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Research Report

MTT FORMAZAN REPLACED WST-8 AS A BETTER SIMPLE SCREENING METHOD FOR DETECTION OF GLUCOSE-6-PHOSPHATE DEHYDROGENASE DEFICIENCY

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ABSTRACT

We have previously developed a new method using a new formazan substrate WST-8, as a simple and rapid screening test for detection of glucose-6-phosphate dehydrogenase (G6PD) deficiency accomplished by the naked eye. However, it was little difficult to distinguish between faint orange colors developed by heterozygous females and pink colors of normal hemolyzed blood, since both have similar tones, but this was the only simple and rapid screening test can be applied in the field. To solve this problem, we established a newer and simple screening method has been established by replacing a different formazan substrate, MTT (3-(4,5-dimethyl-2- thiazolyl)-2,5-diphenyl-2H tetrazolium bromide) in combination with a hydrogen carrier, 1-methoxy phenazine methosulfate to replace WST-8. MTT formazan exhibits a purple color, thus allowing for the ability to easily distinguish the pink colors of hemolyzed blood. However, MTT has been reported to react with hemoglobin non-specifically and to interfere with the interpretation of the color reaction. In our examinations by mixing MTT with hemolyzed blood, we found that the non-specific reaction was very slow, and that the addition of a small amount of blood (5 ~ 10 µl) into a reaction mixture (800 µl) did not interfere the reaction of G6PD activity. In this new MTT method, a strong purple color was generated in normal blood samples at 20~30 min after incubation, which could be distinguished by the naked eye from G6PD-deficient blood samples with less than 50% residual activity and has the same sensitivity and negative predictive value as WST-8 (ca. 85%). In addition, quantitative measurement using a spectrophotometer was also possible despite the fact that MTT formazan is water-insoluble.

Keywords: *G6PD-deficiency, new screening method, formazan substrate, MTT, purple color development*

ABSTRAK

Kami sebelumnya telah mengembangkan metode tes skrining sederhana dan cepat untuk mendeteksi defisiensi glukosa-6-fosfat dehidrogenase (G6PD) menggunakan substrat formazan WST-8 yang dapat diamati langsung dengan mata telanjang. Namun mengalami sedikit kesulitan dalam membedakan antara warna oranye pudar yang dihasilkan oleh perempuan heterozigot dan warna merah muda yang disebabkan oleh hemolisis pada darah normal karena memiliki warna dasar yang sama, tetapi ini merupakan satu satunya tes skrining cepat yang dapat digunakan di lapangan. Untuk mengatasi hal ini, kami mengembangkan metode skrining G6PD baru dan sederhana dengan menggunakan substrat formazan lain, yaitu MTT (3-(4,5-dimethyl-2- thiazolyl)-2,5-diphenyl-2H tetrazolium bromide) yang dikombinasikan dengan1-methoxy phenazine methosulfate sebagai pengganti WST-8 . Formazan MTT akan menghasilkan warna ungu, sehingga dengan mudah dapat dibedakan dengan warna merah muda yang disebabkan oleh hemolisis pada darah normal. Walaupun disebutkan bahwa MTT dapat bereaksi non-spesifik dengan hemoglobin dan mengganggu interpretasi reaksi warna. Namun dari hasil penelitian kami dengan mencampurkan MTT dengan darah hemolisis, menunjukkan bahwa reaksi non-spesifik yang terjadi sangat lambat, dengan demikian bila penambahan hanya dengan sejumlah kecil sampel darah (5 ~ 10 µl) ke dalam campuran reaksi

(800 µl) tidak akan mengganggu reaksi aktivitas G6PD. Dengan metode MTT yang baru ini, akan menampilkan warna ungu yang kuat pada sampel darah normal dalam waktu 20 ~ 30 menit setelah inkubasi sehingga dengan mata telanjang dapat langsung dibedakan dengan sampel darah defisiensi G6PD dengan aktivitas enzim kurang dari 50% dan memiliki sensitivitas dan nilai prediksi negatif yang sama dengan WST-8 (sekitar 85%). Selain itu pengukuran kadar G6PD secara kuantitatif dapat dilakukan dengan menggunakan spektrofotometer walaupun disebutkan bahwa formazan MTT tidak larut dalam air.

