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SHORT COMMUNICATION

IN VITRO EFFECTS OF SOME ANESTHETIC DRUGS ON ENZYMATIC ACTIVITY OF HUMAN RED BLOOD CELL GLUCOSE 6-PHOSPHATE DEHYDROGENASE

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In vitro effects of some anesthetic drugs on enzymatic activity of human red blood cell glucose 6-phosphate dehydrogenase. S. ALTIKAT, M. ÇİFTÇİ, M.E. BÜYÜKOKUROĞLU. Pol. J. Pharmacol., 2002, 54, 67–71.

The study investigated *in vitro* effects of halothane, isoflurane, ketamine, sevoflurane, prilocaine, diazepam, and midazolam on enzymatic activity of human red blood cell glucose-6-phosphate dehydrogenase (G6PD; E.C. 1.1.1.49). G6PD was purified from human red blood cells by 2',5'-ADP-sepharose 4B affinity gel. Enzymatic activity was spectrophotometrically measured at 340 nm according to the method of Beutler. I_{50} values were determined from drug activity (%) – drug concentration curves. I_{50} values were as follows: 0.72 mM for isoflurane, 1.82 mM for sevoflurane, 0.38 mM for diazepam, and 0.0019 mM for midazolam. But halothane, ketamine and prilocaine had no inhibitory effect on the G6PD activity in *in vitro*.

Key words: *glucose-6-phosphate dehydrogenase, human red blood cell, general anesthetics, in vitro*

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INTRODUCTION

Glucose 6-phosphate dehydrogenase (G6PD) deficiency is X-linked hereditary disorder, and approximately 400 million persons are affected by this condition. It is fully expressed in males and homozygous females. Some drugs used in the therapy of this disease (primaquine, aspirin, sulfonamides etc.) evoke production and accumulation of toxic peroxides, cause oxidation of hemoglobin and red blood cell membrane, and the use of these kinds of drugs results in excessive hemolysis in the patients with G6PD deficiency. G6PD catalyzes first step of penthose phosphate metabolic pathway which is an exclusive source of NADPH in red blood cells [2]. The most important role of NADPH in erythrocyte consists in regeneration of reduced glutathione, which prevents hemoglobin denaturation, preserves the integrity of the red blood cell membrane sulfhydryl groups, and detoxifies peroxides and oxygen free radicals in the red blood cells [5, 14]. NADPH production is decreased in G6PD deficiency.

G6PD deficiency is frequently seen in African, Mediterranean, Middle East and Far East nations and their lineages with a frequency ranging from 5% to 40% [1, 8, 14]. In Turkey, cases of this disorder have been observed in the Çukurova Region and Başkale district of Van, and its highest incidence was noted in the Jewish Kurd population (62% of males) [6].

Halothane and isoflurane are widely used inhalation anasthetics in the world. Moreover, isoflurane is the most favorable drug in current use. Sevoflurane has recently been introduced to use as inhalation anesthetic [11]. Ketamine is a phencyclidine derivative applied as intravenous anesthetic, and it may be sufficient alone for short operations such as diagnostic and minor surgical interventions [8]. Benzodiazepines, diazepam and midazolam, are known as hypnotic and sedative drugs, and they are used in anesthesia as premedication, or intraoperative sedation for balanced anesthesia [13]. Medium acting local anesthetic prilocaine is used for regional anesthesia [11].

Effects of many drugs on G6PD enzymatic activity have already been investigated [3]. The above-mentioned anesthetics are widely used in clinical practice but data on their effects on G6PD activity lacking. Therefore, we decided to investigate the *in vitro* effects of halothane, isoflurane, ketamine, sevoflurane, prilocaine, diazepam and midazolam on G6PD activity in human red blood cells.

MATERIALS and METHODS

Materials

2',5'-ADP-sepharose 4B was purchased from Pharmacia (Sweden). $NADP^+$, glucose 6-phosphate, protein assay reagent, chemicals for electrophoresis were purchased from Sigma (USA). All other chemicals used were of analytical grade and purchased from either Sigma (USA) or Merck (Germany).

Preparation of hemolysate

Fresh human blood collected in tubes with EDTA was centrifuged at 2500 × g for 15 min and the plasma and leucocyte coat were removed. The packed red cells were washed with KCl solution (0.16 M) three times, with centrifugation at 2500 × g after every washing and discording of supernatants. The erythrocytes were hemolyzed with 5 vol. of ice-cold water and centrifuged at 4°C, 10 000 × g for 30 min to remove the ghosts and intact cells [10, 12].

