading to RBC damage and lysis [21]. Furthermore, high glucose can induce the acetylation of the RBC membrane tubulin, resulting in reduced RBC deformability and osmotic fragility [23]. In addition, high blood glucose can increase the non-enzymatic glycation of the RBC membrane protein, making the RBC membrane rigid and easy to remove, thereby reducing the RBC lifespan [24].

Although our study found that the RBC lifespan in diabetic patients with poor glycaemic control was reduced, HbA1c did not affect the RBC lifespan in the stepwise multiple linear regression analysis. The result was unexpected and was inconsistent with Ma’s study [7]. Ma showed that the RBC lifespan was negatively correlated with HbA1c [7]. However, we do not know which statistical analysis method was used in this previous study. Furthermore, the study did not include other related parameters such as the blood glucose.
We know that HbA1c is the product of the irreversible reaction of blood glucose and haemoglobin glycation by non-enzymatic proteins, so blood glucose is one of the main factors affecting glycated haemoglobin. In consistence, our study found that the FBG was closely related to HbA1c by a stepwise multiple regression analysis. Moreover, apart from the blood glucose, the RBC lifespan could affect the HbA1c level. In the present study, the RBC lifespan of the diabetic patients was reduced and negatively correlated with FBG. Therefore, with an increase in blood glucose, on one hand, hyperglycaemia will lead to an increase in the non-enzymatic protein glycation between haemoglobin and blood glucose, thereby increasing the HbA1c level. On the other hand, hyperglycaemia shortens the RBC lifespan, thus reducing the reaction time of glycation between haemoglobin and blood glucose, which will result in the reduction of the HbA1c production. Therefore, the final HbA1c level should be lower than the predicted value (the value that counteracts the reduction of the HbA1c level caused by a decrease in the RBC lifespan) if we do not consider the change in the RBC lifespan in type 2 diabetic patients.

5. Conclusions

We measured the RBC lifespan in type 2 diabetic patients and healthy controls by an endogenous CO measurement using a rapid and simplified CO breath test technique. The data were part of an initial investigation and require further large-scale confirmatory studies. We suggest that HbA1c, which is used as an indicator of the chronic complications of diabetes mellitus, particularly as a gold standard for the diagnosis of diabetes by ADA, needs to be carefully re-examined and adjusted with the RBC lifespan.

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Competing interests

The authors declare no commercial or financial conflict of interest.

| Table 3. Stepwise multiple linear regression analysis of factors affecting the RBC lifespan. Multiple stepwise linear regression was used to identify the effect of age, FBG, HbA1c, haemoglobin, haematocrit, ALT, AST, creatinine, TG, TC, HDL-C, LDL-C and RBC count on the RBC lifespan. The stepping criteria employed for entry and removal was based on the significance level of the F-value, which was set as 0.05. Further, p values of <0.05 were considered significant. The analysis showed that FBG was the best predictor of the RBC lifespan. |
|---|---|---|---|
| Non-standardized coefficients | Standardized coefficient | t | p |
| B | Standard error | Beta |  |
| Constant | 111.478 | 5.346 | 20.854 | 0.000 |
| FBG (mmol l⁻¹) | −1.999 | 0.563 | −0.396 | −3.554 | 0.001 |

| Table 4. Stepwise multiple linear regression analysis of factors affecting haemoglobin A1c. Multiple stepwise linear regression was used to identify the effect of age, FBG, RBC lifespan, haemoglobin, haematocrit, ALT, AST, creatinine, TG, TC, HDL-C, LDL-C and RBC count on HbA1c. The stepping criteria employed for entry and removal was based on the significance level of the F-value set, which was as 0.05. Further, p values of <0.05 were considered significant. The analysis showed that FBG was the best predictor of HbA1c. |
|---|---|---|---|
| Non-standardized coefficients | Standardized coefficient | t | p |
| B | Standard error | Beta |  |
| Constant | 2.936 | 0.416 | 7.060 | 0.000 |
| FBG (mmol l⁻¹) | 0.612 | 0.44 | 0.861 | 13.989 | 0.000 |
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