Kata kunci: *defisiensi G6PD, metode skrining baru, substrat formazan, MTT, perubahan warna ungu.*

INTRODUCTION

Glucose-6-phosphate dehydrogenase (G6PD) deficiency is one of the most frequent hereditary disorders, with an estimated 400 million people affected worldwide, particularly in tropical areas including malaria endemic regions.[1] The G6PD gene spans 18 kb on the X chromosome (Xq28), containing an open reading frame of 1,545 base pairs encoded in 13 exons and 12 introns. To date, more than 400 G6PD biochemical variants have been described, and 186 mutations among them have been discovered at the molecular level.[2]

The most frequent clinical manifestation of G6PD deficiency is acute hemolytic anemia, which is usually triggered by taking specific oxidative drugs such as primaquine.[1] Primaquine has been used for the radical treatment of vivax malaria and for gametocytocidal action against falciparum malaria. Primaquine-induced hemolytic crisis is thus a serious problem in chemotherapeutic malaria control efforts. Therefore, primaquine should be administered to malaria patients only after normal G6PD activity is confirmed.

A number of surveys on malaria and G6PD deficiency of individuals living in malaria endemic areas of Southeast Asian countries [3-10] have been done using the Acridine Orange staining method for rapid diagnosis of malaria[11-12] and the WST-8 method[13] for rapid detection of G6PD deficiency. By using these methods, the results of a blood examination could be informed within 30 mins to the malaria patients and prescribed antimalarial drugs, including primaquine, on-site if their G6PD activity was normal.

Presently, several screening methods for detection of G6PD-deficiency in the field have been reported. The fluorescent spot test [14-15] is the most widely used screening method. However, this method requires an ultraviolet lamp in a dark room, and since it provides only a qualitative result, it is very difficult to identify heterozygous females. Other methods, such as the Formazan ring method [16] and the Sephadex gel method,[17] that do not require any equipment or electricity have been used in epidemiological studies.[18-22] Both of these methods have used a formazan substrate, MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H tetrazolium bromide) and a hydrogen carrier, phenazine methosulfate (PMS). Unfortunately, both methods also provide only qualitative results and, thus, it was extremely difficult to diagnose heterozygous females. In addition, PMS is strongly photo-sensitive, and special attention is needed to protect against exposure to ordinary light during screening.[19]

Another formazan method, using WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitro phenyl)-5-(2,4-disulfophenyl)-2H tetrazolium monosodium salt) and 1-methoxy PMS [13] have been reported to overcome the disadvantages in the MTT/PMS methods. The 1-Methoxy PMS is a photo-resistant hydrogen carrier, and WST-8 formazan is highly water-soluble; both are easy to assay qualitatively and quantitatively. However, this method also has a disadvantage: a faint orange color developed by 30~50% G6PD residual activity (*i.e*, heterozygous female samples) is quite similar in tone to the pink color of hemolyzed blood, which is not so easy to distinguish by the naked eye, making it difficult to confidently identify heterozygous females.[9]

As MTT formazan exhibits a purple color, the WST-8 in previous method[13] was replaced by MTT formazan to be easier distinguish faint orange colors from pink colors (Figure 1). A newer rapid screening and detection method of G6PD deficiency by using MTT/1-methoxy PMS and naked eye without the interference of non-specific reactions between MTT and hemoglobin is reported herein.