Ammonium sulfate fractionation and dialysis

Hemolysate proteins were precipitated with ammonium sulfate (35–65%). Ammonium sulfate was added slowly to allow for complete dissolution. The solution was centrifuged at 5000 × g for 15 min, the precipitate was dissolved in 50 mM phosphate buffer (pH 7.0), and dialyzed against 50 mM K-acetate/50 mM K-phosphate buffer (pH 7.0) at 4°C for 2 h with two changes of buffer [10].

Preparation of affinity gel

Two grams of the dried 2',5'-ADP-sepharose 4B gel were used to prepare 10 ml column. Gel was washed with distilled water to remove foreign bodies and air was eliminated from the swollen gel. Gel was suspended in 0.1 M K-acetate/0.1 M K-phosphate buffer (pH 6.0); then, packed in a small column (1 × 10 cm) and equilibrated with the same buffer. Gel was washed with equilibration buffer using peristaltic pump. The flow rates for washing and equilibration were 50 ml/h [10].

Purification of G6PD by affinity chromatography

A dialyzed sample was loaded on 2',5'-ADP-sepharose 4B affinity column and the gel was washed

with 25 ml of 0.1 M K-acetate/0.1 M K-phosphate (pH 6.0), with 25 ml of 0.1 M K-acetate/0.1 M K-phosphate (pH 7.85), and finally, with 0.1 M KCl/0.1 M K-phosphate (pH 7.85). Elution was carried out with 80 mM K-phosphate + 80 mM KCl + 0.5 mM $NADP^+$ + 10 mM EDTA (pH 7.85) solution at a flow rate of 20 ml/h. Eluates were collected in 2 ml tubes and each was tested for enzymatic activity. All of the procedures were performed at 4°C [9,10].

Measurements of G6PD activity

G6PD activity was measured at 37°C according to Beutler's method. This method depends on the reduction of $NADP^+$ by G6PD, in the presence of glucose 6-phosphate. The activity measurement was made by monitoring the increase in absorbtion at 340 nm due to the reduction of $NADP^+$ at 37°C. One enzymatic activity unit was defined as the reduction of 1 μmol of $NADP^+$ per minute at 37°C and pH 8.0 [2].

Protein determination

Quantitative protein determination was spectrophotometrically measured at 595 nm according to Bradford's method, with bovine serum albumin as a standard [4].

SDS polyacrylamide gel electrophoresis

SDS polyacrylamide gel electrophoresis (SDS-PAGE) of the purified enzyme was conducted according to Laemmli's method [7]. It was carried out in 3% and 10% acrylamide concentration for stacking and running gel, respectively, containing 0.1% SDS (Fig. 1) [10].

Inhibitor study

Halothane, isoflurane, ketamine, sevoflurane, prilocaine, diazepam, and midazolam were used as inhibitors. Activies were measured in cuvettes at the following inhibitor concentrations: 0.172, 0.344, 0.516, 0.688 and 1.376 mM for halothane; 0.162, 0.324, 0.648, 0.972 and 1.296 mM for isoflurane; 3.6, 7.2, 10.9, 14.5 and 18.2 mM for ketamine; 0.151, 0.303, 0.606, 1.51 and 1.82 mM for sevoflurane; 0.38, 0.76, 1.52, 3.04 and 6.08 mM for prilocaine; 0.105, 0.175, 0.35, 0.71 and 1.41 mM for diazepam; 0.0904, 0.18, 0.361, 0.452 and 0.904 mM for midazolam. Drugless activity was accepted as 100%. For each drug activity (%) – drug concentration curve was drawn for five different inhibitor concentration.

Statistical analysis

Data were presented as means ± SD. Three parallel measurements were analyzed by Student's *t*-test. Means were compared by Kruskal-Wallis one way analysis of variance. p values < 0.05 were accepted as statistically significant. Drug concentrations which produce 50% inhibition (I_{50}) were calculated from activity (%) – drug concentration curves.