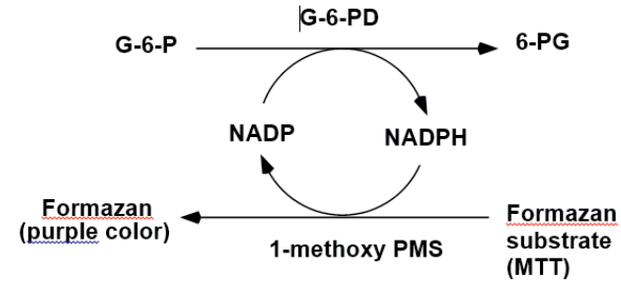

Figure 1. Principle of chemical reactions for detection of G6PD activity by using a formazan substrate, MTT

MATERIALS AND METHODS

Chemicals

Glucose-6-phosphate (G6P) and nicotinamide adenine dinuleotide phosphate (NADP) were obtained from Boehringer Co. (Mannheim, Germany). MTT, 1-methoxy PMS and the WST-8 diagnostic kit were purchased from Dojindo Laboratories (Kumamoto, Japan).

Preparation of Reaction Mixtures for the MTT Method

The transparent type of microcentrifuge tube should be used in this method. The reaction mixture in a 1.5-ml microcentrifuge tube consisted of: (1) 20 µl of the substrate mixtures containing 50 mM G6P, 4 mM NADP in 400 mM Tris-HCl buffer with 100 mM $MgCl_2$ (adjusted pH to 7.2~7.5), (2) 20 µl of 5 mM MTT in H_2O, (3) 20 µl of 1 mM 1-methoxy PMS in H_2O, and (4) 740 µl of H_2O. These substrates and dye solutions can be stored at least for 6 months at 4 °C in the dark or for several years at –20 °C.

Procedures

Normal blood (G6PD activity, 9.0 IU/g Hb), hemizygous male blood (1.0 IU/g Hb) and heterozygous female blood (4.1 IU/g Hb) were obtained from Indonesian donors (the senior author and two volunteers, respectively). Written informed consents were obtained from the two volunteers after the explanation of this study to them based on the guidelines ofthe Declaration of Helsinki. All IUs were measured by an ultraviolet spectrophotometric method using a biochemical assay kit (345-B, Trinity Biotech, Ireland).

The reaction was commenced after adding 5 µl of whole blood to the reaction tube and mixing by shaking several times. The reaction tube was then left to stand and color photographs were taken at various intervals. Development of purple color was observed by measuring the absorbance at 550 nm[23] of the reaction tubes at various intervals using an ultraviolet spectrophotometer, Hitachi U-2800 (Tokyo, Japan).

Examination of the non-specific reaction between MTT and hemoglobin was performed by mixing 5~25 µl normal blood into 800 µl of 0.125 mM MTT in H_2O (the same concentration of MTTs in the reaction mixture for the screening method) and the color change was observed at different intervals.

RESULTS

Non-specific Reaction with Hemoglobin

Fairbanks and Beutler [23]and Hirono *et al.*[17] have reported that MTT reacts with hemoglobin non-specifically, and its dark red or brown color strongly interferes with the interpretation of the color reaction. In our examinations of a 1.5-ml tube containing 800 µl of 0.125 mM MTT in H_2O, addition of 20~25 µl blood reacted with MTT albeit very slowly and the color of hemoglobin was changed to dark red at 6 hrs after incubation (Figure 2). Subsequently, small brownish precipitates were formed in the tubes at 8~10 hrs after incubation. However, these non-specific reactions were not observed when a small amount of blood (5~10 µl) was loaded (see tube 1 in Figure 3D). These results indicated that the interference by the non-specific reaction was negligible when 5~10 µl of blood were mixed with the 800-µl reaction mixture. Indeed, absorbance at 550 nm in the negative control did not change even in the presence of the same concentration of MTT (figure 4).

Qualitative Findings

Figure 3 shows the development of a purple color that was generated by different G6PD activities in 1.5-ml tubes. Appearance of the purple color in normal blood was observed at about 10 min after incubation at room temperature. At 30 min after incubation, a dark purple color in the normal blood sample (tube 4 in Figure 3A) was clearly distinguished from only a faint purple color produced by heterozygous female sample (tube 3 in Figure 3A). At 1 hr (Figure 3B) and 2 hrs (Figure 3C) after incubation, purple colors in the normal blood and heterozygous female blood became stronger, respectively, but at 3 hrs after incubation, small aggregates of MTT formazan formed, and the intensity of the purple color gradually decreased as the aggregates precipitated (tube4 in Figure 3D). Hemizygous male blood showed no change in color (tube 2 in Figure 3A-D), nor did the negative control (tube 1 in Figure 3A-D). However, the samples of heterozygous female blood (tube 3 in Figure 3A-D) showed a slow color development toward purple, and it was possible to visually differentiate them from positive and negative controls.

Time-course of Purple Color Development

Figure 4 shows the time-course of the purple color development in normal blood and in the negative controls without substrate. The color reaction of the normal blood reached a maximum after a 1.5~2-hr incubation, while the color development in the negative control did not occur during the 3-hr incubation (Figure 4). At 3 hrs after incubation, however, absorbance in the normal blood sample decreased as the formazan aggregates precipitated to the bottom of the tube. These results indicated that a quantitative measurement was possible until 2 hrs after incubation although MTT formazan was water-insoluble. All results taken together suggested that judgment of G6PD activity by the new MTT method should be performed between 30 to 60 min of incubation, particularly for accurate identification of heterozygous female samples.

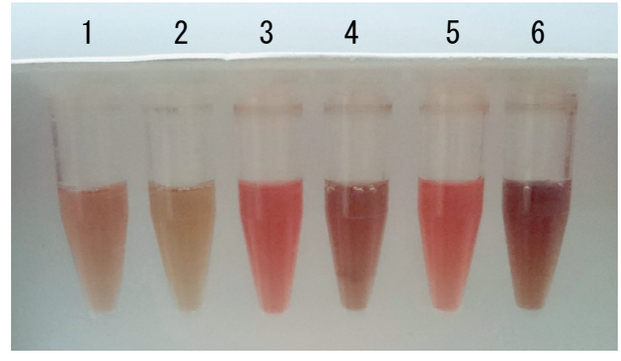

Figure 2. Development of dark red color by non-specific reactions between MTT and hemoglobin at 6 hrs after incubation.

Amount of blood loaded; 15 µl in tubes 1-2; 20 µl in tubes 3-4; 25 µl in tubes 5-6. Tubes 1, 3 and 5, controls without MTT; tubes 2, 4 and 6, 0.125 mM MTT in 800 µlH_2O. Note that hemoglobin colors are changed to dark red in tubes 4 and 6.