RESULTS

G6PD was purified 9300 times with a yield of 51.6% by using ammonium sulfate precipitation and 2',5'-ADP-sepharose 4B affinity gel. SDS-PAGE of the purified enzyme is presented in Figure 1. Isoflurane, sevoflurane, diazepam and midazolam inhibited the *in vitro* G6PD activity. The differences in the mean values between the treatment groups were statistically significant ($p < 0.005$) according to the analysis of variance (Tab. 1). I_{50} values determined from activity (%) – drug concentration curves were: 0.72 mM for isoflurane, 1.82 mM for sevoflurane, 0.38 mM for diazepam, and 0.0019 mM for midazolam. But halothane, ketamine and prilocaine had no inhibitory effect on the G6PD activity *in vitro* (Tab. 2). The differences in the median values between the treatments were not great enough to exclude the possibility that the differences were due to random sample variability, so there was not

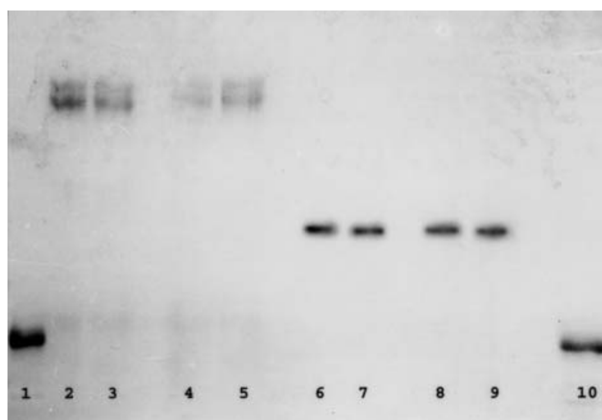

Fig. 1. SDS –polyacrylamide gel electrophoresis of G6PD purified by affinity gel. (lanes 1 and 10 contained human carbonic anhydrase-1, lanes 2–5 – buffalo milk lactoperoxidase, lanes 6–9 – human G6PD)

Table 1. G6PD activity at different drug (inhibitor) concentrations. Drugless activity was accepted as 100%

Drug	Concentration (mM)	Activity % (mean ± SD)
Isoflurane	drugless	100 ± 0.00
	0.162	86 ± 1.00*
	0.324	70 ± 1.73*
	0.648	48 ± 1.73*
	0.972	40 ± 0.00*
	1.229	28 ± 0.00*
Sevoflurane	drugless	100 ± 0.00
	0.151	98 ± 0.00*
	0.303	88 ± 1.73*
	0.606	72 ± 2.00*
	1.51	61 ± 0.00*
	1.82	50 ± 1.00*
Diazepam	drugless	100 ± 0.00
	0.105	95 ± 1.00*
	0.175	80 ± 0.00*
	0.35	45 ± 1.73*
	0.71	25 ± 1.00*
	1.41	9 ± 0.00*
Midazolam	drugless	100 ± 0.00
	0.0904	72 ± 0.00*
	0.18	57 ± 1.00*
	0.361	28 ± 1.73*
	0.452	17 ± 0.00*
	0.904	4 ± 1.00*

N = 3, * $p < 0.01$ vs. control, Student's *t*-test

Table 2. G6PD activity at different drug (non-inhibitor) concentrations. Drugless activity was accepted as 100%

Drug	Concentration (mM)	Activity % (mean ± SD)
Halothane	drugless	100 ± 0.00
	0.172	102 ± 2.00*
	0.344	98 ± 1.73*
	0.516	99 ± 1.00*
	0.688	101 ± 1.73*
	1.376	99 ± 0.00*
Prilocaine	drugless	100 ± 0.00
	0.38	97 ± 1.00*
	0.76	96 ± 1.73*
	1.52	98 ± 1.73*
	3.04	96 ± 0.00*
	6.08	97 ± 1.00*
Ketamine	drugless	100 ± 0.00
	3.6	98 ± 1.73*
	7.2	102 ± 1.00*
	10.9	104 ± 4.00*
	14.5	93 ± 2.646*
	18.2	95 ± 4.359*

N = 3, * $p > 0.05$ vs. control, Student's *t*-test

a statistically significant difference ($p > 0.05$) according to analysis of variance.

DISCUSSION

The most common red blood cell enzymatic defect througout the world is G6PD deficiency [14]. Hemolysis occurs in older erythrocytes after exposure to oxidant drugs or chemicals. Anemia, jaundice and reticulocytosis develop, when G6PD deficiency is more severe, while hemolysis may lead to hemoglobinuria and acute renal failure [1, 6, 8].