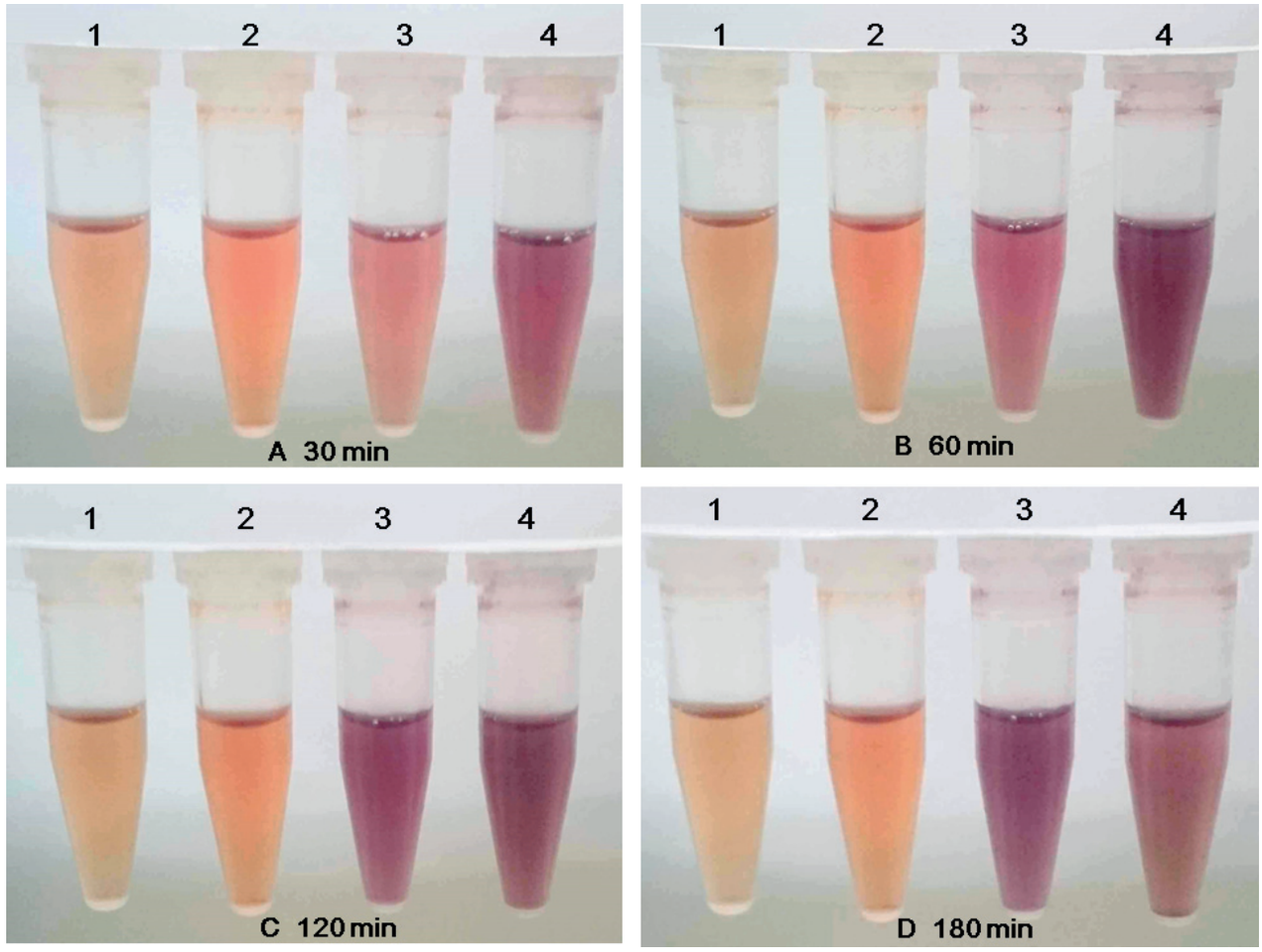

Figure 3. Purple color development in reaction tubes with blood samples of different G6PD activities. Five µl blood is loaded in each tube.
Tube 1, normal blood without the substrates (negative control); tube 2, hemizygous male; tube 3, heterozygous female; tube 4, normal blood (positive control).
Note that at 30 min after incubation, development of a strong purple color is seen in the positive control (tube 4), while a weak color development in tube 3 can be distinguished from the negative control (tube 1). At 3 hrs after incubation, the purple color of the positive control (tube 4) decreased in compared to those at 1~2 hrs since the water-insoluble MTT formazan gradually aggregates and then precipitates at the bottom of the tube. No change in color was observed in the negative control (A-D), even in the presence of MTT.

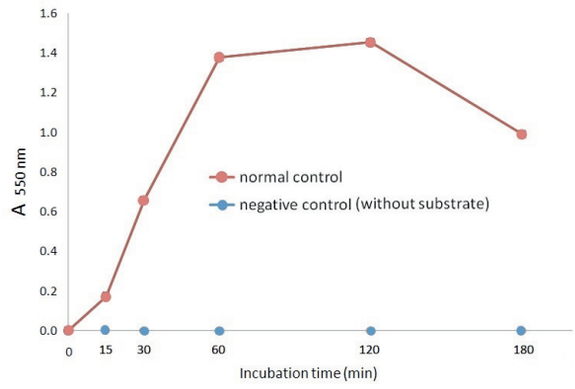

Figure 4. Time-course of purple color development at 550 nm absorbance as measured by ultraviolet spectrophotometer

G6PD activities corresponding to normal blood sample (tube 4 in Figure 3). Values represent the means of three determinations. At 3 hours after incubation, the absorbance at 550 nm decreased due to the precipitation of formazan aggregates. Note that no change in absorbance was seen in the negative control in the presence of MTT.

DISCUSSION

The International standard method for detection of G6PD-deficiency is UV spectrophotometric assay by measurement of absorbance at 340 nm using an UV spectrophotometer with a biochemical assay kit (345-B, Trinity Biotech, Ireland). But this method can be performed only at special hospitals or special institutions. A number of methods for rapid diagnosis of G6PD-deficiency have

been reported. Among them, the fluorescent spot test,[14-15] some MTT formazan methods [16-17, 22] and the WST-8 formazan method [13] have been adopted for application in the field. Recently, two rapid chromatographic diagnostic test kits are commercially available, *i.e*, BinaxNow G6PD (Alere Inc., USA)[24] and the CareStart G6PD (Access Bio, USA).[25] Both are qualitative assays utilizing formazan color development, but quantitative point-of-care tests are currently under development and validation.[26]

In the MTT/PMS methods, many researchers have attempted to resolve the interference problem caused by the non-specific reaction using many techniques for separation of MTT from hemoglobin in reaction mixtures, such as absorption of G6PD enzyme on anion-exchange cellulose paper,[23] of hemoglobin on cation-exchange cellulose paper,[16]and of G6PD enzyme absorbed on DEAE-Sephadex gel,[17] or dissolving all reagents in agar plates and separating from blood (the Formazan ring method[16]). However, our research on non-specific reactions revealed that many special efforts mentioned above are unnecessary. Interestingly, we found that the interference caused by the non-specific reaction can be neglected as a small amount of blood sample is loaded, and that a quantitative measurement is also possible, similar to that of the WST-8 method. Therefore, the new MTT method does not require any technique for separation of MTT from hemoglobin, and the only necessary action is to simply mix reagents in reaction tubes.

All MTT methods are basically qualitative assays due to the fact that the MTT formazan is water-insoluble. Extraction of the produced formazan by organic solvents, such as ether-acetone solution, dimethyl sulfoxide (DMSO) or sodium dodecyl sulphate, is possible.[23] As shown in Figure 4, however, a quantitative assay is possible by the MTT method without the extraction process. Nonetheless, the new MTT method may be more practical if it is used for screening for G6PD deficiency in malaria endemic regions by the naked eye without any equipment. MTT is a cheaper dye than WST-8, and it is more widely commercially available worldwide than WST-8.

Our field trial using the MTT method among the Dayak and the Melayu peoples in Batang Lupar District, Kalimantan Island was successful (unpublished). In this surveillance, 26 deficient individuals among 416 volunteers have been detected. Among those, 22 venous blood samples were confirmed mutations by sequencing. These results may indicate that this new screening method using MTT/1-methoxy PMS is a better method for field detection of G6PD deficiency than the WST-8 method since exhibits a strong purple color which shows production of MTT formazan and describes the high activity of G6PD enzyme which could be easily distinguished by the naked eye.

CONCLUSIONS

We found that the interference by non-specific reactions between MTT and hemoglobin can be neglected as a small amount of blood sample is loaded. Therefore, MTT could be used as a formazan substrate, instead of WST-8, for better rapid screening of G6PD deficiency. This method is easy, rapid and reliable screening method, especially for field application.

CONFLICT OF INTEREST

We have no conflict of interest to declare.

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