General anesthetics are used as an adjunct to surgical procedures to render a patient unaware of and unresponsive to painful stimulation. They are given systemically and exert their main effects on the central nervous system [11]. General anesthetics usually produce analgesia, amnesia, loss of consciousness, inhibition of sensory and autonomic reflexes, and skeletal muscle relaxation. General anesthetics are usually given by inhalation or by intravenous injection [13]. Several drugs are used intravenously, alone or with other drugs, to achieve anesthesia or as components of balanced anesthesia [13]. Balanced anesthesia includes the administration of medications preoperatively for sedation and analgesia, the use of neuromuscular blocking drugs intraoperatively, and the use of both intravenous and inhaled anesthetic drugs [13].

Halothane was the first introduced halogenated agent and it was widely used. But its use is now declining in favor of isoflurane and other drugs. Isoflurane and sevoflurane are the most favorable volatile anesthetic drugs due to their low toxicity [8]. However, the results of this study have shown that halothane has no effect on G6PD activity, but

isoflurane and sevoflurane have. Because of this, the use of halothane may be appropriate in a patient with G6PD deficiency. Additionally, the use of diazepam or midazolam, which have inhibitory effects on *in vitro* G6PD enzymatic activity together with isoflurane or sevoflurane may increase severity of hemolysis. Ketamine may be chosen instead of midazolam or diazepam for balanced anesthesia, as intravenous anesthetic drug. Furthermore, prilocaine should be chosen for local anesthesia if it is required.

In conclusion, we think that the results of this study may be useful in choosing of anesthetic drugs for use in a patient with G6PD deficiency. The use of a G6PD inhibitor as general anesthetic drugs may worsen health of the patients with G6PD deficiency and may lead to fatal outcome. If it is required to administer these inhibitory drugs to the patients with G6PD deficiency, their dosage should be very well ordered to decrease the side-effects. Investigation of *in vivo* effects of these drugs on G6PD activity are very important from clinical point of view and, for this reason, further detailed studies are required.

REFERENCES

1. Berkow R.: The Merck Manual of Diagnosis and Therapy. 15th edn., Merck & Co. Inc., USA, 1987.
2. Beutler E.: Red Cell Metabolism, Manual of Biochemical Methods. Academic Press, London, 1971.
3. Beutler E.: Glucose 6-phosphate dehydrogenase deficiency and other enzyme abnormalities. In: Williams Hematology. Eds. Beutler E., Lichtman M.A., Coller B.S., Kipps T.J., 5th edn., Mc Graw-Hill Company, New York, 1995, 564–581.
4. Bradford M.M.: A rapid and sensitive method for the quantition of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem., 1976, 72, 248–51.
5. Deutsch J.: Glucose-6-phosphate dehydrogenase. In: Methods of Enzymatic Analysis. Vol 3., Eds. Bergmeyer H. U., Bergmeyer J., VCH Verlagsgesellschaft, Weinheim, 1983, 190–196.
6. Kayaalp S. O.: Rasyonal tedavi yönünden tıbbi farmakoloji (Turkish). Hacettepe-Tas Yayıncılık, Ankara, 1998.
7. Laemmli D. K.: Clevage of structural proteins during an assembly of the head of bacteriophage T_4. Nature, 1970, 227, 680–683.
8. Laurence D. R., Bennett P. N., Brown M. J.: Clinical Pharmacology. 8th edn., Churchill Livingstone, Singapore, 1997.
9. Morelli A., Benatti U., Gaetani G.F., De Flora A.: Biochemical mechanisms of glucose-6-phosphate dehydrogenase deficiency. Proc. Nat. Acad. Sci. USA, 1978, 75, 1979–1983.
10. Ninfali P., Orsenigo T., Barociani L., Rapa S.: Rapid purification of glucose 6-phosphate dehydrogenase from mammal's erythrocytes. Prep. Biochem., 1990, 20, 297–309.
11. Rang H.P., Dale M.M., Ritter J.M.: Pharmacology. Churchill Livingstone, China, 1999.
12. Shereve D.S., Levy H.R.: On the molecular weight of human glucose-6-phosphate dehydrogenase. Biochem. Biophys. Res. Commun., 1977, 78, 1369–1375.
13. Trevor A.J., Miller R.D.: General anesthetics. In: Basic and Clinical Pharmacology. Ed. Katzung B.G., Appleton and Lange, USA, 1989, 304–322.
14. Weksler B.B., Moore A., Tepler J.: Hematology. In: Cecil Essentials of Medicine Eds. Andreoli T.E., Carpenter C.C.J., Plum F., Smith L.H. Jr., 2nd edn., WB Saunders Company, Philadelphia, 1990, 341–363.

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